Salivary proteomics as a tool to understand ingestive behavior:

An experimental study in sheep (*Ovis aries*), goat (*Capra hircus*) and mice (*Mus musculus*)

Elsa Cristina Carona de Sousa Lamy

Dissertação apresentada à Universidade de Évora para a obtenção do grau de Doutor em Zootecnia

Orientador: Professora Doutora Elvira Sales Baptista

Co-orientador: Professora Doutora Ana Varela Coelho

Esta tese não inclui as críticas e sugestões feitas pelo júri

Évora
2008

U.E Serviços Académicos	N.º 6015662
31 / 7 / 08 Filomena	Sector: DEPG

Salivary proteomics as a tool to understand ingestive behavior:

An experimental study in sheep (*Ovis aries*), goat (*Capra hircus*) and mice (*Mus musculus*)

Elsa Cristina Carona de Sousa Lamy

171 884

Esta tese não inclui as críticas e sugestões feitas pelo júri

Aknowledgments

I wish to express my sincere gratitude to my supervisor, Prof. Elvira Sales Baptista, for introducing me to the interesting field of animal ingestive behavior, for the confidence she deposited on me and for all the encouraging words. Thank you also for the valuable comments and constructive criticism during the completion of this thesis.

I am much indebted to my other supervisor, Prof. Ana Varela Coelho, whose expert scientific knowledge on proteomics and generous support have been invaluable for the success of my research. Thank you for being so supportive, helpful and positive and for inputting me the passion for laboratorial work and scientific research.

To all the colleagues from the Mass Spectrometry laboratory. Tanks for the friendly way as you included me in your group and for your constant availability to help me solving experimental problems. A particular thanks to Gonçalo Costa, who accomplished this work from the beginning, for his help and support.

To Prof. Maria Ivone da Clara, for always open me the door of her lab. To all the people that is doing their research there, for al the support and the friendship.

To Prof. Alfredo Pereira for kindly help me with statistical analysis.

To Prof. José Caeiro Potes, for the availability in performing the animal surgeries, and for all the support and help.

To Eng. Luisa Fialho, for technical support in tissue processing for the histological studies.

To Eng. Ürs Zuber, unfortunately already died, for the enormous help, in providing the facilities for keep the animals, during the initial steps of this work.

To ICAM (Instituto de Ciências Agrárias Mediterrâneas), in the person of Prof. Maria do Rosário Oliveira, for the accessibility in providing financial support.

I would like also to acknowledge the free offers of Tupafin Ato and Tannin C products to SilvaChimica company.

To all my old friends and all the new friends that I made during this time. For their support to carry on. I would like to particularly tanks Dr. Joana Reis, who have the patience of to revise the language of the general discussion.

To my three hairy friends, Linda, Luna and Miró, which accompanied me great part of the time and supported some moments of bad humor, always showing the same friendship and fidelity.

Aos meus pais e avó. Por todo o amor, apoio e confiança, em todas as etapas da minha vida.

Finally, to my husband Fernando, who was with me from the first moment. His comprehensive support, love and patience, on every hard moments have made this work possible.

This thesis was supported by FCT (Fundação para a Ciência e a Tecnologia of Ministério da Ciência, Tecnologia e Ensino Superior) fellowship.

Preface

The present thesis is the result of studies initiated in 2002, in the sequence of the research project "Effects of phenolic compounds in taste sensitivity: Cell kinetics studies, chemical and morphological adaptations in the oral cavity" (POCTI 33039/CVT/99-00).

Research was performed at Instituto de Ciências Agrárias Mediterrânicas-ICAM of the University of Évora and at the Mass Spectrometry Laboratory of the Instituto de Tecnologia Química e Biológica-ITQB, under the supervision of Prof. Elvira Sales Baptista and Prof. Ana Varela Coelho.

The project starts from the hypothesis that taste perceptions would be a result of species-specific features that could occur at morphological and chemical level, and proposed to acess those adaptations, which could be ultimately expressed at behavioral level. The state of the art and the first observed results lead us to focus the research on the role of salivary proteins in ingestive behavior and in the adapatative changes in saliva protein composition induced by ingestion of plant secondary metabolites, such as tannins.

Salivary protein composition of sheep (*Ovis aries*), goats (*Capra hircus*) and mice (*Mus musculus*) was studied. Sheep and goats represent two small ruminant species that are usually found together in Mediterranean pastures, which choose to feed different plants or plant parts. This different ingestive behavior between this species has been the target of several studies, but that saliva role has received less attention. Mice were used in this research in order to compare the role of salivary proteins in the adaptations to diet of animal species with different digestive physiology. Additionaly, rodents salivary glands are widely used as a model for protein secretion, with a large amount of reference data available, so studies in mice would allow physiological and behavioral comparative discussions.

The present thesis consists of seven chapters. In the first chapter the state of the art is presented. The five subsequent chapters contain papers that are accepted by or intended for international journals. Most of these papers are written jointly with other scientists, representing the work that we did together with other colleagues. Finally, in chapter seven the main results are integrated and discussed and the general conclusions are presented.

In chapter 2 the effects of tannins on mice major salivary glands histo-morphology were studied. The changes in saliva protein composition are expected to derive from changes in salivary glands, which are under nervous control. The effects produced by tannins were compared with those produced by the sympathetic nervous system agonist isoproterenol in

mice major salivary glands. Additionally, in this chapter we compared the effects produced by hydrolysable tannins and condensed tannins.

In chapter 3, we studied the effect of dietary tannins in mice whole saliva composition. Some of the effects of tannins in saliva from rodents were already known, from the bibliography, namely the induction of salivary proline-rich proteins, most of which form insoluble complexes with tannins. These proteins constitute a high proportion of the salivary proteins present in mice fed tannins and, as such, avoid the detection of the proteins expressed in low amounts, for which expression levels were not so well studied. In that way, the insoluble complexes formed between salivary proteins and tannins in the mouth, were removed, before whole saliva analysis. Salivary proteins were separated by SDS-PAGE and changes in salivary protein profile in response to condensed and hydrolysable tannins were accessed through the inclusion of quebracho and tannic acid, respectively, in the diet. Although similarities between the effects of hydrolysable and condensed tannins were observed, a pronounced effect seemed to be produced by condensed tannins.

In chapter 4 mice whole saliva proteome was characterized. Two-dimensional electrophoresis was used to separate proteins according to their molecular masses and isoelectric points, and mass spectrometry was subsequently used for their identification by. Following the previous studies results , condensed tannins (quebracho) were used to study the effects of these compounds in saliva proteome.

Chapter 5 presents sheep and goat parotid saliva protein profile obtained through protein separation by SDS-PAGE and identification by mass spectrometry. Before the feeding experiments, one of the parotid ducts, of each animal, was cannulated in order to collect glandular saliva. Parotid protein profiles from the two species were compared and the results discussed in the light of their different feeding behavior.

In chapter 6, sheep and goat parotid saliva proteomes were characterized by the same proteomic techniques used in chapter 4. Besides comparison between the two ruminant species, a feeding trial in which quebracho tannin was added to the diet was performed, with the aim of looking for salivary protein expressions affected by tannin consumption.

Finally, in chapter seven, as already mentioned, the main results are integrated and discussed and the general conclusions are presented. Based on the results obtained in this thesis further studies are suggested .

Abstract

The oral cavity is the part of the animal internal medium that first comes into contact with food. Numerous chemical and mechanical receptors in the mouth respond to the food chemical and physical properties and monitor the changes during processing. This leads to central perception of taste and texture of food, which, together with odor, are important determinants in the decision of to ingest or not. Saliva plays an important role in the perception of taste and texture sensations. Its composition can modulate food perception and, simultaneously, be modulated by the type of diet.

This thesis is focused on the study of the role of salivary proteins on ingestive behavior. Tannins are used as a model to access changes in salivary protein profile induced by dietary compounds. These plant secondary metabolites produce aversive taste/oral sensations influencing animal diet choices. The levels of dietary tannins tolerated vary according to the physiological mechanisms that animals possess to avoid their potential negative effects. Saliva, and more particularly salivary proteins, has been pointed as a defense mechanism against tannins. Three animal species were studied: sheep, goat and mice. The first two species are ruminant species and present similar digestive characteristics, but differ between them in the levels of dietary tannins tolerated. Mice, on the other hand, represent a rodent mammalian specie with different digestive characteristics.

We have studied the effect of tannins on mice salivary gland histomorphology (chapter 2) since these are the sites of salivary protein production. Both condensed and hydrolysable tannins produced major effects in the acinar structures, with condensed tannins having a stronger effect. The similarities between these effects and the ones produced by isoproterenol suggested that tannins act through activation of sympathetic nervous system.

The effects of quebracho tannin and tannic acid on mice whole saliva protein composition were studied by comparing the SDS-PAGE profile of control animals to the ones from animas fed with these compounds during 10 days (chapter 3). Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (MS) data were used to identify salivary proteins. One isoform of salivary amylase was observed to increase in response to both types of tannins.

Despite a considerable number of studies on mice salivary glands and saliva, a proteome of mice whole saliva was not, at our knowledge, characterized to date. In chapter 4 we used two-dimensional electrophoresis coupled to MALDI-TOF MS for this purpose. A total of 26 proteins were identified. The effects of the ingestion of quebracho tannin for a period of ten days were studied in the salivary protein fraction, which does not precipitate tannins. The expression levels

of one isoform of alpha amylase and of an unidentified protein were observed to increase, whereas acidic mammalian chitinase and Muc10 decreased. Additionally, two protein spots were induced, that were not identified by MS, but, based on their staining characteristics, we suggest them to be proline-rich proteins,

Sheep and goat parotid saliva proteomes were characterized and compared, and a total of 40 parotid salivary proteins were identified, with differences between the two species being reported (chapters 5 and 6). , Two-dimensional electrophoretic protein saliva maps of animals in control diets and after 10 days quebracho tannin consumption were compared (chapter 6). Changes in salivary protein expression levels induced by tannins were observed, some of which are common to sheep and goats and others specific of each species.

Altogether the results suggest that salivary proteome study can be important in understanding feeding behavior and that proteome is influenced in the short-medium term by diet composition. Although differences among mice, sheep and goats exist, in response to tannin ingestion, a common feature to the three species is the increase in the expression levels of proteins usually increased in stress situations. This, together with the histomorphometric data, which point to an action of tannins in sympathetic nervous system, suggests that the mechanisms involved in salivary protein regulation by tannins may be related to a "stress response" imposed by these compounds.

Resumo

A cavidade oral é o local de primeiro contacto entre o alimento e o ambiente interno do animal. Numerosos receptores químicos e mecânicos, presentes na boca, respondem às propriedades químicas e físicas dos alimentos e monitorizam as alterações durante o seu processamento. Isto tem como consequência a percepção do gosto e textura dos alimentos, os quais em conjunto com o olfacto, são determinantes na decisão de ingerir. A saliva desempenha um papel de extrema importância neste processo, pois a sua composição pode modular a percepção dos alimentos, e, em simultâneo, ser modulada pelo tipo de dieta.

A presente tese tem como objectivo o estudo do papel das proteínas salivares no comportamento ingestivo. Para avaliar as alterações na secreção de proteínas salivares induzidas por compostos presentes na dieta utilizaram-se três tipos de taninos: ácido tânico, chestnut (taninos hidrolisáveis) e quebracho (taninos condensados). Estes metabolitos secundários das plantas produzem sensações gustativas/orais aversivas que influenciam a escolha da dieta, por parte dos animais. Os níveis de taninos, presentes na dieta, tolerados pelas diferentes espécies animais variam de acordo com os mecanismos fisiológicos que cada uma possui para evitar potenciais efeitos negativos. A saliva, e mais especificamente as proteínas salivares, têm sido apontadas como componentes de mecanismos de defesa que contrariam os efeitos dos taninos. Para avaliar a importância da saliva no comportamento ingestivo, e mais especificamente no consumo desses compostos, foram estudadas três espécies: ovelhas (*Ovis aries*), cabras (*Capra hircus*) e murganhos (*Mus musculus*). As duas primeiras apresentam características digestivas semelhantes, mas diferem entre elas nos níveis de taninos que toleram; os murganhos, por outro lado, representam uma espécie de mamíferos com diferentes características digestivas.

No capítulo 2 estudaram-se os efeitos dos taninos ao nível da histomorfologia das glândulas salivares de murganhos. É conhecido que, em roedores de laboratório, proteínas salivares, como as proteínas ricas em prolina (PRPs), são induzidas por agonistas do sistema nervoso simpático (ex. isoproterenol), e que essa indução está inter-relacionada com um aumento do tamanho das estruturas acinares das glândulas parótidas e submandibulares. O efeito do consumo de taninos apresenta semelhanças com o efeito provocado pelo tratamento com isoproterenol, no que diz respeito a um aumento da massa glandular e à indução de PRPs. De modo a estudar os efeitos dos taninos, a nível da histomorfologia das glândulas salivares, murganhos foram submetidos a dietas com três taninos pertencentes a diferentes classes estruturais (ácido tânico, chestnut e quebracho), ou injectados com isoproterenol, durante dez dias. O tamanho dos ácinos das glândulas salivares, parótidas e submandibulares, aumentou significativamente, tendo sido esse aumento maior para as glândulas parótidas,

comparativamente às glândulas submandibulares, e maior para os animais que consumiram quebracho, comparativamente com os outros tipos de taninos. O tratamento com qualquer um dos três tipos de taninos também resultou num aumento significativo do tamanho dos ácinos das gandulas sublinguais, ao contrário do tratamento com isoproterenol, que não produziu alterações significativas nestas estruturas. Os resultados obtidos por nós estão de acordo com outros estudos que sugerem que os taninos actuam a nível do sistema nervoso simpático, mais concretamente ao nível dos receptores beta-adrenérgicos. No entanto, e devido à observação de efeitos produzidos ao nível das glândulas sublinguais, não são de excluir mecanismos adicionais da acção dos taninos. Para além disso, são apresentadas evidências de que os efeitos produzidos pelos taninos dependem da estrutura destes compostos, e é possível que os murganhos necessitem de produzir uma maior quantidade de proteínas salivares, como defesa contra a acção de taninos condensados, comparativamente a taninos hidrolisáveis.

Presentemente sabe-se que, dois grupos de proteínas salivares, histatinas e proteínas ricas em prolina, apresentam uma elevada afinidade para taninos, diminuindo a actividade biológica destes compostos. A possibilidade de existirem outras proteínas salivares com funções de defesa contra taninos é desconhecida. No capítulo 3 caracterizaram-se e compararam-se os perfis proteicos da saliva mista de murganhos submetidos a três tipos de dieta: controlo, com a incorporação de taninos hidrolisáveis (5% ácido tânico) ou com a incorporação de taninos condensados (5% quebracho). As proteínas foram separadas de acordo com as suas massas moleculares, por electroforese em gel de poliacrilamida Sodium Dodecyl Sulphate-Polyacrylamide Gel Electroforesis (SDS-PAGE), e analisadas utilizado um espectrómetro de massa com ionização do tipo MALDI (Matrix Assisted Laser Desorption/Ionization) associado a um analisador de massas, do tipo "tempo de voo", TOF (Time of Flight). A identificação das proteínas presentes nas bandas isoladas foi feita através do método "Peptide Mass Fingerprinting" (PMF). Uma vez que as proteínas mais abundantes dificultam a observação e identificação de proteínas com menores níveis de expressão, recorreu-se à centrifugação para remoção das proteínas precipitadas pelos taninos. Foi possível identificar dez proteínas salivares diferentes, algumas das quais apresentando diferentes isoformas. A adição de taninos à dieta provocou alterações no perfil proteico da saliva. Uma isoforma de alfa-amilase foi sobre-expressa em consequência do consumo de ambos os tipos de taninos. Por outro lado, a proteína aldeido redutase foi identificada apenas no grupo que consumiu quebracho.

Apesar do número considerável de estudos realizados com glândulas salivares e saliva de roedores de laboratório, a caracterização do proteoma da saliva destas espécies não se encontra ainda descrita. No capítulo 4 pretendeu-se caracterizar o proteoma da saliva mista de murganho, recorrendo à separação proteica por electroforese bi-dimensional, seguida da identificação das proteínas por PMF, e avaliação das alterações provocadas pelo consumo de taninos na composição proteica da saliva que não é precipitada por esses compostos. Das 26

proteínas identificadas, observou-se, por um lado, um aumento dos níveis expressos de uma isoforma de alfa-amilase e de uma proteína não identificada e, por outro, uma diminuição dos níveis de expressão da proteína quitinase acídica e da mucina Muc10, após os animais consumirem uma dieta enriquecida em taninos (7% quebracho) durante 10 dias. Adicionalmente, foi induzida a expressão de proteínas detectadas em 2 spots localizados na região básica dos géis 2D. Estes spots apareceram cor-de-rosa após utilização de um protocolo modificado de coloração com Coomassie Brilliant Blue R-250, não tendo sido possível a sua identificação por PMF, o que sugere corresponderem a PRPs.

Hofmann propôs uma classificação dos ruminantes em três diferentes "feeding types", "grazers" (ex: ovelha), "intermediate feeder" (ex: cabra) e "browsers", de acordo com a composição da dieta e com as características morfológicas do seu sistema digestivo. Foram referidas as diferentes dimensões das glândulas parótidas, e associadas à diferente quantidade de saliva produzida. Vários autores referiram a presença de proteínas com afinidade para taninos, na saliva dos "browsers" e a sua ausência na saliva dos "grazers". No entanto, pouco mais se sabe acerca da composição proteica da saliva de ruminantes. No capítulo 5 o perfil proteico da saliva secretada pelas glândulas parótidas de cabras e ovelhas foi comparado. As proteínas foram separadas por SDS-PAGE em géis lineares de 12,5% poliacrilamida e identificadas por PMF. Diferenças significativas foram observadas para a região de massas moleculares compreendida entre 25 e 35 kDa: uma banda presente nas duas espécies, mas com intensidades significativamente diferentes; três bandas presentes apenas nos géis de cabra; e uma banda presente apenas nos géis de ovelha. A identificação das proteínas constituintes destas bandas não foi possível.

No capítulo 6 pretendeu-se aumentar o conhecimento obtido no capítulo 5 e avaliar os efeitos dos taninos na composição da saliva secretada pelas glândulas salivares de cabras e ovelhas. O proteoma da saliva parótida destas duas espécies foi caracterizado, recorrendo a uma metodologia semelhante à descrita no capítulo 4. De um total de 205 spots presentes nos géis de cabra e de 260 spots presentes nos géis de ovelha, 106 e 117, respectivamente, foram identificados. Os perfis proteicos das duas espécies apresentaram uma proporção elevada de proteínas plasmáticas. Tal como havia sido observado para proteínas separadas por SDS-PAGE, no capítulo 5, também nos géis 2D as diferenças mais marcadas, entre as duas espécies, foram observadas na região de massas moleculares compreendida ente 25 e 35 kDa. Para além destas, verificaram-se outras diferenças, quer ao nível da presença/ausência de alguns spots, quer ao nível da intensidade de spots presentes em ambas as espécies, quer ainda ao nível do tipo de proteínas identificadas. Após o consumo de taninos (2.5% quebracho) durante 10 dias, a concentração proteica da saliva parótida de ambas as espécies aumentou e o proteoma deste fluido sofreu alterações. Algumas das alterações, induzidas pelos taninos, foram semelhantes em cabras e ovelhas, mas muitas delas foram específicas para cada espécie.

No conjunto, estes resultados sugerem que o estudo do proteoma da saliva pode ser importante na compreensão do comportamento ingestivo e que esse proteoma é influenciado, no curto-médio prazo, pela composição da dieta. Apesar das diferenças entre murganhos, cabras e ovelhas, na resposta a consumo de taninos, uma característica comum às três espécies foi o aumento da expressão de proteínas normalmente aumentadas em situação de stress. Isto, em conjunto com os resultados histomorfométricos, que apontam para uma acção dos taninos ao nível do sistema nervoso simpático, sugere que os mecanismos envolvidos na regulação das proteínas salivares, por parte destes metabolitos secundários, possam estar relacionados com uma "reposta a stress".

CONTENTS

Aknowledgments i

Preface iii

Abstract v

Resumo vii

CHAPTER 1: General Introduction 1

1. Feeding behaviour 1

1.1. Control of food intake 1

1.2. Ruminant ingestive behaviour 2

1.2.1 Feeding types 2

1.2.2 Regulation of food intake in ruminants 4

1.3. Diet selection – Preference vs. aversion 5

1.4. The effects of tannins in ingestive behaviour 6

2. Food perception – Taste and astringency 9

2.1. Sensory attributes of food 9

2.2. Taste perception 10

2.2.1. Structures involved in taste reception 10

2.2.2. Taste reception 13

2.2.3. Neural taste pathways 14

2.3. Taste code in the brain 15

2.4. Astringency 16

2.5. Relation between food perception in mouth and behavioural response 18

2.6. Differences among species in food perception and behaviour 19

3. Saliva 21

3.1 Salivary glands morphology 21

3.2 Saliva secretion 24

3.2.1. Nervous control 24

3.2.2. Secretion of water and electrolytes 27

3.2.3. Secretion of proteins 30

3.2.3.1. Secretion by acinar cells 30

3.2.3.2. Secretion from duct cells 32

3.2.3.3. Secretion of proteins from blood origin 33

3.3. Factors affecting salivary volume and composition 33

3.4. Taste-saliva interactions 35

3.5. Particularities of saliva secretion in ruminants 37

3.5.1. Electrolyte composition 38

3.5.2. Nervous control of ruminant salivary secretion 39

4. Salivary proteins 41
4.1. Proteomics in the study of saliva 41
4.2. Protein functions 42
4.2.1 Teeth mineralization 44
4.2.2. Oral mucosal protection and antimicrobial activity 44
4.3. Glandular origin and amounts of salivary proteins in humans, rodents and ruminants 45
4.4. Salivary proteins involved in food consumption 50
4.5 Changes in salivary protein composition induced by food constituents 50

CHAPTER 2: Salivary glands histomorphological modifications in mice submitted to tannin enriched diets: comparison with sialotrophic effects of sympathetic agonists stimulation 83

CHAPTER 3: Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins 105

CHAPTER 4: Characterization of mouse whole saliva proteome and its adaptation to tannin rich diets 127

CHAPTER 5: Comparison of Electrophoretic Protein Profiles from Sheep and Goat Parotid Saliva 157

CHAPTER 6: Comparative analysis of sheep and goat saliva proteome: a new tool for research on the ingestive behavior of herbivores 175

CHAPTER 7: General discussion 225
1. Salivary glands respond to tannin ingestion trough an increase in size of secretory structures 225
2. Proteome characterization of mice, sheep and goats and comparison among the species 226
3. Aversive substances change salivary proteome in ruminants and rodents 231
4. Main conclusions 233
5. Implications 234

Chapter 1

General Introduction

1. Feeding behaviour

1.1. Control of food intake

Feeding behaviour is composed by the several animal actions directed toward the search and intake of food. It is not a single behaviour and like other motivated behaviour systems, can be viewed as a sequence of constituent component behaviours performed in a series. In the beginning of twenty century, the ethologist and animal physiologist Wallace Craig distinguished between the "appetitive" component of behaviour that lead to seek, find and approach food or water, and the "consummatory" response – eating or drinking – that occurs at the end point of terminal component of the sequence. These two phases are different in both their biology and their developmental origins. Craig's distinction between appetitive and consummatory behaviour components became a key concept of Konrad Lorenz's ethological theory (Kalikow, 1983).

One of the most potent drives for feeding is its rewarding nature. Two different theories were suggested to explain it, each one based in only one of the regulatory mechanisms, hedonic and homeostatic. The "drive reduction" theory (Hull, 1943) considers the homeostatic mechanisms, suggesting that the internal physiological demands are responsible for motivation. According to this theory, hunger is a consequence of a deficit in the organism. The other, referred as the "positive incentive" theory (Bolles and Moot, 1972), suggests that an animal is motivated to ingest a food item by anticipate the pleasure obtained by the ingestion of that food. Internal and external stimulus can originate motivation, for example, the thought or sight of attractive food can elicit a sensation of hunger. In what concerns homeostatic mechanisms, physiological theories for hunger and eating were developed. The glucostatic theory states that the initiation of eating is the result of a decline in blood glucose, whereas, the lipostatic theory hypothesized that some peripheral signal, probably from adipose tissue, would feedback to central satiety centres to modulate food intake and body weight (Mayer, 1955). Despite this simplistic distinction between homeostatic and hedonic mechanisms, reality is more complicated and in fact both mechanisms interact to modulate feeding behaviour. Rewarding effects of food are potentially modulated by internal states, and homeostatic indicators of satiety modulate the hedonic value of a food. In other words, a food stimulus that is pleasurable when the animal is hungry may be unpleasant after satiation. Gustatory, olfactory and visual neurons stop responding to taste, odour and sight of a food eaten to satiety, yet they continue to respond to other foods (Critchley and Rolls, 1996a).

Of the various central nervous system structures in behavioural control, the hypothalamus has been identified as one of the more important in the control of food intake. Mammalian brains have evolved several potent and intercalated neuronal systems that drive feeding behaviour. Initially, the brain mechanisms regulating the homeostatic drive to ingestion were fixated on a model, considering the

lateral hypothalamus responsible for eating (feeding centre) and the ventromedial nucleus responsible for the cessation of ingestion (satiety centre). Nowadays it is known that besides them, systemic mediators, such as leptin and ghrelin, and neuromodulators, such as neuropeptide Y, opioids, norepinephrine, among others, are also involved in the control of eating (best reviewed in Saper et al., 2002; Chaptini and Peikin, 2008). However, and due to the complexity of the pathways involved, a clear and comprehensive understanding of feeding-reward interactions is far from being completely understood.

There are enormous variations in the feeding strategies of different animal species. Laboratory rodents are greatly used for studies of food intake in mammals, and most of the general considerations, referred until this point, also concern them. However, for other species, particularly ruminants, the complexity of the digestive system and the consequent metabolic peculiarities, result in particularities of feeding behaviour, which will be presented in the next section.

1.2. Ruminant ingestive behaviour

1.2.1 Feeding types

The low nutrient concentrations in most plants means that, in comparison to other organisms, herbivores must either harvest and process large volume of foods or they must feed selectively to increase diet quality (Foley et al., 1999).

Hofmann (1989) proposed a classification of ruminants in three feeding types based on functional-anatomical and histological investigations: browsers or concentrate selectors, grazers and intermediate feeders. This feeding type criterion was used by Hofmann to predict adaptative strategies in forage selection, avoidance and utilisation (Fig. 1.1). In general, grazers have a diet based on monocotyledons. These plants are fibrous, having high amounts of cellulose and lignin. Concentrate selectors select for fresh, juicy foliage, forbs and other dicotyledonous matter with a high proportion of easily digestible plant material relatively rich in energy and protein, especially plant cell contents and little plant cell wall (fibre) constituents. The drawback of these diets is their high levels of plant secondary metabolites, namely tannins. Intermediate feeders can behaviour as browsers or grazers seasonally, according with vegetable species available (Fig. 1.1). Although this classification is widely accepted, Hofmann's evolutionary theory has been criticised by other researchers that point it some weaknesses, such as the lack of evidence for some of Hofmann's assumptions (reviewed in Pérez-Barberia et al., 2005).

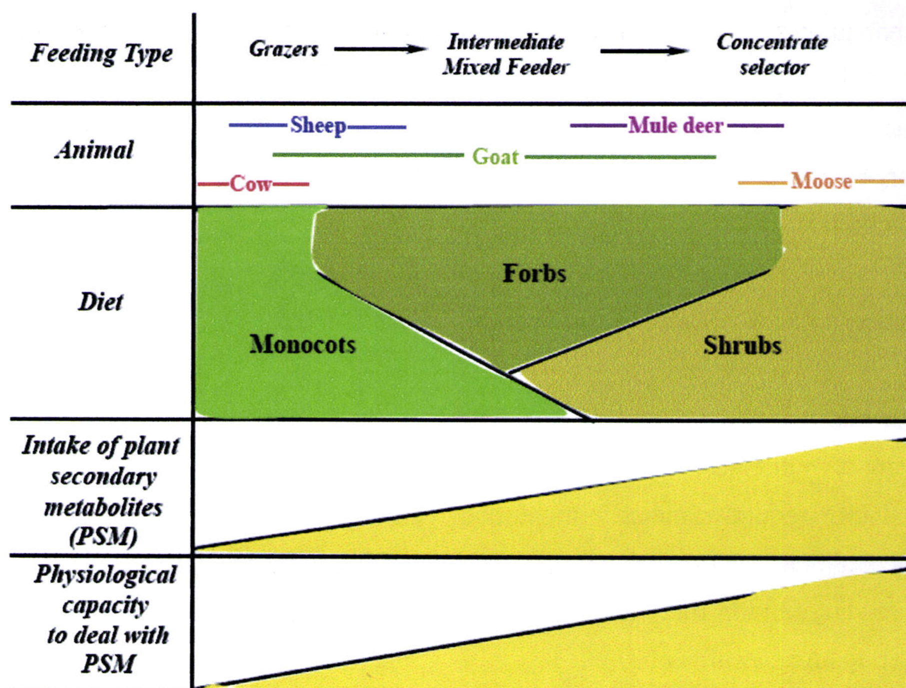


Figure 1.1 – The continuum of mammalian foraging strategies from grazers to concentrate selectors, which have increasing use of plant secondary metabolites (PSM) (Adapted from Iason, 2005)

Sheep (*Ovis aries*) and goats (*Capra hircus*) are two small ruminant species, domesticated about 10 000 years ago, with great economic importance in Mediterranean areas. Although the physiological similarities between them, they present differences in feeding behaviour and in the composition of diets they choose. Most of the contrasts in sheep and goat ingestive behaviour change with the maturity stage of grasses and legumes (Gong et al., 1996). Goats are mainly considered opportunistic (or intermediate) feeders whereas sheep are grazers (Hofmann, 1989; Van Soest, 1994). However, these classifications are not consensual and some authors consider that both sheep and goats can be classified as intermediate feeder (Pfister and Malechek, 1986). Whatever the classification, all are unanimous in consider that goats eat proportionally more browse than sheep. For example, in a mixed Mediterranean environment, browse represents at least 40% of goats diet (Landau et al., 2000). In tropical environments, the percentage of shrubs in goat diet may be higher than 80% (Mellado et al., 2004). Even in the green season, browse continues to represent a considerable amount of goats' selected diet, by opposition to sheep that only consume grasses (Nefzaoui et al., 1995; Trabalza-Marinucci et al., 1992; Nastis, 1997; Kababya et al., 1998; Perevolotsky et al., 1998). Goats usually adapt their ingestive behaviour to the food items available and select diet compounds in order to maintain the proportions between nutrients and secondary plant metabolites relatively constant through the year (Kababya et al., 1998). Although goats prefer vegetal species that present high nutritive values, when available, they can perform well in environments that are inadequate to most ruminants (Silanikove, 2000). Under natural conditions, goats are generally active, selective, walk long distances in search for feed and choose a diet based in both grass and browse. Under confined conditions, however, goats will become heavy browsers of trees and shrubs and less discriminating in

their grazing habits, due to the reduced supply of available herbage. On the other hand, sheep are less selective and utilise pasture more effectively, but in hard environments their productivity decreases greatly (Devendra, 1990). This explains that, in Mediterranean systems, the number of goats, relatively to sheep, increases with a dryness increase or with an increase in the proportion of poor quality vegetation.

1.2.2 Regulation of food intake in ruminants

Compared with non-ruminants, ruminants have some particular characteristics in terms of digestion and metabolism of nutrients. They consume an enormous amount of high fibre and low energy content diets, which require ruminal fermentation. This results in some partiularities in food intake regulation. For example, unlike non-ruminants, blood glucose concentration in ruminants does not increase with feeding, and in that way, mechanisms related to the glucostatic theory cannot account for ruminants (Nagamine et al., 2003).

Several factors were found to affect ruminant food intake. Studies based on each factor isolated resulted in three main "feedback" theories: 1) "physical" theory, which suggested that intake is depressed when rumen is filled (Allen, 1996); 2) "chemostatic" theory, for which the concentration of nutrients and energy (including volatile fatty acids) are involved in control intake (Anil et al., 1993; Illius and Jessop, 1996); and 3) "oxygen efficiency" theory, stated that ruminants eat the amount of forage that gives the optimum yield of net energy per unit of oxygen consumed (Ketelaars and Tolkamp, 1996). Additionally, ruminal and blood osmolality were also observed to influence food intake (Carter and Grovum, 1990). However, these factors seem to act in concert, rather than isolated, and their integration has been reviewed (Forbes, 1996; Fisher, 2002). Convergence of the several stimuli (e.g. distention, temperature, osmolality, volatile fatty acids levels), in ruminants, occurs in the spinal cord and in the brain (Forbes, 1996; Nagamine et al., 2003). Hindbrain provides a potential site for integration of abdominal stimulation and more general whole-body energy status. As it happens for non-ruminant species, also in ruminants the lateral hypothalamic area and the venteromedial hypothalamus are structures involved in the hunger and satiety control, respectively (Baile and Della-Fera, 1981; Nagamine et al., 2003). In the forebrain, visceral information, modulated by such factors such as hindbrain energy status, is integrated with information from the sensory system (e.g. taste, olfaction, vision) and conscious brain. Previous experiences with the food in question are also integrated in forebrain (Provenza, 1995). Together this information results in the behaviour presented by the ruminant (Forbes, 1996; Forbes, 2001).

1.3. Diet selection – Preference vs. aversion

Diet selection was considered within a framework of feeding behaviour that views both diet selection and food intake as an outcome of the animal's internal state and knowledge of the feeding environment (Kyriazakis et al., 1999). Preference is demonstrated by the animal relative consumption of one plant over another when given free choice (Frost and Ruyle, 1993). Animals learn to avoid plants or plant parts through two interrelated systems: affective and cognitive.

The affective system integrates the taste of food with postingestive feedback. This system modulates the intake of food items depending on whether the postingestive feedback is aversive or positive. The strength of aversion to toxic sources is known to depend on the strength of post-ingestive physiological effects (du Toit et al., 1991). Simultaneously, the cognitive system integrates the odour and sight of food with its taste. Animals use these senses to differentiate among foods, and to select or avoid the ones for which post-ingestive feedback is either positive or aversive, respectively (Provenza et al., 1992). Cognitive system can begin to act even before birth. Exposure to different flavours in amniotic fluid and mother's milk may underlie individual differences in food acceptability through the life span (Mennella et al., 2004; 2005a; 2005b) and animals may associate food flavours and gastrointestinal consequences while in utero (Hepper, 1988). Fetal taste experiences may affect adult food preferences in herbivores such as goats, sheep and cattle (Bradley and Mistretta, 1973; Nolte et al., 1991). The experiences learned early in life can explain why animals are reluctant to ingest unfamiliar foods and explore unfamiliar environments. In young animals the mother and young companions are the most important models. Despite these, during their lives animals eat more foods than only the ones they have learned to eat early. This is achieved by trial and error, which is an important mechanism to learn about foods. Besides foods' nutritive value, positive or negative consequences can also depend on the ability of the animal to deal with phytotoxins (Provenza et al., 1992; 2003).

Plants contain variable levels of plant secondary compounds that require detoxification mechanisms within consumers. The herbivore's challenge is to acquire sufficient nutrients while avoiding the consumption of lethal doses of such phytochemicals. Therefore, preferences are likely indicative of underlying physiological adaptations that could promote further behavioural, physiological and ultimately genetic differences between the species. Differences in how animals are built morphologically and how they function physiologically, and marked variations are common even among closely related animals in needs for nutrients and abilities to cope with toxins (Launchbaugh et al., 1999; Provenza et al., 2003). Some factors responsible for the different nutritional needs and tolerance to secondary compounds are sex, age, body condition, physiological state, as well as individual genetics (Burritt and Frost, 2006). Since these differences contribute to differences in post-

ingestive consequences, they will consequently contribute to the variety in preferences among animals.

Despite all the learned behaviour described in the early paragraphs, herditary also plays a role in ingestive behaviour. Animals have an innate perceptions of palatability for either specific plants or for plant attributes such as sweetness, energy density or texture, what goes in accordance with a theory known as hedyphagia, which is based on the idea that animals which prefer the flavour of nutritious foods will succeed and reproduce (Launchbaugh et al., 1999). An inherent preference for nutritious plants and avoidance of toxic plants would contribute significantly to animal fitness (Provenza, 1995). Launchbaugh et al. (1999) studied learned and innate diet selection components. To evaluate if the goats preferences, for some plant species that are avoided by sheep, are innate or due to social and/or mother learning, these authors grafted lambs onto nanny goats so that each nanny raised one kid and one lamb. It was found that even with the same "example", goats presented a different behaviour, consuming higher amounts of plants usually preferred by adult goats.

There is a biological variation in responsiveness to bitter taste (Bufe et al., 2005) what in part may justify the different preferences for different animals of the same specie. With respect to individual variations, factors such as the physiological state lead to variations in taste responses, what means that, for a particular compound, an animal can have a response in a given time and another some days after.

1.4. The effects of tannins in ingestive behaviour

Concentrate selectors (browsers) have a diet with a high degree of herbaceous dicotyledons and tree shrubs, which are rich in tanniniferous phenolic substances. Tannins are a diverse group of polyphenols with a molecular weight higher than 500 Da, that have in common the ability to precipitate proteins and form strong complexes with other macromolecules, such as polyssacharides. Tannins can be divided in two main groups, according to their structure: hydrolysable and condensed. However there are tannin molecules presenting characteristics of both groups. In general, hydrolysable tannins are compounds containing a central core of glucose or other polyhydric alcohol to which molecules of gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) are linked by ester bonds. Condensed tannins, are structurally more complex, being oligomers and polymers of the flavonoids flavan-3-ols, that may differ in the nature and proportions of their constitutive units (e.g., catechin, epicatechin, epicatechin gallate, epigallocatechin), in the degree of polymerization (varying between 2 and more than 100), and in the type of linkages between units. The chemistry of tannins has been extensively reviewed (Haslam, 1998).

Generally, there is an inverse relationship between the concentration of tannins in plant material and voluntary feed intake by ruminants (Kumar and Vaithiyanathan, 1990). The negative effects of tannins on palatability and digestibility are multiple, such as reduction in protein digestibility, either due to the complexation of dietary protein by tannins or inactivation of digestive tract enzymes (Robbins et al., 1987). Reduced palatability is related with the astringency feeling caused by the interaction of tannins with salivary proteins and oral mucosa, and gut irritation and systemic toxicity (Kumar and Singh, 1984; Reed, 1995). However, tannins do not present only negative effects. Despite excessive intake of tannins is associated with a reduction in nitrogen retention in ruminants, the presence of low amounts of these compounds can be beneficial for these animals by having a positive effect on protein digestibility (Nsahlai et al., 1999; Bengaly et al., 2007). This result from the capacity of tannins to form complexes with plant proteins, which remain stable at the conditions found in rumen, being later degraded in the gut. This protects dietary protein from being metabolized by ruminal microorganisms allowing the direct use of supplementary amino acids by the animal.

According with McArthur et al. (1993) browsers tolerate certain levels of tannins if that is the cost of ingesting a more nutritive diet. By selecting a variety of nutrient-rich plants, irrespective of their anti-nutritive contents, these animals can overcome seasonal constraints coming both from plant maturation and lignification. In fact, tannin consumption is not always completely avoided and, in preference trials, it was observed that browser species select in order to voluntary ingest some dietary tannins (Clauss et al., 2003).

Diet selected by sheep and goats differs in the levels of tannins. In general, goats are able to consume larger amounts of tannin-rich browse than sheep under similar conditions (Gilboa et al., 1995, cited by Silanikove et al., 1996a). However, it is necessary to keep in mind that there are several breeds of goats, existing in different climates and accustomed to different levels of dietary fibre and plant secondary compounds. As it was referred before, breeds of goats that are indigenous to semi-arid and arid areas are able to consume more browse and to utilize high-fibre low-quality food efficiently (Silanikove et al., 1993), whereas goats from Mediterranean areas, which are in confinement, choose to eat more tannin-free legume hay than tannin-containing browse, when offered as a sole food (Perevolotsky et al., 1993). The greater ability of goats to consume large amounts of tannin-rich plant material, comparing with sheep, without exhibiting toxic effects can be in part related to their ability to select, avoiding consuming browse in amounts exceeding their capacity to detoxify tannins, and/or probably due an enhanced capacity to detoxify tannins (Silanikove et al., 1996a). The effect of tannins on feed intake is well demonstrated by the dose-response relationship between polyethylene glycol (PEG) supplementation and feed intake in sheep and goats (Silanikove et al., 1996b). PEG complex tannins, reducing their biological activity and allowing a higher intake by animals. Levels of condensed tannins from 3 (Provenza, 1995) to 5% (Cooper and Owen-Smith, 1985) were referred as acting as deterrents for these domestic species. However, the effects of tannins on

feeding behaviour are dependent on the type of tannin and animal specie (Robbins et al., 1991; Hagerman and Robbins, 1993; Clauss et al., 2005).

From the possible mechanisms to detoxify tannins, microbial adaptation to these compounds increased substantially the efficiency of degradation of tannin rich foods in goats' rumen. At the ruminal level *Streptococcus caprinus*, a specific inhabitant of the caprine rumen has the ability to degrade tannin-protein complexes and this was a mechanism suggested to explain the ability of some breed of goats to thrive on tannin-rich forage (Brooker et al., 1994).

Defence mechanisms against tannins can also result from adaptations at oral cavity level. In what concerns salivary glands, large and mainly serous ones (such as parotid and inferior molar glands) can be related to the production of copious saliva for buffering the volatile fatty acids produced in the rumen. Kay (1987) observed an increase in salivary gland weight as percentage of body mass as the animals go from grazers to browsers. Since a positive correlation between salivary gland weight (as percentage of body mass) and volume of saliva secreted seems to exist (Hofmann, 1989), it is predictable that browsers may secrete large volumes of saliva than grazers. The differences in salivary glands mass among the three Hofmann's feeding types were sustained by studies on a great number of bovid species: the average percentage values for the total weight of the salivary glands relative to the respective body mass were 0,36 (for concentrate selectors), 0,26 (for intermediate feeders) and 0,18 (for grazers). The average values for the weight of the parotid gland alone relative to body mass amount to 0,18-0,22 (for concentrate selectors), 0,08-0,15 (for intermediate feeders) and 0,05-0,07 (for grazers) (Frey and Hofmann, 1998). Additionally, parotid saliva protein concentration was proposed to be higher in browsers than in intermediate feeders and grazers and higher in intermediate feeders than in grazers (Göritz et al., 1994; Stolte and Ito, 1996). Besides total protein amount, salivary glands from concentrate selectors (browsers) have been thought to provide specific proteins to bind anti-nutritive polyphenolic compounds present in plants (Austin et al., 1989; Hagerman and Robbins, 1993; Fickel et al., 1998; Clauss et al., 2005). The most studied family of salivary proteins with tannin binding properties has been the proline-rich protein family. The presence of tannin-binding proteins in the several animal species studied has been reviewed by Shimada (2006).

In what concerns the ruminant species studied in this thesis, Seth et al. (1976) and Domingue et al. (1991) referred a higher secretion volume of saliva in goat in comparison to sheep. This higher salivary secretion would allow goats to have a more efficient recycling of urea to the rumen and to prevent a fall in rumen pH even at pick fermentation, which can occur in consequence of browse consumption. The ratio between parotid gland weights to body weight is higher in goats than in sheep (Vaithiyanathan et al., 2001). These higher parotid glands from goats, comparing to grazers, have been suggested as an important factor for a superior digestion capacity in goats (Silanikove, 1997) and it was proposed to be associated to secretion of tannin-binding salivary proteins (Provenza and

Malenchek, 1984; Gilboa, 1995). However, Distel and Provenza (1991) did not detect proline-rich proteins in the saliva of goats fed tannin containing plants, indicating that if specific tannin-binding proteins exist in the saliva of goats, they most likely differ from those found in other mammals, such as mule deer, rats and mice.

From what was stated, until now, it is possible to conclude that the effects of diet on intake behavior can occur in the short term (within seconds) and/or in the long term (days and weeks). The short term effects are related with the sensations produced in the mouth, which will be detailed in next section.

2. Food perception – Taste and astringency

Palatability, which is a key factor in food choice, is dependent on foods' physical and chemical properties, such as texture, odour and taste that together form the flavour. An exhaustive study of receptors and processing of signals in the central nervous system is not the aim of this thesis, however, due to the dependence of feeding behaviour on the sensations and perception of food, we will give a brief overview of the oral receptors and how the chemical signals are conveyed and processed to give the resulting perception.

2.1. Sensory attributes of food

When an animal is close to a potential food object, it can use all of its external senses and by take at least some fraction of the food into the mouth it can add, to the perception process, the sensory system of the oral cavity. In the mammalian mouth several classes of receptors are present: chemical receptors, which include taste receptors; mechanoreceptors, which mediate sensations of touch and proprioception; thermoreceptors, which sense the temperature of the body and objects that come into contact; nociceptors, which signal sensations of pain. All these types of receptors contribute to the total sensation and perception of the food ingested.

Taste perception involves several aspects, such as intensity, quality and hedonic value of the taste sensation. Among commonly recognized types of taste sensed by humans, there are four classically accepted basic taste qualities: sweet, salt, sour and bitter. A fifth stimulus category, called umami, has been proposed by Kikunae Ikeda, in 1909, and accepted recently (Lindemann et al., 2002). Umami is the Japanese term for a savoury sensation and is associated to the taste of monosodium glutamate, being associated to proteinaceous foods.

Some authors also suggested that fat may represent an additional taste quality (Rolls et al., 1999; Mattes, 2001; Laugerette et al., 2007), based on the finding of a population of neurons that respond

when fat is in the mouth. In 2005, a team of French researchers experimenting on rodents claimed to have evidence for a sixth taste, for fatty substances, based on the expression of the fatty acid receptor/transporter CD 36 in taste receptor cells (Laugerette et al., 2005). These receptors bind long-chain fatty acids, and facilitate their transfer into the cell.

The basic tastes can interact to enhance or suppress the final perception (Nakamura et al., 2002), and the distinction between taste and other oral sensations is not always clear. Although specific chemical receptors are not detected, recent studies have shown that capsaicin, which elicits a spicy and burning sensation, can alter neural responses to tastants. These alterations seem to occur at the level of the nucleus of solitary tractus and may be triggered by trigeminal stimulation (Rolls et al., 2003; Kadohisa et al., 2004), or being independent of trigeminal transmission (Simons et al, 2003). Also tannic acid, associated to astringency has a neural representation, at cortex level (Critchley and Rolls, 1996b). Other oral perceptions include metallic (Borocz-Szabo, 1980; Lawless et al., 2005), coldness (de Wijk et al., 2003), viscosity (Guinard and Mazzucchelli, 1996), etc.

2.2. Taste perception

Taste is organized along a neural dimension of nutrients versus toxins, which corresponds to a behavioural dimension of acceptance versus rejection, and to a hedonic dimension of appetitive versus aversive (Scott and Verhagen, 2000). Across the several levels of the gustatory system there is a sequence from recognition, to analysis and to integration of the information, which result in a specific behaviour.

2.2.1. Structures involved in taste reception

Taste perception in mammals is mediated by specialized epithelial derived cells (taste receptor cells), which are arranged in taste buds. In mammals, taste buds are mainly found embedded into the stratified scamous epithelium of epiglottis and in lingual papillae. It is also possible to find taste buds in an area known as *geschmacksstreifen* of the palate (boundary between the hard and soft palate), pharynx and larynx (Miller and Spangler, 1992). Types and densities of lingual papillae can vary wildely between animal species (reviewed in table 2.1), and can fulfill different roles. These differences can be related to differences in taste sensitivity and food preferences (presented in section 2.5). Fungiform, circunvallate and foliate papillae contain taste buds, whereas filiform, conical and lenticular papillae have only a structural role (Fig. 2.1).

Table 2.1. – Number of papilla and taste bud density from different animal species

Species	Lingual papillae			Number of taste buds/papilla		
	Foliate (Fo)	Fungiform (Fu)	Circumvallate (C)	Fo	Fu	C
Herbivores (ruminants)						
Blackbuck (*Antilope cervicapra*)	0[1]	#	30[1]			
Cattle (*Bos Taurus*)	0[2,4]	#	24-30[3] 8-17[5]		4-13[2]	612[3] 445 +/- 279 [2]
Sheep (*Ovis aries*)	0[5]		18-24[5]			
Barbary sheep (*Ammotragus lervia*)	0[6]		30[6]			
Goat (*Capra hircus*)			12-18[5]			
Formosan serow (*Capricornis crispus swinhoei*)	0[7]	202-340[7]	23[7]			
Camel (*Camelus*)			7[8]			
Dromedario (*Camelus dromedarius*)			7[9]			
Alpaca (*Lama pocos*)	0[10]	169-297[10]	5-9[10]			
Japanese serow (*Capricornis crispus*)	0[11]	371.1+/- 43.8[11]	24.0 +/-2.9[11]			
Herbivore (monogastrics)						
Black rhinus (*Diceros bicornis*)	0[12]		60[12]			
Horse (*Equus caballus*)	#[13]	#[13]	2-3[13]			
Donkey (*Equus asinus*)	8-10[14]	100-200[14]	2-3[14]	0[14]	#[14]	#[14]
Omnivores						
Man (*Homo sapiens*)	10-15[15]	171-253[16]	12[15] 7-9[18]		3[16] 0-9[17]	200-250[19]
Squirrel monkey (*Rhesus monkey*)					4-5[20]	550-800[20]
Wild boar (*Sus scrofa*)		637[21]			26.2[21]	
Pig (*Sus scrofa domesticus*)		826-850[22] 818[21]	1-2[23]		4-22[22]	733[22]
Rodents						
Mouse (*Mus musculus*)		80[24]	1[1]	100[24]	125[24]	150-200[24]
Rat (*Rattus norvegicus*)		184[25]			187[25]	573-576[26]
Flying squirrel (*Petaurista leucogenys*)	0[1]		3[1]			
Hamster (*Mesocricetus auratus*)		120-130[27]	1[27]	230[27]	130[27]	168[27]

Carnivores						
Dog (*Canis lupus familiaris*)			4-6[28]		5[28]	
Cat (*Felis catus*)		250[29]	10-12 7-8[29]		4-44[29]	

[1] Emura et al., 1999; [2] Dasgupta *et al.*, 1990; [3] Davies *et al.*,1979; [4] Chamorro *et al.* 1986; [5] Agungpriyono et al. 1995; [6] Emura et al., 2000a; [7] Atoji et al., 1998; [8] Brücher, 1884, cited by Butendieck and Vargas, 1998; [9] Sonntag, 1922, cited by Butendieck and Vargas, 1998; [10] Butendieck and Vargas, 1998; [11] Funato et al., 1985; [12] Emura et al., 2000b; [13] Pfeiffer et al., 2000; [14] Abd-Elnaeim et al.,2002; [15] Kobayashi et al., 1994; [16] Cheng and Robison, 1991; [17] Arvidson, 1979; [18] Jung et al., 2004; [19] Suzuki, 2007; [20] Bradley et al., 1985; [21] Chamorro et al., 1993; [22] Mack et al., 1997; [23] Montavon and Lindstrand, 1991; [24] Zhang et al., 2008; [25] Miller and Preslar, 1975; [26] Hosley and Oakley, 1987; [27] Miller and Smith, 1984; [28] Holland et al., 1989; [29] Robinson and Winkles, 1990.

Lenticular papillae are present only in ruminant species. On the other hand, foliate papillae are absent in the majority of ruminant species, although rudimentary forms of these papillae were observed in cattle (Chamorro et al., 1986) and lesser mouse deer (Agungpriyono et al., 1995). The number of circunvallate papillae also differs among several animal orders: a reduced number is observed in rodents and some omnivores, a slight increase in man and carnivores and a markedly higher number in herbivores.

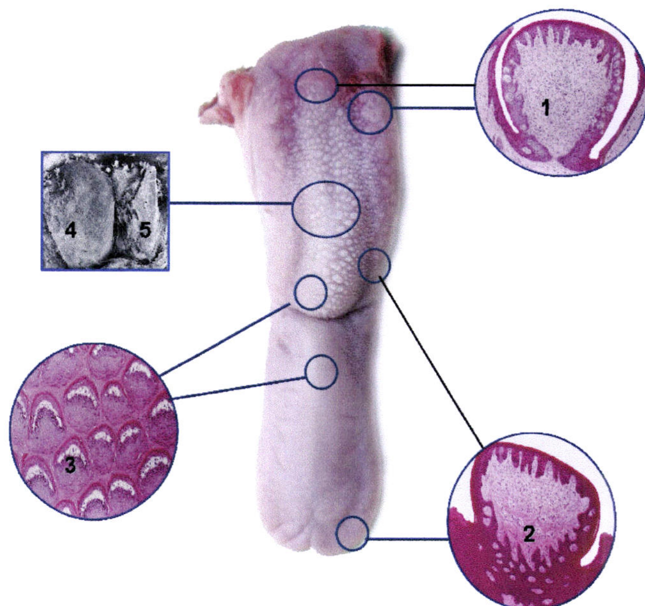


Fig.2.1 –Tongue with the distribution of lingual papillae. The example of adult sheep (original picture): 1–circumvallate papillae; 2–fungiform papillae; 3–filliform papillae; 4–lenticular papillae; 5-conical papillae; microscopic images of the papilla are presented in the lateral boxes (1, 2 and 3 original light microscopy images; 1 and 2 – longitudinal sections; 3 – transversal section; image 4 and 5 scanning electronic images from Tadjalli and Pazhoomand, 2004).

Each taste bud comprises 50-100 highly sensitive taste receptor cells (TRC) and nervous fibres (Mack et al., 1997; Bradbury, 2004). Researchers recognize that there are functionally distinct populations of cells in mammalian taste buds: types I, II, III and IV (Roper, 1989) (Fig. 2.2.).

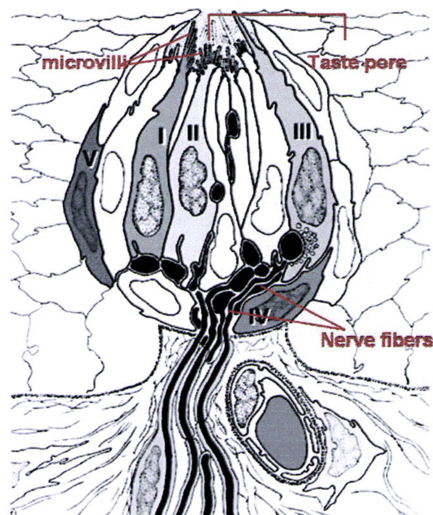


Fig 2.2 - Schematic representation of taste buds, with the different population of cells. Type I – supporting cells; type II – cells responsible for the reception of the chemical stimulant; type III – cells that form synapses with nerve terminals; type IV – basal cells, i.e., progenitor cells that restock the taste bud during its normal course of cell turnover. (adapted from http://www.tu-dresden.de/media/mitarbeiter/witt/tastebud.htm)

2.2.2. Taste reception

Taste recognition takes place at the receptor cells, associated with type II cells. In general terms, these cells receive chemical signals and produce changes in membrane potential and/or intracellular free calcium concentration, which evoke ATP production and/or neurotransmitter release (e.g., serotonin, glutamate and acetylcholine) onto gustatory afferent nerve fibres (Simon et al., 2006). The afferent fibres innervating the receptor cells transmit taste information, such as intensity and quality to the central nervous system (Matsuo, 1999a). In Figure 2.3 the models for detection of the five basic tastes are presented. Detection of salty and sour taste seems to be mediated by ion channels. Cation influx through the channels elicits membrane depolarization, leading to the production of action potentials, which result in neurotransmitter release. On the other hand, detection of sweet, bitter and umami are initiated by the interaction of sapid molecules with G-protein coupled receptors (GPCRs) in the apical membranes of taste receptor cells. Two families of mammalian taste G-protein coupled receptors, T1R and T2Rs, have been found to be implicated in sweet, bitter and umami detection (Baylis and Rolls, 1991; Nelson et al., 2002; Li et al., 2002; Margolskee, 2002). Taste substances activate these receptors, which stimulate intracellular secondary pathways resulting, at the end, in the rise of intracellular Ca^{2+} followed by neurotransmitter release.

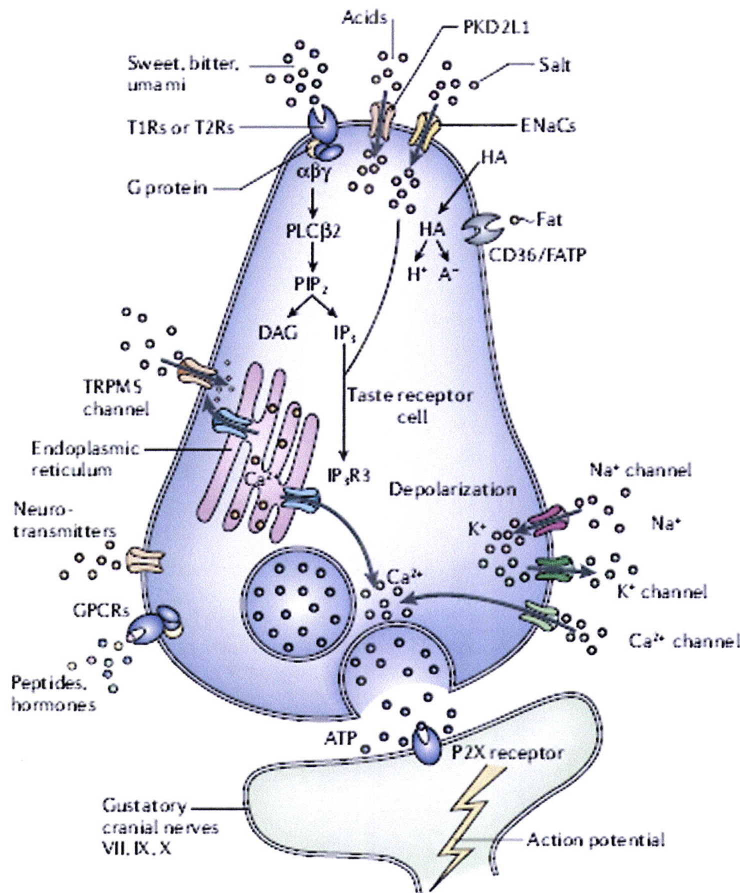


Figure 2.3– Schematic presentation of a generic taste receptor cell (TRC). In the apical membrane the taste receptors are represented. In practice these receptors are not necessarily in the same individual TRC. Besides G-protein coupled receptors for bitter, sweet and umami tastes (represented by T1Rs or T2Rs), ion channels, involved in sour and salty tastes are also on the apical membrane (ENaCs and PKD2L1, respectively). HA represents the undissociated form of the acid. Protons, sodium and calcium may permeate the cell through PKD2L1 channels. In the basolateral membrane are also present G-protein coupled receptors (GPCRs) and ion channels, which have been shown to be responsive to peptides and hormones, and neurotrasmitters, respectively. The intracellular pathways, which ultimately result in the release of calcium and production of ATP, are also represented (Simon et al. 2006).

More detailed information about the mechanisms involved in taste transduction can be found in: Lindemann (2001), Margolskee (2002), Scott (2005), Sugita (2006), Chandrashekar et al. (2006), Simon et al. (2006).

2.2.3. Neural taste pathways

The analysis and integration of taste information that follows reception and transduction involves several neural structures. There are differences among the species in the anatomical organization taste pathways. The anatomical organization of the rat central taste pathways is schematically illustrated in Fig. 2.4.

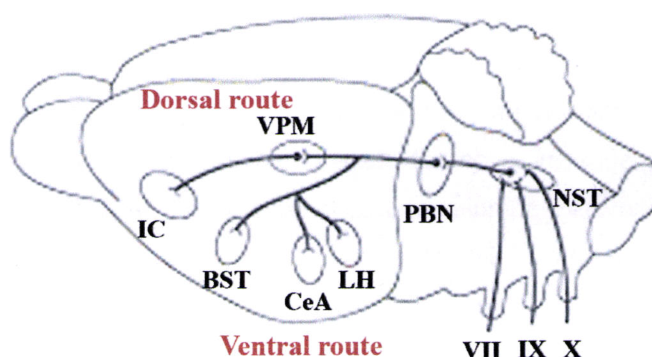


Figure 2.4 – Anatomical overview of the central taste pathways in rodents. Taste information carried by the three cranial nerves **VII**, **IX** and **X** is transmitted to the nucleus of the solitary tract (**NST**) (first order taste relay neurons), which is thought to manage a basic level of taste discrimination, to control somatic reflexes of acceptance or rejection, and to regulate autonomic reflexes that anticipate digestive processes that include salivation, gastric reflexes, and cephalic phase releases of other digestive enzymes and insulin. From NST, information passes to the parabrachial nucleus (**PBN**) (second order taste relay neurons), responsible for the associative process involved in appetitive and aversive conditioning, and the mediation of sodium appetite. The PBN projects both dorsally to the thalamic ventral posteromedial nucleus (**VPM**), and ultimately to insular cortex (**IC**), and ventrally to the limbic system in the forebrain. In the ventral route PBN projects to the lateral hypothalamic area (**LH**), the central nucleus of the amygdala (**CeA**) and the bed nucleus of the stria terminalis (**BST**) (Adapted from Matsuo et al., 1999a).

Taste buds are innervated by afferent fibres belonging to three cranial nerves: the taste buds located in the papilla on the posterior tongue are innervated by glossopharyngeal nerve (IX); the ones located on the anterior and lateral tongue are innervated by chorda tympani branch of the facial nerve (VII); and taste buds located on larynx and pharynx, which are not associated with papillae are innervated by vagal nerve (X) (Matsuo, 1999a; Gilbertson et al., 2000). In the dorsal route, in the thalamic areas, occurs the early phases of the integration of components that ultimately leads to an appreciation of flavour. The insular cortex appears to be the site for a cognitive evaluation of gustatory quality and intensity. The function of the ventral projections may be related to feeding and drinking behaviour and/or hedonic assessment of the chemical (Matsuo, 1999a; Scott and Verhagen, 2000).

In primates the gustatory zone of the nucleus of the solitary tract (NST) appears to project directly to the thalamus, bypassing the synaptic connection with the parabrachial nucleus (PBN) seen in rodents. The PBN seems to be dedicated to convey general visceral information to specialized thalamic nuclei. Several reviews detail anatomical taste pathways in primates (Spector, 2000; Rolls, 2005; Lemon and Katz, 2007). Neural taste pathways are not so well studied in ruminants, so it is not possible to present a comparison with the species described.

2.3. Taste code in the brain

As described previously food can possess a multitude of tastes and sensory properties. Even the same basic taste modality can have great chemical diversity. For example, bitter taste is elicited by structurally diverse compounds, but no clear definition of the molecular properties that confer bitterness has been proposed (Belitz and Wieser, 1985). Some authors demonstrated that the same cell can present several bitter receptors (Adler et al., 2000; Matsunami et al., 2000), suggesting that

individual taste cells respond to several different bitter compounds. However, behavioural and physiological studies indicate that different bitter stimuli can be discriminated, suggesting that the same individual taste cells can discriminate among bitter stimuli (Caicedo and Roper, 2001), or, at least, that different bitter substances do not share homogeneity in taste processing (Brasser et al., 2005). Besides diversity in taste stimulus and taste cells, individual taste buds of all areas contain cells responding to several taste qualities (Lindemann, 2001; Scott, 2004; 2005; Chandrashekar et al., 2006; Sugita, 2006). These findings are incompatible with taste specialized regions in the tongue and lead to the questions such as: "how is the neural processing of taste information organised so that discrimination and the consequent behavioural response could be elicited?"

This complex issue is accessed by two models of spatial coding: "labelled line" theory, according which taste is carried as a line by specialist channels of cells and neurons, and "across-fibre" pattern theory, which states that taste is carried as a pattern of activity across a neural population (reviewed by Erickson, 2008). Labelled-line model suggests that each individual taste cell exclusively recognizes a specific taste quality and a taste cell with particular specificity will be attached to a specific sensitive nervous fibre with the correspondent specific coding of that quality in the brain. The "across-neuron" model assumes that every taste stimulus will elicit a response in every taste fibre and the pattern of activity over many receptors codes taste. Recent data support a comprehensive labelled-line mode of taste coding for four of the five basic taste modalities (Huang et al, 2006): bitter, sweet, umami and sour. There is also emerging evidence that not only space but also timing of neural events contributes to the representation of taste (for a recent review see Lemon and Katz, 2007).

2.4. Astringency

Astringency was defined as the complex sensations, produced in oral cavity, due to shrinking, drawing, or puckering of the epithelium as a result of exposure to substances such as alums or tannins [American Society for the testing of materials (ASTM), 1989]. Definition of astringency was not always consensual, with some authors defending this as a taste and others as a tactile sensation. Schiffman et al. (1992) and Yamashita et al. (1996) considered that astringency drives from the interaction of the astringent compounds with chemoreceptors. According to them, there are electrophysiological evidences that particular gustatory nerves, namely the chorda tympani (VII) and glossopharyngeal (IX) nerves, transmit the information of astringency and in that way this must be considered another taste quality. However, most evidences favours astringency classification as a tactile sensation, in which normal lubrication of oral surfaces is affected (Prinz and Lucas, 2000; Siebert and Chassy, 2004), at least in part, by salivary protein precipitation and the removal of mucins from oral surfaces (Lyman and Green, 1990; Green, 1993). Thorngate and Noble (1995) suggested that mechanoreceptors could be involved in the perception of astringency. There are several aspects supporting astringency as a tactile sensation, such as the fact that astringency do not lead to an

adaptation, by opposite to basic tastes, but rather increases after repeated ingestion, being the rate of increase higher with reduced times between two ingestion episodes (Guinard et al., 1997; Kallithraka et al., 2001). Astringent substances can also be differentiated from taste substances due to the linearity of the relationships between perceived intensity and concentration of astringent substances, by opposite to taste substances (Breslin et al., 1993). Perceived astringency changes by interaction with different chemical compounds: various sorts of lubricants such as gums, polysaccharides and proteins (Colonna et al., 2004) and also sweet taste were referred to decrease astringency (Lyman and Green, 1990).

Polyphenols, namely tannins are compounds frequently associated to bitter taste and astringent sensations. The involvement of these compounds in astringency has been greatly studied due to the importance they represent in human and animals foods and beverages. The bitter and astringent characteristics are more or less intense according to the chemical properties of the polyphenol. In general terms, as the degree of polymerisation increases astringency perception increases and bitterness intensity decreases (Robichaud and Noble, 1990; Peleg et al., 1999). The type of linkage in the phenolic molecule also influences the two types of sensations (Peleg et al., 1999).

A great number of studies on interaction between proteins and astringent compounds have been performed with tannins, some of them with purified salivary proteins (Horne et al., 2002), with gelatine (Hagerman and Butler, 1981), bovine serum albumin (de Freitas and Mateus, 2001), mucins from bovine submandibular glands (Monteleone et al., 2004) among others. Although tannins are generally considered to be nonspecific protein binding agents, Hagerman and Butler (1981) showed that tannins may efficiently precipitate one protein in the presence of a large excess of another protein. The specificity of interaction is a function of the size, conformation and charge of the protein molecule (Hagerman and Butler, 1981), being also strongly influenced by the structural type and degree of polymerization of the tannin (Hagerman et al., 1998).

Several salivary proteins have been reported to interact with tannins: salivary α-amylase is inhibited (McDougall et al., 2005; Kandra et al., 2004) and precipitated by these compounds (de Freitas and Mateus, 2001), salivary histatins were shown to be able to precipitate condensed tannins (Yan and Bennick, 1995; Naurato et al., 1999; Wróblewski et al., 2001) and salivary proline rich proteins (PRPs) were shown to have a high binding affinity for tannins in general (Bacon and Rhodes, 2000). Salivary mucins were also proposed to cross-link and precipitate out of saliva by tannins (Green, 1993).

Salivary PRPs have been the most studied salivary proteins in terms of interaction with tannins. These are proteins with a high affinity for tannins, forming stable complexes with them. This stronger affinity, when compared with the majority of salivary proteins (Hagerman and Butler, 1981; Baxter et al., 1997) is greatly due to the particular characteristics of these proteins, namely, their high content

of proline, their hydrophobicity and their conformational open and flexible structures. Siebert and Chassy (2004) referred that the dimensions of tannin-PRPs complexes are proportional to the amount of proline residues in the protein, and in that way, larger PRPs can bind tannins strongly than smaller molecules.

The interaction between tannins and proteins involve two steps: a) complexation; b) aggregation and precipitation (see Luck et al., 1994; Baxter et al., 1997; Charlton et al., 2002 for a detailed explanation). In PRPs, the pyrrolidine ring of the prolyl residues provide a multiplicity of hydrophobic binding sites in PRPs and exert a strong and selective influence on the recognition processes, which occur with polyphenol subtract. Hydrophobic interactions are the first involved in the process of complexation, with hydrogen bonding acting as a second effect. During complexation, the aggregates formed are soluble and consist of single peptide molecules with polyphenols bound. With increasing amounts of polyphenol, there is a point at which two peptides are cross linked, forming a polyphenol-coated dimmer, which starts to precipitate. As it precipitates more molecules can be added, aggregating into larger insoluble complexes.

The process of polyphenol complexation may be reversible or irreversible (Haslam, 1998). Reversible interaction occurs in the absence of the influence of other external agents, such as oxygen, metal ions, acid or basic conditions. Other factors, such as pH, temperature and the presence of salts also influence the precipitation of proteins. The formation of precipitates occurs through non covalent forces, however, the referred factors can led to the formation of covalent bonds making the precipitation process irreversible.

Astringency is a complex phenomenon and other factors (besides the ones early described) can contribute to the final sensation. For example, the fraction of astringent substances remaining in solution (after a great part have been precipitated by salivary proteins) can interact with taste receptors, contributing to changes of taste perception, or even form soluble complexes that modulate saliva viscosity (Kallithraka et al., 2001).

2.5. Relation between food perception in mouth and behavioural response

Taste beyond its purely sensory function is also inextricably linked to a larger set of behaviours, namely ingestive behaviour. Taste oriented consummatory responses are contrastive (attractive vs. aversive) and the question "how is taste input perception integrated with other sensory proprieties, emotions and memories to be linked to behavioural output?" remains to be completely answered.

Basic taste qualities can signal basic food: attributes that provide guidance among the nutritious vs. toxic balance when making intake decisions salty and sour detection is needed to control salt and acid

balance; bitter detection warns of plant secondary metabolites, produced by plants as defensive mechanisms against predation; sweet and umami perception are coupled with carbohydrate or protein-rich foods.

Salty and sour tastes are generally attractive and aversive modalities, respectively. However, those tastes also evoke the converse responses, depending on their concentrations. Therefore, it is interesting to address how such a switching of responses occurs, and whether it depends upon changes in the precise firing patterns of action potentials. In terms of bitter taste, and without contradict what was referred above, that bitter taste elicits aversive behaviour, it is important to note that the sensitivity for this taste does not accurately mirror the body's systemic reactivity to compounds eliciting it (Glendinning, 1994).

Additionally, the complexity is increased by the learned behaviour of attraction or rejection, obtained by experience. Until the moment is not clearly understood whether or not the taste learning is associated with construction of new neuronal circuitries, and which neurones or which molecules play a role in it (Sugita, 2006). At some stage in taste processing, taste representations are brought together with inputs from different sensitive modalities. On the one hand, activation of sapid stimuli is concurrent with the activation of oral somatosensory system. Taste buds are intercalated and surrounded by general sensory nerve endings from the three cranial nerves referred before (VII, IX and X). The somatosensory receptors transduce information about the thermal, chemical and physical properties of foods. The somatosensory inputs also target the NST and, in that way, the referred properties may also affect the response of taste receptor cells for basic tastants. Additionally, olfactory and effects of internal state (e.g. attention and expectation) add further complexity of neural taste responses (Simon et al., 2006; Jones et al., 2006). In the central nervous system, representations of taste, smell, sight, and mouth feel of food converge and this convergence allows the sensory properties of each food to be represented and defined in detail (Rolls, 2005).

2.6. Differences among species in food perception and behaviour

We will focus particularly on bitter and astringency, since these are the sensations that limit food choices, and particularly the feeding behaviour of the species studied in this thesis.

The bitter rejection response probably did evolve as a general mechanism for avoiding dietary poisons. However, this should be looked carefully, since bitterness per se does not predict accurately the potential toxicity of foods. In fact, thresholds for bitter taste in foods can be greater than, equal to, or less than those for toxicity. Glendinning (1994) hypothesized that mammals in different trophic groups have evolved different strategies for coping with the unpredictable bitterness/toxicity relationship. Carnivores, which rarely encounter bitter and potentially toxic foods, have a high bitter

threshold and tolerance to ingested poisons. On the other hand, omnivores, which encounter bitter and potentially toxic foods somewhat less frequently than herbivores, have an intermediate bitter threshold and tolerance to ingested poisons. A key prediction of this model is that the adaptiveness of the bitter rejection response is dependent on the relative occurrence of bitter and potentially toxic compounds in a specie diet. For carnivores, the lower thresholds of bitter detection would reduce drastically their chances of ingesting a toxic food. At the other extreme, it would be no advantageous that herbivores presented such higher bitter taste sensitivity at the expense of to limit drastically the range of potential foods.

Some mechanisms were proposed to be responsible for a reduction in bitter taste sensitivity. One of them is the production of salivary proteins that bind distasteful compounds, which could thereby lower their free concentration in oral cavity (Glendinning, 1992). Other mechanism can be the habituation for bitter taste, due to the constant presence of particular bitter compounds (Glendinning et al., 2002). Both mechanisms are directly related with taste detection. Additionally there are other physiological mechanisms, such as the higher capacity of detoxification, presented by some animals, that although do not act at oral cavity level, also can lower bitter taste sensitivity, probably due to a learned behaviour: the animal does not relate the bitter taste with any adverse post-ingestive effect (Provenza et al., 2003).

In what concerns the ruminant species studied by us, there are a limited number of studies about the sensitivity for the basic tastes. Results from experiments on gustation in goats and sheep have demonstrated that there are differences between the species with regard to gustatory chemoreception (Bell and Kitchell, 1966). Robertson et al (2006) found no difference between species in the pattern of response for the different flavours, althought between species there are differences in the level of response. Goatcher and Church (1970) found that when a bitter solution, such as quinine, is presented to normal goats and sheep, goats can detect the bitter taste at lower concentrations than sheep. However, this is not immediately translated in rejection behaviour and at low concentrations it seems even that they show a preference for bitter taste. With increased quinine concentrations the preference declines and for high concentrations these animals start to show rejection. Despite the higher taste thresholds for bitter taste, sheep show a strong rejection to bitter compounds than goats (Goatcher and Church, 1970). In the scenario presented by Glendinning (1994) this could be due to the fact that goats are intermediate feeders and, as such, they must have the capacity to adapt to highly diverse diet composition and the capacity of a high selectivity for the diet. They may need to have a high bitter taste perception in order of to choose the more nutritive meal from a mixture of plants, when in favourable conditions. On the other hand, in hard conditions, were browse is abundant, they should not reject every bitter compound at the risk of being underfed.

Taste sensitivity, and particularly bitter taste sensitivity also has a genetic background. Individual differences for bitter taste sensitivity have been greatly studied in humans. A gene was found that

contributes considerably to the perception of some bitter compounds (Mennella et al., 2005). Polymorphisms in mice bitter receptor genes were found, which play a major role in the differences in bitter sensitivities presented by the different strains of laboratory mice (Nelson et al., 2005).

3. Saliva

In mammals, saliva can play several functions, which includes assisting in lubrication, deglutition, digestion, anti-microbial defence, antibody secretion, protection against mechanical and chemical injuries, hydration of oral cavity, oropharynge and oesophagus. In ruminants saliva has particular important roles, acting as a pathway for recycling nitrogen to the rumen and also due to the high amounts of a watery buffered fluid necessary for maintenance of ruminal activity. In humans, the importance of saliva is clearly observed for individuals with hyposalivation, which suffer from oral pain, increase in dental caries and infections by opportunistic microorganisms (Aps and Martens, 2005; Mese ad Matsuo, 2007). Besides all the mentioned functions, saliva has an essential role in ingestive behaviour, since a number of saliva constituents affect the perception of taste, flavour and texture of foods. The study of this last role is the main objective of this thesis.

For further understanding of the functions of saliva and its role in ingestive behavior, the next sections focuses on the mechanisms of salivary secretion, on its effects in taste and how different foods can change saliva secretion and composition. Attention will be paid to the particularities presented by ruminants in terms of saliva secretion (section 3.5.) and protein composition (section 4).

3.1 Salivary glands morphology

Whole mouth saliva is made up of the contributions from major and minor salivary glands and from gingival crevicular sulcus (area located between teeth and marginal free gingival). It also contains bacteria and their metabolites, epithelial cells, erythrocytes, leukocytes and food debris.

The salivary glands were described by Galen, in the second century A.D. but little attention has been paid to their function until the 17th century. The development of the knowledge about salivary secretion has a long and interesting history (see Garrett, 1998a for a historical framing).

Nearly all mammals have three paired sets of major salivary glands (Fig. 3.1): the parotid, submandibular and sublingual glands and a host of minor salivary glands that underlie all the oral mucosa with the exception of the gingival and the dorsum of the body of the tongue. This division in major and minor salivary glands is based on their morphology and the volume of saliva produced. Parotid glands (1 in Fig. 3.1) are constituted only by serous acini, and their saliva is a thin watery fluid. Submandibular and sublingual glands (2 and 3 respectively in Fig 3.1) contain both serous and

mucous acini, being classified as mixed glands, which secrete a more viscous mucus containing saliva. The proportion of mucous cells in sublingual glands is higher than in submandibular glands and, in some species, these can be considered almost mucous glands. Additionally, there are some species' particularities, namely the inferior molar glands (serous glands), present in ruminants (Kay, 1960) (Fig. 3.1 c) and the zygomatic gland, present in carnivores and few wild ruminant species (Frey and Hofmann, 1998; Frey et al., 2001).

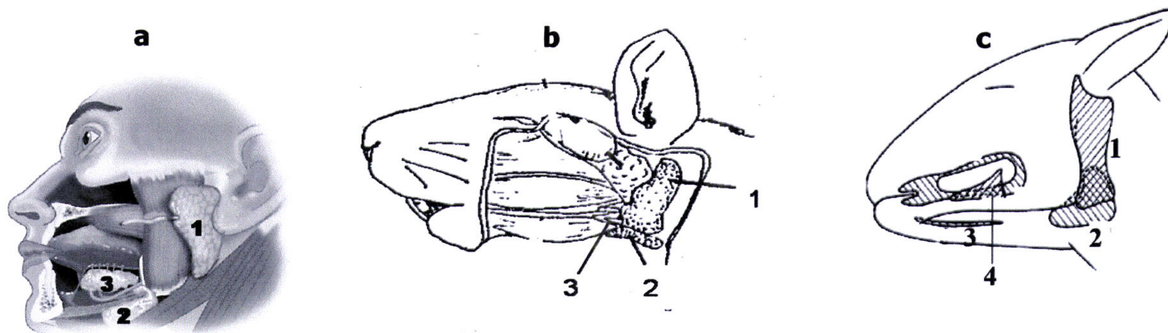


Fig. 3.1 – Anatomical localization of the major salivary glands. **a**–humans (Aps and Martens, 2005); **b**–mice (http://kentsimmons.uwinnipeg.ca/16cm05/16labman05/lb8pg3.htm); **c**–sheep (Kay, 1960); 1-parotid, 2-submandibular, 3-sublingual; 4-inferior molar.

As exocrine glands, major salivary glands present a duct system, which besides transport saliva from glands to the mouth, also participates in defining its composition (Fig. 3.2). Minor glands (for example, labial, buccal, von Ebner salivary glands, palatine, among many other), high in number, do not have a collective orifice (Aps and Martens, 2005).

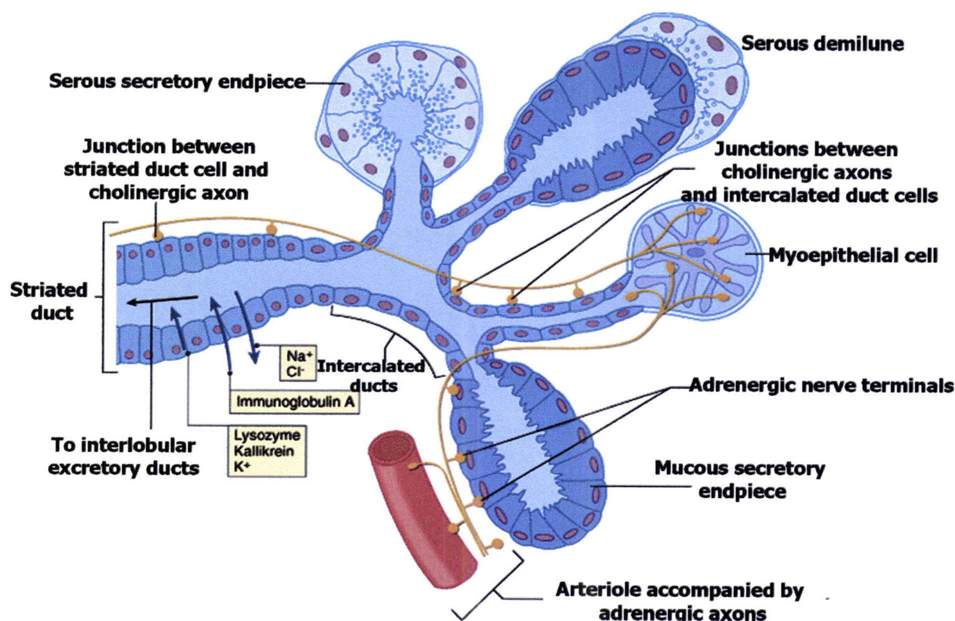


Figure 3.2 – Representative scheme of a basic unity of salivary glands (Telser et al. Elsevier's Integrated Histology – www.studentconsult.com)

The fluid produced in the acini is secreted into intercalated ducts (ID), followed by striated ducts (SD). The saliva passes from SD to excretory ducts (ED) and from these to the main excretory duct (ME) of each major salivary gland. The transition between SD and ED is not necessarily abrupt and sometimes there are ducts of intermediate morphology. Both SD and ED are readily identified in paraffin sections, either by their basal striation or their extra lobular position associated to a thick mantle of fibrous connective tissue, respectively (Fig. 3.3).

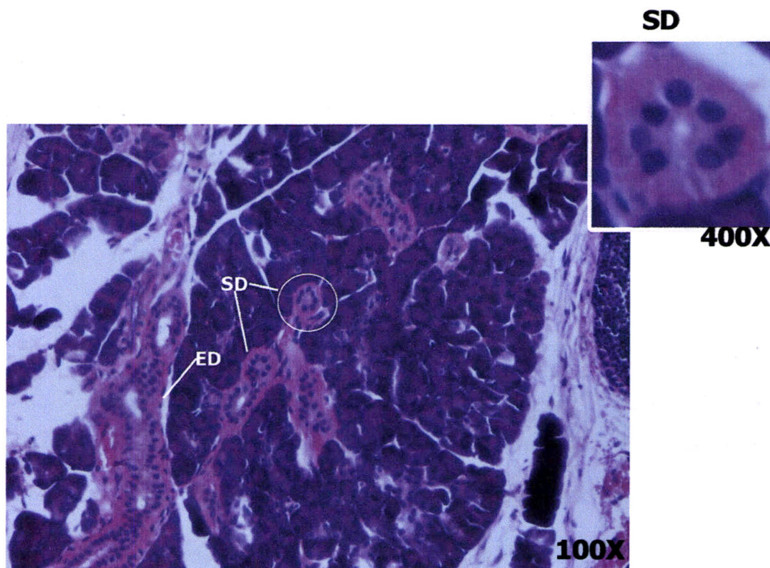


Fig. 3.3 – Histological image of mice parotid gland. H&E (Original picture). **SD** – striated ducts; **ED** – excretory ducts.

Although this is the general structure presented by the secretory units, some species particularities can occur. In submandibular glands of many families of rodents an extra type of ducts – the granular convoluted tubules (CT) – is observed (Figure 3.4) following the ID. These ducts show a typical secretory structure, with numerous secretory granules, having the particularity of contributing considerably to the final protein content of saliva. They exhibit a clear intersexes morphological diversity, being bigger in males than in females (Kurabuchi et al., 1999; Garcia et al., 2002).

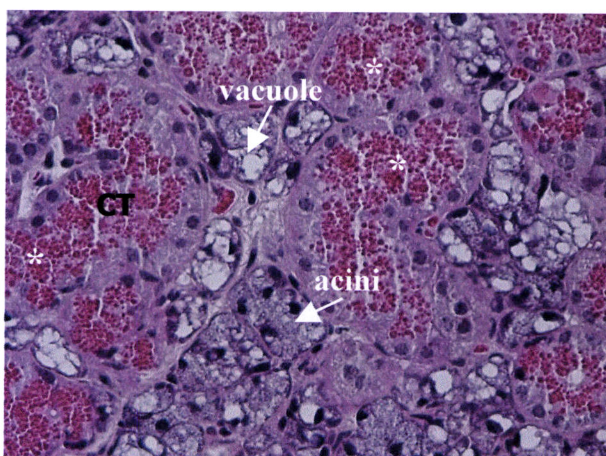


Figure 3.4 – Histological image of male mice submandibular gland, showing granular convoluted tubules (CT). H&E, 200X (Original picture). It is possible to observe the considerable glandular dimension of these structures and the high number of secretory granules inside the cells (*).

Other particularities include the absence of both intercalated and striated ducts in animals such as cats and ferret, with the saliva formed in acini being directly secreted into excretory ducts (Jacob and Poddar, 1989; Tandler and Poulsen, 1977).

Apart from the general characteristics of salivary glands, described above, these are highly diversified structures exhibiting a complex degree of heterogeneity, both in location, development, microscopic structure and function (Young and van Lennep, 1978; Phillips and Tandler, 1996; Phillips et al., 1998). Patterns or combinations of cell types correlate with phylogeny and tend to be consistent within mammalian orders (Tandler and Philips, 1998). However, the distribution in the broad dietary categories carnivore, herbivore and omnivore are not enough to explain the structural diversity. The great diversity in chemical composition presented by the different diets inside of each group can be an explanation. For instance, in herbivores, which are the main focus of the present study, diet can include plant material that varies greatly in terms of phytotoxins, digestibility, caloric content, facility in to access food items, which are all factors that affect digestive physiology. Looking closely at dietary habits, a general conclusion that emerges from Tandler and co-workers comparative studies (Tandler et al., 1986; 1997; 1998; 2001) is that in species of mammals that have specialized diets, the major salivary glands exhibit differences when compared with relatives that are dietary generalists. In ruminants, more specifically, salivary glands size, and particularly that of the parotid, has been related to dietary niche (Hofmann, 1989). In that way, salivary gland weight (and with more emphasis parotid gland weight) relative to body weight are thought to increase with the digestibility of the diet that is naturally consumed.

3.2 Saliva secretion

3.2.1. Nervous control

Salivary secretion is a reflex exclusively mediated by the autonomic nervous system. No hormone usually initiates salivary secretion (Mese and Matsuo, 2007), despite a certain hormonal regulation have been proposed, at least in maintaining protein synthesis (Johnson et al., 1987; Asztély et al., 1996). Besides this distinctive characteristic, and inversely to what is observed for the majority of body systems, the effects of parasympathetic and sympathetic innervations are not antagonic but rather exert relatively independent effects in which the activity of one branch may synergistically augment the effect of the other (Emmelin, 1987; Huang et al., 2001). Due to the phenomenon of the mouth drying in stress situations, during a great number of years it was thought that sympathetic stimulation inhibited salivary secretion. Garrett et al. (1987) presents a comprehensible explanation to demystify this. During a stress episode any inhibition of saliva secretion, is mediated by central inhibitory influences from higher centres, rather than a direct inhibition at glandular level. Moreover, the vasoconstriction associated to the sympathetic innervation is separated from the action of the

sympathetic system on glandular tissue. This is because sympathetic secretory nerves are separated from sympathetic nerves of the blood vessels and come under different central control mechanisms. In other words, the secretory sympathetic nerves are part of the reflex pathway, whereas the vascular sympathetic nerves are part of a more generalised vascular control system, under the influence of the vasomotor centres (Emmelin, 1987; Garrett, 1987).

The effect of autonomic innervation was mainly studied on animal models through direct nerve stimulation or inhibition and through the use of sympathetic and parasympathetic agonists and antagonists. The effects of isoproterenol, as a sympathetic agonist, which parallel the effects of tannins on salivary glands and salivary proteins, will be detailed further. A minority of salivary glands are additionally capable of secreting saliva in the absence of impulses from nerves, a phenomenon referred to as spontaneous secretion (Proctor and Carpenter, 1998). In humans, it was reported a spontaneous secretion by palatine mucosa (minor glands) (Mese and Matsuo, 2007).

The frequently called unstimulated secretion, which occurs in the absence of apparent sensory stimuli related to eating, have two components: the spontaneous secretion by some minor glands, referred above, and a resting secretion, that results from a small amount of nervous control evoked by dryness of the oral mucosa and low-grade mechanical stimulation caused by movements of the jaw and the tongue (Mese and Matsuo, 2007).

A broad perspective on salivary reflex is provided by Matsuo (1999b), Pedersen et al. (2002) and Proctor and Carpenter (2007) and a schematic diagram of the nervous control of saliva secretion is presented in Fig. 3.5. The two branches of the autonomic nervous system are located in different regions. The parasympathetic primary centre is located in the medulla oblongata and comprises two different nucleuses: the rostral part of the parasympathetic centre constitutes the superior salivatory nucleus and connects with the sublingual and submandibular glands; the caudal part constitutes the inferior salivatory nucleus that is connected with the parotid gland. The sympathetic primary salivary centres are situated in the upper thoracic segments of the spinal cord, although it remains unclear the precise location (Matsuo, 1999b; Proctor and Carpenter, 2007). Autonomic parasympathetic efferent fibres to parotid glands are present in the glossopharyngeal nerve (cranial nerve IX), whereas efferent fibres to submandibular and sublingual glands are present in the chorda lingual nerve (cranial nerve VII). Sympathetic efferent nerves conduct signals to salivary glands via the superior cervical ganglia.

