Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by Escherichia coli

Miguel Maria Mendonça Rodrigues

Orientador(es) | Elsa Cristina Lamy
Fernando Capela e Silva
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"*Lo máximo que podemos obtener, sea de lo que sea, es la alegría*"

Fernando Savater

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Resumo - Alterações no Proteoma da Saliva de Suínos com Diarreia Causada por *Escherichia coli*

A infeção pela bactéria Gram-negativa *Escherichia coli* é uma das principais causas de diarreia em suínos e representa atualmente um problema significativo para os produtores. A saliva é um fluido que pode ser recolhido por métodos não invasivos e não stressantes e que contém analitos que se alteram em caso de doença. Estas alterações podem fornecer informações sobre a fisiopatologia da doença e podem ser utilizadas como auxiliares de diagnóstico ou na monitorização da terapêutica. O objetivo do presente trabalho é identificar potenciais alterações no proteoma salivar de suínos com diarreia causada por E. coli.

Para tal, utilizámos dois grupos de suínos Large White pós-desmame, um grupo de controlo e outro grupo infetado (grupo *E. coli*). Recolhemos amostras de saliva de ambos os grupos e depois utilizámos duas técnicas de separação de proteínas, sendo elas a SDS-PAGE e a eletroforese bidimensional. Para além destas técnicas, foi utilizada uma técnica para a identificação de proteínas, denominada espetrometria de massa.

A concentração total de proteínas no grupo infetado foi três vezes superior à do grupo de controlo. Na análise de SDS-PAGE, temos níveis mais elevados de lipocalinas salivares e bandas de IgA e, em contrapartida, níveis mais baixos de proteínas ligadoras de odorantes, inibidor de protease de origem submandibular e proteína induzível por prolactina. Na análise do perfil bidimensional, temos níveis mais elevados de lipocalinas salivares, adenosina deaminase, bandas de IgA e péptidos de albumina e, em contrapartida, níveis mais baixos de alfa-amilase, anidrase carbónica, carbonato desidratase VI e albumina total. Em seguida, foi efetuado um teste de validação em que os suínos com diarreia por *Escherichia coli* apresentavam níveis consideravelmente mais elevados de atividade da adenosina deaminase salivar em comparação com o grupo de controlo (grupo saudável).

Relativamente a este estudo, algumas destas proteínas desempenham um papel importante em processos fisiológicos e em condições fisiológicas/patológicas, foi observado que estas proteínas sofrem alterações ao nível do proteoma salivar. Por esta razão, é possível afirmar que estas técnicas são valiosas para a identificação de novos biomarcadores na saliva que contribuem para a descoberta de novos diagnósticos alternativos de doenças no futuro.

Palavras-chave: *Escherichia coli*; Proteoma salivar; Suínos; Diarreia; Biomarcadores.

Abstract

Infection with the Gram-negative bacterium *Escherichia coli* is one of the main causes of diarrhoea in pigs and currently represents a significant problem for producers. Saliva is a fluid that can be collected by non-invasive, non-stressful methods and contains analytes that change in disease. These changes can provide information on the pathophysiology of the disease and can be used as an aid to diagnosis or monitoring of therapy. The objective of the present work aims to identify potential alterations in the salivary proteome of pigs with diarrhoea caused by *E. coli*.

For that reason, we used two groups of Large White post-weaning pigs, one control group and other infected group (*E. coli* group). We took samples of saliva from both groups and after we used two techniques for protein separation by isoelectric point/molecular mass and molecular mass. These are 2-DE gel electrophoresis and SDS PAGE, respectively. In addition to these techniques, a more sophisticated technique was used for protein identification called mass spectrometry.

The total concentration of proteins in the infected group was three times higher than the control group. In the SDS-PAGE analysis, we have higher levels of salivary lipocalins and IgA bands and, in contrast, lower levels of odorant-binding proteins, protease inhibitor from the submandibular origin and prolactin inducible protein. In the two-dimensional profile analysis, we have higher levels of salivary lipocalins, adenosine deaminase, IgA bands and albumin peptides and, in contrast, lower levels of alpha-amylase, carbonic anhydrase, carbonate dehydratase VI and whole albumin. After this, a validation test was made in which pigs with diarrhoea by *Escherichia coli* had considerably greater levels of salivary adenosine deaminase activity in comparison to the control group (healthy group).

Regarding this study, some of these proteins play a important role in physiological processes and in physiological/pathological conditions, it has been observed that these proteins suffer alterations at salivary proteome level. For this reason, it is possible to say that these techniques are used to identify new biomarkers in saliva which contributes for the discovery of new alternative diagnoses of diseases in the future.

Keywords: *Escherichia coli*; Salivary proteome; Pigs; Diarrhoea; Biomarkers.

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Abbreviation List

1-DE - One-dimensional gel electrophoresis

2-DE - Two-dimensional gel electrophoresis

ACh - Acetylcholine

ADA - Adenosine deaminase

ANS - Autonomic nervous system

APP - Acute phase proteins

APR - Acute phase reaction

APS - Ammonium persulfate

BCA - Bicinchoninic acidic protein assay

BSA - Bovine serum albumin

CA - Carbonic anhydrase

CHAPS - 3-(3-cholamidopropyl) dimethylammonium propane sulfonate

CM - Coliform mastitis

CO_2 - Carbon dioxide

CRP - C - reactive protein

DNA - Deoxyribonucleic acid

DSC2 - Desmocollin-2

DTT - Dithiothreitol

***E. coli* -** *Escherichia coli*

EHEC - Enterohemorrhagic *E. coli*

EIEC - Enteroinvasive *E. coli*

EMA - European Medicine Agency

EPEC - Enteropathogenic *E. coli*

ETEC - Enterotoxigenic *E. coli*

ExPEC - Extraintestinal pathogenic *E. coli*

FTIR - Fourier-transform infrared spectroscopy

GO - Gene ontology

H^+ - Proton

H_2O - Water

HCl - Hydrochloric acid

HCO_3^- - Bicarbonate

IEF - Isoelectric focusing

IgA - Immunoglobulin A

IgG - Immunoglobulin G

IPG - Immobilized pH gradient

LCN - Lipocalin

LDH - Lactate dehydrogenase

LPS - Lipopolysaccharide

MALDI-TOF MS - Matrix-assisted laser desorption-ionization time of flight mass spectrometry

MED - Mediterranean Institute for Agriculture, Environment and Development

mRNA - Messenger ribonucleic acid

MW - Molecular weight

NA - Noradrenaline

OBP - Odorant binding protein

OD - Oedema disease

PAIs - Pathogenicity islands

PIP - Prolactin inducible protein

PRPs - Proline-rich proteins

PWD - Post-weaning disease

RI - Reference interval

SDS - Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

STEC - Shiga toxin-producing *E. coli*

Stx2e - Shiga toxin type 2e

TEMED - N, N, N′, N′-Tetramethyl ethylenediamine

TFA - Trifluoroacetic acid

USI - Urinary system infection

VCL - Metavinculin

WR - Working reagent

Preamble

In many animal and human diseases, saliva is currently an important and crucial source of biomarkers. In general, the composition of this particular fluid can be modified due to physiological processes (stress) or due to pathological processes (inflammation, changes in the immune system). Thus, this may lead to the use of salivaryanalytes as biomarkers in these processes mentioned above [1, 286]. The collection of salivahas many advantages, such as: it is painless, easy and non-invasive, does not require specialised staff and can be collected at any time and in any place [2, 286].

In this animal species, blood collection is stressful and painful. That is why saliva is such a precious fluid in pigs [2, 286]. Previously, it was observed that experimental lipopolysaccharide (LPS) administration in pigs' results in proteomic alterations and sepsis [3, 286].

In sepsis, aldolase A and serpin 12 showed a significant increase in saliva [4, 286]. Apart from these proteins, mentioned above, other proteins showed changes in pigs such as cornulin, heat shock protein 27 and lactate dehydrogenase (LDH). The first ones were significantly increased while immunoglobulin J chain showed a decrease. These changes were observed in a situation of compromised animal welfare [5, 286].

One of the main causes of diarrhoea in piglets is enterotoxigenic *Escherichia coli* (ETEC) [6, 286], affecting pig production economically to a considerable extent [7, 286], producing several virulence factors, such as colonization factors (adhesins) or toxins [286]. Watery diarrhoea and stimulation of the intestinal lining are caused by enterotoxins while adhesins promote adhesion to the small intestine [6, 286]. All these factors lead to sepsis [8, 286].

To evaluate the changes in the intestine of pigs diagnosed with *E. coli* diarrhoea, proteomic studies were carried out [6,7, 286]. Nevertheless, to our knowledge, no studies have been made in saliva.

The central aim of this study is to evaluate possible changes in the salivary proteome of pigs diagnosed with *Escherichia coli* diarrhoea compared with a control group [286]. SDS PAGE and 2-DE gel electrophoresis were used to separate proteins and mass spectrometry was used to identify it [286].

PART I

LITERATURE REVIEW

1. Colibacillosis in Pigs

1.1 Introduction

One of the most prevalent infections in humans and animals is *Escherichia coli* (*E. coli*), which is a ubiquitous bacterium with a common oportunistic pathogeniccharacter that raises many medical and scientific research questions [9].

Colibacillosis in pigs, caused by *E. coli*, is a multi-factorial disease that is determined in three main pathological conditions, such as oedema disease, new-born diarrhoea, and post-weaning diarrhoea. The individual causative microorganisms, the age of the animals and the pathophysiology can be considered as references to distinguish them. [10]. These diseases, mentioned above, have been considered a major problem since pigs have been bred and produced [11].

Many substantial economic costs are accrued due to diarrhoea and oedema disease caused by these bacteria. Other factors are considered, including morbidity, mortality, decreased weight gain and, in addition, the costs associated with treatment, vaccines and dietary supplements [6,7]. PWD is also called enteric post-weaning colibacillosis. This has similar names, such as "bowel oedema" or "gut oedema", since one of the most relevant clinical complaints is swelling of the stomach submucosa and mesocolon. Both PWD and OD can run simultaneously in the same animal or in an epidemic situation. PWD adversely affects producers and occurs according to geography and time. In addition, thereis an enteric form of *E. coli*, which can appear two to three weeks after weaning, resultingin extremely severe diarrhoea or sudden death [11].

Escherichia coli is a pathogenic opportunistic microorganism widely present in the vertebrate intestinal system. This is a facultative anaerobic bacterium which, in humans and animals, causes pathogenic colibacillosis because it carries specific virulencegenes.

Isolates of ETEC (enterotoxigenic *E. coli*) and STEC (Shiga toxigenic) are the major causative agents of post-weaning diarrhoea (PWD) and oedema disease (OD) in pigs, respectively [12]. There are several bacterial-related characteristics that promote bacterial adhesion to the mucosal surface of the small intestine of pigs, in contrast to intestinal peristalsis. These are termed fimbrial adhesins [13].

1.2 Etiology

1.2.1 Classification of the bacteria

Theodor Eschrich (1857-1911) was a German paediatrician who discovered the bacterium *Escherichia coli* and thus the genus of this bacterium was named after him. *Escherichia coli* belongs to the Gram-negative, facultative anaerobic rods known as Enterobacteriaceae. This family contains some "intruders" of the gastrointestinal system as well as other strains that cause many intestinal but also extraintestinal diseases in pigs [11,14].

E. coli, since its discovery, has maintained its status with respect to its genus. However, its taxonomic status, beyond genus, has undergone significant changes in information. Species of this type of bacterium were first identified on the basis of biochemical profiles similar to those of *E. coli*. Subsequent studies (based on genotypic and genomic analyses) have demonstrated a relevant phylogenetic distance from the rest of the genus [15].

In addition to the above characteristics, the genus *Escherichia* contains bacteria that do not form spores and are members of the order Enterobacteriales. The common dimensions of *E. coli* are 2 mm in length and a diameter ranging from 0.2 to 1 mm. In both the human and animal intestine, this bacterium is a natural and essential component. Intestinal and extra-intestinal diseases (such as urethritis) are caused by the role of some *E. coli* serotypes [16].

There are more sophisticated methods, such as MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Mass Spectrometry) and FTIR (Fourier Transform Infrared Spectroscopy), which are used for microbial typing and identification, as opposed to traditional phenotypic and molecular identification. These first ones, present some advantages such as high-speed analysis and low costs, being widely used in diagnosis in both human and veterinary clinics [17] as well as microorganism research for quick bacterial identification, categorization, and extensive subspecies-level screening. However, the categorisation and identification of *E. coli* can be improved [18].

1.2.2 *Escherichia coli* pathotypes

Based on the potential pathologies they can induce, *E. coli* pathotypes have been identified as: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC) are among the most relevant groups of intestinal *E. coli* causing diarrhoea. Shiga toxin-producing *E. coli* (STEC), of which enterohaemorrhagic *E. coli* (EHEC) is a subset, and enterotoxigenic *E. coli* (ETEC) also form part of this group. *E. coli* pathotypes are differentiated primarily by the O- and H- antigens present on the surface of the bacteria's membrane [20].

When serological testing began to subtype *E. coli* strains, pathovars were identified for the first time. In 1947, a serological typing system was developed in whichthe somatic or polysaccharide side chains of the organism (O antigen), the capsular antigen (K antigen) and the flagellar protein (H antigen) were used. Thus, there are alreadyaround 200 different O groups of *E. coli* and 53 types of H identified. For monitoring of associated *E. coli* and enteric diseases as well as for outbreak definition, subtyping has been divided mostly into serogroups (based on O antigens) and serotypes (based on a mixture of O, K and H) [14].

One of the examples of the strain used is serogroup O149. This shares only one of the antigens while serotype O149:H10 shares both antigens. Pathogenic *E. coli* can belong to a limited number of serotypes. However, direct identification of genes coding for bacterial characteristics associated with disease pathophysiology (called virulence factors) has largely superseded serotyping for diagnostic reasons [11].