The multimodal convergence that enables single neurons to respond to different combinations of gustative, olfactive, texture, temperature and visual inputs is probably also present in the control of salivary secretion. There are evidences of central mechanisms modulating salivary secretion. The primary salivary centres receive inputs from neural structures in the lower brainstem and forebrain. The structures in the lower brainstem are related to oral sensory inputs (taste and oral sensations), whereas the forebrain structures are related to the regulation of feeding, drinking and body

temperature (Matsuo 1999b). The nervous control of saliva secretion provides a rapid and highly adaptative response to food present in the oral cavity, influencing saliva volume and composition (further detailed in points 3.3 and 3.4). For an overview of the effects of autonomic nervous system on salivary secretion see Table 3.1.

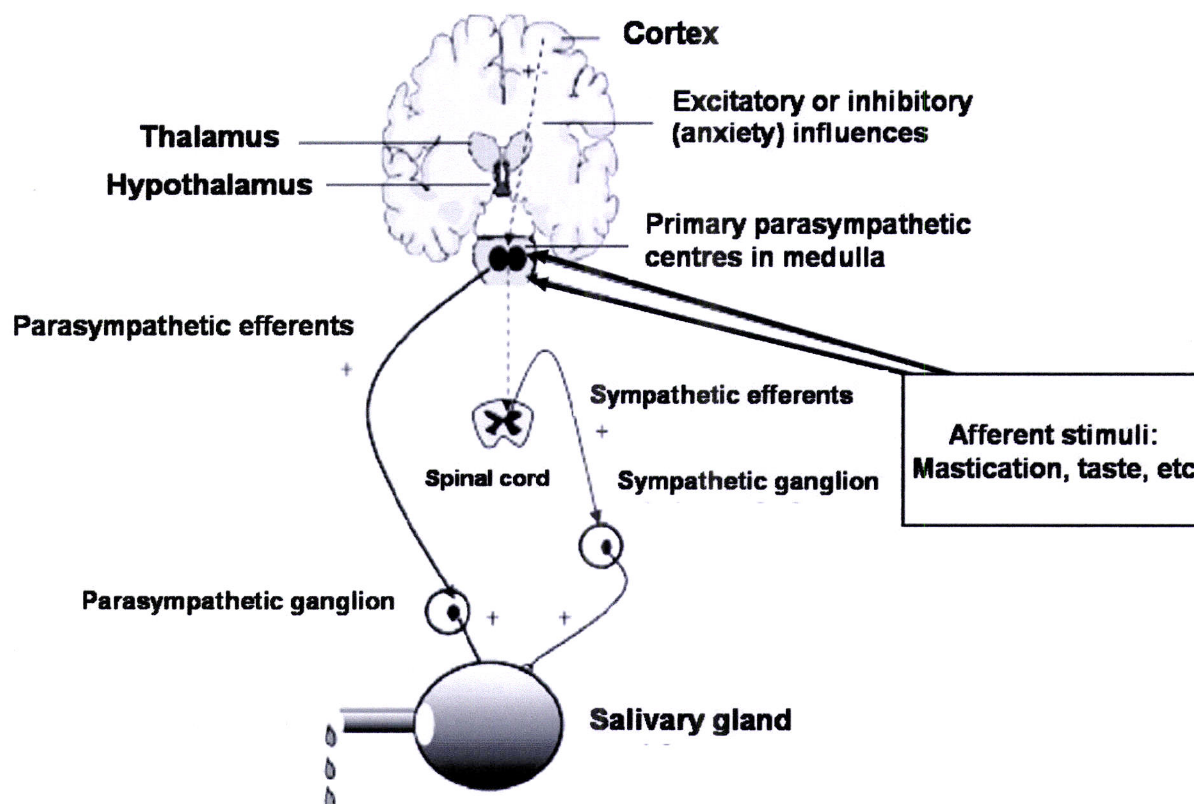


Fig. 3.5 – Schematic diagram of the nervous control of salivary secretion. Broken lines represent possible neural connections without histological confirmation. Lower broken line - the nerves projecting from the medulla to the sympathetic centre in the upper thoracic segments of spinal cord and from here sympathetic efferent nerves conduct signals to salivary glands; Upper broken line - nerves projecting from the cortex to the parasympathetic centres in the medulla (Proctor and Carpenter, 2007)

Sympathetical stimulation can both occur through alpha- and beta-adrenoceptors located on salivary gland cell membranes. An alpha-adrenergic stimulation of the salivary glands causes a calcium influx in the secretory cell, whereas beta-adrenergic stimulation generates cAMP. Parasympathetic stimulation occurs through cholinergic receptors, with a consequent increase in the intracellular calcium levels. Discharge of macromolecules and of electrolytes and fluid is controlled differentially by the two divisions of the autonomic nervous system. There is a tendency to dichotomize the respective roles of the nerves, attributing salivary protein secretion, almost entirely, to sympathetic nerve impulses, and fluid secretion to parasympathetic nerve impulses. However, this is an oversimplification, since sympathetic nerve impulses on α-adrenoceptors also increase fluid secretion and parasympathetically mediated impulses can give rise to a substantial protein secretion (Emmelin, 1987; Ishikawa et al., 2006; Proctor and Carpenter, 2007).

As well as the main neurotransmitters acetylcholine and adrenaline, there are other non-adrenergic, non-cholinergic (NANC) transmitters within nerves in salivary glands. Neuropeptide Y (NPY), neurokinin A (NKA), substance P (SP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), neuronal nitric oxide synthase (nNOS) and calcitonin gene-related peptide (CGRP) have all been detected within either parasympathetic, sympathetic or sometimes both nerves (for a more detailed explanation, see Ekström, 1998).

In salivary glands, not only the acinar cells receive innervation (see Fig. 3.2). Also myoepithelial cells respond to both sympathetic and parasympathetic impulses (Garrett and Emmelin, 1979; Garrett, 1987), in this case, with the particularity of the sympathetic stimulation being achieved exclusively by α-adrenergic receptors (Garrett, 1987). Ductal cells also receive innervation. Parasympathetic nerve endings were identified around ducts and blood vessels (Snell and Garrett, 1957). Also evidence of salivary gland duct sympathetic stimulation exists (Anderson et al., 1995).

A synthesis about the action of the two branches of the autonomic nervous system on saliva secretion is presented in table 3.1.

Table 3.1 –Effects of autonomic nervous system on saliva production. (Adapted from Garrett, 1987).

Parasympathetic impulses	Sympathetic impulses
1. tend to occur with more prevalence than sympathetic impulses;	1. tend to occur more intermittently than parasympathetic impulses;
2. may occur in isolation;	2. act essentially on cells receiving parasympathetic impulses, which tends to produce synergetic effects;
3. cause variable degrees of exocytosis from some cells;	3. tend to modulate the composition of saliva by increasing exocytosis from certain cells;
4. induce contraction of myoepithelial cells;	4. usually induces contraction of myoepithelial cells;
5. cause vasodilatation as part of the secretory process;	5. may occur in a separate population of nerves;
6. may have a direct influence on re-synthesis.	6. often do not cause much mobilization of fluid.

3.2.2. Secretion of water and electrolytes

In 1954 Thayssen et al. made a significant contribution to the elucidation of the mechanisms of salivary secretion, proposing the now classic "two-steps hypothesis" concerning the secretion of water and electrolytes (Poulsen, 1998). In a first step, which occurs in the acini, the formation of isotonic plasma-like primary saliva, with a composition independent of the rate of secretion, takes place. In the second step, changes in electrolyte concentrations occur as saliva passes trough the duct system.

The salivary electrolytes all originate from serum, and are actively transported into the acini and striated ducts. Not only one mechanism is actually accepted for acinar saliva secretion. In Figure 3.6 a schematic representation of three mechanisms for first phase of saliva secretion is presented. The fundamental points of divergence among the three mechanisms are in terms of the entry of Cl^- and HCO_3^- into the cell and the anion excreted to the lumen. Considerable experimental evidence indicates that the first mechanism (Fig. 3.6a) can account for most of the secretion from rat, rabbit and presumably human, major salivary glands. However, there is also evidence that the two other mechanisms can also make significant contributions to salivary fluid secretion. For a more detailed explanation of each mechanism, see: Poulsen (1998), Turner and Sujiya (2002) and Melvin et al. (2005).

Several recent studies have detected Na^+/HCO_3 co-transporter activity in acinar cells, suggesting a potential role for these co-transporters in salivary secretion and/or intracellular pH regulation in at least non-ruminant salivary glands (Melvin et al., 2005).

All of the mechanisms are based on an osmotic coupled principle, according with which water follows salt secretion to the lumen. In 1992 a 28-kDa integral protein, the aquaporin, was discovered as a water channel (Agre et al., 1993). Several studies (summarized in Ishikawa et al., 2006) indicated that aquaporins have a significant role in water secretion from salivary glands acinar and ductal cells and about five aquaporins have been identified from mammalian salivary gland cells. Aquaporins selectively conduct water molecules in and out, while preventing the passage of ions and other solutes.

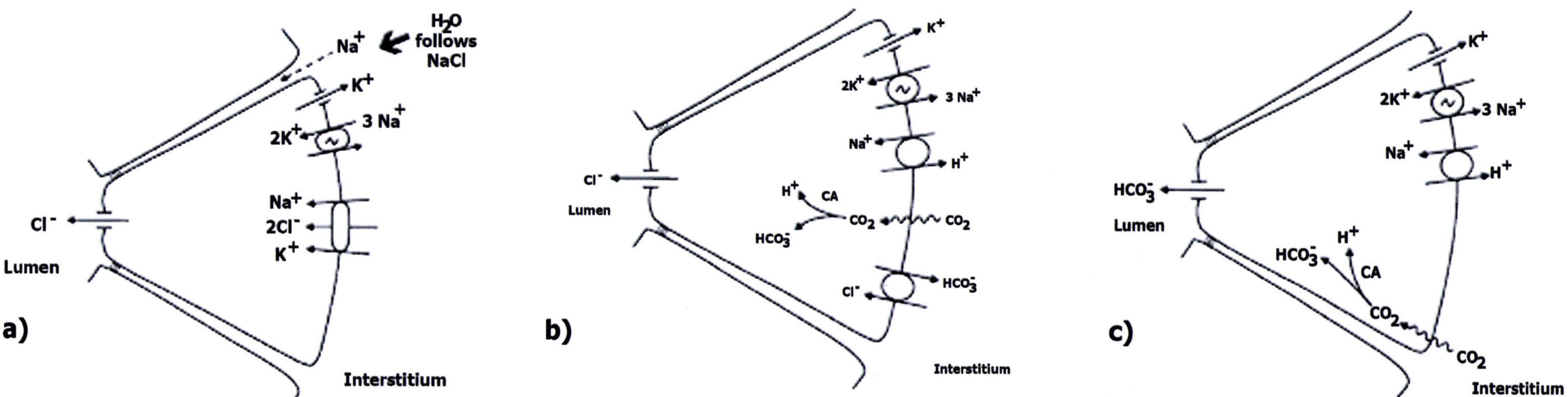


Figure 3.6 – Schematic representation of three models for primary salivary fluid secretion (Turner and Sujiya, 2002). Model **a)** in this model the cotransporter $Na^+/K^+/2Cl^-$ mainly accounts for the capture of Cl into the cell and provides the major driving force for fluid secretion; in model **b)** a secretion of Cl^- for lumen also occurs, but the entry of Cl^- into the acinar cell is due to Cl^-/HCO_3^- exchanger; model **c)** represents the fluid secretion directly derived by HCO_3^-. HCO_3^- is secreted rather than Cl^- due to the little discrimination that luminal Cl^- channels display among anions.

In the "second phase" of saliva secretion, exchanges of electrolytes occur in the duct system mainly in striated and excretory ducts. In humans and in a great number of animal species, a reabsorption of Na^+ and Cl^- and a certain excretion of K^+ and HCO_3^- occurs in duct cells, essentially without water movements. As a consequence of these exchanges, the final saliva is hypotonic to plasma. However, in ruminants the electrolyte composition of final saliva differs from the one of humans and several non-ruminant species (see section 3.5).

3.2.3. Secretion of proteins

3.2.3.1. Secretion by acinar cells

In a general mode, proteins are synthesised in rough endoplasmic reticulum and are transported through a succession of membrane-bounded compartments, including the Golgi complex, condensing vacuoles, and secretory granules (Von Zastrow and Castle, 1987). The secretory granules migrate to particular locations within the cell close to the apical membrane prior to the release of their contents in the acinar lumen. The fusion of these secretory vesicles with the plasma membrane and protein release can occur in the form of constitutive exocytosis or being regulated to allow the controlled release of vesicle contents in response to a physiological signal (Burgoyne and Morgan, 2003).

Saliva protein composition derives mainly from acinar cells, which are the primary secretory units. The complex processes of protein secretion by salivary glands were mainly understood based on some studies using glandular saliva collections and mainly in glandular tissues and salivary cell lines. Rat parotid has been the gland most extensively used as a model for studies of salivary proteins secretion due to its serous characteristics (Takuma and Ichida, 1986; Takuma et al., 2000; Castle, 1998; Castle et al., 2002; among many other authors). At least four different secretory pathways were referred for salivary protein secretion, two of which regulated and two independent of stimulation (Gorr et al., 2005).

The two regulated pathways differ between them in the level of stimulation required and in the relative composition of salivary proteins (Castle and Castle, 1996). The major regulated pathway, which accounts for 80-90% of total protein secretion from parotid acinar cells (Castle, 1998; Castle and Castle, 1998), involves large secretory granules that are exocytosed in response to muscarinic-cholinergic and adrenergic stimulation (Fig. 3.7, route "R" in black). The minor regulated pathway (Fig. 3.7, route "MR" in blue) was described by different authors (Castle and Castle, 1996; Huang et al., 2001) and is a derivative of the maturing secretory granule. It is high sensitive to low doses of agonists and presents higher responses to muscarinic comparatively to β-adrenergic agonists. Composition derived from this pathway mainly includes polypeptides that are least efficiently retained for storage in granules. It was suggested that this pathway can also have the function of relocate

membrane components, serving as a sensitive means to up regulate machinery that is utilized at the cell surface for secretion (Castle et al., 2002).

Apart from the regulated secretion, parotid tissue exhibits a small continuous output of salivary proteins in the absence of stimulation. Constitutive-like secretion has been traditionally accepted as the pathway for salivary protein secretion in the absence of stimulation (Castle and Castle, 1996; 1998; Huang et al., 2001; Castle et al., 2002). More recently, a secretory pathway, which originates directly in the trans-Golgi network, equally to constitutive pathway observed in other exocrine glands, was also proposed for salivary acinar cells (Gorr et al., 2005), (Fig. 3.7, route "C" in green). Gorr et al. (2005) suggested that this pathway exists for the delivery of basolateral plasma membrane proteins and extracellular matrix components, although no secretory proteins specific of this pathway have already being identified.

The origin of the constitutive-like pathway (Fig. 3.7, route "CL" in blue) has been proposed to be the same of the minor regulated pathway, diverging at a certain point (Huang et al., 2001). According to these authors, both pathways originate by a common step of vesicular budding that is linked to maturation of secretory granules and derives from both condensing vacuoles and immature granules. After this branch point, constitutive-like carrier proceed to a junction with endosomes, whereas in the minor regulated pathway occurs the formation of minor regulated carrier vesicles, which are maintained as a storage pool until they are induced to undergo exocytosis.

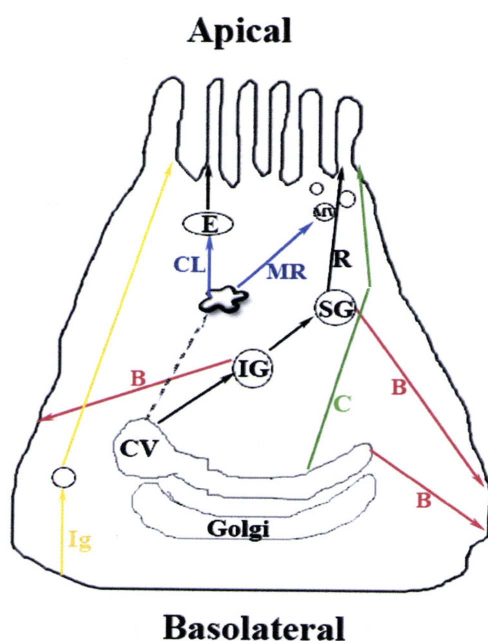


Fig. 3.7 – Protein secretory pathways in salivary acinar cells.
CV- condensing vacuoles; **IG** – immature granules; **SG** – secretory granules; **E** – endosomes; arrows represent the several routes of protein secretion: **R**- regulated pathway; **MR** – minor regulated pathway; **C** – constitutive; **CL** – constitutive-like; **Ig** – immunoglobulin transport; The direction of the arrows shows that protein secretion can occur both through apical membrane, which represents the greatest percentage of protein passage, and through basolateral membrane, signaled by red arrows "**B**". (Adapted from Huang et al., 2001 and Gorr et al., 2005, with few modifications)

Most of the movement of salivary proteins occurs across the apical membrane of the secretory cells into saliva. However, some movement of proteins into blood circulation, have also been reported (Isenman et al., 1999; Voutetakis et al., 2004). Although it was first thought that this might resulted from a paracellular passage (Proctor et al., 1989), it was now assumed that passage can occur via the constitutive vesicular route from the basal or basolateral surfaces of parenchymal cells (Garrett, 1998b) (Fig. 3.7, route "B" in red). In rats, salivary amylase, from acinar cells and kallireins from the granular tubules was referred to enter the blood via the constitutive vesicular route from the basal sides of the cells (Garrett et al., 1995; Garrett, 1998b). Kivela et al. (1997) also suggested that carbonic anhydrase VI also passes from salivary glands to blood, probably across the basolateral membrane. The capacity of passage from salivary glands to blood is likely to vary for the different constituents (Garrett, 1998b). Whether or not such movement is purposeful or a merely incidental passage remains an open question.

The fusion of secretory granules, which are mainly located on apical region, with plasma membrane is regulated by intracellular signals produced in response to the neurotransmitters, or agonists, in a dose-dependent mode. The intracellular cAMP is the principal second messenger resulting from β-adrenergic activation, and is responsible for initiation not only of granule exocytose, but also protein re-synthesis. Secretion obtained by muscarinic-cholinergic and/or α-adrenergic agonists is based in intracellular Ca^{2+}, as second messenger, and appear to have a direct stimulatory effect on non-storage granule vesicular secretion, rather than stimulation of mature granules. For more detailed information see reviews from: Proctor (1998), Turner and Sugiya (2002) and Ishikawa et al. (2006).

The intracellular mechanisms which lead to changes in rates of protein synthesis are at present uncertain and it is unclear whether nerve-mediated stimuli induces changes in rates of translation, transcription or in both. In fact, it appears that both transcriptional and translational control is exerted on salivary secretory protein synthesis in rat. It may be that individual secretory proteins show different degrees of dependence on these two controls (Proctor, 1998).

3.2.3.2. Secretion from duct cells

Duct cells also secrete proteins into saliva. For example, the protease glandular kallikrein has been immunocytochemically identified in secretory granules in the striated ducts of certain salivary glands (Tandler et al., 2001). It was also referred the secretion of nerve growth factor, by the cells from the excretory ducts of the mouse sublingual gland (Ayer-Le Lievre et al., 1989). As well, epidermal growth factor is secreted by the cells of the excretory ducts of rat submandibular glands (Sakabe et al., 1988) and some glycoproteins are secreted by the striated and intercalated ducts (Lima et al., 1977; Hand, 1979). Gresik (1994) also reported the presence of nerve growth factor and epidermal growth factor in secretory granules of the granular convoluted tubules of mice and other rodents.

3.2.3.3. Secretion of proteins from blood origin

The passage of blood proteins to glandular saliva is not completely understood although glandular "permeability" was first observed in nineteen century (for a detailed review see Garrett, 1998b). Three main mechanisms can be involved in the passage of substances through the epithelial membranes: a passive diffusion process (for high lipid soluble molecules non-ionized neither bound to proteins), an active process against a concentration gradient, and ultrafiltration through pores in the membrane (for small polar molecules with molecular weight less than 300 Da) (Aps and Martens, 2005). Some salivary glands, from some animal species [e.g. rabbit (Gamble et al., 1988) and cat (Mann et al., 1979) submandibular glands] have fenestrated capillaries, what could represent an easy way of exit of molecules from capillaries. Both transcellular and paracellular routes were proposed for explaining the passage of blood molecules to saliva, but this is still not certain. However, a great number of studies were focused on paracellular permeation, and passage through tight junctions was observed to occur (Garrett and Parsons, 1976; Parsons et al., 1977; Parsons and Garrett, 1977; Mazariegos et al., 1984; Hashimoto et al., 2000). The junctions seem to have a plasticity enabling passage of larger molecules (up to 40 kDa) into saliva under certain conditions (especially sympathomimetics) when accompanied by secretion (Mazariegos et al., 1984; Segawa, 1994).

The passage of immunoglobulins from blood into saliva, namely in what concerns IgA occurs through a particular mechanism of receptor-mediated epithelial transport. Immunoglobulins present in saliva are produced in the plasma cells that are located adjacent to the acini and ducts of the salivary glands (Korsrud and Brandtzaeg, 1980). Polymeric IgA (pIgA) consists of two IgA monomers linked to a J-chain. In the salivary glands, at basolateral epithelial cell level, polymeric-imunoglobulin receptors to which the pIgA will bind are present. The complex formed is then internalized into endocytic vesicles and transported to the apical surface of the cell, from where it is secreted in the form of S-IgA (Teeuw et al., 2004). The same mechanism is shared by IgM. It is important to state that Immunoglobulin G (IgG) may be also present in saliva, at much lower amounts, but their occurrence has been proposed to be mainly due to a passive diffusion (mainly through gingival crevices), despite a fraction might also originate in plasma cells (Brandtzaeg, 1998). However, in this last case, the passage into saliva would be probably due to some glandular permeability, rather than to a receptor-mediated transport.

3.3. Factors affecting salivary volume and composition

Several factors affect salivary flow rate and composition. Circadian rhythm (Dawes, 1972), sex (Ikemoto and Matsushima, 1984; Inoue et al., 2006), physiological factors (Dawes, 1987), drugs (Scully, 2003), exercise (Dawes, 1981) are factors affecting salivary secretion. Additionally it was observed that diseases such are Sjögren's syndrome (van der Reijden et al., 1996), bulimia nervosa (Riad et al., 1991), diabetes (Mata et al., 2004), vitamin deficiency (Glijer et al., 1985), and several

others systemic diseases (von Bültzingslöwen et al., 2007) also affect salivary flow and saliva composition.

The size of salivary glands has been frequently associated with their salivary flow rate. However, differences exist among the several different glands. In humans, parotid and submandibular gland size correlates with unstimulated saliva flow rate (Inoue et al., 2006; Ono et al., 2006), whereas sublingual gland size does not (Ono et al. 2006). For stimulated salivation, the correlation continues to exist for parotid and submandibular glands, despite this correlation being greater for parotid glands (Ono et al., 2007). A correspondence between parotid secretion rate and the size of the gland was also proposed for ruminants such as domestic sheep (Kay, 1960).

Saliva is intimately related with food consumption. The most copious flow of saliva is produced before, during and after eating. In section 3.2.1, it was presented the neural pathways involved in salivary secretion and the convergence of information from the several senses. Factors such as the thought of food and/or sensory inputs, including visual, olfactory, oropharyngeal and oesophageal senses (gustatory, mechanical and thermal), all initiate saliva secretion (Pedersen et al., 2002; Mese and Matsuo, 2007). Although the precise routes of the reflex arcs in the central nervous system are not known, evidences exist that the fundamental neural circuit of the gustatory-salivary reflex is situated in the lower brain stem (Matsuo, 1999b). Similarly, reflex salivation evoked by sensory inputs from oral mechanoreceptors, thermoreceptors and nociceptors may also have its reflex arc in the lower brainstem. These fundamental salivatory reflex arcs and/or sensory relay neurons are under descending control from higher centres of the brain, such as the cerebral cortex, hypothalamic feeding centre and limbic system. This implies that salivary volume and content do not simply reflect sensory inputs, but will be modified by facilitatory or inhibitory effects caused by, for example, the emotional state (Mese and Matsuo, 2007). It was observed that even before food presentation, the thought of food induces salivation and that this induction appears to be stronger in hunger conditions (Wooley and Wooley, 1973).

Eating and, more precisely, mastication is the main cause of reflex salivation. The reflex is associated with the stimulation of intra-oral receptors, which may be mechanoreceptors in the periodontal ligament (Hector and Linden, 1987) and in the oral mucosa (Scott, 1998). Masticatory force has been reported to influence salivary flow (Yeh et al., 2000). Salivation was observed to increase with mastication frequency and force of chewing and with the number of teeth involved (Jensen-Kjeilen et al., 1987). The reduction in bite force is the main reason for the decrease in salivary flow rate observed in elderly persons (Ikebe et al., 2007). The degree to which mastication influences salivary flow differs among the several glands, with parotid having a more pronounced response. Parotid glands secrete a much higher volume of saliva when food is solid and dry than in liquid diets (Ito et al., 2001). Structural and functional changes in the parotid glands (weight, salivary activity, and neurotransmitter concentrations) from rats (Johnson et al., 1984; Scott et al., 1990; Kurahashi and

Inomata, 1999) and rabbits (Anderson et al., 1985) were obtained after a period on a more liquid diet. It was found, for rats, that the order of increasing salivary flow rate from the parotid glands was solid > powder ≥ liquid, while that from submandibular gland was powder > solid ≥ liquid (Ito et al., 2001).

Food can also influence salivation after being consumed and digested. The increases in plasma osmolality were observed to decrease salivary flow rates in dogs (Miyoshi et al, 1969), sheep (Warner and Stacy, 1977), cattle (Silanikove and Tadmor, 1989), goats (Olsson, 1976), humans (Ship and Fischer, 1997) and rats (Ito et al., 2001). The decrease of salivary secretion induced by hyperosmotic stimulation is reportedly elicited by changes in the transepithelial osmotic gradient in the salivary glands (Nakahari et al., 1997).

3.4. Taste-saliva interactions

Soft foods, which exert only little mechanical stimulation in the mouth, can also influence saliva production, acting through chemical stimuli (Engelen et al., 2003). Some authors proposed that the effect of gustatory stimulation of food can even be more important than the mechanical stimulation of chewing for the saliva flow rate (Gavião et al., 2004). In fact, a combination of gustatory and mechanical stimulation was seen to elicit high saliva flow rates (Mackie et Pangborn, 1990). In humans, it was observed a dose-dependent increase in salivary flow in response stimulation with increasing concentrations of tastants and the overall order of relative salivary flow responses from highest to lowest flows is citric acid (sour) > monosodium glutamate (umami) > sodium chloride (salt) > sucrose (sweet) > magnesium sulphate (bitter) (Hodson and Linden, 2006). On the other hand, other studies found a salivary flow induced by bitter taste lightly lower than the one elicited by salty and higher than the one elicited by sweet (Chauncey and Shannon, 1960), although the substance used in this later study to elicit bitter taste was quinine. In rats it was observed (Matsuo et al., 1994) that the volume of saliva secreted in response of aversive stimuli, such as sour and bitter tastes, is significantly higher than the volume secreted in response to sweet and salty tastes. This mechanism was proposed to be related to the need of to rapidly wash away distasteful substances from receptor sites. In the same way, oral irritants induce the flow of saliva, probably to protect the mucosa (Martin and Pangborn, 1971).

Saliva can influence taste reception (point 2). Taste buds are in contact with saliva and saliva acts in the protection of taste receptor cells (Spielman, 1990; Hershkovich and Nagler, 2004). The composition of saliva bathing taste receptor cells is variable in space and time. Saliva may be mixed with other fluids, reported to be produced by taste buds cells (Mese and Matsuo, 2007). Additionally, the distribution of saliva within the mouth varies, both with unstimulation/stimulation conditions (Sas and Dawes, 1997), as well as with the proximity of excretion ducts. In general, taste receptors at the foliate and circumvallate papillae are influenced by flow from the von Ebner's glands, the ducts of

which opens on the clefts of the papillae (Gurkan and Bradley, 1987; Spielman et al., 1993; Li and Snyder, 1995). On the other hand, taste receptors at the anterior part of the tongue are more influenced by whole saliva.

In the initial process of taste perception, saliva acts as a solvent for taste substances and transports them to the sites of taste reception. During this process salivary constituents may interact chemically with taste substances and modulate taste sensitivity (Spielman, 1990; Matsuo et al., 1997). Both organic and inorganic composition of saliva can affect the five basic taste qualities. In terms of the inorganic composition of saliva, salivary buffers (e.g. bicarbonate ions) will lower the intensity of sour tastes, since they will decrease the concentration of free hydrogen ions, responsible for this taste (Matsuo and Yamamoto, 1992; Matsuo et al., 1994; Christensen et al., 1987). Neyraud and Dransfield (2004) suggested that the role played by saliva in the perception of bitter, sour and salty tastes is greatly attributable to the concentrations of free cations in saliva. Salivary ions, in addition to being taste stimuli, are thought to play other roles in taste perception, including acting as carriers of current during depolarization of taste cells. Salivary ionic composition varies markedly in response to different gustatory stimuli and may therefore influence early electrical events in gustation (Dawes, 1984). Additionally, the mixing of saliva with food can have a diluting effect, and in that way it can also influence the flavour release (Spielman, 1990).

Salivary constituents also modulate salty taste through a direct effect on taste reception sites. The continuous stimulation of taste receptors with the Na^+ present in saliva leads to an adaptation of the peripheral gustatory system to this constituent. Psychophysical studies in humans have shown that salivary Na^+ elevates the taste thresholds and elevate the supra-thresholds intensities of NaCl, what means that a salty taste is perceived above background salt concentrations in saliva to which taste receptors are adapted (Bartoshuk, 1978; Delwiche and O'Mahony, 1996).

Salivary proteins are also important in food perception. For example, alpha-amylase initiates the digestion of starch and this may have some influence on taste of carbohydrates. Despite the lack of studies observing the effects of amylase in taste perception, Gjörstrup (1980) described changes in amylase concentrations induced by taste: elevation of alpha-amylase concentrations, in rabbit saliva, was observed after administration of citric acid. Becerra et al. (2003) also reported variations in alpha-amylase (among other salivary proteins) after a complex stimulation induced by fruit flavoured candies. However, all these results may be viewed with care, since these changes can reflect changes in the relative contribution of each gland to the whole saliva, namely a relative increase in parotid saliva proportion, rather than a real increase in amylase secretion (Neyraud et al., 2006). Other salivary enzyme that was suggested to interact with food components, changing their original taste, is lingual lipase, which can break down dietary triglycerides to fatty acids and other small molecules, which, in turn, can stimulate taste receptors in rats and result in fat perception (Kawai and Fushiki, 2003). Von Ebner's gland protein, which is abundantly expressed in the small von Ebner's salivary

glands of the tongue was referred to bind with lipophilic molecules, such are some bitter taste substances, influencing taste perception (Gurkan and Bradley, 1988). The salivary carbonic anhydrase VI has also been described as associated with taste sensitivity. This protein seems to contribute to taste function by protecting taste receptor cells (Leinonen et al., 2001). Henkin et al. (1999) demonstrated that patients with taste loss have lower salivary carbonic anhydrase VI concentrations and more apoptosis in taste receptor cells. Human carbonic anhydrase VI was suggested to be identical to the already identified salivary gustin, a protein involved in taste perception (Thatcher et al., 1998).

Proline rich proteins constitute near 70% of the total protein content of human parotid saliva (Bennick, 1982). This proteins are induced in rats and mice by tannin consumption and the administration of the β-adrenergic agonist isoproterenol (Mehansho et al., 1983; 1985; Jansman et al., 1994; Ann et al., 1987). They constitute another example of salivary proteins involved in taste perception, since they seem to be able to reduce the aversive bitter/astringent properties of tannins (Glendinning, 1992). The large salivary proteins, such as mucins, can also influence the lubrication properties of saliva and hence the perception of food attributes such as smoothness and astringency (Kallithraka et al, 2001).

3.5. Particularities of saliva secretion in ruminants

The salivary glands of ruminants and other foregut fermenters, such as camels and kangaroos, differ from other mammalian salivary glands (Steward et al., 1996). The amounts of saliva produced are much higher than in non ruminant species, being saliva the only source of fluid to rumen. For example, one sheep produce at least 15L/day (Kay, 1960). Some differences exist among the different animals in terms of total salivary volume, but for the same animal the volumes among the different days do not present a significant variation (Kay, 1960). The ruminant parotid saliva is unusually rich in mineral ions, particularly sodium, phosphate and bicarbonate, comparing with other species, having a pH of 8.2 (McDougall, 1948). These mineral ions are associated with the alkalinity, providing buffering capacity for ruminal fermentative activities, and possibly providing additional phosphorus source for rumen bacteria (Breves et al., 1987). Ruminant saliva contains considerable concentrations of urea, which are related with ruminal ammonium concentrations and with urea concentrations in blood. For example in a resting cow, urea can represent about 77% of the total nitrogenous present in whole saliva (Bailey and Balch, 1961).

A large fraction of whole saliva (about 50% - 60%) is supplied by the parotid glands. The submandibular gland secretes only about one-eight as much saliva as the parotid gland and most of this saliva is secreted during periods of feeding (Kay, 1960). This is contrary to what is observed in

humans, for which submandibular saliva contributes to the greatest percentage of fluid during rest (Denny et al., 2008).

Adult parotid glands are purely serous glands, submandibular glands are mixed and sublingual glands are mucous (Kay, 1960). At birth the salivary glands are not completely developed. In young ruminants the parotid glands frequently contains groups of mucous cells and the sublingual gland groups of serous cells. Additionally to the three pairs of major salivary glands, sheep and goats also present other three paired glands, which secretion is important in the formation of whole saliva. The inferior molar glands are wedge-shaped serous glands, with a secretion similar to parotid secretion, lying in the cheeks opposite the inferior molar teeth (Fig. 3.1 c). Buccal are mainly composed of mucous cells whereas labial are mixed glands. The buccal glands are largely confined to the superior and inferior non-papillated part of the epithelium of the cheek, the inferior group lying beside the inferior molar glands. The labial glands are most numerous at the corners of the mouth (Kay, 1960).

Histomorphologically, the ruminant salivary glands also present particularities, comparing to the other mammals salivary glands. The parotid gland, which is a compound acinar gland in the great majority of the mammals, is a compound tubular gland in ruminants (Van Lennep et al., 1977). The difference in shape of the secretory end pieces seems to be related to differences in the function of ruminant parotid glands, comparing to the other mammals parotid glands, such as the greater amount of water and electrolytes secreted, rather than protein secretion, which is the main role of other mammals parotid glands. For sheep parotid glands, Van Lennep et al. (1977) observed that the acinar cells possess extensively folded basolateral plasma membranes and a moderate number of mitochondria. In addition, a well developed intercellular space in close proximity with secretory canaliculi, lined with many microvilli, were observed, what seems to be related to the rapid equilibration of water across the epithelium. Apart from these general characteristics, some differences exist among the different ruminant species, what was proposed to be related with the feeding type classification proposed by Hofmann, referred in point 1 (browsers, gazers and intermediate feeders). Stolte and Ito (1996) observed that concentrate selectors parotid gland cells are more close to protein-secreting cells than the ones from grazers.

3.5.1. Electrolyte composition

In what concerns salivary secretion regulation, results from Compton et al. (1980), in sheep parotid glands, indicate that salivary secretion in ruminants can be accounted for in terms of the standard two-stage model proposed to non-ruminant mammals (point 3.2.2). However, some particularities exist for ruminants. Ruminant parotid glands secrete continuous and spontaneously in the absence of stimulation (Kay, 1960). A spontaneous saliva secretion was also referred for cat sublingual and rat and rabbit submandibular glands (Smaje et al., 1973), although it has a less important role in these species. In humans, Schneyer et al. (1956) indicated that major salivary glands do not secrete in a

total absence of stimulation. Only some human minor salivary glands secrete spontaneously, being their contribution for the total volume of unstimulated whole saliva reduced. The capacity of ruminant parotid secretory cells to drive fluid and electrolyte secretion even at resting state can be related to the high numbers of high-conductance, voltage and Ca^{2+}-activated K^+ channels present in the secretory cells (Ishikawa and Cook, 1993), or even to the existence of a different type of K^+ channels distinct from Ca^{2+} activated K^+ channels (Hayashi et al., 2003).

The parotid glands of adult ruminants secrete an almost isotonic fluid, rather than the hypotonic saliva referred to humans and non-ruminants, with amounts of bicarbonate and phosphate significantly higher. It should be pointed that, although in normal conditions, ruminant parotid saliva is isotonic to plasma, as it was referred, the flow rate, blood tonicity and, more specifically the sodium state of the animal, affect saliva tonicity (Beal, 1979; Compton et al., 1980; Patterson et al., 1982; Carter and Grovum, 1990). Phosphate seems to derive only from acinar secretion (primary fluid) and potassium and bicarbonate appear to be secreted both by acinar and ductal cells. Sodium and chloride enter saliva only at the primary "stage" (acinar secretion) and can be reabsorbed in the ducts. However, sodium reabsorption by the duct cells only occurs when the animal is depleted from sodium (Compton et al., 1980). The different salivary glands also present different electrolyte composition. The submandibular saliva has much lower buffering power, due to lower concentrations of Na^+, HCO_3^- and HPO_4^{2-}. The concentrations of K^+ and Cl^- are about the same (Kay, 1960).

In contrast to the salivary glands of most common laboratory and non-ruminant species, for which the primary secretion is Cl^- rich and mainly driven by the secondary active transport of Cl^- across the basolateral membrane of the secretory cells (Fig. 3.6.a), in ruminants the Cl^- concentration in the primary fluid is low (Compton et al., 1980) and data from the cattle (Lee and Turner, 1992) and sheep (Steward et al., 1996) parotid gland, suggest that salivary secretion is driven almost exclusively by HCO_3^- transport. The mechanism by which HCO_3^- leaves the cell across the luminal membrane is similar to most of mammalian; however, the mechanism for HCO_3^- uptake across the basolateral membrane presents differences (Steward et al., 1996). Carbon dioxide can represent a source of intracellular HCO_3^-, as it was represented in Fig. 3.6c. However, studies from Blair-West et al. (1980) suggested that this should not represent the main mechanism. Later on, an active transport of HCO_3^- across the basolateral membrane was suggested (Steward et al., 1996). The presence of Na^+/HCO_3^- co-transporters was demonstrated in bovine parotid acinar cells (Yamaguchi and Ishikawa, 2005) and they may have a greater importance for these species than for non-ruminant species.

3.5.2. Nervous control of ruminant salivary secretion

The sites in the ruminant central nervous system, which control salivation, are less well known than in humans and laboratory animals. Grovum and Gonzalez (2000) stimulated electrically the sheep brain

to elicit secretion by the parotid and submandibular salivary glands, and described the topography of both the superior (submandibular) and inferior (parotid) salivary centres.

It is well established that parotid and submandibular glands are differently regulated in sheep. Both parotid and submandibular glands receive dual parasympathetic and sympathetic innervation. However, whereas parotid glands secrete continuously, during resting, eating and rumination, ruminant submandibular glands produce saliva only during feeding (Carr, 1984). The continuous secretion of parotid saliva, in ruminants comprises a low rate of spontaneous secretion, which is independent of neural activity (Kay, 1958). The amount of parotid saliva produced daily are much higher [about eight (Kay, 1958) or ten (Carr, 1984) fold] than the one from submandibular glands. The differences in daily production of the two types of saliva appear to be a consequence of the different spectrum of stimuli by which the respective glands are excited (Carr, 1984). Parasympathetic stimulation results in an increase of fluid secretion both in parotid and submandibular glands, whereas sympathetic stimulation results in the secretion of low volumes of saliva, but with a higher protein content (Carr, 1984).

Sheep are known to produce parotid saliva with widely varying volumes and protein concentration, depending upon circumstances, such as if the animal is resting, eating or ruminating. It is maximal stimulated at the onset of eating but volume secreted rapidly decline during the meal (Carr and Titchen, 1978; Carter and Grovum, 1990; Meot et al., 1997). Eating effects on the parotid gland volume vary both according to the nature of the diet consumed and the duration of a meal, inversely to what occurs for submandibular secretion, for which eating is always a potent stimulus to secretion. The total amount of saliva produced on a meal of fresh grass is higher than the one produced on a dry food meal, inversely to what occurs with submandibular saliva secretion, for which volumes are higher on dry foods (Carr and Titchen, 1978; Carr, 1984). The decrease in parotid saliva secretion, as feeding progress, was hypothesized to be due to high levels of rumino-reticular distension and/or an increase in blood osmolality (Carr and Titchen, 1978). During rumination parotid flow rate increases again, but at lower levels than at the begining of feeding (Meot et al., 1997). The presence of a sustained background of efferent activity in the parasympathetic innervation of the gland in response to buccal, oesophageal and gastric mobility was suggested to account for this increase (Carr, 1984).

In what concerns parotid protein concentration, it is high during feeding but low during rumination (Patterson et al., 1982). As well as for the other non-ruminant mammals, also for ruminant parotid the parasympathetic and sympathetic nervous system act in concert, increasing the flow of saliva and its protein content (Patterson and Titchen, 1979; 1980; Patterson et al., 1982), being this increase much greater than that which would be predicted from simple summation of the two responses (Edwards and Titchen, 1992). This synergy was referred to be due to the interactions between muscarinic-cholinergic and adrenergic receptors (Edwards and Titchen, 2003). The increase in the secretion of parotid salivary protein when sheep are feeding is probably due to concurrent activation of the

parasympathetic and sympathetic innervations. Patterson et al. (1982) suggested that the difference in protein concentration between parotid saliva during feeding and rumination can be related to buccal and oesophageal physic and sapid stimulation.

In ruminant salivary glands, parasympathetic nerve transmission may involve not only the classical autonomic transmitter acethylcholine, but also non-cholinergic non-adrenergic (NANC) transmitters (Ekström, 1998). Vasoactive intestinal peptide (VIP) seems to be the neuropeptide greatly involved in NANC parasympathetic stimulation (Reid and Titchen, 1988; Edwards and Titchen, 2003). VIP is involved in the control of parotid and submandibular salivary secretion and, more particularly protein secretion induced by parasympathetic stimulation (Ekström et al., 1983; Reid and Titchen; 1988) and the effect of VIP on salivary protein production seems to be mediated via the production of nitric oxide (NO) (Edwards et al., 1996; Hanna and Edwards, 1998).

Concluding, the relative importance of each of the salivary glands to digestive processes differs between ruminant and non-ruminant species. Ruminant parotid and other serous salivary glands (inferior molar glands) are stimulated by feeding and rumination, whereas mixed glands, including submandibular, sublingual and labial glands are stimulated by feeding activity but not by rumination. This form of secretion leads to the suggestion that ruminant serous glands are preferentially related to the provision of an appropriate liquid environment for the non-secretory rumino-reticulum, facilitating the digestive processes during feeding and rumination. On the other hand, submandibular, sublingual and other minor mucous glands seem to be related to a primary role of to lubricate the mouth and oesophagus (Carr, 1984).

4. Salivary proteins

4.1. Proteomics in the study of saliva

Saliva contains a large array of proteins, which may have a variety of functions, and which presence and relative amounts change with a variety of factors. The separation of proteins by two-dimensional electrophoresis (2-DE), together with their identification by mass spectrometry (MS), is one of the core technologies of proteome research. 2-DE originates from the work of O'Farrell and Klose in the 1970's (Klose, 1975; O'Farrell, 1975) and is the only method currently available that has a unique capacity of simultaneously separating the thousands of proteins found in biological samples. Proteins are firstly separated according to their isoelectric point, followed by separation according with their molecular masses. The 2-DE can be used to compare quantities of proteins in related samples, such as those from altered environments or from mutant and wild type, thus allowing the response of classes of proteins to be determined. This application, however, has become significant only in the middle eights, when Görg et al. (1985; 1988) developed the currently employed 2-DE technique,

where carrier ampholyte-generated pH gradients have been replaced by immobilized pH gradients and tube gels replaced by gels supported by a plastic backing.

Mass spectrometers consist of three basic components: an ion source, a mass analyzer, and an ion detector. The ion source allow for the transfer of molecules from solution or solid phase into gaseous phase. MS has been used for the analysis of proteins and peptides since the independent development of two ionization techniques, electrospray ionization (ESI) (Yamashita and Fenn, 1984) and matrix assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988). After ionization, the sample reaches the mass analyzer, which separates ions by their mass-to-charge *(m/z)* ratios. Separated ions move on electric or magnetic fields direct toward to a detector, which registers the numbers of ions at each individual *m/z* value. Time-of-flight (TOF) is a type of mass analyzer frequently coupled to MALDI ionization source. MALDI-TOF mass spectrometry has a good mass accuracy, at tenth ppm level, high resolution and sensitivity, and for that reason, it is widely used in proteomics to identify proteins by a process called peptide mass fingerprinting (PMF). In this approach, proteins of interest are digested with a sequence-specific protease such as trypsin and the generated peptides are analysed by MS. The determined masses are then compared against a database comprising peptide masses from a virtual digest with the same sequence-specific protease of all deposited proteins (Guerrera and Kleiner, 2005).

In humans, more than 1400 salivary proteins from major salivary glands have been identified through proteomic approaches (reviewed in Denny et al., 2008). More recently the proteome of minor salivary glands secretion was published (Siqueira et al., 2008). Among the various proteomic approaches, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) coupled to mass spectrometry (MS) was used in the study of this biological fluid (Ghafouri et al., 2003; Yao et al., 2003; Huang, 2004; Vitorino et al., 2004; Hirtz et al., 2005; Walz et al., 2006). In other animal species, techniques such as SDS PAGE (Spielman and Bennick, 1989; Patterson et al., 1992; Ekström e al., 1994; Ekström et al., 1996) and two-dimensional electrophoresis (Williams and Marshall, 1998; Williams et al., 1999a; 1999b) were also employed for salivary protein separation. However, these studies did not resulted in an exhaustive characterization of salivary protein profile of the species studied. Mau et al. (2006) used mass spectrometry to identify salivary proteins from goats and cattle but separation was only according protein mass.

4.2. Protein functions

Functions of saliva are dependent, to a large extent, upon its protein composition. In human saliva the proteins are mainly present as families or structurally close related family members, being this composition the result of allelic variations, gene duplication, alternative splicing events, and post-translational modifications (Oppenheim et al., 2007). The different isoforms of the same salivary

protein may have more than one function and, on the other hand, the same function may be shared by different families of proteins (Fig. 4.1). In addition, many salivary proteins bind to each other forming heterotypic complexes, or are modified by enzymatic cross-linking (Yao et al, 1999) and proteolytic processing (Lamkin et al., 2001), what may result in particular biological functions. This functional redundancy may help to ensure that a given function is always present, i.e., different proteins may guarantee the presence of an active protein under a broader range of physiological conditions what would not be possible for a single protein (Nieuw Amerongen and Veerman, 2002; Huq et al., 2007). Despite the several described functions, the biological role of most of the identified salivary proteins remains to be completely understood.

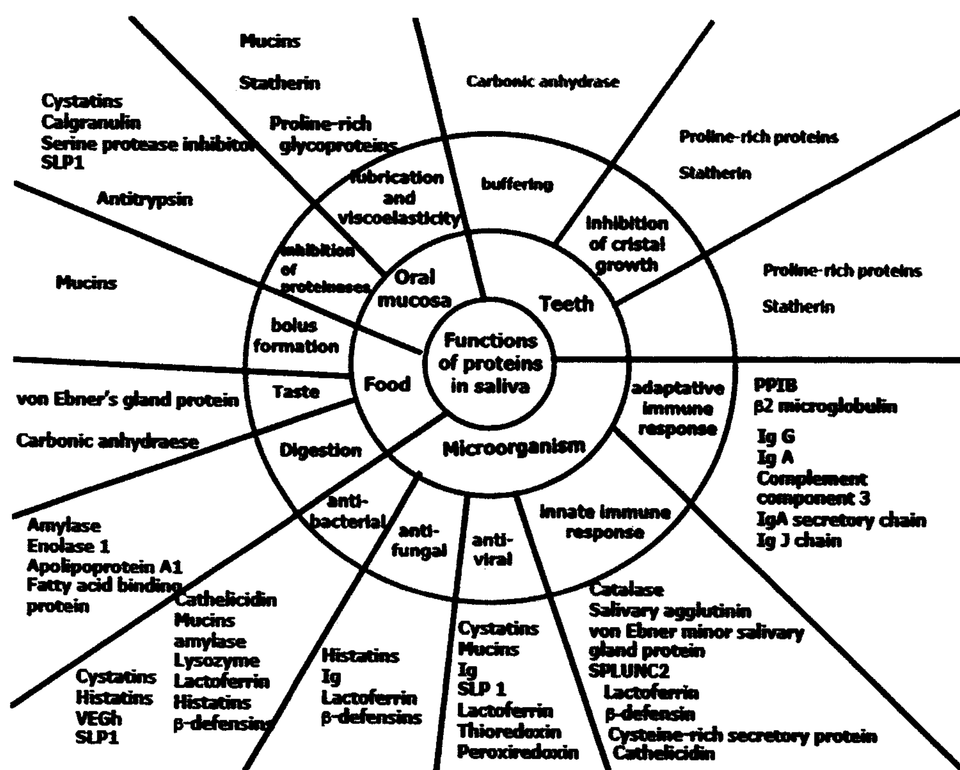


Fig. 4.1 – Schematic presentation of the main functions of several salivary proteins (adapted from Huq et al., 2007)

Most of the factors described in point 3 as affecting saliva secretion, also affect salivary protein concentration and composition, namely circadian rhythm (Dawes, 1974; Hardt et al., 2005; Den et al., 2007), mastication (Johnson, 1982; Dawes and Kubieniec, 2004), duration of stimulation (Dawes, 1969), taste (Neyraud et al., 2006) and diet composition. In humans, and in what concerns protein amount, whole saliva is mainly constituted by glycoproteins (mucins and proline-rich glycoproteins), enzymes (amylase and carbonic anhydrase VI), proline-rich proteins and a variety of peptides (cystatins, sthaterins, histatins and some proline-rich peptides) (Dodds et al., 2005; Huq et al., 2007). As can be seen in Fig. 4.1, it is clear that the greater proportion of proteins present protective functions, either in terms of mucosal oral protection, maintenance of tooth integrity or antimicrobial activity.

4.2.1 Teeth mineralization

Specific salivary proteins, namely statherins and proline-rich proteins, protect teeth against demineralization by inhibiting the precipitation of calcium phosphate salts. Functions such as calcium binding (Bennick et al., 1982), inhibition of hydroxyapatite formation and formation of dental-acquired pellicle (Hay and Moreno, 1979) have been attributed to acidic PRPs. The statherins are peptides involved in maintaining the mineral balance of the tooth. These peptides also mediate the binding of a variety of microorganisms to teeth and oral mucosa (Dodds et al., 2005). Sometimes statherins form heterotypic complexes with highly glycosylated mucins, acting in mucosal protection (Soares et al., 2003).

4.2.2. Oral mucosal protection and antimicrobial activity

Proteins such as lysozyme, lactoferrin, peroxidase, chitinase and immunoglobins are related to antimicrobial and anti-inflammatory activity. Lysozyme is a protein ubiquitously present in body fluids, and it acts antimicrobially by catalyzing the hydrolysis of cell wall polysaccharides, leading to the lysis of bacteria. In addition, non-enzymatic bactericidic activity has been referred for this protein, which has been attributed to activation of bacterial autolysins (Nieuw Amerongen and Veerman, 2002). Lactoferrin is a member of the transferrin family of iron binding proteins. Lactoferrin can inhibit the spread of bacteria by chelating iron, under certain conditions, making it unavailable for microorganisms, namely for gram-negative bacteria, viruses and fungi. In addition, lactoferrin has the capability to induce mucosal defense by inflammatory mediators (Komine et al., 2007). Salivary peroxidases have antimicrobial effects by catalyzing the formation of bactericidic compounds, e.g. hypothiocyanate, by peroxidation of thicyanate (Nieuw Amerongen and Veerman, 2002). Chitinase seems to be involved in the protection against colonization of oral epithelial cells by yeast (Van Steijn, 1999). Salivary IgA is the most abundant immunoglobulin in saliva and represents the main adaptive immune mechanism in the oral cavity, having an important role in the neutralization and elimination of pathogens (Teeuw et al., 2004). This protein is secreted by both parotid and submandibular/sublingual glands but the main contribution of secretory IgA for total saliva comes from minor salivary glands (Eliasson et al., 2006). The particular mechanism of secretion for this protein has been reviewed in section 3.2.3.3.

Histatins and defensins are also antimicrobial peptides (Nieuw Amerongen and Veerman, 2002). The antimicrobial properties of the different histatins may be related to the affinity of these peptides for a large repertoire of chemically and structurally different ligands, whereas defensins are part of the innate immune system, presenting a broad spectrum antibacterial and antifungal activity. Defensins also have properties that may serve to link innate immunity with the acquired immune system.

The salivary antimicrobial proteins may act in both additive and synergistic interactions: examples are the positive interactions between secretory IgA and peroxidase, lactoferrin and peroxidase, lactoferrin and lysozyme and histatins (Pedersen et al., 2002; Soares et al., 2004).

Salivary cystatins comprise a family of multifunctional proteins playing a different role in the oral environment, as can be observed by their distribution, in Fig. 4.1. These proteins are mainly secreted by the submandibular/sublingual glands and at lower amounts by the parotid glands. Some types of cystatins seem to play an important role in the control of proteolytic events in vivo, whereas other types are involved, together with statherin, in the mineral balance of teeth (Baron et al., 1999).

Mucins are the major group of proteins secreted by submandibular/sublingual glands. Human saliva contains two genetically distinct mucin types, designated MG1 and MG2 (Levine et al., 1987; Bolscher et al., 1995; Tabak, 1995). MG2 are relatively small (Mr 125 kDa) monomeric species, having low viscoelastic properties. MG1, on the other hand, have high molecular masses (~1 MDa). Due to their high carbohydrate content (>80%), their large dimensions, and their extended thread-like structure, mucins form hydrophilic viscoelastic gels. These gels function as barriers, protecting the underlying epithelium against mechanical damage and preventing direct entrance of noxious agents, including bacteria and viruses, into the underlying vulnerable epithelium (Nieuw Amerongen and Veerman, 2002).

Salivary agglutinins are other heavily glycosylated proteins, that share some features with MG2. Under native conditions, these proteins occur associated with a variety of salivary proteins (Soares et al, 2004).

4.3. Glandular origin and amounts of salivary proteins in humans, rodents and ruminants

The saliva protein composition varies among the different glandular secretions and among the different animal species. Most of the reports on concentration of each of the salivary proteins are found for humans. Several studies on animal saliva also measured the concentration of some salivary proteins, however, many of them worked with stimulated (parasympathetically and/or sympathetically) saliva, due to the difficulty in collecting saliva in control conditions. Table 4.1 presents concentration values determined for several salivary proteins, in humans and the animal species studied in this thesis.

Table 4.1 – Salivary protein concentrations (g/mL) and distribution by whole and/or glandular fluids in humans, ruminants and laboratory rodents

Protein	Saliva	Human	Ruminant	Laboratory rodents [§]
Acidic chitinase	Whole	nd [52,¥]		
	Parotid	nd [52,¥]		++[41]
	Submand/subling	nd [52,¥]		
Acidic epididymal glycoprotein	Whole			
	Parotid	Not present		++ [50]
	Submand/subling			nd [42]
Albumin	Whole	29-238[1,2]	nd [14]	
	Parotid	2.06 [36] 1.9 [47]		nd [48]
	Submand/subling	2.70 [36]		
Amylase	Whole	380-500[3,4]		+++
	Parotid	650-2,600[3,4]	Not present	++++ [33, 50] 610 - 67150* [46]
	Submand/subling			++
Androgen binding protein	Whole			
	Parotid	Not present		
	Submand/subling			++[31]
Carbonic Anhydrase VI	Whole	6.8 [15]	7.8 [16]	
	Parotid	47 [54]	5.61 – 33.0 [55]	
	Submand/subling			
Common salivary protein 1	Whole	nd[49]		
	Parotid			+[21]
	Submand/subling			+/-[21]
Complement system components (C3, C4 and factor B)	Whole	C3 – 0.52 – 15 [53] C4 – 0.086 – 4.8 [53] Factor B – 0.042 – 0.62 [53]		
	Parotid			
	Submand/subling			
Cystatin	Whole	240-280[3,4]		0.03 – 155 [51]
	Parotid	1.6-4[3,4] 0.2-1.2[18]		

	Submand/subling	92-280[3,4] 89-570[18]		(+/--) to (++)* [29]
DNase I	Whole		+[13]	+[13]
	Parotid	+/--[13]	++++[13]	+++[13, 50]
	Submand/subling	+/--[13]	+/-[13]	+/-[13]
Epidermal growth factor (EGF)	Whole	$2.7 X 10^{-3}$ [34]		
	Parotid	$2.4X10^{-3}$-$3.1X10^{-3}$ [34]	$495X10^{-6}$– $942X10^{-6}$ [57]	
	Submand/subling			
Extra-parotid glycoprotein	Whole			
	Parotid			
	Submand/subling	+ [63]		
Fibronectin	Whole	0.2-2[5,6,7] 0.12[8]		
	Parotid	2-6[5,6,7]		
	Submand/subling	0.3-2.0[5,6,7]		
Histatins	Whole	2-30[9]	Not present	Not present
	Parotid	30-55[9] 65 [62]		
	Submand/subling	13-70[9] 147 [62]		
IgG	Whole	0.4-14.4[1, 10, 11]		242.8[12]
	Parotid		~2.0[14]	
	Submand/subling		~66[14]	
Kallikrein	Whole	nd [49]		3.90 [51]
	Parotid			
	Submand/subling			nd
Lactoferrin	Whole	194[1, 17]		++[33] 87 [40]
	Parotid	12[1, 17] 5.4-8.1[28] 1.81 [36]		
	Submand/subling	13[1, 17] 1.63 [36]		
Lysozyme	Whole			++[33] 972 [40]

	Parotid	7[1, 17] 1.82 [36]		
	Submand/subling	21[1, 17] 8.75 [36]		
MG1	Whole	80-500[4] 233-280[17]		
	Parotid			
	Submand/subling	80-560[4]	++++[20]	
MG2	Whole	10-200[3] 133-143[17]		
	Parotid			
	Submand/subling	5-243[3]	++++[20]	
NGF	Whole	901.4 [35]		
	Parotid	885.9 [35]		
	Submand/subling	1.07×10^{-3} [35]		1.04 – 6.1 [60]
Odorant binding protein	Whole	nd[φ] [61]	nd [56]	nd [66]
	Parotid			
	Submand/subling			
Parotid agglutinin	Whole			
	Parotid	10[22]		
	Submand/subling			
Parotid secretory protein[#]	Whole		15-30% of total protein [58]	
	Parotid		nd [59]	+++[32, 50]
	Submand/subling		nd [59]	
Peroxidase/ catalase	Whole	1.9[19]		
	Parotid	+[45]		nd [44] 11-1224 [46]
	Submand/subling	+/-[45]		nd [44]
Prolactin-inducible protein/ mouse submaxillary gland protein	Whole			
	Parotid	+/-- [43]		+/- [43]
	Submand/subling	+ [43]		+/- [43]
Proline-rich proteins	Whole	90-180[3, 23, 24]		
	Parotid	230-1,251[3, 23, 24]		(+/--) to (++++) [64, 65]
	Submand/subling	270-1,335[3, 23, 24]		

sIgA	Whole	19-439[2, 3, 10, 25, 26, 30]		223.8 [12]
	Parotid	20-230[2, 3, 10, 25, 26, 30] 131 ± 7.0 [36]	~1.19 [14]	
	Submand/subling	41-56[2, 3, 10, 25, 26] 130 [36]	~506 [14]	70-115 [37] 55-573 [38] 487 [39]
Statherin	Whole	2-12[4, 27]		
	Parotid	16-147[4, 27]		
	Submand/subling	20-150[4, 27]		

§ rat, mice and/or hamster;

¥ doubt about if it is the referred protein or a protein with the same activity;

in ruminants named as Bovine salivary protein 30 (BSP30);

φ the salivary odorant-binding protein reported in humans is lipocalin-1

nd – present but level no reported; when protein concentration was no reported the relative amounts were represented by signals, ranging from very low (+/--) to very high (++++);

* the high level presented in table was obtained after sympathetic (or through symathomimetic agonists) stimulation;

[1] Mandel (1980); [2] Henskens et al. (1996), [3] Aguirre et al. (1987); [4] Oppenheim et al. (2007); [5] Babu and Dabbous (1986); [6] Tynelius-Brattihall (1988); [7] Tynelius-Brattihall et al. (1986); [8] Llena-Puy et al. (2004); [9] MacKay et al. (1984); [10] Cole et al. (1978); [11] Brandtzaeg (2007); [12] Ebersole et al. (1979); [13] Takeshita et al. (2000); [14] Scicchitano et al. (1986); [15] Parkkila et al. (1993); [16] Nishita et al. (2007); [17] Rayment et al. (2000); [18] Veerman et al. (1996); [19] Thomas et al. (1994); [20] Leppi and Spicer, 1967; [21] Girard et al., 1993; [22] Ericson and Rundegren (1983); [23] Hay and Moreno, (1979); [24] Kousvelari et al. (1980); [25] Rudney et al. (1991); [26] Stuchell and Mandel (1978); [27] Hay et al. (1984); [28] Dipaola and Mandel (1980); [29] Shaw and Barka (1989); [30] Carpenter and Proctor (2000); [31] Hwang et al. (1997); [32] Owerbachx and Hjorthz (1980); [33] Koller et al. (2000); [34] Thesleff et al. (1988); [35] Nam et al. (2007); [36] Lin et al. (2003); [37] Matsuo et al. (2000); [38] Carpenter et al. (1998); [39] Carpenter et al. (2005); [40] Muratsu and Morioka (1985); [41] Goto et al. (2003); [42] Mizuki and Kasahara (1992); [43] Mirels et al. (1998); [44] Redman and Field (1993); [45] Riva et al. (1978); [46] Anderson et al. (1984); [47] Sweeney and Beeley (1990); [48] Williams et al. (1999a); [49] Xie et al. (2005); [50] Johnson et al. (1995); [51] Bedi (1991); [52] Van Steijn et al. (1999); [53] Andoh et al. (1997), [54] Fernley et al. (1995); [55] Fernley et al. (1991); [56] Mau et al. (2006); [57] Onaga et al. (2006); [58] Rajan et al. (1996); [59] Haigh et al. (2008); [60] Murphy et al. (1977); [61] Vitorino et al. (2004); [62] Gusman et al. (2004); [63] Rathman et al. (1990); [64] Mehansho et al. (1983); [65] Mehasho et al. (1985); [66] Pes and Pelosi (1995)

From Table 4.1 it is possible to conclude that different species present differences in salivary protein composition. Despite the lack of information about the presence/absence for some of the proteins, from each of the three species, it is evident that laboratory rodents present at least two salivary proteins involved in sexual behavior, which are not present in human saliva: acidic epididymal glycoprotein and androgen-binding protein. Their presence may be due to the anatomy of these animals, namely due to the communication between oral, nasal and vomeronasal areas and to the already demonstrated role of saliva in mice sexual communication (Marchlewska-Koj et al., 1990). They are also evident the high levels of alpha-amylase in non-ruminants, which are not present in ruminant saliva.

4.4. Salivary proteins involved in food consumption

Some salivary proteins are involved in food ingestion (Figure 4.1), either in terms of food perception or in terms of food digestion. This issue started to be presented in point 3.4. Some proteins were related to taste perception. Two examples are carbonic anhydrase VI, which was also referred as gustin (Thatcher et al., 1998) and for which a role in maintaining taste buds integrity was suggested (Henkin et al., 1999), and von Ebner gland protein, which was also referred to be involved in taste perception (Spielman, 1990).

The major digestive enzyme that was identified in the non-ruminant saliva was α-amylase, which is a protein with approximately 58-62 kDa (Zajácz et al., 2007), that catalyzes the hydrolysis of α (1,4) glycosidic binding between glucose residues of polyssacharides. This protein is absent in ruminant saliva (Table 4.1) and is found in mammals which have starch or glycogens as part of the diet. A mixture of more than 5 isoforms of this protein has been reported to be present in saliva (Liang et al., 1999).

4.5 Changes in salivary protein composition induced by food constituents

Food constituents may modulate salivary protein composition. Although some salivary proteins, such as cystatins, salivary proline rich proteins and histatins, in Fig. 4.1, do not appear included in the group related to food, they are also involved in food consumption, since for some animal species, their levels were found to be related to the presence of particular food constituents. Studies on capsaicin (Katsukawa et al., 2002), gymenic acid and gurmarin (Katsukawa et al., 1999) and papain (Naito et al., 1992; Ninomiya et al., 1994) containing diets demonstrated the induction of particular salivary proteins in order to counteract the negative effects that these dietary substances may produce. Once these salivary proteins are present, animals are able to ingest foods containing these aversive substances.

PRPs were first detected in human saliva (Mandel et al., 1965, cited by Carlson, 1993). Their presence has also been referred for saliva or salivary glands of several animal species, such as mouse (Mehansho et al., 1985), rat (Mehansho et al., 1983), rote vole (Juntheikki et al., 1996), hamster (Mehansho et al., 1987), rabbit (Mole et al., 1990; Ferreira et al., 1992), musk ox (Gehrke, 2001), mule deer (Austin et al., 1989, Hagerman and Robbins, 1993), roe deer (Fickel et al., 1998), moose (Juntheikki, 1996), macaque (Ann and Lin, 1993), monkey (Oppenheim et al., 1979), pig (Patamia et al., 2005). Whereas in humans salivary PRPs represent about 70% of the total protein secreted by parotid gland, in rodents they are absent or present in low amounts in the absence of beta adrenergic stimulation or tannin ingestion (Mehansho et al., 1983; 1985). They are characterized by their high amount of three amino acids: proline, glutamine and glycine (a total of 80% of all amino acids) and a

small content, or absence, of aromatic and sulfur-containing amino acids (Bennick, 1982; Carlson, 1993). Lu and Bennick (1998) referred the presence of more than 22 salivary proteins belonging to PRPs family. All salivary PRPs are characterized by four general regions: a signal peptide region, a transition region, a repeat region and a carboxyl-terminal region (Clements et al., 1985). Proline–rich proteins can be grouped, according their charge and glycosylation status, in acidic, basic and glycosylated. Acidic PRPs are involved in tooth mineralization, as it was referred in this chapter, whereas glycosylated PRPs provide a lubricating function in mouth (Hatton et al., 1985), and bind microorganisms (Gillece-Castro et al., 1991). Basic PRPs, for which no other specific role has been presented, has been suggested to bind and precipitate dietary tannins (Mehansho et al., 1985; Lu and Bennick 1998). The induction of these salivary proteins in mice impairs the aversion produced by tannin-enriched diets (Glendinning, 1992).

Histatins, for which anti-microbial functions have already been presented, in this chapter, have also been proposed as part of the organism's defence against tannins (Yan and Bennick, 1995), however, their presence was only found in humans (Table 4.1) and certain monkeys.

Besides tannin binding proteins allow the consumption of tannin-rich diets by the animals, they provide a quantitative and qualitative nitrogen saving (Robbins et al., 1991; McArthur et al., 1995). Since they have a higher affinity to tannins than the other salivary proteins, less amounts of protein are required to bind these secondary plant metabolites. In addition, proteins such as PRPs and histatins are rich in non-essential amino acids and in that way these are excreted instead of essential amino acids from dietary proteins. The complexes between tannins and tannin binding proteins remain resistant to the conditions found in digestive tract, both in non-ruminants and ruminant animals (Yang and Russell, 1992).

5. References

Abd-Elnaeim, M.M.M., Zayed, A.E., Leiser, R. 2002. Morphological characteristics of the tongue and its papillae in the donkey (*Equus asinus*): a light and scanning electron microscopical study. Ann. Anat. 184: 473-480.

Adler, E., Hoon, M.A., Mueller, K.L., Chandrashekar, J., Ryba, N.J., Zuker, C.S. 2000. A novel family of mammalian taste receptors. Cell 100: 693-702.

Agre, P., Preston, G.M., Smith, B.L., Jung, J.S., Raina, S., Moon, C., Guggino, W.B.,Nielsen, S. 1993. Aquaporin CHIP: the archetypal molecular water channel. Am. J. Physiol. 265: F463-F476.

Aguirre, A., Levine, M.J., Cohen, R.E., Tabak, L.A. 1987. Immunochemical quantitation of alpha-amylase and secretory IgA in parotid saliva from people of various ages. Arch. Oral Biol. 32: 297-301.

Agungpriyono, S., Yamada, J., Kitamura, N., Nisa, C., Sigit, K., Yamamoto, Y. 1995. Morphology of the dorsal lingual papillae in the lesser mouse deer, *Tragulus Javanicus*. J. Anat. 187: 635-640.

Allen, M.S. 1996. Physical constraints on voluntary intake of forages by ruminants. J. Anim. Sci. 74: 3063.

ASTM, 1989. American Society for the Testing of Materials. 1989. Standard definition of terms relating to sensory evaluation of materials and products. In Annual book of ASTM standards (Vol. 15.07), Philadelphia.

Ann, D.K., Clements, S., Johnstone, E.M., Carlson, DM. 1987. Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein m-RNAs. J Biol. Chem. 262: 899-904.

Ann, D.K., Lin, H.H. 1993. Macaque salivary proline-rich protein: structure, evolution, and expression. Crit. Rev. Oral Biol. 4: 545-551.

Anderson, L.C., Garrett, J.R., Johnson, D.A., Kauffman, D.L., Keller, P.J., Thulin, A. 1984. Influence of circulating catecholamines on protein secretion into rat parotid during parasympathetic stimulation. J. Physiol. 352: 163-171.

Anderson D.J., Hector M.P., Linden R.W.A. 1985. The possible relation between mastication and parotid secretion in the rabbit. J Physiol. 364: 19-29.

Anderson, L.C., Garrett, J.R., Zhang, X., Proctor, G.B., Shori, D.K. 1995. Differential secretion of proteins by rat submandibular acini and granular ducts on graded autonomic nerve stimulations. J. Physiol. 485: 503-511.

Andoh, A., Fujiyama, Y., Kimura, T., Uchihara, H., Sakumoto, H., Bamba, T. 1997. Molecular characterization of complement components (C3, C4, and factor B) in human saliva. J. Clin. Immunol. 17: 404-407.

Anil, M.H.J.N., Mbanya, H.W., Forbes, J.M. 1993. Responses in the voluntary intake of hay or silage by lactating cows to intraruminal infusions of sodium acetate, sodium propionate or rumen distension. Br J. Nutr. 69: 699-712.

Aps, J.K.M., Martens, L.C. 2005. The physiology of saliva and transfer of drugs into saliva. Forensic Science International. 150: 119-131.

Arvidson, K. 1979. Location and variation in number of taste buds in human fungiform papillae. Scand. J. Dent. Res. 87: 435-442.

Asztély, A., Tobin, G., Ekström, J. 1996. The role of circulating catecholamines in the depletion of parotid acinar granules in conscious rats in the cold. Exp. Physiol. 81: 107-117.

Atoji, Y., Yamamoto, Y., Suzuki, Y. 1998. Morphology of the tongue of a male formosan serow (*Capricorns crispus swinhoei*). Anat. Histol. Embryol. 27: 17-19.

Austin, P.J., Suchar, L.A., Robbins, C.T., Hagerman, A.E., 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J. Chem. Ecol. 15: 1335-1347.

Ayer-Le Lievre, C., Ebendal, T., Olson, L., Persson, H. 1989. Detection of nerve growth factor and its mRNA by separate and combined immunohistochemistry and in situ hybridization in mouse salivary glands. Histochem. J. 21: 1-7.

Babu, J.P., Dabbous, M.K. 1986. Interaction of salivary fibronectin with oral streptococci. J. Dent. Res. 65: 1094-1100.

Bacon, J.R., Rhodes, M.J. 2000. Binding affinity of hydrolyzable tannins to parotid saliva and to proline-rich proteins derived from it. J Agric. Food Chem. 48: 838-843.

Baile, C.A., Della-Fera, M.A. 1981. Nature of hunger and satiety control systems in ruminants. J. Dairy Sci. 641: 1140-1152.

Bailey, C.B., Balch, C.C. 1961. Saliva secretion and its relation to feeding in cattle. 2. The composition and rate of secretion of mixed saliva in the cow during rest. Br. J. Nutr. 15: 383-402.

Baron, A., DeCarlo, A., Featherstone, J. 1999. Functional aspects of human salivary cystatins in the oral environment. Oral Dis. 5: 234-240.

Bartoshuk, L.M. 1978. The psychophysics of taste. Am. J. Clin. Nutr. 31: 1968-1077.

Baxter, N.J., Lilley, T.H., Haslam, E., Williamson, M.P. 1997. Multiple interactions between polyphenols and salivary proline-rich proteins repeat result in complexation and precipitation. Biochemistry 36: 5566-5577.

Baylis, L.L., Rolls, E.T. 1991. Responses of neurons in the primate taste cortex to glutamate. Physiol.Behav. 49: 973-979.

Beal, A.M. 1979. Parotid salivary flow and composition during infusion of acetylcholine and atropine into the carotid artery of conscious sodium-replete sheep. Q. J. Exp. Physiol. Cogn. Med. Sci. 64: 89-107.

Bedi, G.S. 1991. The Effects of Autonomic Drugs on the Concentration of Kallikrein-like Proteases and Cysteine-proteinase Inhibitor (Cystatin) in Rat Whole Saliva. J. Dent. Res. 70: 924-930.

Belitz, H.D., Wieser, H. 1985. Bitter compounds: occurrence and structure activity relationships. Food Rev. Int. 1: 271-354.

Bell, F.R., Kitchell, R.L. 1966. Taste reception in the goat, sheep and calf. J. Physiol. 183: 145-151.

Bengaly, K., Mhlongo, S., Nsahlai, I.V. 2007. The effect of wattle tannin on intake, digestibility, nitrogen retention and growth performance of goats in South Africa. Livestock Research for Rural Development 19. [Retrieved May, 2008, from http://www.cipav.org.co/lrrd19/4/beng19050.htm]

Bennick, A. 1982. Salivary proline-rich proteins. Mol. Cell Biochem. 45: 83-99.

Blair-West, J.R., Fernley, R.T., Nelson, J.F., Wintour, E.M., Wright, R.D. 1980. The effects of carbonic anhydrase inhibitors on the anionic composition of sheep´s parotid saliva. J. Physiol. 299: 29-44.

Bolles, R.C., Moot, S.A. Derived motives. Annu. Rev. Psychol. 23: 51-72.

Bolscher, J., Veerman, E., van Niew Amerogen, A., Tulp, A., Verwoerd, D. 1995. Distinct populations of high-M(r) mucins secreted y different human salivary glands discriminated by density gradient electrophoresis. Biochem. J. 309: 801-806.

Borocz-Sczabo, M. 1980. The influence of iron contamination on the sensory properties of liquid foods. Acta Aliment. 9: 341-356.

Bradley, R.M., Mistretta, C.M. 1973. The gustatory sense in foetal sheep during the last third of gestation. J. Physiol. 231: 271-282.

Bradley, R.M., Stedman, H.M., Mistretta, C.M. 1985. Age does not affect numbers of taste buds and papillae in adult rhesus monkeys. Anat. Rec. 212: 246-249.

Bradbury, J. 2004. Taste perception: cracking the code. PLoS Biol. 2: E64.

Brandtzaeg, P. 1998. Synthesis and secretion of human salivary immunoglobulins. In: Garrett, J.R., Ekström, J. Anderson, L.C. (Eds.). Glandular mechanisms of salivary secretion. Karger, Basel, pp.167-200.

Brandtzaeg, P. 2007. Do salivary antibodies reliably reflect both mucosal and systemic immunity? Ann. N.Y. Acad. Sci. 1098: 288-311.

Brasser, S.M., Mozhui, K., Smith, D.V. 2005. Differential covariation in taste responsiveness to bitter stimuli in rats. Chem. Senses 30: 793-799.

Breslin, P.A.S., Gilmore, M.M., Beauchamp, G.K., Green, B.G. 1993. Psychophysical evidence that oral astringency is a tactile sensation. Chem. Senses 18: 405-417.

Breves, G., Rosenhagen, C., Hoeller, H. 1987. Saliva secretion of inorganic phosphorous in phosphorous depleted sheep. J. Vet. Med. A 34: 42-47.

Brooker, J.D., O'Donovan, L.A., Skene, I, Clarke, K., Blackall, L., Muslera, P. 1994. Streptococcus caprinus sp. nov., a tannining-resistant ruminal bacterium from feral goats. Letters in Applied Microbiology 18: 313-318.

Bufe, B., Bresln, P.A., Kuhn, C., Reed, D.R., Tharp, C.D., Slack, J.P., Kim, U.K., Drayna, D., Meyerhof, W. 2005. The molecular basis of individual differences in phenylthiocarbamide and propylthiuracil bitterness perception. Curr. Biol. 15: 322-327.

Burgoyne, R.D., Morgan, A. 2003. Secretory granule exocytosis. Physiol. Rev. 83: 581-632.

Burritt, E., Frost, R. 2006. Animal behavior principles and practices. In: Targeted grazing: a natural approach to vegetation management and landscape enhancement. [Retrieved May, 2007, from http://cnr.uidaho.edu/rx%2Dgrazing/Handbook.htm]

Butendieck, E., Vargas, L. 1998. Presencia y distribution de las papilas linguales en la alpaca (*Lama pacos* Linnaeus, 1758). Arch. Med. Vet. 30: 29-36.

Caicedo, A. Roper, S.D. 2001. Taste receptor cells that discriminate between bitter stimuli. J. Physiol. 291: 1557-1560.

Carlson, D.M. 1993. Salivary proline-rich proteins: biochemistry, molecular biology, and regulation of expression. Crit. Rev. Oral Biol. Med. 4: 495-502.

Carpenter, G.H., Garrett, J.R., Hartley, R.H., Proctor, G.B., 1998. The influence of nerves on the secretion of immunoglobulin A into submandibular saliva in rats. J. Physiol. (Lond.) 512: 567–573.

Carpenter, G.H., Proctor, G.B., Garrett, J.R. 2005. Preganglionic parasympathectomy decreases salivary SIgA secretion rates from the rat submandibular gland. Journal of Neuroimmunol. 160: 4-11.

Carpenter, G.H., Proctor, G.B. 2000. Double electrophoretic separation and lectin analysis of the component chains of secretory immunoglobulin A from human saliva. Electrophoresis 21: 1446-1453.

Carr, D.H., Titchen, D.A. 1978. Post prandial changes in parotid salivary secretion and plasma osmolality and the effects of intravenous infusions of saline solutions. Q.J. Exp. Physiol. 63: 1-21.

Carr, D.H. 1984. The regulation of parotid and submandibular salivary secretion in sheep. Q. J. Exp. Physiol. 69: 589-597.

Carter, R.R., Grovum, W.L. 1990. A review of the physiological significance of hypertonic body fluids on feed intake and ruminal function: salivation, motility and microbes. J. Anim. Sci. 68: 2811-2832.

Castle, A.M., Huang, A.Y., Castle, J.D. 2002. The minor regulated pathway, a rapid component of salivary secretion, may provide docking/fusion sites for granule exocytosis at the apical surface of acinar cells. J. Cell Sci. 115: 2963-2973.

Castle, D., Castle, A. 1998. Intracellular transport and secretion of salivary proteins. Crit. Rev. Oral Biol. Med. 9: 4-22.

Castle, J.D., Castle, A.M. 1996. Two regulated secretory pathways for newly synthesized parotid salivary proteins are distinguished by doses of secretagogues. J Cell Sci. 109: 2591-2599.

Castle, J.D. 1998. Protein secretion by rat parotid acinar cells. Pathways and regulation. Ann. N. Y. Acad. Sci. 842: 115-124.

Chamorro, C.A., de Paz, P., Sandoval, J., Fernandez, J.G. 1986. Comparative scanning electron-microscopic study of the lingual papillae in two species of domestic mammal (*Equus caballus* and *Bos taurus*). 1. Gustatory papillae. Acta Anat. (Basel) 125: 83-87.

Chamorro, C.A., de Paz, P., Fernández, J.G., Anel L. 1993. Fungiform papillae of the pig and wild boar analyzed by scanning electron microscopy. Scanning Micros. 7: 313-320.

Chandrashekar, J., Hoon, M.A., Ryba, N.J., Zuker, C.S. 2006. The receptors and cells for mammalian taste. Nature 444: 288-294.

Chaptini, L., Peikin, S. 2008. Neuroendocrine regulation of food intake. Curr. Opin. Gastroenterol. 24: 223-229.

Charlton, A.J., Baxter, N.J. Kham, M.L., Moir, A.J., Haslam, E., Davies, A.P., Williamson, M.P. 2002. Polyphenol/peptide binding and precipitation. J. Agric. Food Chem. 50: 1593-1601.

Chauncey H.H., Shannon, I.L. 1960. Parotid gland secretion rate as a method for measuring response to gustatory stimuli in humans. Proc. Soc. Exp. Biol. Med. 103: 459-463.

Cheng, L.H., Robinson, P.P. 1991. The distribution of fungiform papillae and taste buds on human tongue. Arch. Oral Biol. 36: 583-589.

Christensen, C.M., Brand, J.G., Malamud, D. 1987. Salivary changes in solution pH: a source of individual differences in sour taste perception. Physiol. Behav. 40: 221-227.

Clauss, M., Lason, K., Gehrke, J., Lechner-Doll, M., Fickel, J., Grune, T., Streich, W.J., 2003. Captive roe deer (*Capreolus capreolus*) select for low amounts of tannic acid but not quebracho: fluctuation of preferences and potential benefits. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136: 369-382.

Clauss, M., Gehrke, J., Hatt, J.M., Dierenfeld, E.S., Flach, E.J., Hermes, R., Castell, J., Streich, W.J., Fickel, J. 2005. Tannin-binding salivary proteins in three captive rhinoceros species. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 140: 67-72.

Clements, S., Mhansho, H., Carlson, D.M. 1985. Novel multigene families encoding highly repetitive peptide sequences. Sequence analyses of rat and mouse proline-rich protein cDNA. J. Biol. Chem. 260: 13471-13477.

Cole, M.F., Bowen, W.H., Sierra, L., Espinal, F., Aguirra, M. Kingman, A., Kemp, L.J., Gomez, I., Reilly, J.A., Hsu, D., Ciardi, J.E., Gillespie, G. 1978. Immunoglobulins and antibodies in plaque fluid and saliva in two populations with contrasting levels of caries. Adv. Exp. Med. Biol. 107: 383-392.

Colonna, A.E., Adams, D.O., Noble, A.C. 2004 Comparison of procedures for reducing astringency carry-over effects of red wines. Aust J Grape Wine Res 10: 26-31.

Compton, J.S., Nelsoni, J., Wright, R.D., Young, J.A. 1980. A micropuncture investigation of electrolyte transport in the parotid glands of sodium-replete and sodium-depleted sheep. J. Physiol. 309: 429-446.

Cooper, S.M., Owen-Smith, N. 1985. Condensed tannins deter feeding by browsing ruminants in a South African savanna. Oecologia 67: 142-146.

Cripps, A.W., Lascelles, A.K. 1976. The origin of immunoglobulins in salivary secretion of sheep. Aust J Exp Biol Med Sci 54: 191–195.

Critchley, H.D., Rolls, E.T. 1996a. Hunger and satiety modify the responses of olfactory and visual neurons in the primate orbitofrontal cortex. J. Neurophysiol. 75: 1673-1686.

Critchley, H.D., Rolls, E.T. 1996b. Response of primate taste cortex neurons to the astringent tastants tannic aid. Chem. Senses 21: 135-145.

Dasgupta, K., Singh, A., Ireland, W.P. 1990. Taste bud density in circunvallate and fungiform papillae of the bovine tongue. Histol. Histopathol. 5: 169-172.

Davies, R.O., Kare, M.R., Cagan, R.H. 1979. Distribution of taste buds on fungiform and circumvallate papillae of bovine tongue. Anat. Rec. 195: 443-446.

Dawes, C. 1969. The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human parotid saliva. Arch Oral Biol. 14: 277-294.

Dawes, C. 1972. Circadian rhythms in human salivary flow rate and composition. J. Physiol. 220: 529-545.

Dawes, C. 1974. Rhythms in salivary flow rate and composition. Int. J. Chronobiol. 2: 253-279.

Dawes, C. 1981. The effects of exercise on protein and electrolyte secretion in parotid saliva. J. Physiol. 320: 139-148.

Dawes, C. 1984. Stimulus effects on protein and electrolyte concentrations in parotid saliva. J. Physiol. 346: 579-588.

Dawes, C. 1987. Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. J. Dent. Res. 66: 740-744.

Dawes, C., Kubieniec, K. 2004. The effects of prolonged gum chewing on salivary flow rate and composition. Arch. Oral Biol. 49: 665-669.

de Freitas, V., Mateus, N. 2001. Structural features of procyanidin interactions with salivary proteins. J. Agric. Food Chem. 49: 940-945.

de Wijk, R.A., Engelen, L., Prinz, J.F. 2003. The role of intra-oral manipulation in the perception of sensory attributes. Appetite 40: 1-7.

Delwiche, J., O'Mahny, M. 1996.Changes in secreted salivary sodium are sufficient to alter salt taste sensitivity: use of signal detection measures with continuous monitoring of the oral environment. Physiol. Behav. 59: 605-611.

Den, R., Toda, M., Nagasawa, S., Kitamura, K., Morimoto, K. 2007. Circadian rhythm of human salivary chromogranin A. Biomed Res 28: 57-60.

Denny, P., Hagen, F.K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G.S., Boontheung, P., Cociorva, D., Delahunty, C.M., Denny, T., Dunsmore, J., Faull, K.F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S.C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J.A., Ogorzalek Loo, R.R., Malamud, D., Melvin, J.E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S.K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M.A., Takashima, J., Than, S., Wang, J., Whitelegge, J.P., Witkowska, H.E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D.T., Yates, J.R. 3rd, Fisher, S.J. 2008. The Proteomes of Human Parotid and Submandibular/Sublingual Gland Salivas Collected as the Ductal Secretions. J. Proteome Res. 7: 1994-2006.

Devendra, C. 1990. Comparative aspects of digestive physiology and nutrition in goats and sheep. In: Devendra, C., Imazumi, E. (Eds.) Ruminant Nutrition and Physiology in Asia. pp 45-60.

Dipaola, C., Madel, I.D. 1980. Lactoferrin concentration in human parotid saliva as measured by an Enzyme Linked Immunosorbent Assay (ELISA). J. Dent. Res. 59: 1463-1465.

Distel, R.A., Provenza, F.D., 1991. Experience early in life affects voluntary intake of blackbrush by gotas. J. Chem. Ecol. 17: 431-450.

Dodds, M.W., Johnson, D.A., Yeh, C.K. 2005. Health benefits of saliva: a review. J. Dent. 33: 223-233.

Domingue, B.M.F., Dellow, D.W., Barry, T.N. 1991. The efficiency of chewing during eating and ruminating in goats and sheep. Br. J. Nutr. 65: 355-363.

du Toit, J.T., Provenza, F.D., Nastis, A. 1991. Conditioned taste aversions: how sick must a ruminant get before it learns about toxicity in foods. Applied Animal Behaviour Science. 30: 35-46.

Ebersole, J.L., Taubman, M.A., Smith, D.J. 1979. The effect of neonatal thymectomy on the level of salivary and serum immunoglobulins in rats. Immunology 36: 649-657.

Edwards, A.V., Titchen, D.A. 1992. Synergism in the autonomic regulation of parotid secretion of protein in sheep. J. Physiol. 451: 1-15.

Edwards, A.V., Tobin, G., Ekström, J., Bloom, S.R. 1996. Nitric oxide and release of the peptide VIP from parasympathetic terminals in the submandibular gland of the anaesthetized cat. Exp. Physiol. 81: 349-359.

Edwards, A.V., Titchen, D.A. 2003. Autonomic control of protein production by the parotid gland of the sheep. Auton Neurosci-Basic 103: 38-49.

Ekström, J., Mansson, B., Tobin, G. 1983. Vasoactive intestinal peptide evoked secretion of fluid and protein from rat salivary glands and the development of supersensitivty. Acta Physiol. Scand. 119: 169-175.

Ekström, J., Marshall, T., Tobin, G., Williams, K.M. 1994. Electrophoretic analysis of cat parotid saliva in response to parasympathetic stimulation or infusion of bethanechol. Acta Physiol. Scand. 152: 57-61.

Ekström, J., Marshall, T., Tobin, G., Williams, K.M. 1996. Electrophoretic analysis of rat parotid salivary composition: investigation of the parasympathetic atropine-resistant secretion. Acta Physiol. Scand. 156: 75-79.

Ekström, J. 1998. Parasympathetic non-adrenergic, non-cholinergic mechanisms in salivary glands and their role in reflex secretion. Eur. J. Morphol. 36: 208-212.

Eliasson, L., Birkhed, D., Osterberg, T., Carlén, A. 2006. Minor salivar gland secretion rates and immunoglobulin A in adults and the elderly. Eur. J. Oral Sci. 114: 494-499.

Emmelin, N. 1987. Nerve interactions in salivary glands. J. Dent. Res. 66: 509-517.

Emura, S., Tamada, A., Hayakawa, D., Chen, H., Yano, R., Shoumura, S. 1999. Morphology of the dorsal lingual papillae in the blackbuck, *Antilope cervicapra*. Okajimas Folia Anat. Jpn. 76: 247-253.

Emura, S., Tamada, A., Hayakawa, D., Chen, H., Shoumura,S. 2000a. Morphology of the dorsal lingual papillae in the barbary sheep, *Ammotragus lervia*. Okajimas Folia Anat. Jpn. 77: 39-45.

Emura, S., Tamada, A., Hayakawa, D., Chen, H., Shoumura, S. 2000b. Morphology of the dorsallingual papillae in the black rhinoceros (*Diceros Bicornis*). Anat. Histol. Embryol. 29: 371-374.

Engelen, L., De Wijk, J.F., Prinz, A., Van Der Bilt, A., Bosman, F. 2003. The relation between saliva flow rate after different stimulations and the perception of flavor and texture attributes in custard desserts. Physiol Behav 78:165-169.

Erickson, R.P. 2008. A study of the science of taste: on the origins and influence of the core ideas. Behav. Brain Sci. 31: 59-105.