To describe the genesis of the disease, virulence mechanisms are used, which are demonstrated by the presence of combinations of virulence factors. The term "pathotype" is used to categorise different forms of *E. coli* based on these same mechanisms. The major types or categories of pathogenic *E. coli* are numbered based on this specific approach. These are: ETEC, enteropathogenic *E. coli* (EPEC), extra-intestinal pathogenic *E. coli* (ExPEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing E. coli (STEC) and enterohemorrhagic *E. coli* (EHEC) [11].

1.2.3 Virulence factors

The degree of contagiousness of *E. coli* is characterised by its virulence genes such as toxins, adhesins or serogroups O [7]. A wide range of serogroup O was related to colibacillosis while a smaller proportion was documented for more specific diseases such as post-weaning diarrhoea and oedema disease [21].

Adhesins, toxins, iron acquisition components, lipopolysaccharides, polysaccharide capsules and invasins are numerous components associated with the virulence of *E. coli*. Certain structures in bacteria serve as storage for these components, such as plasmids, pathogenicity islands and other mobile genetic components [22].

In enteric infections, structures such as plasmids are important in the development of enteric diseases. Interactions between host and pathogen are created by virulence plasmid-containing genes, which are usually large (> 40 kb) and few copies in size. Interactions between host and pathogen can occur in the most diverse ways, as many different *E. coli* pathotypes have plasmids that confer a variety of characteristics that contribute to this diversity [23].

1.3 *Escherichia coli* and Public Health implications

Piglets diagnosed with diarrhoeal disease have serious health problems that can lead to death. The source of this problem is infection with enterotoxigenic *Escherichia coli* (ETEC). Apart from the development and maturation of the pigs, which are intrinsically affected, there are also associated economic problems in production. Being a zoonosis, this disease can be transmitted to humans, damaging their health in general [24].

Recently, surveillance investigations have proven that monogastric farm animals are and should be considered important reservoirs and hosts for the spread of STEC. Up to two months after infection, pigs have been shown to harbour and excrete STEC [25]. Antibiotic resistance in both commensal and pathogenic *Escherichia coli* is the result of the administration of antibiotics in food or water [11].

Escherichia coli bacteria can infect both humans and animals and contributing significantly to their unique microbiota. For this reason, it is said that *E. coli* have a "special" place in the microbiological community. As mentioned earlier, this zoonosis is of major concern characterised by the spread of virulent and resistant *E.coli* from animals to humans. This occurs through direct contact with the bacteria or contact with animal excreta or even through the food chain. Both veterinary and human medical therapies fail, and the cause of failure are the resistance genes of which *E. coli* isa substantial source. Over time, an increasing number of these genes have been found in *E. coli* isolates. It is also known that these genes have been acquired through a process called horizontal gene transfer. *E. coli* functions as both donor and recipient of resistancegenes in relation to the enterobacterial gene pool. This means that it can pick up resistancegenes from other bacteria and transfer these to others. Hence, microbial resistance of thisbacterium is a serious worldwide problem we are facing today. Thus, it must be considered a topic of study and interest on a public level [26].

1.4 Epidemiology

Epidemiology is a science that studies disease and its spread in populations. Through epidemiological studies, it is possible to identify host characteristics and risk factors that predict a specific disease and its components, such as clinical signs, severity and prevalence [27].

The most significant reservoirs of *E. coli* include livestock, pet animals and poultry. On poultry and livestock farms, *E. coli* is spread mainly through drinking water, feed, aerosols, fomites and carriers. When diets are increased to include more protein and less fibre, growth of ETEC/STEC in weaned piglets is promoted [28].

1.5 Post-Weaning *Escherichia coli* Diarrhoea and Oedema Disease

PWD and OD often affect the same age group of piglets. Hence, it can be said that they can be treated together, since the bacteria causing the diseases share common virulence traits of the diseases. In addition, there are some strains of *E. coli* that cause both diseases [11].

Passive lactogenic protection decreases with the age of suckling piglets. Thus, older piglets, which have not yet been weaned, are more susceptible to *E. coli* diarrhoea and OD as the levels of protective antibodies decrease in the mother's milk. The loss of maternal antibodies at weaning is a factor in the susceptibility of pigs to PWD and OD. It is concluded then, that most of the problems occurring on farms, are in the post weaning period [11].

The ability to develop immune responses such as tolerance or defence against mucosal antigens is a capacity that new-born piglets possess. In weaned pigs, there is an absence of passive lactogenic immunity and hence an active immunisation of the intestinal mucosa is required to obtain protection. Vaccines therefore suggest a good solution to promote protective immunity. They must be able to activate the mucosal immune system as well as antigen-specific immunoglobulin (A and M) responses [2]

Weaning is a sudden and rather traumatic transitional event for piglets and is a crucial production/reproduction phase for them. After birth, 21 to 28 days weaning occurs. This occurs in modern pig industries. Dietary, psychosocial as well as morpho-functional changes in the intestinal system are all factors that make weaning a difficult phase for the animals. The intestinal villi of piglets are altered due to a transition from a liquid to a solid diet causing a reduction in growth rate, episodes of hunger and acute anorexia. The consequence of this is the secretion of digestive enzymes and reduced intestinal absorption of nutrients. The prevention of proteolysis and the protection of pathogens before they reach the large intestine results in insufficient secretion of hydrochloric acid (HCl). This results in changes in the stomach. Inflammatory conditions can arise as well with acute immune changes. This results from the underdevelopment of the piglets' immune system at weaning and the marked loss of the passive protection provided by themother's milk. PWD is one of the most economically significant pathologies in pig farming and can develop when a variety of interrelated factors have a negative impact onthe health of the animal. Examples of these factors are treatment costs, slow animal growth and high mortality rates [30].

PWD is a disease, mostly occurring in the first weeks after weaning, characterised by a significant decrease in feed intake and diarrhoea. Piglets dehydrate very quickly and become prostrate. It may result in unexpected death or after a brief associated illness. This pathology is usually caused by enterotoxigenic *E. coli* (ETEC), which expresses F4 (K88) or F18 adhesion fimbriae (genes expression), which facilitate colonisation of the intestinal mucosa, and, in addition, there is a production of enterotoxins that cause secretory diarrhoea. F4ac is the most common of the three F4 fimbria antigenic variants (F4ab, F4ac and F4ad). However,two antigenic variants of the F18 fimbriae have been found (F18ab, formerly F107, and F18ac, also known as 2134P or 8813) [31].

Considerable losses of weaned piglets usually result from a serious condition known as pig’s oedema disease. This is also known as oedema intestinal disease. This disease is a common cause of death in these animals and for the most part, they show clinical signs within two weeks of weaning. The syndrome may manifest itself in older animals when they are fed combinations of medicated feed shortly after weaning. Isolated occurrences of this have been documented. The pathology appears as sporadic and sudden

occurrences, lasting 4 to 14 days, with a mortality rate of 50 to 90% [32].

1.5.1 Physiopathology

Growth of beta-haemolytic ETEC strains in the small intestine of piglets (strains expressing fimbrial adhesins F4 (K88) or F18 33 is associated with post-weaning diarrhoea. The presence and function of the specific F4- or F18 receptors (F4R or F18R) are key to controlling the vulnerability of animals to ETEC infection. These receptors aid in the attachment of epithelial cells in the small intestine and subsequent colonisation by ETEC [33].

The two sequential stages of the pathophysiology of oedema disease are enteral colonisation and initial food enterotoxaemia. Both are accompanied by changes in the gut microbiota. Secondarily, the systemic distribution of Shiga toxin type 2e (Stx2e) causes diffuse fibrinoid vasculopathy at the central neurological and digestive level. During the second pathogenic phase, there is characteristic vascular damage (arteriolopathy/arteriopathy), increased vascular permeability, fluid loss and vasculogenic tissue oedema [34].

1.5.2 Clinical Signs

POST-WEANING DIARRHOEA

Compared to diarrhoea observed in neonatal piglets, post-weaning diarrhoea is considered less severe. Piglets in the latter mentioned stage, suffer from diarrhoea from suckling to the post-weaning stage. The diarrhoea appears in a yellowish or greyish form which causes dehydration and results in emaciation. This can last up to a week. Mortality of up to 25% can be recorded where most pigs in a group become sick over a period of days. Although there are fluctuations in peak diarrhoea levels between pig farms, it mainly occurs in the first three weeks after weaning. When pigs come into the house, severe cases of the disease can occur six to eight weeks after weaning [11].

OEDEMA DISEASE

OD occurs mainly in the first weeks after weaning. The disease may be sporadic or affect the entire animal population when the rod is infected. The first sign of the disease may be an abrupt death without showing clinical signs. Some affected animals show clinical signs such as: inappetence, oedema of the eyelids and forehead, strange squeaking, uncoordination and respiratory distress. Some of the animals may fall to the ground and succumb quickly. Mostly there is no fever or diarrhoea. Sometimes accompanied by a slight itching, the subcutaneous oedema may disappear when the animal recovers. Whether the animals are dyspnoeic or not, their breathing is accompanied by a snorting sound. During the final period, some pigs have waterydiarrhoea with fresh blood clots. Although clinically normal animals, but which have slower growth and acquire vascular lesions, may suffer from subclinical OD. The development of chronic OD appears in a small percentage of pigs that have recovered from acute OD or *E. coli* PWD attacks caused by pathotypes that also produce Shiga toxintype 2e (Stx2e). Cerebrospinal angiopathy was a name given before the relationship between this disease and OD became clear. After intestinal disease, the growth of the animals ceases for periods of days or even weeks. Sick pigs regularly show unilateral neurological abnormalities such as twisting of the head or atrophy of the limb muscles with gradual weakening and circular movements. Subcutaneous oedema is very infrequentin these animals [11].

1.5.3 Lesions

POST-WEANING DISEASE

Poor health, high dehydration, cyanosis or sunken eyes are some of the changes that pigs that die of *E. coli* PWD can show. The stomach is often enlarged due to dry feeding. Hyperaemia is present at the bottom of the stomach. In the small intestine, hyperaemia, dilatation, and oedema are also present. The food content has a distinctive shape and varies from mucoid to watery consistency. Congestion of the mesentery is high. The mucoid to aqueous contents of the large intestine are usually light green or yellowish in colour. In an outbreak, pigs which die, show marked malnutrition and a strong smell of ammonia. Superficial ulcerations with atypical forms may also appear both in the large intestine and in the gastric fundus.

The stool may be pasty and yellowish in consistency. OD lesions are minimal or absent if the strain causing OD produces Stx2e [11].

OEDEMA DISEASE

The macroscopic lesions of OD are oedema of the eyelids, throat, subcutaneous tissue, and intestinal system, while the microscopic lesions are arteriolar cerebral and intestinal necrosis. High mortality results from neurological problems that are caused by systemic vascular lesions in the brainstem that include oedema, infarction and encephalomalacia [35].

1.5.4 Diagnosis

POST-WEANING DISEASE

The diagnosis of PWD in pigs is made by clinical signs, microscopic lesions and also bacteriological tests [36].

For the diagnosis of colibacillosis, identification of serogroups O and virulence genes is widely advised [37].

OEDEMA DISEASE

Aujesky's disease/pseudorabies, Streptococcus suis or meningitis caused by Haemophilus parasuis are considered differential diagnoses for diseases affecting the central nervous system. On farms suspected of S. suis infections, animals which are not responding well to the recommended treatment with amoxicillin should be screened for STEC. It should be noted that, in the infection of oedema disease, meningitis is not detected when it is examined histologically. The clinical signs may be identical, from several non-infectious reasons, namely: lack of water, vitamin E or selenium deficiency or handling stress. The pathological condition should also be considered in the list of differential diagnoses when sudden death is observed in the first weeks of life after weaning. Being a crucial tool, diagnosis serves to determine the primary cause of the disease and the conclusion should not be drawn solely on the basis of clinical indicators and holding history [38].

1.5.5 Immunity

Pigs, like other animals, possess a full complement of innate and adaptive immune effectors that act as crucial signalling agents to trigger immune reactions. There are factors that interfere with the developmental processes of the immune system such as weaning stress [39].

The serum IgA and IgG are important immunoglobulins in humoral immunity. High concentrations and long half-lives in serum IgG occur in response to external infection. During the weaning phase, because of stress and immaturity of the piglets' immune system, serum IgG concentrations are often low [40].

1.5.6 Treatment

Dehydration of piglets, with enteric colibacillosis, results from loss of fluids from the piglets. Sometimes, in many situations, rehydration of patients and administration of saline solution is essential [41].

Non-antimicrobial substitutes are now being investigated to help restore intestinal balance as well as to aid the transition to weaning. These are zinc oxide, essential oils, prebiotics and probiotics [42].

There are some antimicrobials that have a history of positive results in treating pigs diagnosed with enteric colibacillosis, such as enrofloxacin, apramycin, ceftiofur, neomycin, gentamicin and amoxicillin/clavulanic acid. Also signs of progressive resistance to apramycin, neomycin, trimethoprim-sulphonamide and colistin have been observed in ETEC strains [43]. First-line antimicrobials should be preferred over other antimicrobials, and antibiotic administration should adhere to EMA (European Medicine Agency) recommendations for prudent antimicrobial use in veterinary medicine (i.e., Category D "prudence" that includes antibiotics that should be used as first-line treatments, whenever possible). These antibiotics can be used safely in animals, which means that prolonged treatment regimens (and overuse of antibiotics) should be avoided as well as using group therapy only in circumstances where individual therapy is not feasible [44,45].

1.5.7 Prevention and Control

In general, the pigs' barn's husbandry procedures involve:

- Management and alimentary/nutrition procedures;
- Upkeep of hygienic standards and prevention of pathologies;
- Considerations for animal welfare (such as providing enough room and maintaining a comfortable temperature for piglets) [46].

There are oral or parenteral vaccines that promote immunisation of pigs in cases of *E. coli* infection. [47].