Ericson, T., Rundegren, J. 1983. Characterization of a salivary agglutinin reacting with a serotype c strain of Streptococcus mutans. Eur. J. Biochem. 133: 255-261.

Fernley, R.T., Wright, R.D., Coghlan, J.P. 1991. Radioimmunoassay of carbonic anhydrase VI in saliva and sheep tissues. Biochem. J. 274: 313-316.

Fernley, R.T., Farthing, J., Cooper, E.J. 1995. Radioimmunoassay for salivary carbonic anhydrase in human parotid saliva. Arch. Oral Biol. 40: 567-569.

Ferreira, F. D., Robison, R., Hand, A. R., Bennick, A. 1992. Differential expression of proline-rich proteins in rabbit salivary glands. J. Histochem. Cytochem. 40: 1393–1404.

Fickel, J., Göritz, F., Joest, B.A., Hildebrandt, T., Hofmann, R.R., Breves, G., 1998. Analysis of parotid and mixed saliva in roe deer. J. Comp. Physiol. B 168: 257-264.

Fisher, D.S. 2002. A review of a key factors regulating voluntary feed intake in ruminants. Crop Sci. 42: 1651-1655.

Foley, W.J., Iason, G.R., McArthur, C. 1999. The role of plant secondary metabolites in the nutritional ecology of mammalian herbivores: how far have we come in 25 years? In: Nutritional Ecology of Herbivores, pp. 103-209. J.G. Jung and G.C. Fahey (eds). Savoy, IL: American Society of Animal Science

Forbes, J.M. 1996. Integration of regulatory signals controlling forage intake in ruminants. J.Anim. Sci. 74: 3029-3035.

Forbes, J.M. 2001. Consequences of feeding for future feeding. Comp. Biochem. Physiol. A Mol. Int. Physiol. 128: 461-468.

Frost, B., Ruyle, G.B. 1993. Range management terms and definitions. Rangeland Management 15: 15-25.

Frey, R., Hofmann, R.R. 1998. Salivary glands of the takin (*Budorcas taxicolor*, Mammalia, Bovideae) with special consideration of the glandula zygomatica. Zool Anz. 237: 139-153.

Frey, R., Markgraf, U., Hofmann, R.R. 2001. Evolutionary morphology of the zygomatic gland and lacrimal Bulla in Roe Deer (*Capreols capreolus* Linnaeus, 1758 – Mammalia, Cervidae). Zoologischer Anzeiger. 240: 181-195.

Funato, H., Atoji, Y., Suzuki, Y., Sugimura, M. 1985. Morphological studies on the tongue of wild Japanese serrow, *Capricornis crispus*. Res. Bull. Fac. Agr. Gifu Univ. 50: 205-219.

Gamble, J. Smaje, L.H., Spencer, P.D. 1988. Filtration coefficient and osmotic reflection coefficient to albumin in rabbit submandibular gland capillaries. J. Physiol. 398: 15-32.

Garcia, H.F., Garcia-Poblete, E., Moro-Rodriguez, E., Catala-Rodriguez, M., Rico-Morales, M.L., Garcia-Gomez de las Heras, M.S. 2002. Histomorphometrical study of the submandibular gland ductal system in the rat. Histol Histopathol. 17: 813-816.

Garrett, J.R., Parsons, P.A. 1976. Movement of horseradish peroxidase in rabbit submandibular glands after ductal injection. Histochem. J. 8: 177-189.

Garrett, J.R., Emmelin, N. 1979. Activities of salivary myoepithelial cells: a review. Med. Biol. 57: 1-28.

Garrett, J.R. 1987. The proper role of nerves in salivary secretion: a review. J. Dent. Res. 66: 387-397.

Garrett, J.R., Chao, J., Proctor, G.B., Wang, C., Zhang, X.S., Chan, K.M., Shori, D.K. 1995. Influences of secretory activities in rat submandibular glands on tissue kallikrein circulating in the blood. Exp. Physiol. 80: 429-440.

Garrett, J.R. 1998a. Historical introduction to salivary secretion. In: Garrett, J.R., Ekström, J. Anderson, L.C. (Eds.). Glandular mechanisms of salivary secretion. Karger, Basel, pp.1-20.

Garrett, J.R. 1998b. Movements of organic molecules from blood to saliva and from glands to blood. In: Garrett, J.R., Ekström, J., Anderson, L.C. (Eds): Glandular mechanisms of salivary secretion. Front Oral Biol. Karger, vol 10, pp 153-166

Gavião, M.B.D., Engelen, L., Van Der Bilt, A. 2004. Chewing behavior and saliva secretion. Eur J Oral Sci. 112: 19-24.

Gehrke, J. 2001. Investigations of tannin-binding salivary proteins of roe deer and other ruminants. PhD Thesis, University of Potsdam, Potsdam (German with English summary).

Ghafouri, B., Tagesson, C., Lindahl, M., 2003. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. Proteomics 3: 1003-1015.

Gilboa, N. 1995. Negative effects of tannins in livestock and their neutralization. PhD. Thesis, The Hebrew Univ. of Jerusalem, Israel (Hebrew with English summary).

Gilbertson, T.A., Damak, S., Margolskee, R.F. 2000. The molecular physiology of taste transduction. Curr. Opin. Neurobiol. 10: 519-527.

Gillece-Castro, B.L., Prakobphol, A., Burlingame, A.L., Leffler, H., Fisher, S.J. 1991. Structure and bacterial receptor activity of a human salivary proline-rich glycoprotein. J. Biol. Chem. 266: 17358-17368.

Girard, L.R., Castle, A.M., Hand, A.R., Castle, J.D., Mirels, L. 1993. Characterization of common salivary protein 1, a product of rat submandibular, sublingual and parotid glands. J. Biol. Chem. 268: 26592-26601.

Gjörstrup, P. 1980. Taste and chewing as stimuli for the secretion of amylase from the parotid gland of the rabbit. Acta Physiol. Scand. 110: 295-301.

Glendinning, J.I., 1992. Effect of salivary proline-rich proteins on ingestive reponses to tannic acid in mice. Chem. Senses 17: 85-116.

Glendinning, J.I. 1994. Is the bitter rejection response always adaptative? Physiol. Behav. 56: 1217-1227.

Glendinning, J.I., Davs, A., Ramaswamy, S. 2002. Contribution of different taste cells and signaling pathways to the discrimination of "bitter" taste stimuli by an insect. J. Neurosci. 22: 7281-7287.

Glijer, B., Peterfy,C., Tenenhouse, A. 1985. The effect of vitamin D deficiency on the secretion of saliva by rat parotid gland in vivo. J. Phsiol. 363: 323-334.

Goatcher, W.D., Church, D.C. 1970. Taste responses in ruminants. IV Reactions of pygmy goats, normal goats, sheep and cattle to acetic acid and quinine hydrochloride. J Anim Sci. 31: 373-382.

Gong, Y., Hodgson, J., Lambert, M.G., Gordon, I.L. 1996. Short-term ingestive behaviour of sheep and goats grazing grasses and legumes. 1. Comparison of bite weight, bite rate, and bite dimensions for forages at two stages of maturity. New Zeal J Agr Res. 39: 63-73.

Görg, A., Postel, W., Günther, S., Weser, J. 1985. Improved horizontal two-dimensional electrophoresis with hybrid isoelectric focusing in immobilized pH gradients in the first dimension and laying-on transfer to the second dimension. Electrophoresis 6: 599–604.

Görg, A., Postel, W., Günther, S. 1988. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9: 531–546.

Göritz, F., Hildebrandt, T.H., Hofmann, R.R., Pitra, C., 1994. Comparative salivary studies using a new technique yielding uncontaminated saliva from CS, IM and GR ruminant species Proc. Soc. Nutr. Physol. 3: 319.

Gorr, S.-U., Venkatesh, S.G., Darling, D.S. 2005 Parotid secretory granules: crossroads of secretory pathways and protein storage. J. Dent. Res. 84: 500-509.

Goto, M., Fujimoto, W., Nio, J., Iwanaga, T., Kawasaki, T. 2003. Immunohistochemical demonstration of acidic mammalian chitinase in the mouse salivary gland and gastric mucosa. Arch. Oral Biol. 48: 701-707.

Green, B.G. 1993. Oral astringency: a tactile component of flavor. Acta Psychol. Amst. 84: 119-125.

Gresik, E.W. 1994. The granular convoluted tubule cell of rodent submandibular glands. Microsc. Res. Tech. 27: 1-24.

Grovum, W.L., Gonzalez, J.S. 2000. Regions in the brainstem and frontal cortex where electrical stimulation elicits parotid and submandibular saliva secretion in sheep. Brain Res. 852: 1-9.

Guerrera, I.C., Kleiner, O. 2005. Application of mass spectrometry in proteomics. Biosci. Rep. 25: 71-93.

Guinard, J.-X., Mazzucchelli, R. 1996 The sensory perception of texture and mouthfeel. Trends Food Sci. Technol. 7: 213-219.

Guinard, J.-X., Zoumas-Morse, C., Walchak, C. 1997. Relation between parotid saliva flow and composition and perception of gustatory and trigeminal stimuli in foods. Physiol. Behav. 63: 109-118.

Gurkan, S., Bradley, R.M. 1988. Secretions of von Ebner's glands influence responses from taste buds in rat circunvallate papilla. Chem. Senses 13: 655-661.

Gusman, H., Leone, C., Helmerhorst, E.J., Nunn, M., Flora, B., Troxler, R.F., Oppenheim, F.G. 2004. Human salivary gland-specific daily variations in histatin concentrations determined by a novel quantitation technique. Arch. Oral Biol. 49: 11-22.

Hagerman, A.E., Butler, L.G. 1981. The specificity of proanthocyanidin-protein interactions. J. Biol. Chem. 256: 4494-4497.

Hagerman, A.E., Robbins, C.T. 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. Can. J. Zool. 71: 628-633.

Hagerman, A.E., Rice, M.E., Ritchardt, N.T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4-8) catechin (procyanidin). J. Agric. Food Chem. 4: 2590-2595.

Haigh, B., Hooda, K., Broadhurst, M., Medele, S., Callaghan, M., Smolenski, G., Dines, M., Wheeler, T. 2008. The bovine salivary proteins BSP30a and BSP30b are independently expressed BPI-like proteins with anti-*Pseudomonas* activity. Mol Immunol 45: 1944–1951.

Hanna, S.J., Edwards, A.V. 1998. The role of nitric oxide in the control of protein secretion in the parotid gland of anaesthetized sheep. Exp. Physiol. 83: 533-544.

Hand, A.R. 1979. Synthesis of secretory and plasma membrane glycoproteins by striated duct cells of rat salivary glands as visualized by radioautography after 3H-fucose injection. Anat. Rec. 195: 317-339.

Hardt, M., Witkowska, E., Wobb, S., Thomas, L.R., Dixon, S.E., Hall, S.C., Fisher, S.J. 2005. Assessing the effects of diurnal variation on the composition of human parotid saliva: quantitative analysis of native peptides using iTRAQ reagents. Anal. Chem. 77: 4947-4954.

Hashimoto, S., Ochiai, S., Muramatsu, T., Shimono, M. 2000. Tight Junctions in the Rat Parotid Gland Eur J Morphol. 38: 263-267.

Haslam, E. 1998. Practical polyphenols. From structure to molecular recognition and physiological action. Cambridge University Press.

Hatton, M.N., Loomis, R.E., Levine, M.J., Tabak, L.A. 1985. Masticatory lubrication. The role of carbohydrate in the lubricating property of a salivary glycoprotein-albumin complex. Biochem. J. 230: 817-820.

Hay, D.I., Moreno, E.C. 1979. Differential adsorption and chemical affinities of proteins for apatitic surfaces. J. Dent. Res. 58 (Spec. Issue B): 930-942.

Hay, D.I., Smith, D.J., Schluckebier, S.K., Moreno, E.C. 1984. Relationship between concentration of human salivary statherin and inhibition of calcium phosphate precipitation in stimulated human parotid saliva. J. Dent. Res. 63: 857-863.

Hayashi, M., Komazaki, S., Ishikawa, T. 2003. An inwardly rectifying K^+ channel in bovine parotid acinar cells: possible involvement of Kir2.1. J. Physiol. 547: 255-269.

Hector, M.P., Linen, R.W. 1987. The possible role of periodontal mechanoreceptors in the control of parotid secretion in man. Q. J. Exp. Physiol. 72: 285-301.

Henkin, R.I., Martin, B.M., Agarwal, R.P. 1999. Decreased parotid saliva gustin/carbonic anhydrase VI secretion: an enzyme disorder manifested by gustatory and olfactory dysfunction. Am. J. Med. Sci. 318: 380-391.

Henskens, Y.M., van der Weijden, F.A., van den Keijbus, P.A., Veerman, E.C., Timmerman, M. F., van der Velden, U., Amerongen, A.V. 1996. Effect of periodontal treatment on the protein composition of whole and parotid saliva. J. Periodontol. 67: 205-212.

Hepper, P.G. 1988. Adaptative fetal learning: prenatal exposure to garlic affects postnatal preferences. Anim. Behav. 36: 935-936.

Hershkovich, O., Nagler, R.M. 2004. Biochemical analysis of saliva and taste acuity evaluation in patients with burning mouth syndrome, xerostomia and/or gustatory disturbances. Arch. Oral Biol. 49: 515-22.

Hirtz, C., Chevalier, Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., de Périère, D., 2005. Complexity of the human whole saliva proteome. J. Physiol. Biochem. 61: 469-480.

Hofmann, R.R. 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants. A comparative view of their digestive systems. Oecologia 78: 443-457.

Hodson, N.A., Linden, R.W.A. 2006. The effect of monsodium glutamate on parotid salivary flow in comparison to the response to representatives of the other four basic tastes. Physiol Behav. 89: 711-717.

Holland, V.F., Zapighi, G.A., Simon, S.A. 1989. Morphology of fungiform papillae in canine lingual epithelium: location of intercellular junctions in the epithelium. J. Comp. Neurol. 279: 13-27.

Horne, J., Hayes, J., Lawless, H.T. 2002. Turbidity as a measure of salivary protein reactions with astringent substances. Chem. Senses 27: 653-659.

Hosley, M.A., Oakley, B. 1987. Postnatal development of the vallate papilla and taste buds in rats. Anat. Rec. 218: 216-222.

Huang, A.Y., Castle, A.M., Hinon, B.T., Castle, D. 2001. Resting (basal) secretion of proteins is provided by the minor regulated and constitutive-like pathways and not granule exocytosis in parotid acinar cells. J Biol Chem. 276: 22296-22306.

Huang, C.M. 2004. Comparative proteomic analysis of human whole saliva. Arch. Oral Biol. 49: 951-962.

Huang, A.L., Chen, X., Hoon, M.A., Chandrashekar, J., Guo, W., Tränkner, D., Ryba, N.J., Zuker, C.S. 2006. The cells and logic for mammalian sour taste detection. Nature 442: 934-938.

Hull, C.L. 1943. Principles of behavior: an introduction to behavior Theory. Oxford: Appleton-Century.

Huq, N.L., Cross, K.J., Ung, M., Myroforidis, H., Veith, P.D., Chen, D., Staton, D., He, H., Ward, B.R., Reynols, E.C. 2007. A review of the salivary proteome and peptidome and saliva-derived peptide therapeutics. Int. J. Res. Ther. 13: 547-564.

Hwang, J.M., Hofstetter, J.R., Bonhomme, F., Karn, R.C. 1997. The microevolution of mouse salivary androgen-binding protein (ABP) paralleled subspeciation of *Mus musculus*. J. Hered. 88: 93-97.

Iason, G. 2005. The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. Proc. Nutr. Soc. 64: 123-131.

Ikebe, K., Matsuda, K.I., Morii, K., Hazeyama, T., Kagawa, R., Ogawa,T., Nokubi, T. 2007. Relationship between bite force and salivary flow in older adults. Oral Surg. Oral Med. Oral Pathol. Radiol. Endod. 104: 510-515.

Ikemoto, S., Matsushima, Y. 1984. Sex and strain differences of mouse variant salivary proteins. Differentiation. 26: 55-58.

Illius, A.W., Jessop, N.S. 1996. Metabolic constraints on voluntary intake in ruminants. J. Anim. Sci. 74: 3052-3062.

Inoue, H., Ono, K., Masuda, W., Morimoto, Y., Tanaka, T., Yocota, M., Inenaga, K. 2006. Gender difference in unstimulated whole saliva flow rate and salivary gland sizes. Arch Oral Biol. 51: 1055-1060.

Isenman, L., Liebow, C., Rothman, S. 1999. The endocrine secretion of mammalian digestive enzymes by exocrine glands. Am. J. Physiol. Endocrinol. Metab. 276: 223-232.

Ishikawa, T., Cook, D.I. 1993. A Ca(2+)-activated Cl- current in sheep parotid secretory cells. J. Membr. Biol. 135: 261-271.

Ishikawa, Y., Cho, G., Yuan, Z., Skowronski, M.T., Pan, Y., Ishida, H. 2006 Water channels and zymogen granules in salivary glands. J. Pharmacol. Sci. 100: 495-512.

Ito, K., Morikawa, M., Inenaga, K. 2001. The effect of food consistency and dehdrationon reflex parotid and submandibular salivary secretion in conscious rats. Arch. Oral Biol. 46: 353-363.

Jacob, S., Poddar, S. 1989. Ultrastructure of the ferret sublingual gland. Acta Anat. (Basel) 135: 344-346.

Jansman, A.J.M., Frohlich, A.A., Marquardt, R.R. 1994. Production of proline-rich proteins by the parotid glands of rats is enhanced by feeding diets containing tannins from Faba beans (*Vicia faba* L). J. Nutr. 124: 249–258.

Jensen-Kjeilen, J.C., Brodin, P., Aars, H., Berg, T. 1987. Parotid salivary flow in response to mechanical and gustatory stimulation in man. Acta Physiol. Scand. 131: 169-175.

Johnson, D.A. 1982. Effect of a liquid diet on the protein composition of rat parotid saliva. J. Dent. Res. 61: 691-696.

Johnson, D.A. 1984. Changes in rat parotid salivary proteins associated with liquid diet-induced gland atrophy and isoproterenol-induced gland enlargement. Arch. Oral Biol. 29: 215-221.

Johnson, D.A., Alvares, O.F., Etzel, K.R., Kalu, D.N. 1987. Regulation of salivary proteins. J. Dent. Res. 66: 576-582.

Johnson, D.A., Lopez, H., Navial, J.M. 1995. Effects of Protein Deficiency and Diet Consistency on the Parotid Gland and Parotid Saliva of Rats. J. Dent. Res. 74: 1444-1452.

Jones, L.M., Fontanini, A. Katz, D.B. 2006. Gustatory processing: a dynamic system approach. Curr Opin Neurobiol 16: 420-428.

Jung, H.-S., Akita, K., Kim, J-Y. 2004. Spacing patterns o tongue surface-guatatory papilla. Int. J. Dev. Biol. 48: 157-161.

Juntheikki, M.R. 1996. Comparison of tannin-binding proteins in saliva of Scandinavian and North American moose (*Alces alces*). Biochem. Syst. Ecol. 24: 595-601.

Juntheikki, M.-R., Julkunen-Tiitto, R., Hagerman, A. 1996. Salivary tannin-binding proteins in root vole *(Microtus oeconomus).* Biochem. Syst. Ecol. 24: 25-35.

Kababya, D., Perevolotsky, A., Bruckental, I., Landau, S. 1998. Selection of diets by dual-purpose Mamber goats in Mediterranean woodland. J Agr Sci. 131: 221-228.

Kadohisa, M., Rolls, E.T., Verhagen, J.V. 2004. Orbitofrontal cortex neuronal representation of temperature and capsaicin in the mouth. Neuroscience 127: 207-221.

Kalikow, T.J. 1983. Konrad Lorenz's ethological theory: Explanation and ideology, 1938–1943. J. Hist. Biol. 16: 39-73.

Kallithraka, S., Bakker, J., Cliford, M.N., Vallis, L. 2001. Correlations between saliva protein composition and some T-I parameters of astringency. Food Qual Prefer 12: 145-152.

Kandra, L., Gyémánt, G., Zajácz, A., Batta, G. 2004. Inhibitory effects of tannin on human salivary alpha-amylase. Biochem. Biophys. Res. Commun. 31: 1265-1271.

Karas M., Hillenkamp F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 Daltons. Anal. Chem. 60: 2299-2301.

Katsukawa, H., Imoto, T., Ninomiya, Y. 1999. Induction of salivary gurmarin-binding proteins in rats fed gymnema-containing diets. Chem. Senses 24: 387-392.

Katsukawa, H., Shang, Y., Nakashima, K., Yang, K.H., Sugita, D., Mishima, K., Nakata, M., Ninomiya, Y., Sugimura, T. 2002. Salivary cystatins influence ingestion of capsaicin-containing diets in the rat. Life Sci. 71: 457-467.

Kawai, T., Fushiki, T. 2003. Importance of lipolysis in oral cavity for orosensory detection of fat. Am. J. Physiol. 285: R447-R454.

Kay, R.N.B. 1958. Continuous and reflex secretion by the parotid gland in ruminants. *J. Physiol.* 144: 463-475.

Kay, R.N.B. 1960. The rate of flow and composition of various salivary secretions in sheep and calves. *J Physiol.* 150: 515-537.

Kay, R.N.B. 1987. Weights of salivary glands in some ruminant animals. J. Zool. Lond. 211: 431-436.

Ketelaars, J.J.H.M., Tolkamp, B.J. 1996. Oxygen efficiency and the control of energy flow in animals and humans. J. Anim. Sci. 74: 3036-3051.

Kivela, J., Parkkila, S., Waheed, A., Parkkila, A.K., William, S.S., Rajaniemi, H. 1997. Secretory carbonic anhydrase isoenzyme (CA VI) in human serum. Clin Chem 43: 2318-2322.

Klose, J. 1975. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26: 231–243.

Kobayashi, K., Kumakura, M., Shinkai, H., Ishii, K. 1994. Three-dimensional fine structure of the lingual papillae and their connective tissue cores in the human tongue. Kaibogaku Zasshi. 69: 624-635.

Koller, M.M., Purushotham, K.R., Maeda, N., Scarpace, P.J., Humphreys-Beher, M.G. 2000. Desipramine induced changes in salivary proteins, cultivable oral microbiota and gingival health in aging female NIA Fischer 344 rats. Life Sci. 68: 445-455.

Komine, K., Kuroishi, T., Ozawa, A., Komine, Y., Minami, T., Shimauchi, H., Sugawara, S. 2007. Cleaved inflammatory lactoferrin peptides in parotid saliva of perodontitis patients. Mol Immunol 44: 1498-1508.

Korsrud, F.R., Brandtzaeg, P. 1980. Quantitative immunohistochemistry of immunoglobulin- and J-chain-producing cells in human parotid and submandibular salivary glands. Immunology 39: 129-140.

Kousvelari, E.E., Baratz, R.S., Burke, B., Oppenheim, F.G. 1980. Immunochemical identification and determination of proline-rich proteins in salivary secretions, enamel pellicle, and glandular tissue specimens. J. Dent. Res. 59: 1430-1438.

Kumar, R., Singh, M. 1984. Tannins, their adverse role in ruminant nutrition. J. Agric. Food Chem. 32: 447-453.

Kumar, R., Vaithiyanathan, S. 1990. Occurrence, nutritional significce and effect on animals of tannins in tree leaves. Anim. Feed Sci. Technol. 30: 21-23.

Kurahashi, M., Inomata, K. 1999. Effects of dietary consistency and water content on parotid amylase secretion and gastric starch digestion in rats. Arch. Oral Biol. 44: 1013-1019.

Kurabuchi, S., Da, J.T., Gresik, E.W., Hosoi, K. 1999. An unusual sexually dimorphic mosaic distribution of a subset of kallikreins in the granular convoluted tubule of the mouse submandibular gland detected by an antibody with restricted immunoreactivity. Histohem. J. 31: 19-28.

Kyriazakis, I., Tolkamp, B.J., Emmans, G. 1999. Diet selection and animal state: an integrative framework. Proceedings of the Nutrition Society. 58: 765-772.

Lamkin, M.S., Miglari, D., Yao, Y., Troxler, R.F., Oppenheim, F.G. 2001. New in vitro model for the acquired enamel pellicle: pellicles formed from whole saliva show inter-subject consistency in protein composition and proteolytic fragmentation patterns. J. Dent. Res. 80: 385-388.

Landau, S. Perevolotsky, A., Bonfil, D., Barkai, D., Silanikove, N. Utilization of low quality resources by small ruminants in Mediterranean agro-pastoral systems: the case of browse and aftermath cereal stubble. Livest Prod Sci 64: 39-49.

Laugerette, F., Passilly-Degrace, P., Patris, B., Niot, I., Febraio, M., Montmayeur, J.P., Besnard, P. 2005. CD 36 involvement in orsensory detection of dietary lipids, spontaneous fat preference, and digestive secretions. J. Clin. Invest. 115: 3177-3184.

Laugerette, F., Gaillard, D., Passilly-Degrace, P., Niot, I., Besnard, P. 2007. Do we taste fat? Biochimie 89: 265-269.

Launchbaugh, K.L., Walker, J.W., Taylor, C.A. 1999. Foraging behaviour: experience or inheritance? In: Launchbaugh, K.L., Sanders, K.D. (Eds), Grazing behaviour of livestock and wildlive. Univ. of Idaho, Moscow, ID, pp. 28-33.

Lawless, H.T., Stevens, D.A., Chapman, K.W., Kurtz, A. 2005. Metallic taste from electrical and chemical stimulation. Chem. Senses 30: 185-194.

Lee, S.I., Turner, R.J. 1992. Secretagogue-induced 86RbV efflux from bovine parotid is HCO3-dependent. Am J Physiol 264: R162-R168.

Leinonen, J., Parkkila, S., Kaunisto, K., Koivunen, P., Rajaniemi, H. 2001. Secretion of carbonic anhydrase isoenzyme VI (CA VI) from human and rat lingual serous on Ebner's glands. J Histochem Cytochem 49: 657-662.

Lemon, C.H., Katz, D.B. 2007. The neural processing of taste. BMC Neurosci 8: S5.

Leppi, T.J., Spicer, S.S. 1967. The histochemistry of carbohydrate-ric substances in certain ungulate salivary glands. Anat. Rec. 159: 179-191.

Levine, M.J., Reddy, M.S., Tabak, L.A., Loomis, R.E., Bergey, E.J., Jones, P.C., Cohen, R.E., Stinson, M.W., A-Hashimi, I. 1987. Structural aspects of salivary glycoproteins. J. Dent. Res. 66: 436-441.

Li, X.J., Snyder, S.H. 1995. Molecular cloning of Ebnerin, a von Ebner's gland protein associated with taste buds. J. Biol. Chem. 270: 17674-17679.

Li, X., Staszewski, L., Xu, H., Durik, K., Adler, E. 2002. Human receptors for sweet and umami taste. Proc. Natl. Acad. Sci. USA 99: 4692-4696.

Liang, H., Wang, Y., Wang, Q., Ruan, M.S. 1999. Hydrophobic interaction chromatography and capillary zone electrophoresis to explore the correlation between isoenzymes and salivary alpha-amylase and dental caries. J. Chromatogr. B Bomed. Sci. Appl. 724: 381-388.

Lima, T.G., Haddad, A., Fava-de-Moraes, F. 1977. Glycoprotein secretion in the mouse submandibular gland as revealed by radioautography alter L-3H-fucose injection. Cell Tissue Res. 183: 519-530.

Lin, A.L., Johnson, D.A., Stephan, K.T., Yeh, C.K. 2003. Alteration in salivary function in early HIV infection. J. Dent. Res. 82: 719-724.

Lindemann, B. 2001. Receptors and transduction in taste. Nature 413: 219-225.

Lindemann, B., Ogiwara, Y., Ninomiya, Y. 2002. The discovery of umami. Chem. Senses 27: 843-844.

Llena-Puy, M.C., Montañana-Llorens, C., Forner-Navarro, L. 2004. Condiciones optimas de manipulación para la cuantificación de fibronectina en saliva. Med. Oral 9: 191-6.

Lu, Y., Bennick, A. 1998. Interaction of tannin with human salivary proline-rich proteins. Arch. Oral Bol. 43: 717-728.

Lyman, B.J., Green, B.G. 1990. Oral astringency: effects of repeated exposure and interactions with sweeteners. Chem. Senses 15: 151-164.

Mack, A., Singh, A., Gilroy, C., Ireland, W. 1997. Porcine lingual taste buds: a quantitative study. Anat. Rec. 247: 33-37.

MacKay, B.J., Pollock, J.J., Iacono, V.J., Baum, B.J. 1984. Isolation of milligram quantities of a group of histidine-rich polypeptides from human parotid saliva. Infect. Immun. 44: 688-694.

Mackie D.A., Pangborn R.M. 1990. Mastication and its influence on human salivary flow and alpha amylase secretion. Physiol Behav. 47: 593-595.

Mandel, I.D. 1980. Sialochemistry in diseases and clinical situations affecting salivary glands. Crit. Rev. Clin. Lab. Sci. 12: 321-366.

Mann, G.E., Smaje, L.H., Yudilevich, D.L. 1979. Transcapillary exchange in the cat salivary gland during secretion, bradykin infusion and after chronic duct ligation. J. Physiol. 297: 355-367.

Marchlewska-Koj, A., Pochron, E., Sliwowska, A. 1990. Salivary glands and preputal glands of mice as source of estrus-stimulating pheromone in female mice. J. Chem. Ecol. 16: 2817-2822.

Margolskee, R.F. 2002. Molecular mechanisms of bitter and sweet taste transduction. J. Biol. Chem. 277: 1-4.

Martin S., Pangborn RM. 1971. Human parotid secretion in response to ethyl alcohol. J. Dent. Res. 50: 485-490.

Mata, A.D., Marques, D., Rocha, S., Francisco, H., Santos, C., Mesquita, M.F., Singh, J. 2004. Effects of diabetes mellitus on salivary secretion and its composition in the human. Mol. Cell Biochem. 61: 137-142.

Matsunami, H., Montmayeur, J.P., Buck, L.B. 2000. A family of candidate taste receptors in human and mouse. Nature 404: 601-604.

Matsuo, R., Yamamoto, T. 1992. Effects of inorganic constituents of saliva on taste responses of the rat chorda tympani nerve. Brain Res. 583: 71-80.

Matsuo, R., Yamamoto, T., Ikehava, A., Nakamura, O. 1994. Effect of salivation on neural taste responses in freely moving rats: analysis of salivary secretion and taste responses of the chorda tympani nerve. Brain Res. 649: 136-146.

Matsuo, R., Yamauchi, Y., Morimoto, T. 1997. Role of submandibular and sublingual saliva in maintenance of taste sensitivity recorded in the chorda tympani of rats. J. Physiol. 498: 797-807.

Matsuo, R. 1999a. Interrelation of taste and saliva. In: Neural mechanisms of salivary gland secretion. Garrett, J.R., Ekström, J., Anderson, L.C. (Eds) Front. Oral Biol., Karger, vol 11: 185-195.

Matsuo, R. 1999b Central connections for salivary innervations and efferent impulse formation. In: Neural mechanisms of salivary gland secretion. Garrett, J.R., Ekström, J., Anderson, L.C. (Eds) Front. Oral Biol., Karger, vol 11: 26-43.

Matsuo, R., Garrett, J.R., Proctor, G.B., Carpenter, G.H. 2000. Reflex secretion of proteins into submandibular saliva in conscious rats, before and after preganglinic sympathectomy. J. Physiol. 527: 175-184.

Mattes, R.D. 2001. The taste of fat elevates postprandial triacylglycerol. Physiol. Behav. 74: 343-348.

Mau, M., Muller, C., Langbein, J., Rehfeldt C., Hildebrandt, J.P., Kaiser, T.M., 2006. Adhesion of bovine and goat salivary proteins to dental enamel and silicate. Arch. Tierz. Dummerstorf 5: 439-446.

Mayer, J., 1955. Regulation of energy intake and the body weight: the glucostatic theory and the lipostatic hypothesis. Ann. N. Y. Acad. Sci. 63: 15-43.

Mazariegos, M.R., Tice, L.W., Hand, A.R. 1984. Alteration of tight junctional permeability in the rat parotid gland after isoproterenol stimulation. J. Cell Biol. 98: 1865-1877.

McArthur, C., Hagerman, A.E., Robbins, C.T., Hanley, T.H. 1993. Diet selection by a ruminant generalist browser in relation to plant chemistry. Can. J. Zool. 71: 2236-2243.

McArthur, C., Sanson, G.D., Beal, A.M. 1995. Salivary proline-rich proteins in mammals - roles in oral homeostasis and counteracting dietary tannin. J. Chem. Ecol. 21: 663- 691.

McDougall, E.I., 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Bichem. J. 43: 99-109.

McDougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A., Stewart, D. 2005. Different polyphenolic components of soft fruits inhibit alpha amylase glucosidase. J. Agric. Food Chem. 53: 2760-2766.

Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc. Natl. Acad. Sci. USA 80: 3948–3952.

Mehansho, H., Clements, S., Sheares, B.T., Smith, S., Carlson, D.M. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. J. Biol. Chem. 260: 4418–4423.

Mehansho, H., Ann, D.K., Butler, L.G., Rogler, J., Carlson, D.M. 1987. Induction of proline-rich proteins in hamster salivary glands by isoproterenol treatment and an unusual growth inhibition by tannins. J. Biol. Chem. 262: 12344-12350.

Mellado, M., Rodríguez, A., Olvera, A., Villarreal, J.A., Lopez, R. 2004. Diets of nubian and granadina goats grazing on arid rangeland. Rangeland Ecol. Manag. 57: 630-634.

Melvin, J.E., Yue, D., Shuttleworth, T. Begenisich, T., 2005. Regulation of fluid and electrolyte secretion in salivary gland acinar cells. Annu. Rev. Physiol. 67: 445-469.

Mennella, J.A., Griffin, C.E., Beaucamp, G.K. 2004. Flavor programming during infancy. Pediatrics. 113: 840-845.

Mennella, J.A., Turnbull, B., Ziegler, P.J., Martinez, H. 2005a. Infant feeding practices and early flavour experiences in Mexican infants: an intra-cultural study. J. Am. Diet Assoc. 105: 908-915.

Mennella J.A., Pepino M.Y., Reed D.R. 2005b. Genetic and environmental determinants of bitter perception and sweet preferences. Pediatrics. 115: 216-222.

Meot, F., Cirio, A., Boivin, R. 1997. Parotid secretion daily patterns and measurement with ultrasonic flow probes in conscious sheep. Exp. Physiol. 82: 905-923.

Mese, H., Matsuo, R. 2007. Salivary secretion, taste and hyposalivation. J. Oral Rehabil. 34: 711-723.

Miller, I.J. Jr, Preslar, A.J. 1975. Spatial distribution of rat fungiform papillae Anat. Rec. 181: 679-684.

Miller, I.J., Smith, D.V. 1984. Quantitative taste bud distribution in the hamster. Physiol. Behav. 32: 275-285.

Miller, I.J. Jr., Spangler, K.M. 1992. Taste bud distribution and innervation on the palate of the rat. Chem. Senses 7: 99-108.

Mirels, L., Hand, A.R., Branin, H.J. 1998. Expression of gross cystic disease fluid protein-15/ prolactin-inducible protein in rat salivary glands. J. Histochem. Cytochem. 46: 1061-1071.

Mizuki, N., Kasahara, M. 1992. Mouse submandibular glands express an androgen-regulated transcript encoding an acidic epididymal glycoprotein-like molecule. Mol. Cell. Endocrinol. 89: 25-32.

Mole, S., Butler, L. G., Iason, G. 1990. Defense against dietary tannin in herbivores-a survey for proline-rich salivary proteins in mammals. Biochem. Syst. Ecol. 18: 287–293.

Montavon, P., Lindstrand, K. 1991. Immunohistochemical localization of neuron-specfic enolase and calcitonin gene-related peptide in pig taste papillae. Regul. Pept. 36: 235-248.

Monteleone, E., Condelli, N., Dinnella, C., Bertuccioli, M. 2004. Prediction of perceived astringency induced by phenolic compounds. Food Qual Prefer 15: 761-769.

Muratsu, K., Morioka, T. 1985. Levels of salivary lysozyme, lactoperoxidase, and lactoferrin in diabetic hamsters. Infect. Immun. 48: 389-394.

Murphy, R.A., Saide, J.D., Blanchard, M.H., Young, M. 1977. Nerve growth factor in mouse serum and saliva: role of the submandibular gland. Proc. Natl. Acad. Sci. USA 76: 2330-2333.

Nagamine, I., Sunagawa, K., Kuriwaki, J., Shinjo, A. 2003. Changes in single unit activity in the lateral hypothalamic area of goats during feeding. J. Anim. Sci. 81: 529-536.

Naito, Y., Suzuki, I., Hasegawa, S. 1992. Induction of cystatin S in rat submandibular glands by papain. Comp. Biochem. Physiol. B. 102: 861-865.

Nakahari, T., Steward, M.C., Yoshida, H., Imai, Y. 1997. Osmotic flow transients during acetylcholine stimulation in the perfused rat submandibular gland. Exp. Physiol. 82: 55-70.

Nakamura, T., Tanigake, A., Miyanaga, Y., Ogawa, T., Akiyoshi, T., Matsuyama, K., Uchida, T. 2002. The effect of various substances on the suppression of the bitterness of quinine-human gustatory sensation, binding, and taste sensor studies. Chem. Pharm. Bull. 50: 1589-1593.

Nam, J.W., Chung, J.W., Kho,H.S., Chung, S.C., Kim, Y.K. 2007. Nerve growth factor concentration in human saliva. Oral Dis 13: 187-192.

Nastis, A. 1997. Feeding behaviour of goats and utilization of pasture and rangelands. CIHEAM – Options Mediterraneennes 25: 39-45

Nefzaoui, A., Ben Salem, H., Abdouli, H., Ferchichi, H. 1995. Palatability for goat of some Mediterranean shrubs. Comparison between browsing time and cafeteria technique. Ann. Zootech. 44: 117-117.

Nelson, G., Chandrashekar, J., Hoon, M.A., Feng, L., Zhao, G., Ryba, N.J., Zuker, C.S. 2002. An amino acid taste receptor. Nature 416: 199-202.

Nelson T.M., Munger, S.D., Boughter, J.D. Jr 2005. Haplotypes at the Tas2r locus on distal chromosome 6 vary with quinine taste sensitivity in inbred mice. BMC Genet. 6: 32.

Neyraud, E., Dransfield, E. 2004. Relating ionization of calcium chloride in saliva to bitterness perception. Physiol. Behav. 81: 505-510.

Neyraud, E., Sayd, T., Morzel, M., Dransfield, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. J. Proteome Res. 5: 2474-2480.

Nieuw Amerongen, A.V., Veerman, E.C. 2002. Saliva - the defender of the oral cavity. Oral Dis. 8: 12-22.

Ninomiya, Y., Kajiura, H., Naito, Y., Mochizuki, K., Katsukawa, H., Torii, K. 1994. Glossopharyngel denervation alters responses to nutrients and toxic substances. Physiol. Behav. 56: 1179-1184.

Nishita, T., Tanaka, Y., Wada, Y., Murakami, M., Kasuya, T., Ichihara, N., Matsui, K. Asari, M. 2007. Measurement of carbonic anhydrase isozyme VI (CA-VI) in bovine sera, saliva, milk and tissues. Vet. Res. Commun. 31: 83-92.

Noble, A.C. 1995. Application of time-intensity procedures for the evaluation of taste and mouthfeel. Am. J. Enol. Viticult. 46: 128-133.

Nsahlai, I.V., Umunna, N.N., Osuji, P.O. 1999. Influence of feeding sheep on oilseed cake following the consumption of taniferous feeds. Livest Prod Sci. 60: 59-69.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007–4021.

Onaga, T., Shimizu, Y., Hayashi, H. , Tsuji, M., Endoh, D., Okada, H. 2006. Localization and secretion of epidermal growth factor in the parotid gland and its intragastric kinetics in sheep. Life Sci. 79: 1616–1629.

Olsson, K. 1976. Influence of altered CSF solute composition on parotid salivary secretion in goats. Acta Physiol. Scand. 97: 196-201.

Ono, K., Morimoto, Y., Inoue, H., Masuda, W., Tanaka,T., Inenaga, K. 2006. Relationship of the unstimulated whole saliva flow rate and salivary gland size estimated by magnetic resonance image in healthy young humans. Arch Oral Biol. 51: 345-349.

Ono, K., Inoue, H., Masuda, W., Morimoto, Y., Tanaka, T., Yolota, M., Inenaga, K. 2007. Relationship of chewing-stimulated whole saliva flor rate and salivary gland size. Arch Oral Biol 52: 427-431.

Oppenheim, F.G., Kousvelari, E., Troxler, R. 1979. Immunological cross-reactivity and sequence homology between salivary proline-rich proteins in human and macque monkey (*Macaca fascicularis*) parotid saliva. Arch. Oral Biol. 24: 595-599.

Oppenheim, F.G., Salih, E., Siqueira, W.L., Zhang, W., Helmerhorst, E.J. 2007. Salivary proteome and its genetic polymorphisms. Ann. N.Y. Acad. Sci. 1098: 22-50.

Owerbachx, D., Hjorthz, P. 1980. Inheritance of a parotid secretory protein in mice and its use in determining salivary amylase quantitative variants. Genetics 95: 129-141.

Parkkila, S., Parkkila, A.K., Vierjoki, T., Ståhlberg, T., Rajaniemi, H. 1993. Competitive time-resolved immunofluorometric assay for quantifying carbonic anhydrase VI in saliva. Clin. Chem. 39: 2154-2157.

Parsons, P.A., Garrett, J.R. 1977. Movement of horseradish peroxidase in submandibular glands of dog after ductal injection. Med. Biol. 55: 249-260.

Parsons, P.A., Klinger, A.H., Garrett, JR. 1977. Adrenergic influences on the permeability of rabbit submandibular salivary glands to blood-borne horseradish peroxidase. Histochem. J. 9: 419-433.

Patamia, M., Messana, I., Ptruzzelli, R., Vitali, A., Inzitari, R., Cabras, T., Fanali, C., Scarano, E., Contucci, A., Galtieri, A., Castagnola, M. 2005. Two proline-rich peptides from pig (*Sus scrofa*) salivary glands generated by pre-secretory pathway underlying the action of a proteinase cleaving ProAla bonds. Peptides 26: 1550-1559.

Patterson, J., Titchen, D.A. 1979. Sympathetic and beta-adrenergic stimulation of parotid salivary secretion in sheep. Q. J Exp. Physiol. Cogn. Med. Sci. 64: 175-184.

Patterson, J., Titchen, D.A. 1980. beta-Adrenergic stimulation of parotid salivary secretion in sheep. J. Physiol. 298: 44P-45P.

Patterson, J. Brightling, P., Titchen, D.A. 1982. beta-Adrenergic effects on composition of parotid salivary secretion of sheep on feeding. Q.J. Exp. Physiol. 67: 57-67.

Pedersen, A.M., Bardow, A., Jensen, S.B., Nauntofte, B. 2002. Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis. 8: 117-129.

Peleg, H., Gacon, K., Schlich, P., Noble, A.C. 1999. Bitterness and astringency of flavan-3-ol monomers, dimmers and trimers. J Sci Food Agric. 79: 1-6.

Perevolotsky, A., Brosh, A., Ehrlich, O., Gutman, M., Henkin, Z., Holzer, Z. 1993. Nutritional value of common oak (*Quercus calliprinos*) browse as fodder for goats: experimental results in ecological perspective. Small Rumin. Res. 11: 95-106.

Perevolotsky, A., Landau, S., Kababia, D., Ungar, E.D. 1998. Diet selection in dairy goats grazing woody Mediterranean rangeland. Appl Anim Behav Sci. 57: 117-131.

Pérez-Barberia, F.J., Elston, D.A., Gordon, I.J., Illius, A.W. 2005. The evolution of phylogenetic differences in the efficiency of digestion in ruminants. Proc. R. Soc. Lond. B 271: 1085-1090.

Pes, D., Pelosi, P. 1995. Odorant-binding proteins of the mouse. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 112: 471-479.

Pfeiffer, C.J., Levin, M., Lopes, M.A. 2000. Ultrastructure of the horse tongue: further observations on the lingual integumentary architecture. Anat. Histol. Embryol. 29: 37-43.

Pfister, J.A., Malechek, J.C. 1986. The voluntary forage intake and nutrition of goats and sheep in the semi-arid tropics of northeastern Brazil. J. Anim. Sci. 63: 1978-1086.

Phillips, C.J., Tandler, B. 1996. Salivary glands, cellular evolution and adaptative radiation in mammals. Eur J. Morphol. 34: 155-162.

Phillips, C.J, Weiss, A., Tandler, B. 1998. Plasticity and patterns of evolution in mammalian salivary glands: comparative immunohistochemistry of lysozyme in bats. Eur. J. Morphol. 36 Suppl: 19-26.

Poulsen, J.H. 1998. Secretion of electrolytes and water by salivary glands. In: Garrett, J.R., Ekström, J., Anderson, L.C. (Eds): Glandular mechanisms of salivary secretion. Front. Oral Biol. Basel, Karger, vol 10: 55-72.

Prinz, J.F., Lucas, P.W. 2000. Saliva tannin interactions. J. Oral Rehabil. 27: 991-994.

Proctor, G.B., Asking, B., Garrett, J.R. 1989. Effects of secretory nerve stimulation on the movement of rat parotid amylase into the circulation. Arch. Oral Biol. 34: 609-613.

Proctor, G.B. 1998. Secretory protein synthesis and constitutive (vesicular) secretion by salivary glands, In: Glandular mechanisms of salivary secretion. Garrett, J.R., Ekström, J. Anderson, L.C. (eds.) Karger, pp.21-35.

Proctor, G.B., Carpenter, G.H. 1998. The function of salivary proteins and the regulation of their secretion by salivary glands. Biomed Reviews. 9: 3-15.

Proctor, G.B., Carpenter, G.H. 2007. Regulation of salivary gland fuction by autonomic nerves. Auton. Neurosci. 133: 3-18.

Provenza, F.D., Malechek, J.C. 1984. Diet selection by domestic goats in relation to blackbrush twig chemistry. J. Appl. Ecol. 21: 831-841.

Provenza, F.D., Pfister, J.A., Cheney, C.D. 1992. Mechanisms of learning in diet selection with reference to phytotoxicosis in herbivores. J Rang Manag. 45: 36-45.

Provenza, F.D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. J. Range Manag. 48: 2-17

Provenza, F.D., Villalba, J.J., Dziba, L.E., Atwood, S.B., Banner, R.E. 2003. Linking herbivore experience, varied diets, and plant biochemical diversity. Small Ruminant Res. 49: 257-274.

Provenza, F.D., Villalba, J.J., Haskell, J., MacAdam, J.W., Griggs, T.C., Wiedmeier, R.D. 2007. The value to herbivores of plant physical and chemical diversity in time and space. Crop Sci. 47: 382-398.

Rabilloud, T. 2002. Two-dimensional gel electrophoresis in proteomics: Old, old fashioned, but it still climbs up the mountains. Proteomics 2: 3-10.

Rajan, G.H., Morris, C.A., Carruthers, V.R., Wilkins, R.J., Wheeler, T.T. 1996. The relative abundance of a salivary protein, BSP30, is correlated with susceptibility to bloat in cattle herds selected for high or low bloat susceptibility. Anim. Genet. 27: 407–414.

Rathman, W.M., van Zeyl, M.J., van den Keijbus, P.A., Veerman, E.C., Nieuw Amerongen, A.V. 1990. Characterization of monoclonal antibodies to human salivary (glyco)proteins. Cellular localization of mucin, cystatin-like 14 kD protein and 20 KD glycoprotein in the human submandibular gland. J. Biol. Buccale 18: 19-27.

Rayment, S.A., Liu, B., Offner, G.D., Oppenheim, F.G., Troxler, R.F. 2000. Immunoquantification of human salivary mucins MG1 and MG2 in stimulated whole saliva: factors influencing mucin levels. J. Dent. Res. 79: 1765-1772.

Redman, R.S., Field, R.B. 1993. Chronology of peroxidase activity in the developing rat parotid gland. Anat. Rec. 235: 611-621.

Reed, J.D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73: 1516-528.

Reid, A.M., Titchen, D.A. 1988. Atrpine-resistant secretory responses of the ovine parotid gland to reflex and direct parasympathetic stimulation. Q. J. Exp. Physiol. 73: 413-424.

Riad, M., Barton, J.R., Wilson, J.A., Freeman, C.P., Maran, A.G. 1991. Parotid salivary secretory pattern in bulimia nervosa. Acta Otolaryngol. 111: 392-395.

Riva, A., Puxeddu, P., del Fiancco, M., Testa-Riva, F. 1978. Ultrastructural localization of enogenous peroxidase in human parotid and submandibular glands. J. Anat. 127: 181-191.

Robbins, C.T., Hanley, T.A., Hagerman, A.E., Hjeljord, O., Baker, D.L., Schwartz, C.C., Mautz, W.W. 1987. Role of tannins in defending plants against ruminants: reduction in protein availability. Ecology 68: 98-107.

Robbins, C.T., Hagerman, A.E., Austin, P.J., McArthur, C., Hanley, T.A., 1991. Variation in mammalian physiological responses to a condensed tannin and its ecological implications. J. Mammal. 72: 480-486.

Robinson, P.P., Winkles, P.A 1990. Quantitative study of fungiform papillae and taste buds on the cat's tongue. Anat. Rec. 226: 108-111.

Robertson, E., Gordon, I., Pérz-Barberia, F. 2006. Preferences of sheep and goats for straw pellets treated with different food-flavouring agents. Small Rum. Res. 63: 50-57.

Robichaud, J.L., Noble, A.C. 1990. Astringency and bitterness of selected phenolics in wine. 53: 343-353.

Rolls, E.T., Critchley, H.D., Browning, A.S., Hernadi, A., Lenard, L. 1999. Responses to the sensory properties of fat of neurons in the primate orbitofrontal cortex. J. Neurosci. 19: 1532-1540.

Rolls, E.T., Verhagen, J.V., Kadohisa, M. 2003. Representations of the texture of food in the primate orbitofrontal cortex: neurons responding to viscosity, gritness and capsaicin. J. Neurophysiol. 90: 3711-3724.

Rolls, E.T. 2005. Taste, olfactory, and food texture processing in the brain, and the control of food intake. Physiol. Behav. 19: 45-56.

Roper, S.D. 1989. The cell biology of vertebrate taste receptors. Annu. Rev. Neurosci. 12: 329-353.

Rudney, J.D., Krig, M.A., Neuvar, E.K., Soberay, A.H., Iverson, L. 1991. Antimicrobial proteins in human unstimulated whole saliva in relation to each other, and to measures of health status, dental plaque accumulation and composition. Arch. Oral Biol. 36: 497-506.

Ruth S.M., Roozen J.P., Nahon D.F., Cozijnsen J.L., Posthumus M.A. 1996. Flavour release from rehydrated french beans (*Phaseolus vulgaris*) influenced by composition and volume of artificial saliva. Z-Lebensm-Unters-Forsch 203: 1-6.

Sakabe, K., Seiki, K., Fujii-Hanamoto, H., Kawshima, I. 1988. Progestin and estrogen receptors: characterization and localization in rat submandibular glands, with special reference to epidermal growth factor. Endocrinol Jpn. 35: 709-723.

Saper, C.B., Chou, T.C., Elmquist, J.K. (2002) The need to feed: homeostatic and hedonic control of eating. Neuron 36: 199-211.

Sas, R., Dawes, C. 1997. The intra-oral distribution of unstimulated and chewing-gum stimulated parotid saliva. Arch. Oral Biol. 42: 469-474.

Schneyer, L.H., Pigman, W., Hanahan, L. Gilmore, R.W. 1956. Rate of flow of human parotid, sublingual and submaxillary secretions during sleep. J. Dent. Res. 35: 109-114.

Scicchitano, R., Sheldrake, R.F., Husband, A.J. 1986. Origin of immunoglobulins in respiratory tract secretion and saliva of sheep. Immunology 58: 315-321.

Scott, J., Berry, M.R, Gunn, D.L., Woods, K. 1990. The effects of a liquid diet on initial and sustained, stimulated parotid salivary secretion and on parotid structure in the rat. Arch. Oral Biol. 35: 509-514.

Scott, B.J. 1998. The masticatory-parotid salivary reflex in edentulous subjects. J. Oral Rehabil. 25: 28-33.

Scott, T.R., Verhagen, J.V. 2000. Taste as a factor in the management of nutrition. Nutrition 16: 874-885.

Scott, K. 2004. The sweet and the bitter of mammalian taste. Curr. Opin. Neurobiol. 14: 423-427.

Scott, K. 2005. Taste recognition: food for though. Neuron 48: 455-464.

Scully, C. 2003. Drug effects on salivary glands: dry mouth. Oral Diseases 9: 165-176.

Schiffman, S.S., Suggs, M.S., Simon, S.A. 1992. Astringent compounds suppress taste responses in gerbil. Brain Res. 595: 1-11.

Segawa, A. 1994. Tight junctional permeability in living cells: dynamic changes directly visualizad by confocal laser micoscopy. J. Electron Microsc. (Tokyo) 43: 290-298.

Seth, O.N., Rai, G.S., Yadav, P.C., Pandey, M.D. 1976. A note on the rate of secretion and chemical composition of parotid saliva in sheep and goats. Indian. J. Anim. Sci. 46: 660-663.

Shimada, T., 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32: 1149-1163.

Shaw, P.A., Barka, T. 1989. α-Adrenergic induction of a cysteine-proteinase-inhibitor mRNA in rat salivary glands. Biochem. J. 257: 685-689.

Ship, J.A., Fischer, D.J. 1997. The relationship between dehydration and parotid salivary gland function in young and older healthy adults. J. Gerontol. A: Biol. Sci. Med. Sci. 52: M310-M319.

Siebert, K.J., Chassy, A.W. 2004. An alternative mechanism for the astringent sensation of acids. Food Qual. Prefer. 15: 13-18.

Siqueira, W.L., Salih, E., Wan, D.L., Helmerhorst, E.J., Oppenheim, F.G., 2008. Proteome of human minor salivary gland secretion. J. Dent. Res. 87: 445-450.

Silanikove, N., Tadmor, A. 1989. Rumen volume, saiva flow rate, and systemic fluid homeostasis in dehydrated cattle. Am. J. Physiol. 256: R809-R815.

Silanikove, N., Tagari, H., Shkolnik, A. 1993. Comparison of rate passage fermentation rate and efficiency of digestion of high fiber diet in desert black Bedouin goats as compared to Swiss Saanen goats. Small Ruminant Res. 12: 45-60.

Silanikove, N., Gilboa, N., Perevolotsky, A., Nitsan, Z. 1996a. Goats fed tannin-containing leaves do not exhibit toxic syndromes. Small Ruminant Res. 21: 195-201.

Silanikove, N., Gilboa, N., Nir, I., Perevolotsky, A., Nitsan, Z. 1996b. Effect of a daily supplementation of polyethylene glycol on intake and digestion of tannin-containing leaves (*Quercus calliprinos, Pistacia lentiscus,* and *Ceratonia siliqua*) by goats. J. Agric. Food Chem. 44: 199-205.

Silanikove, N. 1997. Why goats raised on harsh environments perform better than other domesticated animals. In: Recent advances in small animal nutrition. pp: 185-194. Lindberg, J.E., Gonda, H.L., Ledin, I. (Eds.) Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 34.

Silanikove, N. 2000. The physiological basis of adaptation in goats to harsh environments. Small Ruminant Res. 35: 181-193.

Simon, S.A., Araujo, I.E., Gutierrez, R., Nicolelis, A.L. 2006. The neural mechanisms of gustation: a distributed processing code. Nature 7: 890-901.

Simons, C.T., Boucher, Y., Carstens, E. 2003. Suppression of central taste transmission by oral capsaicin. J. Neurosci. 23: 978-985.

Smaje, L.H. 1973. Spontaneous salivation in the rabbit submandibular gland. J. Physiol. 231: 179-193.

Snell, R.S., Garrett, J.R. 1957. The distribution of cholinesterase in the submaxillary and sublingual salivary glands of the rat. J. Histochem. Cytochem. 5: 236-245.

Soares, R.V., Siqueira, C.C., Bruno, L.S., Oppenheim, F.G., Offner, G.D., Troxler, R.F. 2003. MG2 and lactoferrin form a heterotypic complex in salivary secretions. J. Dent. Res. 82: 471-475.

Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G., Troxer, R.F. 2004. Salivary micelles: identification of complexes containing MG2, sIgA lactoferrin, amylase, glycosylated proline-rich protein and lysozyme. Arch. Oral Biol. 49: 337-349.

Spector, A.C. 2000. Linking gustatory neurobiology to behavior in vertebrates. Neurosci Behav Reviews 24: 391-416.

Spielman, A.I., Bennick, A. 1989. Isolation and characterization of six proteins from rabbit parotid saliva belonging to a unique family of proline-rich proteins. Arch. Oral Biol. 34: 117-130.

Spielman, A.I. 1990. Interaction of saliva and taste. J. Dent. Res. 69: 838-843.

Spielman, A.I. 1993. Protein analysis of human von Ebner saliva and a method for its collection from the foliate papillae. J. Dent. Res. 72: 1331-1335.

Steward, M.C., Poronnik, P., Cook, D.I. 1996. Bicarbonate transport in sheep parotid secretory cells. J. Physiol. 494: 819-830.

Stolte, M., Ito, S., 1996. A comparative ulrastructural study of the parotd gland acinar cells of nine wild ruminant species (mammalian, artiodactyla). Eur. J. Morphol. 34: 79-85.

Stuchell, R.N., Mandel, I.D. 1978. Studies of secretory IgA in caries-resistant and caries susceptible adults. Adv. Exp. Med. Biol. 107: 341-348.

Sugita, M. 2006. Taste perception and coding in the periphery. Cell Mol. Life Sci 63: 2000-2015.

Suzuki, T. 2007. Cellular mechanisms in taste buds. Bull. Tokyo Dent. Coll. 48: 151-161.

Sweeney, D., Beeley, J.A. 1990. An enzyme-linked immunoassay for human salivary albumin. Arch. Oral Biol. 35: 741-746.

Tadjalli, M., Pazhoomand, R. 2004. Tongue papillae in lambs: a scanning electron microscopic study. Small Ruminant Res. 54: 157-164.

Takeshita, H., Mgi, K., Yasuda, T., Nakajima, T., Nakashima, Y., Mori, S., Hoshino, T. 2000. Mammalian deoxyribonucleases I are classified into three types: pancreas, parotid, and pancreas-parotid (mixed), based on differences in their tissue concentrations. Biochem. Biophys. Res. Commun. 269: 481-484.

Takuma, T., Ichida, T. 1986. Does cyclic cAMP mobilize Ca^{2+} for amylase secretion from rat parotid cells? Biochim. Biophys. Acta 887: 113-117.

Takuma, T., Arakawa, T., Tajima, Y. 2000. Interaction of SNARE proteins in rat parotid acinar cells. Arch. Oral Biol. 45: 369-375.

Tandler, B., Poulsen, J.H. 1977. Ultrastructure of the cat sublingual gland. Anat. Rec. 187: 153-171.

Tandler, B., Nagato, T., Phillips, C.J. 1986. Systematic implications of comparative ultrastructure of secretory acini in the submandibular salivary gland in artibeus (Chiroptera: Phyllostomidae). J. Mammal. 67: 81-90.

Tandler, B., Naato, T.,Phillips, C.J. 1997. Ultrastructure of the parotid salivary glands in seven species of fruit bats in the genus Artibeus. Anat. Rec. 248: 176-188.

Tandler, B., Phillips, C.J. 1998. Microstructure of mammalian salivary glands and its relationship to diet. In: Glandular mechanisms of salivary secretion. Garrett, J.R., Ekström, J. Anderson, L.C. (Eds.) Karger., pp.21-35.

Tandler, B., Gresik, E.W., Nagato, T., Phillis, C.J. 2001. Secretion by striated ducts of mammalian major salivary glands: review from an ultrastructural, functional and evolutionary perspective. Anat. Rec. 264: 121-145.

Teeuw, W., Bosch, J.A., Veerman, E.C.I., NieuwAmerongen, A.V. 2004. Neuroendocrine regulation of salivary IgA synthesis and secretion: implications for oral health. Biol. Chem. 385: 1137-1146.

Thatcher, B.J., Doherty, A.E., Orvisky, E., Martin, B.M., Henkin, R.I. 1998. Gustin from human parotid saliva is carbonic anhydrase VI. Biochem Biophys. Res Commun. 250: 635-641.

Thesleff, I., Viinikka, L., Saxén, L., Lehtonen, E., Perheentupa, J. 1988. The parotid gland is the main source of human salivary epidermal growth factor. Life Sci. 43: 13-18.

Thomas, E.L., Jefferson, M.M., Joyner, R.E., Cook, G.S., King, C.C. 1994. Leukocyte myeloperoxidase and salivary lactoperoxidase: identification and quantitation in human mixed saliva. J. Dent. Res. 73: 544-555.

Thorngate, J.H., Noble, A.C. 1995. Sensory evaluation of bitterness and astringency of 3R(-)-epicatechin and 3S(+)-catechin. J. Sci Food Agric. 67: 531-535.

Trabalza-Marinucci, M., Verini-Supplizi, A., Valfe, F., Olivieri, O. 1992. Voluntary hay intake by Sardinian ewes during last weeks of pregnancy and lactation. Small Ruminant Res. 7: 203-213.

Turner, R.J., Sugiya, H. 2002. Salivary glands and saliva. Number 1: Understanding salivary fluid and protein secretion. Oral Dis. 8: 3-11.

Tynelius-Bratthall, G., Ericson, D., Araujo, H.M. 1986. Fibronectin in saliva and gingival crevices. J. Periodontal Res. 21: 563–568.

Tynelius-Bratthall, G. 1988. Crevicular and salivary fibronectin before and after gingivitis treatment. J. Clin. Periodontol. 15: 283-287.

Vaithiyanathan, S., Mishra, J.P., Sheikh, Q., Kumar, R., 2001. Salivary glands tannins binding proteins of sheep and goat. Indian J. Anim. Sci. 71: 1131-1134.

van Lennep, E.W., Kennerson, A.R., Compton, J.S. 1977. The ultrastructure of the sheep parotid gland. Cell Tiss. Res. 179: 377-392.

Van Soest, P.J. 1994. Nutritional ecology of the ruminant. 2nd ed. Cornell University Press, New York.

van Steijn, G.J., Amerongen, A.V., Veerman, EC.I., Kasanmoentalib, S., Overdijk, B. 1999. Chitinase in whole and glandular human salivas and in whole saliva of patients with periodontal inflammation. Eur. J. Oral Sci. 107: 328-337.

van der Reijden W.A., van der Kwaak, J.S., Veeran, E.C., Nieuw Amerongen, A.V. 1996. Analysis of the concentration and output of the whole salivary constituents in patients with Sjögren's syndrome. Eur. J. Oral Sci. 104: 335-340.

Veerman, E.C., van der Keybus, P.A., Vissink, A., Nieuw Amerongen, A.V. 1996. Human glandular salivas: their separate collection and analysis. Eur. J. Oral Sci. 104: 346-352.

Vitorino, R., Lobo, M.J., Ferrer-Correira, A.J., Dubin, J.R., Tomer, K.B., Domngues, P.M., Amado, F.M., 2004. Identification of human whole saliva protein components using proteomics. Proteomics 4: 1109-1115.

Von Bültzingsöwen, I., Sollecito, T.P., Fox, P.C., Daniels, T., Jonsson, R., Lockhart, P.B., Wray, D., Brennan, M.T., Carrozzo, M., Gandera, B., Fujibayahi, T., Navazesh, M., Rhodus, N.L., Schiødt, M. 2007. Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations. Oral Surg. Oral Med. Pathol. Oral Radiol. Endod. 103 (Suppl): S57.e1-S57.e15.

Von Zastrow, M., Castle, J.D. 1987. Protein sorting among two distinct export pathways occurs from the content of maturing exocrine storage granules. J. Cell Biol. 105: 2675-2684.

Voutetakis, A., Wang, J., Baum, B.J. 2004. Utilizing endocrine secretory pathways in salivary glands for systemic gene therapeutics. J. Cell Physiol 199: 1-7.

Walz, A., Stühler, K., Wattenberg, A., Hawranke, E., Meyer, H.E., Schmalz, G., Blüggel, M., Ruhl, S., 2006. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole saliva by two-dimensional gel electrophoresis. Proteomics 6: 1631-1639.

Williams, K.M., Marshall, T., 1998. Electrophoretic characterization of the major cat parotid salivary protein. Anal. Chim. Acta 372: 249-255.

Williams, K.M., Ekström, J., Marshall, T., 1999a. High-resolution electrophoretic analysis of rat parotid salivary proteins. Electrophoresis 20: 1373-1381.

Williams, K.M., Ekström, J., Marshall, T., 1999b. The protein composition of ferret parotid parotid saliva as revealed by high-resolution electrophoretic methods. Electrophoresis 20: 2818-2823.

Wooley, S.C., Wooley, O.W. 1973. Salivation to the sight and thought of food: a new measure of appetite. Psychosom Med. 35: 136-142.

Warner, A.C., Stacy, B.D. 1977. Influence of ruminal and plasma osmotic pressure on salivary secretion in sheep. Q.J. Exp. Physiol. Cogn. Med. Sci. 62: 133-142.

Wróblewski, K., Muhandiran, R., Chkrabartty, A., Bennick, A. 2001. The molecular interaction of human salivary histatins with polyphenolic compounds. Eur. J. Biochem. 268: 4384-4397.

Xie, H. Rhodus, N.L., Griffin, R.J., Carlis, J.V., Griffin, T.J., 2005. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol. Cell Proteomics 4: 1826-1830.

Yamaguchi, S., Ishikawa, T. 2005. Electrophysiological characterization of native Na+-HCO3-cotransporter current in bovine parotid acinar cells. J. Physiol. 568: 181-197.

Yamashita, M., Fenn, J.B. 1984. Negative ion production with the Electrospray Ion Source. J. Phys. Chem. 88: 4671.

Yamashita, S., Kiyohara, S., Ohno, M., Hara, Y. 1996. Specificity of glossopharyngeal nerve responses to astringent compounds in Xenopus. Chem. Senses 21: 459-465.

Yan, Q., Bennick, A. 1995. Identification of histatins as tannin-binding proteins in human saliva. Biochem. J. 311: 341-347.

Yang, C.-M.J., Russell, J.B. 1992. Resistance of proline-containing peptides to ruminal degradation in vitro. Appl. Environ. Microbiol. 58: 3954-3958.

Yao, L., Berg, E.A., Costello, C.E., Troxler, R.F., Oppenheim, F.G., 2003. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomic approaches. J. Biol. Chem. 14: 5300-5308.

Yao, Y., Lamkin, M.S., Oppenheim, F.G. 1999. Pellicle precursor proteins: acidic proline-rich proteins, statherin and histatins and their crosslinking by oral transglutaminase. J. Dent. Res. 78: 1696-1703.

Yeh, C.-K., Johnson, D.A., Dodds, M.W.J., Sakai, S., Rugh, J.D., Hatch, J.P. 2000. Association of salivary flow rates with maximal bite force. J. Dent. Res. 79: 1560-1565.

Young, J.A., van Lennep, E.W. 1978. The morphology of salivary glands. London. Academic Press.

Zajácz, A., Gyémánt, G., Vittori, N., Kandra,L. 2007. Aleppo tannin: structural analysis and salivary amylase inhibition. Carbohyd. Res. 342: 717-723.

Zhang, G.H., Zhang, H.V., Deng, S.P., Qin, Y.M., Wang, T.H. 2008. Quantitative study of taste bud distribution within the oral cavity of the postnatal mouse. Arch. Oral Biol. 53: 583-589.

Chapter 2

Salivary glands histomorphological modifications in mice submitted to tannin enriched diets: comparison with sialotrophic effects of sympathetic agonists stimulation

E. Lamy, F. Capela e Silva, A.V. Coelho, E. Sales Baptista

The work presented here has been prepared as a manuscript for publication on Journal of Animal Physiology and Animal Nutrition

Abstract

In laboratory rodents, salivary proteins such as proline-rich proteins are induced by sympathetic agonists, such as isoproterenol and this induction is inter-related with increases in size of acinar structures from parotid and submandibular glands. The consumption of dietary tannins presents similarities to isoproterenol in increasing glandular weight and inducing proline-rich protein secretion. In order to study the effects of tannins at histomorphological level, mice were either fed with three structurally different types of tannins (tannic acid, chestnut and quebracho) or treated with isoproterenol, during 10 days. Acini of parotid and submandibular glands increased significantly, being the increase higher for parotid compared to submandibular glands, and higher in the quebracho compared with the other tannin groups. Sublingual acinar size also increased after tannin consumption, by opposition to isoproterenol-treated animals. Our results go in accordance with other studies suggesting that tannins act at beta-adrenergic receptor level, although some additional mechanisms might also be involved. Moreover, we present evidences that the effects produced by tannins are dependent on their structure being possible that mice will have the need to produce a greater amount of protein to counteract condensed rather than hydrolysable tannins.

Keywords: condensed tannin; histology; hydrolysable tannin; isoproterenol; mice; salivary glands

---

1. Introduction

Saliva is intimately related with food consumption. Food constituents may modulate salivary protein composition relatively quickly, since salivary secretion is a reflex exclusively mediated by the autonomic nervous system (Emmelin, 1987). Muscarinic-cholinergic and alpha-adrenergic stimulations have been proposed to be mainly involved in total volume secretion and beta-adrenegic stimulation in the production of a protein rich saliva (see Proctor and Carpenter, 2007 for a review).

The avoidance of tannins by animals is mainly due to the aversive sensations of astringency/bitter taste and to their anti-nutritive/toxic characteristics (Glendinning, 1992). The presence of salivary tannin-binding proteins, which has been suggested as the "first line of defense" against tannins, was observed in several herbivores and omnivorous mammalian species (reviewed by Shimada, 2006). Proline-rich proteins (PRPs), the main studied tannin binding proteins, can form complexes with tannins in the oral cavity, which remain stable in the gastrointestinal tract (Mitaru et al., 1984; Skopec et al., 2004), preventing these plant secondary metabolites to interact with dietary or endogenous proteins (Robbins et al., 1991; Hagerman and Robbins, 1993). In rodents, salivary PRPs are inexistent (or present in low amounts) for animals feed on regular laboratorial diets, but their synthesis can be induced by the beta-agonist isoproterenol (Mehansho and Carlson, 1983; Mehansho et al., 1983;

1987) or by feeding tannins (Mehansho et al., 1983; 1985; Jansman et al., 1994; Juntheikki et al., 1996; Shimada et al., 2006).

The induction of salivary PRPs by chronic administration of isoproterenol, and their secretion into saliva, has been associated with morphological and histo-morphological changes in parotid and submandibular glands (Wells and Humphreys-Beher, 1985; López Solís and Wilson, 1986; López Solís et al., 1987; 1989; 1990; 1993). Chronic administration of isoproterenol for several days elicits massive growth of salivary glands. This is due, in the first 2-3 days to the stimulation of DNA synthesis and cell proliferation (hyperplasia), followed, in the next days, by an enlargement of cell size (hypertrophy) (Chisholm and Adi, 1995; Ochiai et al., 2002), what, in the last case, may also reflect the increase in number and size of secretory granules (Matsuura and Hand, 1991; Matsuura and Suzuki, 1997). Not only acinar but also intercalated duct cells have been proposed to proliferate following isoproterenol administration (Hand and Ho, 1985). After the stop of stimulation with this sympathomimetic agent, it is observed a regression of the enlarged tissue. Apoptosis (Chilson and Adi, 1995), and at a fewer extent necrosis (Ochiai et al. 2002), seem to be responsible for this involution.

A few reports have shown that feeding rats and mice on tannin containing diets can produce salivary gland enlargement, similarly to what occurs with isoproterenol administration (Mehansho et al., 1983, 1985, 1987; Humphreys-Beher et al., 1987; Jansman et al., 1994).

The rodent parotid and submandibular gland acinar enlargement produced by isoproterenol, an agonist of autonomic sympathetic nervous system, has been studied by a considerable number of investigators (Barka and Burke, 1977; Vugman and Hand, 1995; Chisholm and Adi, 1995, among others). These morphological changes have been associated to an increased synthesis and secretion of salivary proteins, namely alpha-amylase and salivary proline-rich proteins (PRPs) (Robinovitch et al., 1977; Vugman and Hand, 1995; López Solís et al., 1987, 1989, 1990, 1993). Parotid and submandibular glands weight increase was also referred for rats (Mehansho et al., 1983; Jasman et al., 1994) and mice (Mehansho et al., 1985) consuming tannins, being this increase linear with increasing amounts of condensed tannins (Jansman et al., 1994). Similarities in the effects produced by these plant secondary metabolites, at cellular level, and the ones from isoproterenol administration were suggested (Mehansho et al., 1983; 1985). Topical administration of gallotanins in mice mouth, for several hours or days, resulted in the production of salivary proline rich polypeptides, which were suggested to be markers of induced trophic growth in parotid glands (Gho et al., 2007). All these data suggest that the molecular and cellular effects produced by high-tannin diets, both on the parotid salivary tissue and saliva are indistinguishable from those produced by isoproterenol stimulation (Tu et al., 1993; Ann et al., 1997). Isoproterenol sialotrophic effect is thought to be mediated by its direct interaction with β-adrenergic receptors, located on the basolateral surface of acinar cells, followed by an increase in cAMP, which acts as an intracellular messenger in the activation of several enzymes

associated with exocytosis, synthesis of secretory proteins, cell proliferation (Brenner and Wulf, 1981), among other phenomena (Baum et al., 1981; Vugman and Hand, 1995). Although it was observed that beta-adrenergic antagonists inhibited parotid gland hypertrophy induction by shorgum tannins (Mehansho et al., 1992), the mechanism linking dietary tannins to the sialotrophic response is not well understood (González et al., 2000; Gho et al., 2007) and it is still possible to hypothesize that differences between isoproterenol and tannin-rich diets as sialotrophic agents may well exist.

We proposed to study the changes on the morphometric parameters of the three pairs of major salivary glands, at histological level. Such study would allow a better elucidation of the effects of tannin consumption at glandular level and the establishment of comparable morphometric parameters for future experimental research. We will compare the effects produced by different type of stimulations: hydrolysable tannins (ex. the gallotannin tannic acid and the ellagitannin chestnut) and condensed tannins (quebracho) among them and with the sympathomimetic agonist isoproterenol. We will also access the modifications after withdrawing the stimulation.

2. Materials and methods

2.1 Animals

For each experiment inbred Balb/c mice, 4-week-old, were obtained from the licensed bioterium of Instituto Gulbenkian de Ciência (Oeiras, Portugal). The animals were housed in mice cages, type IV (Techniplast) (5 mice per cage), according to European Union (EU) recommendations and revision of Appendix A of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS Nº 123) and maintained on a 12:12 h, light/dark cycle at a constant temperature of 22ºC with *ad libitum* access to water and to a standard diet with 21.86% crude protein (dry basis) in the form of pellets (RM3A-P; Dietex Interational, UK). Animals were submitted to a 7-day acclimation period to minimize stress associated with transportation. This period was followed by a 7-day pretrial period to allow adaptation to the ground diet using during the experimental period. The standard pellet diet was ground daily with a blender to obtain a meal with visibly homogeneous fine-sized particles. Before the feeding-trial period, animals were individually weighed and allocated to the experimental groups, each group has no significant differences in body mass (20.47 ± 1.27 for animals used in the first experiment and 23.29 ± 3.22 for animals used in the second experiment).

All procedures involving the animals were approved by the scientific committee, supervised by a Federation of European Laboratory Animal Science Association (FELASA)- trained scientist and conforming to the regulations of the Portuguese law (*Portaria 1005/92*), following European Union Laboratory Animal Experimentation Regulations.

2.2 Feeding trials

2.2.1 Effects of tannic acid and isoproterenol

Immediately after the pretrial period, we started the 10-day "administration" period, followed by a 9-day "recovery" period. A total of 33 female mice were divided in three experimental groups. The control group (n=5) received a tannin-free diet, the same standard ground diet as in the pre-trial period, and were daily intra-peritoneally injected with 1mL of a saline solution (NaCl 0.9%). The tannic acid group (n=14) received the standard ground diet plus tannic acid (Merk, Ref 1.00773.100; hydrolysable tannin) added to obtain a mixture with 3g tannin/100g (3%) wet weight of the standard diet. The animals from this group received the same daily saline injection described for control group. The isoproterenol group (n=14) received the same standard ground diet, as the control group, but were daily injected with 50 μg/ g live weight of isoproterenol (*dl*-isoproterenol-HCl, Sigma), dissolved in 1mL of saline solution (NaCl 0.9%). At day 11, five animals from each group were injected intraperitoneally with anesthetic (xylazine hydrochloride combined with ketamine hydrochloride) and euthanized with an overdose. The three main salivary glands were dissected, washed briefly with phosphate buffer 0.1M, pH 7.4, and fixed in 10% neutral buffered formaline, to carry out further routine histological procedures. On the other hand, the remaining animals from tannic acid and isoproterenol groups initiated the "recovery period", which consisted in the absence of stimulation, either by isoproterenol or tannic acid. At days 3, 6 and 9 after stimuli cessation, three animals from each group were euthanized, and salivary glands dissected, as previous described, to examine the progression in the "regression" of salivary glands. During all the experimental period food and water were provided *ad libitum* and the diets were prepared daily with a blender, as described for the pretrial period. The glands were always collected between 9:00 and 12:00 am to avoid circadian variations.

2.2.2 Effects of different structural types of tannins

Twenty male mice were divided in four groups: control (n=5), tannic acid (n=5), chestnut (n=5) and quebracho (n=5) groups. Tannins were added to the powdered standard diet to obtain a mixture with 6g tannin/100g (6%) wet weight of the standard diet [Tannic acid from Merk, Ref 1.00773.100; chestnut extract (Tannino C) from SilvaChimica SRL; 77% of hydrolysable tannins; quebracho extract (Tupafin-Ato) from SilvaChimica SRL; 72% of condensed tannins]. At day 11 the animals were euthanized and salivary glands removed as described for the first experiment.

2.3 Histology

After embedding the fixed salivary glands in paraffin wax, using routine procedures, a series of sections of 5μm thick were cut with a microtome, and the slides were stained with hematoxylin and eosin (H&E). Salivary glands were observed through light microscopy with a Nikon Eclipse 600 microscope (Kanagawa, Japan). For each animal, ten digital pictures from random areas, of each salivary gland, were collected with a Nikon DN100 camera (Kanagawa, Japan). For each animal, the areas and perimeters of a minimum of 100 acini from parotid, submandibular and sublingual glands, and a minimum of 100 submandibular granular convoluted tubules (GCTs) (transverse sections) were randomly choose and measured by using SigmaScan Pro 5.0 software (SPSS, Chicago, IL, USA). Only the histological structures whose limits were clear defined were considered for measure.

2.4 Statistical analysis

Histomorphometric data were tested for normality and homocedaticity by Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA was performed. When normality was not achieved for each treatment, the non-parametric Kruskal-Wallis Z multiple comparison procedure was used. For normally distributed data, the means significantly different to post-hoc comparison of means (Turkey-Kramer test) and regarded as significantly different when $P<0.05$. All statistical analysis procedures were performed by NCSS 2001 software package (Kaysville, UT, USA).

3. Results

3.1. Effects of tannic acid and isoproterenol

Histomorphological analysis showed that both isoproterenol and tannic acid induced changes in female mice parotid and submandibular salivary glands. At acinar level (Table 1), isoproterenol treatment resulted in a great enlargement of parotid acinar (about 5-fold increase) and submandibular acinar (about 3-fold increase) areas, when compared with tannic acid consumption that only increased in a less pronounced way the parotid acinar area (about 2-fold increase) (Fig.1.1). In submandibular glands (Fig. 1.2), isoproterenol treatment also produced a significant increase in the size of seromucous acini (about 3-fold), whereas tannic acid did not produced changes in these submandibular structures. No changes were observed in acinar sublingual glands after both treatments (Table 1).

Once the stimulation was withdraw, the glandular dimensions started to decrease. At three days following the cessation of tannin consumption, the size of parotid acini had returned to control values (Fig. 2A), whereas for the individuals treated with isoproterenol such a recovery was not complete

even after 9 days treatment cessation (Fig. 2D). Nevertheless, at the third day of recovery period a significant reduction was already observed for the animals whose received isoproterenol (about half of the size than at the 11th day trial) and at the sixth day the parotid acinar size, from these animals, was similar to the one from the animals that received 3% tannic acid for ten days. At the third day of recovery period it was possible to observe apoptotic bodies in some acinar cells of the animals that received isoproterenol (Fig. 2E), suggesting that apoptosis may be a mechanism involved in the deletion of acinar cells and in the returning of parotid glands to normal levels.

Through qualitative evaluation, it was evident that the amount of visible GCTs was considerably decreased in the animals treated with isoproterenol for ten days. In these, GCTs appeared scattered among the hypertrophic acinar cells (Fig. 1.2C). However, for the well limited GCTs measured no significant changes in the dimensions were obtained (Table 2). With the stop of isoproterenol administration, the visible amount of these structures seemed to start returning to control levels, even since the third day after the last isoproterenol administration (Fig. 2F).

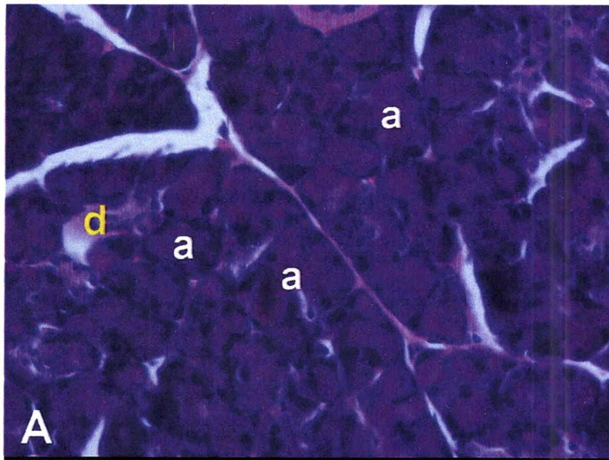
Table 1. Comparison (N=5 animals) of acinar structures among control tannic-acid (3%) enriched-diet and isoproterenol treatment (Mean ± SD)

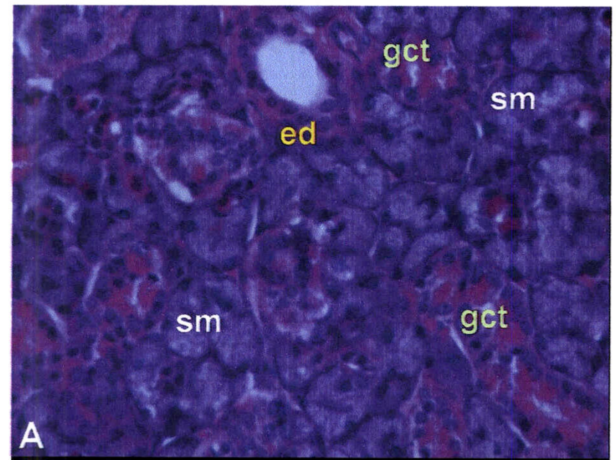
		Treatment						
		Administration period			Withdraw period			
		Control	Tannic acid 3%	Isoproterenol	3-d TA	3-d I	6-d I	9-d I
Parotid	Area (μm^2)	558.09 ± 184.49^{a}	921.29 ± 334.18^{b}	2466.47 ± 1491.52^{c}	533.2916 ± 176.4158^{a}	1242.35 ± 519.06^{d}	985.11 ± 390.03^{b}	775.32 ± 230.98^{e}
	z-value	**(C,TA)** 9.8172*; **(C,I)** 21.3607*; **(TA,I)** 8.9816*			**(TA, 3-d TA)** 8.4938* **(C, 3-d TA)** 0.7514	**(I, 3-d I)** 4.5583* **(C, 3-d I)** 13.3783*	**(I, 6-d I)** 9.0668* **(C, 6-d I)** 13.1506*	**(I, 9-d I)** 11.8763* **(C, 9-d I)** 7.3078*
	Perimeter (μm)	93.62 ± 15.99^{a}	122.14 ± 24.36^{b}	195.02 ± 60.43^{c}	93.23 ± 15.99^{a}	141.64 ± 29.74^{d}	127.60 ± 26.08^{b}	113.68 ± 17.36^{e}
	z-value	**(C,TA)** 9.9650*; **(C,I)** 21.4080*; **(TA,I)** 8.8880*			**(TA, 3-d TA)** 7.9927* **(C, 3-d TA)** 0.0419	**(I, 3-d I)** 4.3129* **(C, 3-d I)** 13.6878*	**(I, 6-d I)** 8.5367* **(C, 6-d I)** 13.8145*	**(I, 9-d I)** 11.1974* **(C, 9-d I)** 8.1131*
Submandibular	Area (μm^2)	551.94 ± 226.75^{a}	580.40 ± 224.36^{a}	1685.14 ± 874.53^{b}	-----	1289.88 ± 556.39^{c}	1266.89 ± 670.19^{c}	948.35 ± 352.39^{d}
	z-value	**(C,TA)** 0.5842; **(C,I)** 17.1239*; **(TA,I)** 13.7740*			-----	**(I, 3-d I)** 2.0606* **(C, 3-d I)** 11.7497*	**(I, 6-d I)** 2.9716* **(C, 6-d I)** 12.4779*	**(I, 9-d I)** 7.2309* **(C, 9-d I)** 9.9383*
	Perimeter (μm)	94.07 ± 20.22^{a}	97.70 ± 18.61^{a}	159.92 ± 41.93^{b}	-----	143.05 ± 32.65^{c}	140.34 ± 34.66^{c}	123.13 ± 23.09^{d}
	z-value	**(C,TA)** 0.8799; **(C,I)** 16.7239*; **(TA,I)** 13.1347*			-----	**(I, 3-d I)** 1.9851* **(C, 3-d I)** 11.5020*	**(I, 6-d I)** 2.9490* **(C, 6-d I)** 12.1411*	**(I, 9-d I)** 7.1149* **(C, 9-d I)** 9.6552*
Sublingual	Area (μm^2)	1059.68 ± 416.49^{a}	1021.87 ± 420.65^{a}	957.42 ± 370.73^{a}	-----			
	z-value	**(C,TA)** 0.7546; **(C,I)** 1.8939; **(TA,I)** 1.1920			-----	-----	-----	-----
	Perimeter (μm)	126.60 ± 25.88^{a}	124.79 ± 27.49^{a}	121.46 ± 24.74^{a}				
	z-value	**(C,TA)** 0.6139; **(C,I)** 1.5391; **(TA,I)** 0.9681			-----			

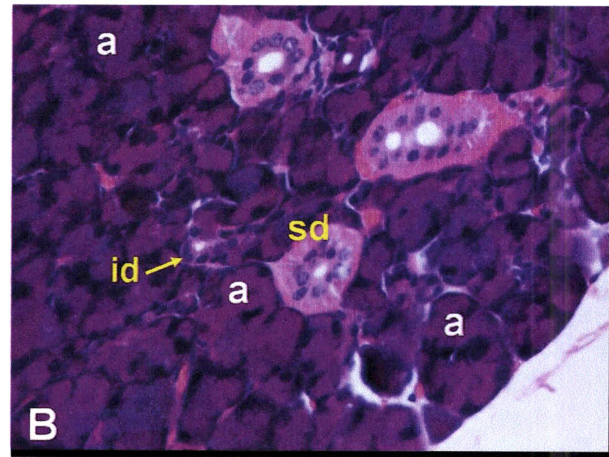
C- Control; TA-Tannic acid; I-Isoproterenol; 3-d TA – Three days recovery from tannic acid; 3-d I – three days recovery from isoproterenol treatment; 6-d I – six days recovery from isoproterenol treatment; 9-d I – nine days recovery from isoproterenol treatment

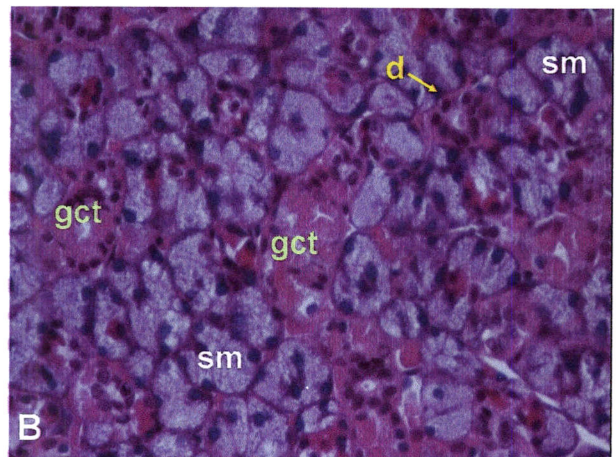
Same lowercase letters indicate no differences among the values of the columns in the same line (Kruskal-Wallis Z multiple comparison procedure)

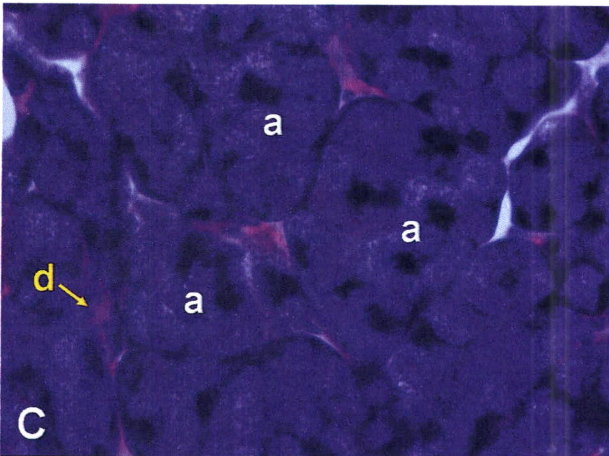
*Differences are significant for z-value>1.9











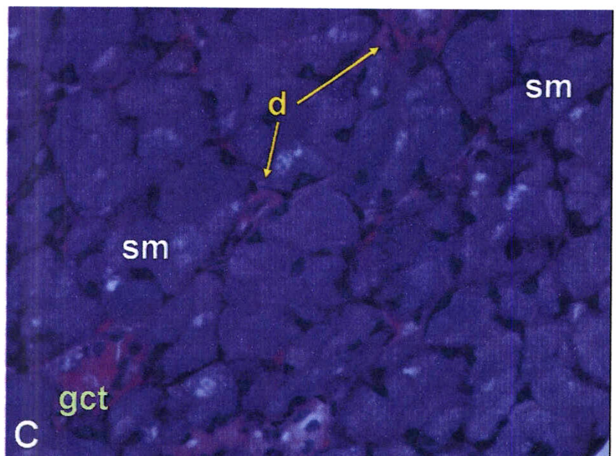


Figure 1.1 – Changes in female mice parotid glands after 10-day of stimulation:

Figure 1.2 – Changes in female mice submandibular glands after 10-day of stimulation.