The best way to prevent PWD in the past was antimicrobial strategies. However, due to increasing resistance to antimicrobials, urgent and strict rules prohibiting their use are needed. Thus, immunoprophylaxis may be effective through passive immunity following oral administration of plasma immunoglobulins containing allogeneic antibodies (only relevant very soon after delivery). Active immunisation (after the development of autologous antibodies against specific adhesins and/or toxins) can be done according to oral vaccination with live or attenuated ETEC strains that produce F4 and F18 fimbriae but lack toxin genes [48].

Over time, alternatives have been sought for the treatment of PWD that require no or little use of antibiotics and high doses of zinc. Today, there is extensive research and studies regarding feed-related measures such as additives. In addition, important management measures have been put in place. There is a large list of alternative measures in the treatment of these animals. These are probiotics, prebiotics, symbiotics, postbiotics, proteobiotics, plants and plant extracts (essential oils and tannins), macroalgae (in particular polysaccharides derived from macroalgae), dietary fibres antimicrobial peptides, specific amino acids, food fatty acids, milk replacers, milk components, animal feed, vaccines, bacteriophages and single-domain antibodies (nanobodies), which have a range of therapeutic properties [49].

2. Saliva and Salivary Glands

2.1 Saliva

Saliva is a physiological component in the digestion of humans and animals alike. In addition, it is now recognised as an important source of biomarkers for the diagnosis of many diseases. It is susceptible to physiological changes due to stress, inflammation, immune responses, resulting in molecules that serve as biomarkers in pathological situations [50]. The collection of biological samples from saliva has several advantages, such as: it is painless, it is carried out through simple and non-invasive techniques, there is no need for a specialised person to perform the technique and it can be collected anywhere at any time [51]. Saliva is useful in human medicine and veterinary medicine [52]. It is of particular importance to note that these advantages are valuable in this animal species, as blood collection is highly stressful and unpleasant in pigs [51]. The focus of this thesis includes diseases in pigs and given the usefulness of saliva for veterinary studies, subsequent sections will detail more about this particular fluid.

2.2 Global Concept of Saliva

Saliva is found in the oral cavity and consists of a "watery liquid" formed by a complex mixture of excretory substances from the salivary glands, as well as other substances from the oropharynx, the respiratory system, the upper respiratory system, gastrointestinal reflux, gingival fluid, food deposits and blood-derived compounds. These substances can be of organic or inorganic origin [53–55].

Saliva is a fluid with multiple functions in the human body and is essential for the preservation of general human health as well as dental health [56].

In most animals, saliva is a slightly acidic fluid. A pH of 6 to 7 is common in the saliva of pigs [57]. It consists of a physiological/biological fluid consisting of the secretions from the three major salivary glands (parotid, submandibular and sublingual) as well as the four minor salivary glands (labial, buccal, lingual and palatal). There are other structures that also contribute to a range of secretions. These are the gingival crevicular fluid, cellular debris, plaque, bacteria, nasal and bronchial secretions, lining cells, blood and exogenous substances. Most of the saliva content is water (99%), followed by proteins

(0.3%) and finally, in a smaller amount, we have the organic and inorganic compounds. This is when a physiological stimulation occurs such as chewing, taste of food or olfactory stimuli). Among the most common inorganic components are sodium, potassium, calcium, magnesium, chloride and carbonates whereas amylase, peroxidase, lipase, mucins, lysozyme, lactoferrins, kallikreins, cystatins, hormones, and growth factors are the most common organic compounds [58]. The presence and/or absence of these proteins varies from animal to animal.

Salivary secretion is influenced by hormones and reflexes and is regulated by the autonomic nervous system. Here we can distinguish two distinct components: the afferent component of the reflex pathways where the salivation centre is included while the efferent component refers to the activation of the salivary glands [59].

2.3 Anatomy and Physiology of Salivary Glands

2.3.1 Anatomy

The oral cavities of pigs are long, narrow and do not vary between animal breeds, unlike in humans [60]. Despite these variations, humans and pigs have the three main types of salivary glands. These are the parotid, submandibular and sublingual gland. Apart from these, there are other smaller glands which are in the buccal, labial, palatal and lingual areas of the oral cavity. Note that the oral glands of pigs are divided into dorsal and ventral buccal glands (Figure 1) [60].

The parotid gland of the pig is a medium-sized structure, triangular and covered in adipose tissue. The parotid duct penetrates the buccinator muscle and enters the vestibule of the parotid papilla. This occurs at the level of the upper fourth premolar to the first molar teeth. The mandibular gland (glandula mandibularis) is protected (covered)by the parotid gland. The duct enters the oral cavity at the lingual frenulum (or sublingual caruncle) and runs along the area between the mandibles under the mylohyoid muscle. Two sublingual salivary glands are present in the pig. The main sublingual duct exits through the oral cavity through the same orifice as the mandibular duct. This consists of the monostomatic sublingual gland. The polystomatous gland (larger than the previous one), on the other hand, secretes into the sublingual recess through pores [60].

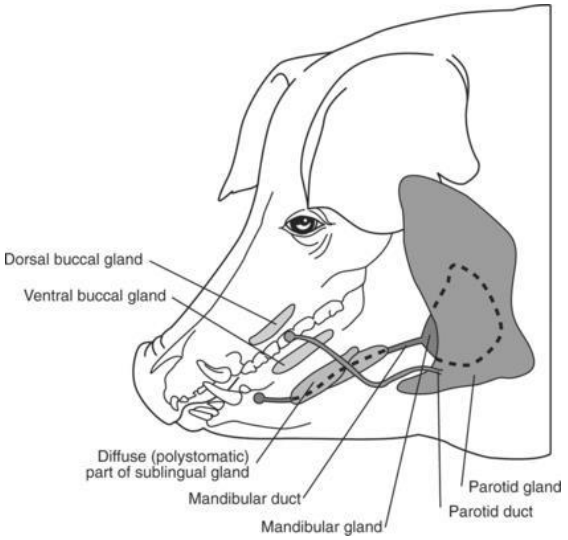


Figure 1. Schematic representation of the major salivary glands of pigs [285].

2.3.2 Physiology

Salivary glands are composed of two types of secretory cells responsible for the secretion produced: serous and mucous. The serous cells are responsible for secretion with a higher proportion of water and lower protein concentration, whereas mucous cells are mainly responsible for mucin production. Parotid glands are composed of serous acini, whereas submandibular contain both serous and mucous cells, classified as mixed glands. In the case of sublingual glands, which are also considered mixed glands, they are mainly composed of mucous acinar cells with some serous demilunes. In a functional way, human salivary glands are innervated. This is described based on animal/veterinary research[61,62]. The acinar cells and associated myoepithelial cells are innervated via the sympatheticand parasympathetic 'branches' of the autonomic nervous system (ANS). There is no difference between the two branches [62,63]. The parasympathetic nerve impulses cause the saliva they produce to be high-flowing and low in protein, whereas with sympathetic impulses the saliva is low-flowing and high in protein. However, some changes may arise. The exocytosis of the salivary cells is stimulated by parasympathetic stimuli. Thus, there is a release of protein in saliva. However, these stimulatory responses seem to be of special interest and important in the secretion of mucins, which result from the secretion of the mucous glands. Salivary flow can be caused by sympathetic adrenergic stimulation, although this is not part of the salivary reflex [62,64,65]. When sensory stimulation occurs or when parasympathetic activity is activated, the average saliva flow rate in people is known

to vary between 0.25 and 0.90 ml/min, with an increase to 0.4 ml/min [62,66,67]. Salivary secretions at rest may change based on several factors, such as age, number of teeth, gender, body weight, circadian rhythm or certain medications, as well as some diseases such as hepatitis, malnutrition, depression, neurological diseases, diabetes, chronic pain disorders, among others [62,67].

The two main types of neurotransmitters released by the autonomic nervous system nerve fibres that innervate the salivary glands are acetylcholine (ACh) andnoradrenaline (NA), which respectively produce cholinergic and adrenergic responses in the body. Regarding acetylcholine, this is the main neurotransmitter that is released in thesynaptic cleft between pre- and postganglionic neurons as well as between postganglionic parasympathetic neurons and the salivary glands. As for noradrenaline, it occurs in the sympathetic postganglionic neurons and the effector salivary glands [62].

2.4 General Animal Saliva

Significant advances in veterinary care and animal research are due to animal saliva, as it has been the subject of several studies over the years. Thus, consequently, investigations about the proteome of body fluids in humans and animals (also animals of economic interest) have been carried out and published [68].

Studies and research on this fluid have shown that the proteomic composition may vary in different animal species [69–71].

To identify pathophysiologically relevant changes for the detection and understanding of diseases, proteomics research focused heavily on the human salivary profile until very recently. [69,72–77]. In the saliva of some ruminants, such as goats and sheep, there is no scientific evidence of the presence of cystatins, proline-rich proteins (PRPs) or histatins, these being very relevant in human saliva [69,77,78].

Acute phase proteins (APR) refer to a set of structurally/morphologically unrelated proteins that are qualitatively and quantitatively modified in the blood and other body fluids. Acute phase reactions (APR) (also known as local inflammation), initiate processes leading to a systemic response, which is a physiological response to disease and injury, common to ruminants [79].

Investigations have been carried out at the bovine level, such as: most of the analytes detected in bovine saliva can be altered in the presence of feed [80], salivary analytes related to inflammation and stress are altered at the salivary level during the peripartum period of dairy cows [81], in cows with mastitis, several response patterns have been demonstrated as a result of modification of protein expression and metabolic pathways due to changes in the salivary and serum proteome [82], salivary oxytocin fluctuations in cows exist in different physiological or productive circumstances, such as calving or weaning, respectively [83], the increases in the concentrations of antioxidants and oxidants, in calves, were observed in a grouping situation where the balance of these molecules is significantly altered [84], in claudication in cows, it is possible to note that the change in some salivary analytes reflects an improvement in the claudication condition after a certain therapy is given [85] and, finally, other studies have shown that the concentrations of sIgA in the saliva of calves changes as a result of changes in feeding, play activity and time of day [86], etc.

Studies in sheep and goats have shown that saliva changes under certain conditions, namely in stress [87,88], by ingesting certain polyphenols such as tannins [89], daily rhythms [90], and ingestion behaviour [91].

In horses, studies have already proven that the proteome of saliva changes in several conditions such as gastrointestinal pathologies like equine gastric ulcer syndrome [92] and acute equine abdomen [93–95] in endurance exercise [96,97], in the circadian rhythm and season variance [98], behavioural reactions [99] and systemic inflammations [100].

Salivary metabolites can influence and determine the health status of humans, according to some studies. The dog is an example of an important experimental model for human diseases and thus could confirm the above mentioned. In addition to "accompanying" dogs in analysis, diagnosis and treatment in veterinary medicine, their saliva is a non-invasive fluid and represents a novelsource of information on the molecular mechanisms underlying various pathological conditions [101].

It has also been studied that the salivary proteome changes in dogs under certain pathological conditions [102]. Hypothyroidism is an endocrine disease very often diagnosed in dogs. The detection of the pathology by the salivary method is fundamental since this disease is characterised by a decrease in thyroid hormones and consequently results in a negative impact on the quality of life of the animal [103]. In dogs, also in dental pathologies, saliva suffers changes to [104]. Other recent and not recent studies have also proven the alteration of salivary proteomics in this species [105–139].

Finally, there are studies in another kind of species of animals where saliva changes in certain conditions, like in cats, rodents, and even in wildlife animals, such as Asian elephants [140–150].

In short, all these studies carried out in animal saliva over all these years have revealed the importance that saliva, specifically salivary biomarkers, has in alternative diagnosis to other body fluids for the detection of numerous pathologies in veterinary medicine.

2.5 Saliva Collection and it's Pros and Cos

Saliva presents certain drawbacks and limitations. The collection of saliva samples is simple and comfortable, both in humans and animals (including pigs and dogs). This collection can often be difficult, or even intrusive, in some situations, depending on the species and habits of the animals [62].

Only a few techniques to collect this biofluid, which are used in humans, are also available for animals due to several restrictions, including the size and habits of the animals. In veterinary care and animal research, cannulation of the salivary glands or

mechanical stimulation of the animals are techniques mostly used. Collection techniques vary depending on the species and the objectives of the study/work [62].

To obtain saliva from pigs, what is used is a pair of metal tweezers with a sponge on the edge, with which pressure is applied in the animal's mouth for it to chew [151].

For decades, one of the traditional techniques for collecting saliva from pigs was the "rope-chew" method, using cotton ropes [152] and in numerous biochemical tests, the cotton technique (cotton wool for medical purposes) assisted by a thread was used [153]. Both these methods used a medical cotton wool attached to a rope or a strong support [152,153].

After a clear description of the technique, anyone can collect saliva samples without the need for specialised personnel. Sampling is more convenient than e.g., blood, urine, and faeces sampling, leading to a larger collection of animal saliva. Finally, stress and anxiety of the animal result from the discomfort of the blood sample, which can alter analytical parameters, causing delays in analysis, for example. Since saliva collection is painless, the likelihood of negative effects is reduced [62].

What also reduces the possibility of accidents and infections is the fact that saliva collection does not require the use of needles, which is also an advantage over blood collection [154].

Another advantage that saliva presents is its simplicity of storage compared to blood or stool samples (which require a more sophisticated procedure or treatment) [154,155].

As mentioned earlier, saliva has many advantages such as: the collection is painless, quick, and simple and safer for the animal and the person performing the procedure. Hence, its use is frequent in clinical settings as well as in human and animal research. However, some restrictions must be mentioned before using a saliva-based technique for the diagnosis, prognosis and monitoring of animal diseases. Thus, it is crucial to consider normalisation methods, which analyte is best for each disease and any factors/motives that may be affecting the results.

In the simpler methodologies, there are some disadvantages to collecting this type of sample, such as contamination (e.g., presence of food) and, of course, intra-species genetic variation also helps to vary the protein composition of saliva [286].