(A) control group, **(B)** tannic acid group and **(C)** isoproterenol group. **a**- acinus; **d** – duct ; **id** – intercalated duct ; **sd** – striated duct ; **ed** – excretory duct; **sm** – seromucous acinus; **gct** – submandibular granular convoluted tubules;
200X. H&E; These photomicrographs are representative of multiple sections examined from five mice per condition.

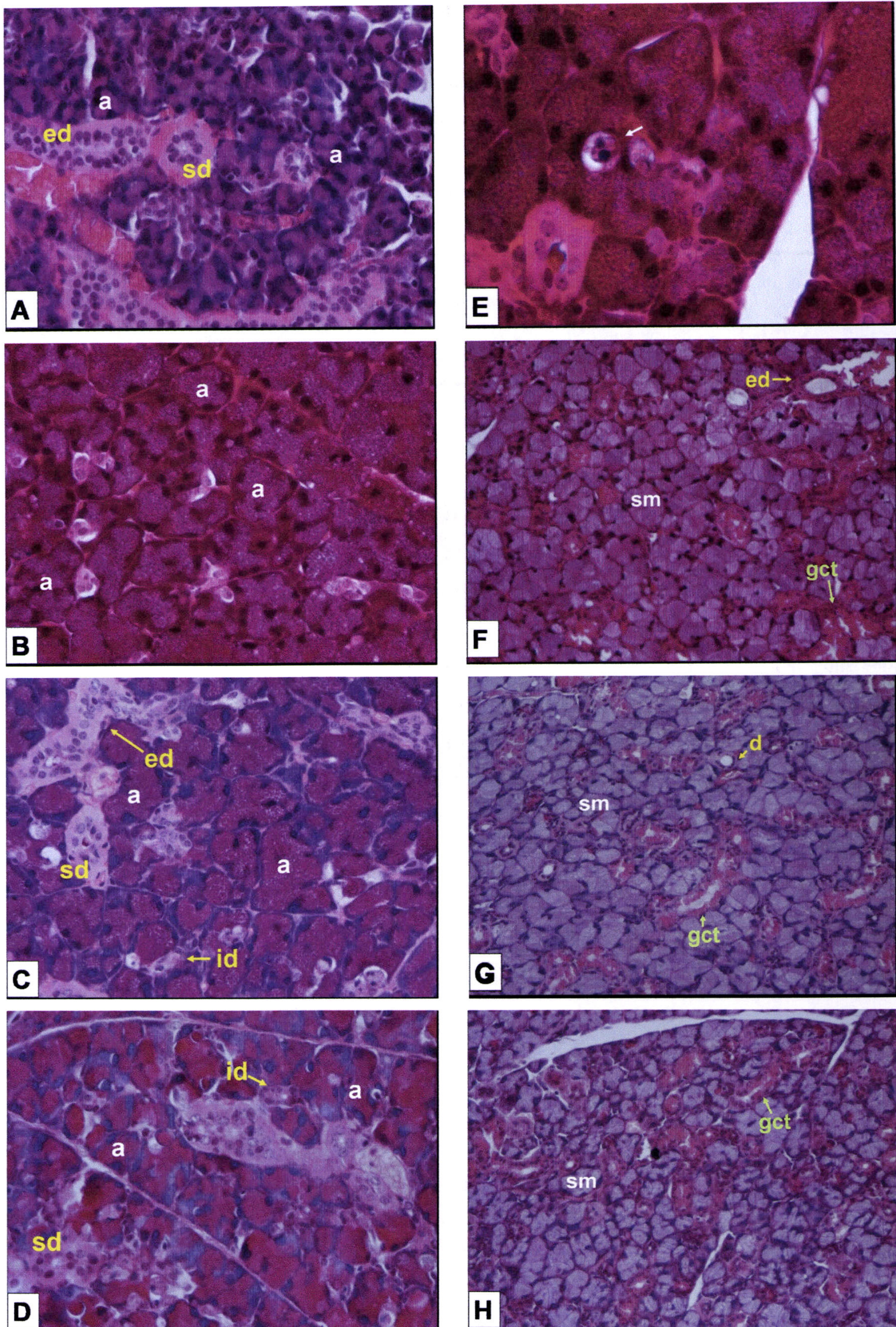


Figure 2 – Changes in female mice parotid and submandibular glands after withdraw of the stimulation. **(A)** parotid gland 3-days following tannic acid withdraw; **(B,C,D) parotid glands following isoproterenol withdraw:** **B** at 3-days; **C** at 6-days; **D** at 9-days; **(E)** Apoptotic bodies in parotid acinar cell (arrow) at 3-days after isoproterenol treatment stopped; **(F,G,H) submandibular glands following isoproterenol withdraw:** **F** at 3-days; **G** at 6-days; **H** at 9-days; H&E. (A,B,C,D) – 200X; (E) – 400X; (F,G,H) – 100 H&E (200X)

(Previous page. Cont.)
a- acinus; **d** – duct ; **id** – intercalated duct ; **sd** – striated duct ; **ed** – excretory duct; **sm** – seromucous acinus; **gct** – submandibular granular convoluted tubules; these photomicrographs are representative of multiple sections examined from five mice per condition.

Table 2. Comparison (N=5 animals) of submandibular granular convoluted tubules (GCTs) among control, tannic-acid (3%) enriched-diet and isoproterenol treatment (Mean ± SD)

	Treatment			z-value		
	Control	**Tannic acid**	**Isoproterenol**	**C,TA**	**C,I**	**TA, I**
Area (μm^2)	831.39 ± 467.59	795.07 ± 351.62	789.71 ± 509.90	0.0726	0.9107	0.8682
Perimeter (μm)	124.44 ± 41.68	118.29 ± 33.69	112.55 ± 39.53	0.4174	1.5676	1.1959

C – control; TA – tannic acid; I – isoproterenol
* Differences are significant for z-value>1.96

3.2. Effects of different structural types of tannins

All the three structurally different tannins, used in this experiment, produced an enlargement in parotid, submandibular, and sublingual acini (Figs. 3.1 and 3.2). The enlargement in parotid acini was significantly higher for the animals consuming quebracho tannin (almost a 3-fold increase in acinar area, compared with control group) than for the groups consuming the same amount of tannic acid or chestnut tannins (2-fold increase, compared with control group) (Table 3). On the contrary, the consumption of tannins had no effect on the size of submandibular GCTs (Table 4) and no such pronounced differences in the distribution of these structures, as the ones observed for isoproterenol treatment in the first experiment, seemed to occur (Figs. 3.1 and 3.2).

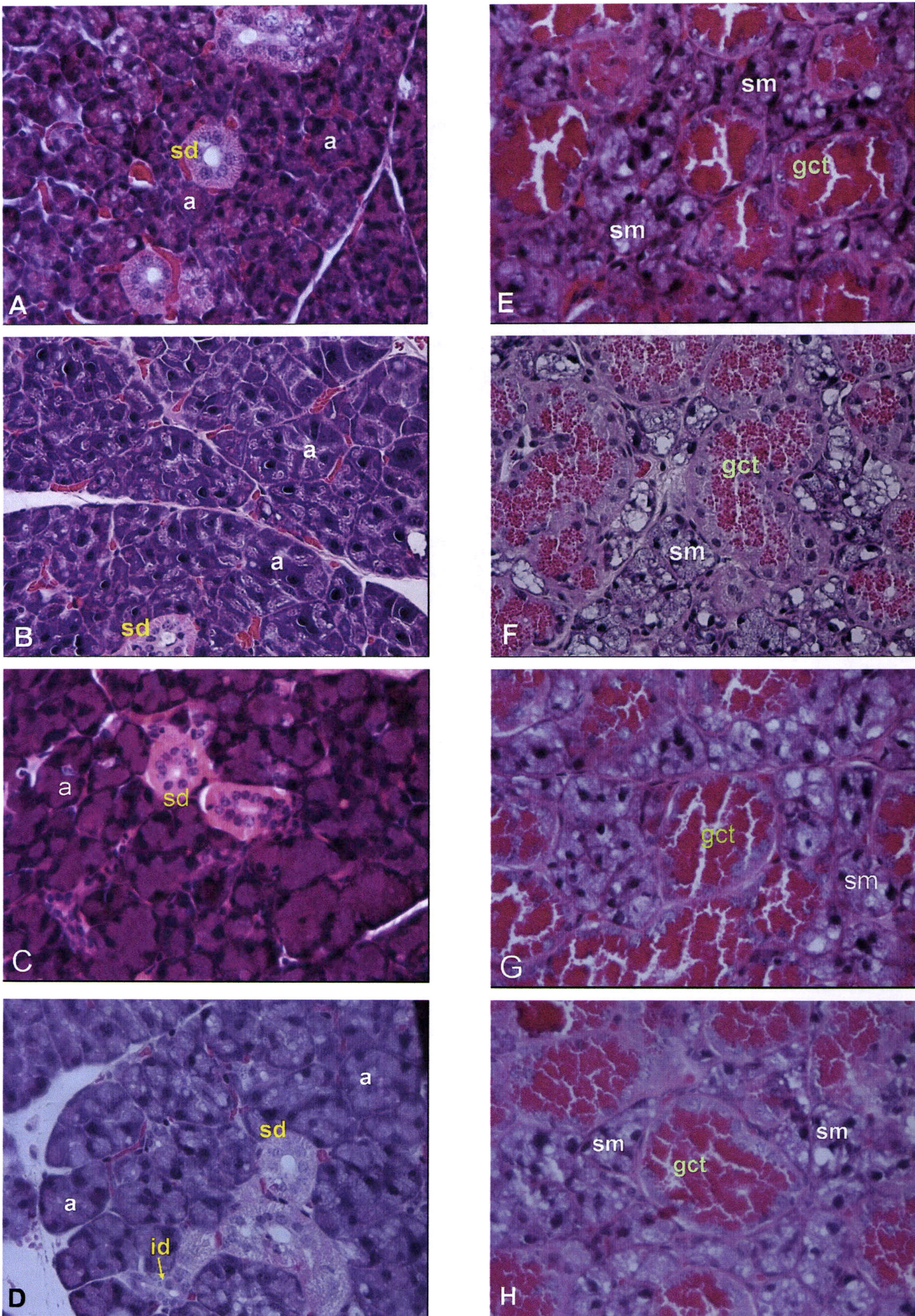


Figure 3.1 –Changes in male mice parotid (A to D) and submandibular (E to H) glands: (A and E) control group; **(B and F)** tannic acid group; **(C and G)** chestnut group ; **(D and H)** quebracho group
a- parotid serous acinus; **sm** – submandibular seromucous acinus; **gct** – submandibular granular convoluted tubules; **d** – duct ; **id** – intercalated duct ; **sd** – striated duct ; **ed** – excretory duct; H&E. 200X.
These photomicrographs are representative of multiple sections examined from five mice per condition.

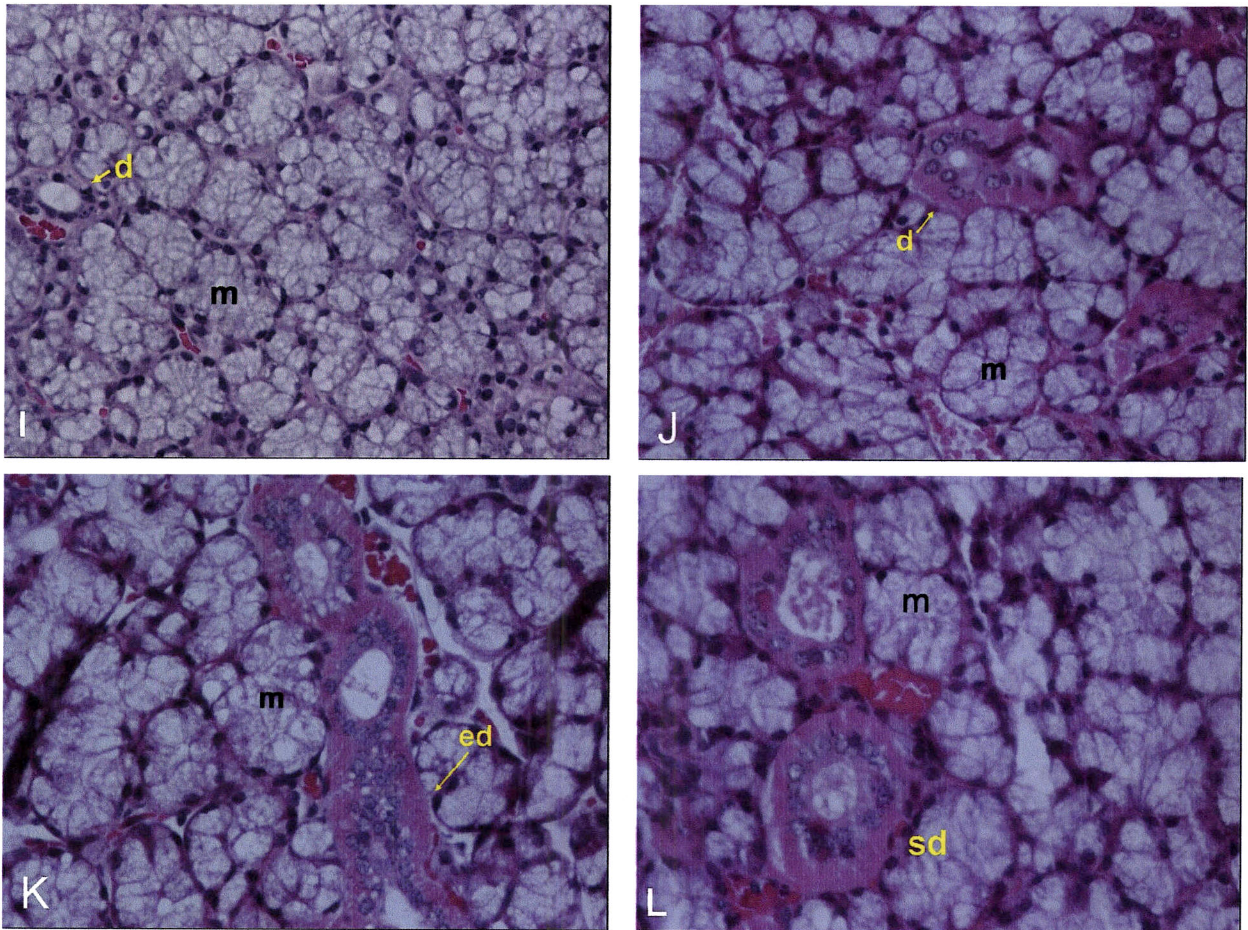


Figure 3.2 –Changes in male mice sublingual glands: **(I)** control group; **(J)** tannic acid group; **(K)** chestnut group ; **(L)** quebracho group. ; **m** – sublingual mucous acinus ; **d** – duct ; **sd** – striated duct ; **ed** – excretory duct H&E. 200X.
These photomicrographs are representative of multiple sections examined from five mice per condition

Table 3. Comparison (N=5 animals) of acinar structures among control and the three structurally different tannin types (Mean ± SD)

		Treatment			
		Control	Tannic acid	Chestnut	Quebracho
Parotid	Area (μm^2)	560.56 ± 85.89^a	945.40 ± 360.66^b	1062.37 ± 432.01^b	1400.53 ± 605.22^c
	z-value	(C,TA) 12.9851*; (C,CH) 10.0033*; (C,Q) 18.9667*; (TA,CH) 1.5632; (TA,Q) 6.9698*; (CH,Q) 3.4632*			
	Perimeter (μm)	95.01 ± 15.95^a	123.70 ± 22.91^b	130.72 ± 26.74^b	151.49 ± 32.78^c
	z-value	(C,TA) 13.0418*; (C,CH) 9.8620*; (C,Q) 18.9301*; (TA,CH) 1.3910; (TA,Q) 6.8878*; (CH,Q) 3.5689*			
Submandibular	Area (μm^2)	756.27 ± 308.61^a	746.42 ± 252.76^{a,b}	842.65 ± 336.88^{b,c}	952.41 ± 449.62^c
	z-value	(C,TA) 0.1904; (C,CH) 2.6471*; (C,Q) 4.4064*; (TA,CH) 1.3180; (TA,Q) 2.4335*; (CH,Q) 1.6682			
	Perimeter (μm)	110.04 ± 22.58^a	110.19 ± 19.26^{a,b}	115.11 ± 23.50^b	123.07 ± 29.29^c
	z-value	(C,TA) 0.3131; (C,CH) 2.0785*; (C,Q) 4.3946*; (TA,CH) 0.8821; (TA,Q) 2.3145*; (CH,Q) 2.1152*			
Sublingual	Area (μm^2)	1051.20 ± 546.84^a	1210.82 ± 625.43^b	1225.09 ± 588.85^b	1234.21 ± 670.20^b
	z-value	(C,TA) 3.4686*; (C,CH) 3.6585*; (C,Q) 2.6925*; (TA,CH) 0.5167; (TA,Q) 0.1494; (CH,Q) 0.2740			
	Perimeter (μm)	124.63 ± 32.97^a	134.24 ± 36.46^b	135.36 ± 34.58^b	136.61 ± 39.70^b
	z-value	(C,TA) 3.5151*; (C,CH) 3.6167*; (C,Q) 3.0048*; (TA,CH) 0.4427; (TA,Q) 0.4061; (CH,Q) 0.0278			

C – control; TA – tannic acid; CH – Chestnut; Q – Quebracho;
Same lowercase letters indicate no differences among the values of the columns in the same line (Kruskal-Wallis Z multiple comparison procedure)
* Differences are significant for z-value>1.96

Table 4. Comparison (N=5 animals) of submandibular GCTs among control and the three structurally different tannin types (Mean ± SD)

	Treatment				Z value
	Control	Tannic acid	Chestnut	Quebracho	
Area (μm^2)	3566.50 ± 1933.80	3343.44 ± 1751.26	3817.33 ± 2195.39	3739.74 ± 2416.27	(C,TA) 1.1472 (C,CH) 1.1010 (C,Q) 0.0274 (TA,CH) 1.9153 (TA,Q) 0.8832 (CH,Q) 0.9416
Perimeter (μm)	235.84 ± 75.41	227.15 ± 67.83	245.57 ± 81.13	242.39 ± 92.79	(C,TA) 1.2902 (C,CH) 1.4129 (C,Q) 0.0811 (TA,CH) 2.3145* (TA,Q) 0.9471 (CH,Q) 1.2463

C – control; TA – tannic acid; CH – Chestnut; Q – Quebracho;
* Differences are significant for z-value>1.96

4. Discussion

In the present study we could confirm that both isoproterenol and tannins increase the size of the acini from parotid and submandibular glands and that these changes are induced by both hydrolysable and condensed tannins. Selye et al. (1961) were the first authors reporting that long-term

administration of isoproterenol elicits massive growth of salivary glands, most notably the parotid and submandibular glands in rodents. Since then, hundred of studies have been published about the mechanisms of this growth.

Apparently, the effect of isoproterenol treatment was stronger than the one from tannins, although statistical comparisons with chestnut and quebracho groups were not performed, due to the use of different sexes in each experiment. Although parotid sexual dimorphism is not as conspicuous as in the mouse submandibular gland, it was referred that the acini and intercalated ducts of parotid glands are more developed in male mice than in females (Ribeiro et al., 2001). Additionally, differences between male and female in the regulation of some salivary proteins in parotid glands were also referred (Kurabuchi and Hosoi, 2004). The proportion of increase in acinar areas and perimeters seemed to be unchanged when stimulating with tannic acid with 3 or 6%, tendency that we are unable to confirm statistically. The increases in salivary acinar areas produced by isoproterenol are concordant with the ones referred in bibliography (Onofre et al., 1997; Ochiai et al., 2002).

The mouse submandibular gland possesses two morphologically and biochemically distinct exocrine compartments with different modes of secretory activity: acini and GCTs. Both were analyzed in the present study. In what concerns seromucous acini, an increase in their dimensions was induced both by isoproterenol, in the first experiment, and chestnut and quebracho tannins, in the second experiment. In submandibular acini, differences were not observed after tannic acid treatment, what lead us to hypothesize the existence of some differences between this and the other two types of tannins in their action at submandibular acinar level. We were unable to find studies reporting differences among the different structural types of tannins in their effects in salivary gland weight/structure. However, it was observed, in ruminants, that different types of tannins produce different effects: some species are tolerant to a structural type of tannin, but not to a different type, whereas others seem to tolerate all structural types (Clauss et al., 2003). It had been proposed that this scenario could be due to the amounts and types of tannins usually found in their regular diets (McArthur et al., 1995). For rats, it was referred that condensed tannins from grape seed and quebracho produced significant effect on growth depression and feed intake, whereas hydrolysable tannins were devoid of such a deleterious effect (Joslyn and Glick, 1969).

The morphological characteristics of apoptosis include pycnotic nuclear chromatin, cytoplasmic condensation, intact organelles, and the presence of membrane-bound apoptotic bodies (Ochiai et al., 2002). By day 3, post-treatment, apoptotic cells were observed in the animals that received isoproterenol. Apoptosis were already proposed to account for cell deletion in salivary glands enlarged by isoproterenol (Chisholm and Adi, 1995; Ochiai et al., 2002). Nine days post-isoproterenol administration the salivary glands had not the control values of size, suggesting that a higher period of time is necessary for a full recovery, what goes in accordance with other studies (Ochiai et al., 2002). The recovery from tannin treatment was quicker, and only based on hematoxylin-eosin staining

we were unable to observe apoptotic bodies at day 3 post-treatment. The lower growth induced by these compounds may explain the quicker recovery, and apoptosis (if accounting for this regression) may have occurred earlier.

The GCT is a specialized type of secretory canal that lies between the intercalated and striated ducts and is well-marked in the submandibular glands of rodents (Pinkstaff, 1980; Tandler, 1993). Systemic application of isoproterenol for a period of ten days did not result in changes in the dimension of these structures. Chisholm and Adi (1995) also reported an absence of cell proliferation in these glandular structures after isoproterenol treatment, as well as in other duct cells. However, isoproterenol produced an apparent reduction in the amount of GCTs. Similar features had been already observed in female rats after one week isoproterenol treatment (Thulesen et al., 2002). We may speculate that this decrease is due to the great enlargement of acini, which constrict GCTs, hiding them. A real disappearing is less plausible, since other studies observed maintenance, in salivary secretion of proteins synthesized in these structures, after chronic isoproterenol administration (Thulesen et al., 2002).

The marked sexual dimorphism in mice submandibular gland level was first referred by Laassagne, in 1940, and since then it was demonstrated that such dimorphism become clear evident within the gland at four weeks of age (Jayasinghe et al., 1990), which is the age of the animals used in the present study. This dimorphism have been greatly studied (Pinkstaff, 1998) and seems to result from sex-related differences in gene expression (Treister et al., 2005). In accordance with the bibliography (Jayasinghe te al., 1990; Chai et al., 1993; Pardini and Taga, 1996), we were also able to observe the occupancy of a relatively higher proportion of the gland space by acinar structures, in females, together with a lower size and number of GCTs, compared with male mice (Fig. 2A vs. Fig. 4 and Fig. 5E). Moreover, the cells of the female GCTs appear to contain fewer secretory granules than the ones from males, what had already been observed by Jayasinghe et al. (1990).

Isoproterenol is a powerful β-adrenergic agonist, which is active in the stimulation of β1- and β2-adrenergic responses (Zaagsma and Nahorski, 1990). The submandibular acini and GCTs were surrounded by both an adrenergic and a cholinergic plexus. Whereas acinar exocytotic secretion is greatly stimulated through β-adrenergic receptors, the granular secretion from the convoluted tubules is predominantly stimulated through α-adrenergic receptors (Hosoi et al., 1978; Vreugdenhil et al., 1980). This may be the explanation for the great effect that isoproterenol had on acinar cells rather than on GCT cells. According with studies showing that systemic administration of isoproterenol and topical administration of tannic acid to mice mouths both resulted in the production of a particular group of polypeptides (Gho et al., 2007), it is possible to think that tannin consumption has an effect at sympathetic nervous level, resulting in the activation of adrenergic receptors from the β-, rather that the α-type. This explains the lack of effect of tannins in GCT observed by us. Despite all the

similar effects produced by tannins and isoproterenol, in what concerns sublingual glands only tannins induced an increase in the acinar dimensions. This difference points to the possibility of tannins do not act exclusively through beta adrenergic receptor stimulation, inversely to isoproterenol.

Tannic acid and chestnut, the two hydrolysable tannins used in the second experiment, produced a lower increase in parotid acinar size than the condensed tannin quebracho. The mice and rat parotid gland enlargement induced by isoproterenol had been associated to the secretion of proline-rich salivary proteins (Mehansho et al., 1983; 1985; Gho et al., 2007). The productions of these proteins were also observed after tannin consumption. We may speculate that the different structural types of tannins have different "negative" effects on the animal, and condensed tannin consumption results in a greater need of tannin-binding proteins than hydrolysable tannins consumption.

5. References

Ann, D.K., Lin, H.H., Kousvelari, E. 1997. Regulation of salivary gland specific gene expression. Crit. Rev. Oral Biol. Med. 8: 244-252.

Barka, T., Burke, G.T. 1977. Secretory behaviour of hypertrophic and hyperplastic salivary gland. Histochem. J. 9: 453-466.

Baum, B.J., Freiberg, J.M., Ito, H., Roth, G.S., Filburn, C.R. 1981. beta-Adrenergic regulation of protein phosphorylation and its relationship to exocrine secretion in dispersed rat parotid acinar cells. J. Biol. Chem. 256: 9731-9736.

Brenner, G.M., Wulf, R.G. 1981. Adrenergic beta receptor mediating submandibular salivary gland hypertrophy in the rat. J. Pharmacol. Exp. Ther. 218: 608-612.

Chai, Y., Klauser, D.K., Denny, P.A., Denny, P.C. 1993. Proliferative and structural differences between male and female mouse submandibular glands. Anat. Rec. 235: 303-311.

Clauss, M., Lason, K., Gehrke, J., Lechner-Doll, M., Fickel, J., Grune, T., Jürgen Streich, W. 2003. Captive roe deer (*Capreolus capreolus*) select for low amounts of tannic acid but not quebracho: fluctuation of preferences and potential benefits. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136: 369-382.

Chisholm, D.M., Adi, M.M. 1995. Cell proliferation and apoptosis in isoprenalina-induced sialosis in the rat submandibular glands. Int. J. Exp. Pathol. 76: 263-269.

Emmelin, N. 1987. Nerve interactions in salivary glands. J. Dent. Res. 66: 509-517.

Gho, F., Peña-Neira, A., López-Solís, R.O. 2007. Induction of salivary polypeptides associated with parotid hypertrophy by gallotannins administered topically into the mouse mouth. J. Cell Biochem. 100: 487-498.

Glendinning, J.I. 1992. Effect of salivary proline-rich proteins on ingestive responses to tannic acid in mice. Chem. Senses 17: 1-12.

González, M.J., Lillo, S.P., Alliende, C., López Solís, R.O. 2000. Cell-enlargement-related polypeptides are induced via β1-adrenoceptors in mouse parotids. Exp. Mol. Pathol. 69: 91-101.

Hagerman, A.E., Robins, C.T. 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. Can. J. Zool. 71:628–633.

Hand, A.R., Ho, B. 1985. Mitosis and hypertrophy of intercalated duct cells and endothelial cells in the isoproterenol-treated rat parotid gland. J. Dent. Res. 64: 1031-1038.

Hosoi, K., Aoyama, K., Ueha, T. 1978. Regulation of the secretory process of granular components from the convoluted tubular cells of the mouse submandibular gland. J Dent Res 57: 87-90.

Humphreys-Beher, M.G., Robinson, G.D., Al-Zahid, S., Schneyer, C.A. 1987. Neural and dietary modulation of proline-rich protein and 4 -galactosyltransferase biosynthesis in rat parotid glands. Archs Oral Biol. 32: 573-578.

Jansman, A.J.M., Frohlich, A.A., Marquardt, R.R. 1994. Production of proline-rich proteins by the parotid glands of rats is enhanced by feeding diets containing tannins from Faba beans (*Vicia faba* L). J. Nutr. 124: 249–258.

Jayasinghe, N.R., Cope, G.H., Jacob, S. 1990. Morphometric studies on the development and sexual dimorphism of the submandibular gland of the mouse. J. Anat. 172: 115-127.

Joslyn, M.A., Glick. 1969. Comparative effects of gallotanins and related phenolics on the growth of rats. J. Nutr. 98: 119-126.

Juntheikki, M.-R., Julkunen-Tiitto, R., Hagerman, A.E. 1996. Salivary tannin-binding proteins in root vole (*Microtus oeconomus* Pallas). Biochem. Syst. Ecol. 24: 25–35.

Kurabuchi, S., Hosoi, K. 2004. Immunocytochemical localization of mK1, a true tissue kallikrein, in the mouse parotid gland: sexual dimorphism and effects of castration and hypophysectomy. Odontology 92: 73-76.

López Solís, R.O., Wilson, D.M. 1986. Changes in the polypeptide composition of mouse parotid glands after stimulation of secretion and DNA synthesis by isoproterenol. Biochim. Biophys. Acta 881: 422-429.

López Solís, R.O., Alliende, C., González, J., Miranda, D. 1987. Changes in the polypeptide composition related to the growth response in chronically isoproterenol-stimulated mouse parotid glands. Bioch. Biophys. Acta 930: 463-469.

López Solís, R.O., 1989. Secretory character of a group of isoproterenol-induced polypeptides in mouse parotid glands. J. Cell Physiol. 141: 660-666.

López Solís, R.O., Miranda, D., Wilson, D.M., González Burgos, J. 1990. Effect of isoproterenol analogs on the polypeptide composition of mouse parotid glands: relationship to enhanced growth. Biochim. Biophys. Acta 1054: 311-316.

López Solís, R.O., González, M.J., Castillo, L., Peña y Lillo, S., Alliende, C. 1993. Transcriptional and posttranscriptional control in synthesis of growth marker polypeptides in mouse parotids. Am. J. Physiol. 265: G514-G520.

Matsuura, M., Hand, A.R. 1991. Quantitative immunocytochemistry of rat submandibular secretory proteins during chronic isoproterenol administration and recovery. J. Histochem. Cytochem. 39: 945-954.

Matsuura, S., Suzuki, K. 1997. Immunohistochemical analysis of DNA synthesis during chronic stimulation with isoproterenol in mouse submandibular gland. J. Histochem. Cytochem. 45: 1137-1145.

McArthur, C., Sanson, G.D., Beal, A.M. 1995. Salivary proline-rich proteins in mammals: role in oral homeostasis and counteracting dietary tannins. J. Chem. Ecol. 21: 663-691.

Mehansho, H., Carlson, D.M. 1983. Induction of protein and glycoprotein synthesis in rat submandibular glands by isoproterenol. J. Biol. Chem. 258: 6616–6620.

Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc. Natl. Acad. Sci. USA 80: 3948–3952.

Mehansho, H., Clements, S., Sheares, B.T., Smith, S., Carlson, D.M. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. J. Biol. Chem. 260: 4418–4423.

Mehansho, H., Ann, D.K., Butler, L.G., Rogler, J., Carlson, D.M. 1987. Induction of proline-rich proteins in hamster salivary glands by isoproterenol treatment and an unusual growth inhibition by tannins. J. Biol. Chem. 262: 12344–12350.

Mehansho, H., Asquith, T.N., Butler, L.G., Rogler, J.C., Carlson, D.M. 1992. Tannin-mediated induction of proline-rich protein synthesis. J. Agric. Food Chem. 40: 93-97.

Mitaru, B.N., Reichert, R.D., Blair, R. 1984. The binding of dietary protein by sorghum tannins in the digestive tract of pigs. J. Nutr. 114: 1787–1796.

Ochiai, S., Hashimoto, S., Shimono, M. 2002. Acinar cell hypertrophy, proliferation and cell death in rat submandibular glands induced by long-term isoproterenol administration. Acta Histochem. Cytochem. 35: 383-393.

Onofre, M.A., de Souza, L.B., Campos, A. Jr, Taga, R. 1997. Stereologic study of acinar growth in the rat parotid gland induced by isoproterenol. Arch. Oral Biol. 42: 333-338.

Pardini, L.C., Taga, R. 1996. Stereological study of the sexual dimorphism in mouse submandibular glands. Okajimas Folia Anat. Jpn. 73: 119-124.

Pinkstaff, C.A. 1980. The cytology of salivary glands. Int. Rev. Cytol. 63: 141-261.

Pinkstaff, C.A. 1998. Salivary gland sexual dimorphism: a brief review. Eur. J. Morphol. 36 (Suppl): 31-34.

Proctor, G.B., Carpenter, G.H. 2007.Regulation of salivary gland function by autonomic nerves. Auton. Neurosci. 133: 3-18.

Ribeiro, T.T., Cestari, T.M., Taga, R. 2001. Morphometric dimensions of the mouse parotid glands of both sexes. Ital. J. Anat Embryol. 106: 27-34.

Robbins, C.T., Hagerman, A.E., Austin, P.J., McArthur, C., Hanley, T.A. 1991. Variation in mammalian physiological responses to a condensed tannin and its ecological implications. J. Mammal. 72: 480-486.

Robinovitch, M.R., Keller, P.J., Johnson, D.A., Iversen, J.M., Kauffman, D.L. 1977. Changes in rat parotid salivary proteins induced by chronic isoproterenol administration. J. Dent. Res. 56: 290-303.

Selye, H., Veilleux, R., Cantin, M. 1961. Excessive stimulation of salivary gland growth by isoproterenol. Science 133: 44-45.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32: 1149-1163.

Shimada, T., Saitoh, T., Sasaki, E., Nishitani, Y., Osawa, R. 2006. Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins. J. Chem. Ecol. 32: 1165-1180.

Skopec, M.M., Hagerman, A.E., Karasov, W.H. 2004. Do salivary proline-rich proteins counteract dietary hydrolyzable tannin in laboratory rats? J. Chem. Ecol. 30: 1679–1692.

Tandler, B. 1993. Structure of the duct system in mammalian major salivary glands. Microsc. Res. Tech. 26: 57-74.

Thulesen, J., Bor, M.V, Thulesen, S., Nexø, E., Pulsen, S.S., Jørgensen, P.E. 2002. Altered secretion and processing of epidermal growth factor in adrenergic-induced growth of the rat submandibular gland. Regul. Pept. 106: 105-114.

Treister, N.S., Richards, S.M., Suzuki, T. Jensen, R.V., Sullivan, D.A. 2005. Influence of androgens on gene expression in the BALB/c mouse submandibular gland. J. Dent. Res. 84: 1187-1192.

Tu Z.J., Lazowsky K.W., Ehlenfeldt, R.G., Wu, G., Lin, H.H., Kousvelari, E., Ann, D.K. 1993. Isoproterenol/tannin-dependent R15 expression in transgenic mice is mediated by an upstream parotid control region. Gene Expression 3: 289-305.

Vreugdenhil, A.P., de Lange, G.L., Nieuw Amerongen, A.V., Roukema, P.A. 1980. Morphological changes in the salivary glands upon stimulation by receptor-selective agonists. II. Submandibular glands of the mouse. J. Biol. Buccale 8: 73-86.

Vugman, I., Hand, A.R. 1995. Quantitative immunocytochemical study of secretory protein expression in parotid glands of rats chronically treated with isoproterenol. Microsc. Res. Tech. 31: 106-117.

Wells, D.J., Humphreys-Beher, M.G. 1985. Analysis of protein synthesis in rat salivary glands after chronic treatment with beta-receptor agonist and phosphodiesterase inhibitors. Biochem. Pharmacol. 34: 4229-4237.

Zaagsma, J., Nahorski, S.R. 1990. Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical "β3-adrenoceptor"? Trends Pharmacol. Sci. 11: 3-7.

UNIVERSIDADE

Chapter 3

Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins

G. da Costa*, E. Lamy*, F. Capela e Silva, J. Andersen, E. Sales Baptista, A.V. Coelho

**The two first authors have contributed equally to this work.*

The work presented here has been published in Journal of Chemical Ecology (2008) 34: 376-387.

Abstract

Tannins are plant secondary metabolites characterized by protein-binding affinity. They have astringent/bitter properties that act as deterrents, affecting diet selection. Two groups of salivary proteins, proline rich proteins and histatins are effective precipitators of tannin, decreasing levels of available tannins. The possibility of other salivary proteins having a co-adjuvant role on host defense mechanisms against tannins is unknown. In this work we characterized and compared the protein profile of mice whole saliva from animals fed on three experimental diets: tannin-free diet, diet with the incorporation of 5% hydrolysable tannins (tannic acid) or diet with 5% condensed tannins (*quebracho*). Protein analysis was performed by one-dimensional gel electrophoresis combined with Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry to allow the dynamic study of interactions between diet and saliva. Since abundant salivary proteins obscure the purification and identification of medium and low expressed salivary proteins, we used centrifugation to obtain saliva samples free from proteins that precipitate after-tannin binding. Data from Peptide Mass Fingerprinting has allowed us to identify ten different proteins, some of them showing more than one isoform. Tannin-enriched diets were observed to change the salivary protein profile. One isoform of α-amylase was over expressed with both types of tannins. Aldehyde reductase was only identified in saliva of the *quebracho* group. Additionally, a hypertrophy of parotid salivary glands acini was observed by histology, along with a decrease in body mass in the first four days of the experimental period.

Key Words: Salivary proteins, amylase, SDS-PAGE, mass spectrometry, defense mechanisms against tannins, taste.

1. Introduction

Tannins are plant secondary metabolites (PSMs) with an enormous structural diversity, depending on their origin, and are found worldwide in many different families of higher plants. One of the characteristic properties of these natural high molecular mass polyphenols is their high capacity to bind proteins, forming complexes that tend to precipitate (Haslam, 1998). In the mouth, the precipitates are perceived via mechanoreceptors as rough, puckering or drying oral sensations, characteristic of astringency (Breslin et al., 1993; Green, 1993; Prinz and Lucas, 2000). The astringent properties of tannins, together with the bitter taste frequently associated with these PSMs (Lesschaeve and Noble, 2005), may result in the avoidance of some plants or plant parts by herbivores. Taste perception is generally considered to be an adaptive response in assessing nutritional value and/or coping with toxicity in potential foods (Le Magnen, 1986). Animals with a relatively high occurrence of bitter and potentially toxic compounds in their diet (e.g., browsing

herbivores) are believed to have developed a high bitter taste threshold and tolerance to poisonous plants (Glendinning, 1994). The cause for this tolerance can be rooted in physiological postingestive adaptative mechanisms, namely, modifications of saliva composition or flow rate, which can contribute to mitigate the negative biological effects of PSMs.

Saliva contains a complex mixture of proteins with different biological roles in digestion, host defense and lubrication (Humphrey and Williamson, 2001). Its composition is diverse among animal species and changes with circadian rhythm (Hardt et al., 2005a) and diet (Katsukawa and Ninomiya, 1999; Neyraud et al., 2006), among other factors. As the secretion of salivary fluid and proteins is controlled by autonomic nerves (Proctor and Carpenter, 2007), saliva plasticity represents a rapid mechanism that allows animals to adapt to random dietary changes, conferring an important advantage. Rats and mice consuming tannin-rich diets showed both parotid gland hypertrophy and increased synthesis and accumulation of salivary proline-rich proteins (PRPs) (Mehansho et al., 1983, 1985, 1987; Jansman et al., 1994). These molecular and cellular effects produced by high-tannin diets, both on the salivary gland tissue and on the saliva of mice and rats, are indistinguishable from those produced by recurrent stimulation with the beta-adrenergic agonist isoproterenol (Ann et al., 1987; Ann et al., 1997). Recently, Gho et al. (2007) reinforced this by observing the induction of a group of salivary polypeptides (designated by isoproterenol-induced polypeptides) both by tannins and isoproterenol. Mice salivary PRPs induction was suggested as acting as a countermeasure against tannins through the formation of tannin-protein complexes that remain insoluble in the conditions found in the digestive compartments (Hagerman et al., 1998; Lu and Bennick, 1998). Apart from these protein species, little is known about possible changes in other salivary proteins induced by tannins, namely proteins that do not form insoluble complexes with these PSMs. Besides PRPs, isoproterenol also induces changes in the expression levels of salivary proteins, such as cystatins (Shaw and Yu, 2000) and amylase (Gallacher and Petersen, 1983), among others. Therefore, the possibility of changes in relative amounts of other proteins cannot be excluded. The characterization of such modifications may be valuable for the better understanding of mammalian physiological countermeasures against tannins. Rodents have been traditionally used as an animal model for studies of the anatomy of the salivary glands and physiology and several proteins have been reported as constituents of their saliva: PRPs (Lin and Ann, 1991), namely the parotid salivary protein (PSP), (Madsen and Hjorth, 1985) and amylase (Hagenbüchle et al., 1980). However, to our knowledge, a systematic characterization of rodent saliva proteome has not been reported yet. The most complete rodent salivary protein profile reported to date is a 2D map of rat parotid saliva, in which the identification of detected proteins was inferred from their determined molecular masses in the gel (Williams et al. 1999).

Peptide mass fingerprinting (PMF), using Matrix-Assisted Laser Desorption Ionization –Time of Flight (MALDI-TOF) mass spectra is a current strategy for the identification of proteins expressed in certain physiological conditions, allowing comparisons of different treatments. In the present study, our first objective was to identify salivary proteins from mice whole saliva, using MALDI-TOF mass

spectrometry after separation by SDS-PAGE, and subsequently to evaluate if ingestion of tannins induced changes in the expression of these proteins. We tested the influence of hydrolysable (tannic acid) and condensed (quebracho) tannins on saliva samples from which insoluble tannin-protein complexes had been removed prior to the analysis, in order to better assess proteins expressed in lower concentrations. Light microscopy was also used to study morphological changes in parotid glands.

2. Methods and Materials

2.1 Animals

Thirty-one inbred male Balb/c mice, four weeks of age, were obtained from the licensed bioterium of Instituto Gulbenkian de Ciência (Oeiras, Portugal). The animals were housed in mice cages, type IV (Techniplast) (10 to 11 mice per cage), according to EU recommendations and revision of Appendix A (ETS 123) and maintained on a 12 h light/12 h dark cycle at a constant temperature of 22ºC with *ad libitum* access to water and to a standard diet with 21.86% crude protein (dry basis) in the form of pellets (RM3A-P; Dietex International, UK). The animals were individually marked and submitted to a seven- day acclimation period to minimized stress effects associated with transportation. This period was followed by a seven-day pre-trial period, to allow adaptation to the ground diet used during the feeding trials. The standard pellet diet was ground daily with a blender, to obtain a meal with visibly homogeneous fine-sized particles. Before the feeding-trial period the animals were individually weighed and allocated to three experimental groups, each group having no significant differences in body mass (24.5 ± 3 g).

All procedures involving the animals were approved by the scientific committee, supervised by a FELASA trained scientist and conforming to the regulations of the Portuguese law (*Portaria 1005/92*), following European Union Laboratory Animal Experimentation Regulations.

2.2 Feeding Trials

Immediately after the pre-trial period, we started the ten-day experimental period, the first day of which we consider day one. The control group (n=10) received a tannin-free diet, the same standard ground diet as in the pre-trial period. The tannic acid group (TA) (n=10) and the *quebracho* group (Q) (n=11) received the standard ground diet plus tannic acid (Merck Ref 1.00773.100) (hydrolyzable tannin) or *quebracho* extract (Tupafin-Ato, SilvaChimica SRL; 72% ±1.5 of condensed tannins) respectively, added in order to obtain a mixture with 5g tannin/100g wet weight of the standard diet. Food and water were provided *ad libitum* and the diets were prepared daily using a blender, as

described for the pre-trial period. Body mass changes during the first eight days of the experimental period were determined daily.

2.3 Saliva and Salivary Gland Collection and Sample Preparation

On day eleven, individual mice whole saliva was collected. The mice remained in the cages, with food and water available, until the time of collection. Saliva production was induced with an intraperitoneal injection of pilocarpine (Sigma; 5 mg kg BW^{-1}), dissolved in 1 ml 9% (m/v) sodium chloride, and prepared immediately before use, as described by Muenzer et al. (1979). Saliva was individually collected by aspiration from all the mice, directly from their mouths, using a micropipette. Saliva samples were immediately frozen in liquid nitrogen and stored at –80 ºC until required for further use. Prior to protein quantification, saliva samples were centrifuged at 16,000*g* for 5 min at 4ºC to remove particulate matter and salivary proteins that could be precipitated by tannins. Only the soluble fraction was used for further analyses. All the animals were injected intraperitoneally with anaesthetic (xylazine hydrochloride combined with ketamine hydrochloride) and euthanized with an overdose. Both parotid salivary glands were dissected, washed briefly with phosphate buffer 0.1M, pH 7.4, and fixed in 10% neutral buffered formalin, in order to carry out further routine histological procedures.

2.4 Protein Quantification and Gel Electrophoresis

Total protein concentration was determined by the BCA Protein Assay Method (Pierce) using a microplate reader (SpectroMAX 340, Molecular Devices, Union City, CA, USA). One dimension SDS-PAGE was run with 20 µg of saliva total protein after reduction using dithiothreitol (USB) and alkylation with iodoacetamide (Sigma). Denatured protein samples were loaded on bis-tris polyacrilamide 4-12% gradient precast gels (100 x 100 x 1 mm) (Nupage Invitrogen) with MES SDS (Nupage Invitrogen) used as a running buffer. Molecular mass markers (BioRad, Ref 161-0317) were run simultaneously with the samples in each gel in order to calibrate the molecular masses of the protein sample bands. Protein bands were stained with colloidal Coomassie blue (Neuhoff et al., 1988) and with Comassie blue R-250 following Beeley et al. (1991). Digital images of the gels were acquired using a densitometer with internal calibration (Molecular Dynamics, Amersham Biosciences Europe GmbH, Freiburg, Germany) and gels were subjected to linescan analysis using Imagequant Software 5.0 (Amersham Biosciences Europe GmbH, Freiburg, Germany). Sensitivity 9.0 and kernel 4.0 were the software parameters used to assign the detected significant bands in the protein profiles obtained. Only bands present in at least 50% of individuals were considered.

2.5 Protein Identification

The protein bands were excised from all gels and the polypeptides subjected to digestion with a sequence grade modified trypsin (Promega) according to Pandey et al. (2000). Sample peptides were assayed for Peptide Mass Fingerprinting (PMF) in a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometer. Peptide co-crystallization was achieved applying 0.5 μl of the peptide digest on the MALDI plate and adding an equal volume of re-crystallized matrix α-cyano-4-hydroxycinnamic acid 10 mg/ml prepared in acetonitrile 70 % (v/v) with 0,1 % TFA (v/v). The mixture was allowed to air dry (dried droplet method). Average spectra were obtained in the mass range between 800 and 4000 Da, using three spectra acquired with 500 laser shots in the positive ion reflectron mode. Spectra were processed and analyzed by the Data Explorer (version 4.0, Applied Biosystems, Foster City, CA, USA). Monoisotopic peptide masses were used to search for protein identification with Mascot software (Matrix Science, UK) (Perkins et al., 1999). Searches were performed in the NCBI non-redundant protein sequence database. A minimum mass accuracy of 100 ppm, one missed cleavage in peptide masses and carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively, were considered. Criteria used to accept the identification were significant homology scores achieved in Mascot, significant sequence coverage values, and similarity between the protein molecular mass calculated from the gel and for the identified protein (Roxo-Rosa et al., 2006).

2.6 Histology

In order to confirm that the doses of tannins used were enough to induce significant changes in glandular morphology, salivary glands were observed through light microscopy using a Nikon Eclipse 600 microscope (Kanagawa, Japan). After embedding the fixed parotid glands in paraffin wax, using routine procedures, a serie of sections of 5 μm thick were cut using a microtome, and the slides were stained with haematoxylin and eosin. Three animals from each group were used and for each animal ten pictures from random parotid areas were collected using a Nikon DN100 camera (Kanagawa, Japan), at 200X magnification. For each animal, the area and perimeter of 150 parotid acini were randomly chosen and measured using SigmaScan Pro5.0 software (SPSS Inc., Chicago, Illinois).

2.7 Statistical Analysis

Body mass data were tested for normality and homocedasticity by Kolgomorov-Smirnof and Levene tests, respectively.

Body mass was analyzed according to a general linear model procedure with two fixed factor and one nested factor:

$$Y_{ijkl}=\mu + T_i + W_k + T(A)_{i(j)} + T^{*}W_k + \varepsilon_{ijkl}$$

Where Y_{ijkl} are the observed values for body mass, μ is the observed mean value, T_i is the fixed effect of treatment, W_k is the fixed effect of weighing day, $T(A)_{i(j)}$ is the nested effect and ε_{ijkl} is the random error or residual effect.

Means significantly different were submitted to post-hoc comparisons of means (Tukey-Hsu test) and regarded as significantly different when $p<0.05$. All statistical analysis procedures were performed by NCSS 2001 software package (Kaysville, UT, USA).

3. Results

3.1 Body Mass

The animals were weighed daily to search for changes in body masses that reflect physiological adaptation of the animals to tannin diets, including the induction of PRP's (Mehansho et al., 1983; Skopec et al., 2004).

It was possible to observe that tannic acid and quebacho groups lost weight during the first three and four days, respectively, after which they recovered, whereas the control group presented an increase in body mass in the first two days and remained nearly stable after that. Statistically significant differences were observed in days three and four, with the animals from the tannin groups presenting lower mean body masses than animals from control group (Table 1). Despite the increases in body masses after day four, for animals either from tannic acid and *quebracho* groups, that increase was less pronounced in tannic acid group, with the animals from this last remained with lower body masses until the end of the trial.

Table 1. Mean body masses in grams of mice fed with different diets through the first eight days of the experiment (mean ± SD)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control	24.90 ± $2.14^{a,A}$	24.93 ± $2.22^{a,A}$	25.58 ± $2.15^{b,A}$	25.65 ± $2.04^{b,A}$	25.56 ± $2.05^{b,A}$	25.64 ± $1.99^{b,A}$	25.70 ± $2.13^{b,A}$	25.75 ± $1.95^{b,A}$
Tannic acid	24.24 ± $2.29^{a,A}$	23.38 ± $2.31^{b,c,B}$	23.12 ± $2.14^{b,B}$	23.27 ± $2.23^{b,c,B}$	23.32 ± $2.22^{b,c,B}$	23.69 ± $2.20^{c,d,B}$	24.06 ± $2.15^{a,d,B}$	24.02 ± $2.18^{a,d,A}$
Quebracho	25.04 ± $1.36^{a,A}$	25.38 ± $1.34^{a,b,A}$	25.20 ± $1.32^{a,b,C}$	24.98 ± $1.31^{a,C}$	25.71 ± $1.40^{b,d,e,A}$	25.97 ± $1.32^{c,d,e,A}$	26.40 ± $1.37^{c,e,A}$	26.14 ± $1.29^{c,e,A}$

Same lowercase letters indicate no differences among the values of the columns in the same line (Tukey-Hsu test; $P<0.05$).
Same uppercase letters indicate no differences among the values of the lines in the same columnn (Tukey-Hsu test; $P<0.05$).

3.2 Histology

A dramatic increase in the acinar area and the perimeter of the parotid salivary gland was observed in the animals receiving tannin-enriched diets over a period of ten days (Fig. 1).

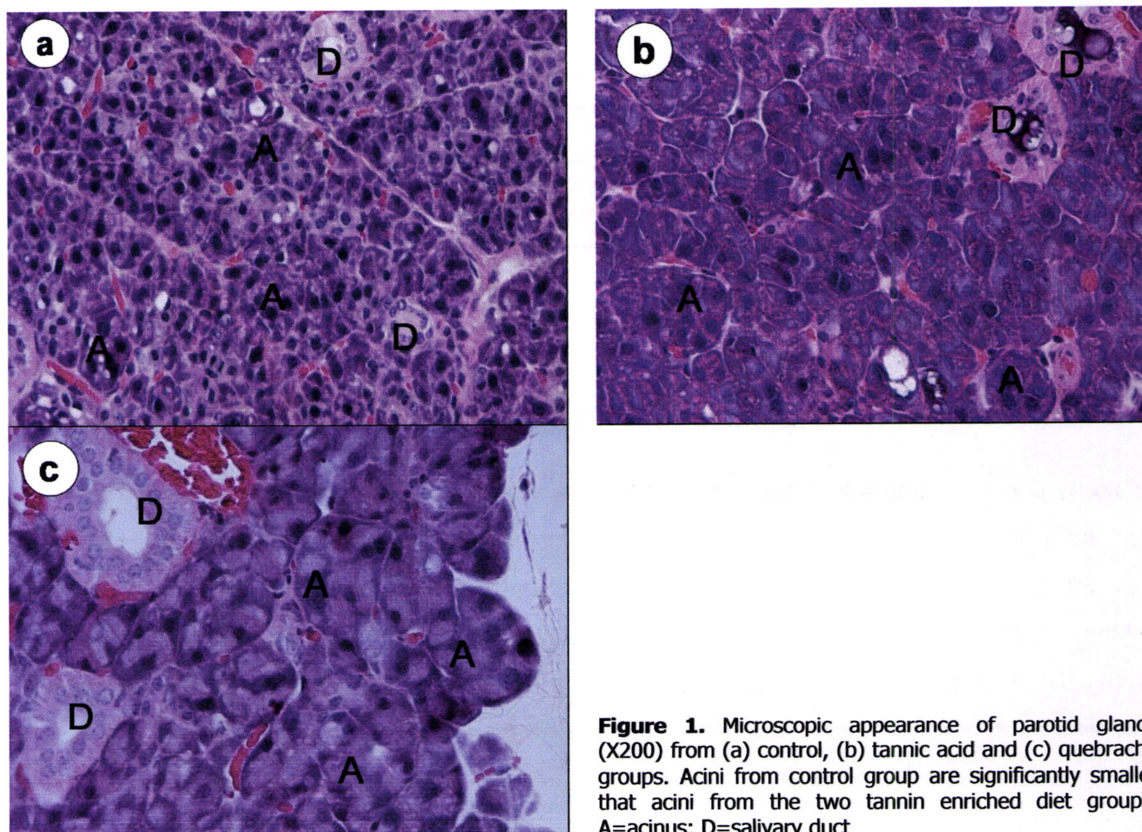


Figure 1. Microscopic appearance of parotid glands (X200) from (a) control, (b) tannic acid and (c) quebracho groups. Acini from control group are significantly smaller that acini from the two tannin enriched diet groups. A=acinus; D=salivary duct

Levels of 5 g of hydrolysable or condensed tannin per 100 g wet weight in the diet seem to produce a hypertrophy of the secretor tissue (Table 2).

Table 2. Comparison of acinar areas and perimeters between control and tannin enriched dietary groups (Mean ± SD)

	Diet							
	Control		Tannic acid		Quebracho		t(C,TA)	t(C,Q)
	X	SD	X	SD	X	SD		
Area (pixel)	15674.9	5787.0	28021.5	10729.3	33303.8	13696.1	-12.4*	-14.5*
Perimeter (pixel)	504.6	96.9	676.0	129.2	739.2	153.3	-13.0*	-15.8*

* Differences are significant for $P<0.05$. Independent sample t-test for equality of means was used to test differences in acinar area and perimeter between each diet group
C=control; TA=tannic acid; Q=quebracho

3.3 Saliva Protein Concentration

The total protein saliva concentration, after the ten days of the feeding trial, was significantly higher in the control group than in the quebracho and in tannic acid groups (Table 3).

Table 3. Whole saliva protein concentration (mean ± SD)

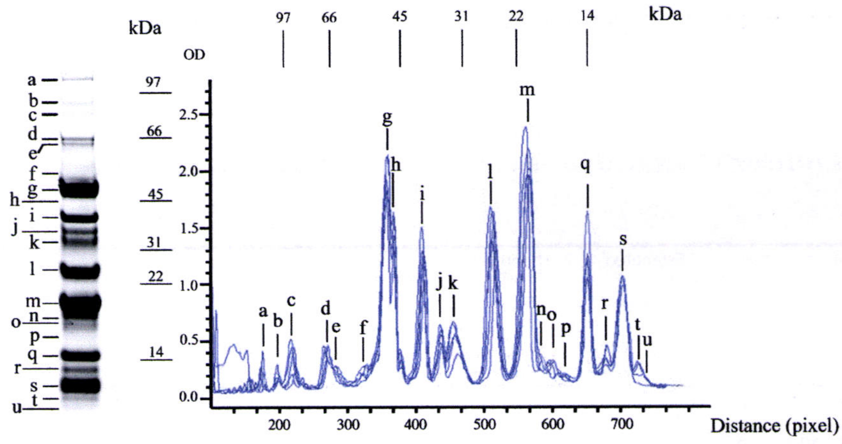
	Diet						t(C,TA)	t(C,Q)
	Control		Tannic acid		Quebracho			
	X	SD	X	SD	X	SD		
Protein concentration (μg/mL)	2522.20	601.99	1889.63	377.65	1652.61	303.56	2.82*	4.12*

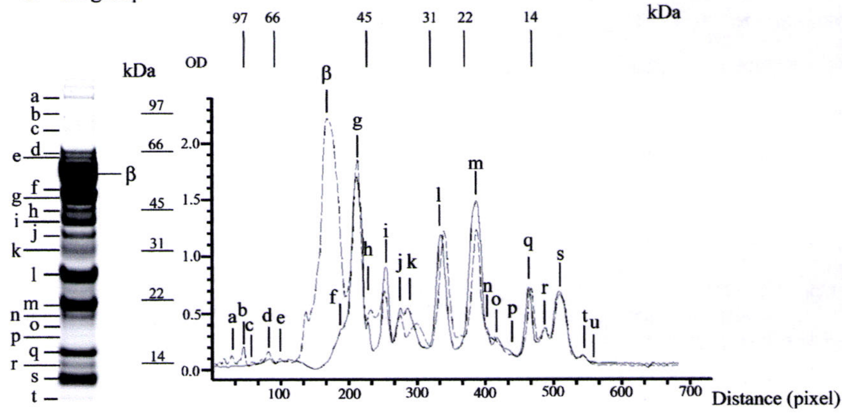
* Differences are significant for $P<0.05$. Independent sample t-test for equality of means was used to test differences in protein concentration between each diet group.
C=control; TA=tannic acid; Q=quebracho

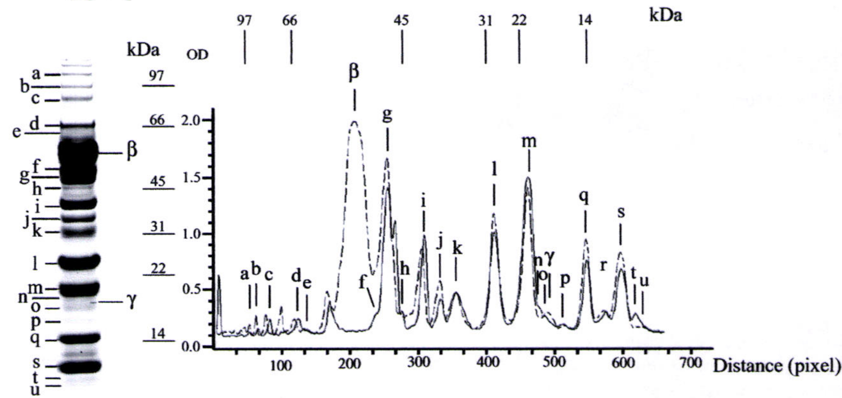
3.4 Saliva Protein Profile

We started by characterizing the saliva profile of the control group using the Imagequant 5.0 software to assign the significative bands in the protein profiles obtained. These results are in agreement with visual observation of the gel pattern. Despite slight inter-individual variations in whole saliva composition, 21 protein bands (from ***a*** to ***u***) were consistently present in all the animal saliva protein profiles (n=10). Figure 2-a shows a typical 1-DE pattern of the control group saliva and also an overlay of the ten linescan resulting from the analysis of the control group saliva protein profiles. The molecular masses of the proteins were calculated by the gel analysis software, after superimposing the linescan of the molecular mass markers on each sample linescan.

With regard to the control group, inter-individual protein profile variability was assessed for the TA (n= 10) and Q (n= 11) groups. Only slight variation was found among the animals on the tannin-fed diets. Comparing the saliva protein profile of the control group with the TA group, it was possible to identify one additional band (band β - Fig. 2-b) around molecular mass 51 kDa that was not visible in the control saliva protein profile. The saliva protein profile of the Q group, when compared with the control saliva protein profile, presented two additional bands (Fig. 2-c). The very intense protein band presented in the saliva protein profile of TA group around 51 kDa (band β) and an additional band (band γ) of reduced intensity and molecular mass around 20 kDa.


A - Control group
kDa
97
66
45
31
22
14
OD
Distance (pixel)


B - TA group
kDa
97
66
45
31
22
14
OD
Distance (pixel)


C - Q group
kDa
97
66
45
31
22
14
OD
Distance (pixel)

3.5 Protein Identification

The identification of all the protein bands excised from the control group saliva protein profile can be seen in Table 4.

Table 4 Proteins identified in saliva from animals of each dietary group

Band ID	Dietary group [a)]	Protein name	Estimated[b)] MW (kDa)	Theoretical [c)] MW (KDa)	Score	Coverage (%)	Peptides match/ peptides submitted	Swiss-Prot code	Reference
a	C, TA, Q	Acidic chitinase	99	52	119	35	15/42	Q91XA9	(1)
b	C, TA, Q	Muc10 protein	78	29	77	31	15/79	Q80XS5	(2)
c	C, TA, Q	Salivary amylase 1	66	58	81	34	21/72	Q921Y7	(3-6)
d	C, TA, Q	Salivary amylase 1	59	58	74	25	16/69	Q921Y7	(3-6)
e	C, TA, Q	Salivary amylase 1	54	58	136	39	22/78	Q921Y7	(3-6)
f	C, TA, Q	Salivary amylase 1	51	58	179	49	23/45	Q921Y7	(3-6)
β	TA, Q	Salivary amylase 1	51	57	115	31	15/32	Q921Y7	(3-6)
g	C, TA, Q	Salivary amylase 1	48	58	339	59	24/55	Q921Y7	(3-6)
h	C, TA, Q	Glandular kallikrein	45	29	103	39	22/83	P36368	(7)
i	C, TA, Q	Similar to carbonic anhydrase 6	40	37	92	39	18/45	Q80YB7	(5,6)
j	C, TA, Q	Similar to carbonic anhydrase 6	37	37	91	39	18/44	Q80YB7	(5,6)
k	C, TA, Q	Apolipoprotein A-I	33	31	71	20	7/23	Q00623	(3,5)
l	C, TA, Q	Parotid secretory protein	26	25	78	37	67/45	P07743	(5)
m	C, TA, Q	Parotid secretory protein	20	25	83	42	7/42	P07743	(5)
n	C, TA, Q	Parotid secretory protein	18	25	71	42	7/53	P07743	(5)
γ	Q	Aldehyde reductase	18	37	86	27	7/18	Q80XJ7	
o	C, TA, Q	Parotid secretory protein	16	25	63	37	5/50	P07743	(5)
p	C, TA, Q	n.d.	15						
q	C, TA, Q	Parotid secretory protein	14	25	83	42	7/43	P07743	(5)
r	C, TA, Q	Immunoglobulin heavy chain variable region	12	13	65	52	6/42	Q683Y7	(3,5,6)

s	C, TA, Q	14 kDa submandibular gland protein	9	17	94	58	6/35	P02816	(8)
t	C, TA, Q	Androgen binding protein β subunit	9	10	104	54	7/41	Q7TNM9	(9)
u	C, Q	Androgen binding protein γ subunit	7	10	210	54	7/38	Q8JZX1	(9)
	C, Q	Androgen binding protein β subunit	7	13		48	9/38	Q7TNM9	

[a] Dietary groups: C-control; TA – tannic acid; Q – quebracho
[b] Molecular mass measured based on electrophoretic mobility
[c] Determined molecular weight for identified protein
The nonidentified proteins are labeled as n.d.; Articles reporting the presence of the correspondent protein in saliva: (1) Stjein et al., 1999; (2) Denny et al., 1996; (3) Huang, 2004; (4) Yao et al., 2003; (5) Vitorino et al., 2004; (6) Hardt et al., 2005; (7) Kim et al., 1991; (8) Myal et al., 1998; (9) Karn et al., 2003

The only band whose identification was not possible was band ***p***, probably due to the presence of a low amount of protein. From the twenty-one protein bands excised, we were able to identify ten different polypeptides. The other ten bands were assigned to four of the already identified polypeptides: four additional bands to salivary amylase 1, one additional band to carbonic anhydrase VI, four additional bands to parotid secretory protein (PSP) and one additional band to androgen-binding protein. This fact could be attributed to the existence of post-translational modifications and/or protein fragments. Band ***u*** was assigned to a mixture of β and γ subunits of the androgen binding protein.

The two protein bands that were additionally detected in the saliva profile of the animals submitted to a tannin-enriched diet (β and γ) were also used for protein identification following the PMF approach. As can be seen in Table 4, these two protein bands were successfully identified as amylase 1 and aldehyde reductase, respectively. Aldehyde reductase was only observed in the Q group.

4. Discussion

Tannins act as feeding deterrents, due both to their aversive chemosensory properties (such as bitter taste and astringency) and the negative post-ingestive effects they exert (Glendinning, 1994). A reduction in body mass in the first days of tannin administration was previously reported (Mehansho et al., 1983; 1985; Jansman et al., 1994; Shimada et al., 2004; Shimada et al., 2006). In mice and rats this effect is reversed after 2-3 days, when a hypertrophy of parotid glands occurs, coincident with a dramatic increase in salivary PRPs production (Mehansho et al., 1983). In the present work, tannin-fed mice decreased in body mass in the first days of the experiment (Table 1). This effect ceased to after 3-4 days of trial. Despite the body mass recovery of tannin-fed mice, animals from tannic acid group remained with lower body masses until the end of the trial, what suggests a greater capacity to adapt to *quebracho* tannin. The parotid hypertrophy usually associated with tannin consumption was also observed in this experiment. This trophic effect in the parotid glands has been also associated with the expression of a number of isoproterenol-induced salivary proline-rich polypeptides (IISPs) (Lopez-Solis and Kemmerling, 2005; Gho et al., 2007), which seems to be related

with ß1 adrenergic activity (Gonzalez et al, 2000). We therefore found those changes relevant, demonstrating that the tannin levels used were enough to induce an effect at glandular level.

This study provides a deeper knowledge of the changes in whole saliva protein composition that occur when mice consume tannin-enriched diets. The great majority of the studies suggest a defense response associated with the induction of salivary proteins (mainly PRPs) that precipitate these PSMs, preventing them from exerting negative effects (Bennick, 2002). We predicted that tannin consumption could also influence other salivary proteins. To ensure that the minor expressed salivary proteins would not be masked by over-expression of the tannin-binding proteins, we removed the insoluble fraction that is expected to contain the tannin-protein complexes. We assumed that this purpose was achieved since total protein concentration from the tannin-fed groups was lower compared with the control group. The lack of observation of pink bands in the Coomassie stained gels (according to Beeley et al., 1991) supports the idea that we efficiently removed the majority of PRPs.

Although a great number of studies concerning salivary proteins have been performed on mice, a global perspective relating whole saliva protein profile to protein identification is, to the best of our knowledge, presently unknown. Williams et al. (1999) provide a 2-DE protein profile, referring to the presence of PRPs, amylase, acidic epididymal glycoprotein, deoxyribonuclease, parotid secretory protein and common salivary protein although a protein identification was not performed but rather inferred from the position of protein spots. Our results evidenced the presence of ten different salivary proteins, some of them showing more than one isoform. This situation is well documented in human saliva (Hirtz et al. 2005b), where a simultaneous low diversity in terms of variety of accessions and a high complexity in terms of number of protein bands identified by the same accession are reported. This degree of redundancy displayed by extensive salivary protein polymorphisms seems to be important in saliva plasticity. Moreover, the presence of several families of structurally and functionally closely related molecules indicates that these proteins have been subjected to evolutionary pressures, which may reflect nature's selection for improved function (Oppenheim at al., 2007).

The identified proteins have already been described in saliva: acidic chitinase, mucin apoprotein, amylase 1, carbonic anhydrase VI, apolipoprotein A-I, parotid secretory protein, immunoglobulin heavy chain, submandibular gland 15 kDa protein, androgen-binding protein and glandular kallikrein, as referenced in Table 4. The first seven proteins listed above have multiple functions in saliva and are involved in the formation of enamel pellicle, present hydrolytic activity and act as a first line of defense against microorganisms (Huang, 2004; Yao et al., 2003; Vitorino et al., 2004; Hardt et al., 2005b). The submandibular gland 15 kDa protein is known as a gross cystic disease fluid protein that was initially described in humans as a protein secreted by the T47D human breast cancer cell line. This protein is produced in many exocrine glands such as sweat, salivary and lachrymal; however its function remains unknown (Myal et al., 1998). Androgen-binding protein secreted by mice submandibular salivary glands has been hypothesized as having a major role in mate selection. This

member of the secretoglobins family is secreted into saliva in two different dimer forms: an α subunit disulfide bridged to either a β or a γ subunit (Karn and Laukaitis, 2003). However, cellular site(s) of synthesis, mode of function, and evolution patterns of this biologically important protein are otherwise unknown (Dlouhy et al., 1987). Glandular kallikrein, mainly secreted by submandibular glands, is a peptidase belonging to the serine proteases family, which brings about maturation of growth factors and polypeptide hormones, by conversion of inactive precursors to biologically active peptides (Kim et al., 1991). Parasympatic stimulation causing an increase in saliva flow will promote low outputs of this protein without degranulation of acinar or granular tubules cells (Shori and Asking, 2001).

The control group electrophoresis gel pattern presents five different bands identified as salivary amylase 1, between masses 48 and 66 kDa - one very intense band at a lower molecular mass (band ***g***), and four weak or medium intense bands at higher molecular mass values. Different bands could correspond to different isoforms of the protein. Glycosilation and deamidation of amylase 1 have already been described (Bank et al., 1991; Hirtz et al., 2005a). The glycosilation could be a possible explanation for the higher molecular mass of the amylase bands observed in the gel, comparative to amylase native form. Knowing that glycosylated asparagine residues almost always occur in the sequence Asn-X Ser/Thr, two potential N-glycosilation sites for mice α-amylase are 412-414 and 461-463 (peptide residue numbered taking into account that the fifteen aminoacid peptide signal is lost during the secretion process). These two sites were also referred for human α-amylase (Bank et al., 1991; Hirtz et al., 2005a). The peptide containing the first site is detected for all of our amylase bands, so a glycosilation should not occur in that position. On the other hand, N-glycosilation can occur in the second potential site, since the peptide containing it systematically fails to be detected in mass spectra. Although some authors (Bank et al., 1991) considered that N-glycosilation is more prone to occur in the first potential site, Hirtz et al. (2005a) found results similar to ours for human α-amylase. The lack of experimental results on the specific detection of glycoproteins does not allow the confirmation of this hypothesis since the absence of peptide 458-466 can also be explained based on experimental limitations. Bank et al. (1991) proposed that amylase deaminated isoforms correspond to the lower molecular mass bands observed in the saliva SDS-PAGE profile. Using the software Findmod (http://www.expasy.ch/tools/findmod/), it is possible to assign two peaks in the salivary amylase mass spectra of band ***g*** as deaminated peptides, both in the control group and in the Q and AT groups. These post-translational modifications can occur at one of two residues of peptide 46-76 and at one of three residues of peptide 414-436. These modified peptides were also observed for band β, present only in the animal groups submitted to the tannin-enriched diet. The deamination of these residues does not seem to be characteristic of any of the amylase isoforms observed.

Although salivary proteins can bind tannins as a mode of defensive action, other physiological mechanisms, including enzymatic or immune responses, are not to be excluded. The only effect common to both types of tannins on the salivary profile of proteins was an increase in amylase. The

over-expression of amylase 1 induced in both groups (band ***β***, Fig.2) overlaps the medium molecular mass and less intense band of this protein in the control group (band ***f***, Fig.2). This suggests that only the medium molecular mass isoform of amylase 1 is over-expressed in the tannin-rich diet fed groups or that a new amylase isoform is induced in these groups. Furthermore, protein band ***g*** (Fig.2), the most intense band in the control group identified as amylase 1, did not show any significant expression level change with tannin treatment. Several differences can be observed between the tryptic peptide mass spectra of band ***β*** and of band ***g***. We could not assign these peaks to described modifications of salivary amylase 1, but it suggests that a different isoenzyme of salivary amylase 1 is being expressed in the Q and AT group.

The increase in salivary α-amylase levels may be a consequence of tannin stimulation of sympathetic pathways. Isoproterenol and tannins lead to similar changes in salivary glands and it has been suggested that these changes are due to stimulation of the β1 adrenergic receptors (Waters et al., 1998). Beta adrenergic agonists are capable of stimulating salivary α-amylase release (Gallacher and Petersen, 1983). There is a recurrent pattern across studies that show that salivary α-amylase levels increase in response to physical and psychological stress (Chatterton et al. 1996; for a comprehensive review see Granger et al., 2007). As a result, salivary α-amylase has been used as a surrogate marker of the autonomic/sympathetic nervous system component of stress in humans. Other oral stimulators influencing texture and flavour perception were also observed to increase alpha-amylase expression. Amylase increased with taste stimulation in rabbits (Gjorstrup, 1980) and humans (Neyraud et al., 2006) and a strong correlation between α-amylase and texture perception was observed by Engelen et al. (2007).

We suggest that, despite the primary biological function of salivary α-amylase being the digestion of polysaccharides, the increase of its expression is not a direct consequence of dietary carbohydrates, but a result of adrenergic stimulation. Indirectly, this increase can also represent a co-adjuvant for the inhibition of tannin biological activity. Tannins are very potent inhibitors of salivary amylase 1 (Kandra et al., 2004; McDougall et al., 2005), although affinity of α-amylase for tannins is not as high as PRPs affinity (De Freitas and Mateus, 2001). Tannins have been described as responsible for enlarged pancreas (Ahmed et al., 1991; Mahmood and Smithard., 1993), stimulation and secretion of an increased amount of amylase, which, to some extent, may counteract the inhibition of this enzyme during gut digestion. Although α-amylase in saliva and the pancreas are produced by independent sources, we believe that a similar function could be involved, and amylase content may increase when animals are fed with tannins to counteract the inhibition of this enzyme in the mouth.

Salivary amylase is stored in large dense-core secretory granules that undergo stimulated secretion in response to extracellular stimulation (Gorr et al., 2005). The α-amylase increase observed in the present study could result from the exocytosis of acinar secretory mature granules, as usually occurs in the first hours after isoproterenol administration (Henriksson, 1982). Moreover, chronic stimulation

with isoproterenol is reported to decrease (Madsen and Hjorth, 1985; Ann et al., 1987) or maintain (Bedi, 1993) amylase levels. To the best of our knowledge this is the first reference of over-expression of salivary amylase 1 after the ingestion of polyphenols and, surprisingly, not all the amylase isoforms changed with tannins but only one. Band ß could be an induction of a newly produced isoform or could represent an over-expression of one of the previously visible isoforms.

Another difference that was detected was the expression of protein band γ (n=8) in the *quebracho* tannin-fed diet group. This protein was identified as aldehyde reductase. Aldehyde reductase belongs to the aldo-keto reductases (AKR) superfamily. They are carbonyl-reducing enzymes, along with the short-chain dehydrogenases/reductases (SDR), and are responsible for the reduction of aldehydes, ketones, and quinones to their corresponding hydroxyl derivatives (Hoffmann and Maser, 2007). Although salivary levels of enzymes belonging to the human aldo-keto reductase superfamily were found to be high in humans subjected to a continuous intake of coffee, which has a high content of polyphenols (Sladeck, 2003), we tend to analyze this result with caution. First of all, this enzyme, although ubiquitous in nature (present in plants, fungi, insects, fish, bacteria), has been found mainly in the intracellular media and in tissues. In mice they were found in several tissues, such as the liver (Ahmed et al., 1978), the lung (Nakayama et al., 1986) and the ovary (Iwata et al., 1990). A search in the UCLA human salivary proteome project database (http://www.hspp.ucla.edu) found only two salivary proteins from the AKR family 1 in saliva (Aldose reductase-like and Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase). Secondly, *quebracho* used in this study is a plant extract, containing only 72% of tannins. The presence of carbonyl-group bearing substances, such as small phenolics, other PSMs or even quinones, resulting from oxidation during the extraction process, is not to be discarded. The presence of aldehyde reductase could be a consequence of chemical species other than tannin.

We conclude that the amylase up-regulation is an unspecific adaptation of saliva to dietary tannins that could be a consequence of the stimulation of sympathetic pathways and of amylase inhibition. Indirectly and through these mechanisms tannin adverse effects could be reduced, probably due to the preferential involvement of one amylase isoform more prone for this activity.

Despite the similarities of results obtained for tannic acid and *quebracho*, we think that further studies with other forms of tannins would help to clarify whether a specific protein defense response exists for the type of tannin.

5. References

Ahmed, N.K., Felsted R.L., Bachur, N.R. 1978. Heterogeneity of anthracycline antibiotic carbonyl reductases in mammalian livers. Biochem. Pharmacol. 27: 2713-2719.

Ahmed, A.E., Smithard, R., Ellis, M. 1991. Activities of enzymes of the pancreas, and the lumen and mucosa of the small intestine in growing broiler cockerels fed on tannin-containing diets. Br. J. Nutr. 65: 189-197.

Ann, D.K., Clements, S., Johnstone, E.M., Carlson, D.M. 1987. Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs, J. Biol. Chem. 262: 899-904.

Ann, D.K., Lin, H.H., Kousvelari, E. 1997. Regulation of salivary gland specific gene expression. Crit. Rev. Oral Biol. Med. 8: 244-252.

Bank, R.A., Hettema, H.E., Arwert, F., Amerongen, A.V., Pronk, J.C. 1991. Electrophoretic characterization of post-translational modifications of human parotid salivary alpha-amylase. Electrophoresis 12: 1032-1041.

Bedi, G.S. 1993. The effect of adrenergic agonists and antagonists on the expression of proteins in rat submandibular and parotid glands. Crit. Rev. Oral Biol. Med. 4: 565-571.

Beeley, J.A., Sweeney, D., Lindsay, J.C., Buchanan, M.L., Sarna, L., Khoo, K.S. 1991. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human parotid salivary proteins. Electrophoresis 12: 1032-1041.

Bennick, A. 2002. Interaction of plant polyphenols with salivary proteins. Crit. Rev. Oral Biol. Med. 13: 184-196.

Breslin, P.S.A., Gilmore, M.M., Beauchamp, G.K., Green, B.G. 1993. Psychophysical evidence that oral astringency is a tactil sensation. Chem. Senses. 18: 405-415.

Chatterton R.T. Jr, Vogelsong, K.M., Lu Y.C., Ellman A.B., Hudgensg, A. 1996. Salivary alpha-amylase as a measure of endogenous adrenergic activity. Clin. Physiol. 16: 433-448.

de Freitas, V., Mateus, N. 2001. Structural features of procyanidin interactions with salivary proteins. J. Agric. Food Chem. 49: 940-945.

Denny, P.C., Mirels, L., Denny, P.A. 1996. Mouse Submandibular Gland Salivary Apomucin Contains Repeated N-Glycosylation Sites. Glycobiology 6: 43-50.

Dlouhy, S.R., Taylor, B.A., Karn, R.C. 1987. The genes for mouse salivary androgen-binding protein (ABP) subunits alpha and gamma are located on chromosome 7. Genetics 115: 535-543.

Engelen, L., van den Keybus, P.A., de Wijk, R.A., Veerman, E.C., Amerongen, A.V., Bosman, F., Prinz, J.F., van der Bilt, A. 2007. The effect of saliva composition on texture perception of semi-solids. Arch. Oral Biol. 52: 518-525.

Gallacher, D.V., Petersen, O.H. 1983. Stimulus-secretion coupling in mammalian salivary glands. Int. Rev. Physiol. 28: 1-52.

Gho, F., Pena-Neira, A., Lopez-Solis, R.O. 2007. Induction of salivary polypeptides associated with parotid hypertrophy by gallotannins administered topically into the mouse mouth. J. Cell Biochem. 100: 487-498.

Gjorstrup, P. 1980. Taste and chewing as stimuli for the secretion of amylase from the parotid gland of the rabbit. Acta Physiol. Scand. 110: 295-301.

Glendinning, J.I. 1994. Is the bitter rejection response always adaptive? Physiol. Behav. 56: 1217-1227.

Gonzalez, M.J., Peña y Lillo S., Alliende C., Lopez-Solis, R.O. 2000. Cell-enlargement-related polypeptides are induced via beta(1)-adrenoceptors in mouse parotids. Exp. Mol. Pathol. 69: 91-101.

Gorr, S.-U., Venkatesh, S.G., Darling, D.S. 2005. Parotid secretory granules: crossroads of secretory pathways and protein storage. J. Dent. Res. 84: 500-509.

Granger, D.A., Kivlighan, K.T., El-Sheikh, M., Gordis, E.B., Stroud, L.R. 2007. Salivary alpha-amylase in biobehavioral research: recent developments and applications. Ann. N Y Acad. Sci. 1098: 122-144.

Green, B.G. 1993. Oral astringency: a tactil component of flavor. Acta Psychol (Amst.) 84: 119-125.

Hagerman, A.E., Rice, M.E., Ritchard, N.T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin (4-8) catechin (procyanidin). J. Agric. Food Chem. 46: 2590-2595.

Hagenbüchle, O., Bovey, R., Young, R.A. 1980. Tissue-specific expression of mouse α-amylase genes: Nucleotide sequence isoenzyme mRNAs from pancreas and salivary gland. Cell 21: 179-187.

Hardt, M., Witkowska, H.E., Webb, S., Thomas, L.R., Dixon, S.E., Hall, S.C., Fisher, S.J. 2005a. Assessing the effects of diurnal variation in the composition of human parotid saliva: quantitative analysis of native peptides using iTRAQ reagents. Anal Chem. 77: 4947-4954.

Hardt, M., Thomas, L.R., Dixon, S.E., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska, H.E., Fisher, S.J. 2005b. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry 44: 2885-2899.

Haslam, E. 1998. Practical polyphenols: from structure to molecular recognition and physiological action. University Press, Cambridge.

Henriksson, R. 1982. Beta 1- and beta 2-adrenoceptor agonists have different effects on rat parotid acinar cells. Am. J. Physiol. 242: G481-G482.

Hirtz, C., Chevalier, F., Centeno, D., Rofidal, V., Egea, J.C., Rossignol, M., Sommerer, N., Periere, D.D. 2005a. MS characterization of multiple forms of alpha-amylase in human saliva. Proteomics 5: 4597-4607.

Hirtz, C., Chevalier, F., Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., Periere, D.D. 2005b. Complexity of the human whole saliva proteome. J. Physiol. Biochem. 61: 469-480.

Hoffmann F., Maser E. 2007. Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. Drug Metab. Rev. 39: 87-144.

Huang, C.M. 2004. Comparative proteomic analysis of human whole saliva. Arch. Oral Biol. 49: 951-962.

Humphrey, S. P., Williamson, R. T. 2001. A review of saliva normal composition, flow and function. J. Prosthet. Dent. 85: 162–169.

Iwata N., Inazu N., Satoh, T. 1990. Immunological and enzymological localization of carbonyl reductase in ovary and liver of various species. J. Biochem. (Tokyo) 107: 209-212.

Jansman, A.J., Frohlich, A.A., Marquardt, R.R. 1994. Production of proline-rich proteins by the parotid glands of rats is enhanced by feeding diets containing tannins from faba beans (*Vicia faba* L.). J. Nutr. 124: 249-258.

Kandra, L., Gyémánt, G., Zajacz, A., Batta, G. 2004. Inhibitory effects of tannin on human salivary alpha amylase. Biochem. Biophy. Res. Comm. 319: 1265-1271.

Karn, R.C., Laukaitis, C.M. 2003. Characterization of Two Forms of Mouse Salivary Androgen-Binding Protein (ABP): Implications for Evolutionary Relationships and Ligand-Binding Function. Biochemistry 42: 7162-7170.

Katsukawa, H., Ninomiya, Y. 1999. Capsaicin induces S-like substances in submandibular saliva of the rat. J. Dent. Res. 78: 1609-1616.

Kim, W.S., Nakayama, K., Nakagawa, T., Kawamura, Y., Haraguchi, K., Murakami, K. 1991. Mouse submandibular gland prorenin-converting enzyme is a member of glandular kallikrein family. J. Biol. Chem. 266: 19283-19287.

Le Magnen, J. 1986. Hunger. Problems in the Behavioural Sciences. University Press, Cambridge.

Lesschaeve, I., Noble, A.C. 2005. Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. Am. J. Clin. Nutr. 81: 330S-335S.

Lin, H.H., Ann, D.K. 1991. Molecular characterization of rat multigene family encoding proline-rich proteins. Genomics 10: 102-113.

Lopez-Solis, R.O., Kemmerling, U. 2005. Codominant expression of genes coding for different sets of inducible salivary polypeptides associated with parotid hypertrophy in two inbred mouse strains. J. Cell Biochem. 95: 99-107.

Lu, Y., Bennick, A. 1998. Interaction of tannin with human salivary proline-rich proteins. Arch. Oral Biol. 43: 717-728.

Madsen, H.O., Hjorth, J.P. 1985. Molecular cloning of mouse PSP mRNA. Nucleic Acids Res. 13: 1-13.

Mahmood, S., Smithard, R. 1993. A comparison of effects of body weight and feed intake on digestion in broiler cockrels with effects of tannins. Br. J. Nutr. 70: 701-709.

Mcdougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A., Stewart, D. 2005. Different polyphenolic components of soft fruits inhibit alpha-amylase and alpha-glucosidase. J. Agric. Food Chem. 53: 2760-2766.

Mehansho, H., Hagerman, A.E., Clements, S., Butler, L.G., Rogler, J., Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghum with high tannin levels. Proc. Natl. Acad. Sci. USA 80: 3948-3952.

Mehansho, H., Hagerman, A.E., Clements, S., Butler, L., Rogler, J., Carlson, D.M. 1985. Indution of proline rich glycoproteins synthesis in mouse salivary glands by isoproterenol and by tannins. J. Biol. Chem. 260: 4418-4423.

Mehansho, H., Rogler, J., Carlson, D.M. 1987. Dietary tannins and salivary proline rich proteins: interaction, inductions and defense mechanisms. Ann. Rev. Nutr. 7: 423-440.

Muenzer, J., Bildstein, C., Gleason, M., Carlson, D.M. 1979. Purification of proline-rich proteins from parotid glands of isoproterenol-treated rats. J Biol. Chem. 254: 5623-5628.

Myal, Y., Iwasiow, B., Cosby, H., Yarmill A., Blanchard, A., Tsuyuki, D., Fresnoza, A., Duckworth, M.L., Shiu, R.P.C. 1998. Analysis of tissue- and hormone-specific regulation of the human prolactin-inducible protein/gross cystic disease fluid proein-15 gene in transgenic mice. J. Mol. Endocrinol. 21: 217-223.

Nakayama T., Yashiro K., Inoue Y., Matsuura K., Ichikawa H., Hara A., Sawada H. 1986. Characterization of pulmonary carbonyl reductase of mouse and guinea pig. Biochem. Biophys. Acta 882: 220-227.

Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W. 1988. Improved staining of proteins in polyacrilamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brillant Blue G-250 and R-250. Electrophoresis 9: 255-262.

Neyraud, E., Sayd, T., Morzel, M., Dransfield, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes, J. Proteome Res. 5: 2474-2480.

Oppenheim, F.G., Salih, E., Siqueira, W.L., Zhang, W. Helmerhorst, E.J. 2007. Salivary proteome and its genetic polymorphisms. Ann N Y Acad. Sci. 1098: 22-50.

Pandey, A., Andersen, J.S. Mann, M. 2000. Use of mass spectrometry to study signaling pathways. Sci STKE 37: PL1.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20: 3551-3567.

Prinz, J.F., Lucas, P.W. 2000. Saliva tannin interactions. J. Oral Rehabil. 27: 991-994.

Proctor, G.B., Carpenter, G.H. 2007. Regulation of salivary gland function by autonomic nerves. Auton. Neurosci. 133: 3-18.

Roxo-Rosa, M., da Costa, G., Luider, T.M., Scholte, B.J., Coelho, A.V., Amaral, D.M., Penque, D. 2006. Proteomic analysis of nasal cells from Cystic Fibrosis (CF) patients and non-CF control individuals: search for novel biomarkers of CF lung disease. Proteomics 6: 2314-2325.

Shaw, P.A., Yu, W.A. 2000. Autonomic regulation of cystatin S gene expression in rat submandibular glands. Auton. Neurosci. 83: 49-57.

Shimada, T., Saitoh, T., Matsui, T. 2004. Does acclimation reduce the negative effects of acorn tannins in the wood mouse *Apodemus speciosus*? Acta Theriol. 49: 203-214.

Shimada, T., Saitoh, T., Sasaki, E., Nishitani, Y., Osawa R. 2006. Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins. J. Chem Ecol. 32: 1165-1180.

Shori, D.K., Asking, B. 2001. Dynamics of protein and fluid secretion from the major salivary glands of rat: relevance of research findings to clinically observed defective secretion in cystic fibrosis. Pflugers Arch. 443 (Suppl 1): 535-543.

Skopec, M.M., Hagerman, A.E., Karasov, W.H. 2004. Do salivary proline-rich proteins counteract dietary hydrolysable tannin in laboratory rats? J. Chem. Ecol. 30: 1679-1692.

Sladeck, N. 2003. Human aldehyde dehydrogenase: Potencial pathological, pharmacological and toxicological impact. J. Biochem. Mol. Toxicol. 17: 7-23.