2.6 Factors that Influence Saliva Composition

A diverse range of biomarkers are used in production animals, such as pigs, for diagnostic testing, animal health monitoring, serological surveillance, and farm management. The reference interval (RI) and interpretation of biomarker test results will be influenced by non-pathological variables (non-pathological elements) which pose a problem for the use of reasonable and reliable biomarkers. Biological or analytical, their impact is determined by various physiological and environmental factors as well as intra-species genetic diversity [156].

Saliva, as a biological fluid, and due to its non-invasive and non-stressful collection approach, allows routine monitoring of the animals' health status and can be carried out by non-specialist personnel. The widespread application of salivary biomarkers in the clinical evaluation of production animals (pigs) as well as further study validation (including the establishment of a reference range) is still needed. There should be continued use of new veterinary clinical biomarkers and furthermore, standardisation of clinical trials, publication of various reference intervals and creation of clear recommendations for interpretation of results are crucial [156].

Salivary oxidative stress concentrations are dramatically affected by 1% blood contamination in human studies. These blood samples are obviously coloured, making it easy and effective to remove all contaminated samples from the tests being performed. An example given is the fact that in a group of people diagnosed with gingivitis and a control group, due to micro-lesions in the periodontium, there was a blood leakage that interfered with the concentrations of salivary biomarkers. In humans, saliva samples should be taken before dental hygiene procedures or clinical examinationsto be performed in the oral cavity. This is so that we can have a good assessment of salivary oxidative stress [157].

2.7 Research in Salivary Proteomics

The omics techniques are used to quantify and qualify proteins and study several aspects at the same time (proteins, genes, etc.) whereas more traditional techniques study one protein at a time [287]. Proteomics offers better resolution for identifying molecular masses [288]. This is speaking at the time of personalised medicine or so- called precision medicine. Through high-throughput and efficient techniques in conjunction with bioinformatics tools, proteomics is a method of identifying and measuring the total content of proteins in cells, tissues, or biological fluids. Saliva has several physiological and practical advantages for human health monitoring and isconsidered a special fluid when compared to blood or other biological fluids [158].

Technological advances (more equipment and more excellent resolution) have allowed more approaches and greater knowledge about the area of proteomics, more specifically, the area of saliva.

Proteomics is, by definition, the study of all the proteins presents in a biological sample at a particular time and under particular conditions. As such, salivary proteomics refers to the study of the proteins present in saliva.

Gel-based and gel-free approaches are techniques in which salivary proteomics can be studied. Total proteins in the samples are digested (with a protease), and peptides are then examined by mass spectrometry utilising gel-free techniques. For the identification of "all" the proteins present, using gel-based approaches, the separation of proteins precedes their digestion and identification. Separation of proteins in the gel can be unidimensional, i.e., taking advantage of only one characteristic of the proteins(molecular mass, SDS-PAGE, or charge, in the case of isoelectric focusing), or bi- dimensional, when both protein characteristics are used for higher separation. Based on the characteristics of the proteins, they pass through two successive separation phases (two dimensions) when the two-dimensional electrophoresis (2DE) technique is used. In the first dimension, the proteins (which have been separated by isoelectric focusing (IEF))migrate until they reach the isoelectric point (moment when the charges cancel each other out); the second dimension is polyacrylamide gel electrophoresis, in which proteins that have been separated by IEF are separated based on their molecular masses [159].

There are a few protein technique approaches that are used to study saliva, namely gel-based,gel-free, and mass spectrometry.

- Gel Based Proteomics

In 1975, O'Farrell introduced the method known as two-dimensional polyacrylamide gel electrophoresis (2-DE) [156]. Over time, the functionality of this technique has been improved but the essential idea has not changed. In simple terms, this technique explains that in a first dimension, through isoelectric focusing, proteins are divided according to their isoelectric point and in a second dimension they are divided according to their molecular weight. Since these two parameters are not related, it is possible to have a uniform distribution of protein spots on a two-dimensional protein gel. The resulting "map" of these protein spots gives a "fingerprint" of the sample protein [160].

- Gel Free Proteomics

Some (recently developed) "shotgun" proteomic techniques can be used to examine certain hydrophobic proteins and peptides [229]. Liquid chromatography in conjunction with tandem mass spectrometry are techniques used by these 'shotgun' methods to identify and separate peptides that are derived from the enzymatic digestion of a whole protein extract (instead of using 2-DE). This technique does not separate or identify the proteins themselves. Instead, tandem mass spectrometricanalysis is used where proteins are split and analysed after being separated into peptides through the action of proteolytic enzymes. Through mass spectrometric identification of these peptides, the content of the initial sample can be determined. Instead of a completegel-based analysis, significantly faster and more economical, a peptide-based proteome analysis can be performed. This is because peptides can be separated by liquid chromatography more easily [160].

In this method (mass spectrometry) an ion source is used, a mass analyser that calculates the mass-to-charge (m/z) ratio of the ionised analytes and adetector that counts the number of ions at various m/z values [161].

The identification of proteins and the methods of analysis have undergone a constant evolution over time. This is how proteomics was "born". With advances in technology, it has become possible to identify disease specific biomarkers as well as the detection and characterisation of large and small proteins in biological samples. The source of biomarkers is mainly characterised by the presence of minor proteins in the salivary proteome[162].

Further research is needed in the future to improve and develop the current studies [163–179].

Proteomics technologies can use a variety of biological fluids, including saliva, urine, and blood. Today, the protein content of these fluids is being identified and characterised to gain new insights into the evolutionary adaptations of farm animal species as well as domestic ones (such as pigs). Their normal physiological state is also being characterised. The results of these studies can be applied to animal welfare and the improvement of animal productivity, as well as providing biomarkers of disease and stress.

The search for disease biomarkers, the understanding of some diseases and the evaluation/understanding of the mechanisms involved in some physiological and pathophysiological processes have been the purposes for which salivary proteomics has been most used in humans [180].

One- and two-dimensional electrophoresis as well as immunoblotting are techniques that have helped to validate (through scientific experiments) the complex and dynamic nature of the pig salivary proteome. Immunoglobulins as well as 2-DE gel proteomic maps of salivary protein were discovered through these investigations [181]. The production of various protein isoforms results from fragmentation, bacterial degradation, endogenous truncation, glycosylation and phosphorylation. Thirteen proteins were identified by mass spectrometry [181]. With improved databases and comparative analyses, 20 distinct proteins were identified. This is because, normalisation techniques were used to find a panel of biomarkers that could distinguish between clinically healthy and diseased pigs [182]. In both serum and saliva of pigs, numerous proteins can be found that serve as indicators of general health. Unlike amylase, lipocalins, which make up 25% of the human salivary proteome, are the most prevalent in the porcine salivary proteome.

Also present in the saliva of these animals are proline-rich proteins. Their identification on gels depends on their staining (pink colouration) [183].

Carbonic anhydrase VI, -1-antichymotrypsin and haptoglobin were detected by 2-DE gel electrophoresis in saliva from pigs with rectal prolapse. Interestingly, compared to serum-derived haptoglobin glycoproteins, the haptoglobin glycoproteins in salivashowed very different results [184].