Vitorino, R., Lobo, M.J., Ferrer-Correira, A.J., Dubin, J.R., Tomer, K.B., Domingues, P.M., Amado, F.M. 2004. Identification of human whole saliva protein components using proteomics. Proteomics 4: 951-962.

Waters, C.A., Morand, J.N., Schatzman, R.C., Carlson, D.M. 1998. Induction of p34cdc2 in mouse parotid glands upon activation of beta 1-adrenergic receptors. Cell Mol. Biol. (Noisy-le-grand) 44: 333-342.

Williams, K.M., Ekstrom, J., Marshal, T. 1999. High resolution electrophoretic analysis of rat parotid salivary proteins. Electrophoresis 20: 1373-1381.

Yao, Y., Berg, E., Costello, C.E., Troxler, R.F., Oppenheim, F.G. 2003. Identification of proteins components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J. Biol. Chem. 278: 5300-5308.

Chapter 4

Characterization of mouse whole saliva proteome and its adaptation to tannin rich diets

E. Lamy*, G. Graça*, G. da Costa, F. Capela e Silva, E. Sales Baptista, A.V. Coelho

** The two first authors have contributed equally to this work.*

The work presented here has been prepared as a manuscript for publication on BBA – Proteins and Proteomics

Abstract

Laboratory rodents are greatly used as animal models and may be useful to understand the physiological mechanisms of intake. Mice saliva and/or salivary glands have been studied for a variety of purposes, but no general characterization of saliva proteomes has been made. We used two-dimensional electrophoresis coupled to mass spectrometry to characterize mice whole saliva proteome, and to access how quebracho tannin ingestion affects the fraction of proteins that do not form insoluble complexes with these plant secondary metabolites. From the 26 different proteins identified, in control mice whole saliva, the expression levels of one isoform of alpha-amylase and one non-identified protein increased, whereas acidic mammalian chitinase and Muc10 decreased after tannin consumption. Additionally, two basic spots that stained pink with CBB R-250 were induced, suggesting that some salivary proline-rich proteins may remain uncomplexed or form soluble complexes with tannins.

Keywords: Alpha-amylase; mass spectrometry; mice, mucins; saliva; tannin; two-dimensional electrophoresis

1. Introduction

Saliva is not merely an ultrafiltrate of plasma, but contains the entire library of proteins, hormones, antibodies and other molecular compounds, which are typically measured in routine blood tests. Due to its relatively simple and non invasive collection, saliva is an alternative to blood or urine testing. Coupled with the use of proteomics saliva has received growing interest as disease diagnostic fluid (Segal and Wong, 2008).

As the proteome is dynamic and varies according to specific physiological and/or developmental conditions, the salivary protein composition may be used to monitor other physiological changes behind disease situations. In nutrition research, proteome analysis is expected to provide a wealth of useful information to better explore and understand the effects of nutrients or food components on metabolic pathways, the regulatory mechanisms of intake, the pathogenic mechanisms and pathophysiology of nutritional disorders (Fuchs, et al., 2005; Kussmann and Blum, 2007; Thongboonkerd, 2007). The knowledge of the protein profiles and characteristics of body fluids, such as plasma/serum, have been proposed to be useful as biomarkers of the nutritional status and disease and individualized requirements of nutrients (Wang et al., 2006). Several studies showed that nutrition alters plasma and body fluid proteomes in humans and animals (Aldred et al., 2006; Linke et al., 2004; Gianazza et al., 2003).

It is known for quite some time that salivary protein profile is probably species-specific, showing differences in particular proteins, such as amylase or proline rich proteins (PRPs). Also the composition of the diet seems to contribute to changes in saliva, not only in the flow rate and electrolytes (Dawes, 1984), but also in the protein composition (Mazengo et al., 1994). The presence of particular compounds in diet may influence the salivary proteome, resulting in the secretion of particular types of proteins or in the change in particular proteins amounts. It was recently observed by us that dietary tannins affect mouse salivary protein composition (da Costa et al, 2008). Moreover, different basic taste qualities were observed to change differently human saliva proteome (Neyraud et al., 2006).

Due to the easiness of collection and to the importance for pharmaceutical and medical research, saliva proteome analysis was mainly performed in humans. By opposition, there is a lack of information concerning saliva proteome in animal models, such as laboratory rat and mice. Many of the goals in nutritional research, namely the understanding of intake regulatory mechanisms, would be better achieved using animal models. The possible physiological factors regulating macronutrient diet selection are unknown, but are though to include responses to both the orosensory and postingestive effects of food (Smith, 2000), which may be genetically determined (Bachmanov and Beauchamp, 2008). The development of genetic engineered mice, combined with the publication of the first drafts of the human and murine genome maps, the speed and convenience in terms of the logistic of experiments resulting in the use of laboratory rodents, and the possibility of the usage of a higher number of individuals in each experiment, made them a robust model for analysis of diet paradigms.

In humans a high number of salivary proteins have been identified so far. More than 1100 proteins from glandular ducts collections have been identified: 914 proteins from parotid gland collection and 917 proteins from submandibular/sublingual collection (Denny et al., 2008). Additionally, 56 proteins were identified from minor salivary glands secretion, 12 of which had not been reported in any salivary secretion (Siqueira et al., 2008).

The great number of proteins, and protein isoforms, is responsible for a variety of functions. Some proteins can have more than one function. For example, mucins main functions are in lubrication, hydration and protection of oral tissues (Wu et al., 1994), but they may simultaneously act against microorganisms (Zalewska et al., 2000; Wei et al., 2007). On the other hand, the same function can be performed by different salivary proteins. This high variability, coupled with the autonomic nervous control, provides a rapid adaptation to changes in the oral cavity.

Tannins are plant secondary metabolites (PSMs) present in several plant species, which may produce negative effects by causing toxicity and/or being anti-nutritive for the animals consuming them. The

extent of their effects varies according to their levels and to the physiological mechanisms the animal possesses to deal with them (Iason, 2005). Moreover, they affect palatability through bitter/astringent sensations. Some animal species present salivary proteins that act as defence mechanism against the harmful effects of these compounds, whereas in other species these proteins are induced when the amounts of dietary tannins increase (Bennick, 2002; Shimada, 2006). Since these PSMs are responsible for negative operant conditioning in feeding behaviour, they are used in the present paper as a model of aversive compounds, to access changes in mice salivary proteome induced by diet.

In the first part of this study we will present a characterization of mice whole saliva proteome, providing a two-dimensional electrophoresis (2-DE) map that might be further used for comparison. In the second part changes in mice whole saliva proteome with quebracho-tannin consumption will be studied.

2. Material and Methods

2.1. Animals

Twenty inbred male Balb/c mice, five weeks of age, were obtained from the licensed bioterium of Instituto Gulbenkian de Ciência (Oeiras, Portugal). The animals were housed in mice cages, type IV (Techniplast) (10 mice per cage), according to EU_recomendations and revision of Appendix A of European Convention for the Protection of Vertebrate Animals used for Experimental and other scientific Purposes (ETS No 123) and maintained on a 12 h light/12 h dark cycle at a constant temperature of 22ºC with *ad libitum* access to water and to a standard diet with 21.86% crude protein (dry basis) in the form of pellets (RM3A-P: Dietex International, UK). The animals were individually marked and submitted to a seven-days acclimation period to minimize stress effects associated with transportation. This period was followed by a seven-day pre-trial period, to allow adaptation to the ground diet used during feeding trials. The standard pellet diet was ground daily with a blender, to obtain a meal with visibly homogeneous fine-sized particles. Before the feeding-trial period the animals were indiviadually weighed and allocated to two experimental groups, with no significant differences in body mass (25.5 ± 1.7 g).

All procedures involving the animals were approved by the scientific committee, supervised by a FELASA trained scientist and conforming to the regulations of the Portuguese law (*Portaria 1005/92*), following European Union Laboratory Animal Experimentation Regulations.

2.2 Feeding Trials

A ten-day experimental period was initiated immediately after the pre-trial period. The control group (n=6) received a tannin-free diet, consisting on the same standard ground diet administred during the pre-trial period. The quebracho group (n=6) received the standard groud diet plus *quebracho* extract (Tupafin-Ato, SilvaChimica SRL; 72% ± 1.5 of condensed tannins), added in order to obtain a mixture with 7g tannin/100g wet weight of the standard diet. Food and water were provided *ad libitum* and the diets were prepared daily using a blender, as described for the pre-trial period.

2.3 Saliva collection and sample preparation

After the ten-day feeding trial – on day eleven – individual mice whole saliva was collected as described by da Costa et al. (2008). Saliva samples were immediately frozen in liquid nitrogen and stored at –80ºC until requirement for further use. Prior to protein quantification, saliva samples were centrifuged at 16,000 *g* for 5 min at 4ºC to remove particulate matter and salivary proteins that could be precipitated when complexed with tannins. Only the soluble fraction was used for further analyses.

2.4 Determination of total protein content

Whole saliva protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as standard.

2.5 Two-dimensional gel electrophoresis separation

Whole saliva samples containing 100 µg total protein were mixed with rehydration buffer [7M urea (Amersham Biosciences); 2M Thiourea (Sigma); 4% CHAPS (w/v) (3-[3-cholamidopropyl dimethilamonio]-1 propanessulphonate) (Sigma); 2% (v/v) IPG buffer (Amersham Biosciences); 60 mM dithiothreitol (DTT) (USB) and bromophenol blue 0,002% (w/v) (Amersham Biosciences)]. Samples were subjected to isoelectric focusing (IEF), at 20º C, in 13 cm IPG strips pH 3-10 NL (Amersham Biosciences) using an IPGphor Isoelectric Focusing System (Amersham Biosciences) with the program: 2h at 0V, 12h at 30V (active rehydration), 1h at 200V, 1h at 500V, 1h at 1000V, 1h at gradient linear 1000-8000V and 6h at 8000V. After focusing, proteins in the IPG strips were reduced by soaking with 1% (w/v) DTT/50 mM tris-HCl, pH 8.8/6M urea/30% (v/v) glycerol/ 2% (w/v) SDS at room temperature for 15 min., then alkylated with 65 mM iodoacetamide/50 mM tris-HCl, pH 8.8/6M urea/30% (v/v) glycerol/ 2% (w/v) SDS for 15 min. at room temperature. The equilibrated strips were then horizontally applied on top of a 12% SDS-PAGE gel (1 X 160 X 200 mm) and proteins were separated vertically, using a Protean II xi cell (Bio-Rad), at 18ºC, applying a constant current of 5 mA/gel during the first hour, after which it was step changed to 15 mA/ until the end of the run.

Broad range molecular mass markers (BioRad, Ref 161-0317) were run simultaneously with the sample, in some control and tannin group gels, to calibrate molecular masses of the protein spots. Gels were stained with Coomassie Coloidal G-250 stain, following the procedure of Blakesley and Boezi (1977). Additionally, a PRP specific stain/destain procedure described by Beeley et al. (1991) was used in some gels, in order to access the induction of these proteins by tannins. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250, dissolved in 40% (v/v) methanol, 10% (v/v) acetic acid *overnight* and destained with 10% acetic acid (v/v) for 48h.

2.6 Gel analysis

Digital images of the 2-DE gels were acquired using a scanning densitometer with internal calibration (Molecular Dynamics), using LabScan software (Amersham Biosciences Europe GmbH, Freiburg, Germany). Gel analysis was performed using Image Master Platinum v.6 software (Amersham Biosciences Europe GmbH, Freiburg, Germany). Spots volume normalization, in the various 2-DE maps, was carried out using the relative spot volumes (% Vol).

Spot detection was performed, first by using automatic spot detection, followed by manual editing for spot splitting and noise removal. The gel containing the greatest number of protein spots for each diet condition was chosen as the reference gel. All other gels from the same experimental condition were matched to the reference gel by placing user landmarks on approximately 10% of the visualised protein spots to assist in automatic matching. After automatic matching completion, all matches were checked for errors by manual edition.

2.7 Protein identification

2.7.1 In-gel digestion

Stained spots were excised, washed in acetonitrile and dried in a speedvac (Thermo Savant). Gel pieces were re-hydrated with a digestion buffer (50mM NH_4HCO_3) containing trypsin (Promega, Madison, WI, USA) and incubated overnight at 37ºC. The digestion buffer containing peptides was acidified with formic acid, desalted and concentrated using C8 microcolumns (POROS R2, Applied Biosystems, Foster City, CA, USA).

2.7.2 Peptide Mass Fingerprinting

The peptides were eluted with a matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile;0.1% (v/v) TFA. The mixture was allowed to air-dry (dried droplet method). Mass spectra were obtained using a Voyager-DE STR (Applied Biosystems, Foster

City, CA, USA) Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer in the positive ion reflectron mode. External calibration was made by using a mixture of standard peptides (Pepmix 1, LaserBiolabs, Sophia-Antipolis, France). Spectra were processed and analyzed by the MoverZ software (Genomic Solutions Bioinformatics, Ann Harbour, MI, USA). Peakerazor software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant *m/z* peaks and for internal calibration. Monoisotopic peptide masses were used to search for protein identification using Mascot software (Matrix Science, London, UK). Database searches were performed against SwissProt, MSDB and NCBInr. The following criteria were used to perform the search: (1) mass accuracy of 100 ppm; (2) one missed cleavage in peptide masses; and (3) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. Criteria used to accept the identification were: significant homology scores achieved in Mascot; significant sequence coverage values and similarity between the protein molecular mass calculated from the gel and for the identified protein.

2.7.3. Prediction of post translational modifications

Potential posttranslational modifications (PTMs) were predicted using the FindMod (http://www.expasy.ch/tools/findmod/) and GlycoMod (http://www.expasy.org/cgi-bin/glycomod) search engines (Gasteiger et al., 2005), which work by examining peptide mass fingerprint results of the identified proteins for the presence of PTMs. This is done by looking at mass differences between experimentally determined peptide masses and theoretical peptide masses calculated for the specified protein sequence. Additionally, NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict putative serine, threonine and tyrosine phosphorylation sites using a neural network-based method trained on a large dataset of known phosphorylation sites (Blom et al, 1999). Glycosylation and phosphorylation presented in Swissprot database were also considered. The presence of signal peptides in each identified protein was searched using Signal IP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Only the predicted PTMs associated to peptides not matched to the identified protein were considered.

2.8. Statistical analysis

All data were analysed for normality (Kolmogorov-Smirnoff test) and for homocedasticity (Levene test). The values of salivary protein concentration were normally distributed and independent sample T-tests were performed to access differences between diet treatments.

Spot relative volume (% Vol) did not present normal distribution or homocedasticity. The difference in the expression levels between control and *quebracho* group, for each protein spot was statistically

accessed using the Mann-Whitney test. Means were considered significantly different when $P<0.05$. All statistical analysis procedures were performed by SPSS 15.0 software package.

3. Results

3.1. Saliva protein concentration

After the ten-day feeding trial, the total protein saliva concentration was significantly lower in the *quebracho* group than in the control group (Table 1).

Table 1 – Whole saliva protein concentration from control (N=6) and quebracho group (N=6) mice

	Diet				
	Control		Quebracho		*P*
	X	SD	X	SD	
Protein concentration (μg/mL)	2920.5	289.0	1940.6	138.3	0.00012[a]

X: mean; SD: standard deviation
[a] Differences are significant for $P<0.05$

3.2 Whole saliva protein pattern

We constructed a two-dimensional map of mice whole saliva with a non-linear pI range of 3-10 and a molecular mass range from about 10 to 100 kDa (Fig 1). A total of 86 protein spots were reproducibly displayed in Coomassie Coloidal G-250 stained gels. From them, 53 protein spots, corresponding to 26 polypeptides were identified by peptide mass fingerprinting (PMF) (Table 2). From the non identified spots, three were observed in the acidic extreme of the gel, signalled in Fig. as a, b and c. Those spots may contain acidic proteins and the electrophoretic conditions used probably did not allow their separation.

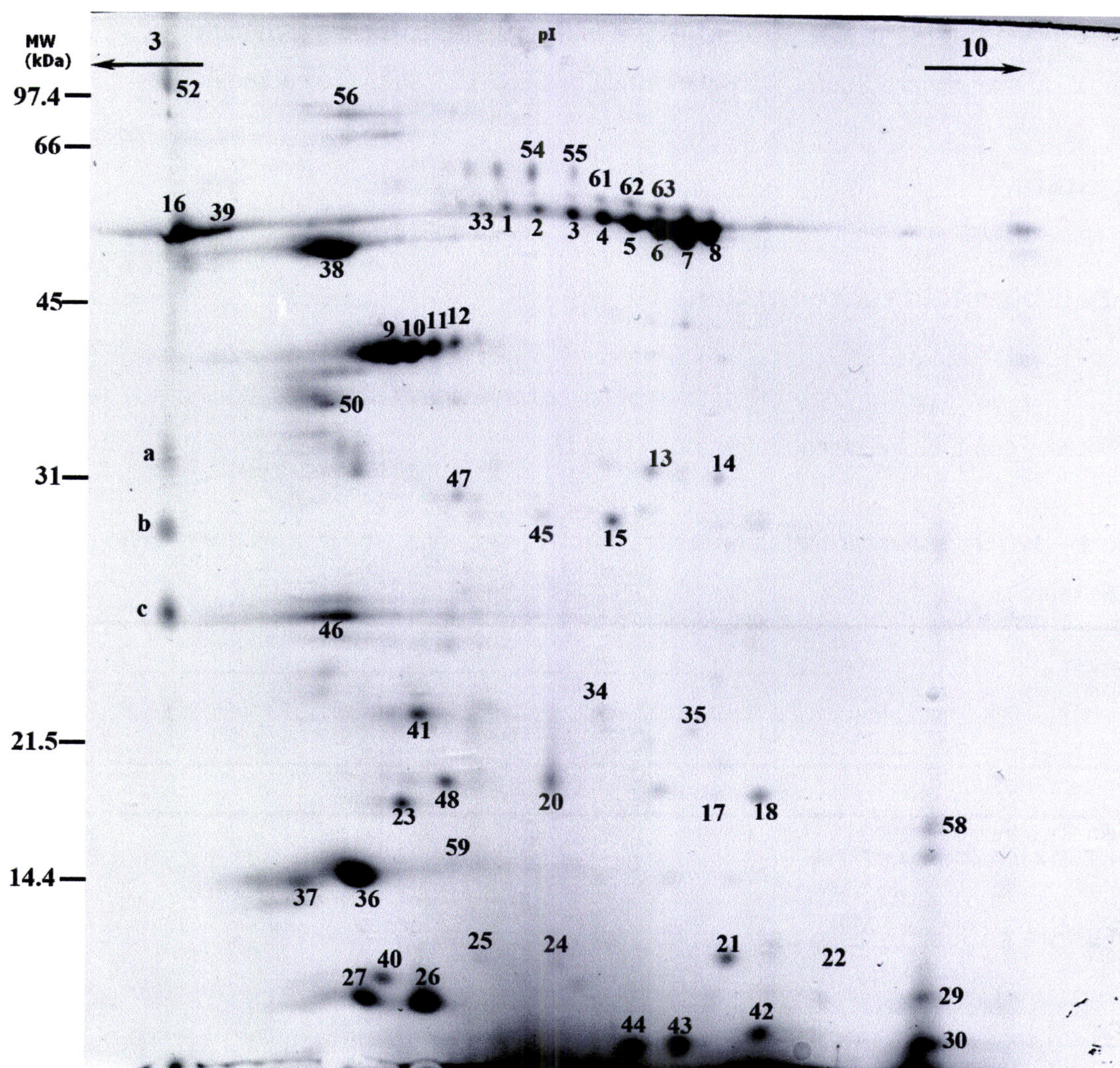


Figure 1 – Two-dimensional proteomic profile of mice whole saliva. Aliquots containing 100 μg of proteins from control animals were subjected to IEF in a 3-10 NL range, separated by molecular masses in 12% polyacrylamide gels and stained with Coomassie Coloidal G-250. Molecular markers are represented on the left side of the gel. Numbered spots correspond to the identified proteins (with the exception of spots a, b and c)

Several protein spots were identified with the same accession code. These correspond to the proteins alpha-amylase, androgen-binding protein subunits α, β and γ, carbonic anhydrase VI, cysteine-rich secretory protein 1, demilune cell and parotid protein 1, glandular kallikrein K22, parotid secretory protein and vomeromodulin (Table 2). The different spots with the same identification differ among them in the observed pI and/or molecular masses. These differences may be due to the presence of post-translational modifications, protein fragments and isoforms.

Table 2 – Mice whole saliva proteins identified by Peptide Mass Fingerprinting (PMF)

Spot	Protein	Accession code *SwissProt*	*Score*[a]	Pep.[b]	Seq. cov. (%)	Est.[c] MW (kDa)/ pI	Theor.[d] MW (kDa)/ pI	Biological function[e]
38	Acidic mammalian chitinase precursor	**Q91XA9**	170	12	39	53/4,8	52/4.9	Protection/defense
1	Amy 1 protein (salivary amylase)	**Q921Y7**	88	9	23	58/5,5	58/6.5	Metabolism Carbohydrates
2			193	12	32	58/5,6		
3			193	17	46	58/5,7		
4			217	17	48	58/5,8		
5			230	18	52	58/5,9		
6			270	17	50	58/6,0		
7			226	18	51	58/6,1		
8			232	18	50	58/6,2		
16			155	13	38	58/3,4		
33			107	8	28	58/5,4		
39			148	11	22	58/3,7		
61			132	12	31	58/5,8		
62			163	13	39	58/5,9		
63			149	10	32	58/6,0		
36	Androgen binding protein α subunit	**Q9WUM8**	78	4	47	15/5,0	10/5.3	Sexual behaviour and/or regulation
37			78	4	47	15/4,8		
56	BC048546 protein fragment (α-2-macroglobulin)	**Q8K040**	101#	9	32	97/5,0	45/5.13	Protein degradation/ inhibition
9	Carbonic anhydrase VI	**Q7TNG9**	177	15	52	44/5,0	36/6.1	Metabolism Carbohydrates
10			156	15	52	44/5,1		
11			193	13	53	44/5,2		
12			134	11	52	44/5,3		
13	Cysteine-rich secretory protein 1 precursor	**Q03401**	82*	8	34	33/6,0	28/6.4	Sexual behavior and/or regulation
14			79*	6	33	33/6,3		
34	Demilune cell and parotid protein 1 (Dcpp 1)	**Q64097**	70*	5	47	21/5,9	18/6.1	Protection/defense
35			82*	6	47	20/6,2		
18	Demilune cell and parotid protein 2 (Dcpp 2)	**Q6PCW3**	97	5	52	17/6,8	16/7.8	
50	Deoxyribonuclease I - percursor	**P49183**	90	6	23	39/4,9	32/4.8	DNA replication and repair
40	Glandular kallikrein k13 (mGK-13)	**P36368**	71	5	55	12/5,1	29/8.3	Protein degradation/ inhibition
47	Glandular kallikrein k5 (mGK-5)	**P15945**	109	7	31	32/5,4	29/5.3	
20	Glandular kallikrein k6 (mGK-6)	**P15947**	79	6	22	18/5,7	29/5.0	
21	Glandular kallikrein k9 (mGK-9)	**P15949**	84	7	29	12/6,4	29/7.6	
15	Glandular kallikrein k22 (mGK-22)	**P15948**	111	7	27	30/5,9	28/6.2	
45			77 *	6	24	31/5,6		
25	Lacrimal androgen binding protein ε	**Q6UGQ3**	86	6	45	12/5,4	13/5.7	Sexual behavior and/or regulation
52	Mucin apoprotein precursor	**Q61002**	84*	7	20	97/3,4	30/10.4	Protection/defense
30	Muc 10	**Q8VC95**	85*	7	28	11/9,2	20/10.2	
23	Odorant binding protein Ia	**P97336**	182	11	61	17/5,2	17/5.2	Sexual behavior and/or regulation
48	Odorant binding protein Ib - fragment	**P97337**	118	8	66	18/5,4	17/5.5	
17	Parotid secretory protein precursor	**P07743**	77	7	27	17/6,3	25/4.9	Protection/defense
46			82	7	27	25/4,9		
59	Prolactin-inducible protein homolog precursor (14 kDa submandibular gland protein)	**P02816**	116	7	58	15/5,4	17/4.7	
58	Prorenin-converting enzyme (MK 13b) precursor	**O88309**	76*	5	24	17/9,3	29/7.5	Protein degradation/ inhibition

26	Salivary androgen binding protein β subunit - fragment	Q7TNM8	102	7	83	11/5,2	10/5.3	Sexual behavior and/or regulation
27			80	5	76	11/5,0		
22	Salivary androgen binding protein subunit γ	Q8JZX1	87	7	51	11/7,7	13/7.7	
29			153	8	51	11/9,1		
42			121	9	44	11/7,2		
43			157	10	49	10/6,1		
44			69*	5	35	10/6,0		
41	Similar to odorant bindin protein 1F	Q9D3N5	81*	6	45	21/5,2	19/5.5	
54	Vomeromodulin precursor	Q80XI7	81#	8	22	66/5,6	62/5.5	Sexual behavior and/or regulation
55			68#	8	21	66/5,7		

[a] Score is significant ($p<0.05$) when is higher than 75 (search performed with no taxonomic restriction), 67 (* search restricted to mammal database), or 61 (# search restricted to rodent database);
[b] Number of peptides from experimental Peptide Mass Fingerprint whose masses match those from a theoretical PMF determined from a known sequence
[c] Molecular mass measured based on electrophoretic mobility;
[d] Determined molecular mass present in database;
[e] Biological function based on their annotations in the database and/or bibliography.

The spots identified as salivary alpha amylase (Table 2), have apparent molecular masses around 58 kDa and pI ranging approximately between 3.4 and 6.2. The theoretical molecular mass of the native form of alpha amylase is 56kDa and the pI is 6.4. Glycosylation, with neutral and acidic (sialic acid) carbohydrates, and post-secretion spontaneous deamidation of amylase 1 were described by Bank et al. (1991) and Hirtz et al. (2005b). Knowing that glycosylated asparagines residues almost always occur in the sequence Asn-X-Ser/Thr, two potential N-glycosylation sites for mice alpha-amylase are 427-429 and 475-477 (Supplementary Table 1). These two sites were also referred to human (Bank et al., 1992; Hirtz et al., 2005b) and discussed for mice salivary alpha-amylase (da Costa et al., 2008). The glycosylations, with different oligosaccharides, can be a reason for the higher apparent molecular mass of the amylase isoforms obtained compared with the native alpha amylase form, and for the different pI presented by the several protein spots. Moreover the peptides containing the potential N-glycosylation sites were only observed in the peptide maps of the spots 2, 5 and 8.

3.3 Effects of quebracho consumption on whole saliva protein patterns

In an attempt to investigate changes in whole saliva protein profile induced by aversive substances, such as tannins, 2-DE whole saliva profiles from the mice group that were fed a 7% *quebracho* tannin supplemented diet were compared with the ones from the control group. Some consistent changes were observed.

Three protein spots, were observed to change significantly in terms of relative volume (Table 3). The levels of one isoform of alpha-amylase (spot 62) and of the unidentified spot 24 increased, whereas the levels of chitinase (spot 38) decreased. Also two new protein spots (Q1 and Q2) not observed in the gels from control group, were consistently present in the gels from quebracho group, whereas spot 30, corresponding to Muc 10, was absent (Fig. 2).

Table 3– Comparison between control (N=6) and quebracho-fed animals (N=6) in protein expression levels (% Vol) (mean ± SD)

Spot Nº	Control	Quebracho	P^a	Protein
24	17.07 ± 41.23	110.87 ± 59.55	0.0099	Not identified
38	475.26 ± 272.43	110.99 ± 132.25	0.015	Acidic mammalian chitinase precursor
62	0.28 ± 0.02	0.44 ± 0.05	0.00015	Amy 1 protein (salivary amylase)

[a] Differences are significant for $P<0.05$

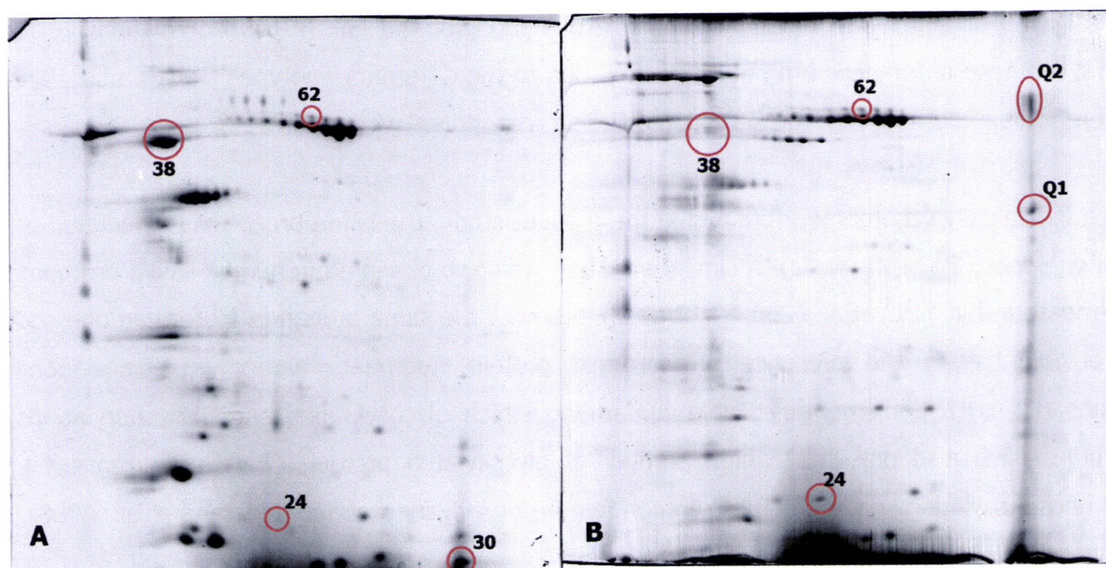


Figure 2 - Changes in the proteome of mice whole saliva after quebracho consumption. A) representative gel of an individual from control group; B) representative gel of an individual of quebracho group. A decrease in expression levels was observed for spot 38, whereas spots 24 and 62 increased after quebracho consumption. Spot 30 was only observed in the gels from control group, whereas spots Q1 and Q2 were only observed in 2-DE gels from quebracho group. These last spots were dark pink stained.

When both control and quebracho gels were subjected to Coomassie Brilliant Blue R-250 modified staining procedure for proline-rich proteins (Beeley et al. 1991) the Q1 and Q2 spots, with an apparent pI of 9.5 and apparent molecular masses of 42 and 64 kDa, respectively, appeared with a slightly dark pink colour, whereas the remaining spots appeared as blue spots in both gels (supplementary Fig.1).

4. Discussion

4.1. Characterization of mice whole saliva 2-DE profile

Using two-dimensional electrophoresis and mass spectrometry, a proteome profile of mice whole saliva comprising 26 unique proteins was established.

Williams et al. (1999) obtained 2-D gels from rat parotid saliva, using a pI range similar to that used in our study (pH 3-10). Comparing them with the gels obtained in the present study, it is possible to observe a similar distribution for some spots namely for the spots we have identified as amylase, deoxyribonuclease (DNase), parotid secretory protein and demilune cell and parotid protein (equivalent to the rat common salivary protein). However, several differences are observed between the two patterns when comparing our Coomassie Colloidal stained gels with the published silver stain gels. The presence of basic and acidic proline-rich proteins (PRPs) was suggested in rat parotid saliva 2D pattern (Williams et al., 1999). These proteins are not constitutively expressed in mice salivary glands (Mehansho et al., 1985), what can be an explanation for the fail in to observe them in our control gels. All these differences are not surprising, due to the different genotypes (rat vs. mice) and to the different glandular origin of the secretions studied (parotid vs. whole saliva).

In the mice whole saliva proteome, obtained in the present study, the same protein was identified for more than one spot (Fig. 1; Table 2). A similar situation was also observed in human saliva proteome and was empathized in Hirtz et al. (2005a). The expression of the same protein in more than one spot may be attributable to the presence of isoforms, protein fragments and/or post-translational modifications (PTMs) (Supplementary Table 1), among which glycosylations and phosphorylations. Human whole saliva also presents a high number of glycosylated proteins (Ramachandran et al., 2006) and phosphorylation seems to be a common feature of salivary proteins (Messana et al., 2008).

The identified proteins can be sorted into functional categories (Fig. 3). A large group includes proteins sex-linked, either related to sexual behaviour or hormonally regulated. This includes cysteine-rich secretory protein (CRSP), androgen binding protein (ABP), odorant binding protein (OBP) and vomeromodulin. CRSP is synthesized in the granular convoluted tubular cells of the mouse submandibular gland strongly under androgen control (Haendler et al., 1993). In humans the cysteine-rich protein 3 was also identified in saliva (Wilmarth et al., 2004; Vitorino et al., 2004). Mouse salivary ABPs are secreted into saliva in two different dimeric forms: an α-subunit disulfide-bridged to either a β or a γ subunit (Karn and Laukaitis, 2003). All of the three subunits were identified in the present study. These proteins bind steroid hormones (Karn and Laukaitis, 2003) and a role as pheromones or in the modulation of odorant detection has been proposed (Emes et al., 2004). Female mice shows mating preference for males based on its genotype for this protein, suggesting that this protein can be related with the sexual selection in this specie (Laukaitis et al., 1997, 2005). The presence of OBP was already reported for rat saliva (Marchese et al., 1998) but, from our knowledge, this is the first study identifying this protein in mice whole saliva. OBPs, secreted in the nasal epithelium, belong to the lipocalins superfamily and may be involved in the activation of odorants (Hajjar et al., 2006). In human whole saliva 2-DE maps, lipocalin 1 was observed within four spots (Vitorino et al., 2004). OBPs are also present in male pig submandibular gland and are sex-specific, being absent in females, and a function in chemical communication between sexes has been

proposed (Marchese et al., 1998). Additionally, Van't Hof et al. (1997) suggested that lipocalin 1 can play a role in the control of inflammatory processes in oral and ocular tissues. BLAST application (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al 1997) was used to confirm the identification for spots 54 and 55. Homology to a rat ligand interacting mucosal protein was achieved. A role in smell perception, similar to the one of the odorant binding proteins is suggested.

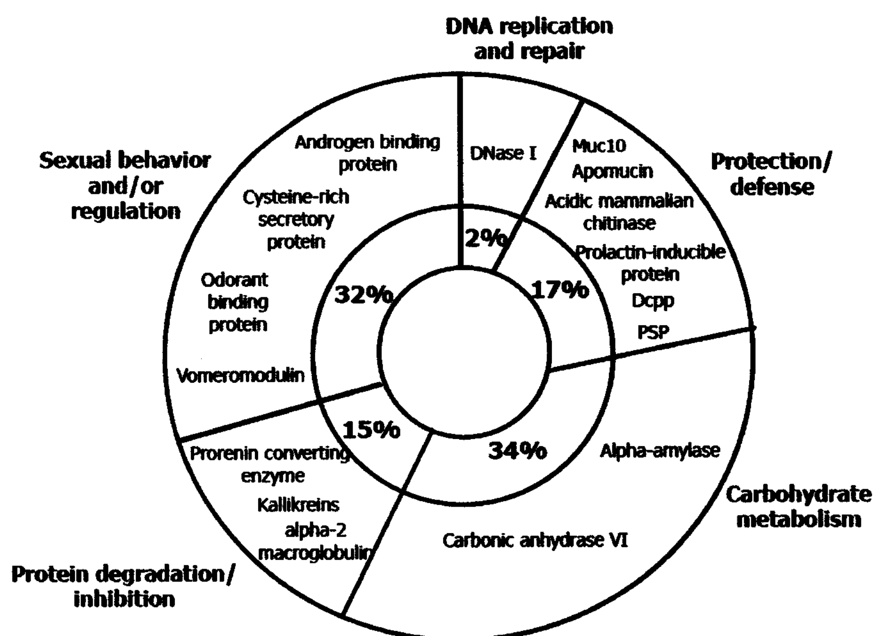


Figure 3 – Functional category of identified salivary proteins. Sorting of the functions of proteins was arbitrarily based on their annotations in SwissProt database and in bibliography. Relative amount of spots identified for each functional category.

Fourteen spots were identified as salivary amylase. A higher number of salivary amylase spots with a similar distribution is also observed in human whole saliva (Hirtz et al., 2005b), what suggests similarities for human and mouse in the digestive functions of saliva.

Carbonic anhydrase VI identified in four distinct spots is expressed in considerable relative amounts, with a distribution similar to the one observed in human whole saliva. 2-DE pattern (Vitorino et al., 2004; Hardt et al., 2005; Hu et al., 2005; Walz et al., 2006). This protein is believed to provide a greater buffering capacity to saliva, tears and milk (Ogawa et al., 2002; Nishita et al., 2007), and have also been related with taste function (Thatcher et al., 1998).

Defensive functions were suggested for five of the identified proteins: two mucins, two forms of demilune cell and parotid protein (Dcpp) and parotid secretory protein. Mucins are present in human whole saliva, constituting approximately 16% of the total proteins, having a protective role and contributing to oral coating and lubrication (Rayment et al., 2000). The reduced number of mucins

identified in the present work can be due to the difficulty of assessing these proteins because of their large molecular mass, high viscosity, and poor solubility in aqueous solvents (Veerman et al., 2003).

Dcpp was first described, in mouse, by Bekhor et al. (1994). This protein is the mouse equivalent of the rat common salivary protein 1 (Girard et al., 1993) and is mainly secreted by the sublingual gland and in lesser quantities from the submandibular and parotid glands. Mullins et al. (2006) suggested that these salivary proteins could display antimicrobial activity or provide a defensive coating to enamel. Wilmarth et al. (2004) observed the presence of common salivary protein 1 in human whole saliva.

Parotid secretory proteins (PSP), together with amylase, were referred as being the major salivary proteins secreted by mouse parotid glands (Owerbach and Hjorth, 1980). Mouse PSP has been shown to bind to bacteria and bacterial membrane proteins in vitro (Robinson et al., 1997) and inhibits the growth of *Candida albicans* (Khovidhunkit et al., 2005). Antibacterial activity was also demonstrated for human PSP (Geetha et al., 2003). BSP30, a bovine PSP homolog, was detected in cattle saliva (Rajan et al., 1996). Khovidhunkit et al. (2005) suggested that PSP may be part of a host defence system against microbial infection in the oral cavity and in circulation.

The submandibular 14kDa protein identified for mice presents, at the amino acid levels, 80% homology to the rat prolactin inducible protein (PIP) (Mirels et al., 1998) and 51% homology with the human PIP, which is secreted in several human fluids, including saliva (Lee, 2001). The ability of PIP to bind to bacteria, keratin and CD4, as well as the regulation of PIP gene expression by interleukins lead to the suggestion of a role in mucosal immunity or in non immune mucosal defence (Lee, 2001). Besides, PIP also binds to hydroxyapatite, the predominant component of tooth enamel and Mirels et al. (1998) suggested a participation of this protein in pellicle enamel formation. Since the expression of PIP, in human whole saliva, is increased by prolactin and steroids, Ghafouri et al. (2003) suggested that the levels of this protein in human saliva might reflect activities in the neuroendocrine and neuroimmune systems and, in that way, can be a new biological stress marker.

Seven protein spots were identified as corresponding to five different kallikrein forms. This protein belongs to a family of serine proteases, which are involved in hormones and growth factors processing (Blaber et al., 1987). It is of interest the great amount and diversity of kallikreins present in mice saliva. These proteins were also found in human (Jenzano et al., 1992) and rat (Bedi, 1991) saliva, but in lower amounts. Several kallikreins are expressed in mouse submandibular glands (Blaber, 1993). The relative proportion of the various tissue kallikreins secreted by rat submandibular glands was observed to be differentially influenced by the two branches of the autonomic nervous system. Kallikreins in sympathetic induced saliva arrive from exocytosis of pre-packaged granules, in granular tubules, whereas the kallikreins in parasympathetic induced saliva were likely to be secreted through a

constitutive vesicular rout (Garrett et al., 1998). In this study, we use pilocarpine to stimulate mice saliva secretion, and in that way we may hypothesize that the forms identified can arrive from a constitutive vesicular route, as it occurs in rats.

For spot 56 the results obtained indicated a homology with the human and rat alpha-2-macroglobulin. Alpha-2-macroglobulin, which had been also identified in human whole saliva (Xie et al., 2005), inhibits endogenous proteases, as well as bacterial proteases delivered in soft tissues during inflammatory processes (Sandholm, 1986).

4.2 Changes in whole saliva proteome induced by quebracho tannin consumption

It is generally believed that tannins are synthesized by plants to act as deterrents to animals because of their bitter and astringent properties (McArthur et al., 1991). The ingestion of polyphenols is associated with dry, puckering sensation in the mouth known as astringency (Haslam and Lilley, 1988). The challenge to animals is to be able to eat plant foods without suffering ill effects from tannins. Saliva may play an important role in defence against tannins for mice, perhaps by minimizing their unpalatable astringent properties (Glendinning, 1992). In this study, mice consuming a condensed tannin rich diet presented whole saliva with a lower protein concentration, what suggests that the stable insoluble complexes formed between salivary proteins and dietary tannins in the mouth (Bennick, 2002), were lost during centrifugation in the sample preparation step (da Costa et al., 2008). This allows us to analyse the protein expression changes induced by quebracho consumption in the salivary proteins soluble fraction, which are less well studied.

The production of salivary tannin binding proteins is a defence mechanism presented by some animals species, from which salivary proline rich proteins (PRPs) are the more studied. The presence of PRPs was observed in salivary glands of polyphenols fed animals, namely rats (Mehansho et al., 1983) and mice of BALB/c strain (Mehansho et al., 1985). The salivary PRPs induced in BALB/c mice, after ingestion of polyphenols, showed a very high affinity to tannins, leading to the formation of insoluble complexes (Mehansho et al., 1983), impeding these secondary plant metabolites of being free to exert negative effects in the animal digestive tract. From the protein spots that newly appeared in the quebracho group, spots Q1 and Q2 stained dark-pink, suggesting these may be PRPs (Beeley et al., 1991). Identification of PRPs by mass spectrometry is a challenge due to the primary sequence of these proteins. It was demonstrated previously, by Leymarie et al. (2002), for PRP 3, that tryptic digests of this family only produce 3 or 4 visible fragments, which have a higher mass than the *m/z* range of the mass spectra acquired. The pI around 9,5, presented by Q1 and Q2 spots, is in agreement with main bibliography that argue that basic PRPs act as a defence against dietary tannins (Bennick, 2002). Although the majority of PRPs was supposed to form insoluble complexes with tannins, being removed by centrifugation during sample preparation step, it is possible that some

PRPs-tannin soluble complexes are also formed (Richard et al., 2006). The observation of PRPs in two-dimensional gels and its lack of observation in the previous one-dimensional gels (da Costa et al., 2008) may be due to their mixture with other proteins with similar molecular masses but with different staining characteristics that impeded the observation of the pink bands characteristic after Coomassie staining

Muc10 spot was absent in the 2D-E profile of mice whole saliva from the quebracho group. Since salivary mucins can form complexes with tannins (Asquith et al., 1987), it is possible that the insoluble ones had been removed from the saliva during the centrifugation steps of laboratorial sample preparation. Moreover, changes in other salivary mucins may be not accessed using this methodology due to the high molecular mass of the majority of mucins that impede them to enter electrophoresis gels with such polyacrylamide concentration.

The levels of chitinase were observed to consistently decrease in the individuals that consumed quebracho for ten days. The presence of this protein in mice saliva has been observed by one-dimensional electrophoresis, but its level did not change after ten-day tannin consumption (da Costa et al., 2008). The presence of this protein in saliva had been already reported in mice (Goto et al., 2003) and human (Van Steijn et al., 1999; 2002) saliva. A digestive (Goto et al., 2003) and/or a defensive role against chitinous pathogens (Van Steijn et al., 1999; 2002; Goto et al., 2003) has been proposed. In humans the levels of this enzyme increased in cases of periodontitis (Van Steijn et al., 2002).

The increase in the level of one isoform of alpha amylase was already observed, by one-dimensional SDS-PAGE, in mice subjected either to quebracho or tannic acid enriched diets (da Costa et al., 2008). The increase of salivary amylase levels, after tannin consumption, was suggested as a co-adjuvant for the inhibition of the biological activity of these plant secondary metabolites and/or as a response to counteract the amounts of this enzyme that was inactivated by binding with tannins (da Costa et al., 2008). In the referred study only the levels of one isoform were observed to increase, similarly to what is observed in the present study, what supports the thought of different functions for the different isoforms. Despite additional methodologies being necessary to elucidate these differences, the present study adds the information that the increased isoform of amylase has a more acidic isoelectric point, that the remainder amylase protein spots.

In conclusion, the majority of proteins identified in mice whole saliva, in our previous study, using one dimensional electrophoresis (da Costa et al., 2008) were also identified in the present study, with few exceptions. The lack of identification of the proteins Apolipoprotein A-I and Immunoglobulin chains may be related to the amounts of total protein used. Further studies with high protein amounts may be useful to a deepest knowledge on mice salivary protein composition. Two-dimensional

electrophoresis allowed the identification of a higher number of proteins and the establishment of a 2-DE map that may further used for comparison studies. It was possible to observe similarities between mice and humans 2-DE profiles, both of which are omnivores, but many differences were also observed. This goes in accordance with the thought of species-specific salivary proteome and demonstrates the importance on the characterization of a 2-DE mice saliva map. By introducing quebracho tannin in mice diet, we demonstrated that the mice whole saliva 2-DE profile changes with the presence of aversive compounds in diet and that proteomics may be useful in nutritional studies to monitor these changes.

Supplementary Table 1 – Predicted posttranslational modifications (PTMs) and protein processing for the proteins identified by peptide mass fingerprinting (PMF)

Protein	Accession code	Spot	Modified residues					Signal[2] and/or propeptide	Ref.
			Glycosylation		Deamidation*	Phosp.	Proteolitic processing		
			N-glyc.[1]	O-glyc.					
Androgen binding protein α subunit	Q9WUM8	36; 37	37-39					1-22	Karn and Laukaitis; 2003
Apomucin	Q61002	52	103-105; 116-118; 129-131; 142-143; 155-157; 168-170; 177-179; 186-188; 195-197; 204-206; 239-241	Several Thr and Ser residues in the region 103-211				1-22	Denny et al., 1996, Perez-Vilar and Hill, 1999
Carbonic anhydrase VI	Q7TNG9	9; 10; 11; 12	255-257			22; 24; 47; 66		1-17	Feldstein and Silverman, 1984; Murakami and Sly, 1987; Vitorino et al., 2004
Cysteine-rich secretory protein 1	Q03401	13; 14	145-147					1-25	-----
Demilune cell and parotid protein 1 (Dcpp 1)	Q64097	34; 35	44-46					1-23	Girard et al., 1993
DNase I	P49183	50	40-42; 128-130					1-22	-----

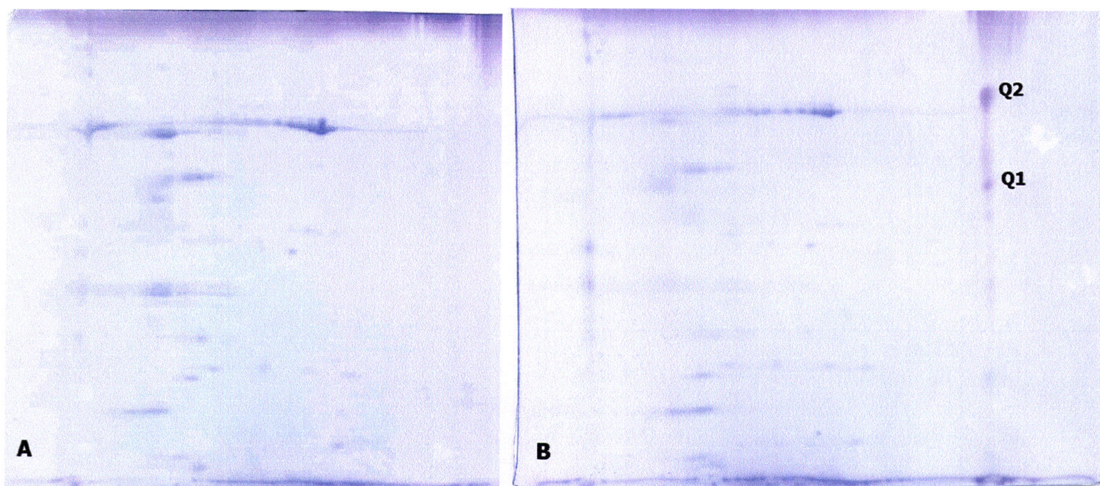
Muc 10	**Q8VC95**	30	103-105	Several Thr and Ser residues in the region 103-211				1-22	Denny et al., 1996; Perez-Vilar and Hill, 1999
Parotid secretory protein	**P07743**	17	----				185-186	1-20	Poulsen et al., 1986
		46					-----		
Prorenin-converting enzyme (MK 13b) *	**O88309**	40	104-106				164-165	1-24	Timm, 1997
Salivary-amylase	**Q921Y7**	1	427-429; 475-477		417; 419; 431			1-15	Bank et al., 1991; Hirtz et al., 2005b
		2			-----				
		3			417; 419; 431				
		4 5			364; 365; 379; 417; 419; 431				
		6 7			50; 56; 364; 365; 379				
		8 61			364; 365; 379				
		62 63 16 33 39			-----				

* according with FindMod;

[1] According with the existence of a consensus region and the absence of the peptide from tryptic peptide map obtained by MALDI TOF MS;

[2] Predited from Signal IP 3.0 (http://ww.cbs.dtu.dk/services/SignalIP/);

This table only includes information about the most probable PTMs according to the bibliography. Here are indicated the residues for which these PTMs are predicted, according with mass spectra analysis; blank spaces were left for the modifications that were not probable for the protein, either due to the lack of consensus regions, in the protein sequence for that modification, or due to the presence of the potentially modified peptide in mass spectra.



Supplementary Figure 1 – Mice whole saliva 2-DE gels stained with Coomassie Brilliant Blue R-250; A) representative gel of an individual from control group; B) representative gel of an individual of quebracho group. Spots Q1 and Q2 can be observed as dark-pink spots in the basic region of the gel

5. References

Aldred, S., Sozzi, T., Mudway, I., Grant, M.M., Neuert, H., Kelly, F.J., Griffths, H.R., 2006. α Tocopherol supplementation elevates plasma apolipoprotein A1 isoforms in normal healthy subjects. Proteomics. 6: 1695-1703.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller W., Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Asquith, T.N., Uhlig, J., Meansho, H., Putman, L., Carlson, D.M., Butler, L. 1987. Binding of condensed tannins to salivary proline-rich glycoproteins: the role of carbohydrate. J Agric. Food Chem. 35: 331-334.

Bachmanov, A.A., Beauchamp, G.K. 2008. Amino acid and carbohydrate preferences in C57BL/6ByJ and 129P3/J mice. Physiol. Behav. 93: 37-43.

Bank, R.A., Hettema, E.H., Arwert, F., Amerongen, A.V., Pronk, J.C. 1991. Electrophoretic characterization of posttranslational modifications of human parotid salivary alpha-amylase. Electrophoresis 12: 74-79.

Baxter, N.J., Lilley, T.H., Haslam, E., Williamson, M.P. 1997. Multiple interactions between polyphenols and salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry 36: 5566-5577.

Bedi, G.S. 1991. The effects of autonomic drugs on the concentration of kallikrein-like proteases and cysteine-proteinase inhibitor (cystatin) in rat whole saliva. J. Dent. Res. 70: 924-30.

Beeley, J.A., Khoo, K.S., Lamey, P.J. 1991. Two-dmensional electrophoresis of human parotid salivary proteins from normal and connective tissue disorder subjects using immobilised pH gradients. Electrophoresis 12: 493-499.

Bekhor I., Wen Y., Shi S., Hsieh, C.H., Denny, P.A., Denny, P.C. 1994. cDNA cloning, sequencing and in situ localization of a transcript specific to both sublingual demilune cells and parotid intercalated duct cells in mouse salivary glands. Arch Oral Biol. 39: 1011-1022.

Bennick, A. 2002. Interaction of plant polyphenols with salivary proteins. Crit. Rev. Oral Biol. Med. 13: 184-196.

Blaber, M., Isackson, P.J., Bradshaw R.A. 1987. A complete cDNA sequence for the major epidermal growth factor binding protein in the male mouse submandibular gland. Biochemistry 26: 6742-6749.

Blaber, M., Isackson, P.J., Holden, H.M., Bradshaw, R.A. 1993. Synthetic chimeras of mouse growth factor-associated glandular kallikreins. II. Growth factor binding properties. Protein Sci. 2: 1220-1228.

Blakesley, R.W., Boezi, J.A. 1977. A new staining technique for proteins in polyacrylamide gels using coomassie brilliant blue G-250. Anal. Biochem. 82: 580-582.

Blom, N., Gammeltoft, S., Brunak, S. 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J. Mol. Biol. 294: 1351-1362.

da Costa, G., Lamy, E., Capela e Silva, F., Andersen, J., Sales Baptista, E., Coelho, A.V. 2008. Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. J. Chem. Ecol. 34: 376-387.

Dawes, C. 1984. Stimulus effects on protein and electrolyte concentrations in parotid saliva. J. Physiol 346: 579-588.

Denny, P.C., Mirels, L., Denny, P.A. 1996. Mouse submandibular gland salivary apomucin contains repeated N-glycosylation sites. Glycobiology 6: 43-50.

Denny, P., Hagen, F.K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G.S., Boontheung, P., Cociorva, D., Delahunty, C.M., Denny, T., Dunsmore, J., Faull, K.F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S.C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J.A., Ogorzalek Loo, R.R., Malamud, D., Melvin, J.E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S.K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M.A., Takashima, J., Than, S., Wang, J., Whitelegge, J.P., Witkowska, H.E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D.T., Yates, J.R. 3rd, Fisher, S.J. 2008. The Proteomes of Human Parotid and Submandibular/Sublingual Gland Salivas Collected as the Ductal Secretions. J Proteome Res. 7: 1994-2006.

Emes, R.D., Riley, M.C., Laukaitis, C.M., Goodstadt, L., Karn, R.C., Ponting, C.P. 2004. Comparative evolutionary genomics of androgen-binding protein genes. Genome Res. 14: 1516-1529.

Feldstein, J.B., Silverman, D.N. 1984. Purification and characterization of carbonic anhydrase from the saliva of the rat. J. Biol. Chem. 259: 5447-5453.

Fuchs, D., Winkelmann, I., Johnson, I.T., Mariman, E., Wenzel, U., Daniel, H. 2005. Proteomics in nutrition research: principles, technologies and applications. Br. J. Nutr. 94: 302-314.

Garrett, J.R., Proctor, G.B., Zhang, X.S., Anderson, L.C., Shori, D.K. 1998. Constitutive secretion of kallikreins in vivo from rat submandibular glands. Eur. J. Morphol. 36 (Suppl): 86-91.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A. 2005. Protein Identification and Analysis Tools on the ExPASy Server; (In) John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press.

Geetha, C., Venkatesh, S.G., Dunn, B.H., Gorr, S.U. 2003. Expression and anti-bacterial activity of human parotid secretory protein (PSP). Biochem. Soc. Trans. 31: 815-818.

Gianazza, E., Veber, D., Eberini, I., Beccellato, F.R., Mutti, E., Sironi, L., Scalabrino, G., Cobalamin, G., 2003. Cobalamin (vitamin B12)-deficiency-induced changes in the proteome of rat cerebrospinal fluid. Biochem. J. 374: 239-246.

Girard, L.R., Castle, A.M., Hand, A.R., Castle, J.D., Mirels, L. 1993. Characterization of common salivary protein 1, a product of rat submandibular, sublingual, and parotid glands. J. Biol. Chem. 268: 26592-26601.

Goto, M., Fujimoto, W., Nio, J., Iwanaga, T., Kawasaki, T. 2003. Immunohistochemical demonstration of acidic mammalian chitinase in the mouse salivary gland and gastric mucosa. Arch. Oral Biol. 48: 701-707.

Glendinning, J.I. 1992. Effect of salivary proline-rich proteins on ingestive responses to tannic acid in mice. Chem. Senses 17: 1-12.

Haendler, B., Krätzschmar, J., Theuring, F., Schleuning, W.D. 1993. Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland. Endocrinology 133: 192-198.

Hajjar, E., Perahia, D., Débat, H., Nespoulous, C., Robert, C.H. 2006. Odorant binding and conformational dynamics in the odorant-binding protein. J. Biol. Chem. 281: 29929-29937.

Hardt, M., Thomas, L.R., Dixon, S.E., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska H.E., Fisher, S.J. 2005. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry 44: 2885-2899.

Haslam, E., Lilley, T.H. 1988. Natural astringency in food stuffs – a molecular interpretation. Crit. Rev. Food Sci. Nutr. 27: 1-40.

Hirtz, C., Chevalier, F., Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., de Périère, D. 2005a. Complexity of the human whole saliva proteome. J. Physiol. Biochem. 61: 469-480.

Hirtz, C., Chevalier, F., Centeno, D., Rofidal, V., Egea, J.C., Rossignol, M., Sommerer, N., Deville de Périère, D. 2005b. MS characterization of multiple forms of alpha-amylase in human saliva. Proteomics 5: 4597-4607.

Hu, S., Xie, Y., Ramachandran, P., Loo, R.R.O., Li, Y., Loo, J.A., Wong, D.T. 2005. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics 5: 1714-1728.

Iason, G. 2005. The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. Proc. Nutr. Soc. 64: 123-131.

Jenzano, J.W., Su, H.W., Featherstone, G.L., Lundblad, R.L. 1992. Molecular diversity of tissue kallikrein in human saliva. Agents Actions Suppl. 38: 137-144.

Karn, R.C., Laukaitis, C.M. 2003. Characterization of two forms of mouse salivary androgen-binding protein (ABP): implications for evolutionary relationships and ligand-binding function. Biochemistry 42: 7162-7170.

Khovidhunkit, W., Hachem, J.P., Medzihradszky, K.F., Duchateau, P.N., Shigenaga, J.K, Moser, A.H., Movsesyan, I., Naya-Vigne, J., Kane, J.P., Feingold, K.R., Grunfeld, C. 2005. Parotid secretory protein is an HDL-associated protein with anticandidal activity. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288: R1306-R1315.

Kussmann, M., Blum, S. 2007. OMICS-derived targets for inflammatory gut disorders: opportunities for the development of nutrition related biomarkers. Endocr. Metab. Immune Disord. Drug Targets 7: 271-287.

Laukaitis, C.M., Critser, E.S., Karn, R.C. 1997. Salivary androgen-binding protein (ABP) mediates sexual isolation in *Mus musculus*. Evolution 51: 2000-2005.

Laukaitis, C.M., Dlouhy, S.R., Emes, R.D., Ponting, C.P., Karn, R.C. 2005. Diverse spatial, temporal, and sexual expression of recently duplicated androgen-binding protein genes in *Mus musculus*. BMC Evol. Biol. 5: 40-55.

Leymarie, N., Berg, E.A., McComb, M.E., O'Connor, P.B., Grogan, J., Oppenheim, F.G., Costello, C.E. 2002. Tandem mass spectrometry for structural characterization of proline-rich proteins: application to salivary PRP-3. Anal. Chem. 74: 4124-32.

Linke, T., Ross, A.C., Harrison, E.H. 2004. Profiling of rat plasma by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, a novel tool for biomarker discovery in nutrition research. J. Chromatogr A. 1043: 65-71.

Marchese, S., Pes, D., Scaloni, A., Carbone, V., Pelosi, P. 1998. Lipocalins of boar salivary glands binding odours and pheromones. Eur. J. Biochem. 252: 563-568.

Mazengo, M.C., Söderling, E., Alakuijala, P., Tiekso, J., Tenovuo, J., Simell, O., Hausen, H. 1994. Flow rate and composition of whole saliva in rural and urban Tanzania with special reference to diet, age, and gender. Caries Res. 28: 468-476.

McArthur, C., Hagerman, A.E., Robbins, C.T. 1991. Physiological strategies of mammalian herbivores against plant defenses. In: R.T. Palo and C.T. Robbins (eds.) Plant defenses against mammalian herbivory. Pp 103-114. CRC Press, Boca Raton, FL.

Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc Natl Acad Sci U S A 80: 3948-3952.

Mehansho, H., Clements, S., Sheares, B.T., Smith, S., Carlson, D.M. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. J Biol Chem. 260: 4418-4423.

Messana, I., Inzitari, R., Fanali, C., Cabras, T., Castagnola, M. 2008. Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us? J. Sep. Sci. 31: 1948-1963.

Murakami, H., Sly, W.S. 1987. Purification and characterization of human salivary carbonic anhydrase. J. Biol. Chem. 262: 1382-1388.

Mirels, L., Hand, A.R., Branin, H.J. 1998. Expression of gross cystic disease fluid protein-15/Prolactin inducible protein in rat salivary glands. J. Histochem. Cytochem. 46: 1061-1071.

Mullins, J.J., Mullins, L.J., Dunbar, D.R., Brammar, W.J., Gross, K.W., Morley, S.D. 2006. Identification of a human ortholog of the mouse Dcpp gene locus, encoding a novel member of the CSP-1/Dcpp salivary protein family. Physiol. Genomics 28: 129-140.

Neyraud, E., Sayd, T., Morzel, M., Dransfield, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. J. Proteome Res. 5: 2474-2480.

Nishita, T., Tanaka, Y., Wada, Y., Murakami, M., Kasuya, T., Ichihara, N., Matsui, K., Asari, M. 2007. Measurement of carbonic anhydrase isozyme VI (CA-VI) in bovine sera, saliva, milk and tissues. Vet. Res. Commun. 31: 83-92.

Ogawa, Y., Matsumoto, K., Maeda, T., Tamai, R., Suzuki, T., Sasano, H., Fernley, R.T. 2002. Characterization of lacrimal gland carbonic anhydrase VI. J. Histochem. Cytochem. 50: 821-827.

Owerbach, D., Hjorth, J.P. 1980. Inheritance of a parotid secretory protein in mice and its use in determining salivary amylase quantitative variants. Genetics 95: 129-141.

Perez-Vilar, J., Hill, R.L. 1999. The Structure and Assembly of Secreted Mucins, J. Biol. Chem. 274: 31751-31754.

Poulsen, K., Jakobsen, B.K., Mikkelsen, B.M., Harmark, K., Nielsen, J.T., Hjorth, J.P. 1986. Coordination of murine parotid secretory protein and salivary amylase expression. EMBO J. 5: 1891-1896.

Rajan, G.H., Morris, C.A., Carruthers, V.R., Wilkins, R.J., Wheeler, T.T., 1996. The relative abundance of a salivary protein, BSP30, is correlated with susceptibility to bloat in cattle herds selected for high or lowbloat susceptibility. Anim. Genet. 27: 407–414.

Ramachandran, P., Boontheung, P., Xie, Y., Sondej, M., Wong, D.T., Loo, J.A. 2006. Identification of N-linked glycoproteins in human saliva by glycoprotein capture and mass spectrometry. J Proteome Res. 5: 1493-1503.

Rayment, S.A., Liu, B., Offner, G.D., Oppenheim, F.G., Troxler, R.F. 2000. Immunoquantification of human salivary mucins MG1 and MG2 in stimulated whole saliva: factors influencing mucin levels. J. Dent. Res. 79: 1765-1772.

Richard, T., Lefeuvre, D., Descendit, A., Quideau, S., Monti, J.P. 2006. Recognition characters in peptide-polyphenol complex formation. Biochim. Biophys. Acta. 1760: 951-958.

Robinson, C.P., Bounous, D.I., Alford, C.E., Nguyen, K.H., Nanni, J.M., Peck, A.B., Humphreys-Beher M.G. 1997. PSP expression in murine lacrimal glands and function as a bacteria binding protein in exocrine secretions. Am J Physiol. 272: G863-G871.

Sandholm, L. 1986. Proteases and their inhibitors in chronic inflammatory periodontal disease. J. Clin. Periodontol. 13: 19-26.

Segal, A., Wong, D.T. Salivary diagnostics: enhancing disease detection and making medicine better. Eur. J. Dent. Educ. 12 Suppl 1: 22-29.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32:1149-1163.

Siqueira, W.L., Salih, E., Wan, D.L., Helmerhost, E.J., Oppeneim, F.G. 2008. Proteome of human minor salivary gland secretion. J. Dent. Res. 87: 445-450.

Smith, G.P. 2000. The controls of eating: a shift from nutritional homeostasis to behavioral neuroscience. 16: 814-820.

Thatcher, B.J., Doherty, A.E., Orvisky, E., Martin, B.M., Henkin, R.I. 1998. Gustin from human parotid saliva is carbonic anhydrase VI. Biochem Biophys. Res. Commun. 250: 635-641.

Thongboonkerd, V. 2007. Proteomics. Forum Nutr. 60: 80-90.

Timm, D.E. 1997. The crystal structure of the mouse glandular kallikrein-13 (prorenin converting enzyme). Protein Sci. 6: 1418-1425.

Van Steijn, G.J., Amerongen, A.V., Veerman, E.C., Kasanmoentalib, S., Overdijk, B. 1999. Chitinase in whole and glandular human salivas and in whole saliva of patients with periodontal inflammation. Eur. J. Oral Sci. 107: 328-337.

Van Steijn, G.J., Amerongen, A.V., Veerman, E.C., Kasanmoentalib, S., Overdijk, B. 2002. Effect of periodontal treatment on the activity of chitinase in whole saliva of periodontitis patients. J. Periodontal Res. 37: 245-249.

van't Hof, W., Blankenvoorde, M.F., Veerman, E.C., Amerongen, A.V. 1997. The salivary lipocalin von Ebner's gland protein is a cysteine proteinase inhibitor. J. Biol. Chem. 272: 1837-1841.

Veerman, E.C., van den Keijbus, P.A., Nazmi, K., Vos, W., van der Wal, J.E., Bloemena, E., Bolscher, J.G., Amerongen, A.V. 2003. Distinct localization of MUC5B glycoforms in the human salivary glands. Glycobiology 13: 363-366.

Vitorino, R., Lobo, M.J.C., Ferrer-Correia, A.J., Dubin, J.R., Tomer, K.B., Domingues, P.M. Amado, F.M.L. 2004. Identification of human whole saliva protein components using proteomics. Proteomics 4: 1109-1115.

Walz, A., Sühler, K., Watteberg, A., Haranke, E., Meyer, H.E., Schmalz, G., Blüggel, M., Ruhl, S. 2006. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to human whole saliva by two-dimensional gel electrophoresis. Proteomics 6: 1631-1639.

Wang, J., Li, D., Dangott, L.J., Wu, G., 2006. Proteomics and its role in nutrition research. J. Nutr. 136: 1759-1762.

Wei, G.X., Campagna, A.N., Bobek, L.A. 2007. Factors affecting antimicrobial activity of MUC7 12-mer, a human salivary mucin-derived peptide. Ann. Clin. Microbiol. Antimicrob. 6:14-23.

Williams, K.M., Ekström, J., Marshall, T., 1999. High-resolution electrophoretic analysis of rat parotid salivary proteins. Electrophoresis 20: 1373-1381.

Wilmarth, P.A., Riviere, M.A., Rustvold, D.L., Lauten, J.D., Madden, T.E., David, L.L. 2004. Two-dimensional liquid chromatography study o the human whole saliva proteome. J. Proteome Res. 3: 1017-1023.

Wu, A.M., Csako, G., Herp, A. 1994. Structure, biosynthesis, and function of salivary mucins. Mol. Cell Biochem. 137: 39-55.

Xie, H., Rhodus, N.L., Griffin, R.J., Carlis, J.V., Griffin, T.J. 2005. A catalogue of human saliva proteins identified by free flow electrophoresis-based petide separation and tandem mass spectrometry. Mol. Cell Proteomics. 4: 1826-1830.

Zalewska, A., Zwierz, K., Zólkowski, K., Gindzienski, A. 2000. Structure and biosynthesis of human salivary mucins. 47: 1067-1079.

Chapter 5

Comparison of Electrophoretic Protein Profiles from Sheep and Goat Parotid Saliva

E. Lamy, G. da Costa, F. Capela e Silva, J. Potes, A.V. Coelho, E.S. Baptista

The work presented here has been published in Journal of Chemical Ecology (2008) 34: 388-397.

Abstract

Saliva provides a medium for short-term adaptation to changes in diet composition, namely the presence of plant secondary metabolites. Salivary proteins have biological functions that have particular influence on oral homeostasis, taste, and digestive function. Some salivary proteins, such as proline-rich proteins, are present in browsers, but absent in grazers. In spite of the significance of salivary proteins, the expression patterns of these proteins in many herbivores are unknown. We investigated the SDS-polyacrylamide gel electrophoresis profile of parotid salivary proteins from two domesticated species, one a grazer, the sheep, *Ovis aries*, and the other a mixed feeder, the goat, *Capra hircus*, both fed on the same conventional diet. With 12.5% polyacrylamide linear gels, we observed quite uniform patterns of salivary proteins within the two species. Twenty-one major bands were observed in the goat profile, and 19 were observed in the sheep profile. Each band was subjected to peptide mass fingerprinting (PMF) for purposes of identification, allowing for 16 successful protein identifications. Marked differences were observed between the species in the region of 25-35 kDa molecular weights: one band was present in significantly different intensity; three bands were present only in goats; and one band was present only in sheep. This is the first report of a comparison of the protein salivary composition of sheep and goats, and suggests that future research should be conducted to reveal a physiological function for salivary proteins related to the differences in feeding behavior of these species.

Keywords: *Capra hircus*, Feeding behaviour, MALDI-TOF MS, *Ovis aries,* Parotid saliva, Protein identification, Salivary proteins, SDS-PAGE

---

1. Introduction

Salivary function is closely related to oral health and digestion. Humphrey and Williamson (2001) organized the functions of saliva into five major categories: (1) lubrication and protection; (2) buffering action and clearance; (3) maintenance of tooth integrity; (4) antibacterial activity; and (5) taste and digestion. Saliva modulates taste perception through the transportation of taste substances and the protection of taste receptors, as well as through the chemical interaction of salivary constituents with taste substances. Salivary flow rate and composition are influenced by the quality of taste stimuli (Spielman, 1990), drugs and physiological factors (Aps and Martens, 2005), and, at the same time, salivary flow rate and composition affect taste perception (Matsuo, 2000). Some salivary proteins have been reported to be involved in feeding behavior, namely von Ebner's gland salivary protein (VEGP) (Kock et al., 1994), salivary cystatins (Katsukawa et al., 2002), and salivary kallikreins (Yamada et al., 2006). Levels of tannin-binding salivary proteins (TBSPs) in animal saliva are associated with tannin levels in the diet (Mehansho et al., 1983, 1987, 1992; Austin et al., 1989; Hagerman and Robbins, 1993; Fickel et al., 1998; Makkar and Becker, 1998; Clauss et al., 2003a,b).

A recent review by Shimada (2006) stresses the importance of gathering basic information on salivary proteins as a way of understanding the relationships between feeding niches and saliva composition.

Sheep and goats are both generalist herbivores. They have similar body sizes and frequently graze together in major farming systems (Bartolome et al., 1998; El Aich and Waterhouse, 1999). Although they are competing species that co-exist in the same niche and have access to the same forage items, they often show different feeding behavior, selecting and ingesting diets that overlap to variable degrees (Ngwa et al., 2000; Pande et al. 2002). In the context of the three feeding types proposed by Hofmann (1989), sheep are considered grazers, whereas goats are viewed as intermediate feeders, capable of dealing with large amounts of browse in their diets. There are several possible explanations for these differences in feeding behavior between sheep and goats. According to the detoxification limitation hypothesis (Freeland and Janzen, 1974, recently reviewed by Marsh et al., 2006), goats could have a greater ability to eliminate plant secondary metabolites (PSMs), when compared to sheep. A second explanation is based on one of the deductive generalizations of Hofmann's morphophysiological hypothesis, which suggests that goats may have large salivary glands producing large amounts of fluid, helping to digest browse and providing a medium of defense against PSMs.

To our knowledge, salivary protein expression patterns have not been reported from sheep and goats, and only a limited number of salivary proteins have been identified for these two species. Austin et al. (1989) used electrophoretic approaches to search for TBSPs in the whole saliva of sheep, cattle, and deer, but did not characterize the entire protein profile in the saliva. The aim of the present study is to gain a better understanding of the parotid saliva protein composition of the domestic sheep, *Ovis aries*, and of the goat, *Capra hircus*. To this end, we used one-dimensional SDS-PAGE for protein separation and MALDI-TOF MS for protein identification by Peptide Mass Fingerprinting (PMF).

2. Methods and Materials

2.1 Animals

To obtain saliva samples, we used adult females that had been reared in separate sheep and goat flocks and had grazed on Mediterranean rangeland. The collections were made during six different periods, over the course of one year. In each period, five Merino sheep, *Ovis aries*, and five Serpentina goats, *Capra hircus*, were used and kept in the same location in separated crates for 15 d preceding the saliva collection. During this period, all animals were fed with vetch-oat hay, *Vicia sativa* x *Avena sativa*, and had water and food available *ad libitum*. The objective of the pre-trial period was to keep sheep and goats in similar conditions so as to minimize diet effects when comparing the two species. Before each saliva collection period, polyethylene urinary cat stylet

catheters (1.0 or 1.3 x 130 mm) were introduced into one of the parotid ducts of each animal, which had previously been anaesthetized intravenously with Xylazine/Ketamine (0.1/5.0 mg/kg). To facilitate the positioning of the catheter, an intravenous 14G cannula was previously inserted into the masseter muscle from the inside to the outside. The catheters were then introduced into the parotid papilla, from the exterior to the interior of the mouth (Fickel et al., 1998), by using guide wires. The free end of the catheter, which protruded 1 cm, was fixed to the cheek skin by transfixation knots. To avoid any possible effect of the anaesthetics on the saliva composition (Edwards and Titchen, 2003; Edwards et al., 2003), sample collection was initiated one day after surgery. In the morning and before food distribution, samples were collected during each of the following three days. At least 2 ml of parotid saliva were collected from each animal by aspiration with a syringe.

2.2 Saliva Collection and Preparation for SDS-polyacrylamide Gel Electrophoresis

Each saliva sample was collected into capped polypropylene sample tubes. All saliva samples were immediately frozen in liquid nitrogen and stored at –70°C. Prior to protein quantification, samples were centrifuged at 16,000 × *g* for 5 min at 4°C to remove particulate matter. Protein concentration of the parotid saliva was determined by the BCA-method (Pierce, Rockford, IL, USA), in which bovine serum albumin (BSA) is used as the standard. For the analysis, 10 µl of either BSA (0-2.0 mg/ml) or saliva was mixed with 200 µl of the BCA reagent and incubated for 30 min at 37°C. Absorbance was measured at 565 nm by using a microtiter plate reader (SpectroMAX 340, Molecular Devices, Union City, CA, USA). Before SDS-PAGE separation, the salivary proteins were concentrated with a 5 kDa cut-off ultra-filtration microfuge tube (Millipore, Eschborn, Germany; Ref: UFV5BCC00).

2.3 SDS-Polyacrylamide Gel Electrophoresis

Individual samples of parotid saliva from sheep and goats were run simultaneously in each gel for comparison. Proteins were separated with 12.5% SDS-polyacrylamide gels (200x200x1 mm) in a Protean II xi slab gel apparatus (BioRad, California, United States). Saliva samples with 70 µg of protein were mixed with 4X concentrated SDS sample buffer (0.125 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 20% glycerol with traces of bromophenol blue). The mixture was heated at 90°C for 5 min and immediately cooled on ice until gel application. Electrophoresis was carried out by using a running buffer [0.025 M Tris, 0.192 M glycine, 1% (w/v) SDS] at pH 8.3, with 100 V constant current. After the sample entered the separation gel, the voltage was changed to 250 V. Molecular mass protein standards (from 15 to 200 kDa) (PageRuler Protein Ladder, SM0661, Fermentas, Ontario, Canada) were also included in each gel for reference.

2.4 Gel Staining and Densitometry

Gels were fixed and stained overnight in a solution of 0.1% Coomassie brilliant blue R-250 in 50% v/v methanol and 10% v/v acetic acid and destained with several changes of 10% v/v acetic acid, following the protocol of Beeley et al. (1991) for proline-rich proteins. Digital images of the gels were obtained by using a densitometer (Molecular Dynamics, Amersham Biosciences Europe GmbH, Freiburg, Germany), and the gels were subjected to linescan analysis by using IMAGEQUANT 5.0 software with parameter sensitivity 9.0 and kernel 4.0 (Amersham Biosciences Europe GmbH, Freiburg, Germany) in order to assign the significant bands in the protein profiles.

2.5 Protein Identification

For protein identification, the peptide mass fingerprinting (PMF) approach was used. Stained bands were excised, washed, reduced with dithiothreitol, alkylated with iodoacetamide, and dried in a speedvac. Gel pieces were re-hydrated with digestion buffer (50 mM NH_4HCO_3) containing trypsin (Promega, Madison, WI, USA) and incubated overnight at 37ºC. The buffered peptides were acidified with formic acid, desalted, and concentrated with C8 microcolumns (POROS R2, Applied Biosystems, Foster City, CA, USA). The peptides were eluted with matrix solution containing 10 mg/m[illegible] -cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid.. The mixture was allowed to air-dry (dried droplet method). Mass spectra were obtained with a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer in the positive ion reflectron mode. External calibration was made by using a mixture of standard peptides (Pepmix 1, LaserBiolabs, Sophia-Antipolis, France). Spectra were processed and analyzed with MoverZ software (Genomic Solutions Bioinformatics, Ann Arbor, MI, USA). Peakerazor software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant *m/z* peaks and for internal calibration. Monoisotopic peptide masses were used to search for protein identification by using Mascot software (Matrix Science, London, UK; http://www.matrixscience.com). Database searches were performed against MSDB (a non-identical protein sequence database maintained by the Proteomics Department at the Hammersmith Campus of Imperial College, London; http://csc-fserve.hh.med.ic.ac.uk/msdb.html) and SwissProt. The following criteria were used to perform the search: i) mass accuracy of 50-100 ppm; ii) one missed cleavage in peptide masses; and iii) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. Criteria used for protein identification in the Mascot software were: i) significant homology scores achieved in Mascot; ii) significant sequence coverage values; and iii) similarity between the protein molecular mass calculated from the gel and for the identified protein.

3. Results

3.1 Salivary Protein Profile

Salivary protein concentrations in both animals ranged from 30 to 2,000 µg/ml. Twenty-one and 19 protein bands (Fig. 1) were reproducibly displayed in goat and sheep parotid saliva protein profiles, respectively. There was a similar pattern to the parotid saliva profiles for the two animal species, except for the 25 to 35 kDa molecular mass range. In this range, the parotid saliva electrophoretic profile from sheep had two visible bands (**o** and **s**, corresponding to approximately 32 and 26 kDa, respectively), whereas the profile from goat had four bands (**p**, **q**, **r**, and **s**, corresponding to approximately 30, 28, 27, and 26 kDa, respectively). Band **o**, which was a very intense band in the sheep profile, was not present in goat saliva. Bands **p, q** and **r**, observed in goats, were absent from sheep. Moreover, the protein band **s**, common to both species, was much more intense in sheep. Fig.2 emphasizes the differences between goats and sheep parotid saliva protein profile, which are common to the different individuals from the same specie.

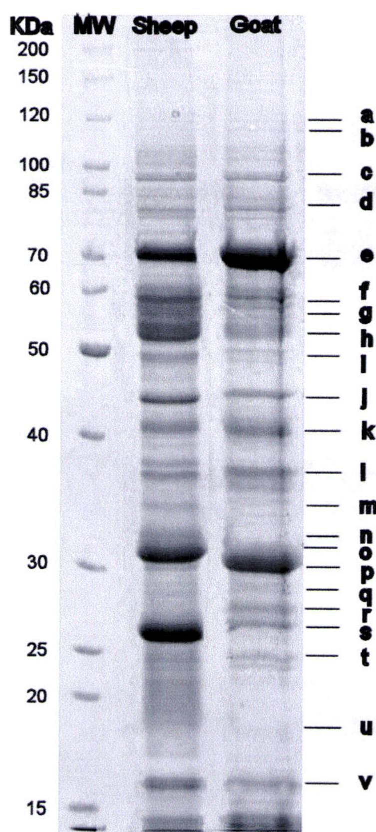


Fig. 1 Typical profiles of sheep and goat parotid salivary proteins in a 12.5% linear gel. Each lane represents the profile from an individual saliva sample. Molecular markers (MW) are represented on the left side of the figure. The protein bands are identified by letters **a** to **v**.

3.2 Salivary Protein Identification

Mass spectra from a total of 21 and 19 peptide digests of well-resolved bands from goats and sheep, respectively, were analyzed. The MSDB database was searched by using a taxonomic restriction for "other mammals," and 16 different proteins were identified (Table 1). In most instances, the same proteins were identified in the two species. However, the identification of the bands **h, n, o, q, r, u** and **v** were not possible, due perhaps to a low amount of protein in some bands or to the existence of several different proteins in the same band, or even to a lack of homologous proteins in the searched protein sequence databases. Peptide map comparison for goat and sheep band **f** shows the presence of some peaks corresponding to the catalase predicted tryptic peptide masses, however more peaks with relevant intensities were observed. This suggests that catalase is also present in goat **f** band, probably mixed with other unidentified proteins. Similar results were obtained when peptide maps for band **s** were compared between goat and sheep. Apolipoprotein A-I was likely present in sheep band **s**, but the band may also contain other unidentified proteins. This interpretation was supported both by the higher intensity of this band in sheep when compared with the corresponding one from goat and by the presence of mass peaks not observed in goat band **s** peptide map.

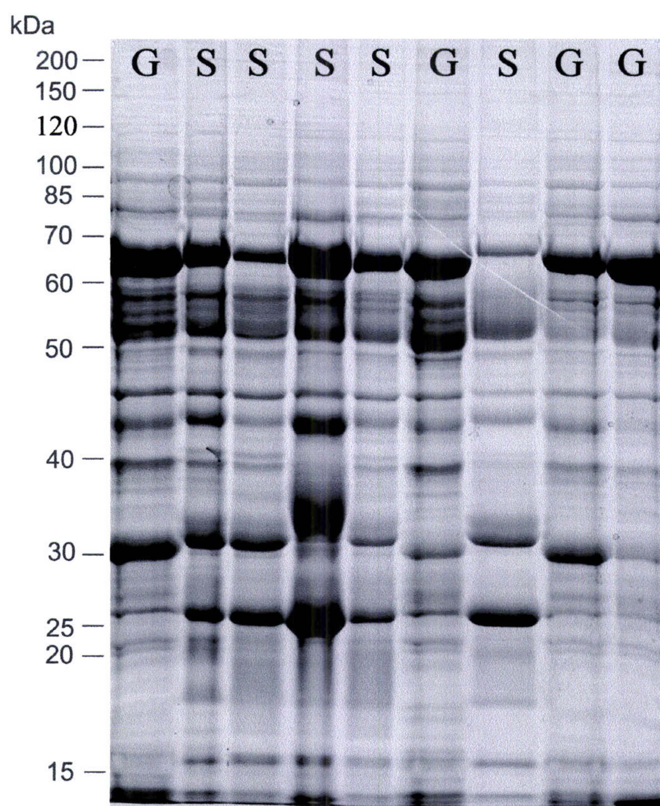


Fig.2 SDS-PAGE of goat and sheep parotid saliva. The samples were obtained from four different goat (G) and five different sheep (S) in the same dietary conditions. The similarities between the individuals from the same specie and the differences among species are evident.

4. Discussion

Electrophoretic profiles of salivary proteins have been reported for several species, such as rats (Ekström et al., 1996; Williams et al., 1999a), ferrets (Williams et al., 1999b), and cats (Marshall et al., 1993), but the bulk of the studies on salivary profiles have been performed on humans (Ghafouri et al., 2003; Vitorino et al., 2004; Wilmarth et al., 2004; Hardt et al., 2005; Hirtz et al., 2005; Hu et al., 2005; Guo et al., 2006; Walz et al., 2006). According to our knowledge, this is the first study in which the SDS-PAGE electrophoretic profiles of parotid salivary proteins from sheep and goats have been characterized, with MALDI-TOF MS used to identify the more representative proteins.

The proteins identified in the present study (Table 1) can be sorted into three main functional categories. The largest group includes salivary proteins exhibiting immune response or oral protection functions: Complement C3 precursor, gelsolin precursor, serotransferrin precursor, catalase, immunoglobulin, annexin A1, cathepsin H precursor, and glutathione S-transferase P. Among these, catalase and glutathione S-transferase P have a more specific role in detoxification. They are associated with feeding behaviour because their presence has been associated with plant consumption (Felton and Duffey, 1991; Rodman and Miller, 1992; Sreerama et al., 1995; Lampe et al., 2000). Annexin A1 has also been related to taste perception (Neyraud et al., 2006).

A second functional category includes proteins involved in protein biosynthesis: Elongation factor 2, heat shock protein HSP 90-beta, and protein disulfide-isomerase A3 precursor. The third functional category includes typical serum proteins that, among other functions, are concerned with transport: Serotransferrin precursor, serum albumin precursor, and apolipoprotein A-I precursor. The functions of actin and deoxyribonuclease 1 in saliva are not well understood. Some authors have considered deoxyribonuclease 1 as a digestive enzyme (Takeshita et al., 2000), despite others having previously suggested that deoxyribonuclease 1 activity in human parotid saliva is insufficient to fulfil any digestive function (Yaegaki et al., 1982). The presence in saliva of cytoplasmatic proteins, such as actin may be a consequence of the apocrine-like type of secretion reported for ruminant parotid glands (Stolte and Ito, 1996).

Carbonic anhydrase VI is the only protein that has been previously reported from sheep parotid glands and this is the only sheep salivary protein sequence deposited in databases (Fernley et al., 1988a,b). Carbonic anhydrase VI has a role in electrolytic equilibrium and in the buffer properties of saliva (Kimoto et al., 2006). Its presence in saliva has also been associated with the development of adequate taste function (Henkin et al., 1999).

Our results suggest a strong similarity between the electrophoretic profiles of sheep and goat salivary proteins. From the 16 proteins identified, only one, band **p**, is not common to both species (Table 1).

The similarity likely reflects the phylogenetic proximity of the two species, and the consumption of an equal diet during the study. Not surprisingly, we found more pronounced differences when we compared our results with the salivary protein composition of carnivores (Marshall et al., 1993; Williams et al., 1999b) and omnivores (Beeley et al., 1991; Williams et al., 1999a; Hardt et al., 2005). In the dietary habits ranging from carnivores through omnivores to those animals that are exclusively herbivores, plant allelochemical levels progressively increase. It has been suggested (McArthur et al., 1995) that during the evolution from meat-eater to plant-eater, selective pressure encouraged salivary proteins with defense functions against anti-nutritive and/or toxic substances present in plants. Saliva is one of the behavioral and physiological mechanisms that mammals have evolved for coping with hazards related to feeding. For herbivores, this can mean having to deal with toxic and anti-nutritive substances, whereas for omnivores the major risk faced is that of foodborne illness. The trade-off between costs/nutritional benefits could be reflected in the salivary profiles of different trophic groups, with differences in the proportion of proteins. In humans and rodents the proportion of serum proteins, relative to total salivary proteins, is lower than the proportion observed in the present study for sheep and goats. Saliva with a composition similar to serum can be more useful for ruminants than for humans or rodents. The lack of digestive enzymes in ruminant saliva has been widely reported and probably reflects digestive characteristics, such as the low levels of starch in the diet and the importance of ruminal fermentation of structural carbohydrates. An adequate digestion is achieved by the rhythms of salivary secretion and by a more marked role of saliva in providing and maintaining a buffered environment for ruminal fermentation, contributing to half of the bicarbonate entering the rumen (Owens et al., 1998). The digestive differences between ruminants and omnivores such as humans and rodents referred to above, can also explain why the latter possess other salivary proteins, which we have not found in sheep and goats.

Despite the similarities, the differences found between sheep and goats parotid salivary protein profiles are also meaningful. From the bands common to both profiles, differences in intensity were only observed for band **s**, identified as apolipoprotein A-I, which was more intense in the sheep profile. The large number of peaks in the peptide map for band **s** suggests additional unidentified proteins of similar mass in the same gel band. A more pronounced difference was observed in the region of 25-35 kDa. Band **p**, which was only observed in the goat profile, contained cathepsin H. This protein is involved in the degradation of proteins in lysosomes and no role in digestion has been attributed to it. As previously discussed, the presence of this cytoplasmic protein in saliva may result from the apocrine-like type of secretion characteristic of ruminants (Stolte and Ito, 1996). In additional to the cathepsin peaks, a large number of other peaks were present in the peptide map, suggesting the presence of unidentified proteins in this intense gel band as well. It is possibile that cathepsin is also present in sheep parotid saliva in low concentrations, which were insufficient to allow a band observation in Coomassie stained gels. For band **o**, which was only observed in the sheep profile and bands **q** and **r**, only observed in the goat profile, we were unable to obtain identification.

Some authors (Austin et al., 1989; Fickel et al., 1998) refer to the presence of salivary PRPs in browser ruminant species and to their absence in sheep saliva. Since sheep are grazers and goats are intermediate feeders, one possibility is that goats could have salivary PRPs. Human basic PRPs, which are the group of PRPs with a higher affinity for tannins, have molecular masses between 14 and 45 KDa (Bedi and Bedi, 1995), which correspond to mass values of the unidentified bands. We tested the presence of PRPs by staining the gels with Coomassie Brilliant Blue R-250, following the protocol of Beeley et al. (1991), but were unable to observe the characteristic pink bands. The absence of salivary PRPs in sheep and goat parotid saliva may reflect the low tannin diet consumed by the two species during the experiment. Further studies with the incorporation of high levels of tannins into the diet may be useful in assessing the induction of this particular group of salivary proteins.

This study provides a first step to the full characterization of the goat and sheep parotid saliva protein profile, and it provides useful preliminary information that can be further used to study the immediate oral adaptation to the diet. Based on the differences between the species, even when fed under a similar feeding situation, we believe that salivary protein composition can play an important role in feeding choices. The complexity of parotid saliva is evident from the great number of protein bands, the lack of identification of some of them, and the large number of tryptic peptides obtained for each one. This highlights the importance of the use of more powerful separation techniques. Moreover, we think that more dynamic information can be obtained by studying these two species subjected to different diets. We intend to use two-dimensional electrophoresis (2-DE) coupled to mass spectrometry (MS) and MS/MS to study potential changes in the parotid saliva proteome caused by the consumption of tannin-enriched diets.

Table 1 Proteins identified from SDS-PAGE analysis of parotid saliva of goat, *Capra hircus*, and sheep, *Ovis aries*

Band ID	Animal species	Protein name	Score[a]	Coverage (%)[b]	Pep[c]	MSDB Accession number	MW a kDa	MW t kDa	Ref.[d]
a	goat sheep	Complement C3 precursor (fragment) Complement C3 precursor (fragment)	68 128	22 33	9/38 12/28	O46544_SHEEP	121	40	(1)
b	goat sheep	Elongation factor 2 Elongation factor 2	85 86	21 24	13/35 16/53	EF2_BOVIN	117	96	(2)
c	goat sheep	1) Gelsolin precursor + 2) Heat shock protein HSP 90-beta 1) Gelsolin precursor + 2) Heat shock protein HSP 90-beta	1) 149 2) 60 1) 102 2) 109	1) 32 2) 19 1) 27 2) 25	1) 20/53 2) 12/53 1) 16/52 2) 18/52	1)Q3SX14_BOVIN 2)HS90B_BOVIN	100	1) 81 2) 84	(2)
d	goat sheep	Serotransferrin precursor Serotransferrin precursor	62 78	16 17	10/36 12/37	AAA96735	90	80	(2-4)
e	goat sheep	Serum albumin precursor Serum albumin precursor	75 81	16 17	7/14 8/13	ABSHS	77	71	(2-8)
f	goat sheep	Unidentified Catalase	128	32	16/50	CATA_BOVIN	70	60	(2-3)
g	goat sheep	Protein disulfide-isomerase A3 precursor Protein disulfide-isomerase A3 precursor	94 73	23 19	13/36 9/32	JC2385	67	55	(2)
h	goat sheep	Unidentified Unidentified							
i	goat sheep	Ig heavy chain C region Ig heavy chain C region	88 60	39 31	8/30 7/45	C30554	58	52	(2-4)
j	goat sheep	Actin cytoplasmic 1 (Beta-actin) Actin cytoplasmic 1 (Beta-actin)	90 98	33 42	10/30 14/47	ATBOB	51	42	(8) (13)
k	goat sheep	Carbonic anhydrase VI Carbonic anhydrase VI	63 133	38 58	9/41 12/32	CAH6_SHEEP	45	36	(2) (4-9)
l	goat sheep	Annexin A1 Annexin A1	76 109	36 41	9/38 11/36	S28228	40	40	(2) (4) (8) (10)
m	goat sheep	Deoxyribonuclease-1 Deoxyribonuclease-1	62 60	30 38	5/17 6/34	B26325	37	29	(11, 14, 15)
n	goat sheep	Unidentified Unidentified							
o	goat sheep	Not present Unidentified							
p	goat sheep	Cathepsin H precursor Not present	55[e],**	23	7/41	Q3T0I2[e],*	30	38	(12)
q	goat sheep	Unidentified Not present							
r	goat sheep	Unidentified Not present							
s	goat sheep	Apolipoprotein A-I precursor Unidentified	174	44	17/51	AAI02942	25	30	(2-4,6)
t	goat sheep	Glutathione S-transferase Pi Unidentified	96 100	64 64	10/47 10/43	AF186248	22	24	(2) (4) (6-8)
u	goat sheep	Unidentified Unidentified							
v	goat sheep	Unidentified Unidentified							

[a]The minimum Mascot score for a probability less than 5% for the match to be a random event is 59.
[b]Percentage of identified protein sequence covered by matched peptides.
[c]Number of peptides from experimental Peptide Mass Fingerprint whose masses match those from a theoretical PMF determined from a known sequence / Number of peptides from experimental Peptide Mass Fingerprint submitted for Mascot search.
[d]Articles reporting the presence of the identified protein in saliva: (1) Andoh et al., 1997; (2) Xie et al., 2005; (3) Huang, 2004; (4) Wilmarth et al., 2004; (5) Hardt et al., 2005; (6) Ghafouri et al., 2003; (7) Vitorino et al., 2004; (8) Hu et al., 2005; (9) Fernley et al., 1988a; (10) Neyraud et al., 2006; (11) Tenjo et al., 1993; (12) Saliva Proteome Project – http://fields.scripps.edu/public/project/saliva/; (13) Walz et al. , 2006 ; (14) Nadano et al., 1993; (15) Williams et al., 1999a.
[e*]Swiss-Prot Accession number; ** For Swiss-Prot database searches the minimum score for a probability less than 5% for the match to be a random event is 53.

5. References

Andoh, A., Fujiyama, Y., Kimura, T., Uchihara, H., Sakumoto, H., Okabe, H., Bamba, T. 1997. Molecular characterization of complement components (C3, C4 and factor B) in human saliva. J. Clin. Immunol. 17: 404–407.

Aps, J.K., Martens, L.C. 2005. The physiology of saliva and transfer of drugs into saliva. Forensic Sci. Int. 150: 119–131.

Austin, P.J., Suchar, L.A., Robbins, C.T., Hagerman, A.E. 1989. Tannin binding proteins in the saliva of deer and their absence in the saliva of sheep and cattle. J. Chem. Ecol. 15: 1335–1347.

Bedi, G.S., Bedi, S.K. 1995. Purification and characterization of rat parotid glycosylated basic and acidic proline rich proteins. Prep. Biochem. 25: 119–132.

Beeley, J.A., Sweeney, D., Lindsay, J.C., Buchanan, M.L., Sarna, L., Khoo, K.S. 1991. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of human parotid salivary proteins. Electrophoresis 12: 1032–1041.

Bartolome, J., Franch, J., Plaixats, J., Seligman, N.G. 1998. Diet selection by sheep and goats on Mediterranean heath-woodland range. J. Range Manage. 51: 383–391.

Clauss, M., Lason, K., Gehrke, J., Lechner-Doll, M., Fickel, J., Grüne, T., Jürgen Streich, W. 2003a. Captive roe deer (*Capreolus capreolus*) select for low amounts of tannic acid but not quebracho: fluctuation of preferences and potential benefits. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136: 369-382.

Clauss, M., Lechner-Doll, M., Streich W.J. 2003b. Ruminant diversification as an adaptation to the physicomechanical characteristics of forage. A reevaluation of an old debate and a new hypothesis. Oikos 102: 253–262.

Edwards, A.V. Titchen, D.A. 2003. Autonomic control of protein production by the parotid gland of sheep. Auton. Neurosci. 103: 38–39.

Edwards, C.M., Corkery, P.P., Edwards, A.V. 2003. Submandibular responses to stimulation of the parasympathetic innervation in anesthetized sheep. J. Appl. Physiol. 95: 1598–1605.

Ekström, J., Marshall T., Tobin, G., Williams K.M. 1996. Electrophoretic analysis of rat parotid salivary protein composition: investigation of the parasympathetic atropine resistant secretion. Acta Physiol. Scand. 156: 75-79.

El Aich, A., Waterhouse, A. 1999. Small ruminants in environmental conservation. Small Ruminant Res. 34: 271–287.

Felton, G.W., Duffey, S.S. 1991. Protective action of midgut catalase in lepidopteran larvae against oxidative plant defenses. J. Chem. Ecol. 17: 1715–1732.

Fernley, R.T., Wright, R.D., Coghlan, J.P. 1988a. Complete amino acid sequence of ovine salivary carbonic anhydrase. Biochemistry 27: 2815–2820.

Fernley, R.T., Coghlan, J.P., Wright, R.D. 1988b. Purification and characterization of a high-Mr carbonic anhydrase from sheep parotid gland. Biochem. J. 249: 201–207.

Fickel, J., Goritz, F., Joest, B. A., Hildebrandt, T., Hofmann, R. R., Breves, G. 1998. Analysis of parotid and mixed saliva in Roe deer (*Capreolus capreolus* L.). J. Comp. Physiol. [B] 168: 257–264.

Freeland, W.J., Janzen, D.H. 1974. Strategies in herbivory by mammals: the role of plant secondary compounds. Am. Nat. 108: 269–289.

Ghafouri, B., Tagesson, C., Lindhal, M. 2003. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. Proteomics 3: 1003-1015.

Guo, T., Rudnick, P.A., Wang, W., Lee, C.S., Devoe, D.L., Balgley, B.M. 2006. Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. J. Proteome Res. 5: 1469–1478.

Hagerman, A.E., Robbins, C.T. 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. Can. J. Zool. 71: 628–633.

Hardt, M., Thomas, L.R., Dixon, S.E., Newport G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska, H.E., Fisher, S.J. 2005. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry 44: 2885–2899.

Henkin, R.I., Martin, B.M., Agarwal, R.P. 1999. Decreased parotid saliva gustin/carbonic anhydrase VI secretion: an enzyme disorder manifested by gustatory and olfactory dysfunction. Am. J. Med. Sci. 318: 380–391.

Hirtz, C., Chevalier, F., Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., De Periere, D. 2005. Complexity of the human whole saliva proteome. J. Physiol. Biochem. 61: 469–480.

Hofmann, R.R. 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. Oecologia 78: 443–457.

Hu, S., Xie, Y.M., Ramachandran, P., Loo, R.R.O., Li, Y., Loo, J.A., Wong, D.T. 2005. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics 5: 1714–1728.

Huang, C.M. 2004. Comparative proteomic analysis of human whole saliva. Arch. Oral Biol. 49: 951–962.

Humphrey, S. P. Williamson, R. T. 2001. A review of saliva normal composition, flow and function. J. Prosthet. Dent. 85: 162–169.

Katsukawa, H., Shang, Y., Nakashima, K., Yang, K.H., Ohashi, R., Sugita, D., Mishima, K., Nakata, M., Ninomiya, Y., Sugimura, T. 2002. Salivary cystatins influence ingestion of capsaicin-containing diets in the rat. Life Sci. 71: 457–467.

Kimoto M., Kishino M., Yura, Y., Ogawa, Y. 2006. A role of salivary carbonic anhydrase VI in dental plaque. Arch. Oral Biol. 51: 117–122.

Kock, K., Morley, S.D., Mullins, J.J., Schmale, H. 1994. Denatonium bitter tasting among transgenic mice expressing rat von Ebner's gland protein. Physiol. Behav. 56: 1173–1177.

Lampe, J.W., Chen, C., Li, S., Prunty, J., Grate, M.T., Meehan, D.E., Barale, K.V., Dightman, D.A., Feng, Z., Potter J.D. 2000. Modulation of human glutathione S-transferases by botanically defined vegetable diets. Cancer Epidemiol. Biomarkers Prev. 9: 787–793.

Makkar, H.P.S. Becker, K. 1998. Adaptation of cattle to tannins: role of proline-rich proteins in oak-fed cattle. Animal Sci. 67: 277–281.

Marsh, K.J., Wallis, I.R., Andrew, R.L., Foley, W.J. 2006. The detoxification limitation hypothesis: where did it come from and where is it going? J. Chem. Ecol. 32: 1274–1266.

Marshall, T., Williams, K.M., Ekström, J., Tobin, G., Bayard, C., Vesterberg, O. 1993. Electrophoretic analysis of stimulated cat parotid saliva. Electrophoresis 14: 1328–1332.

Matsuo, R. 2000. Role of saliva in the maintenance of taste sensitivity. Crit. Rev. Oral Biol. Med. 11: 216–229.

McArthur, C., Sanson, G.D., Beal, A.M. 1995. Salivary proline-rich proteins in mammals: roles in oral homeostasis and counteracting dietary tannin. J. Chem. Ecol. 21: 663–691.

Mehansho, H., Hagerman, A., Clements, S., Butler, L.G., Rogler, J.C., Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc. Natl. Acad. Sci. USA 80: 3948–3952.

Mehansho, H., Butler, L.G., Carlson, D.M. 1987. Dietary tannins and salivary proline-rich proteins: interactions, induction and defense mechanism. Ann. Rev. Nutr. 7: 423–440.

Mehansho, H., Asquith, T.N., Butler, L.G., Rogler, J.C., Carlson, D.M. 1992. Tannin-mediated induction of proline-rich protein synthesis. J. Agric. Food Chem. 40: 93–97.

Nadano, D., Yasuda, T., Kishi, K. 1993. Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. Clin. Chem. 39: 448–452.

Neyraud, E., Sayd, T., Morzel, M., Dransfield, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. J. Proteome Res. 5: 2474–2480.

Ngwa, A.T., Pne, D.K., Mafeni, J.M. 2000. Feed selection and dietary preferences of forage by small ruminants grazing natural pastures in the Sahelian zone of Carmeroon. Anim. Feed Sci. Technol. 88: 253–266.

Owens, F.N., Secrist, D.S., Hill, W.J., Gill, D.R. 1998. Acidosis in cattle: a review. J. Anim. Sci. 76: 275–286.

Pande, R.S., Kemp, P.D., Hodgson, J. 2002. Preference of goats and sheep for browse species under field conditions. New Zeal. J. Agr. Res. 45: 97–102.

Rodman, D.H., Miller, D.J. 1992. Enzyme activities associated with salivary glands of the froghopper *Eoscarta carnifex* (F.) (Homoptera, Cercopoidae): possible role of salivary catalase in phytotoxicity. Aust. J. Zool. 40: 365–370.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32: 1149–1163.

Spielman, A.I. 1990. Interaction of saliva and taste. J. Dent. Res. 69: 838–843.

Sreerama, L., Hedge, M.W., Sladek, N.E. 1995. Identification of a class 3 aldehyde dehydrogenase in human saliva and increased levels of this enzyme, glutathione S-transferases, and DT-

diaphorase in the saliva of subjects who continually ingest large quantities of coffee or broccoli. Clin. Cancer Res. 1: 1153–1163.

Stolte, M. Ito, S. 1996 A comparative ultrastructural study of the parotid gland acinar cells of nine wild ruminant species (Mammalia, Artiodactyla). Eur. J. Morphol. 34: 79-85.

Takeshita, H., Mogi, K., Yasuda, T., Nakajima, T., Nakashima, Y., Moi, S., Hoshino, T., Kishi, K. 2000. Mammalian deoxyribonucleases I are classified into three types: pancreas, parotid, and pancreas-parotid (mixed), based on differences in their tissue concentrations. Biochem. Biophys. Res. Commun. 269: 481–484.

Tenjo, E., Sawazaki, K., Yasuda, T., Nadano, D., Takeshita, H., Iida, R., Kishi, K. 1993. Salivary deoxyribonuclease I polymorphism separated by polyacrylamide gel-isoelectric focusing and detected by the dried agarose film overlay method. Electrophoresis 14: 1042-1044.

Vitorino, R., Lobo, M.J., Ferrer-Correira, A.J., Dubin, J.R., Tomer, K.B., Domingues, P.M., Amado, F.M. 2004. Identification of human whole saliva components using proteomics. Proteomics 4: 1109–1115.

Walz, A., Stuhler, K., Wattenberg, A., Hawranke, E., Meyer, H.E., Schmalz, G., Bluggel, M., Ruhl, S. 2006. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. Proteomics 6: 1631–1639.

Williams, K.M., Ekström, J., Marshall, T. 1999a. High-resolution electrophoretic analysis of rat parotid salivary proteins. Electrophoresis 20: 1373-1381.

Williams, K.M, Ekström, J., Marshall, T. 1999b. The protein composition of ferret parotid saliva as revealed by high-resolution electrophoretic methods. Electrophoresis 20: 2818–2823.

Wilmarth, P.A., Riviere, M.A., Rustvold, D.L., Lauten, J.D., Maden, T.E., David, L.L. 2004. Two-dimensional liquid chromatography study of the human whole saliva proteome. J. Proteome Res. 3: 1017–1023.

Xie, H., Rhodus, N.L., Griffin, R.J., Carlis, J.V., Griffin, T.J. 2005. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol. Cell Proteomics 4: 1826–1830.

Yaegaki, K., Sakata, T., Ogura, R., Kameyama, T., Sujaku, C. 1982. Influence of aging on DNase activity in human parotid saliva. J. Dent. Res. 61: 1222–1224

Yamada, A., Nakamura, Y., Sugita, D., Shirosaki, S., Ohkuri, T., Katsukawa, H., Nonaka, K., Imoto T., Ninomiya, Y. 2006. Induction of salivary kallikreins by the diet containing a sweet suppressive peptide, gurmarin, in the rat. Biochem. Biophys. Res. 346: 386–392.

Chapter 6

Comparative analysis of sheep and goat saliva proteome: a new tool for research on the ingestive behavior of herbivores.

E. Lamy, G. da Costa, R. Santos, F. Capela e Silva, J. Potes, A. Pereira, A.V. Coelho, E. Sales Baptista

In this chapter MS/MS results were obtained by Dr. Romana Santos.

The work presented here has been prepared as a manuscript for publication on BBA – Proteins and Proteomics

Abstract

Sheep and goats differ in diet selection, which may reflect different abilities to deal with the ingestion of plant secondary metabolites. Although saliva provides a basis for immediate oral information via sensory cues and also a mechanism for detoxification, our understanding of the role of saliva in the pre-gastric control of the intake of herbivores is rudimentary. We used two-dimensional electrophoresis gel analysis to compare the proteome of parotid saliva in sheep and goats and to access modifications after the intake of a diet containing quebracho tannin. Matrix-Assisted Lazer Desorption Ionization-Time of Flight and Liquid Chromatography tandem mass spectrometry were used to identify proteins. From a total of 260 sheep and 205 goat protein spots, 117 and 106 were identified, respectively. Salivary protein profiles presented a high proportion of serum proteins. Major differences between the two species were detected for proteins within the range of 25-35 kDa. Although no new proteins appeared, quebracho tannin consumption increased the concentration of proteins and changed the proteome of both species. Moreover, the two species presented differences in response to tannin consumption. This study presents the parotid saliva proteome of sheep and goats and highlights the potential of proteomics for investigations relating to intake behavior research.

Keywords: *Capra hircus*; *Ovis aries;* Feeding behaviour; Mass spectrometry; Parotid saliva; Tannins; Two-dimensional gel electrophoresis

1. Introduction

In mammals, the main saliva functions are to lubricate the oral cavity, assisting mastication and deglutition, to protect the oral tissues, and, in some species, to initiate enzymatic digestion. Ruminant saliva is mainly a bicarbonate-phosphate buffer secreted at a mean pH of 8.1 (McDougall, 1948), which aids in buffering the volatile fatty acid produced during the ruminal digestive processes, and plays an important role in electrolyte and water homeostasis. It provides nutrients for microflora (e.g. urea as N source), and a fluid environment for ruminal fermentation and for the transport of ingesta both back to the mouth for remastication and onwards through the stomach to the small intestine. (Carter and Grovum, 1990; Van Soest, 1994).

Apart from the knowledge about the regulation of volume secretion and electrolyte composition (McDougall, 1948; Coats et al., 1958; Kay, 1960; Carr, 1984; Meot et al., 1997), little is known about ruminant salivary protein compositions. Jones et al. (1982) and McLaren et al. (1987) used one-dimensional gel electrophoresis to separate proteins from ruminant saliva, and they reported the presence of more than ten distinct protein bands. From then on, the main research on this issue has

been related to dietary habits (Austin et al., 1989; Hagerman and Robbins, 1993; Juntheikki, 1996; Makkar and Becker, 1998; Fickel et al., 1998; Clauss et al., 2003). The pioneering work of Hofmann (1989), based on detailed comparative morphological studies of the digestive system from 65 ruminant species from four continents, resulted in their classification into three feeding-type categories: browsers (concentrate feeders), grazers and intermediate (mixed feeders) based on the relation between dietary habits and the anatomy of the digestive system. Despite some controversy about the validity of this relationship (Robbins et al., 1995), it continues to be generally accepted that browsers' larger parotid glands produce higher volumes of thin serous saliva with a greater proportion of proteins than those of grazers (Clauss et al., 2005; Hofmann et al., 2008).

Sheep and goats, which are considered as grazers and intermediate feeders respectively, are small ruminants with a significant economic importance in Mediterranean ecosystems, showing different dietary habits. Goats have a higher tolerance than sheep to the amounts of diet plant allelochemicals (Gilboa et al., 1995; Narjisse et al., 1995; Silanikove et al., 1996a). But since only a limited number of reports were found for the salivary protein composition of these two species, no conclusions have yet been drawn with regard to an eventual intervention of these biomolecules in this process. Patterson et al. (1982) used gel electrophoresis for sheep parotid saliva protein separation and reported the detection of four major bands with apparent molecular masses of 150, 120, 45, and 25 kDa. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of goat whole saliva revealed nineteen bands with molecular masses ranging from 10 to 168 kDa (Mau et al., 2006). In both studies, a relation between salivary protein composition and ingestion and/or food composition was suggested. Recently, using SDS-PAGE, sheep and goats parotid saliva protein profiles have been compared (Lamy et al., 2008). Sixteen proteins common to both species were identified, although identification of four of the differently expressed proteins has not been possible.

Salivary proteins with defence functions against plant allelochemicals, such as tannins, seem to be present in browsers, whereas in grazers their levels are reduced or non-existent (Robbins et al., 1987; Austin et al., 1989; Fickel et al., 1998). It was suggested that tannin-binding salivary proteins (TBSP) are constitutively expressed in the parotid saliva of animals which have to deal with high amounts of tannins in their regular diets and are absent from the ones that have tannin free-diets (Robins et al., 1991; Clauss et al., 2005; Shimada, 2006). Additionally, their synthesis can be induced in some species by the consumption of tannin-enriched diets (Mehansho et al., 1983; 1985; Clauss et al., 2005). Sheep have been noted for not having constitutive TBSP in their parotid saliva (Austin et al., 1989; Fickel et al., 1998). Concerning goats, some authors suggest the possibility of tannin-binding protein induction by browse consumption (Provenza and Malechek, 1984; Silanikove et al., 1996b; Natis, 1997), although proline rich proteins were not detected. Vaithiyanathan et al. (2001) also suggested that sheep and goats may have the ability to produce TBSP, but this study was performed on salivary glands rather than saliva and failed to identify these potential TBSPs. Moreover, the consumption of tannins induces changes apart from the induction of TBSPs, as it was observed in

mice, in which the expression of one isoform of salivary amylase was dramatically increased by tannin consumption (da Costa et al., 2008).

The diversity of breeds and habitats on the one hand, and the scarcity of studies on small ruminant saliva on the other, do not allow us to reach any conclusions about the presence of salivary defence mechanisms for these species. Altogether, the data obtained so far reinforces the importance of obtaining more information on ruminant salivary protein composition in order to improve the understanding of specific adaptations in the oral milieu to different diets.

In this study, we have used a proteomic approach to characterize and compare sheep and goat parotid saliva and to access its protein composition changes induced by condensed tannin consumption. Samples collected directly from parotid ducts were analysed by two-dimensional polyacrylamide gel electrophoresis (2-DE), followed by peptide mass fingerprinting (PMF) identification using Matrix-Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectra and/or Liquid Chromatography tandem mass spectrometry (LC-MS/MS). We demonstrate that these two ruminant species present differences in parotid saliva proteome, which are ultimately related to specific dietary adaptations. Our data show changes in the expression level of some proteins when animals are fed with a quebracho-enriched diet.

2. Materials and methods

2.1. Animals and feeding trial

Adult, non-pregnant and non-lactating Merino sheep (*Ovis aries*) (n=5) and Serpentina goats (*Capra hircus*) (n=5) were kept individually in separate crates. The animals were maintained under similar dietary conditions and submitted to a 15-day acclimation period, followed by an experimental period of 13 days. During the acclimation period, they were fed with chopped wheat straw [*Triticum aestivum*, 2.4% crude protein, 84.4% neutral detergent fibre (NDF)], supplemented with a 5g/Kg metabolic weight ($Kg^{0.75}$) standard pelleted maintenance diet (16% crude protein). Half of the amount of the pellet diet was ground in order to allow the animals to adapt before the experimental period, the grinding being necessary to allow a better mixture with the tannin. Water and roughage were available *ad libitum* and orts were weighed daily in order to adjust to a 10% refusal level. On the 14th day of the acclimation period, polyethylene catheters were inserted into one of the parotid ducts of each animal. The insertion of the parotid catheters was performed according to Fickel et al. (1998), with some modifications (Lamy et al., 2008). Parotid saliva sample collection was initiated one day after surgery.

During the 13-day of the experimental period, animals were fed on a diet consisting of 40g/Kg$^{0.75}$ roughage and 5g/Kg$^{0.75}$pellets. During the first two days of the trial period, tannins were not added to the diet to allow the characterization of a control parotid saliva proteome. From day three, quebracho (powdered commercial extract, Tupafin-Ato, SilvaChimica SRL; 72%± 1.5 of condensed tannins) was mixed with the standard ground pelleted diet to a final amount of 2.5% wet weight. The mixture was prepared immediately before distribution, to minimize the decrease of tannin biological activity.

2.2. Saliva collection and sample preparation

Saliva collections were performed daily during the morning (between 10 and 12 a.m.), some minutes after the delivery of the pelleted diet and before roughage distribution. Each saliva sample was collected through a syringe from the parotid catheter, into capped 1.5 mL polypropylene sample tubes. The samples were stored at -70ºC until laboratorial analysis. Prior to protein quantification and electrophoresis separation, samples were centrifuged at 16,000 × *g* for 5 min at 4ºC to remove particulate matter. Samples that were not completely clear were rejected, in order to avoid contamination.

2.3. Quantification of total Protein

Parotid saliva protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as standard.

2.4. Two-dimensional gel electrophoresis separation

An ultra-filtration step previous to isoelectric focusing was performed using 5 kDa cut-off ultra-filtration microfuge tubes (Millipore, Eschborn, Germany; Ref: UFV5BCC00) with a final protein concentration of 1-2 mg/mL. Concentrated and desalted individual saliva samples were aliquoted (150 µg protein in 50 µL) to avoid freeze/thawing cycles, which could affect sample quality (Francis et al., 2000).

Parotid saliva samples, containing 150 µg total protein, were mixed with rehydration buffer [7M urea (Amersham Biosciences); 2M Thiourea (Sigma); 4% (w/v) CHAPS (3-[3-cholamidopropyl dimethilamonio]-1 propanessulphonate) (Sigma); 2% (v/v) IPG buffer (Amersham Biosciences); 60 mM dithiothreitol (DTT) (USB) and bromophenol blue 0,002% (w/v) (Amersham Biosciences)] to a final volume of 250 µL and loaded onto 13 cm pH 3-10 NL IPG strips (Amersham Biosciences) by in-gel rehydration overnight in the Multiphor Reswelling Tray (Amersham Biosciences). Strips were focused for 25 kVh at 20ºC, using a programme of 150V for the first hour, a gradient increase to 300V during 15 min, 300V for 1 hour, a gradient increase from 300V to 3500V during 4 hours and finally

3500V for 6 hours, using the Multiphor II isoelectric focusing system (Amersham Biosciences). After focusing, proteins in the IPG strips were reduced by soaking with 1% (w/v) DTT;50 mM tris-HCl, pH 8.8; 6M urea; 30% (v/v) glycerol; 2% (w/v) SDS at room temperature for 15 min, then alkylated with 65 mM iodoacetamide (Amersham Biosciences); 50 mM tris-HCl, pH 8.8; 6M urea; 30% (v/v) glycerol (USB); 2% (w/v) SDS for 15 min. at room temperature. The equilibrated strips were then horizontally applied on top of a 12% SDS-PAGE gel (1 X 160 X 160 mm) and proteins were separated vertically, using a Protean II xi cell (Bio-Rad), at 18ºC, applying a constant current of 5mA/gel during the first hour, after which it was step changed to 10mA/gel for another hour and then to 20mA/gel until the end of the run. Gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R-250, dissolved in 40% methanol, 10% acetic acid *overnight* and destained with 10% acetic acid for 48h. This procedure described by Beeley et al. (1991) allows the specific pink stain of PRPs.

2.5. Gel analysis

Digital images of the 2-DE gels were acquired using a scanning densitometer with internal calibration (Molecular Dynamics), and the LabScan software (Amersham Biosciences). The acquisition parameters were 300 dpi and green filter. Gel analysis was performed using Image Master Platinum v.6 software (Amersham Biosciences). Spots volume normalization, in the various 2-DE maps, was carried out using the relative spot volumes (% Vol).

Spot detection was performed, first by using automatic spot detection, followed by manual editing for spot splitting and noise removal. The gel containing the greatest number of protein spots for each animal species and diet condition was chosen as the reference gel. All other gels from the same experimental condition were matched to the reference gel by placing user landmarks on approximately 10% of the visualised protein spots to assist in automatic matching. After automatic matching completion, all matches were checked for errors by manual edition.

2.6. Protein identification

2.6.1. In-gel digestion

Stained spots were excised, washed in acetonitrile and dried in a speedvac (Thermo Savant). Gel pieces were re-hydrated with a digestion buffer (50mM NH_4HCO_3) containing trypsin (Promega, Madison, WI, USA) and incubated overnight at 37ºC. The digestion buffer containing peptides was acidified with formic acid, desalted and concentrated using C8 microcolumns (POROS R2, Applied Biosystems, Foster City, CA, USA).

2.6.2. Peptide Mass Fingerprinting

The peptides were eluted with a matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile (Sigma); 0.1% (v/v) TFA (Sigma). The mixture was allowed to air-dry (dried droplet method). Mass spectra were obtained using a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer in the positive ion reflectron mode. External calibration was made by using a mixture of standard peptides (Pepmix 1, LaserBiolabs, Sophia-Antipolis, France). Spectra were processed and analyzed by the MoverZ software (Genomic Solutions Bioinformatics, Ann Harbour, MI, USA). Peakerazor software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant *m/z* peaks and for internal calibration. Monoisotopic peptide masses were used to search for protein identification using Mascot software (Matrix Science, London, UK; http://www.matrixscience.com/). Searches were performed against SwissProt, MSDB and NCBInr protein sequence databases. The following criteria were used to perform the search: (1) mass accuracy of 100 ppm; (2) one missed cleavage in peptide masses; and (3) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. Criteria used to accept the identification were: significant homology scores achieved in Mascot; significant sequence coverage values and similarity between the protein molecular mass calculated from the gel and for the identified protein.

2.6.3. LC-MS/MS

Protein digests were analyzed by LC-ESI linear ion trap-MS/MS using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (Thermo-Finnigan, San Jose, CA). Peptides were concentrated and desalted on a RP precolumn (0.18 × 30mm, BioBasic18, Thermo Electron) and on-line eluted on an analytical RP column (0.18 × 150 mm, BioBasic18, Thermo Electron) operating at 2 μL/min. Peptides were eluted using 33-min gradients from 5 to 60% solvent B (solvent A: 0.1% formic acid, 5% acetonitrile; solvent B: 0.1% formic acid, 80% acetonitrile). The linear ion trap was operated in data-dependent ZoomScan and MS/MS switching mode using the three most intense precursors detected in a survey scan from 450 to 1600 m/z. Singly charged ions were excluded for MS/MS analysis. ZoomScan settings were: maximum injection time, 200 ms; zoom target parameter, 3000 ions; and the number of microscans, 3. Normalized collision energy was set to 35%, and dynamic exclusion was applied during 10 s periods to extend the number of fragmented peptides.

Peptide MS/MS data was evaluated using Bioworks™ 3.3.1 software. Searches were performed against an indexed UniRef 100 database (04/30/2008, 5888655 entries, www.uniprot.org). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 2 Da for precursor ions and 1 Da for MS/MS fragments ions. The

variable modifications allowed were methionine oxidation, and carbamidomethylation of cysteine. Only proteins identifications with two or more distinct peptides, a p<0.01 and Xcorr thresholds of at least 1.5/2.0/2.5 for singly/doubly/triply charged peptides were accepted. Protein identifications were further validated by manual inspections of the MS/MS spectra.

2.6.4. Prediction of post translational modifications

Potential posttranslational modifications (PTMs) were predicted using the FindMod (http://www.expasy.ch/tools/findmod/) and GlycoMod (http://www.expasy.org/cgi-bin/glycomod) search engines (Gasteiger et al., 2005), which work by examining peptide mass fingerprint results of the identified proteins for the presence of PTM. This is done by looking at mass differences between experimentally determined peptide masses and theoretical peptide masses calculated for the specified protein sequence. Additionally, NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict putative serine, threonine and tyrosine phosphorylation sites using a neural network-based method trained on a large dataset of known phosphorylation sites (Blom et al, 1999). Glycosylation and phosphorylation presented in Swissprot database were also considered. The presence of signal peptides in each identified protein was searched using Signal IP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Only the predicted PTMs associated to peptides not matched to the identified protein were considered.

2.7. Statistical analysis

All data were analysed for normality (Kolmogorov-Smirnoff test) and for homocedasticity (Levene test). The values of salivary protein concentration were normally distributed and independent sample T-tests were performed to access differences between species, as well as within each species between diet treatments. Spot % Vol data tested presented neither normal distribution nor homocedasticity. In order to compare species and treatments, the differences in the protein expression levels between species (sheep and goat) and between treatments (control and quebracho) were analysed by non-parametric procedures Kruskal-Wallis test. For a finer comparison between treatments within species and between species within treatments the Mann-Whitney test was used. Means were considered significantly different when $P < 0.05$. All statistical analysis procedures were performed by SPSS 15.0 software package (SPSS Inc., Chicago, USA).

3. Results

3.1. Salivary protein concentration

Ruminant saliva has a high ionic content, particularly in regard to phosphates and bicarbonates, which confer its unique buffer capacity (McDougal, 1948), and a lower protein concentration in comparison with humans (Lin and Chang, 1989) and rodents (da Costa et al. 2008). Therefore, an ultra-filtration step was performed to desalt and concentrate samples prior to two-dimensional electrophoresis separation. This desalting and concentration method was chosen instead of the TCA precipitation method. TCA has been frequently used to solubilize salivary proline-rich proteins (Mehansho et al., 1983; 1985; Fickel et al., 1998), the presence of which in sheep and goat saliva we intend to evaluate during the present study.

The values of parotid saliva protein concentration presented some variability among different animals from the same species and within the same animal, with the same treatment, on different collection days. Sheep and goats did not differ from each other in parotid saliva protein concentration, either before or after being fed a quebracho-enriched diet, and the consumption of tannins for a period of more than ten days resulted in a statistically significant increase in parotid protein concentration for both species (Table 1).

Table 1 – Comparison of parotid saliva protein concentration (μg/mL) between control and tannin-enriched dietary conditions (mean ± SD)

	Goat	Sheep
Control	155.12 ± 74.51	186.29 ± 92.08
P	*0.0042*[a]	*0.01*[a]
Quebracho	355.02 ± 270.77	396.74 ± 82.1

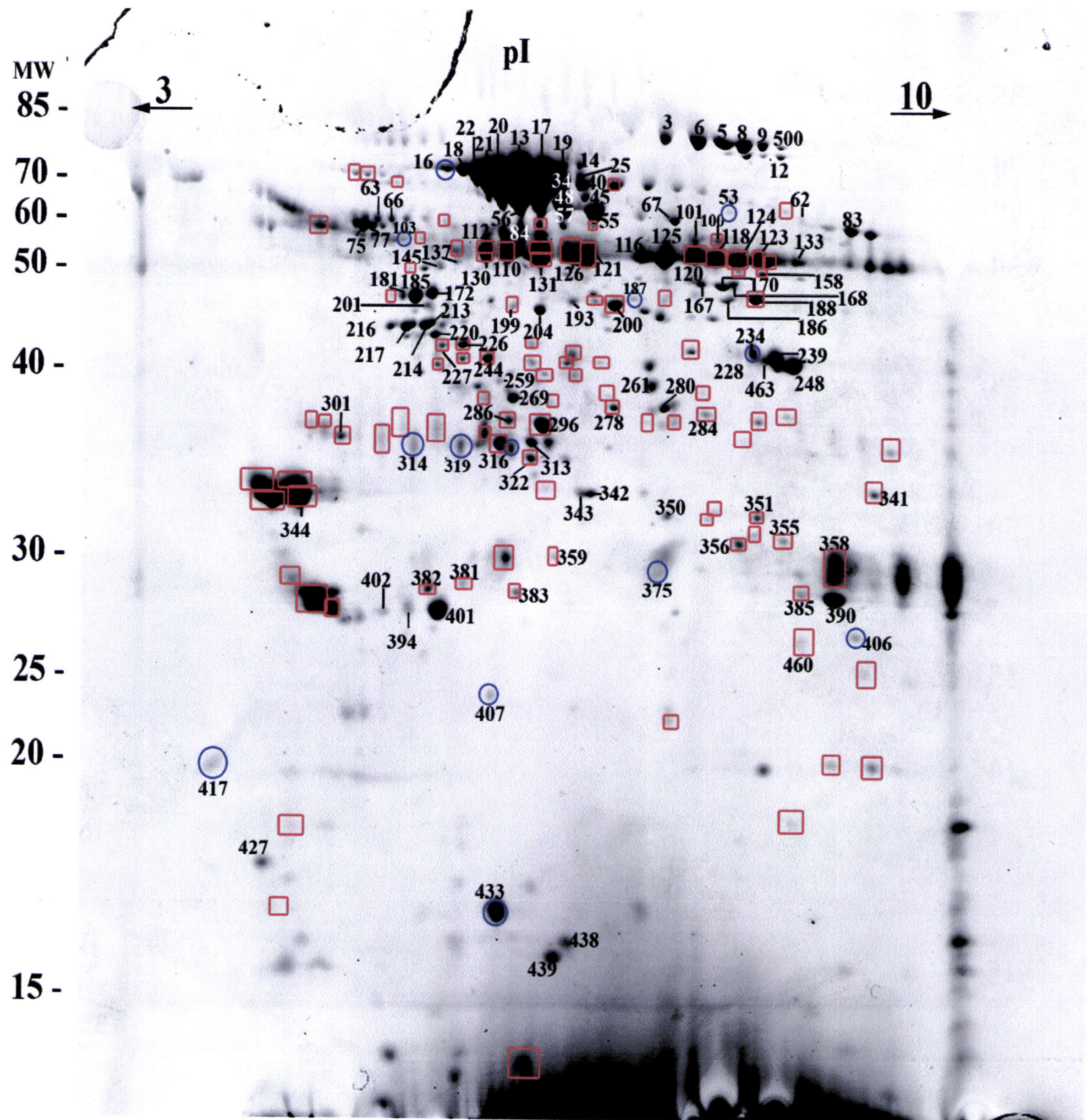
[a] Differences are significant for $P<0.05$

3.2. Characterization of sheep and goat parotid saliva proteome

The collection of parotid saliva through parotid catheters is effective and provides non-contaminated samples, although catheter displacing can occur. In the present study this had the consequence of reducing the number of animals analysed from five to three individuals of each specie.

Samples from control treatment were used to characterize sheep and goat parotid saliva proteome. A total of 260 and 205 protein spots were consistently observed in CBB R-250 stained gels from sheep and goats, respectively, between a pI of 3 and 10 and molecular masses of 15 and 85 kDa. Representative 2-DE gel patterns of sheep and goat parotid saliva from a tannin-free diet (control) are shown in figures 1A and 1B, respectively.

A



B

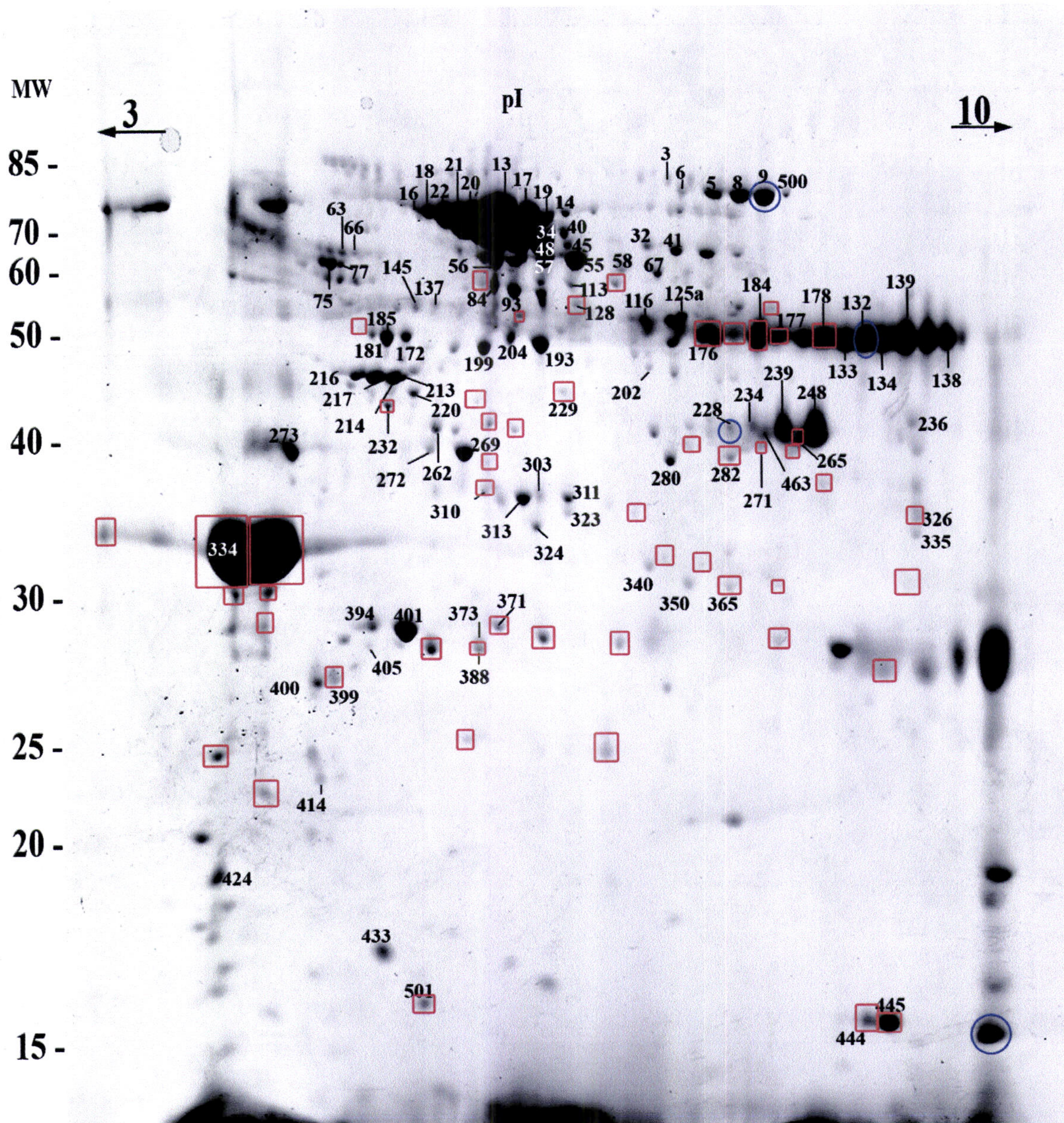


Figure 1 – 2-DE profiles of control parotid saliva. 150 μg of salivary proteins from sheep (**A**) and goats (**B**) were subjected to two-dimensional electrophoresis (IPG strips pH 3-10 NL; 12% SDS-PAGE). The numbered spots are the ones identified by PMF and listed in Table 2. Squares show spots only observed in the specie correspondent to the image where they are represented. Circles show spots that, despite being observed in both species, are expressed at higher levels in the specie correspondent to the image where they are represented. Numbers in the left side correspond to molecular mass markers positions.

After gel analysis, the more intense 180 protein spots from sheep's and 170 protein spots from goats 2-DE gels were excised, tryptic digested and submitted to identification by PMF, using MALDI-TOF mass spectra. Some tryptic digests that resulted in bad mass spectra and/or non-significant identification were further submitted to LC-MS/MS. Table 2 shows the 106 sheep and 99 goat protein spots identified by PMF, including information about protein biochemical function and subcellular localization, whereas Table 3 shows the 11 sheep and 7 goat protein spots identified by LC-MS/MS.

Table 2 - Sheep (*Ovis aries*) and goat (*Capra hircus*) parotid proteins identified by PMF

Sheep									
Spot	Protein	Accession code [a]	Est. MW[b] (kDa)/p*I*	Theor. MW[c] (kDa)/p*I*	MASCOT search results			Biological function/subcellular localization	Ref.[g]
					Pep.[d]	*Score*[e]	Seq. cov. (%)[f]		
213S	Actin cytoplasmic 1	P60713	49/5.5	42 / 5.3	10/26	104	28	Structural; cytoskeletal/ cytoplasm	3, 5, 6, 8, 11, 14, 16
214S			49/5.5		16/33	158	39		
216S			49/5.4		8/16	97	23		
217S			49/5.4		9/23	92	30		
220S			48/5.5		7/21	71	22		
63S	Alpha-1-antiproteinase precursor	P12725	63/5.2	46 / 5.8	8/17	92	19	Protein degradation or inhibitor/secreted; extracellular	3, 5 , 6, 12, 14, 16
66S			63/5.3		6/16	63	14		
278S	Annexin A1	P46193	42/6.2	39 / 6.4	8/13	105	28	Transport/ Membrane	5, 6, 14, 16
280S			42/6.2		15/37	155	52		
383S	Apolipoprotein A1 precursor	P15497	28/5.7	30 / 5.7	16/34	193	49	Transport/ Secreted; extracellular	1, 3, 5, 6, 14, 16
394S			28/5.5		15/23	215	51		
401S			28/5.5		16/51	161	49		
402S			28/5.4		6/15	56	23		
234S	Carbonic anhydrase VI	P08060	44/7.0	36 / 6.3	12/16	149	21	Metabolism; carbohydrates/ Secreted; extracelular	4, 5, 6, 8, 9, 14, 11*, 13, 15, 16
239S			44/7.5		18/30	216	50		
248S			44/8.0		15/29	122	39		
463S			44/7.4		13/20	128	23		
62S	Catalase	P00432	62/8.0	60 / 6.8	6/17	57	10	Redox/ Cytoplasmic	
406S	Cathelicidin-1 precursor	P54230	27/8.0	18 / 7.5	5/17	69	43	Antimicrobial/ Secreted; extracellular	----
433S			16/5.4		6/25	76	46		
427S	Cathelicidin-2 precursor	P82018	17/4.4	20 / 9.4	5/19	65	26	Antimicrobial/ Secreted; extracellular	----
341S	Complement C4 precursor (gamma chain)[#]	P01030	35/9.1	33 / 6.7[#]	12/36	75	16	Defense; immunoresponse/ Secreted; extracellular	6, 13, 16
301S	Deoxyribonuclease	P11937	37/4.9	29 / 4.8	4/7	64	26	DNA cleavage/Secreted; extracellular	17, 18
284S	Haptoglobin	Q2TBU0[2]	41/6.8	45 / 7.8	8/28	66	18	Transport/Secreted; extracellular	5, 6
116S	Ig heavy chain C region	gi|109029[1]	55/6.2	34/6.1	8/29	71	34	Defense; immunoresponse/ Secreted; extracellular	3, 6, 7, 8, 12, 16
118S			55/7.1		6/21	69	26		
120S			55/6.6		8/26	76	33		
121S			55/6.1		12/30	113	46		
123S			55/7.4		10/6	88	34		
125S			55/6.5		9/18	108	42		
126S			55/6.0		7/17	77	33		
130S			55/5.7		8/20	85	34		
131S			55/5.9		10/41	82	32		
261S			42/6.5		7/26	79	26		
5S	Serotransferrin precursor	Q29443	70/6.6	78 / 6.8	10/39	57	14	Transport/ Secreted; extracellular	3, 5, 6, 8, 11, 13, 14, 16
6S			72/6.5		12/33	79	16		
8S			70/7.1		9/26	56	10		
9S			70/7.4		15/42	106	20		
12S			69/7.5		10/25	70	13		
500S			72/7.5		7/15	58	9		
13S	Serum albumin precursor	P14639	67/5.8	69 / 5.8	8/12	100	18	Transport/ Secreted; extracellular	1, 2, 3, 4, 5, 6, 7, 8, 9, 11*, 14, 15
14S			67/6.6		11/14	134	19		
17S			67/5.9		22/35	235	41		
18S			67/5.7		5/9	57	11		

19S		67/6.0	10/18	106	20
20S		67/5.8	9/20	86	15
21S		67/5.7	9/19	86	15
22S		67/5.6	11/17	128	21
25S		67/6.6	14/31	119	20
34S		64/6.0	25/45	254	44
40S		65/6.1	11/35	74	17
45S		63/6.1	20/29	229	35
48S		62/6.0	8/18	55	15
55S		62/6.1	12/15	121	25
56S		64/5.8	8/12	100	18
57S		61/6.0	24/36	267	37
67S		60/6.2	11/17	138	22
84S		59/5.8	21/36	213	33
100S		55/6.6	11/22	105	19
101S		55/6.5	8/18	61	12
110S		55/5.8	6/8	71	11
112S		55/5.7	18/39	157	33
124S		55/7.1	8/20	59	13
137S		55/5.7	16/24	177	29
145S		54/5.5	6/9	64	10
158S		54/7.4	7/15	61	12
167S		53/6.5	11/14	136	19
168S		53/7.2	7/11	67	16
170S		53/7.2	13/25	125	21
172S		51/5.4	9/21	77	12
181S		51/5.2	11/17	124	22
185S		51/5.3	18/26	203	27
186S		51/7.4	6/9	70	12
188S		51/7.4	5/7	58	8
193S		51/6.0	9/11	97	21
199S		51/5.7	12/36	88	15
200S		51/6.1	20/39	199	30
201S		51/5.4	9/16	94	14
204S		51/5.7	19/28	215	31
226S		44/5.5	6/7	79	11
227S		44/5.4	11/16	113	17
244S		40/5.6	15/21	178	23
259S		39/5.7	10/18	105	16
269S		39/5.7	15/16	214	22
286S		39/5.7	10/19	96	16
296S		39/5.9	9/17	95	20
313S		38/5.7	17/31	170	28
316S		38/5.6	20/39	178	32
322S		37/5.7	11/24	100	16
342S		35/6.1	17/29	157	29
343S		35/6.1	17/29	157	29
350S		35/6.5	11/18	120	18
351S		35/7.4	21/34	218	33
355S		34/8.0	10/21	102	21
356S		33/7.0	17/25	188	28
359S		30/6.0	8/13	87	15
381S		30/5.5	9/27	70	13
382S		30/5.4	9/32	57	17

385S			30/8.0		10/20	98	15		
390S			30/8.5		18/44	126	25		
439S			14/6.1		7/17	53	11		
83S	similar to fibrinogen beta chain precursor	**gi119908847**[1]	58/8.3	56 / 8.5	19/43	104	33	Protein modification; polymerization/ Secreted; extracellular	3, 5, 8
438S	Transthyretin precursor (prealbumin)	**P12303**	14/6.0	16 / 5.6	6/19	86	35	Transport/ Secreted; extracellular	—
75S	Vitamin D-binding protein precursor	**Q3MHN5**	61/5.2	53 / 5.4	9/31	87	22	Transport/ Secreted; extracellular	5, 6, 14, 16
77S			61/5.2		5/14	55	17		

Goats

Spot	Protein	Accession code[a]	Est. MW[b] (kDa)/pI	Theor. MW[c] (kDa)/pI	MASCOT search results			Biological function/subcel. localization	Ref.[g]
					Pep.[d]	*Score*[e]	Seq. cov. (%)[f]		
216G	Actin cytoplasmic 1	**P60713**	49/5.4	42 / 5.3	15/38	145	35	Structural; cytoskeletal/ cytoplasm	3, 5, 6, 8, 11, 14, 16
217G			49/5.4		11/41	103	31		
214G	Actin cytoplasmic 1 + Serum albumin precursor	**P60713** + **P14639**	49/5.5	(42/5.3) / (69/5.8)	14/12	132 / 84	36 / 19		
202G	Alpha-enolase	**Q9XSJ4**	50/6.3	47 / 6.4	11/19	150	33	Metabolism-glycolysis; carbohydrates/ cytoplasmic	3, 4, 5, 6, 7, 8, 11, 12, 14, 16
63G	Alpha-1-antiproteinase precursor	**P12725**	63/5.3	46 / 5.8	6/14	65	15	Protein degradation or inhibitor/secreted; extracellular	3, 5 , 6, 12, 14, 16
66G			63/5.3		6/16	63	13		
280G	Annexin A1	**P46193**	42/6.3	39 / 6.4	12/23	139	42	Transport/ Membrane	5, 6, 14, 16
282G			41/7.0		6/11	76	28		
303G	Annexin A3	**Q3SWX7**	39/5.9	36 /6.5	6/24	52	16	Protein degradation or inhibitor/ Membrana	6, 14
371G	Apolipoprotein A1 precusor	**P15497**	28/5.8	30 / 5,7	8/18	88	20	Transport/ Secreted; extracellular	1, 3, 5, 6, 14, 16
394G			28/5.5		15	202	46		
401G			28/5.5		14/32	165	45		
405G			27/5.3		6/16	67	13		
232G	Apolipoprotein A IV precursor	**Q32PJ2**	47/5.4	43 / 5.3	8/18	83	21	Signaling/ Secreted; extracellular	6
228G	Carbonic Anhydrase VI	**P08060**	44/6.9	36 / 6.3	7/24	59	16	Metabolism; carbohydrates/ Secreted; extracelular	4, 5, 6, 8, 9, 14, 11*, 13, 15, 16
234G			44/7.0		5/12	61	13		
239G			44/7.5		8/18	81	22		
248G			44/8.0		13/16	176	37		
265G			43/7.9		11/21	128	27		
271G			43/7.4		7/10	105	18		
463G			44/7.4		6/15	71	16		
433G	Cathelicidin-1 precursor	**P54230**	16/5.4	18 / 7.5	7/37	81	47	Antimicrobial/ Secreted; extracellular	—
424G	Cathelicidin-2 precursor	**P82018**	18/4.4	20 / 9.4	8/17	123	36	Antimicrobial/ Secreted; extracellular	—
501G	Cathelicidin-3 precursor	**P50415**	14/5.6	22 / 10.9	4/12	58	16	Antimicrobial/ Secreted; extracellular	—
335G	Complement C4 precursor (gamma chain)[#]	**P01030**	36/9.1	33 / 6.7[#]	7/15	54	6	Defense; immunoresponse/ Secreted; extracellular	6, 13, 16
273G	Deoxyribonuclease	**P11937**	43/4.9	29 / 4.8	7/20	87	30	DNA cleavage/Secreted; extracellular	17, 18
444G	Hemoglobin subunit beta-A	**P02077**	15/8.5	16 / 6.8	7/27	91	44	Transport/ Secreted; extracellular	6, 16
445G			14/8.8		8/24	113	56		
132G	Immunogobulin gamma 2 heavy chain constant region	**gi147744654**[1]	53/8.5	22 / 6.3	6/15	78	34	Defense; immunoresponse/ Secreted; extracellular	3, 6, 7, 8, 12, 16
134G			53/8.8		7/24	79	40		
138G			53/10		7/30	66	37		
139G			53/9.1		9/36	80	54		

177G			53/7.5		6/23	66	39		
184G			53/7.4		8/37	66	49		
236G			45/9.1		6/18	72	43		
326G			37/9.1		8/30	86	43		
32G	Lactoferrin	**Q5MJE8**[2]	67/6.2	77 / 8.4	8/26	60	15	Transport/ Secreted; extracellular	4, 5, 6, 11, 14, 16
41G			63/6.5		9/33	59	12		
5G	Serotransferrin precursor	**Q29443**	70/6.6	78 / 6.8	10/24	79	13	Transport/ Secreted; extracellular	3, 5, 6, 8, 11, 13, 14, 16
6G			72/6.5		12/21	109	17		
8G			70/7.1		13/30	102	18		
9G			70/7.4		18/59	97	24		
500G			72/7.5		7/15	54	9		
13G	Serum albumin precursor	**P14639**	67/5.8	69 / 5.8	20/38	186	28	Transport/ Secreted; extracellular	1, 2, 3, 4, 5, 6, 7, 8, 9, 11*, 14, 15
14G			67/6.6		8/14	76	12		
16G			68/5.6		18/25	201	30		
17G			67/5.9		24/51	201	31		
18G			67/5.7		20/28	231	34		
19G			67/6.0		14/33	117	22		
20G			67/5.8		16/31	137	25		
21G			67/5.7		17/31	159	28		
22G			67/5.6		12/27	98	24		
34G			64/6.0		27/38	304	42		
37G			67/5.7		10/21	95	15		
40G			65/6.1		14/29	134	27		
45G			63/6.1		13/22	140	22		
48G			62/6.0		21/36	220	33		
55G			62/6.1		8/12	89	15		
56G			64/5.8		23/41	236	37		
57G			61/6.0		8/15	82	12		
58G			60/6.25		7/21	54	14		
67G			60/6.25		17/48	128	28		
83G			58/5.7		7/11	78	11		
84G			59/5.8		12/20	126	21		
93G			58/5.8		18/26	204	28		
113G			58/6.1		6/8	69	9		
116G			55/6.2		8/19	74	13		
125aG			55/6.5		12/18	130	19		
128G			56/6.1		8/12	88	15		
137G			56/5.7		8/16	63	15		
145G			57/5.5		12/21	126	19		
172G			53/5.5		19/23	243	35		
176G			53/6.7		9/22	79	15		
181G			54/5.3		5/8	54	9		
185G			53/5.4		6/8	71	11		
193G			52/6.0		12/30	95	20		
199G			52/5.5		16/32	136	24		
204G			52/5.7		8/19	76	18		
213G			50/5.5		11/21	109	20		
220G			48/5.5		14/17	175	25		
229G			44/5.8		7/13	65	12		
262G			44/5.6		15/24	154	22		
269G			42/5.7		11/23	109	16		
272G			43/5.8		8/11	101	14		
310G			39/5.8		7/16	54	10		
311G			39/6.0		9/13	100	14		

313G			39/5.8		11/32	87	19		
323G			37,3/6.0		8/11	84	11		
324G			36/6.0		12/22	116	16		
340G			34/6.2		6/13	55	11		
350G			33/6.5		12/17	143	20		
373G			30/5.8		6/9	70	8		
388G			29/5.8		6/13	54	11		
399G			27/5.5		7/14	63	11		
400G			27/5.2		9/18	77	13		
414G			22/5.2		5/8	59	8		
75G	Vitamin D-binding protein precursor	**Q3MHN5**	61/5.2	53 / 5.4	11/35	106	23	Transport/ Secreted; extracellular	5, 6, 14, 16
77G			62/5.2		7/22	73	19		

[a] Swiss-Prot Accession codes except where other else stated: [1] NCBInr accession codes and [2] MSDB accession codes;
[b] Est. MW/pI: Molecular mass estimated based on electrophoretic mobility/ pI estimated according **Appendix 1**
[c] Theor. MW/pI: Determined molecular mass for the identified protein/Determined pI for the identified protein
[d] Pep.: Number of peptides from experimental Peptide Mass Fingerprint whose masses match those from a theoretical PMF determined from a known sequence / Number of peptides from experimental Peptide Mass Fingerprint submitted for Mascot search (The list of peptides submitted s presented in **Appendix 2**
[e] Score: The minimum Mascot score for a probability less than 5% for the match to be a random event is 53 for Swis-Prot, 59 for MSDB and 65 for NCBInr database searches;
[f] Seq. cov: Percentage of the identified protein sequence covered by matched peptides;
[g] Ref: Articles reporting the presence of the identified protein in saliva: (1) Ghafouri et al., 2003; (2) Yao et al., 2003; (3) Huang, 2004; (4) Vitorino et al., 2004; (5) Wilmarth et al., 2004; (6) Xie et al., 2005; (7) Hirtz et al., 2005; (8) Hu et al., 2005; (9) Hardt et al., 2005; (10) Guo et al., 2006; (11) Walz et al., 2006 ; (12) Neyraud et al., 2006; (13) Ramachandran et al., 2006; (14) Siqueira et al., 2006; (15) Vitorino et al., 2004b; (16) Nicholas et al., 2006; (17) Williams et al., 1999; (18) Lamy et al. (2008); * studies in which the protein was identified in parotid saliva;
[#] The accession number refer to the complete sequence of complement C4, however, the peptides observed in *m/z* spectra correspond to gamma chain, so theoretical values for molecular mass and pI correspond to gamma chain.

Table 3 - Sheep (*Ovis aries*) and goat (*Capra hircus*) parotid proteins identified using LC-MS/MS data

Spot	Protein	Access. Code[1]	Est. MW (kDa)/pI [1]	Theor. MW (kDa)/pI[1]	p-value[a]	Score[1]	Seq. Cov.[1]	Dist. Pep.[b]	Biological function/subcel. localization[1]	Ref.[c]
					Sheep					
279S	Annexin A1	**P46193**	42 / 6.0	39 / 6.4	1.75E-09	60.25	23.10	6	Transport/ Membrane	(1)
228S	Carbonic anhydrase VI	**P08060**	44 / 6.9	36 / 6.3	2.45E-13	110.30	38.40	7	Metabolism; carbohydrates/ Secreted; extracelular	(1)
293S	Clusterin precursor	**P17697**	38 / 5.5	51 / 5.7	2.10E-08	110.26	14.60	6	Cell growth and/or differentiation / Secreted; extracelular	(2)
	Serum albumin precursor	**P14639**		69 / 5.8	8.23E-11	20.26	4.80	2	Transport/Secreted extracellular	(1)
	Alpha-S1-casein precursor	**P02662**		25 / 5.0	7.63E-06	20.19	10.30	2	Transport/Secreted extracellular	—
298S	Clusterin precursor	**P17697**	38 / 5.5	51 / 5.7	9.64E-12	70.25	9.10	5	Cell growth and/or differentiation / Secreted; extracelular	(2)
	Alpha-S1-casein precursor	**P02662**		25 / 5.0	8.51E-07	20.19	10.30	2	Transport/Secreted extracellular	—
314S	Alpha-S1-casein precursor	**P02662**	37 / 5.5	25 / 5.0	1.62E-10	40.26	17.30	3	Transport/Secreted extracellular	—
	Beta-lactoglobulin precursor	**P02754**		20 / 4.9	1.93E-12	30.31	19.10	2	Transport/Secreted extracellular	—
	Kappa-casein precursor	**P02668**		21 / 6.3	1.74E-09	30.30	14.70	2	Transport/Secreted extracellular	—
	Clusterin precursor	**P17697**		51 / 5.7	2.75E-10	20.29	8.90	2	Cell growth and/or differentiation / Secreted; extracelular	(2)
		P02663		26 / 8.6	9.20E-05	20.18	8.60	2		—

	Alpha-S2-casein precursor								Transport/Secreted extracellular	
317S	Clusterin precursor	P17697	36 / 5.6	51 / 5.7	1.68E-08	20.28	8.90	2	Cell growth and/or differentiation / Secreted; extracelular	(2)
319S			36 / 5.4		1.85E-08	40.25	11.62	3		
384S[d]	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor (BSP30b)	UPI0000615459	28 / 5.0	26 / 4.3	3.49E-07	20.28	6.70	1	Transport/ Secreted; extracellular	(4)
386S[d]			28 / 4.8		2.98E-07	20.28	6.70			
395S[d]			27 / 5.0		1.63E-06	20.28	6.70			
467S	Clusterin precursor	P17697	38/5.4	51 / 5.7	1.31E-07	40.20	5.20	3	Cell growth and/or differentiation / Secreted; extracelular	(2)
Goat										
91G	Leukocyte elastase inhibitor	Q1JPB0	60 / 7.5	42 /5.7	5.82E-11	60.25	11.90	4	Protein degradation or inhibition / Cytoplasmic	(2; 3)
250G			44 / 6.4		3.71E-10	50.26	9.50	3		
127G	Cytosolic non-specific dipeptidase	Q3ZC84	59 / 5.7	53 / 5.6	1.11E-14	80.31	22.10	7	Protein degradation or inhibition / Cytoplasmic	----
	Serum albumin precursor	P14639		69 / 5.8	5.91E-08	20.18	0.47	2	Transport/ Secreted; extracellular	(1)
194G	Serum albumin precursor	P14639	52 / 5.6	69 / 5.8	8.88E-15	80.30	17.30	6	Transport/ Secreted; extracellular	(1)
333G[d]	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor (BSP30b)	P79125	33 / 4.8	26 / 4.3	1.55E-08	20.29	6.30	2	Transport/ Secreted; extracellular	(4)
334G[d]			33 / 4.5		5.50E-08	20.29	6.30	2		
442G	Hemoglobin subunit beta-C	P02078	15 /10	16 /6.7	5.06E-12	40.32	21.83	2	Transport / Secreted; extracellular	(1)
	Hemoglobin subunit beta-A	P02077		16 / 6.8	5.57E-10	40.31	22.07	2		
	Hemoglobin subunit alpha-1	P01967		15 / 8.2	2.75E-08	30.21	14.89	2		

[1] See Table 2 footnotes
[a] p-value: protein probability identification calculated based on the probability of the associated peptides. It represents the likelihood of finding an equally good protein match by chance. It was set to 0.01, i.e. 1% probability that the match was a random event;
[b] Dist. Pep.: number of distinct LC MS/MS fragmented peptides that originated identification. Covalently modified peptides, including N- or C-terminal elongation (i.e. missed cleavages) count as unique peptides;
[c] Ref.: Articles reporting the presence of the identified protein in saliva: (1) Please see references indicated in Table 2 for the same protein; (2) Xie et al., 2005; (3) McNeely et al., 1995;(4) Weeler et al. 2002
[d] The confidence level for this identification is lower than for the other ones, due to the fragmentation of only one peptide. However, it was considered due to the good fragmentation of the peptide (see supplementary Table 2 and supplementary Figure)

Despite the high number of protein spots identified for each species, several resulted in the same identification, that is, only 23 and 24 different proteins were identified for sheep and goats, respectively. Additionally, differences between theoretical and estimated molecular masses and/or pI were also observed for some spots. These two findings suggest that some proteins present several isoforms, maybe due to the presence of PTMs. Glycosylations and phosphorylations are the most widespread PTMs (Temporini et al., 2008) and are the ones responsible for the greatest shifts in MW and pI of the proteins observed in 2-DE gels. For that reason in those situations, we used FindMod, GlycoMod and NetPhos 2.0 applications to predict the presence of these PTMs in proteins identified by PMF. It was found that several proteins may be present in ruminant saliva in phosphorylated and/or glycosylated forms (Supplementary Table 1).

Common salivary protein (BSP30b) was identified by MS/MS in both sheep and goat spots (384S, 386S, 395S, 333G and 334G). The level of confidence in these identifications is lower in comparison

with other proteins because these identifications rely on the fragmentation of a unique peptide in sheep, and two peptides differing in only two amino acids in goat (Supplementary Table 2 and Figure). However, the fact that high peptide scores and probabilities were obtained, together with the agreement of theoretical and observed molecular mass and pI, support the obtained identifications. Moreover, it is interesting to notice that sheep and goat spots differed in terms of the chromatographic pattern (data not shown) and also in terms of the estimated molecular mass (Table 3). This suggests that, although homologous to bovine common salivary protein, there might be some differences between the protein sequence of the two studied species.

The identified proteins belong to several categories, according to "SwissProt and TrEMBL" protein database, including transporters, proteases, protease inhibitors, proteins involved in signalling, defense/immune response, DNA cleavage, carbohydrate metabolism, redox processes and structural proteins. The great proportion of proteins identified corresponds to proteins involved in transport (about 70%). The second large group includes the proteins exhibiting an immune response or protection function, particularly an antimicrobial function. Most of the identified proteins are secreted/extracellular proteins, but also cytoplasmic, such as alpha enolase, cytoplasmic actin, annexins and catalase. Serum albumin was the protein identified for a higher number of spots, in a total of 61 and 53 for sheep and goats, respectively. These spots were distributed through a pH range from 5.2 to 7.0 and presented molecular masses ranging from, approximately, 20 to 70 kDa. The theoretical molecular mass of the protein, without signal peptide and propeptide, is of about 66 kDa, what goes in accordance with the higher observed molecular masses for sheep and goats albumin spots. The entire range of molecular masses observed for albumin spots can be due to the presence of albumin fragments in parotid saliva. Some spots showed a distribution of fingerprint peptide pattern containing peptides from only one of the albumin domains, whereas others containing peptides from only two of the three domains. Additionally there were spots that contained both the N-terminal region and the C-terminal domain with a complete lack of MS signal from the internal sequence region. In general, these peptide distributions are in accordance with the observed masses in 2-DE gels. This is very similar to what was observed for albumin distribution in human urine (Candiano et al., 2006), for which a plasmatic origin associated to the presence of urine proteases were suggested. We may hypothesize a similar situation for salivary albumin, probably having plasmtic origin. Moreover, the bovine plasma proteome presents similar albumin distribution, reinforcing this hypothesis (Wait et al., 2002). Ghafouri et al. (2003) observed several spots, in human whole saliva 2-DE gels, identified as serum albumin distributed in two different molecular masses: 68 and 40 kDa. The spots with apparent molecular masses of 40 kDa were reported as being the N-terminal region of albumin.

3.3. Comparison between sheep and goat parotid saliva proteome

After gel analysis by ImageMaster, and statistical analysis as described in material and methods section, 132 protein spots appeared to be expressed in similar levels (% Vol) in sheep and goats. Some of them were only identified with a confident score for one of the species (Tables 2 and 3). Proteins such as lactoferrin (spots 32G, 41G), alpha enolase (spot 202G), leukocyte elastase inhibitor (spots 91G and 250G), cytosolic non-specific dipeptidase (spot 127G) and annexin A3 (spot 303G) were only positively identified for the spots excised from goats' 2-DE gels, despite these spots being equally expressed for both species. However, it was possible to observe, in the sheep peptide maps, *m/z* peaks from the theoretical digestion of these proteins, suggesting their presence also in sheep parotid saliva. The same is true for the spots identified as catalase (spot 62S), and as a protein similar to fibrinogen (spot 83S), in sheep 2-DE gels. For the proteins cyosolic non-specific dipeptidase (only identified in goats for the spot 127G) and the proteins clusterin, beta-lactoglobulin and caseins (proteins only identified in sheep even for spots 314 and 319, which are spots also present in goats) the same analysis was not possible due to the poor resolution of the *m/z* spectra obtained.

Several protein spots appeared to be differentially expressed between sheep and goats. A total of 167 protein spots were found to be expressed in only one of the species: 111 and 56 protein spots were present exclusively in sheep and goat 2-DE gel maps, respectively (signalled by a square in Fig. 1). Another 17 protein spots differed in terms of expression levels: 13 and 4 protein spots highly expressed in sheep and goat, respectively (Table 4). Proteins differentially expressed by the two species are distinctly signalled by a circle in Fig.1.

Apolipoprotein A-IV, hemoglobin subunits and cathelicidin-3 correspond to protein spots only observed in the goats parotid saliva proteome, whereas clusterin (spots 293S, 298S and 467S), haptoglobin (spot 284S) and transthyretin precursor (spot 438S) were proteins identified for spots only observed in sheep.

Table 4 - Comparison of protein expression levels (% Vol) between sheep (*Ovis aries*) and goats (*Cara hircus*) in control diets (mean ± SD)

Spot Nº	Goat	Sheep	p^a	Protein identified	
				Goat	Sheep
9	0.29 ± 0.23	0.05 ± 0.09	0.027	Serotransferrin precursor	
16	0.01 ± 0.03	0.17 ± 0.05	0.01	Serum albumin precursor	
53	0.002 ± 0.005	0.03 ± 0.01	0.011	n.d.	
103	0.01 ± 0.01	0.03 ± 0.01	0.014	n.d.	
132	0.41 ± 0.20	0.06 ± 0.04	0.014	Immunoglobulin gamma 2 heavy chain constant region	n.d.

187	0.005 ± 0.01	0.03 ± 0.03	0.041	**n.d.**	
228	0.104 ± 0.02	0.09 ± 0.03	0.027	**Carbonic anhydrase VI**	
234	0.098 ± 0.09	0.49 ± 0.30	0.027		
314	0.007 ± 0.13	0.20 ± 0.10	0.011	**n.d.**	**Alpha-S1-casein precursor** **Beta-lactoglobulin precursor** **Kappa-casein precursor** **Clusterin precursor** **Alpha-S2-casein precursor**
317	0.02 ± 0.02	0.21 ± 0.10	0.013	**n.d.**	**Clusterin precursor**
319	0.007 ± 0.02	0.13 ± 0.08	0.011	**n.d.**	**Clusterin precursor**
375	0.08 ± 0.05	0.33 ± 0.16[b]	0.027	**n.d.**	
406	0.02 ± 0.01	0.12 ± 0.08	0.014	**n.d.**	**Cyclic dodecapeptide precursor**
407	0.004 ± 0.006	0.07 ± 0.03	0.013	**n.d.**	
417	0.02 ± 0.03	0.22 ± 0.11[b]	0.013	**n.d.**	
433	0.11 ± 0.06	0.49 ± 0.30	0.014	**Cyclic dodecapeptide precursor**	
442	1.59 ± 1.00	0.26 ± 0.39[b]	0.027	**Hemoglobin subunit beta**	**n.d.**

[a] Differences are significant for $P<0.05$
[b] Due to experimental difficulties it was not possible to identify
n.d. - not identified proteins

Most of the unidentified spots included in Table 4 presented low intensities and that may be the reason for the failure in identification. However, we cannot state that the same protein is not present in both species.

Interestingly, several spots observed in only one of the species were identified with the same accession code in spots from the other specie presenting different apparent molecular masses and pI. This suggests the existence of different isoforms of the same protein between species.

In addition to the differences referred to so far, a pronounced difference is very evident at the acidic end of the gel maps from the two species, in the region between 25 and 35 kDa (Fig. 2). The spots 333G and 334G were identified as BSP30b, and the same identification was obtained for the spots 384S, 386S and 395S. However, the spots positions, in terms of molecular mass observed in goats 2-DE maps (~33 kDa) differ from the position in sheep 2-DE maps (~26-27 kDa). BSP30b is a bovine protein for which no homologous are found in sequence databases for sheep and goats. It is possible

that differences in sheep and goat BSP30b sequences explain the molecular mass differences observed in 2-DE gels. Another intense group of spots (325S, 329S, 344S and 345S) was only observed in sheep gels. These were not identified neither through PMF or MS/MS. By looking to the *m/z* spectra obtained by MALDI-TOF it is possible to observe a great similarity among them and for that reason we may suspect that the spots represent the same protein(s), which sequence(s) are probably not deposited in the searched sequence databases.

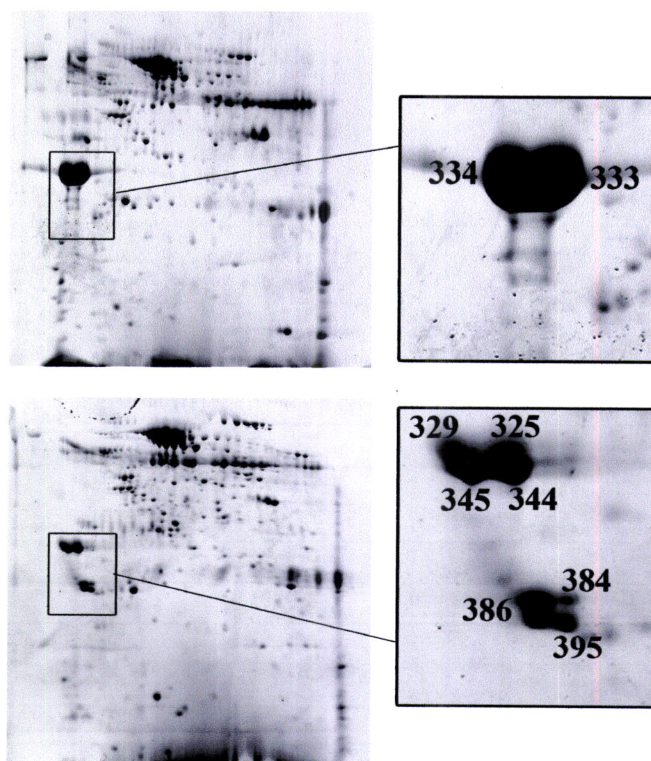
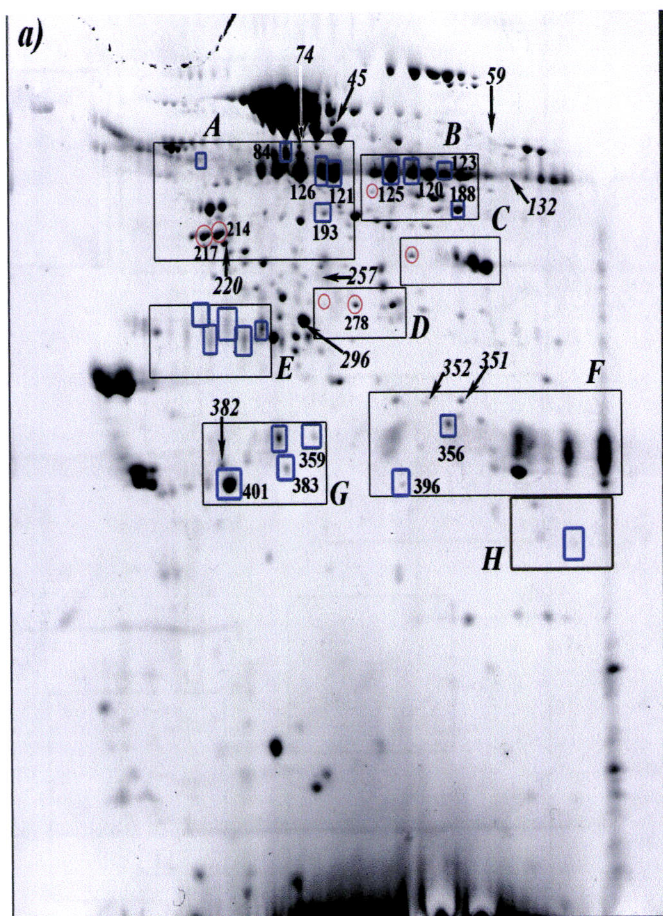


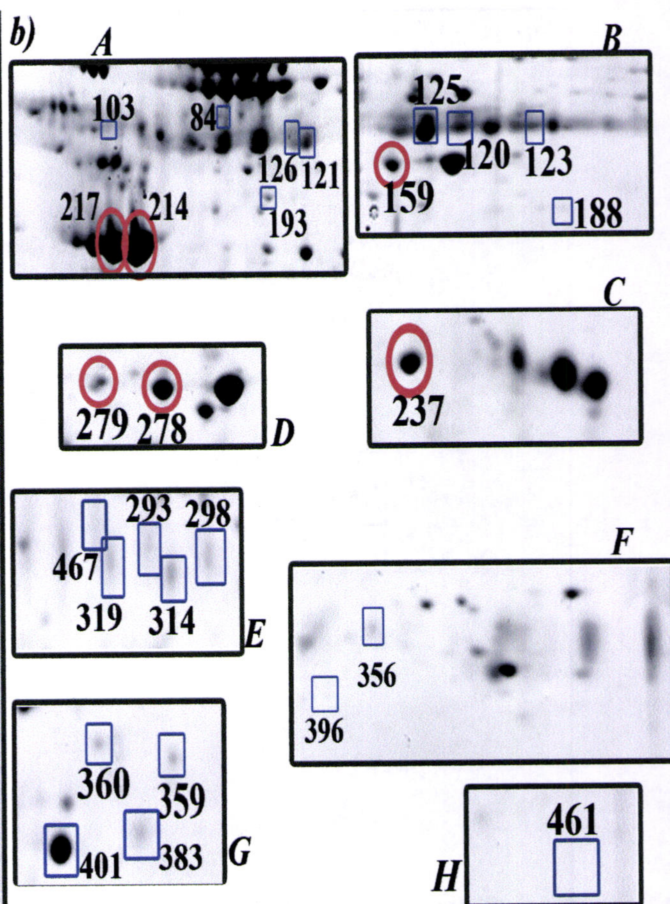
Figure 2 – Regions of marked differences between sheep and goats parotid saliva proteome. Upper images – goats; lower images - sheep

3.4. Effect of tannin consumption

With the quebracho-tannin levels used in this study, we did not observe the induction of new protein spots after 10-days' consumption, for both species. However, four protein spots from goats' and ten protein spots from sheep's 2-DE maps were not observed in any individual after tannin consumption, suggesting a reduction in the relative expression of these proteins / protein isoforms. These spots are signalled by arrows in Fig. 3. From these, only spot 220 disappeared from both species' 2-DE gels. Other isoforms of serum albumin also disappeared after tannin consumption, but only in one of the species (spot 388G and spots 45S, 296S, 351S and 382S).

A





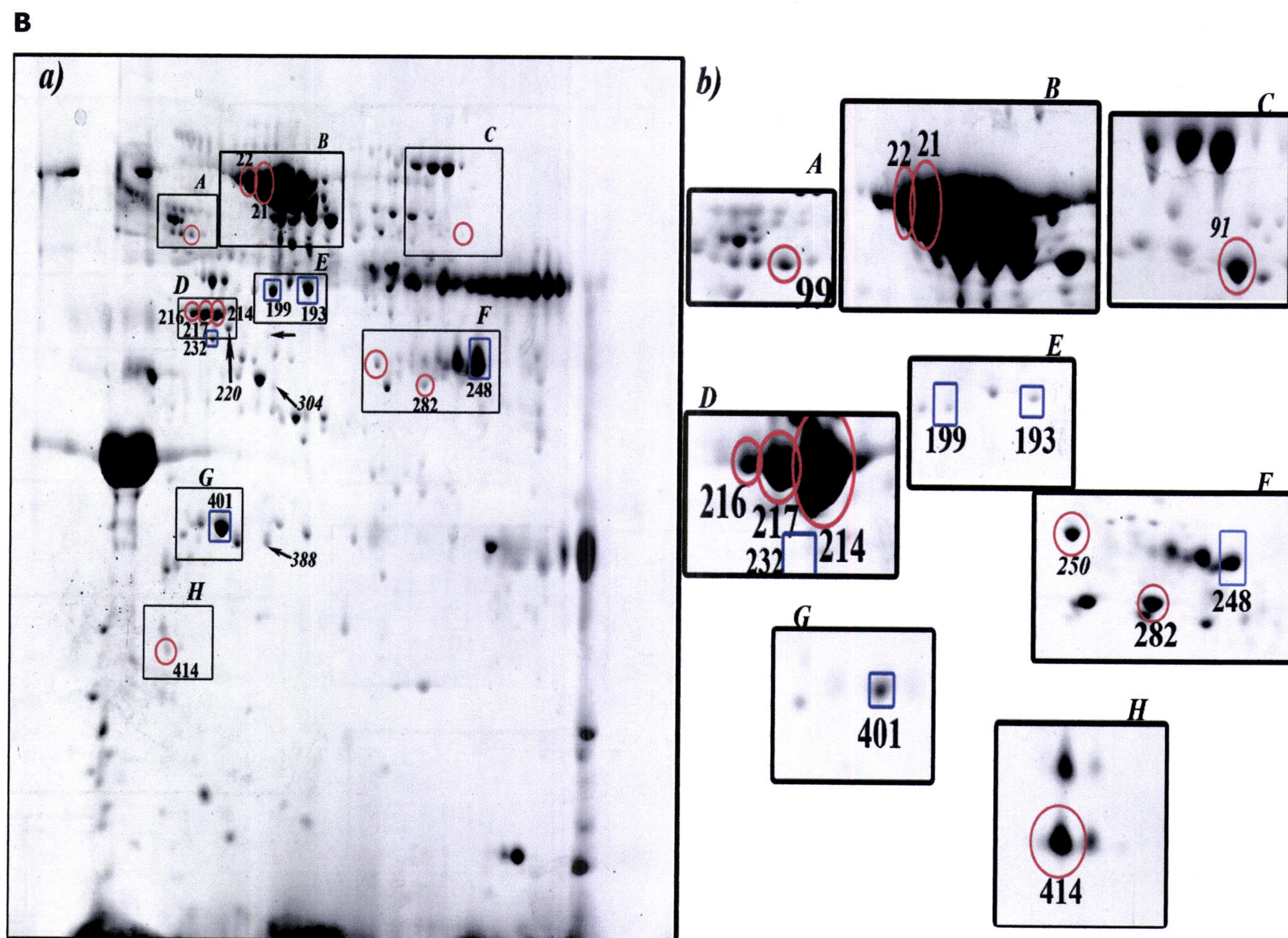


Figure 3 – Changes in parotid saliva 2-DE profile induced by quebracho tannin consumption. 2-DE profiles of parotid saliva from sheep (A) and goats (B) either in control conditions (a) or after 10-day quebracho consumption (b). Spots marked with arrows, in control gels, signal the spots which were not observed in quebracho gels. Spots circled show proteins up-regulated after quebracho tannin consumption, whereas spots squared show down regulated ones. Numbers correspond to the spot identifications listed in Tables 2 and 3.

Quantitative changes were also observed for both species. In both sheep and goats, the levels of the protein actin (spots 214, 217, for both species and 216G) increased after quebracho-tannin consumption. An increase in the expression levels of annexin A1 was also observed in both species, despite the fact that the isoforms that increased were different for the two species (spots 282G, 278S and 279S). The levels of three isoforms of serum albumin precursor (spots 21G, 22G and 414G) were also observed to increase in goats. For spot 414G (serum albumin precursor), the increase was pronounced and the levels obtained after tannin consumption greatly exceeded the levels observed in sheep (Tables 4 and 5). Two isoforms of leukocyte elastase inhibitor (spots 91G and 250G) were also observed to increase only in goats. On the other hand, one isoform of carbonic anhydrase VI (spot 237S) was observed to increase only in sheep. Additionally one spot from goats and one from sheep, which were not identified, were also observed to increase (Table 5 and Fig. 3).

Table 5 – Changes in protein expression levels (% Vol) induced by quebracho-tannin consumption (mean ± SD)

Spot Nº	Control	Quebracho	P*	Protein
Goat – *Decreases in expression level after tannin consumption*				
401	0.31 ± 0.24	0.02 ± 0.02	0.025	Apolipoprotein A1 precursor
232	0.03 ± 0.01	0.006 ± 0.005	0.025	Apolipoprotein A IV
248	1.32 ± 0.31	0.38 ± 0.34	0.025	Carbonic Anhydrase VI
193	0.33 ± 0.19	0.05 ± 0.01	0.025	Serum albumin precursor
199	0.16 ± 0.13	0.03 ± 0.005	0.025	
Goat – *Increases in expression level after tannin consumption*				
214	0.10 ± 0.09	2.12 ± 1.97	0.025	Actin cytoplasmic 1
216	0.07 ± 0.04	0.19 ± 0.04	0.025	
217	0.13 ± 0.14	0.76 ± 0.29	0.025	
282	0.03 ± 0.01	0.49 ± 0.35	0.025	Annexin A1
91	0.01 ± 0.01	0.07 ± 0.02	0.024	Leukocyte elastase inhibitor
250	0.03 ± 0.02	0.24 ± 0.12	0.025	
21	2.21 ± 1.34	5.75 ± 1.35	0.025	Serum albumin precursor
22	0.63 ± 0.57	1.57 ± 0.17	0.025	
414	0.01 ± 0.01	0.34 ± 0.38	0.022	
99	0.03 ± 0.01	0.07 ± 0.01	0.025	n.d.
Sheep – *Decreases in expression level after tannin consumption*				
383	0.06 ± 0.02	0.008 ± 0.01	0.032	Apolipoprotein A1 precursor
401	0.55 ± 0.16	0.06 ± 0.10	0.032	
293	0.10 ± 0.04	0.01 ± 0.02	0.032	Clusterin precursor Serum albumin precursor Alpha-S1-casein precursor
298	0.18 ± 0.11	0.01 ± 0.01	0.032	Clusterin precursor Alpha-S1-casein precursor
314	0.20 ± 0.10	0.02 ± 0.03	0.032	Alpha-S1-casein precursor Beta-lactoglobulin precursor Kappa-casein precursor Clusterin precursor Alpha-S2-casein precursor
319	0.13 ± 0.08	0.02 ± 0.02	0.034	Clusterin precursor
467	0.05 ± 0.02	0.006 ± 0.01	0.032	
120	0.42 ± 0.17	0.21 ± 0.05	0.034	Ig heavy chain C region
121	0.73 ± 0.18	0.27 ± 0.10	0.034	
123	0.29 ± 0.08	0.11 ± 0.10	0.034	
125	0.65 ± 0.18	0.43 ± 0.06	0.034	
126	0.31 ± 0.19	0.03 ± 0.05	0.032	
84	0.26 ± 0.17	0.06 ± 0.03	0.034	Serum albumin precursor
188	0.14 ± 0.10	0.01 ± 0.01	0.034	
193	0.10 ± 0.04	0.007 ± 0.01	0.032	

356	0.10 ± 0.03	0.01 ± 0.02	0.032	
359	0.07 ± 0.03	0.006 ± 0.01	0.032	
396	0.03 ± 0.01	0.003 ± 0.006	0.032	**n.d.**
103	0.03 ± 0.01	0.004 ± 0.008	0.032	**n.d.**
360	0.18 ± 0.07	0.03 ± 0.03	0.034	**n.d.**
461	0.23 ± 0.19	0.007 ± 0.012	0.032	**n.d.**
Sheep – *Increases in expression level after tannin consumption*				
214	0.21 ± 0.10	3.51 ± 2.68	0.034	**Actin cytoplasmic 1**
217	0.19 ± 0.08	1.01 ± 0.92	0.034	
278	0.05 ± 0.02	0.32 ± 0.14	0.034	**Annexin A1**
279	0.01 ± 0.009	0.05 ± 0.001	0.034	
237	0.04 ± 0.03	0.24 ± 0.02	0.034	**Carbonic Anhydrase VI**
159	0.007 ± 0.009	0.10 ± 0.06	0.032	**n.d.**

[a] Differences are significant for $P<0.05$
n.d. - not identified proteins

The decreases in the expression levels, with tannin consumption, were observed for several proteins (Table 5). One serum albumin isoforms (spot 193) and one isoform of apolipoprotein A-I (spot 401) decreased in both species. Additionally, Apolipoprotein A-IV (spot 232G) and carbonic anhydrase VI (spot 248G) also decreased in goats, whereas in sheep decreases were observed for different serum albumin isoforms (spot 84S, 188S, 356S, and 359S), immunoglobulins (spots 120S, 121S, 123S, 125S and 126S), clusterin, beta-lactoglobulin and/or caseins (spots 293S, 298S, 314S, 317S, 319S and 467S) and apolipoprotein A-I (spot 383S). It is interesting to note that such a decrease in expression levels of immunoglobulins, with quebracho-tannin consumption, was not observed for goats.

4. Discussion

4.1. Differences in salivary protein composition between small ruminants and other mammals

Saliva, in humans, has been studied considerably in recent years, by proteome approaches (Ghafouri et al, 2003; Vitorino et al., 2004; Wilmarth et al., 2004; Hardt et al., 2005; Hu et al., 2005; Xie et al., 2005; Walz et al., 2006, among others), allowing the cumulated identification of 1,116 accessions for saliva collected from parotid and submandibular/sublingual glands (Denny et al., 2008). As far as we know, oral fluids in other animals have been much less studied through proteomic techniques. Two-dimensional electrophoresis was used for the separation of parotid salivary proteins from cats (Williams and Marshall, 1998), rats (Williams et al., 1999a) and ferrets (Williams et al., 1999b), submandibular saliva of rats (Yamada et al., 2006) and, in ruminants, mass spectrometry was used to separate goat and bovine salivary proteins involved in teeth protection (Mau et al., 2006).

We have been using saliva as a mean of understanding oral adaptation to diet. When relating saliva composition to ingestive behaviour, comparing the observations from the present study with the results obtained from studies in non-ruminant species, marked differences are evident, which may be related to the different functions of this fluid among the different species.

Parotid saliva from humans presents protein concentrations ranging from 1 to 2 mg/mL (Dawes, 1984; Pogrel et al. 1996). Similar values were referred to for rodents (Williams et al., 1999a; da Costa et al., 2008). In this study, we observed much lower values for sheep (186.29 ± 92.08 µg/mL) and goats (155.12 ± 74.51 µg/mL) parotid saliva concentrations and there was little significant difference between the species. The values observed in the present study fit into the range reported by Patterson et al. (1982) for sheep and by Göritz et al. (1994) for grazers in general. Despite goats being intermediate feeders, it may be that in a tannin free diet they do not need higher levels of protein in their saliva than grazers.

Sheep and goats 2-DE maps showed great similarities in protein profile. As observed for protein concentration, small ruminants' 2-DE patterns obtained also present marked differences from the profiles of other species, such as humans (Hardt et al., 2005; Walz et al., 2006) and rats (Williams et al., 1999a). The differences are mainly in terms of the lack of detection of proteins such as amylase, cystatins, proline-rich proteins and kallikreins, among others, and the observation of a high proportion of serum proteins in sheep and goats parotid saliva proteome. This greater proportion of serum proteins was previously observed in one-dimensional SDS-PAGE separation (Lamy et al., 2008). We found out that 2-DE maps from sheep and goats parotid saliva have greater similarities with 2-DE maps from bovine plasma (Wait et al., 2002) than with non-ruminant saliva 2-DE profiles.

The main quantitative and qualitative differences observed among the different trophic groups may reflect the different roles played by saliva in digestive processes. Saliva is the major digestive secretion in the ruminant, with daily inputs of 6 to 16 litres in sheep (Kay, 1960). Approximately 70 to 90% of all the fluid entering the rumen is saliva (Bailey, 1961) and its importance is mainly in providing the fluid for the transport of feed particles to the lower gut, in maintaining pH conditions between 6 and 7 for adequate microbial fermentation and in avoiding rises in rumen tonicity.

The presence of serum proteins in mixed saliva has been reported as coming from crevicular fluid and some from serum leakage. But as far as glandular secretions are concerned, the presence of serum proteins, in these last, is not well understood. Studies in mammalian salivary glands have suggested that the tight junctions may become permeable to various organic substances, including proteins (Junqueira et al., 1965; Parsons et al., 1977) and that permeability is, at least in part, dependent upon secretory stimulation (Mazariegos et al., 1984; Segawa, 1994; Asztély et al., 1998; Hashimoto et al., 2003). It has been shown that, at the ultrastructural level, ruminant parotid glands present some particularities (VanLennep et al., 1977; Shackleford and Wilborn, 1969; Stolte and Ito, 1996) that

differ from non-ruminants. We can speculate that those differences may be responsible for a higher passage of serum proteins from plasma to saliva, but to answer this question, additional ultrastructural studies would be necessary, which are beyond the scope of the present paper.

Besides the presence of serum proteins, the presence of cytoplasmic proteins, such as actin, is found in sheep's and goat's parotid saliva. This may be explained by the unusual feature of an apocrine-like secretion by the parotid glands of ruminants (Suzuki et al., 1981; Stolte and Ito, 1996), as it was already discussed (Lamy et al., 2008).

4.2. Differences between sheep and goat parotid saliva proteome

The two ruminant species investigated differ in their feeding strategies (Hofmann, 1989). Differences in the protein composition of their parotid saliva were recently observed by one-dimensional SDS-PAGE electrophoresis (Lamy et al., 2008). Mau et al. (2006) also observed differences between grazers (represented by cattle) and intermediate feeders (represented by goats) in one-dimensional SDS-PAGE profiles of whole saliva.

The high number of protein spots observed in 2-DE maps did not result in the identification of a high number of different proteins, but instead in different isoforms of some proteins and it is possible to conclude that a great part of the differences between sheep and goats parotid saliva composition is in terms of the protein isoforms expressed, rather than in terms of different protein composition. Despite similarities between sheep and goats for the proteins identified, there were also a few proteins which were identified in only one of the species. Three proteins were identified for spots only observed in goat parotid saliva proteome: apolipoprotein A-IV, hemoglobin and cathelicidin-3 precursor. In addition, the proteins clusterin, haptoglobin, and transthyretin precursor were identified for spots only observed in sheep 2-DE maps. With the exception of cathelicidin-3, all of these proteins are characteristically present in plasma and we were unable to find an explanation for the presence of each of them in the parotid saliva of one of the species and the absence in the other. Cathelicidin-3 was only identified in goat parotid saliva, but other members of the cathelicidin family were identified in sheep´s fluid, namely cathelicidins 1 and 2, with the particularity of cathelicidin-1 being expressed in higher amounts in sheep parotid saliva compared to goats. Cathelicidins are a widely expressed family of mammalian antimicrobial peptides that have a broad-spectrum activity against bacteria, fungi and envelop viruses (Zannety, 2005), which were already observed to be expressed in murine salivary glands and human whole saliva (Murakami et al., 2002), being considered as "natural antibiotics" (Nizet and Gallo, 2003). It is possible that the cathelicidins forms with higher expression levels in sheep parotid saliva "compensate" for the presence of different forms in goat parotid saliva, or that this difference may relate to differences in microbial ecology in these two ruminant species and consequently different needs in "antibiotic" action.

With regard to the proteins annexin A3, alpha-enolase, leukocyte elastase inhibitor and lactoferrin identification was only obtained in goat spots. These (spots 303, 202, 91, 250, 32 and 41, respectively) may not represent a real difference, since, in the peptide mass spectra of the correspondent sheep spots, several mass peaks of the referred proteins could be observed. The lack of identification may be attributed to the presence of other peptides, which may possibly correspond to different unidentified proteins.

The proteins beta-lactoglobulin, clusterin and three forms of casein were identified for six spots in sheep. The spots common to both species, which were only confidently identified for sheep, were present in higher levels in this species (Table 4). Both beta-lactoglobulin and caseins are proteins present in high amounts in sheep and goats' milk. It has been commonly accepted that the mammary gland is the sole organ in which these proteins are synthesized. However, authors such as Pich et al. (1976) and Onoda and Inano (1997) localized caseins in human and rat organs other than the mammary gland, among which the salivary glands.

From the identified proteins whose differences in expression levels were observed, one isoform of serum transferrin (spot 9) and one isoform of serum albumin (spot 199) were found to be present at higher levels (% Vol) in goats than in sheep. In contrast, one serum albumin isoforms (spot 16), one carbonic anhydrase VI isoforms (spot 234) and the two cathelicidin-1 isoforms (spots 406 and 433) were present at higher levels in sheep than in goats (Table 4). Concerning carbonic anhydrase VI, it is interesting to note that a lower number of isoforms were observed in sheep's 2-DE maps (4 different spots) than in goats' 2-DE maps (7 different spots), but those present in sheep's show a tendency for being expressed at higher levels (data not shown, only statistically significant differences were presented in Table 4). The explanation for all these differential isoform expressions is beyond the scope of this article. Glycosylations and phosphorylations are post-translational modifications that may explain the presence of different spots with the same identification (Table 1 – supplementary material). These modifications are often essential for the function of the proteins (Temporini et al., 2008) and consequently these differences in isoform expression may be thought in terms of physiological differences between the species. To more detailed conclusions on these differences it would be necessary further studies to characterize the activity of each isoform.

In the gel region between molecular masses 25-35 kDa and pI 4-5, differences in sheep and goat parotid protein composition were consistently observed in 2-DE electrophoresis maps (Fig. 2). Differences in the same molecular mass range were previously observed in one-dimensional electrophoresis protein separation (Lamy et al., 2008), and both studies suggest that this may be an important region of differences between the species. With LC-MS/MS we were able to identify BSP30b for the group of spots composed by 384S, 386S and 395S and for the group 333G and 334G. This protein *per se* does not suggest a real difference between the species, but the high number of *m/z*

peaks, which do not correspond to the theoretical tryptic digestion of BSP30b suggests the presence of other(s) different protein(s). Moreover, the group composed by the spots 325S, 329S, 344S and 345S failed to be identified even by LC-MS/MS.

4.3. Effect of tannin ingestion on sheep and goat parotid saliva proteome

The presence of tannin-binding proteins in the saliva of species which have to deal with high levels of these compounds in their regular diet has been reported (Robbins et al., 1991; Mehansho et al., 1992; Clauss et al., 2003). Proline-rich proteins (PRPs) have been, so far, the most studied salivary proteins with defense functions against the potential harmful effects of tannins. Their presence has been reported for some browsers (Fickel et al., 1998; Clauss et al., 2005), whereas they have been reported to be absent in grazers (Austin et al., 1989). To access their presence in sheep and goat parotid saliva, we stained the gels with Coomassie Brilliant Blue R-250, following Beeley et al. (1991) protocol, according with which PRPs stain pink. We did not observe pink spots, suggesting the absence of these proteins both in tannin-free fed animals and after a 10-day period of quebracho consumption, which accords with some authors (Austin et al., 1989; Distel and Provenza, 1991).

Feeding quebracho tannins significantly increased sheep's (up to 396.74 ± 82.1 μg/mL) and goats' (up to 355.02 ± 270.77 μg/mL) parotid saliva protein concentration to almost twice the levels. Gilboa (1995) found higher concentrations of proteins in parotid saliva of goats fed diets with highly condensed tannin levels than in goats fed only wheat straw (550 μg/mL vs. 212 μg/mL). This increase in parotid saliva protein concentrations may be due to the β-adrenergic stimulation (Edwards and Titchen, 1992; 2003) which seems to be elicited by tannin consumption (Waters et al., 1998).

Despite the dramatical increase in protein concentration observed, no new protein spots were detected in the mass range covered by 2-DE gels. However, the proteome has changed in terms of the expression levels of individual spots.

Consumption of quebracho tannin resulted in the increase of expression of actin and in one of the isoforms of annexin A1, in both species. The isoforms of actin that increased with tannin consumption were common to both species, in contrast to what was observed for annexin A1 (spot 282 for goats and 278 for sheep). Again, this may be indicative of some functional differences. Actin filaments are constituents of cytoskeleton, and may participate in the processes involved in protein secretion (Valentijn et al., 1999). In pancreatic acinar cells, it was observed that the actin network under the plasma membrane had a direct involvement in exocytosis (Muallem et al., 1995). The increase in the several forms of actin may be related to the particular "apocrine like" type of salivary secretion presented by ruminants (Stolte and Ito, 1996). With quebracho consumption, salivary protein secretion increased, as was observed from the protein concentration values obtained. It is probable

that actin appeared in parotid saliva through the small portions of cytoplasm that accompany salivary proteins. Annexin A1 levels have already been observed to increase in human whole saliva, following bitter and/or sour stimulation (Neyraud et al., 2006). Tannins have been reported as having astringent/bitter properties and annexin A1 induction may result from a mechanism of inflammatory-like responses, similar to that reported for the increase of annexin A1 levels in human saliva (Neyraud et al., 2006). Leukocyte elastase inhibitor was also suggested as having anti-inflammatory functions (Doumas et al., 2005). Its increase in expression, after tannin consumption, observed for goats may follow the same principle described for annexin A1.

In sheep, five isoforms of immunoglobulins decreased, whereas in goats, immunoglobulins levels remained unchanged. As well, clusterin isoforms, identified in sheep, also decreased after tannin consumption. These last proteins were proposed to interact with immunoglobulins (Wilson et al., 1991). Some studies have suggested that tannins may act at immunitory system level, inhibiting immunoglobulin synthesis (Marzo et al., 1990; Takano et al., 2007). This decrease in immunoglobulin levels, only observed in sheep, goes in accordance to studies demonstrating the lower tolerance that sheep present to tannins, compared with goats (Narjisse et al., 1995).

As far as albumin is concerned, several spots were observed to disappear and/or decrease in both species after tannin consumption. However, in goats, some also increased. These different responses by different isoforms suggest, once more, that they may have different functions.

Apoliprotein A1 decreased in both species. Apolipoprotein A1 is the major protein component of high density lipoprotein (HDL) in plasma. It is known that the availability of volatile fatty acids (VFA) is crucial for the *de novo* synthesis of cholesterol and other lipids in ruminants (Bell, 1981) and that the amount of VFA produced reflect the fiber fermentation capacity of the animal. Since tannins can affect fermentative characteristis in ruminants it is possible that this influence the circulating levels of cholesterol and, consequently, the circulating levels of apolipoprotein A1.

5. Conclusions

Sheep and goats parotid protein profiles seem to be closer to plasma protein profiles than to non-ruminant saliva protein profiles. This may be representative of the primary role of ruminant parotid as electrolyte and fluid secreting gland rather than protein secreting gland as it occurs in non-ruminant animals. Sheep and goat parotid saliva present few differences in terms of individual proteins, contrarily to what occurs when they are compared with humans or rodents. The major differences in parotid protein profile between the two ruminant species are in terms of protein isoforms present. The ingestion of tannins resulted in changes in parotid protein profile in both species. However the majority of changes were different between sheep and goats, what can be related to the different

feeding behaviour of these species. Namely, the lower tolerance to tannins, presented by sheep was illustrated in 2-DE maps, by the decrease in immunoglobulin levels, which has been associated to an action of tannins at immunitory system level.

The present work is a starting point for the use of proteomics to identify physiological adaptations that can be related to ingestive behaviour, so our observations should be interpreted with caution. Firstly, in gel regions with high spot densities, quantification using two-dimensional electrophoresis may be affected by spot overlapping. Also, a larger number of individuals from each specie is needed in order to establish the importance of these findings. The presence of a high number of protein spots identified as serum albumin, some with a considerable intensity, can make difficult the detection of low abundant proteins or different proteins, with molecular masses and pI similar to some albumin spots and, as such, hidden by those spots. A depletion of serum albumin from the samples in human body fluids has been reported to be useful (Plymoth et al., 2003) and we propose, in further studies, to test this methodology for sheep and goat parotid saliva.

Nevertheless, the study of salivary proteome lines up as a promising technique that can contribute to a better understanding of the ingestive behaviour control, namely those related to immediate adaptations responsible for the pre-gastric control of ingestion.

Supplementary Table 1 – Predicted PTMs and protein processing for the proteins identified by PMF

<table>
<tr><th rowspan="3">Protein</th><th rowspan="3">Accession nº</th><th rowspan="3">Spot</th><th colspan="5">Modified residues</th><th rowspan="3">Signal and/or propeptide[3]</th><th rowspan="3">Ref.</th></tr>
<tr><th colspan="2">Glycosylation[1]</th><th colspan="3">Phosphorylation</th></tr>
<tr><th>N-glyc.[2]</th><th>O-glyc.</th><th>Tyr</th><th>Thr</th><th>Ser</th></tr>
<tr><td rowspan="8">Actin cytoplasmic</td><td rowspan="8"><u>P60713</u></td><td>214G</td><td rowspan="7">12-14</td><td rowspan="5">33, 52, 60, 66, 89, 186, 194, 199, 201, 202, 203, 318, 323, 324</td><td rowspan="2">166[§], 53, 218</td><td>66, 149, 318[§]</td><td rowspan="2">52, 60, 141, 145, 232, 233, 234, 235, 323</td><td rowspan="8">1-1</td><td rowspan="8">Stea et al., 2007</td></tr>
<tr><td>216G</td><td rowspan="2">149, 318[§]*</td></tr>
<tr><td>217G</td><td>53, 218, 240</td><td>52, 60, 141, 145, 232, 233, 234, 235, 239, 323</td></tr>
<tr><td>213S</td><td>53, 166[§], 218, 362</td><td>66, 149, 318[§]</td><td>52, 60, 141, 145, 232, 233, 234, 235, 323</td></tr>
<tr><td>214S</td><td>166[§], 218</td><td>66, 149</td><td>141, 145, 232, 233, 234, 235, 323</td></tr>
<tr><td>216S</td><td>33, 52, 60, 66, 186, 194, 199, 201, 202, 203, 318, 323, 324</td><td>166[§], 218, 294</td><td>66, 149, 304</td><td>13*, 141, 145, 232, 233, 234, 235, 323</td></tr>
<tr><td>217S</td><td>33, 52, 60, 66, 89, 106, 186, 194, 199, 201, 202, 203, 239, 249, 318, 323, 324</td><td>53, 166[§], 218, 362</td><td>66, 149</td><td rowspan="2">52, 60, 141, 145, 232, 233, 234, 235, 323</td></tr>
<tr><td>220S</td><td>12-14*</td><td>33, 52,60, 66, 89, 186, 194, 199, 201, 202, 203, 318, 323, 324</td><td>53, 166[§], 218, 294</td><td>66, 149, 304</td></tr>
<tr><td>Alpha-1-antiproteinase</td><td><u>P12725</u></td><td>63G</td><td>68-70; 105-107; 143-145; 269-275</td><td>4, 6, 58, 151, 162, 166, 187, 236, 237, 304, 305, 314, 316, 318, 361, 401, 403, 414</td><td>182, 253</td><td>38, 236, 237, 361, 401</td><td>187, 335</td><td>1-24</td><td>Vaughan et al., 1982; Mistry et al., 1991</td></tr>
</table>

		66G		4, 6, 58, 151, 162, 166, 187, 236, 237, 304, 305, 314, 316, 318, 323, 361, 401, 403, 414,					
		63S		4, 6, 151, 162, 166, 187, 236, 237, 314,316, 318, 323, 361, 401, 403, 414					
		66S		4, 6, 162, 166, 187, 323, 361, 401, 403, 414					
Annexin A1	**P46193**	280G	Does not present a consensus region	28, 64, 145, 182, 265, 291, 295, 302	21, 335	**24**§, 56, 291	28, 244, 302, 304	-----	Solito et al. 2006
		282G		24, 28, 291	176	**24**§, 56, 172, 216, 226, 291	28, 170, 173, 189, 201, 244, 302, 304		
		278S		-----	21, 176, 335	**24**§, 56, 172, 216, 226, 291, **295***	28, 170, 173, 244, **302***, 304		
		280S		5, 24, 28, 56, 64, 70, 145, 170, 172, 173, 182, 229, 241, 244, 265, 272, 273, 291, 295, 302, 304,	-----	**24**§, 56, 216, 226, 291, 295*	**5**§, 244, 302*		
Apolipoprotein A1	**P15497**	371G	Does not present a consensus region	48, 102, 104, 165, 181, 198, 219,	41, **189***	75, 198	29, 30, 48, 10, 165, **190***, 210, 259	Signal - 1-18 Propep - 19-24	Hoeg et al., 1986
		394G		48, 102, 104, 181, 198, 219	41, **189***	198	29, 30, 48, 104, **190***, 259		
		401G		39, 48, 75, 78, 79, 81, 102, 104, 165, 181, 198, 210, 219, 246, 250, 259	41, **138***, **189***	75, 198	20, 30, 104, **190***		

		405G		165, 181, 198, 246	41	75, 198	29, 30, 48, 104, 165, 210, 259		
		383S		39, 48, 102, 104, 165, 181, 190, 198, 210, 219, 246	41, **189***	75, 198	29, 30, 104		
		394S		181, 198, 219	-----	198	29, 30, 104, 210		
		401S		48, 102, 104, 181, 198, 219	**214***	198	29, 30, 104, **210*, 219***		
		402S		48	41	75, 198	29, 30, 48, 104, 210, 259		
Apolipoprotein A IV[7]	**Q32PJ2**	232G	Does not present a consensus region	92, 144, 148, 158, 222, 257, 288, 293, 294, 352, 353, 357	72	92, 158, 202, 210, 324, 357	70, 95, 222, 257, 288,294, 353	1-20	Weinberg and Scanu, 1983
Carbonic anhydrase VI	**P08060**	228G	50-52 239-241	31, 45, 131, 155, 161, 165, 176, 177, 223, 225, 233, 241, 249	129, 132, 154, 159	82, 204, 219	105, 127, 131, 223	Doesnot present signal peptide	Fernley et al. 1988; Feldstein and Silverman, 1984; Murakami and Sly, 1987; Hooper et al., 1995; Vitorino et al. 2004
		234G		223, 225					
		239G		31, 45, 127, 131, 155, 161, 165, 176, 177, 223, 225, 233, 241, 249					
		248G		31, 45, 131, 155, 161, 165, 176, 177, 223, 225, 233, 241, 249			131, 223		
		265G		31, 45, 127, 131, 155, 161, 165, 176, 177, 223, 225, 233, 241, 249			105, 127, 131, 223		
		271G		-----					
		463G		31, 223, 225					
		234S		176, 177, 223, 225, 249	129, 132, 154, 159	82, 204, 219	105, 127, 131, 223		

		239S		31, 45, 127, 176, 177, 223, 225, 233, 249,	129, 132		105, 131, 223		
		248S		31, 45, 176, 177, 223, 225, 249	129, 132, 154, 159		223		
		463S		31, 45, 131, 155, 161, 165, 176, 177, 223, 225, 233, 241, 249	129, 132, 154, 159	82,204, 219	105, 131, 223		
Cathelicidin-1[6]	**P54230**	433G 406S 433S	-----	-----	-----	-----	-----	Signal – 1-29 Propeptide – 30-143	Bagella et al. 1995; Huttner et al., 1998
Cathelicidin-2[6]	**P82018**	424G 427S	-----	-----	-----	-----	-----	Signal – 1-29 Propeptide – 30-130 Propeptide (removed in mature form) – 174-176	Shamova et al., 1999
Cathelicidin-3[6]	**P50415**	501G	-----	-----	-----	-----	-----	Signal – 1-29 Propeptide – 30-130	Bagella et al., 1995
Deoxyribonuclease	**P11937**	273G	18-20 106-108	10, 14, 43, 75, 119, 120, 122, 123	65, 175	123	43, 75, 90, 108, 110, 119, 122, 182	Does not present signal peptide	Lundblad et al. 1977; Paudel and Liao, 1986; Williams et al. 1999a
		301S	**18-20*** 106-108	10, 14, 75, 119, 120, 122, 123	175				
Hemoglobin subunit beta-A	**P02077**	444G	Does not present a consensus region	3, 11, 37, 69, 71, 83, 88	-----	-----	69	Doesnot present signal peptide	Bunn et al., 1978; Shapiro et al., 1980
		445G		3, 11, 37, 69, 71, 83, 88	-----	-----	-----		
Lactoferrin	**Q5MJE8**	32G	187-189, 300-302, 387-389, 495-497, 532-534, 564-566	302, 653, 655	111, 417, 439, 452	103, 232, 353, 362, 395, 571, 596, 682	60, 156, 204, 278, 302, 310, 394, 436, 437, 440, 507, **518***, **519***, 538, 575, 576, 618, 620, 697	Signal peptide [3] – 1-19	Castellino et al., 1970; Ramachandran et al., 2006

		41G	187-189, **252-254***, 300-302, 387-389, 495-497, 532-534, 564-566	-----	111, 246, 417, 439, 452	103, 232, 353, 362, 395, 571, 596, 682	60, 156, 204, 278, 302, 310, 394, 436, 437, 440, 507, 538, 575, 576, 618, 620, 697		
Serotransferrin	**Q29443**	5G	**36-38***, **495-497***, **514-516***, 644-646	-----	113, 261, 449	24, 105, 254, 375, 444, 528, 593, 661, 692	40, 71, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 615, 637	Signal peptide – 1-19	Richardson et al., 1973; Aisen and Listowsky, 1980; Maeda et al., 1980; Yang et al., 2005; Ramachandran et al., 2006
		6G	36-38, 495-497, 514-516, 644-646	25, 40, 123, 135, 138, 143, 197, 278, 330, 352, 357, 358, 375, 444, 457, 458, 472, 475, 528, 615, 621, 661, 666, 685			40, 62, 71, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 615, 637		
		8G		-----		24, 105, **138***, 254, 375, 444, 528, 593, 692,	40, 62, 71, **135***, **143***, 203, 257, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 615, 637		
		9G	**495-497***, **514-516***, **644-646***	-----		24, 105, 254, 375, 444, 528, 593	40, 62, 71, **143***, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 615, 637		
		500G	36-38, 495-497, 514-516, 644-646	-----	113, 261, 417, 449	24, 105, 254, 375, 444, 528, 593, 661, 692	40, 62, 71, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 515, 524, 615, 637		

		5S		-----	261, 449	24, 105, **138***, 254, 375, 444, 528, 593, 692	40, 62, 71, **135***, **143***, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 536, 615, 637		
		6S	36-38, **495-497***, **514-516***, **644-646***	-----	113, 261, 449		40, 62, 71, **135***, **143***, 203, 257, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 615, 637		
		8S	36-38, **495-497***, 514-516, 644-646	24, 278	113, 261, 417, 449	24, 105, 254, 375, 444, 528, 593, 692	40, 62, 71, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 515, 516, 524, 615, 637		
		9S	495-497, 514-516, 644-646	-------	261, 449	24, 105, **138***, 254, 375, **392***, **396***, 444, 528, 593	40, 62, 71, **135***, **143***, 203, 257, 310, 330, 352, 357, 358, **381***, **384***, 440, 511, 515, 516, 524, 615, 637		
		12S		278	113, 261, 417, 449	24, 105, 254, 375, 444, 528, 593, 692	40, 62, 71, 203, 257, 310, 330, 352, 357, 358, 381, 384, 440, 511, 516, 524, 615, 637		
		500S	36-38, 495-497, 514-516, 644-646	24, 375, 593	**113***, **114***, 261, 417, 449	24, 105, **111***, 254, 375, 444, 528, 593, 661, 692			
Vitamin D-binding protein	**Q3MHN5**	75G	86-88, **287-289***	34, 231, 289, 290, 293, 366, 367, 392, 445, 449	84, 163, 280, 460	303, 339, 353, 367	34, 49, 184, 192, 234, 268, 289, 350, 370, 377, 449, 463	Signal – 1-16	Litwiller et al., 1986; Christiansen et al., 2007

		77G		366, 367	84, 136, 163, 280, 460	339, 353, 367	34, 49, 184, 192, 268, 289, 350, 370, 377, 449, 463		
		75S		34, 231, 289, 290, 293, 366, 367, 397, 445, 449	84, 163, 280, 460	303, 339, 353, 367	34, 49, 184, 192, 234, 268, 289, 350, 370, 377, 449, 463		
		77S		34, 88, 89, 92, 231, 234, 289, 290, 293, 366, 367, 392, 396, 397	84, 163, 280, 460	303, 339, 353, 367	34, 49, 89, 92, 184, 192, 221, 234, 268, 289, 350, 370, 377, 449, 463		

[1] Only the peptides for which FindMod did not suggested to be potentially modified were further submitted to GlycoMod;
[2] According with the existence of a consensus region and the absence of the peptide from tryptic peptide map obtained by MALDI TOF MS;
[3] Predited from Signal IP 3.0 (http://ww.cbs.dtu.dk/services/SignalIP/);
[4] For this protein it was referred the possibility of genetic polymorphisms (Kamboh et al. 1994);
[5] For this protein it was referred the possibility of molecular weight heterogeneity (Tsuji et al., 1984);
[6] For these proteins no potential PTMs were searched, since the existence of propeptide and its cleavage explain the differences between the observed ad the theoretical MW and pI;
* with numbers in bold: potential modified residue also predicted submitting the non-matched peptide list to search on FindMod and/or Glycomod;
* with numbers in bold and underlined: residues predicted as phosphorylated by submitting the non-matched peptide list to search on FindMod and which have simultaneously a significant score according NetPhos 2.0;
§ Indicated in SwissProt as potential sites of phosphorylation. Underlined when score is significant also according NetPhos2.0

Supplementary Table 2 – Proteins identified using LC-MS/MS data

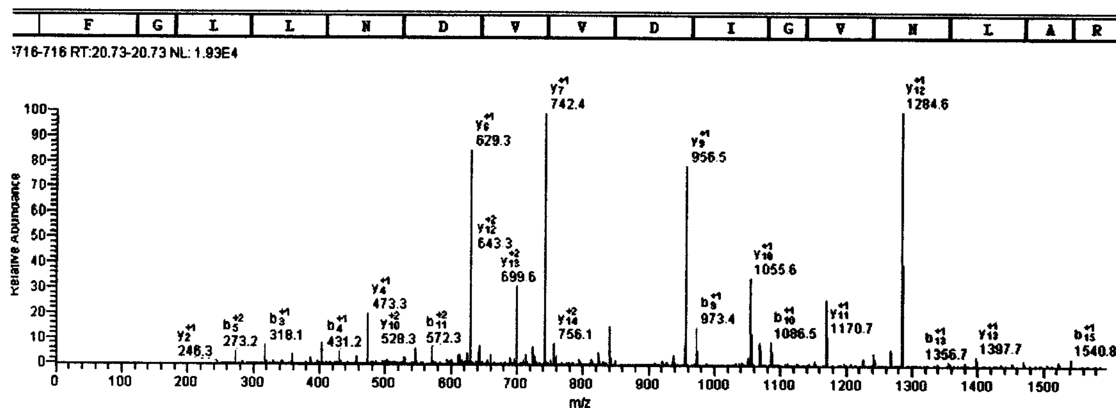
Spot	Protein name	Accession number[a]	Identified peptides	[MH]+	z[b]	Score	p value[c]
91G	Leukocyte elastase inhibitor	**Q1JPB0**	IPELLASGMVDSLTK	1573	2	4.96	2.85E-06
			VLELPYEGK	1047	2	2.31	8.29E-04
			DLSMVILLPDDIQDEATGLK	2186	3	4.06	4.08E-07
			DLSMVILLPDDIQDEATGLKK	2314	2	4.00	5.82E-11
127G	Cytosolic non-specific dipeptidase	**Q3ZC84**	FCLEGMEESGSEGLDALIFAQK	2374	3	5.80	4.13E-12
			WRYPSLSLHGIEGAFSGSGAK	2220	3	4.14	1.03E-06
			YPSLSLHGIEGAFSGSGAK	1877	2	5.15	1.11E-14
			LVPNMTPEVVSEQVTSYLTK	2235	2	2.89	1.17E-10
			TVFGVEPDLTR	1233	2	2.51	9.22E-06
			EGGSIPVTLTFQEATGK	1734	2	3.49	1.18E-04
	Serum albumin precursor	**P14639**	MLAAYLYEVSQLKD	1643	2	4.78	1.97E-08
			DVFLGSFLYEYSR	1595	2	3.68	5.91E-08
			KAPQVSTPTLVEISR	1625	2	3.04	1.27E-07
194G	Serum albumin precursor	**P14639**	ADFTDVTKIVTDLTK	1666	2	4.32	4.04E-09
			DVFLGSFLYEYSR	1595	2	4.03	9.71E-08
			NCELFEKHGEYGFQNALIVR	2367	3	5.89	8.88E-15
			KAPQVSTPTLVEISR	1625	2	3.55	1.62E-09
			CCAKPESERMPCTEDYLSLILNR	2671	3	3.53	6.19E-07
			ATDEQLKTVMENFVAFVDK	2185	2	5.79	1.14E-11
228S	Carbonic anhydrase	**P08060**	QMHFHWGGASSEISGSEHTVDGMR	2643	3	3.28	4.21E-04
			YVIEIHVVHYNSK	1600	2	4.94	4.77E-11
			YNSYEEAQKEPDGLAVLAALVEVK	2636	3	6.10	1.64E-12
			FISHLEDIR	1129	2	2.96	1.75E-04
			GLDIEDMLPGDLR	1443	2	4.04	1.19E-06
			VVEANFMSRPHQEYTLASK	2207	3	3.84	3.90E-08
			LHFYLNNIDQTLEYLR	2052	2	5.88	2.45E-13
250G	Leukocyte elastase inhibitor	**Q1JPB0**	IPELLASGMVDSLTK	1573	2	4.13	1.27E-07
			DLSMVILLPDDIQDEATGLK	2186	2	3.86	3.71E-10
			DLSMVILLPDDIQDEATGLKK	2314	3	3.57	5.20E-09
279G	Annexin A	**P46193**	QAWFIENEEQEYIK	1826	2	4.80	1.24E-04
			GGPGSAVSPYPTFNPSSDVEALHK	2414	3	3.89	1.75E-09
			GVDEATIIEILTK	1401	2	3.66	2.45E-05
			GVDEATIIEILTKR	1557	2	3.48	2.36E-06
			GLGTDEDTLNEILASR	1703	2	4.76	4.04E-07
			VLDLELKGDIEK	1371	2	3.41	3.59E-05
293S	Clusterin precursor (Glycoprotein III) (GpIII) [contains clusterin alpha and beta chain]	**P17697**	KLLLSSLEEAK	1230	2	3.70	4.58E-06
			KLLLSSLEEAKK	1358	2	4.52	1.41E-06
			LLLSSLEEAK	1102	2	3.05	4.80E-06
			IDSLMENDREQSHVMDVMEDSFTR	2884	3	4.62	5.02E-08
			ASSIMDELFQDR	1411	2	3.91	1.82E-06
			RPQDTQYYSPFSSFPR	1975	3	3.90	2.10E-08
	Serum albumin precursor	**P14639**	DVFLGSFLYEYSR	1595	2	4.02	8.02E-08
			FFTFHADICTLPDTEK	1884	2	5.22	8.23E-11
	Serum albumin precursor; n=1	**P02769**	MPCTEDYLSLILNR	1667	2	4.18	5.87E-05
			TVMENFVAFVDK	1399	2	4.53	1.04E-06
	Alpha-S1-casein precursor	**P02662**	FFVAPFPEVFGK	1384	2	3.31	7.63E-06
			YLGYLEQLLR	1267	2	3.65	1.44E-05

Spot	Protein name	Accession number[a]	Identified peptides	[MH]+	z[b]	Score	p value[c]
298S	Clusterin precursor (Glycoprotein III) (GpIII) [contains clusterin alpha and beta chain]	**P17697**	KLLLSSLEEAK	1230	2	3.92	1.07E-05
			KLLLSSLEEAKK	1358	2	4.09	7.91E-06
			LLLSSLEEAK	1102	2	3.22	3.03E-05
			ASSIMDELFQDR	1411	2	4.16	1.05E-05
			RPQDTQYYSPFSSFPR	1975	2	4.97	9.64E-12
	Alpha-S1-casein precursor	**P02662**	FFVAPFPEVFGK	1384	2	3.28	8.51E-07
			YLGYLEQLLR	1267	2	3.68	7.20E-06
314S	Alpha-S1-casein precursor	**P02662**	HQGLPQEVLNENLLR	1759	2	4.51	1.62E-10
			FFVAPFPEVFGK	1384	2	2.75	3.40E-08
			YLGYLEQLLR	1267	2	3.73	6.50E-06
	Beta-lactoglobulin precursor	**P02754**	VYVEELKPTPEGDLEILLQK	2313	3	6.19	1.93E-12
			TPEVDDEALEKFDK	1635	2	4.67	1.63E-06
	Kappa-casein precursor [Contains: Casoxin-C; Casoxin-6; Casoxin-A; Casoxin-B; Casoplatelin]	**P02668**	YIPIQYVLSR	1251	2	2.52	9.91E-04
			SPAQILQWQVLSNTVPAK	1980	2	6.03	1.74E-09
	Clusterin precursor (Glycoprotein III) (GpIII) [Contains: Clusterin beta and alpha chain]	**P17697**	LYDQLLQSYQQK	1526	2	5.20	1.47E-06
			LFNSFPITVTVPQEVSSPNFMENVAEK	3024	3	5.51	2.75E-10
	Alpha-S2-casein precursor [Contains: Casocidin-1]	**P02663**	ALNEINQFYQK	1367	2	3.64	9.20E-05
			FALPQYLK	979	2	2.48	2.98E-03
317S	Clusterin precursor (Glycoprotein III) (GpIII) [Contains: Clusterin beta and alpha chain]	**P17697**	LYDQLLQSYQQK	1526	2	4.67	8.57E-06
			LFNSFPITVTVPQEVSSPNFMENVAEK	3024	3	5.62	1.68E-08
319S	Clusterin precursor (Glycoprotein III) (GpIII) [Contains: Clusterin beta and alpha chain]	**P17697**	ASSIMDELFQDR	1411	2	3.74	5.17E-06
			LYDQLLQSYQQK	1526	2	4.70	3.50E-05
			LFNSFPITVTVPQEVSSPNFMENVAEK	3024	3	4.92	1.85E-08
325S	Alpha-S1-casein precursor	**P02662**	FFVAPFPEVFGK	1384	2	2.97	2.66E-05
			YLGYLEQLLR	1267	2	3.53	1.52E-05

Spot	Protein name	Accession number[a]	Identified peptides	$[MH]^+$	z[b]	Score	p value[c]
384S	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor	**P79125**	FGLLNDVVDIGVNLAR	1714	2	3.87	3.49E-07
386S	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor	**P79125**	FGLLNDVVDIGVNLAR	1714	2	4.02	2.98E-07
395S	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor	**P79125**	FGLLNDVVDIGVNLAR	1714	2	4.62	3.61E-06
442G	Hemoglobin subunit beta-C	**P02078**	FFEHFGDLSSADAVLGNAK LLGNVLVIVLAR	2024 1279	2 2	6.30 4.07	5.06E-12 2.57E-05
	Hemoglobin subunit beta-A; n=2	**P02077**	VKVDEVGAEALGR FFEHFGDLSSADAVMNNAK	1342 2099	2 2	4.18 6.11	1.17E-05 5.57E-10
	Hemoglobin subunit alpha-1	**P01967**	MFLSFPTTK FLANVSTVLTSK	1071 1279	2 2	2.84 3.62	1.07E-04 2.75E-08
467S	Clusterin precursor (Glycoprotein III) (GpIII) [Contains: Clusterin beta and alpha chain]	**P17697**	LLLSSLEEAK LLLSSLEEAKK ASSIMDELFQDR	1102 1230 1411	2 2 2	3.02 2.65 3.93	5.71E-05 1.54E-05 1.31E-07

[a] Accession numbers for searches performed on UniRef 100 database (04/30/2008, 5888655 entries, www.uniprot.org);
[b] z – ion charge;
[c] p-value: protein probability identification calculated based on the probability of the associated peptides. It represents the likelihood of finding an equally good protein match by chance. It was set to 0.01, i.e. 1% probability that the match was a random event

Supplementary Figure: Electrospray mass spectrum (Bioworks™ 3.3.1) produced by tryptic digestion of spot 384S. The peptide sequence is FGLLNDVVDIGVNLAR (theoretical monoisotopic mass 1714 Da). Similar spectra were obtained for spots 386S and 395S.



6. References

Aisen, P., Listowsky, I. 1980. Iron transport and storage proteins. Annu. Rev. Biochem. 49: 357-393.

Austin, P.J., Suchar, L.A., Robbins, C.T., Hagerman, A.E. 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J. Chem. Ecol. 15: 1335-1347.

Asztély, A., Havel, G, Ekström, J. 1998. Vascular protein leakage in the rat parotid gland elicited by reflex stimulation, parasympathetic nerve stimulation and administration of neuropeptides. Regul. Pept. 77: 113-120.

Baella, L., Scocchi, M., Zanetti, M. 1995. cDNA sequences of three sheep myeloid cathelicidins. FEBS Lett. 376: 225-228.

Bailey, C.B. 1961. Saliva secretion and its relation to feeding in cattle. 4. The relationship between the concentration of sodium, potassium, chloride and inorganic phosphate in mixed saliva and rumen fluid. Br. J. Nutr. 15: 489-498.

Beeley, J.A., Sweeney, D., Lindsay, J.C., Buchanan, M.L., Sarna, L., Khoo, K. S. 1991. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of human parotid salivary proteins. Electrophoresis 12: 1032–1041.

Bell, A.E. 1981. Lipid metabolism in liver and selected tissues and in the whole body animals. In: Christie, W.W. (ed) Lipid metabolism in ruminant animals, Pergamon Press, Oxford, pp. 363-410.

Bunn, H.F., Gabbay, K.H., Gallop, P.M. 1978. The glycosylation of hemoglobin: relevant to diabetes mellitus. Science 200: 21-27.

Candiano, G., Musante, L., Bruschi, M., Petretto, A., Santucci, L., Del Boccio, P., Pavone, B., Perfumo, F., Ubani, A., Scolari, F., Ghiggeri, G.M. 2006. Repetitive fragmentation products of albumin and α1-antitrypsin in glomerular diseases associated with nephritic syndrome. J. Am. Soc. Nephrol. 17: 3139-3148.

Carr, D.H. 1984. The regulation of parotid and submandibular salivary secretion in sheep. Q. J. Exp. Physiol. 69: 587-97.

Carter, R.R., Grovum, W.L. 1990. A review of the physiological significance of hypertonic body fluids on feed intake and ruminal function: salivation, motility and microbes. J. Anim. Sci. 68: 2811-2832.

Castellino, F.J., Fish, W.W., Mann, K.G. 1970. Structural studies on bovine lactoferrin. J. Biol. Chem. 245: 4269-4275.

Christiansen, M., Jørgensen, C.S., Laursen, I., Hirschberg, D., Højrup, P., Hoven, G. 2007. Protein chemical characterization of Gc globulin (vitamin D-binding protein) isoforms, Gs-1f, Gs-1s and Gs-2. Biochim. Biophys. Acta 1774: 481-492.

Clauss, M., Lason, K., Gehrke, J., Lechner-Doll, M., Fickel, J., Grune, T., Jürgen Streich, W. 2003. Captive roe deer (*Capreolus capreolus*) select for low amounts of tannic acid but not quebracho:

fluctuation of preferences and potential benefits. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136: 369-382.

Clauss, M., Gehrke, J., Hatt, J.M., Dierenfeld, E.S., Flach, E.J., Hermes, R., Castell, J., Streich, W.J., Fickel, J. 2005. Tannin-binding salivary proteins in three captive rhinoceros species. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 140: 67-72.

Coats, D.A., Denton, D.A., Wright, R.D. 1958. The ionic balances and transferences of the sheep's parotid gland during maximal stimulation. J. Physiol. 14: 108-122.

da Costa, G., Lamy, E., Capela e Silva, F., Andersen, J., Sales Baptista, E., Coelho, A.V. 2008. Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. J. Chem. Ecol. 3: 376-387.

Dawes, C. 1984. Stimulus effects on protein and electrolyte concentrations in parotid saliva. J. Physiol. 34: 579-588.

Denny, P., Hagen, F.K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G.S., Boontheung, P., Cociorva, D., Delahunty, C.M., Denny, T., Dunsmore, J., Faull, K.F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S.C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J.A., Ogorzalek Loo, R.R., Malamud, D., Melvin, J.E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S.K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M.A., Takashima, J., Than, S., Wang, J., Whitelegge, J.P., Witkowska, H.E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D.T., Yates, J.R. 3rd, Fisher, S.J. 2008. The Proteomes of Human Parotid and Submandibular/Sublingual Gland Salivas Collected as the Ductal Secretions. J. Proteome Res. 7: 1994-2006.

Distel, R.A., Provenza, F.D. 1991. Experience early in life affects voluntary intake of blackbrush by gotas. J. Chem. Ecol. 17: 431-450.

Doumas, S., Kolokotronis, A. Stefanopoulos, P. 2005. Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. Infect. Immun. 73: 1271-1274.

Edwards, A.V., Titchen, D.A. 1992. Synergism in the autonomic regulation of parotid secretion of protein in sheep. J. Physiol. 451: 1-15.

Edwards, A.V., Titchen, D.A. 2003. Autonomic control of protein production by the parotid gland of the sheep. Auton. Neurosci. 103: 38-49.

Feldstein, J.B., Silverman, D.N. 1984. Purification and characterization of carbonic anhydrase from the saliva of the rat. J. Biol. Chem. 259: 5447-5453.

Fernley, R.T., Wright, R.D., Coghlan, J.P. 1988. Complete amino acid sequence of ovine carbonic anhydrase. Biochemistry 27: 2815-2820.

Fernley, R.T., Darling, P., Aldred, P., Wright, R.D., Coghlan, J.P. 1989. Tissue and species distribution of the secreted carbonic anhydrase isoenzyme. Biochem. J. 259: 91-96.

Fickel, J., Göritz, F., Joest, B.A., Hildebrandt, T., Hofmann, R.R., Breves, G. 1998. Analysis of parotid and mixed saliva in roe deer. J. Comp. Physiol. B 168: 257-264.

Ghafouri, B., Tagesson, C. Lindahl, M. 2003. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. Proteomics 3: 1003-1015.

Gilboa, N. 1995. Negative effects of tannins in livestock and their neutralization. PhD Thesis. The Hebrew Univ. of Jerusalem, Israel (Hebrew with English summary).

Göritz, F., Hildebrandt, T.H., Hofmann, R.R., Pitra, C. 1994. Comparative salivary studies using a new technique yielding uncontaminated saliva from CS, IM and GR ruminant species. Proc. Soc. Nutr. Physol. 3: 319.

Guo, T., Rudnick, P.A., Wang, W., Lee, C.S., DeVoe, D.L., Balgley, B.M. 2006. Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. J. Proteome Res. 5: 1469-1478.

Hagerman, A.E., Robbins, C.T., 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. Can. J. Zool. 71: 628-633.

Hardt, M., Thomas, L.R., Dixon, S.E., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witowska, H.E., Fisher, S.J. 2005. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafitration, HPLC, and mass spectrometry. Biochemistry 44: 2885-2899.

Hashimoto, S., Murakami, M., Kanaseki, T., Kobayashi, S, Matsuki, M., Shimono, M., Segawa, A., 2003. Morphological and functional changes in cell junctions during secretory stimulation in the perfused rat submandibular gland. Eur. J. Morphol. 41: 35-39.

Hervás, G., Pérez, V., Giráldez, F.J., Mantecón, A.R., Almar, M.M., Frutos, P. 2003. Intoxication of sheep with quebracho tannin extract. J. Comp. Pathol. 129: 44-54.

Hirtz, C., Chevalier, Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., de Périère, D. 2005. Complexity of the human whole saliva proteome. J. Physiol. Biochem. 61: 469-480.

Hoeg, J.M., Meng, M.S., Ronan, R., Fairwell, T., Brewer, H.B. Jr 1986. Human apolipoprotein A-I. Post-translational modification by fatty acid acylation. J. Biol. Chem. 261: 3911-3914.

Hofmann, R.R. 1989 Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. Oecologia 78: 443-457.

Hofmann, R.R., Streich, W.J., Fickel, J., Hummel, J., Clauss, M. 2008. Convergent evolution in feeding types: salivary gland mass differences in wild ruminant species. J. Morphol. 269: 240-257.

Hooper, L.V., Beranek, M.C., Manzella, S.M., Baenziger, J.U. 1995. Differential expression of GalNAc-4-sulfotransferase and GalNAc-transferase results in distinct glycoforms of carbonic anhydrase VI in parotid and submaxillary glands. J. Biol. Chem. 270: 5985-5993.

Hu, S., Xie, Y., Ramachandran, P., Ogorzalek Loo, R.R., Li, Y., Loo, J.A., Wong, D.T. 2005. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics 5: 1714-1728.

Huang, C.M. 2004. Comparative proteomic analysis of human whole saliva. Arch. Oral Biol. 49: 951-962.

Hutner, K.M., Lambeth, M.R., Burkin, H.R., Burkin, D.J. Broad, T.E. 1998. Localization and genomic organization of sheep antimicrobial peptde genes. Gene 206: 85-91.

Jones, W.T., Broadhurst, R.B., Gurnsey, M.P. 1982. Partial characterization of bovine salivary proteins by electrophoretic methods. Biochim Biophy Acta 701: 382-388.

Junqueira, L.C., Toledo, A.M., Ferri, R.G. 1965. Permeability of the stimulated rat submaxillary gland to its blood serum proteins. Arch. Oral Biol 10: 863-868.

Juntheikki, M.R. 1996. Comparison of tannin-binding proteins in saliva of Scandinavian and North American moose (*Alces alces*). Biochem. Syst. Ecol. 24: 595-601.

Kay, R.N.B. 1960. The rate of flow and composition of various salivary secretions in sheep and calves. J. Physiol. 150: 515-537.

Lamy, E., da Costa, G., Capela e Silva, F., Potes, J., Coelho A.V., Sales Baptista, E. 2008. Comparison of electrophoretic protein profiles from sheep and goat parotid saliva. J. Chem. Ecol. 34: 388-397.

Lin, L.Y. Chang, C.C. 1989. Determination of protein concentration in human saliva. Gaoxiong Yi Xue Ke Xue Za Zhi. 5: 389-397 (Article in Chinese with abstract in English).

Litwiller, R., Fass, D., Kumar, R. 1986. The amino acid sequence of the NH_2-terminal portion of rat and human vitamin D-binding protein. Life Sci. 38: 2179-2184.

Lundblad, R.L., Hofman, S., Noyes, C.M., Kingdon, H.S. 1977. Purification and partial characterization of deoxyribonuclease I from bovine parotid gland. J. Dent. Res. 56: 320-326.

Maeda, L., McKenzie, H.A., Shaw, D.C. 1980. Nature of the heterogeneity within genetic variants of bovine serum transferrin. Anim. Blood Groups Biochem. Genet. 11: 63-75.

Makkar, H.P.S., Becker, K. 1998. Adaptation of cattle to tannins: role of proline-rich proteins in oak-fed cattle. Anim. Sci. 67: 277-281.

Marzo, F., Tosar, A., Santidrian, S. 1990. Effect of tannic acid on the immune response of growing chickens. J. Anim. Sci. 68: 3306-3312.

Mau, M., Muller, C., Langbein, J., Rehfeldt C., Hildebrandt, J.P., Kaiser, T.M. 2006. Adhesion of bovine and goat salivary proteins to dental enamel and silicate. Arch. Tierz. Dummerstorf 5: 439-446.

Mazariegos, M.R., Tice, L.W., Hand, A.R. 1984. Alterations of tigt junctional permeability in the rat parotid gland after isoproterenol stimulation J. Cell Biol. 98: 1865-1877.

McDougall, E.I. 1948. Studies on ruminant saliva. 1. The composition and ouput of sheep's saliva. Bichem. J. 43: 99-109.

McLaren, R.D., Mcintosh, J.T., Howe, G.W. 1987. The purification and characterization of bovine salivary proteins by electrophoretic procedures. Electrophoresis 8: 318-324.

McNeely, T.B., Dealy, M., Dripps, D.J., Orenstein, J.M., Eisenberg, S.P., Wahl, S.M. 1995. Secretory leucocyte protease inhibitor: a human salivary protein exhibiting anti-human immunodeficiency virus activity in vitro. J. Clin. Invest. 96: 456-464.

Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc. Natl. Acad. Sci. USA 80: 3948-3952.

Mehansho, H., Clements, S., Sheares, B.T., Smith, S., Carlson, D.M. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. J. Biol. Chem. 260: 4418-4423.

Mehansho, H., Asquith, T.N., Butler, L.G., Rogler, J.C., Carlson, D.M. 1992. Tannin-mediated induction of proline-rich protein synthesis. J. Agric. Food Chem. 40: 93-97.

Meot, F., Cirio, A., Boivin, R. 1997. Parotid secretion daily patterns and measurement with ultrasonic flow probes in conscious sheep. Exp. Physiol. 82: 905-923.

Mistry, R., Snashall, P.D., Totty, N., Guz, A., Tetley, T.D. 1991. Isolation and characterization of sheep alpha 1-proteinase inhibitor. Biochem. J. 273: 685-690.

Muallem, S., Kwiatkowska, K., Xu, X., Yin, H.L. 1995. Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. J. Cell Biol. 128: 589-598.

Murakami, H., Sly, W.S. 1987. Purification and characterization of human salivary carbonic anhydrase. J. Biol. Chem. 25: 1382-388.

Murakami, M. Ohtake, T., Dorschner, R.A., Gallo, R.L. 2002. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. J. Dent. Res. 81: 845-850.

Narjisse, H., Elhonsali, M.A., Olsen, J.D. 1995. Effects of oak (*Quercus ilex*) tannins on digestion and nitrogen balance in sheep and goats. Small Ruminant Res. 18: 201-206.

Nastis, A. 1997. Feeding behaviour of goats and utilization of pasture and rangelands. CIHEAM – Options Mediterraneennes 25: 39-45

Neyraud, E., Sayd, T., Morzel, M., Dransfied, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. J. Proteom. Res. 5: 2474-2480.

Nicholas, B., Skipp, P., Mould, R., Rennard, S., Davres, D.E., O'Connor, C.D., Djukanovic, R. 2006. Shotgun proteomic analysis of human induced spuntum. Proteomics 6: 4390-4401.

Nizet, V., Gallo, R.L. 2003. Cathelicidins and innate defense against invasive bacterial infection. Scand J. Infect. Dis. 35: 670-676.

Onoda, M., Inano, H. 1997. Distribution of casein-like proteins in various organs of rat. J. Histochem. Cytochem. 45: 93-97.

Parsons, P.A., Klinger, A.H., Garrett, J.R. 1977. Adrenergic influences on the permeability of rabbit submandibular salivary glands to blood-borne horseradish peroxidase. Histochem. J. 9: 419-433.

Patterson, J., Brightling, P., Titchen, D.A. 1982. beta-Adrenergic effects on composition of parotid salivary secretion of sheep on feeding. Q. J. Exp. Physiol. 67: 57-67.

Paudel, H.K., Liao, T.H. 1986. Comparison of the three primary structures of deoxyribonuclease isolated from bovine, ovine, and porcine pancreas. Derivation of the amino acid sequence of ovine DNase and revision of the previously published amino acid sequence of bovine DNase. J. Biol. Chem. 261: 16012-16017.

Pich, A., Bussolati, G., Carbonara, A. 1976. Immunocytochemical detection of casein and casein-like protein in human tissues. J. Histochem. Cytochem. 24: 940-947.

Plymoth, A., Löfdahl, C.G., Ekber-Jansson, A., Dahlbäck, M. Lindberg, H., Fehniger, T.E., Marko-Varga, G., 2003. Human bronchoalveolar lavage: Biofluid analysis with special emphasis on sample preparation. Proteomics 3: 962-972.

Pogrel, M.A., Lowe, M.A., Stern, R. 1996. Hyaluronan (hyaluronic acid) in human saliva. Arch. Oral Biol. 41: 667-671.

Provenza, F.D., Malechek, J.C. 1984. Diet selection by domestic goats in relation to blackbrush twig chemistry. J. Appl. Ecol. 21: 831-841.

Ramachandran, P., Bootheung, P., Xie, Y., Sondej, M., Won, D.T., Loo, J.A. 2006. Identification of N-linked glycoproteins in human saliva by glycoprotein capture and mass spectrometry. J. Proteome Res. 5: 1493-1503.

Robbins, C.T., Mole, S., Hagerman, A.E., Hanley, T.A. 1987. Role of tannins in defending plants against ruminants: reduction in dry matter digestion? Ecology 68: 1606-1615.

Robbins, C.T., Hagerman, A.E., Austin, P.J., McArthur, C., Hanley, T.A. 1991. Variation in mammalian physiological responses to a condensed tannin and its ecological implications. J. Mammal. 72: 480-486.

Robbins, C.T., Spalinger, D.E., van Hoven, W. 1995. Adaptation of ruminants to browse and grass diets: are anatomical-based browser-grazer interpretations valid? Oecologia 103: 208-213.

Segawa, A. 1994. Tight junctional permeability in living cells: dynamic changes directly visualized by confocal laser microscopy. J. Electron Microsc. (Tokyo) 43: 290-298.

Shackleford, J.M., Wilborn, W.H. 1969. Ultrastructure of bovine parotid glands. J. Morphol. 127: 453-473.

Shamova, O., Brogden, K.A., Zhao, C., Nguyen, T., Kokryakov, V.N., Lehrer, R.I. 1999. Purification and properties of proline-rich antimicrobial peptides from sheep and goat leukocytes. Infect. Imun. 7:4106-4111.

Shapiro, R., McManus, M.J., Zalut, C., Bunn, H.F. 1980. Sites of nonenzymatic glycosylation of human hemoglobin A. J. Biol Chem. 255: 3120-3127.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32: 1149-1163.

Silanikove, N., Gilboa, N., Nitsan, Z., Perevolotsky, A. 1996a. Effect of a daily supplementation of polyethylene glycol on intake and digestion of tannin-containing leaves (*Quercus calliprinos, Pistacia lentiscus and Ceraonia siliqua*) by goats. J. Agric. Food Chem. 44: 199-205.

Silanikove, N., Gilboa, N., Perevolotsky, A., Nitsan, Z. 1996b. Goats fed tannin-containing leaves do not exhibit toxic syndromes. Small Ruminant Res. 21: 191-207.

Siqueira, W.L., Salih, E., Wan, D.L., Helmerhorst, E.J., Oppenheim, F.G. 2008. Proteome of human minor salivary gland seretion. J. Dent. Res. 87: 445-450.

Solito, E., Christian, H.C., Festa, M., Mulla, A., Tieney, T., Flower, R.J., Buckingham, J.C. 2006. Post-translational modification plays an essential role in translocation of annexin A1 from the cytoplasm to the cell surface. FASEB J. 20: 1498-1500.

Stea, E.A., Routsias, J.G., Samiotaki, M., Panayotou, G., Papaambios, E., Moutsopoulos, H.M., Tzioufas, A.G. 2007. Analysis of parotid glands of primary Sjögren's syndrome patients using proteomic technology reveals altered autoantigen composition and novel antigenic targets. Clin. Exp. Immunol. 147: 81-89.

Stolte, M., Ito, S. 1996. A comparative ulrastructural study of the parotid gland acinar cells of nine wild ruminant species (mammalian, artiodactyla). Eur. J. Morphol. 34: 79-85.

Suzuki, S., Nishinakawa, H., Otsuka, J. 1981. Scanning electron microscopic observations on the goat parotid glands. Jpn. J. Vet. Sci. 43: 181-189.

Takano, F., Takata, T., Yoshihara, Y., Nakamura, Y., Arima, Y., Ohta, T. 2007. Aqueous extract of peanut skin and its main constituent procyanidin A1 suppress serum IgE and IgG1 levels in mice-immunized with ovalbumin. Biol. Pharm. Bull. 30: 922-927.

Temporini, C., Calleri, E., Massolini, G., Caccialanza, G. 2008. Integrated analystical strategies for the study of phoporylation and glycosylation in proteins. Mass Spectrom. Rev. 27: 207-236.

Vaithiyanathan, S., Mishra, J.P., Sheikh, Q., Kumar, R. 2001. Salivary glands tannins binding proteins of sheep and goat. Indian J. Anim. Sci. 71: 1131-1134.

Valentijn, K., Valentijn, J.A., Jamieson, J.D. 1999. Role of actin in regulated exocytosis and compensatory membrane retrieval: insights from an old acquaintance. Biochem. Biophys. Res. Commun. 266: 652-661.

Van Lennep, E.W., Kennerson, A.R., Compton, J.S. 1977. The ultrastructure of the sheep parotid gland. Cell Tiss. Res. 179: 377-392.

Van Soest, P.J. 1994. Nutritional ecology of the ruminant. 2nd ed. Cornell University Press, New York.

Vaughan, L., Lorier, M.A., Carrell, R.W. 1982. Alpha 1-ntitrypsin microheterogeneity. Isolation and physiological significance of isoforms. Biochim. Biophys. Acta 1: 339-345.

Vitorino, R., Lobo, M.J., Ferrer-Correira, A.J., Dubin, J.R., Tomer, K.B., Domngues, P.M., Amado, F.M. 2004. Identification of human whole saliva protein components using proteomics. Proteomics 4: 1109-1115.

Wait, R., Miller, I., Eberini, I., Cairoli, F., Veronesi, C., Battocchio, M., Gemeiner, M., Gianazza, E. 2002. Strategies for proteomics with incompletely characterized genomes: the proteome of *Bos Taurus* serum. Electrophoresis 23: 3418-3427.

Walz, A., Stühler, K., Wattenberg, A., Hawranke, E., Meyer, H.E., Schmalz, G., Blüggel, M., Ruhl, S., 2006. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole saliva by two-dimensional gel electrophoresis. Proteomics 6: 1631-1639.

Waters, C.A., Morand, J.N., Schtzan, R.C., Carlson, D.M. 1998. Induction of p34cdc2 in mouse parotid glands upon activation of beta 1-adrenergic receptors. Cell Mol. Biol. (Noisyle-grand) 44: 333-342.

Weinberg, R.B., Scanu, A.M. 1983. Isolation and characterization of human apolipoprotein A-IV from lipoprotein-depleted serum. J. Lipid. Res. 24: 52-59.

Wheeler, T.T., Haigh, B.J., McCracken, J.Y., Wilkins, R.J., Morris, C.A., Grigor, M.R. 2002. The BSP 30 salivary proteins from cattle, LUNX/PLUNC and vonEbner's minor salivary gland protein are members of the PSP/LBP superfamily of proteins. Biochim. Biophys. Acta 1579: 92-100.

Williams, K.M., Marshall, T. 1998. Electrophoretic characterization of the major cat parotid salivary protein. Anal. Chim. Acta 372: 249-255.

Williams, K.M., Ekström, J., Marshall, T. 1999a. High-resolution electrophoretic analysis of rat parotid salivary proteins. Electrophoresis 20: 1373-1381.

Williams, K.M., Ekström, J., Marshall, T. 1999b. The protein composition of ferret parotid parotid saliva as revealed by high-resolution electrophoretic methods. Electrophoresis 20: 2818-2823.

Wilmarth, P.A., Riviere, M.A., Rustvold, D.L., Lauten, J.D., Madden, T.E., David, L.L. 2004. Two-dimensional liquid chromatography study of the human whole saliva proteome. J. Proteome Res. 3: 1017-1023.

Wilson, M.R., Roeth, P.J., Easterbrook-Smith, S.B. 1991. Clusterin enhances the formation of insoluble immune complexes. Biochem. Biophys. Res. Commun 28: 985-990.

Xie, H. Rhodus, N.L., Griffin, R.J., Carlis, J.V., Griffin, T.J. 2005. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol. Cell Proteomics 4: 1826-1830.

Yamada, A., Nakamura, Y., Sugita, D., Shirosaki, S., Ohkuri, T., Katsukawa, H., Nonaka, K., Imoto, T., Ninomiya, Y. 2006. Induction of salivary kallikreins by the diet containing a sweet-suppresive peptide, gurmarin, in the rat. Biochem. Biophys. Rs. Commun. 346: 386-392.

Yang, Z., Hancick, W.S., Chew, T.R., Bonilla, L. 2005. A study of glycoproteins in human serum and plasma reference standards (HUPO) using multilectin affinity chromatography coupled with RPLC-MS/MS. Proteomic 5: 3353-3366.

Yao, L, Berg, E.A., Costello, C.E., Troxler, R.F., Oppenheim, F.G. 2003. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomic approaches. J. Biol. Chem. 14: 5300-5308.

Zanetti, M. 2005. The role of cathelicidins in the innate host defenses of mammals. Curr. Issues Mol. Biol. 7: 179-196.

Chapter 7

General Discussion

The working hypothesis of this thesis was that animals presenting different feeding preferences also present differences in their salivary protein composition and this composition changes with the consumption of aversive compounds, such as tannins. The aims of the present study were: 1) to access the histo-morphological changes produced by tannins, in the different major salivary glands and to get insights on the nervous system mechanisms involved; 2) to characterize and to compare the salivary proteomes of three species with different dietary habits: mice, sheep and goats; 3) to study the changes in the salivary protein profiles from these species induced by tannin consumption.

The oral cavity is the part of the animal internal medium that first comes into contact with food. Numerous chemical and mechanical receptors in the mouth respond to the food chemical and physical properties and monitor the changes during processing. This leads to central perception of taste and texture of food, which, together with odor, are important determinants in the decision of to ingest or not (Provenza et al., 1995). An important role in this process is played by saliva in the perception of taste and texture sensations (Engelen et al., 2007): its composition can modulate food perception and, simultaneously, be modulated by the type of diet (Dawes, 1970; Mese and Matsuo, 2007).

Differences in taste perception are common in species from different trophic groups, particularly in what concerns aversive sensations, such as sour and bitter tastes and astringency. These differences were thought to be related to the levels of potential harmful compounds found in each animal regular diet and to the adaptations animals present to these compounds (Glendinning, 1994). Even when animals are hungry, they may choose not to eat a food rich in nutrients only as a consequence of the presence of distasteful compounds associated to negative post-ingestive mechanisms (Provenza et al., 1995). On the other hand, the same compound can be accepted by a different animal species that has defense mechanisms against it. Tannins represent one of these groups of aversive compounds, being greatly distributed among the foods consumed by herbivores and omnivores (Haslam, 1998). In species that do not have tannins in their regular diets, these plant secondary metabolites could reduce food digestibility and thus diminishing animal growth rate and health status. On the other hand, some animal species fed on tannin-rich foods do not suffer from the mentioned consequences, probably due to some physiological or metabolic adaptations (Mole et al., 1993; Iason, 2005). Salivary proteins have been pointed as one of the animal's defense mechanisms against these dietary compounds (detailed list of studies are reviewed in Shimada, 2006).

1. Salivary glands respond to tannin ingestion through an increase in size of secretory structures

It is well established that the major part of the total saliva entering the oral cavity are produced by the three pairs of major salivary glands, which are the local of synthesis of many salivary proteins and which are exclusively under nervous regulation (Emmelin, 1987). We started by analyzing changes in

their histomorphology produced by tannin consumption. In chapter 2 we presented evidences that, in mice, the more marked changes produced by tannins occur at the parotid gland level, although changes in submandibular and sublingual glands are also observed. Within each gland, main effects of tannins were observed in acinar structures, which are responsible for a great part of the synthesis and secretion of salivary proteins (Turner and Sugyia, 2002). The effects induced by hydrolysable tannins appear not to be as strong as the ones induced by condensed tannins but both are similar to the ones induced by the sympathetic nervous system agonist isoproterenol, with the exception of effects at the sublingual gland. We suggest that the mechanisms involved in adaptation of the oral cavity to these polyphenols are mainly controlled by sympathetic innervation.

The hypertrophy of parotid glands induced by the presence of tannins in the diet, and their subsequent regression after removal of this compound from the diet, draw attention to the capacity of rapid adaptation of parotid glands and, stress to their importance in protein secretion in tannin consumption behavior.

2. Proteome characterization of mice, sheep and goats and comparison among the species

Proteomics has emerged in the nineties, with advances in mass spectrometry (Yamashita and Fenn, 1984; Karas and Hillenkamp, 1998) as well in two-dimensional electrophoresis methodologies (Görg, 2000). Since then many biological material proteomes have been characterized. Whereas more than 1100 proteins have been identified in human saliva (Denny et al., 2008), other mammalian species salivary proteomes are not so well established. Animal saliva is a key fluid that enables modeling biological processes involved in the response to ingestive and digestive processes.

The study of glandular saliva, rather than whole saliva, has several advantages: (i) the proteins are from known glandular origin, which is important in understanding the physiological control; (ii); it is possible to study proteins expressed at low levels, which in mixed saliva would be more diluted and possibly in insufficient amounts for analysis (Walz et al., 2006); (iii) lower sample contamination by food particles, what is particularly important in ruminants due to the characteristic re-mastication of food; (iv) less protein degradation by the time of permanence in the oral cavity (Helmerhorst, 2007); (v) it is possible to access proteins before a potential interaction with food occurs.

For all these reasons, as well as due to the importance that parotid presents for the total of saliva produced in ruminants (Kay, 1960), we decided to study sheep and goat saliva collected directly from the parotid duct. The same approach was not possible in mice due to the small size of the animals, making parotid duct cannulation difficult. In this case we collected whole saliva directly from mouth with a micropipette, after pilocarpine stimulation.

In chapters 3 and 4 the salivary mice whole saliva protein profile was characterized and the changes in this profile, induced by the consumption of tannins, were evaluated. Taken together, one-and two-dimensional electrophoretic protein separation followed by mass spectrometry allowed the identification of a total of 28 different proteins (Table 1).

Table 1 – Summary of the mice whole salivary proteins identified in the present thesis

Protein	Biological function
Acidic mammalian chitinase precursor	Protection/defense
Androgen binding protein α subunit	Sexual behaviour and/or regulation
Apolipoprotein A1	Transport
BC048546 protein fragment (α-2-macroglobulin)	Protein degradation/inhibition
Carbonic anhydrase VI	Metabolism; Carbohydrates
Cysteine-rich secretory protein 1 precursor	Sexual behavior and/or regulation
Demilune cell and parotid protein 1 (Dcpp 1)	Protection/defense
Demilune cell and parotid protein 2 (Dcpp 2)	
Deoxyribonuclease I - precursor	DNA replication and repair
Glandular kallikrein k13 (mGK-13)	Protein degradation/inhibition
Glandular kallikrein k5 (mGK-5)	
Glandular kallikrein k6 (mGK-6)	
Glandular kallikrein k9 (mGK-9)	
Glandular kallikrein k22 (mGK-22)	
Immunoglobulin heavy chain variable region	Defense; immunoresponse
Lacrimal androgen binding protein ε	Sexual behaviour and/or regulation
Mucin apoprotein precursor	Protection/defense
Muc 10	
Odorant binding protein Ia	Sexual behavior and/or regulation
Odorant binding protein Ib - fragment	
Parotid secretory protein precursor	Protection/defense
Prolactin-inducible protein homolog precursor (14 kDa submandibular gland protein)	
Prorenin-converting enzyme (MK 13b) precursor	Protein degradation/inhibition
Salivary amylase 1	Metabolism; Carbohydrates (digestion)
Salivary androgen binding protein β subunit	Sexual behaviour and/or regulation
Salivary androgen binding protein γ subunit	
Similar to odorant binding protein 1F	
Vomeromodulin precursor	

A high proportion (about 32%) of the spots identified corresponds to proteins linked to sexual behavior, which is under hormonal, namely androgen, regulation (Pratt et al., 1981). Most of these, namely lipocalins and odorant binding proteins (Marchese et al., 1998), as well as androgen binding proteins (Wickliffe et al., 2002), are proteins secreted from the submandibular glands. These glands present considerable morphological sexual dimorphism (Pinkstaff, 1998), known to result in salivary protein secretion sexual dimorphism. For example, the expression of the mouse salivary protein gene

Vcs2 has been reported as being differentially expressed in males and females, what might ultimately result in differences in the expression of the secreted salivary protein (Señorale-Pose et al., 1998). This highlights the importance of animal sex in comparative studies of saliva and salivary glands.

Sheep and goat parotid salivary protein profiles were presented in chapters 5 and 6. A total of 40 different proteins were identified (Table 2).

Several of the proteins identified from sheep and goat 2-DE, by PMF and MS/MS, were matched to bovine entries in protein sequence databases. Moreover, some of the spots remained unidentified even after repeated analysis of the equivalent spots from two or more gels. Although some of these failures may be attributable to low protein abundance or to inefficient proteolysis by trypsin, others are probably a consequence of the limited number of sequences present in public protein databases, for the ruminant species studied. At present (June 2008) Swiss-Prot and TREMBL contain a combined total of 2487 entries for *Ovis aries* and 688 for *Capra hircus*, compared with 15336, 60213 and 71952 for *Bos Taurus*, *Mus musculus*, and *Homo sapiens*, respectively.

Table 2 – Summary of sheep and goat parotid salivary proteins identified in the present thesis.

Protein	sheep*	goats**	Biological function
Actin cytoplasmic 1			Structural protein
Alpha-enolase			Metabolism-glycolysis; carbohydrates
Alpha-S1-casein precursor	X		Transport of calcium and phosphorus (micelles)
Alpha-S2-casein precursor	X		
Alpha-1-antiproteinase precursor			Protein degradation or inhibitor
Annexin A1			
Annexin A3			
Apolipoprotein A1 precusor			Transport
Apolipoprotein A IV precursor		X	
Beta-lactoglobulin	X		
Carbonic Anhydrase VI			Metabolism; carbohydrates
Catalase	X		Redox
Cathelicidin-1 precursor			
Cathelicidin-2 precursor			Antimicrobial
Cathelicidin-3 precursor		X	
Cathepsin H precursor		X	Protein degradation
Clusterin precursor	X		
Complement C3 precursor (fragment)			Defense; immunoresponse
Complement C4 precursor			
Cytosolic non-specific dipeptidase		X	Protein degradation or inhibition
Deoxyribonuclease			DNA cleavage
Elongation factor 2			Translaction ; molecular chaperone
Gelsolin precursor			Actin-modulating protein
Glutathione S-transferase Pi		X	Detoxification

Haptoglobin			Transport
Hemoglobin subunit alpha-1		X	
Hemoglobin subunit beta-A		X	
Hemoglobin subunit beta-C		X	
Heat shock protein HSP 90-beta			Molecular chaperone
Immunogobulin gamma 2 heavy chain constant region		X	Defense; immunoresponse
Ig heavy chain C region			
Kappa-casein precursor	X		Transport of calcium and phosphorus
Leukocyte elastase inhibitor		X	
Protein disulfide-isomerase A3 precursor			
Serotransferrin precursor			Transport
Serum albumin precursor			
Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor (BSP30b)			Antimicrobial
similar to fibrinogen beta chain precursor			Protein modification; polymerization
Transthyretin precursor (prealbumin)			Transport
Vitamin D-binding protein precursor			

*proteins only identified in sheep.
**proteins only identified in goats;

It was already proposed that saliva protein composition varies considerably among species, reflecting diverse diets and modes of digestion (Young and Schneyer, 1981). In this thesis, differences in saliva protein composition among the three species studied were demonstrated. Mice whole saliva concentrations obtained by us (between 2.5 to 2.9 mg/mL) did not differ to a great extent from the mean 2 mg/mL referred for humans (Dawes, 1984; Pogrel et al. 1996) and it is possible that some difference came from the different methodologies used for protein concentration determination (see Williams et al., 1999 for comparison of protein assay methods in saliva samples). These are values much higher than the ones observed in ruminant parotid saliva (100-200 μg/mL). Although we are referring to saliva from different sources (whole vs parotid saliva), it is possible to think that this tendency were still obtained if we had work with mice parotid saliva. This difference, together with the higher ionic content of ruminant parotid saliva, suggests the relatively small importance of ruminant parotid as protein secreting gland and its important role in providing a buffered fluid capable of to maintain an adequate ruminal fermentation environment (McDougall, 1948; Kay, 1960; Van Lennep et al., 1977).

Major differences were observed between mice and small ruminant protein profiles. From the total of 68 individual proteins identified only four identifications are common to mice and small ruminants: apolipoprotein A1, carbonic anhydrase VI, deoxyribonuclease I and immunoglobulin.

One of the most evident differences between mice and small ruminant 2-DE gels is the high number of amylase spots in mice versus the high number of albumin spots in sheep and goats. Different

isoforms of salivary alpha-amylase were observed in mice whole saliva, similarly to what was obtained in studies of human whole saliva proteome (Ghafouri et al., 2003; Huang, 2004; Vitorino et al., 2004; Hirtz et al., 2005b). Despite studies reported the secretion of alpha amylase into the saliva by the nasolabial glands of some ruminants (Church, 1976, cited by Engvall, 1980), the absence of its secretion by ruminant major salivary glands has been greatly proposed (Sissons, 1981). In the present study we also did not observe alpha amylase in sheep and goats parotid saliva.

In ruminants, many of the identified proteins are blood proteins. The mode of secretion of these proteins by salivary glands is not completely understood. In studies on human whole saliva the presence of serum proteins has been referred as coming from gingival crevicular fluid (Rantonen and Meurman, 2000; Yao et al., 2003; Huang, 2004). However, the presence of these proteins was also observed in glandular secretions (Walz et al., 2006), despite its provenience being not clarified. In the studies presented in this thesis, ruminant saliva was collected directly from parotid duct, so the stronger hypothesis is that blood proteins came from parotid glands. Paracellular permeation, and passage of proteins through tight junctions was already reported for parotid glands (Mazariegos et al., 1984; Hashimoto et al., 2000). Nonetheless, the synthesis of serum proteins, such as albumin, by parotid gland is not to exclude. Albumin, which was the protein that we identified in higher number of spots in ruminants (about 50% of the total of spots identified in parotid saliva), is synthesized mainly in the liver, although non hepatic expression has been documented in several other tissues, including mouse retina (Dodson et al., 2001), mouse skeletal muscle (Wagatsuma et al., 2002), human ovarian epithelial cells (Varricchio and Stroberg, 1994), bovine tracheal gland serous cells (Jacquot et al., 1988) and bovine mammary gland (Shamay et al., 2005). Despite the known differences between mammary and salivary glands (McManaman et al., 2006), they are similar secretory tissues which may suggest that some albumin synthesis may occur in salivary glands. The same reasoning may be used to explain our observation of caseins and lactoglobulin in sheep and goat parotid saliva proteome.

The higher proportion of blood proteins in ruminant parotid saliva, comparatively to parotid saliva from humans (Walz et al., 2006) or rodents (Williams et al., 1999) also reinforce the different roles that parotid saliva plays among the different species. It had already been described that salivary glands from ruminants are extensively different in their structure and function when compared with those of other mammalian species (Shackleford and Wilborn, 1968; Van Lennep et al., 1977; Stolte and Ito, 1996). Their unusual morphological characteristics have been used as an indication of the production of large amounts of saliva (Shackleford and Wilborn, 1968, 1969; van Lennep et al., 1977; Pinkstaff, 1980) and are in contrast with the ultrastructure of serous acinar cells of non-ruminant mammals characteristic of protein secreting cells (van Lennep et al., 1977). Even not knowing the mechanism of passage of blood proteins to saliva, it is possible that their amount be flow-related. In this case, the higher parotid saliva secretion rate, that occurs in ruminants compared to human and rodent, might account for a higher passage of blood proteins into glands, what together with the

diminished capacity and need of protein synthesis and secretion from ruminant parotid cells, would induced for a higher proportion of blood proteins in parotid saliva.

Proteins with defense function, such as proteins from immune system or anti-microbial proteins, appeared in a greater proportion in ruminant parotid protein profile than in mice whole saliva protein profile. Apart from the highly intense serum albumin spots, the two spots with great intensity in goats and three of the most intense spots in sheep were identified as BSP30b, which has anti-microbial functions (Wheeler et al., 2003; Wheeler et al., 2007). We hypothesized that the mode of acquisition of energy, by ruminants, through a symbiotic relationship with plant fiber degrading microbes, in the rumen, may hold for a greater need for proteins able to modulate the microbial ecology in the ruminant oral cavity and gastric compartments, in order to maintain optimal digestive functions or prevent pathological infection. Salivary BSP30 is genetically related to mouse parotid secretory protein (PSP) and functional similarities have been proposed (Wheeler et al., 2002), but the expression levels of PSP, in our mouse whole saliva proteome, appeared to be lower than in ruminant parotid saliva proteome. We believe that this difference may not only be due to the different glandular origin of saliva, since it was already reported that BSP30 comprise approximately 30% of total salivary protein in cattle (Rajan et al., 1996).

The protein pattern of mice whole saliva showed low variability among the different individual. Inversely, different ruminant individuals did not present such a high constancy, what was particularly evident in the 2-DEprofiles. The consistency of mice whole salivary patterns may be linked to the genetic homogeneity characteristic of laboratory mice and which was discussed in chapter 4. Several factors can be involved in ruminant individual variability. Apparently age can be one of the most important, as it is responsible for marked changes in the oral cavity, namely in tooth structure (Pérez-Barberia and Gordon, 1998) and saliva production (Vissink et al., 1996; Zussman et al., 2007). We suggest that in further studies care must be taken to work with ruminant animals with similar body condition, weight and age.

3. Aversive substances change salivary proteome in ruminants and rodents

After this characterization studies, we intended to access the role of saliva in taste. When food is presented, the animal must make a decision between eating or not eating based in sensory clues, mostly vision and olfaction. But if they chose to eat, they face another decision: to swallow or spit out. This decision must be taken in a few seconds and is based in taste/texture clues. Saliva secretion is adapted to this rapid response through autonomic nervous control. To better achieve a deeper knowledge on the importance of salivary proteins in this pre-gastric phase of feeding behavior, and to observe how they are diet-regulated, we studied the effects of feeding tannins.

The induction of salivary PRPs by dietary tannins is a well studied change in mice saliva composition (Mehansho et al., 1985; Shimada et al., 2006). Our results showed that not only these salivary proteins, but also amylase levels increase with tannin consumption (chapters 3 and 4). Moreover, we suggest that not all PRPs complex tannins with similar affinities since a fraction of basic PRPs, with observed molecular masses of about 45 and 66kDa, remains soluble. The increase in mice salivary amylase was produced both by hydrolysable and condensed tannins. When we studied the changes in salivary gland histomorphometry (chapter 2), it was possible to observe a greater effect from quebracho (condensed tannin) than from tannic acid and chestnut (hydrolysable tannins). As well, the concentration of proteins that remained soluble after contact with tannins, in the saliva from the mice fed quebracho was lower than the one from the mice fed tannic acid (chapter 3). Considering that animals were in similar experimental conditions, the differences should correspond to differences in amounts of proteins precipitated by tannins, which we believe might be PRPs (Baxter et al., 1997; Hagerman et al., 1998). It is possible that the same levels of quebracho tannins induce higher levels of PRPs than the hydrolysable ones, not affecting differently the other salivary proteins.

With the methodology used, we could not observe an induction of salivary PRPs for any of the two small ruminant species studied. Concerning sheep, most of the bibliography mentioned an absence of these proteins (Austin et al., 1989; Fickel et al., 1998), but for goats, on the other hand, some authors suggested the possibility of their production, mostly based on this species feeding behavior (Provenza and Malechek, 1984; Silanikove et al., 1996; Salem et al., 2000; 2001). Despite this reasonable hypothesis, an irrefutable identification, at our knowledge, was not presented. Most of the studies, on the constitutive presence or induction of salivary PRPs by dietary tannins, in animal species, were based on the fact that these proteins are particularly soluble in 10% trichloacetic acid (Muenzer et al., 1979). The presence or absence of these proteins were reported not through a concrete identification, but rather based on protein TCA solubility, although these properties might be not exclusive of PRPs.

In general terms, quebracho tannin consumption increased the expression levels of salivary amylase in mice and of actin cytoplasmic 1, annexin 1, leukocyte elastase inhibitor and also few serum albumin isoforms. The increase in actin cytoplasmic 1, observed in small ruminant parotid saliva had been discussed in chapter 6 and may be due to an increased activity of small ruminant parotid secretion, in response to tannins, and to the apocrine-like mode of secretion, particular of these species (Stolte and Ito, 1996). Serum albumin has various kinds of functions including antibacterial activity, antioxidant effect, inflammation inhibitory effect, enzymatic activity, in addition to the most described functions as transporters and regulator of osmotic pressure (Kragh-Hansen, 1990). Under inflammatory conditions, tissues such as mammary gland appeared to increase the synthesis and secretion of albumin and a participation in the mammary gland immune system was proposed for this protein (Shamay et al., 2005). As we stated before, similarities between salivary and mammary glands should be considered

and, in that way, the increase in serum albumin secretion by ruminant parotid glands may had occurred in response to potential inflammation produced by tannins.

Salivary amylase, annexin 1 and leukocyte elastase inhibitor all increase in response to stress. Despite the major function of amylase in the digestion of dietary starch and glycogen, this protein can have additional functions. Several studies have generated interest in salivary α-amylase as a surrogate marker of the autonomic/sympathetic nervous system component of the psychobiology of stress (Granger et al., 2007). Annexin 1 levels were also observed to increase in response to stress (Rhee et al., 2000) and to increased cortisol (Mulla et al., 2005), with the same having been observed for a leukocyte protease inhibitor (Abbinante-Nissen et al., 1995). Taking these results together, we may suggest that tannins induce some stress level both in mice and small ruminants. Higher levels of cortisol were observed in the ruminants white-tailed and mule deer after a period of 10% tannin consumption, when compared with animals consuming regular diets (Hudson et al., 2000). Despite no great attention has been given to these results, they are in accordance with our hypothesis of a physiological stress induced by tannin consumption.

The effect of tannins at salivary gland level has been reported as being possibly due to beta-adrenergic receptor activation. Our results, in chapter 2 do not contradict that theory, although they can not prove it. The changes in salivary protein profiles (chapters 3, 4 and 6) are in accordance with a situation of increased levels of blood glucocorticoids. It is known that both glucocorticoids and neurotransmitters actions (namely catecholamines) are inextricably intertwined (Sapolsky et al., 2000). Subsequent studies for elucidating how tannins induce stress responses and to access how important are the role of glucocorticoids in that will be useful.

4. Main conclusions

In conclusion, the results presented in this thesis provide evidences that:

1. Saliva can be a fluid important in accessing feeding behavior, since salivary proteome differs among animals from different feeding niches, reflecting differences in diet composition;
2. salivary protein composition changes in response to aversive substances, such as tannins;
3. these changes were observed for different proteins in mice, compared to sheep and goats;
4. the mechanisms involved in salivary protein regulation by tannins may be related to a "stress response" imposed by these compounds;
5. animal saliva can be useful to monitor animal physiological changes induced by dietary compounds. For example, the inhibitory effects of tannins in immunitary system were observed in sheep parotid saliva proteome.

5. Implications

The results obtained in the present thesis triggered a whole range of questions, which could be potential future research topics.

First, all the studies reported in this thesis, looking for salivary adaptations to diet, were performed with tannins. To be able to understand a more general role of saliva in dietary choices, it would be interesting to perform similar experiments, studying both glandular and salivary proteome levels, with compounds eliciting different tastes and/or tactile sensations.

Differences among different individuals were also observed. It would be important to explore this heterogeneity in the context of different taste sensitivities. This might be achieved by behavioral studies with different taste compounds.

When we started the present research we had no information on what other proteins, besides salivary PRPs, could suffer changes in their expression, influenced by tannins, and we opted by working with male animals. At present, and since no submandibular secreted salivary proteins were observed to vary with tannin consumption, we think it would be interesting to perform the same kind of research in female mice. The expected lower proportion of submandibular salivary proteins might allow the observation of other salivary proteins expressed at lower levels.

We observed a high amount of serum proteins in ruminant parotid saliva. The presence of these proteins in glandular saliva is not well understood and further investigation on their provenience (from blood or synthesized in salivary glands) would be interesting.

We only analyzed the salivary protein fraction separated by two-dimensional electrophoresis in a particular molecular mass and pI range. However, different approaches will be required to compile a comprehensive catalogue of all the proteins present in saliva. Liquid Chromtogaphy – Tandem Mass Spectrometry (LC-MS/MS), which was only used for few protein identifications in chapter 6, could be further used for mice and small ruminant saliva proteome characterization.

The post-translational modification of proteins is a common biological mechanism for regulating protein localization, function and turnover. Considering that some of the changes induced by diet occurred only for certain isoforms, it would be interesting to perform studies allowing the characterization of the protein post translational modifications, to better understand its function.

The studies performed in the present thesis have not the aim of short-time improvements in animal production, but rather intend to provide basic knowledge that can further be used.

6. References

Abbinante-Nissen, J.M., Simpson, L.G., Leikauf, G.D. 1995. Corticosteroids increase secretory leukocyte protease inhibitor transcript levels in airway epithelial cells. Am. J. Physiol. 268:L601-L606.

Austin, P.J., Suchar, L.A., Robbins, C.T., Hagerman, A.E., 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J. Chem. Ecol. 15: 1335-1347.

Baxter, N.J., Llley, T.H., Haslam, E., Williamson, M.P. 1997. Multiple interactions between polyphenols and proline-rich protein repeat result in complexation and precipitation. Biochemistry 36: 5556-5577.

Engvall, A. 1980. α-Amylase activity in rumen fluid of cows producing milk of low and normal fat content. J. Dairy Sc. 63: 2012-2019.

Dawes, C. 1970. Effects of diet on salivary secretion and composition. J. Dent. Res. 49: 1263-1273.

Dawes, C. 1984. Stimulus effects on protein and electrolyte concentrations in parotid saliva. J. Physiol. 346: 579-588.

Denny, P., Hagen, F.K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G.S., Boontheung, P., Cociorva, D., Delahunty, C.M., Denny, T., Dunsmore, J., Faull, K.F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S.C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J.A., Ogorzalek Loo, R.R., Malamud, D., Melvin, J.E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S.K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M.A., Takashima, J., Than, S., Wang, J., Whitelegge, J.P., Witkowska, H.E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D.T., Yates, J.R. 3rd, Fisher, S.J., 2008. The Proteomes of Human Parotid and Submandibular/Sublingual Gland Salivas Collected as the Ductal Secretions. J. Proteome Res. 7: 1994-2006.

Dodson, C.S., Rengarajan, K., Gewant, H.D., Stodulkova, E., Nguyen, H.T., Boatright, J.H., Nickerson, J.M. 2001. Extra-hepatic expression of serum albumin mRNA in mouse retina. Curr. Eye Res. 22: 182-189.

Emmein, N. 1987. Nerve interactions in salivary glands. J. Dent. Res. 66: 509-517.

Engelen, L., van den Keybus, P.A., de Wijk, R.A., Veerman, E.C., Amerongen, A.V., Bosman, F., Prinz, J.F., van der Bilt, A. 2007. The effect of saliva composition on texture perception of semi-solids. Arch. Oral Biol. 52: 518-525.

Glendinning, J.I. 1994. Is the bitter rejection response always adaptative? Physiol. Behav. 56: 1217-1227.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Weiss, W. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis. 21: 1037-1053.

Haslam, E. 1998. Practical polyphenols. From structure to molecular recognition and physiological action. Cambridge University Press.

Huang, C.M., 2004. Comparative proteomic analysis of human whole saliva. Arch. Oral Biol. 49: 951-962.

Hagerman, A.E., Rice, M.E., Ritchardt, N.T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4-8) catechin (procyanidin). J. Agric. Food Chem. 4: 2590-2595.

Hashimoto, S., Ochiai, S., Muramatsu, T., Shimono, M. 2000. Tight Junctions in the Rat Parotid Gland Eur J Morphol. 38: 263-267.

Fickel, J., Göritz, F., Joest, B.A., Hildebrandt, T., Hofmann, R.R., Breves, G. 1998. Analysis of parotid and mixed saliva in roe deer. J. Comp. Physiol. B 168: 257-264.

Ghafouri, B., Tagesson, C. Lindahl, M. 2003. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. Proteomics 3: 1003-1015.

Granger, D.A., Kivlighan, K.T., el-Sheikh M., Gordis, E.B., Stroud, L.R. 2007. Salivary alpha-amylase in biobehavioral research: recent developments and applications. Ann. N.Y. Acad. Sci. 1098: 122-144.

Hagerman, A.E., Rice, M.E., Ritchard, N.T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4-8) catechin (procyanidin). J. Agric. Food Chem. 46: 2590-2595.

Helmerhorst, E.J. 2007. Whole saliva proteolysis: wealth of information for diagnostic exploitation. Ann. N. Y. Acad. Sci. 1098: 454-460.

Hirtz, C., Chevalier, F. Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., de Périère, D., 2005. Complexity of the human whole saliva proteome. J. Physiol. Biochem. 61: 469-480.

Hudson, R., Irving, B., Gedir, J., Donkor, N., Swanson, K., Morley, F. Nutrition of farmed white-tailed & mule deer. Project completion report. AARI Direct Funding Project 99075. Retrieved June 2008 from: http://www.deer.rr.ualberta.ca/library/tannin/index.html

Iason, G. 2005. The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. Proc. Nutr. Soc. 64: 123-131.

Jacquot, J., Goldstein, G., Sommerhoff, C., Benali, R., Puchelle, E., Basbaum, C.B. 1998. Synthesis and secretion of an albumin-like protein by cultured bovine tracheal gland serous cells. Biochem. Biophys. Res. Commun. 155: 857-862.

Kragh-Hansen, U. 1990. Structure and ligand binding properties of human serum albumin. Dan. Med. Bull. 37: 57-84.

Karas, M., Hillenkamp, F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 Daltons. Anal. Chem. 60: 2299–2301.

Kay, R.N.B. 1960. The rate of flow and composition of various salivary secretions in sheep and calves. J Physiol. 150: 515-537.

Marchese, S., Pes, D., Scaloni, A., Carbone, V., Pelosi, P. 1998. Lipocalins of boar salivary glands binding odours and pheromones. Eur. J. Biochem. 252: 563-568.

Mazariegos, M.R., Tice, L.W., Hand, A.R. 1984. Alteration of tight junctional permeability in the rat parotid gland after isoproterenol stimulation. J. Cell Biol. 98: 1865-1877.

McDougall, E.I. 1948. Stdies on ruminant saliva. 1. The composition and output of shep's saliva. Biochem. J. 43: 99-109.

McManaman, J.L., Reyland, M.E., Thrower, E.C. 2006. Secretion and fluid transport mechanisms in the mammary gland: comparisons with the exocrine pancreas and the salivary gland. J. Mammary Gland Biol. Neoplasia. 11: 249-268

Mehansho, H., Clements, S., Sheares, B.T., Smith, S., Carlson, D.M. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. J. Biol. Chem. 260: 4418–4423.

Mese, H., Matsuo, R. 2007. Salivary secretion, taste and hyposalivation. J. Oral Rehabil. 34: 711-723.

Mole, S., Rogler, J.C., Butler, L.G. 1993. Growth reduction by dietary tannins-different effects due to different tannins. Biochem. Syst. Ecol. 21: 667–677.

Muenzer, J., Bildstein, C., Gleason, M., Carlson, D.M. 1979. Purification of proline-rich proteins from parotid glands of isoproterenol-treated rats. J Biol Chem. 254: 5623-5628.

Mulla, A., LeRoux, C., Solito, E., Buckingham, J.C. 2005. Correlation between the anti-inflammatory protein annexin 1(lipocortin 1) and serum cortisol in subjects with normal and dysregulated adrenal function. J. Clin. Endocrinol. Metab. 90: 557-562.

Pérez-Barberia, F.J., Gordon, I.J. 1998. The influence of molar occlusal surface area on the evolutionary intake, digestion, chewing behaviour and diet selection of red deer (*Cervus elaphus*). J. Zool. 245: 307-316.

Pinkstaff, C.A. 1980. Sexual dimorphism of sublingual glands in tree shrews. Basic Appl. Histochem. 24: 95-100.

Pinkstaff, C.A. 1998. Salivary gland sexual dimorphism: a brief review. Eur. J. Morphol. 36 (Suppl): 31-34.

Pogrel, M.A., Lowe, M.A., Stern, R. 1996. Hyaluronan (hyaluronic acid) in human saliva. Arch. Oral Biol. 41: 667-671.

Pratt, R.E., Dzau, V.J., Ouellette, A.J. 1981. Abundant androgen regulated mRNAs in mouse submandibular gland: cell-free translation of renin precursor mRNA. Nucleic Acids Res. 9: 3433-3449.

Provenza, F.D., Malechek, J.C. 1984. Diet selection by domestic goats in relation to blackbrush twig chemistry. J. Appl. Ecol. 21: 831-841.

Provenza, F.D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. J. Range Manag. 48: 2-17.

Rajan, G.H., Morris, C.A., Carruthers, V.R., Wilkins, R.J., Wheeler, T.T. 1996. The relative abundance of a salivary protein bsp30, is correlated with susceptibility to bloat in cattle herds selected for high or low bloat susceptibility. Anim. Genet. 27: 407-414.

Rantonen, P.J., Meurma, J.H. 2000. Correlations between total protein lysozyme, immunoglobulins, amylase and stimulated whole saliva during daytime. Acta Odontol. Scand. 58: 160-165.

Rhee, H.J., Kim, G.Y., Huh, J.W., Kim, S.W., Na, D.S. 2000. Annexin I is a stress protein induced by heat, oxidative stress and a sulfhydryl-reactive agent. Eur J Biochem. 267: 3220-3225.

Salem, A.Z.M., González, J.S., López, S., Ranilla, M.J. 2000. The effect of feeding alfalfa treated with quebracho on parotid salivation in sheep. In: Proc. 51st Annual Meeting of the European Association for Animal Production (EAAP), van Arendonk, J.A.M. (Ed.), The Hague (The Netherlands). Wageningen Press. Wageningen. The Netherlands. pp. 21-24.

Salem, A.Z.M., González, J.S., López, S., Ranilla, M.J. 2001. Evolución de la respuesta en la producción unilateral de saliva parotídea a la inclusión de quebracho en la dieta en ganado ovino y caprino. ITEA, 22 (Vol. Extra): 322-324.

Sapolsky, R.M., Romero, L.M., Munck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21: 55-89.

Señorale-Pose, M., Jacqueson, A., Rougeon, F., Rosinski-Chupin, I. 1998. Acinar cells are target cells for androgens in mouse submandibular glands. J. Histochem. Cytochem. 46: 669-678.

Shackleford, J.M., Wilborn, W.H. 1968. Structural and histochemical diversity in mammalian salivary glands. Ala J. Med. Sci. 5: 180-203.

Shamay, A., Homans, R., Fuerman, Y., Levin, I., Barash, H., Silanikove, N., Mabjeesh, S.J. 2005. Expression of albumin in nonhepatic tissues and its synthesis by the bovine mammary gland. J Dairy Sci. 88: 569-576.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32: 1149-1163.

Shimada, T., Saitoh, T., Sasaki, E., Nishitani, Y., Osawa, R. 2006. Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins. J. Chem. Ecol. 32: 1165-1180.

Silanikove, N., Gilboa, N., Perevolotsky, A., Nitsan, Z. 1996. Goats fed tannin-containing leaves do not exhibit toxic syndromes. Small Ruminant Res 21: 195-201.

Sissons, J.W. 1981. Digestive enzymes of cattle. J. Sci. Food Agric. 32: 105-114.

Stolte, M., Ito, S. 1996. A comparative ultrastructural study of the parotid gland acinar cells of nine wild ruminant species (Mammalia, artiodactyla). Eur. J. Morphol. 34: 79-85.

Turner, R.J., Sugiya, H. 2002. Understanding salivary fluid and protein secretion. Oral Dis. 8: 3-11.

Van Lennep, E.W., Kennerson, A.R., Compton, J.S., 1977. The ultrastructure of the sheep parotid gland. Cell Tiss. Res. 179: 377-392.

Varricchio, F., Stromberg, K. 1994. An albumin-like protein is the major secretory protein of ovarian epithelial cells in vivo and in vitro. Int. J. Gynecol. Cancer. 4: 328-332.

Vitorino, R., Lobo, M.J., Ferrer-Correira, A.J., Dubin, J.R., Tomer, K.B., Domngues, P.M., Amado, F.M., 2004. Identification of human whole saliva protein components using proteomics. Proteomics 4: 1109-1115.

Wagatsuma, A., Fujimoto, K., Yamada, S. 2002. Alteration in albumin level during modified muscular activity. Scand. J. Med. Sci. Sports. 12: 143-149.

Walz, A., Stühler, K., Wattenberg, A., Hawranke, E., Meyer, H.E., Schmalz, G., Blüggel, M., Ruhl, S., 2006. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole saliva by two-dimensional gel electrophoresis. Proteomics 6: 1631-1639.

Williams, K.M., Ekström, J., Marshall, T. 1999. High-resolution electrophoretic analysis of rat parotid salivary proteins. Electrophoresis. 20: 1373-1381.

Yamashita, M., Fenn, J.B. 1984. Negative ion production with the Electrospray Ion Source. J. Phys. Chem. 88: 4671-4675.

Wheeler, T.T., Haigh, B.J., McCracken, J.Y., Wilkins, R.J., Morris, C.A., Grigor, M.R. 2002. The BSP30 salivary proteins from cattle, LUNX/PLUNC and von Ebner's minor salivary gland protein are members of the PSP/LBP superfamily of proteins. Biochim. Biophys. Acta. 1579: 92-100.

Wheeler, T.T., Hood, K., Oden, K., McCracken, J., Morris, C.A. 2003. Bovine parotid secretory protein: structure, expression and relatedness to other BPI (bactericidal/permeability-increasing protein)-like proteins. Biochem. Soc. Trans. 31: 781-784.

Wheeler, T.T., Hood, K.A., Maqbool, N.J., McEwan, J.C., Bingle, C.D., Zhao, S. 2007. Expansion of the Bactericidal/Permeability Increasing like (BPI-like) protein locus in cattle. BMC Genomics. 8: 75.

Wickliffe, J.K., Lee, V.H., Smith, E., Tandler, B., Phillips, C.J. 2002. Gene expression, cell localization, and evolution of rodent submandibular gland androgen-binding protein. Eur. J. Morphol. 40: 257-260.

Yao, L., Berg, E.A., Costello, C.E., Troxler, R.F., Oppenheim, F.G., 2003. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomic approaches. J. Biol. Chem. 14: 5300-5308.

Young, J.A., Schneyer, C.A. 1981. Composition of saliva in mammalian. Aust. J. Exp. Biol. Med. Sci. 59: 1-53.

Vissink, A., Spijkervet, F.K., Van Nieuw Amerogen, A. 1996. Aging and saliva: a review of the literature. Spec. Care Dentist. 16: 95-103.

Zussman, E., Yarin A.L., Nagler, R.M. 2007. Age- and flow-dependency of salivary viscoelasticity. J. Dent. Res. 86: 281-285.

Appendix 1

Determination of spot protein isoelectric point in 2D gels for IPG strips 13 cm 3-10 NL

Spot protein isoelectric point was determined by measuring the length of the Immobiline DryStrip gel and the position of the strip on the second dimension gel. Then, the spot position (as a percentage of gel length) was ploted versus pH and read off the pI from the graph of the pH gradient (Fig. 1)

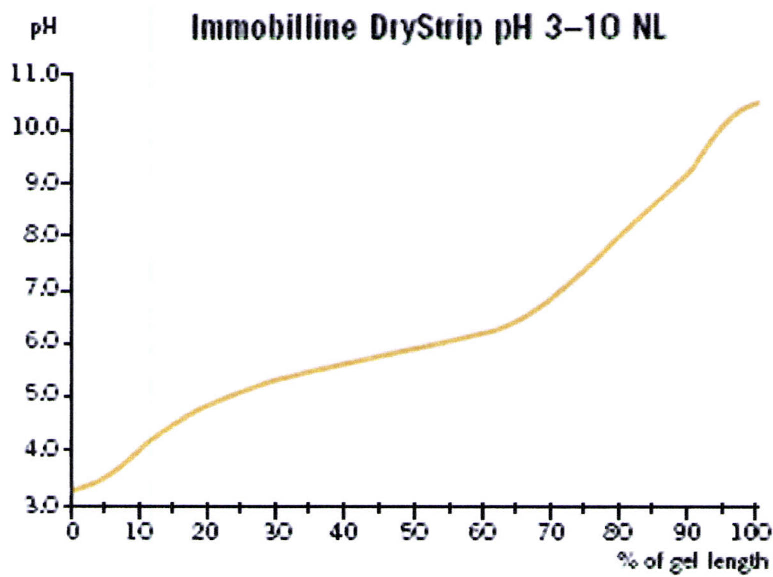


Fig. 1 – pH gradient graph. The pH gradients showed in this figure are valid at 20ºC in 8M urea, with IPGbuffer 3-10 NL. (Immobiline DrySrip gels Data file, 18-1177-60 AB, 2004-06; Amersham Biosciences)

Appendix 2

5G	6G	8G	9G	13G	14G	17_G	18_G	19G
1119.4700	1119.4994	1715.8614	1346.6753	1547.8602	1547.5561	1547.9117	1193.6196	1547.7752
1715.8113	1715.9223	1311.6261	1335.6767	913.5213	913.3454	913.5409	1449.6910	1990.9526
1311.5912	1311.6674	1396.6520	1015.5387	2045.1168	1001.4417	1069.6764	1547.7818	913.4835
1156.4805	1757.8903	1757.8388	1715.9295	1889.0586	1000.5818	1001.6715	1991.0084	1069.5879
2234.9425	1156.5377	1156.4954	1311.6828	1747.7932	1305.5347	847.5836	913.4833	2044.9966
1104.4951	2235.0412	2234.8788	3018.2117	2113.9779	1433.6091	2114.0408	1747.7214	1001.6005
1097.4653	1104.5424	1104.4995	1156.5628	1749.7611	2423.8577	2329.2747	847.5051	1595.7693
1594.6645	1097.5124	1097.4854	2219.1631	2248.0487	1503.5511	1439.9052	2113.8869	1439.8113
1466.5729	1594.7610	1305.6642	2235.1129	1439.8854	1502.7629	1283.8030	2248.0092	1283.7101
1215.5813	1466.6519	1122.5398	1104.5532	1283.7743	1740.6011	1595.8952	1518.7828	1305.7020
771.4667	1396.7115	1594.6992	2183.0496	1595.8544	1466.4314	1305.8173	1595.7977	1503.7517
1137.5201	1215.6279	1466.6020	1097.5376	1552.7604	1595.5321	1433.9131	1439.8293	1625.9179
1282.5154	1088.5401	1215.5909	950.5299	1305.7864	1990.6603	2424.3328	1552.7052	1740.8430
1306.6180	1139.5141	935.5244	1122.6106	1503.8286	2611.6658	1503.8688	1793.8291	1399.6360
1397.6413	1626.7784	1043.5564	1594.7768	1626.0060		1626.0213	1305.7239	1097.5124
1675.7575	1663.8531	1136.4779	1466.6895	1497.9169		1497.9315	1503.7808	1121.4701
1691.6354	1691.7176	1182.5618	1396.7227	1724.9172		1724.9802	1625.9292	1137.5719
1803.8356	1779.8830	1282.5266	1215.6555	1740.9129		1740.9879	1740.8597	1293.6733
1924.9738	1978.8543	1292.6650	857.4780	1138.5478		1466.7956	898.4902	1423.4449
1978.6950	1999.8273	1298.5272	935.5693	1399.7563		1138.5978	1138.5041	1515.7558
2134.1507	2321.0434	1360.6031	989.4771	804.3272		1142.8074	935.4586	1525.7501
2320.8685		1432.7336	1025.5017	1121.5227		1014.6928	1121.4759	1648.9877
2379.8856		1524.7618	1043.5929	1149.6213		2185.2559	1525.7553	1684.9106
2530.0446		1576.7410	1065.5666	1293.1193		1399.6120	1730.7104	1712.9409
		1626.7178	1078.6476	1406.8025		774.2917	1952.8663	1804.9384
		1691.6597	1088.5594	1423.5134		776.3076	1969.8704	1835.0648
		1965.8269	1112.5722	1432.7402		1016.6488	2612.1895	1951.9489
		1978.7720	1139.5462	1449.7593		1121.5582	2925.3336	1968.7455
		2028.8518	1170.5673	1515.8347		1292.7696		2254.1733
		2320.9642	1172.5582	1679.7031		1421.4770		2612.0983
			1188.5616	1684.9781		1423.4909		2863.8148
			1266.6077	1713.0390		1449.7610		2910.0887
			1283.6344	1805.0694		1515.8570		2925.0470
			1292.7680	1952.9357		1529.8775		
			1299.6399	1969.9539		1679.6746		
			1352.7074	2254.3117		1713.0678		
			1360.6751	2612.2960		1805.0855		
			1422.6891	3496.9621		1835.2392		
			1432.8260			1953.0237		
			1473.6941			1970.0483		
			1483.7172			1991.2126		
			1524.8222			2045.2458		
			1538.8003			2248.2006		
			1545.8121			2552.4867		
			1576.8216			2612.3541		
			1601.7787			2909.3378		
			1626.7927			2926.5241		
			1671.9444			3497.0477		
			1691.7527			3575.9899		
			1756.8947			4230.4574		
			1895.0153			4349.4330		
			1978.8982					
			2028.9732					
			2321.0591					
			2335.0895					
			2385.1819					
			2513.2300					
			2604.2121					
			2653.2548					
			3035.2254					
			3074.1342					
			3091.3434					
			3374.7359					
			3553.8390					

20G	21G	22G	28G	32G	34G	37G	40G	41G
1547.9462	1547.7709	1449.6232	1547.9462	1231.4659	1449.6425	1449.7018	1449.6955	1231.5018
1991.2203	913.4651	1547.7397	1991.2203	1201.5947	2850.1986	1547.8328	1547.7888	1201.6712
913.5670	1069.5733	913.4481	913.5670	1881.0713	1547.7348	1991.0554	1990.9994	2402.2355
2045.2487	2044.9618	2044.9534	2045.2487	1879.8846	1990.9304	913.4547	913.4868	1673.9073
1001.7028	1001.5962	2247.8257	1001.7028	2402.1792	913.4458	2329.1392	2045.0451	864.4760
2329.3115	2329.0482	1595.7029	2329.3115	864.4394	1069.5488	1595.7952	2247.9657	1505.8486
1439.9624	1595.7502	1283.6625	1439.9624	1505.8727	2044.9733	1283.7116	1595.7841	1417.6503
1283.8628	1439.8010	1793.7969	1283.8628	1097.5058	1747.6752	1305.7411	1439.8243	1354.6492
1595.9326	1793.8640	1305.6538	1595.9326	2553.2046	1001.5534	1433.8247	1552.7082	1097.5162
1433.8465	1305.7058	1625.8514	1433.8465	808.3380	2113.8418	1724.8860	1793.8766	765.3888
2424.3758	2424.1303	1724.7786	2424.3758	861.0230	2247.8902	882.3980	1305.7295	808.3506
1503.9290	1503.7379	1740.7619	1503.9290	876.9851	2329.0432	1022.4310	1503.7822	856.5298
1626.1106	1625.8993	882.3907	1626.1106	892.9693	1595.7287	1057.5361	1625.9362	1017.5306
1725.0183	1724.7989	1022.3943	1725.0183	1017.4943	1439.7823	1121.4680	1740.8539	1030.5626
1741.0237	1740.8278	1057.5127	1741.0237	1051.6825	1283.6875	1405.7459	1097.5202	1051.6959
1399.8247	2185.0822	1121.4426	1399.8247	1137.5647	1800.7665	1731.8900	1121.4908	1082.6024
728.6153	1399.6960	1405.6736	728.6153	1215.4961	1793.8294	1790.9565	1137.5771	1137.5776
744.6038	869.4596	1433.6218	744.6038	1221.6505	1305.6761	1969.1313	1433.6636	1145.5695
756.5986	966.5056	1504.7110	756.5986	1254.6113	1433.7703	2612.2775	1459.7586	1199.5144
802.3047	1121.4660	1569.7032	802.3047	1459.7268	2424.1186	2909.3629	1515.7865	1215.5049
888.7953	1515.7541	1679.5328	888.7953	1503.7526	1503.7336	2925.3692	1649.0048	1254.6865
920.7325	1525.7346	1731.7780	920.7325	1625.8716	1625.8811		1684.9519	1442.7245
1461.9069	1679.4642	1969.7556	1461.9069	1639.8767	1724.8028		1712.9612	1459.7537
1525.9289	1969.8401	2612.0455	1525.9289	1668.9659	1740.7837		1778.8509	1585.7185
1679.7990	2612.1620	2863.4441	1679.7990	1674.8338	1466.6327		1804.9564	1601.7102
1970.0254	2909.1201	2909.0236	1970.0254	2328.1061	1138.4774		1835.1341	1668.9363
2553.3888	2925.2244	2925.0985	2553.3888	2484.1750	1399.6462		1969.8608	1825.9180
2612.4620	3350.5363		2612.4620		1119.4409		2612.1974	2068.0733
2909.4914	3496.7212		2909.4914		1121.4360		2926.2665	2328.1319
2925.4208	3575.7276		2925.4208		1517.7314			2467.8076
3576.0963	4348.9295		3576.0963		1679.5453			2484.2399
					1730.6337			2537.2598
					1778.7648			2554.2342
					1925.8562			2693.4397
					1952.7899			
					1969.8227			
					2553.2314			
					2612.1007			
					2909.1386			
					2925.1858			
					3496.5802			
					3575.5050			

45G	48G	55G	56G	57G	58G	63G	66G	67G
1547.8220	1449.6040	1547.6423	1177.7191	913.5665	1547.7748	842.5690	1497.7687	1449.7002
1991.0066	2018.7142	913.4271	1547.8462	1121.5917	913.4979	855.1088	1872.8847	1547.8116
913.4900	1547.6918	1001.5654	1991.0695	1283.8211	2045.0994	861.1371	1328.7239	1991.0278
2045.0524	1990.9017	1595.6224	913.5182	1399.6295	1439.8390	871.0905	1200.6302	913.4898
2247.9770	913.4140	1439.7063	2045.1170	1423.5339	1305.7395	877.1128	1168.6103	1069.5984
1595.8137	1069.5252	1503.6407	1360.7102	1439.9466	1503.8187	1168.6556	747.4370	2045.0691
1439.8303	1001.5245	1625.7726	1001.6276	1449.8027	1625.9708	1179.6720	856.5253	1001.6066
1283.7212	2113.8148	1724.6881	2113.9782	1503.9123	741.9373	1200.7036	881.2670	2247.9942
1305.7371	2247.8357	1423.2742	2247.9945	1515.9028	855.0761	1475.8243	942.5583	1595.8057
1433.7659	1595.7298	2044.8035	2329.2447	1595.9167	861.0857	1497.8511	1240.6616	1439.8271
1503.7899	1439.7447	2423.8239	1595.8405	1626.0658	877.0622	1602.8925	1266.6062	1283.7370
1625.9415	1283.6382	2611.8398	1439.8746	1679.7515	893.0323	1687.9481	1602.8100	1793.8742
1740.8882	1800.7480		1283.7760	1725.0040	930.9936	1768.9228	1665.8427	1305.7325
1043.5798	1793.7630		1800.9081	1769.0406	1082.0715	1794.8862	1744.7986	1433.8376
1070.6042	1305.6459		1793.9188	2045.2766	1098.0477	1872.6857	2220.1274	1503.7851
1097.5430	1433.7329		1305.7685		1293.0968	2077.0077	2532.0562	1625.9366
1121.4971	2424.0893		1433.8595		1327.7415	2211.0595		1740.8822
1137.6029	1503.7045		2424.2910		1449.8645	2220.3008		900.5171
1449.7270	1625.8480		1503.8268		1461.8373	2384.0033		1029.6042
1515.7955	1497.7463		1625.9954		1477.7767	2532.2196		1049.5661
1969.9255	1724.7802		1497.8861		1541.7595			1097.5396
2612.2051	728.5059		1740.9004					1121.4939
	816.5641		1466.6987					1137.5051
	876.9837		877.0942					1172.5652
	904.6022		893.0729					1188.5588
	1121.4215		1097.5510					1292.8919
	1525.7238		1121.5270					1315.7743
	1867.9392		1156.5810					1370.7461
	1952.7439		1215.6840					1399.5405
	1969.7733		1449.7379					1423.4489
	2521.1450		1515.8192					1515.7890
	2554.2603		1525.8256					1649.0068
	2589.1021		1540.8437					1668.7979
	2612.0596		1585.8217					1680.7996
	2909.0618		1663.9643					1684.9490
	2925.0637		1679.6623					1712.9829
	3449.4787		1866.1035					1725.9375
	3465.4496		2321.1315					1805.0094
			2553.3592					1835.1342
			2612.2391					1855.0380
			2925.3493					1920.0793
								1922.0877
								1969.8880
								2525.9219
								2612.2218
								2822.3960
								2863.8013
								2926.3649

75G	77G	83G	84G	93G	113G	116G	125aG	128G
3558.4613	3558.3608	913.7848	1547.7728	1547.8790	913.5889	1001.6373	1195.5510	1449.6375
2856.0621	2856.2435	1283.8931	1990.9825	1991.1168	1001.7087	1595.8325	1001.5826	1547.7017
2984.1196	2984.3831	1292.9539	913.4740	913.5275	1301.8862	1439.8598	847.5232	913.4340
2707.0487	1417.7072	1301.9196	1069.5784	1069.6270	1440.0130	1433.8668	1518.7963	1001.5455
1160.5037	953.5837	1305.8078	2045.0287	1195.6656	1503.9578	2424.3926	1595.6719	1595.7332
1417.6348	2769.5148	1423.6060	1001.5917	1001.6443	1596.0181	1503.8087	1305.7448	1503.6914
951.4856	1861.9415	1439.9594	2247.9205	2114.0429	1626.1444	1625.9369	1433.7977	1625.8583
2054.7701	881.2548	1503.8991	1595.7684	2248.1009	1725.1467	1497.8181	1503.6894	1466.5926
2182.8650	942.5651	1595.8636	1439.8072	1518.8646		833.1128	1625.8201	728.5211
1861.8709	1056.5585	1626.0031	1305.7159	1439.8977		855.0866	1497.7269	877.0043
1676.7526	1161.6592	2045.1497	1503.7661	1800.9526		871.0487	1539.7722	1301.6545
813.4915	1240.6549		1625.9171	1793.9886		885.2624	1466.6223	1432.5736
942.5397	1289.7420		1097.5054	1305.8064		1018.0161	836.5447	
1124.5893	1311.7258		1122.6507	1433.9074		1110.4849	1301.6715	
1182.4941	1327.7079		1515.7727	1503.8609		1301.7612	1647.8775	
1198.4560	1497.7414		1648.9877	1626.0171		1572.8937	1924.7238	
1239.6065	1602.8122		1712.9431	1497.9194		1589.8515	2423.9287	
1284.6633	1768.8983		1835.0907	1466.7606		1925.0538	2611.9516	
1289.6988	2432.0040		1920.0324	1178.6481		2612.2165		
1369.6512	2661.6401		2612.1388	1449.7770				
1456.4167	2749.1417			1455.8776				
1741.8032	3161.8779			1569.8623				
1768.8053				1648.0458				
1871.8773				2612.3185				
2096.7515				2909.3344				
2103.0251				2925.3345				
2246.0325								
2310.9521								
2385.9827								
2432.9007								
2659.9191								
2749.0354								
2765.0289								
3161.2574								
3686.5618								

132G	133G	134G	137G	138G	139G	145G	172G	176G
1579.7730	1096.5248	1744.9148	864.1122	1744.7565	1744.9189	913.4885	2850.4433	1195.6763
836.5419	2306.3420	2149.1137	913.3581	2148.9293	2149.1344	2113.7482	2034.9497	1001.6597
2482.3203	2359.2559	836.5388	1001.4203	1291.6760	836.5408	2247.7789	1547.8183	1518.9074
1827.0090	1203.6711	2482.2355	1121.3395	836.4447	2482.2593	1595.7294	1991.0296	1439.9420
1378.6602	1572.8698	1826.9505	1292.6377	1826.8064	1826.9703	1439.7838	913.4834	1595.9094
1924.8727	1158.6320	1378.6908	1301.5535	1924.7225	1378.6885	1800.7365	1069.5952	1433.9311
1301.7283	1407.7618	1924.8910	1305.5533	728.4770	1924.8638	1305.6956	1747.7989	2424.4161
1323.7225	1082.6106	927.4849	1322.5425	744.4779	2991.3775	1433.7734	1195.6282	1503.8841
1339.6729	2932.5974	1027.4894	1423.2092	927.3929	3007.3345	1432.8038	1001.5973	1626.0572
1589.8221	2991.3889	1028.4649	1439.5907	1014.4725	776.2515	1503.7197	847.4983	728.5989
1848.8886	836.5314	1301.7434	1503.5638	1027.3878	858.5283	1625.8561	1666.9230	877.1068
1864.9220	844.4679	1313.7502	1595.5454	1301.6098	927.4780	1466.6225	2113.9439	1178.6547
2468.2626	927.4493	1335.7385	1625.6906	1313.6354	1027.4844	1138.4970	2248.0262	1301.8329
3085.1173	999.4303	1351.7409	1724.6055	1335.6216	1044.5067	1003.6060	1518.8051	1465.9182
3129.1887	1029.6238	1449.8163	1740.7516	1351.6116	1301.7310	1121.4611	2329.2902	1572.9763
	1049.5498	1572.8201	1919.7252	1373.5840	1313.7764	1517.7301	1595.8046	1590.0071
	1165.6144	1589.8537	2044.7101	1395.5591	1335.7446	1684.8328	1439.8466	1670.8872
	1259.7167	1735.8650	2556.9503	1766.7450	1351.6905	1712.8593	1800.8818	1725.1041
	1301.7575	1848.9207	2611.8920	1782.6891	1572.8289	1835.0411	1466.7061	1800.9948
	1313.8073	1864.9025		1848.7780	1580.1960	1952.7492	1178.5654	1868.2175
	1323.6999	2140.0783		1864.7480	1589.8351	1969.7449	1432.6885	2046.2631
	1329.7547	2468.2748		1962.7362	1766.8276	2612.0147	1449.7236	2612.4698
	1338.7013	3129.3103		2170.9040	1848.9074		2612.2558	
	1346.7536	3246.3774		2186.9326	1864.8863		2845.2944	
	1438.7367			2482.0755	1946.8703		2863.6781	
	1455.7089			2938.0276	2134.0671		2909.2964	
	1570.8602			3084.0932	2140.1074		2925.3727	
	1589.8723			3100.0773	2159.9637		3496.6907	
	1652.9619			3246.0282	2181.1280			
	1740.9263			3408.1722	2468.3389			
	1744.9896				2504.2948			
	1757.9508				2938.2185			
	1827.0426				2946.0105			
	1897.9640				3100.2600			
	1924.9481				3129.2822			
	2047.1515				4493.3390			
	2064.1813							
	2136.0578							
	2140.2257							
	2150.1714							
	2349.2321							
	2418.3070							
	2468.3614							
	2482.3231							
	2584.2906							
	2602.3377							
	3129.3975							
	3197.6325							

177G	178G	181G	184G	185G	193G	198G	199G	202G
3287.3450	3287.5582	913.5137	1744.8891	877.0508	2612.2443	2248.0076	2248.0076	804.4865
2149.1227	1579.8120	1001.6152	2437.1717	893.0386	2248.0360	1595.8329	1595.8329	806.5082
836.5429	836.5281	1292.4171	836.5371	913.4964	1595.8181	1439.8690	1439.8690	842.5545
2482.4021	2482.5403	1301.7270	2482.1772	1001.5989	1439.8510	1283.7588	1283.7588	870.6011
1827.0039	1827.1028	1439.7722	1826.9302	1439.7927	1283.7390	3022.3498	3022.3498	965.6402
1924.8764	1378.7473	1595.6805	1924.8776	1547.8115	1793.9484	1552.7379	1552.7379	1045.6541
855.0733	1925.0393	1625.8255	2991.3152	1595.7382	1305.7506	1793.9332	1793.9332	1118.7440
861.2812	927.4876	1919.8597	3007.3128	2044.9065	1433.8674	1305.7693	1305.7693	1259.7898
1301.7430	1027.5223		927.4783		1503.8137	1433.8634	1433.8634	1390.7980
1313.7927	1080.5469		998.5179		1625.9707	1503.8318	1503.8318	1691.9656
1572.8290	1088.6106		1000.5080		1740.8966	1625.9850	1625.9850	1805.0361
1589.8587	1134.5545		1217.5422		1399.6463	1724.8924	1724.8924	1998.0539
1735.9062	1178.5939		1228.6486		1415.7276	1740.9085	1740.9085	2004.1709
2140.0927	1217.5990		1301.7156		1016.5575	1138.5375	1138.5375	2138.1283
2468.3752	1228.7028		1313.7434		1121.4870	1399.6925	1399.6925	2178.1478
2938.3170	1301.7823		1503.7520		1137.4997	1415.7445	1415.7445	2194.2068
3084.3315	1511.0097		1572.8098		1292.8644	1016.6040	1016.6040	2211.2215
3100.2255	1572.9114		1589.8192		1423.4541	1121.5118	1121.5118	2353.3122
3129.4012	1589.9424		1635.7127		1461.8294	1180.6946	1180.6946	2534.3507
3246.3712	1654.4208		1735.8941		1515.7954	1293.1806	1293.1806	
3262.1708	1670.8525		1767.8615		1525.7917	1423.4727	1423.4727	
3408.5676	1684.8676		1809.5679		1541.7761	1461.8467	1461.8467	
3449.4022	1686.8922		1848.9335		1648.9314	1515.8119	1515.8119	
	1715.9562		1864.8862		1663.9018	1541.7898	1541.7898	
	1736.0264		2140.0883		1679.7483	1649.9163	1649.9163	
	1849.0710		2144.9373		1712.9979	1663.8857	1663.8857	
	1881.0753		2468.2108		1952.8965	1679.6519	1679.6519	
	2140.3078		2938.2110		1969.9108	1713.0159	1713.0159	
	2145.1858		3084.3590		1991.9073	1952.8776	1952.8776	
	2160.1336		3100.3410		2525.0810	1969.9194	1969.9194	
	2468.5115		3112.1338			2524.9805	2524.9805	
	2938.5898		3129.3047			2612.2766	2612.2766	
	3084.6786		3246.3069					
	3100.6596		3262.3748					
	3129.6446		3302.2768					
	3246.6660		3408.3676					
	3262.4456		3449.4774					
	3303.7148							
	3408.7915							
	3449.7422							
	3611.7829							

204G	207G	213G	214G	216G	217G	220G	228G	232G
2248.0685	832.3130	728.5549	976.4580	976.4398	976.5664	1449.6841	1215.6161	1311.6438
1518.8699	842.4976	842.5102	1198.7131	1198.6914	1198.8471	913.4868	1231.6004	1001.5546
1439.8979	870.5187	913.4896	1171.5971	800.5216	800.6356	1195.5983	1104.5967	1449.7485
1800.9308	877.0514	1001.5937	1187.5330	1954.0622	1515.9270	1001.5984	976.5176	1047.5403
1433.9333	881.2669	1098.5181	1954.0856	1639.8211	1954.3187	1518.7671	1129.5666	1443.7582
1503.8424	917.2613	1156.5642	1639.8652	1014.4596	998.5839	1595.7649	994.5330	1286.7125
1626.0315	1030.5529	1170.6327	1132.5250	1132.5261	1014.5908	1439.8043	1459.6218	1305.6043
1539.9577	1043.5744	1301.7322	1629.8205	1629.8002	1132.6601	1800.8251	728.5981	1212.5660
1003.6557	1049.5443	1305.7084	1790.9098	1790.8773	2215.3603	1305.7148	825.1274	728.5261
1121.5324	1088.5417	1433.8257	2231.1024	2215.0514	2231.3492	1433.8127	841.0997	877.0076
1138.6234	1104.5303	1439.8108	795.4590	2231.0484	795.5751	1503.7652	857.0684	913.4430
1305.9297	1156.5373	1503.7715	923.5608	795.4571	923.6824	1625.8924	877.0669	960.4568
1327.7771	1173.6521	1516.7119	1516.7183	923.5612	1644.9912	1539.8231	893.0402	977.4911
1466.8990	1199.6959	1518.7723	1644.8276	1516.6890	945.6683	1466.6666	1008.5582	1144.5756
1498.0434	1203.6600	1595.7617	881.2708	1644.7847	1052.8339	856.5269	1025.5786	1150.6245
1647.9848	1239.5466	1625.9118	913.4624	877.0379	1130.6810	1121.4633	1067.5173	1160.5778
1953.0916	1283.5767	1695.8090	1001.5778	893.0061	1158.6906	1350.6971	1097.4909	1167.6510
1970.1522	1311.6658	1790.8946	1121.4723	945.5492	1182.4762		1151.5397	1625.8668
2612.3099	1354.7853	1800.8624	1138.4766	1164.7420	1220.8413		1181.6544	
	1390.7000	1954.0606	1164.7406	1170.4745	1236.8236		1301.6630	
	1399.7516	2211.0853	1182.3522	1182.3265	1285.8162		1359.6753	
	1492.7592	2247.9529	1305.7339	1220.6818	1351.9763		1589.7284	
	1537.7294	2612.1254	1433.7938	1236.6560	1379.9950		1594.6159	
	1715.8971		1439.8120	1241.6213	1456.8651		1827.7423	
	1725.8614		1466.6545	1499.6754	1472.8776			
	1997.0373		1499.6849	1531.7279	1499.8654			
	2212.0828		1503.7709	1537.6513	1529.9060			
	2308.1297		1547.7690	1547.7308	1531.9116			
			1595.7917	1554.6645	1537.8845			
			1625.9258	1627.7614	1547.9280			
			1695.7945	1812.8397	1553.8807			
			1725.8833	1828.8381	1670.0674			
			1800.8644	1850.8571	1698.0639			
			1812.8726	1976.0237	1813.1093			
			1976.0525	1992.0011	1829.0984			
			1992.0271	2013.9900	1944.2361			
			2101.9144	2029.9655	1961.1539			
				2169.6737	1976.2580			
					1992.2161			
					1999.1186			
					2170.0317			

234G	236G	239G	248G	259G	262G	265G	269G	271G
1231.7371	836.5014	1215.7940	1215.7224	1449.6639	913.4946	1231.7625	1595.6542	1231.6346
1104.6820	842.4696	1231.8078	1104.6643	1547.7734	2329.1410	1104.6905	1439.7002	1104.6112
976.5781	870.5003	1104.7369	976.5618	913.4776	1595.7853	976.5869	1283.6183	976.5148
1129.6618	1027.4391	976.6292	2643.3588	1195.5822	1439.8270	1129.6823	1793.7123	1129.5998
1459.8175	1291.7270	1129.7295	1600.9431	1001.5836	1800.8722	994.6054	1305.6214	994.5311
856.5615	1313.7230	994.6474	1129.6427	1518.7647	1793.8850	1443.8259	1433.6906	1459.7298
1301.8171	1378.6064	1459.8775	2105.2558	1466.6454	1305.7410	1459.8322	2423.9674	1065.5449
1359.8222	1388.6753	2223.3677	994.5692	833.0656	1433.8377	1065.6291	1503.6393	856.5268
1572.9284	1570.8218	877.1424	1443.7966	855.0603	2424.1314	1021.6504	1625.7803	1301.7199
1589.9858	1707.7508	911.6168	1459.7765	1060.0528	1503.7871	865.5288	1497.7024	1359.7125
2233.2796	1725.8063	1046.6670	1065.5983	1178.5636	1625.9384	2223.1936	1740.6798	
2468.4518	1812.7576	1054.6719	2207.1973	1432.6261	1497.8501	1203.7795	861.0095	
	1924.8209	1296.8287	2223.2619	2612.0717	1724.8399	1301.8153	876.9706	
	2149.1316	1301.8857	728.5788		1740.8729	1359.8375	1121.3794	
	2206.1502	1347.9315	1398.6561		1138.5098	1398.6629	1461.6696	
	2211.0633	1358.8760	2161.7591		776.2528	1589.9430	1515.6508	
	2268.0770	1738.0367			1119.4958	1670.9105	1525.6337	
	2305.0377	2161.8907			1121.4913	1827.2133	1647.7823	
	2482.1897				1408.6857	2161.8752	1952.6949	
	2938.1310				1679.5905	2548.4703	1969.7194	
	3083.5155				1952.8713	3130.7296	1975.7678	
	3099.0626				1969.8974		1991.6709	
					2509.2500		2552.0699	
					2612.2176		3575.3987	
					3111.4635			
					3496.7215			
					4229.7384			

272G
1595.7062
1439.7523
1793.7566
1305.6728
1433.7394
2424.0804
1503.7075
1625.8521
877.0121
1416.7187
1553.6734

273G
1375.7879
2659.3035
1183.6790
2909.3730
1055.5944
1063.1894
1167.3275
1335.5006
1359.4134
1392.8450
1617.8121
1674.9510
1183.6564
1468.8071
804.4640
1219.7
1585.0
2225.1
2868.5
870.54

280G
2414.2281
1557.8723
1276.6170
1703.8279
1727.8291
1043.6384
2047.8946
1719.7405
908.4657
928.5176
1371.7801
1432.6640
833.0828
855.0654
966.5043
982.5015
1060.0744
1092.6224
1301.7245
1415.6534
1537.8235
1604.8857
2030.5636

282G
1826.6877
2413.8840
1557.7121
1276.4797
1703.6734
2315.8763
818.9989
823.0200
994.0378
1225.0277
1821.8061

303G
1599.7971
1727.8018
958.5409
1377.7226
943.4566
1018.5440
909.4810
931.4591
1001.5864
1204.5178
1220.5528
1315.7382
1427.6317
1503.7301
1547.8039
1616.8047
1625.8695
1703.8606
1744.9103
1861.9422
1959.4536
1975.9011
2303.1158
2542.2554

310G
1449.6922
1547.8093
913.4726
1069.5731
1195.5895
1001.5950
1466.6749
856.5172
1060.0596
1178.5664
1199.6623
1243.6935
1287.7096
1331.7421
1375.7730
1432.6521

311G
1449.5954
1547.7116
913.4212
1069.5294
1747.6237
1001.5302
2113.6810
2247.9040
1466.5750
999.5142
1432.5830
2612.0556
2925.2088

313G
913.5567
1001.6565
1069.6482
1292.5933
1419.7453
1441.6604
1449.7483
1466.7519
1503.8202
1547.9628
1569.7348
1585.6527
1625.9412
1990.9003
2247.8242
2612.2194

323G
1449.6678
1547.7722
913.4695
1069.5765
1195.5668
1001.5840
847.4986
1466.6519
856.5193
1178.5536
1432.6380

324G
913.4938
1800.8278
1305.7427
1433.8406
939.4495
1503.8108
1625.9373
1497.8726
1539.8526
1138.4828
1142.7462
1014.6451
855.0772
966.4069
982.5324
1121.4955
1343.6758
1455.7963
1471.7930
1663.8762
1812.9021
1973.9317

326G
836.4621
842.4404
870.4745
900.3349
913.5020
1023.4063
1027.4082
1040.4113
1056.4080
1086.5458
1093.5775
1097.4028
1110.5154
1164.5307
1168.5017
1178.5331
1184.5020
1291.6726
1313.6513
1378.5547
1391.6195
1428.6882
1556.6763
1744.7431
1782.7862
1826.8226
1880.7565
1924.7132
2053.7985
2148.9507
2210.9155
2232.8528
2238.9492
2324.8075
2482.0244
2937.9982
3083.1240
3099.0251
3245.1205

333G
774.3278
776.3260
804.3665
826.3315
832.3924
842.3186
1233.8124
1339.7926
1364.5964
1381.9138
1397.9085
1403.8980
1407.8987
1489.2104
1574.9251
1588.9358
1596.9148
1617.9764
1675.0559
1709.0201
1723.0221
1730.9303
1746.0720
1813.7557
1820.7259
1831.1971
1837.1150
1851.1282
1859.1133
1942.1795
1964.1294
2133.3330
2138.0743
2145.2629
2173.2731
2224.3128
2238.3056
2245.3087
2380.4932
2414.3925
2442.4764
2461.3489
2531.3905
2604.4358
2621.4326
2635.4279
2657.4954

334G
1730.7188
1233.6262
1722.7879
1251.6414
2442.0978
2132.9659
1192.4797
774.2113
776.2122
826.2213
842.2079
1183.6213
1209.5025
1397.6836
1403.7020
1409.6890
1488.8124
1574.6945
1590.6965
1658.4360
1674.8303
1708.7650
1745.8932
1814.5009
1830.9404
1836.8582
1850.8415
1941.9316
1963.8796
2137.6440
2144.9460
2206.6247
2224.0116
2238.0364
2380.1589
2414.1163
2461.0698
2531.1356
2564.0700
2604.1310
2621.1238
2635.0807
2657.2173
2701.1267

335G
1224.6312
1056.5106
1184.5791
1911.8324
900.4068
955.5061
1110.6053
881.2560
1023.4816
1040.4931
1151.5642
1168.5923
1291.7565
1391.7359
1428.8054

340G
1193.5727
1449.6562
2034.8930
1547.7679
913.4798
1001.5874
1122.6380
1358.6843
1432.6376
1496.6645
1748.6785
1812.7961
1973.3736

350G
913.4657
1793.7869
1305.6907
1433.7797
2424.1096
1503.7491
1625.8883
1497.7887
1740.8142
1539.8050
2311.1354
1014.6299
1121.4583
1131.6100
1867.9674
1952.7624
1969.7590

371G
1266.4968
1394.5813
1260.4677
1017.4219
1430.5811
1052.4796
1305.5070
1589.6361
860.9851
876.9508
1012.4719
1264.4576
1288.4784
1292.4420
1452.5861
1572.6105
1669.6932
1686.7058

373G
1449.6615
2850.1905
1547.7704
1990.9538
913.4802
1069.4864
877.0345
971.5761
2496.1694

376G	388G	389G	394G	399G	400G	401G	405G	414G
855.5284	870.6457	1215.6569	1576.8556	1177.6857	1449.6859	1813.6324	1260.6357	1449.6730
893.4975	913.5836	1231.6442	2188.1525	1449.6902	2850.2828	1266.4724	1273.6852	2850.2482
993.5817	951.5657	1104.6045	1394.7289	1547.7846	2018.8480	1394.5329	1017.5500	1547.7855
1007.5872	1023.7355	976.5196	1388.7558	913.4758	2034.9223	1388.5067	1430.7553	1990.9728
1021.5949	1067.7640	1129.5936	1260.6016	1069.5879	1547.7998	1260.4182	1305.6785	913.4811
1023.6063	1069.7310	2105.1085	1516.7428	1747.7398	1991.0322	1017.3916	1589.7999	785.3913
1034.6259	1419.8677	994.5370	1273.7038	1268.6602	913.4827	1430.5292	856.5245	1122.6278
1051.5968	1440.0221	1443.7252	1017.5228	856.5132	1069.5887	1866.7510	913.4843	2925.0898
1065.6193	1441.8426	1459.7106	1430.7331	1151.5812	1747.7463	1026.4447	927.5003	
1081.6091	1449.8519	2223.1100	1867.0453	1385.7842	1432.6513	1297.5338	1012.5787	
1092.6538	1457.8345	728.5462	1297.7210	1432.6547	1456.7551	1305.4582	1119.4685	
1227.6839	1503.9435	1158.7066	1052.6298	1574.7899	1569.8133	1589.5878	1439.8270	
1235.6330	1547.9295	1185.6927	1305.6731	1730.7375	1730.7125	1772.6941	1479.8218	
1670.7535	1585.9241	1338.7839	1589.7881	2224.1580	2119.0749	2394.0334	1567.7526	
1676.8560	1626.1222	1359.7473	1772.9184		2304.1652	1012.4245	1639.9453	
1681.8759	1991.1283	1398.5925	877.0932		2863.5600	1119.3124	1686.8781	
1691.9023	2045.2810	2233.1907	1012.5748		2909.1774	1129.3618		
1697.9052	2083.2648		1243.5566		2925.2853	1243.4018		
1787.0570			1414.3735			1273.4982		
1798.8746			1452.7877			1283.4008		
1814.0539			1469.7370			1288.4337		
1885.9120			1639.8607			1414.1199		
2002.0667			1669.8259			1452.5326		
2013.9895			1686.9284			1468.5288		
2024.0116			2138.2308			1650.6628		
2039.9854						1669.6457		
2064.0669						1686.6594		
2223.1314						1708.6162		
						1897.7669		
						2122.8965		
						2137.8964		
						2213.9115		

424G	433G	434G	444G	445G	463G	500G	501G
886.4886	2431.4160	771.1964	936.5271	1754.9388	1231.5369	1716.0337	733.4386
939.4613	1863.0111	832.3410	1342.7823	936.5116	1104.5318	1714.8890	825.4450
1850.9463	2207.1900	842.5266	1115.6056	1342.7439	976.4440	1156.6190	842.5151
2737.4832	988.5927	870.5579	1274.7790	1274.7411	1129.5238	1104.6051	870.5607
905.5582	1433.8077	877.0865	2116.1080	2115.9794	994.4628	1097.5876	923.4671
1647.9195	1463.8899	897.4455	1177.7277	1097.5448	1459.5759	1594.8749	978.4809
1479.6724	1121.6499	973.5644	1477.8553	1177.6922	854.9896	1466.7789	988.5306
2120.0290	748.4310	1060.6023	964.5363	1477.8120	861.0043	1215.7143	1045.5807
742.3583	770.4131	1109.5305	1082.6332	856.5446	876.9755	861.1294	1142.8033
1061.5132	1034.6400	1140.6236	1215.7068	864.5112	892.9522	877.1030	1408.6747
1288.7291	1048.6594	1157.6470	1230.7694	958.4959	1053.4793	1088.6129	1494.6850
1316.7920	1104.6220	1165.6443	1272.7559	995.6562	1141.4877	1416.9007	1812.8572
2103.7001	1137.6549	1179.6364	1288.7313	1051.7069	1301.6301	1553.8763	1992.9901
2142.0123	1143.6200	1307.7374	1290.7661	1114.5382	1359.6224	2137.3439	2135.0481
2158.0309	1153.6527	1323.7218	1304.7559	1242.6301	2222.8466	2195.4032	
2202.9317	1159.6123	1383.7539	1306.7574	1324.7315		2318.3481	
2493.1889	1260.6710	1475.8192	1322.7447	1364.7444		2377.4631	
	1316.8142	1493.8022	1491.8164	1380.7113			
	1417.4052	1497.8219	1724.7732	1495.8545			
	1479.8713	1533.8370	1854.9848	1523.8746			
	1495.8899	1574.8378	1866.9847	1551.9094			
	1585.8261	1590.8405	1876.9830	1854.9395			
	1590.8707	1596.8239	1892.9202	1876.8981			
	1619.7919	1612.8057	2054.7033	2233.0826			
	1632.8405	1674.9699	2132.0497				
	1659.8260	1708.9093	2535.2551				
	1676.8446	1715.0513	2622.3515				
	1692.8797	1745.9912					
	1698.8224	1750.9665					
	1819.0349	1837.0300					
	1877.0055	1852.0468					
	1884.9587	1859.0331					
	2134.1620	1865.0230					
	2290.2762	1874.9966					
	2335.2553	1881.0166					
	2696.5677	2022.9446					
	2824.6030	2211.1868					
		2224.2382					
		2233.1909					
		2380.3203					
		2717.2015					

5S	6S	8S	9S	12S	13S	14S	17S	18S
1733.0550	1119.4067	1716.0168	1346.6451	1346.4317	864.1909	1547.8451	1547.7975	913.4725
1716.0568	1715.8549	1156.6115	1335.6705	1335.5097	880.1711	913.5192	913.4796	1001.5746
1311.7671	1311.5986	1104.6080	1604.8302	1715.6871	913.4476	1195.6398	1888.9646	1234.6491
1156.6236	1757.9721	2183.0913	1732.8992	1156.3961	1439.7218	1001.6463	1747.7202	1292.9421
2235.1545	1156.5716	1097.5868	1715.9060	1104.3747	1503.6581	1436.7105	2113.9343	1320.5425
1097.5990	2235.1196	1122.6475	1311.6538	1097.3600	1525.6494	1518.8339	2247.9610	1423.3715
1122.6624	1104.5396	1594.8497	1156.5395	1122.4320	1541.6399	1595.8295	1595.7930	1439.7546
1594.8690	1097.4286	1466.7442	2235.0023	1594.5520	1595.6624	1800.8767	1439.8207	1503.7097
1466.7699	1122.5026	1215.7211	1104.5309	1466.4840	1625.8141	1503.8334	1283.7169	1595.6946
1215.7292	1594.6973	833.1318	1097.4992	1215.4805	1724.7003	1625.9763	3022.4176	1625.8419
935.6239	1466.6076	855.1217	1122.5724	855.1069	1740.7062	1497.8972	1305.7295	1724.7298
1043.6152	1215.5657	871.0905	1594.7345	861.1234	2044.8467	855.0785	1433.8290	2044.9357
1088.6202	728.4811	885.3463	1466.6520	877.1000	2423.9911	1121.5243	1503.7979	
1104.6183	876.9491	1043.5776	1396.6919	893.0881	2557.0285	1178.6194	1625.9497	
1120.6192	1043.5693	1088.6048	1215.6282	935.4342			1497.8354	
1136.6192	1088.5428	1237.7026	1043.4877	1237.4496			1724.8507	
1139.6035	1283.5516	1275.7982	1088.5384	1275.5477			1740.8590	
1172.6213	1360.5682	1283.6799	1120.5283	1282.4263			1138.5235	
1238.7204	1397.5937	1298.7782	1139.5054	1299.4316			2557.2833	
1266.6745	1525.8418	1524.9243	1150.5728	1538.5524			1941.9185	
1275.8241	1538.8066	1538.8525	1266.5789	1626.6193			1399.6996	
1283.7061	1691.7638	1755.0188	1275.7040	1978.5310			1955.8449	
1299.7190	1737.8270	1967.1014	1282.5760	2234.7318			776.2606	
1306.7882	1936.0249	2321.2064	1299.5925	2280.9627			826.2532	
1360.7933	1965.8257	2446.4733	1352.6541	2320.7330			1121.4816	
1396.8303	1978.9534	2512.4129	1360.6645				1423.4312	
1524.9420	1988.0330		1524.8067				1551.7388	
1538.8730	2000.7158		1538.7331				1679.6749	
1674.0441	2257.0741		1545.7727				1730.7087	
1676.0062	2321.1375		1626.7482				1778.8668	
1691.8730	2380.0502		1673.8851				1993.0321	
1748.0742	3091.7058		1691.7065				2540.8378	
1755.0052	3178.5936		1756.8746				2612.1851	
1758.0742			1802.8227				3008.3661	
1925.2589			1818.8619				3497.7708	
1966.0708			1936.0334					
1978.9879			1965.8632					
2321.2254			1978.8345					
2512.4246			2281.1681					
			2321.0088					
			2380.0191					
			2512.1379					

19S	21S	22S	25S	32S	34S	40S	45S
913.4445	864.2971	913.4755	1547.8498	870.5732	1177.6230	1547.7942	1547.6632
1097.4602	880.2772	1001.5804	913.4902	913.5108	2850.3414	1990.9688	1990.8171
1121.3873	913.5689	1069.5752	1195.6308	1030.5489	2018.9030	913.4709	913.4090
1283.6102	1121.5570	1121.4662	1001.6162	1097.5330	1547.7666	1145.5658	1069.4925
1292.8410	1283.8138	1293.1750	1436.6832	1121.4749	1991.0153	2247.9657	1747.6001
1305.6660	1293.1008	1399.5132	1595.8296	1145.5450	913.4657	1436.6523	1001.5172
1399.3846	1399.6628	1423.3911	1305.7418	1154.4281	1069.5560	1518.7557	2113.7274
1423.2836	1423.5182	1439.7763	1503.8088	1179.5973	2045.0316	728.3919	2247.7394
1439.6933	1439.9294	1503.7335	1625.9801	1199.5072	1747.7164	734.4225	1518.6491
1503.6388	1503.8897	1595.7507	881.2690	1215.5046	1001.5792	745.4190	1595.6153
1515.5992	1595.8841	1625.8723	966.4871	1220.6868	2113.9048	808.3478	1439.7001
1595.6406	1626.0610	1679.6333	1055.6120	1439.7581	2247.9624	822.5134	1800.6865
1625.7828	1679.7672	1724.7926	1178.6134	1442.6442	1595.7626	827.4292	1305.6024
1679.3927	1724.9614	2044.9540	1469.7784	1459.6853	1439.7871	856.3283	1433.7045
1724.7186	1741.0474	2424.1486	1678.9596	1503.7103	1283.6828	1056.5912	1503.6515
1941.6742	1942.2050	2557.3708	2037.0234	1595.6925	1800.8143	1107.5162	1625.7908
2044.8328	2045.2309	2612.1537	2093.1444	1625.8358	1793.9056	1121.4766	1740.7227
2557.0276	2557.6172		2343.1517	1953.8548	1305.6981	1138.6543	1539.7066
2612.0379	2612.5405		2612.2953	2342.9259	1433.8200	1154.3351	1142.6126
			2912.8832	2482.6620	1503.7405	1199.4928	1955.6894
			2958.5516		1625.9186	1207.6549	1097.4197
			2974.4636		1497.8276	1215.4906	1121.3892
			3260.6269		1740.8560	1231.5006	1730.5381
					1138.4899	1250.5928	1854.8283
					1955.8702	1401.6532	1867.8688
					808.3381	1417.6348	1938.6345
					1121.4663	1459.7530	2223.9594
					1292.9902	1585.7047	2342.9263
					1399.4344	1854.9925	2611.9103
					1423.3956	2174.9693	
					1459.7213	2343.0545	
					1487.3571	2482.7406	
					1515.7330	2499.1677	
					1517.7572	2570.1894	
					1563.7431		
					1583.6165		
					1661.9058		
					1730.6794		
					1778.8357		
					1855.0004		
					2343.0812		
					2551.2726		
					2595.7805		
					2612.1975		
					3497.7982		

55S	57S	62S	63S	65S	66S	67S	77S
913.5009	1547.7162	2118.9198	854.4252	854.4370	854.4132	1547.7956	2856.0097
1001.5998	1990.8974	1407.6651	1497.6984	1497.7984	1497.6861	913.4913	2707.0378
1069.5719	913.4383	1285.5833	1872.8697	1873.0125	1872.8984	1195.5861	2054.7536
1121.4608	1069.5234	728.5324	1768.9248	1071.6173	1200.5856	1001.5806	2182.8564
1292.7624	1747.6333	877.0511	1200.6103	1233.6508	1168.5748	1518.7299	1861.8674
1399.4845	1001.5472	893.0298	1168.5799	1266.6330	1071.5795	1595.7459	854.4202
1423.3846	2113.8106	1021.5451	1071.5831	1301.7765	1233.5974	1439.7944	1071.5598
1439.7682	2247.8288	1049.5496	747.4063	1602.8766	1266.5779	1305.7144	1082.5437
1503.7193	1518.6965	1119.5834	1233.5808	1730.0324	1308.5904	2424.1924	1289.6810
1515.7084	1595.6851	1168.6919	1266.5831	1812.9326	1340.6223	1503.7500	1323.5944
1595.6986	1439.7180	1439.8344	1602.7677	2296.2217	1602.7747	1625.8994	1768.8029
1625.8725	1283.6178	1503.7654	1625.8091	2321.2781	1720.8687	1724.9086	1872.7864
1724.7288	1800.7577	1625.9619	1753.8384	2518.2519	1769.9271	1178.5627	2094.9024
2044.9222	1793.7930	1661.9084	1812.7958		1812.8579	1301.7188	2310.9097
2557.0798	1305.6540	1705.8450	1919.9556		2296.0834	2612.0942	2748.9999
2612.0617	1433.7486	1740.8648	2518.0380		2518.0170	2826.2181	3161.2137
	1503.6932	1855.0292	2540.0008			3497.6390	
	1625.8422						
	1497.7556						
	1138.4548						
	1142.6616						
	1014.5647						
	1415.6186						
	1955.7820						
	897.3974						
	935.4310						
	1121.4311						
	1156.4686						
	1455.7301						
	1778.9871						
	1854.9425						
	1938.7194						
	1976.6906						
	2320.9315						
	2551.1608						
	2612.0119						

83S	84S	100S	101S	110S	116S	118S	120S	121S
842.5970	1928.7997	1547.8001	1547.8274	913.5137	1656.7725	1611.7276	1767.8781	1767.9517
845.5570	1547.6616	913.4874	913.5223	1001.6152	1611.6668	2161.3109	1611.8348	1158.5050
980.5447	1990.8188	1195.5935	2248.0132	1301.7270	2161.1425	1735.9579	2161.3149	1611.7207
1028.6575	913.3963	1001.5942	1595.8340	1439.7722	1735.8905	2140.2452	1736.0743	2161.2681
1030.6568	1069.4947	2329.2056	1305.7565	1547.7920	2140.1082	2468.2968	2140.2849	1735.9524
1044.6745	2044.8580	1595.7833	1433.8558	1595.6805	2468.2642	1827.0453	2468.4791	2140.1665
1049.6551	1001.5222	1439.8252	1724.8883	1625.8499	1826.9508	3129.3071	1827.1183	1603.7934
1060.7010	847.4207	2424.1494	1740.9002	1919.8597	3129.2967	833.0011	3129.4401	2468.3860
1071.6470	2113.7561	1503.7840	882.4621		850.4537	855.0278	932.7705	1827.0025
1076.6491	2247.7995	1625.9287	1022.4723		927.4734	870.9481	976.8218	3129.4747
1194.7070	1595.6535	1724.8555	1057.5929		1228.6438	885.1016	1020.8467	3018.2629
1221.6505	1439.6947	1178.5767	1121.5210		1301.7111	927.4546	1301.8572	3034.2501
1237.6317	1283.6016	1301.7463	1405.7813		1313.6827	1017.8312	1323.8483	927.5346
1239.6497	1800.6911	1572.8325	1449.7235		1323.6937	1060.0460	1339.8294	1235.6720
1255.6492	1793.7556	1589.8584	1790.9496		1525.7815	1082.6008	1510.6308	1301.7596
1271.6513	1305.6122	1611.6961	1969.1972		1572.7987	1193.5621	1532.9771	1572.8625
1388.8811	1433.7014	1668.7735	2612.2218		1589.8271	1301.7505	1572.9604	1589.8980
1402.8892	1503.6436	2140.1383	2909.2420		1627.6927	1339.5493	1589.9929	2144.1901
1611.8973	1625.7848	2161.1666			1848.8875	1572.8405	1594.9618	2172.1399
1617.8633	1497.7032	2468.3114			1864.8951	1589.8763	1656.9528	2938.3106
1627.8970	1740.6840	2612.1167			2109.9916	1848.9328	1849.0861	2967.1984
1633.8356	854.3834	3129.4154			2172.1941	1864.8560	1865.0479	3084.4152
1662.0840	927.3910				3101.3445	2143.9094	2110.1348	3100.2601
1671.9722	931.3497				3114.0940		2144.3317	3246.4505
1725.8915	1017.5082				3245.7487		2178.2844	3262.4198
1742.9025	1097.4016				3262.2912		3100.5371	3287.4531
1855.1924	1301.6357				3408.2765			3303.5390
1941.7306	1519.6654				3449.2621			3408.4139
1987.1228	1748.5438				3610.3704			3449.5556
2003.1405	1826.8339							3611.5744
2124.2501	1919.8896							
2156.9197	1949.9054							
2211.3665	2140.0137							
2218.2618	2320.8324							
2241.1646	2611.9696							
2257.3233	3129.1552							
2267.3848								
2374.4030								
2385.2143								
2401.3106								
2417.3980								
2562.5172								
2658.6827								
3060.6153								
3216.7641								
3479.8376								

123S
3246.4751
1626.8756
3129.3990
1649.7198
833.0925
855.0784
913.4989
1001.6122
1227.6521
1301.7528
1317.7445
1323.7624
1329.7679
1339.7178
1510.9383
1520.7395
1525.9199
1572.8617
1589.8902
1611.7308
1656.8161
1735.9664
1767.8633
1827.0303
2110.0286
2140.1796
2161.2182
2468.3871
3100.4427
3261.4149
3287.4518
3408.5467
3449.4291
3609.8475

124S
1547.9599
913.5994
1069.7245
1001.7166
1801.0665
1626.0980
1504.9482
1294.8361
861.1867
877.1609
893.1408
945.6237
1121.6040
1192.6719
1199.7731
1224.6639
1278.7858
1351.8736
1449.8599
1508.8803

125S
1611.7702
2161.2423
1736.0055
2140.2062
2468.4239
1827.0750
3129.5275
3645.6820
3018.3571
927.5275
1296.7151
1301.7799
1572.9197
1589.9284
2110.0515
2144.2263
3112.3344
3408.5739

126S
842.6143
870.6010
927.5906
1301.8850
1323.8962
1573.0202
1590.0247
1611.8698
1827.2147
1849.1254
2110.1797
2140.2970
2144.4175
2161.4633
2172.4189
2211.3347
2239.3850
2468.5698
3129.6652
3245.8476

130S
1611.6239
2160.9837
1735.8445
2140.1065
1603.7222
2468.2067
1826.9034
2144.8916
759.4575
927.4567
1130.5342
1301.6921
1322.6879
1572.7691
1589.7765
1661.7577
1848.8723
1864.8588
2210.0419
3128.9495

131S
1656.7872
1767.8296
1158.4942
1611.6645
1735.9049
2140.1081
1603.7331
2468.2646
1826.9478
3129.3091
913.4821
927.4823
1001.6000
1070.5679
1301.7327
1323.6809
1339.6873
1433.7884
1439.7861
1503.7667
1547.7496
1572.8070
1589.8406
1625.9001
1661.7788
1663.7663
1773.7462
1848.9257
1864.8829
2144.1009
2162.1052
2172.0810
2178.1167
2612.1292
3113.2887
3246.3908
3262.3291
3286.4434
3408.4480
3449.4162
3611.3025

133S
870.5892
927.5242
951.6171
992.5247
1301.7656
1323.7494
1339.7314
1572.8562
1589.8616
1735.9794
1757.8852
1773.8667
1826.9305
1848.8930
1864.8715
2140.0386
2145.0794
2239.1494
2468.1645
3129.3165

137S
2850.2348
1547.7653
1990.9477
913.4694
1069.5856
1195.5901
1001.5899
847.5068
2113.9150
2247.9648
1436.6534
1518.7762
1595.7664
1800.8346
1305.7227
1941.8575
1178.5768
1301.7207
1572.8141
1589.8429
1611.6819
1827.0007
2612.0708
3129.3451

145S
1547.8331
913.4864
1195.5922
1001.6213
1436.6951
1518.8773
881.2686
1178.5728
2612.0216

157S
1001.5826
847.5066
1436.6580
1518.7845
1595.7765
1305.7405
2424.1855
1503.7801
1625.9229
1539.8372
2612.0863

158S
1449.6803
1547.7929
913.4779
1195.5878
1001.5935
1595.7654
1439.8025
861.0726
877.0463
893.0224
1178.5644
1432.6212
1465.7742
1919.9933
2612.1235

167S
1195.5925
1001.5846
1436.6874
1518.7987
1595.8303
1439.8542
1305.7381
1433.8386
1503.8221
1625.9558
1539.8590
728.5356
1301.7604
2612.3385

168S
877.0912
893.0682
1293.6954
1301.7549
1305.7805
1439.8326
1503.7852
1595.7755
1625.9768
1942.0413
2557.3814

170S
1195.5341
1001.5411
1436.5785
1518.6817
1439.7432
1800.7203
1305.6629
1433.7473
1503.7027
1625.8442
1497.7624
1539.7318
1138.4546
1017.5243
1023.5485
1107.4887
1121.4214
1132.4427
1178.5114
1284.5098
1301.6485
1632.6725
1812.7868
2224.0513
2612.0489

172S
1547.7404
1990.9249
913.4638
1069.5502
1001.5652
2247.8854
1595.7297
1439.7770
1283.6734
877.0260
1122.6301
1210.7214
1420.6566
1457.6429
1569.7057
1585.6883
2112.9701
2551.0129
2612.0590
2831.0916
2845.1270

181S
2850.3339
1547.8164
1991.0154
913.4946
1195.5930
1001.6006
2113.9863
1518.7983
1595.7806
1439.8501
1800.8680
1122.6842
1178.6211
1210.7844
1748.7447
1812.8791
2612.1805

185S
1177.7214
837.4673
2034.9007
1700.8365
1928.9284
1547.8194
1991.0882
913.5188
1069.6196
1747.7510
1001.6260
847.5380
2113.9380
2248.0132
1518.8245
1439.8494
1800.8733
1488.6541
1017.6295
1457.7423
1569.8096
1585.7950
1730.7427
1973.6391
2551.2277
2612.2520

186S
1001.6178
1595.8226
1439.7792
1433.8403
1503.8126
1625.9720
856.5588
1301.7594
2612.2602

188S
1001.7354
1439.9992
1503.9381
1596.0776
1626.0653
2612.4393
2899.6982

193S
1001.7146
1121.6071
1293.0007
1423.6143
1439.9984
1503.9676
1596.0017
1626.1286
1725.0672
2248.2567
2557.7055
2612.6721

199S
1518.7498
1595.7608
1439.8005
1800.7695
1305.6965
1433.7888
1503.7248
1625.8910
881.2552
1121.4608
1284.5426
1812.8385
2165.0755
2612.1334

200S
2113.9831
1749.7254
2247.9915
1595.8397
1439.8591
1283.7462
1552.7255
1305.7559
1433.8674
939.4542
1503.8298
1625.9949
1497.8675
1740.8934
898.5134
2557.3457
1142.7343
1014.6460
1415.7372
1955.9101
997.6098
1049.5762
1051.6153
1092.5708
1119.4900
1121.5035
1137.5123
1293.0801
1354.5699
1423.4469
1515.7953
1538.7135
1540.7795
1679.7135
1938.9099
1976.8431
2612.2799
3008.4142
3496.5838

201S
1547.6558
1990.8023
913.4116
1001.5293
2113.7020
1595.6433
1439.6958
1283.6116
1800.6616
1088.4871
1122.5721
1730.5372
1748.5442
1789.7021
2550.9239
2611.9067

204S
2113.9326
1749.7248
2248.0054
1941.9592
1595.8158
1439.8555
1283.7402
1552.7019
1793.9120
1305.7521
1503.8052
1625.9679
1497.8639
1740.8694
2557.2958
1142.7493
1399.6887
1415.7117
1955.8850
882.9227
900.0029
917.3074
1121.4998
1292.7831
1515.7941
1679.6595
2612.2261
3497.7113

207S
842.3703
845.3311
860.9341
870.3977
876.9102
892.8848
1030.3779
1044.3931
1049.3728
1060.4111
1062.3947
1071.3610
1076.3815
1087.3178
1093.3517
1237.3525
1239.3320
1388.5109
1402.5387
1725.4813
1742.4889
1776.5074
2002.6531
2401.7746
2417.8213
2561.8314

213S
913.4912
923.5675
945.5490
976.4659
1036.6562
1132.5313
1158.4846
1164.7588
1182.3357
1198.7203
1220.7074
1234.6847
1236.6775
1320.6095
1499.7231
1515.7731
1707.8249
1790.9534
1954.1347
1976.1528
1992.1260
2169.7117
2215.1656
2231.1048
2297.8160
3137.7366

214S
976.4465
1198.7292
1171.5910
1187.5965
1354.6607
1954.1258
1639.8857
1132.5384
1629.8665
1790.9428
2215.1496
2231.1885
795.4650
923.5643
1516.7358
1644.8245
913.4785
945.5514
1158.5560
1164.7591
1182.3208
1185.6409
1407.9003
1499.7182
1547.7908
1627.8229
1812.9383
1976.0929
1992.0667
2169.8703
2507.2569
2523.2809
2539.3316

216S
976.3552
1198.5981
1953.8805
1132.4246
1629.6618
1790.7174
795.4098
1516.5585
799.4110
1182.1935
1499.5267
1812.7037
1975.8693
1991.7788
2021.9471
2230.8337

217S
877.1910
976.5884
1036.8173
1052.8282
1132.6704
1158.6295
1164.9000
1182.4789
1198.8610
1220.8219
1236.8183
1515.9051
1578.7938
1630.0621
1640.0204
1791.0733
1954.3138
1976.1791
1992.2045
2169.8719
2215.3620
2232.3012
3136.8678

220S
1198.6349
1203.5429
1953.9091
1132.4467
872.4784
1790.7911
1516.6388
744.3847
913.4083
1208.6490
1459.6825
1464.5994
1481.6456
1536.7387
1566.6544
1582.6196
1695.6727
1769.8370
1875.9764
1897.9449
2518.9793

226S
870.5189
913.4609
1001.5677
1121.4072
1439.7022
1503.6561
1595.6642
1625.7828

227S
877.0282
893.0023
1142.6343
1292.7904
1301.6494
1305.6235
1423.3034
1433.6981
1439.6843
1503.6357
1515.6476
1595.6163
1625.7580
1663.7116
1724.6463
1740.6680
2328.7210
2423.8750

234S
1215.6853
1231.6955
1203.6864
1104.6426
976.5226
1129.6195
994.5405
1443.7824
1459.7858
1065.5653
1021.5641
865.4618
1078.5575
1186.6588
1359.7931
1398.5689

239S
1215.7237
1231.7153
1104.6655
976.5746
2659.3035
1600.9620
1523.9610
1253.6166
1129.6638
994.5789
1443.8004
1459.7858
909.4879
1021.6093
865.4984
2052.1724
820.4994
801.4381
1007.5924
1017.5879
1062.5891
1068.5697
1078.5921
1151.6419
1173.6483
1195.6444
1264.7187
1398.6281
1481.7771
1812.9709

244S
1518.7634
1595.8114
1800.8113
1305.7126
1433.8164
1503.7849
1625.9180
1497.8229
1740.8497
898.4586
1539.8281
1138.4835
1142.7123
1014.6078
1955.8414
1119.4707
1121.4805
1327.7140
1647.8705
1812.8822
1938.8067

261S	269S	278S	280S	284S	286S	296S	301S	303S
870.5971	1595.7845	2414.1127	1826.8075	870.5848	870.6288	870.5130	1375.7683	909.5353
927.5306	1439.8152	1557.8294	2414.0818	920.5162	924.5623	1121.4153	2659.1890	931.5144
973.5712	1283.7343	1276.5518	1401.7265	942.5015	1121.5450	1138.4320	1183.6545	947.4845
980.5382	1800.8243	1703.7902	1557.8182	958.4903	1283.7947	1292.9893	2909.1298	1001.6413
1064.5998	1305.7276	2047.8655	1276.5633	980.5437	1292.9055	1305.6123	1359.3976	1366.4887
1088.6323	1433.8229	1719.7082	1703.7849	994.5372	1305.8039	1399.4685	1617.7744	1411.5822
1179.6120	1503.7881	908.4154	1727.7712	996.5325	1423.5341	1423.3208	2980.2367	1616.7125
1234.7044	1625.9359	928.4713	2047.8519	1002.5359	1433.9293	1439.7048		1827.7610
1301.7443	1497.8488	1092.5729	1719.6994	1012.5494	1439.9184	1503.6815		1896.3075
1320.5943	1740.8381	1184.5743	908.4038	1014.6583	1497.8681	1515.6701		1913.5248
1572.8298	898.4930	1537.7975	928.4616	1018.5195	1503.8839	1595.6920		1935.3703
1589.8311	1539.8275	1812.7923	1371.7088	1031.6418	1515.8539	1625.8393		1959.4015
1611.6430	1142.7324	1821.9334	1432.6126	1088.6698	1595.8890	1679.5402		1975.7537
1707.6146	1014.6356		1088.5578	1104.6658	1626.0404	1724.7535		
1826.7760	1955.8444		2316.1006	1120.6627	1679.6967	1941.7954		
1848.9799	1121.4859		815.4299	1276.7071	1724.9747	1963.6809		
1864.8662			920.4109	1294.7382	1741.0670	2540.7734		
2140.0723			966.4434	1300.7036	2557.5503	2557.3163		
2144.8249			980.4477	1316.6871				
2239.1399			982.4397	1345.7457				
2468.1741			1061.5141	1367.6987				
3129.3610			1082.5537	1383.6862				
			1092.5610	1703.9090				
			1184.5577	1817.7904				
			1245.6728	1863.0502				
			1261.5713	2432.3426				
			1301.6751	2965.3598				
			1314.5959	2981.1767				
			1323.6230					
			1415.5748					
			1523.7498					
			1529.6826					
			1537.7572					
			1665.8602					
			1838.8792					
			1864.8240					
			2030.4867					

313S	316S	322S	325S	329S	341S	342S	344_sheep
1193.6209	1193.6787	1700.8600	822.4178	842.4859	1641.9870	913.4395	804.2621
1177.6923	1177.7501	1929.0003	842.4810	881.2359	1224.6996	2045.0492	822.3950
837.4412	837.4916	1547.8460	930.5117	930.5167	2680.3752	1595.7834	842.4742
2850.3253	2850.3983	1991.1048	1113.0376	1176.6057	1056.5649	1439.8148	930.4946
2034.8857	2018.9390	913.5361	1176.5863	1251.6592	1184.6776	1283.6937	1176.5928
1700.7957	2034.9271	1195.6659	1251.6405	1289.6623	1911.9601	1552.6789	1251.6500
1928.9018	1700.8641	1001.6438	1289.6654	1381.7588	900.4746	1305.7010	1268.6071
1547.7940	1928.9447	2113.9809	1314.6291	1382.7527	955.5611	1433.8222	1273.6091
1990.9801	1547.8552	1749.6280	1317.6845	1403.7205	1110.6695	1503.7811	1314.6217
913.4792	1991.0807	2248.0532	1337.6845	1419.7134	1086.7132	1625.9341	1317.7038
1069.5872	913.5313	1436.7127	1339.6350	1421.7268	2483.3960	1724.8445	1339.5515
1747.7148	1069.6409	785.2579	1381.7891	1465.7940	2382.1812	1740.8497	1364.3690
1001.5919	1747.7813	829.2888	1403.6987	1487.7040	877.0525	2557.2892	1381.7359
847.4958	1001.6456	881.3153	1419.6691	1509.7700	1039.5496	1941.9243	1382.7290
2113.8981	847.5467	1077.2599	1465.7475	1553.8051	1051.5645	1399.6793	1397.7063
2247.9805	896.4847	1122.7109	1487.7060	1626.8393	1097.5953	1415.6719	1403.6847
1436.6543	2113.9668	1178.6251	1509.7733	1641.8485	1124.6705	1955.8461	1405.6458
1056.6883	2248.0254	1569.8325	1553.8006	1643.8149	1133.6844	1121.4428	1449.7887
1122.6687	1436.7124	1585.8163	1571.7380	1720.8821	1240.6985	1327.6947	1487.6868
1210.7800	1626.0388	1730.7646	1593.6817	1729.9532	1246.7073	1343.6549	1581.8376
1284.5910	1017.6457	1812.9344	1603.5313	2019.9170	1256.7062	1453.6370	1603.4905
1390.6716	1122.7246	1973.7062	1609.6715	2040.9410	1273.8158	1515.7684	1626.4273
1569.7840	1210.8367	2551.2240	1626.4371	2133.1007	1317.8599	1525.7476	1643.7814
1585.7431	1273.7877	2612.2403	1641.8461	2136.9899	1377.8279	1541.7232	1659.7725
1683.7644	1317.8308		1643.8145	2224.0983	1405.9080	1730.6959	1665.7430
1730.7027	1390.7333		1659.8039	2246.0666	1421.9090	1748.7333	1674.8387
1769.6675	1405.9082		1665.7513	2262.0523	1449.9157	1779.8287	1720.8817
1812.8576	1458.7421		1681.7623	2380.1702	1466.7458	1976.7796	1743.8721
2017.8229	1537.9914		1685.8948	2433.3220	1480.7472	3008.3093	1876.9789
2551.1845	1569.8521		1720.8840	2604.1523	1489.7907		1911.8936
2612.1783	1581.9967		1727.7907		1496.7427		1933.8911
	1585.8111		1817.9360		1581.9846		2018.8884
	1670.0719		2019.9078		1691.8240		2133.0370
	1683.8328		2133.0625		1820.9659		2137.8755
	1730.7687		2137.0133		1933.9554		2167.0225
	1812.9221		2150.0670		2320.9234		2181.0580
	1973.5930		2167.0730				2206.6101
	2551.2623		2181.6923				2224.0682
	2612.2522		2224.1492				2238.0332
			2246.0181				2380.1522
			2262.0394				2461.1249
			2289.1298				2515.0661
			2363.8549				2531.1151
			2380.2212				2564.0859
			2604.1658				2590.1216
			2621.1644				2604.1469
							2621.1477
							2635.1106
							2643.0918
							2701.2214

345S	350S	351S	355S	356S	359S	375S	381S	382S
791.3665	1700.6727	1193.5942	1942.0072	1941.9338	1929.0331	1449.6821	1193.5952	1928.7477
802.4406	1928.7317	2329.1373	1439.8800	1439.8384	1547.8777	2034.8635	1700.7473	1547.6593
822.4303	1547.6531	1595.7654	1283.7783	1552.7170	913.5322	1547.7925	1928.8344	1990.8209
842.5121	1990.8349	1439.7901	1305.7907	1793.8970	2045.1655	913.4816	1547.7360	913.4322
881.2649	913.4065	1283.6825	1503.8523	1305.7470	1747.8645	1001.5921	1990.9466	2044.9212
930.5447	2044.8742	1800.7975	1626.0229	1433.8455	1439.9181	1122.6633	913.4582	1441.5902
1176.6496	1888.8175	1793.8583	1740.9271	1503.8120	1595.8996	1194.6326	2044.9932	1457.5847
1179.6062	1595.6439	1305.6947	2557.3730	1625.9647	1503.8777	1358.6602	1888.9197	1569.6480
1233.6824	1439.7082	1433.7967	1415.7550	1497.8724	776.3038	1432.6633	1439.7325	1585.6029
1251.7048	1503.6580	939.3970	1955.9322	1724.8943	1082.6862	1496.6686	881.2356	1699.6848
1381.7889	1625.8303	2424.1569	1121.5463	1740.8727	1323.7641	1748.7076	899.9585	1748.5566
1382.7817	1122.5814	1503.7447	1317.8591	1539.8007	1699.9557	1973.5315	915.2428	1769.5622
1405.6773	1235.5809	1625.8994	1361.8710	2557.2626	2551.3900		917.2506	1786.5315
1419.7466	1730.5451	1724.8258	1405.9069	1142.7488			1077.1758	2550.9594
1430.7249	1748.5351	1740.8096	1449.9195	1014.6594			1122.6380	
1475.7543	1812.7239	1539.8076	1553.9377	1415.7356			1165.5804	
1487.7633	2077.8743	1138.4685	1582.0129	1955.8607			1267.6045	
1503.7498	2550.9370	2557.2184	1597.9768	1121.5118			1342.7361	
1571.7510		1142.6949	1697.9682	1274.6947			1457.6505	
1603.5155		1014.5914	1839.0367	1515.8106			1569.7307	
1626.5082		1955.8267	2078.1746	1525.7842			1585.7155	
1643.8577		995.4994		1697.9380			1730.6308	
1659.8498		1082.5766		1785.8462			1748.6425	
1707.7849		1107.5115		1865.9101			1769.6219	
1720.9335		1121.4456		2060.1372			2060.0063	
1727.8628		1165.5742					2307.1197	
2018.9651		1351.6211					2551.1209	
2133.1358		1423.4005						
2137.9464		1779.8272						
2150.1165		1812.8373						
2167.0924		2094.9905						
2206.7786		2286.1097						
2224.1395		2566.2887						
2246.1064		2584.1734						
2262.0584								
2289.1236								
2363.8895								
2380.2825								
2604.2448								
2621.2078								

383S	385S	390S	394S	396S	401S	402S	406S	427S
1813.9081	1305.6853	1305.6796	1482.8310	856.5734	1482.8097	842.6176	1863.0023	886.5255
2188.1425	1433.7736	1433.7765	1813.8862	864.5226	1255.6520	855.1607	2207.2081	939.4992
1266.6607	1503.7415	1067.4873	1398.7070	870.5828	1670.8337	861.1733	988.5877	1850.9261
1394.7591	1625.8878	939.3938	1576.8416	877.0275	1398.6675	877.1534	1478.7696	2737.4028
1388.7120	1497.8042	1503.7452	2188.0846	880.5029	1576.8191	1017.6653	1433.7954	1647.9025
1260.6242	1740.7919	1625.8964	1266.6484	965.5036	2188.1206	1026.7232	748.4365	855.0833
1273.7040	1142.6747	1497.8079	1394.7434	1000.2021	1932.9490	1060.1932	1260.6680	861.1246
1017.5531	1014.5966	1724.8210	1260.6147	1178.5989	1522.7807	1066.2118	1316.7951	877.1037
1430.7698	1415.6923	1740.8047	1017.5414	1194.5749	1266.6123	1179.7371	1345.7641	1288.7290
1867.0741	1955.7991	898.4602	1430.7507	1216.5702	1388.6788	1260.7544	1585.8520	1317.7850
1026.6110	1121.4587	1138.4594	1867.0490	1238.6657	1260.5823	1305.7918	1643.0225	1487.7839
1297.7499	1134.5183	2557.2154	1026.5977	1254.6284	1017.5207	1323.8479	1676.8426	1872.9223
1305.6755	1515.7381	1941.8859	1297.7173	1524.7772	1026.5763	1398.8608	1883.9429	2033.9479
1589.8394	1525.7704	1142.6849	1305.6428	1540.7367	825.4378	1475.9284	2072.2431	2121.0018
1772.9554	1537.8710	1014.5875	1344.7782	2009.8348	1305.6081	1594.0038	2134.1663	2141.9536
1216.6494	1812.8228	1399.6712	877.0535		1216.6353	1709.0128	2289.2685	2158.9607
1012.5995	1854.9939	1415.6677	1012.5892		1012.5686	1717.0573	2335.2430	2202.9329
1082.6300	1938.7363	1955.8137	1053.6115		1039.4861	1795.0154		2875.2568
1193.6467	2004.9764	749.4247	1119.4674		1056.5000	2037.2079		2891.2155
1230.6920	2078.0435	997.5679	1201.6536		1074.4555	2093.3320		
1243.6014		1084.5908	1213.6624		1119.4396	2211.2584		
1288.6457		1119.4208	1898.0152		1129.4910	2221.2440		
1572.8280		1121.4361	2123.1845		1165.5566	2860.6404		
1633.9046		1354.4962			1197.6159			
1650.9130		1421.6510			1213.6268			
1669.8991		1515.7139			1230.6402			
1686.9185		1525.7200			1276.5686			
1898.0427		1541.6937			1288.6010			
1985.5857		1588.6120			1379.7164			
2001.9303		1649.7991			1407.6866			
2017.9492		1679.5887			1420.6577			
2095.0088		1697.8679			1504.7868			
2123.2293		1785.8030			1598.7957			
2139.1835		1864.8018			1608.8245			
		1898.8215			1614.7862			
		2005.0283			1699.8242			
		2060.0379			1702.8693			
		2078.0580			1706.8017			
		2144.0600			1897.9999			
		2234.1275			1954.9149			
		2466.2262			1970.8301			
		2500.1889			2064.1383			
		2540.8371			2129.0552			
		2579.1677			2170.7101			
					2186.0714			
					2204.0905			
					2210.0408			
					2220.0560			
					2226.0863			
					2309.1121			
					2350.0596			

433S	438S	439S	460S	463S	500S
1862.8248	1552.8502	830.4287	842.5473	1215.6378	1715.9857
2206.9623	1536.7115	842.4997	941.5407	1231.6213	1156.5772
1478.6106	1408.6070	861.0555	1101.4850	1343.6841	1104.5663
1433.6702	1589.6891	870.5220	1146.6077	1359.6787	1097.5452
1463.7567	2426.1564	877.0155	1163.6367	2161.2774	1594.8448
1121.5571	855.4353	1014.5893	1179.6438	1104.6322	1466.7414
748.3816	746.3401	1036.5726	1301.7892	976.5575	1215.6543
896.4569	1267.6130	1121.4558	1317.8175	1600.7181	833.0697
1104.5293	1424.6232	1135.5265	1449.9353	1129.5995	855.0558
1137.5432	1430.6002	1137.5311	1493.9430	994.5579	871.0355
1143.5327	1440.6089	1138.4807	1537.9588	1443.6071	1060.0873
1153.5488	1446.5868	1320.5801	1572.8875	1459.6290	1275.7649
1159.5306	1547.7312	1399.6487	1589.9178	1158.6823	1538.8123
1183.4875	1563.7340	1415.6680	1626.0564	1170.5635	1812.9345
1260.5538	1667.8202	1437.6107	1676.8942	1298.6403	2321.0926
1495.7127	1812.8218	1493.7278	1735.9802	1398.5127	
1586.6661	2019.9705	1864.8063	1812.9757	1622.6309	
1659.6705	2036.9531	1898.8209	1827.0509	2104.6796	
1676.6872	3111.4341	1938.7783	2140.2013	2206.6617	
1738.5582		1955.8098	2145.0791	2222.8222	
1884.8102		1993.7987	2211.2323		
1900.7943		2557.1764	2239.2175		
2133.9540			2342.1346		
2290.0297			2468.3811		
2335.0129			3129.5442		
			3261.5242		