3. Salivary Biomarkers for Clinical Applications

3.1 The Idea of Biomarker and the Potential of Saliva as a Diagnostic Fluid

A biomarker can be defined as: "A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention." [185].

Saliva serves as one of the easily accessible body fluids for biomarker assessment and research. There is a growing interest in using saliva in clinical settings as salivary secretions are simple and easy to collect, no specialised personnel are required to perform the collection and it is also an inexpensive technique. Thus, using saliva as a sample, it is possible to take numerous samples repeatedly at short time intervals [186].

In saliva there are numerous health indicators as well as disease indicators. A variety of organic (peptides, proteins and enzymes) and inorganic (calcium, magnesium, sodium and potassium cations; phosphate and carbonate anions) compounds, as well as cells, including exfoliated epithelial cells, leucocytes and bacteria, can be found in saliva [187].

Saliva (as a diagnostic fluid) has attracted attention and has thus become a very successful translational research case study over the last 20 years. The use of saliva as a diagnostic tool for the identification and prediction of disease progression, has been based on nanotechnologies that have the analytical sensitivity needed to analyse saliva.

3.2 Current Knowledge of Salivary Biomarkers in Pigs

- General Overview

The investigation of porcine salivary proteomics is still under increasing study. However, in this present thesis it is possible to conclude that there is still "much room" for development in proteomics, considering pig saliva as a non-invasive diagnostic fluid. In this species, compared to other body fluids, saliva has many advantages (as mentioned above) [162].

As a diagnostic sample, pig saliva has been widely used to detect diseases (directly or indirectly) by using specific antibodies [57]. Several protein and non-protein analytes were found and analysed in the saliva of pigs, for example, haptoglobin [188], as well as cortisol [189], estrone sulphate [190], progesterone [191], and immunoglobulin [192,193]. With the advancement of technology, one of the indicators used in pig health and welfare monitoring is C-reactive protein (CRP) which was compared in saliva samples from healthy and sick pigs.

Most research on salivary proteins concentrates on humans, but also on rodents and primates. Study methods have been developed for the scientific analysis of certain animals, such as pigs. It is therefore concluded that changes in these proteins may reflect specific infections and pathological conditions as well as changes in the general health of the animals [194]. Furthermore, these changes also reflect the pig as a model animal for research, and knowledge of its physiology can also be studied [195].

Other studies have been made regarding salivary chromogranin, haptoglobin, circadian rhythm, c-reactive proteins, etc [196–205].

- Stress and Welfare

Saliva in pigs has shown studies about stress and animal welfare [150,206,207]. According to Gutierrez there are currently some studies carried out on pig saliva for some

pathologies [208–216]. However, for *Escherichia coli* diarrhoea in pigs in the post-weaning phase, there is nothing studied yet.

In terms of animal welfare, saliva emerges as a better option as its collection is less stressful compared to blood [217].

Regarding stress and welfare studies in pigs, it is possible to say that saliva changes in these conditions, more specifically in relation to alpha-amylase, serum amyloid A, oxytocin, cholinesterase, testosterone, cortisol, etc [218–239].

- Physiological Conditions

The detection of oestrus in sows is possible based on saliva proteomics, according to certain studies [240]. During their production cycle, changes, based on production stage and sex, can be observed in the salivary proteome of healthy fattening pigs [241], semen collection also influences oxytocin levels in breeding male pigs [242] and, also, saliva can change due to porcine breed, sex, and production stage [243]. In the same way, gender can influence saliva proteome too [244].

Several factors can influence leptin in pig saliva such as body weight, food intake and inflammation [245].

- Pathological Conditions

Numerous biomarkers of disease are found in saliva. One of them is called adenosine deaminase. In relation to the purine metabolic pathway, the elimination of adenosine is catalysed by this enzyme. Due to this enzyme, lymphocytes and the cell lines of monocytes and macrophages (immune cells) undergo differentiation and maturation [246].

In recent investigations, studies have reported that changes in the porcine salivary proteome may be caused by experimental induction of sepsis due to administration of lipopolysaccharide (LPS) [247]. In contrast to the changes observed in non-septic inflammation, salivary proteins, aldolase A and serpin 12, were found to be markedly elevated in septic inflammation. It was also shown that pigs with sepsis have altered pathophysiological pathways compared to pigs with non-septic inflammation according to Gene Ontology (GO) investigation. This revealed prospective future biomarkers (including aldose A) as well as helping to clarify several pathophysiological pathways in septic inflammation.

Proteomic investigations were carried out to assess intestinal changes in pigs diagnosed with *E. coli* diarrhoea [248,249]. Nevertheless, there is still much to be discovered regarding saliva research.

Finally, it is possible to summarize that various studies were made regarding several pathological conditions in pigs using saliva as the study method. Some pathologies for which these have already been done, including African Swine Fever, sepsis, post-partum dysgalactia syndrome, lameness, systemic diseases, porcine reproductive and respiratory syndrome virus, among others [250–259]. There are some studies regarding jejunal tissues from piglets diagnosed with enterotoxigenic *Escherichia coli*. However, no study has been done on *Escherichia coli* diarrhoea in post-weaned piglets.

THE FUNDAMENTAL PROBLEM OF THIS WORK

"The Need for Non-Invasive Methods for Diagnosing *Escherichia coli* Diarrhoea in Pigs."

"Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by *Escherichia coli*"[1]

[1] **Rodrigues, M.**; López-Martinez, M.J.; Ortin-Bustillo, A.; Cerón, J.J.; Martinez-Subiela, S.; Muñoz-Prieto, A.; Lamy, E. Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by Escherichia coli. Proteomes 2023, 11, 14. https:// doi.org/10.3390/proteomes11020014 [Annex I]

PART II

OBJECTIVES

The central aim of this study is to evaluate the changes in the salivary proteome of pigs diagnosed with *Escherichia coli* diarrhoea. Two techniques were used for protein separation by isoelectric point/molecular mass and molecular mass. These are 2-DE gel electrophoresis and SDS PAGE, respectively. In addition to these techniques, amore sophisticated technique was used for protein identification called mass spectrometry [286].

PART III

MATERIALS AND METHODS

- MATERIALS REQUIRED

1. Equipment:

Bio-Rad electrophoresis apparatus for vertical slab gels with a size of 0.75mm X 10cm X 12cm;

Power supply;

Micropipette for loading samples.

2. Chemicals/Reagents/Buffers:

Stock acrylamide solution: 30g acrylamide, 0.8g bisacrylamide. Make up to 100ml in distilled water and filter through Whatman No1 filter and store in amber bottle at 4ºC.

2.1 Buffers:

- **Separating gel buffer:** 1.875M Tris-HCl, pH 8.8
- **Stacking gel buffer:** 0.6M Tris-HCl, pH 6.8

10% w/v Ammonium persulfate. Make fresh. Store at 4ºC.

10% w/v Sodium dodecyl sulphate (SDS) – chemical detergent.

N, N, N′, N′-Tetramethyl ethylenediamine (TEMED).

- Sample buffer

0.6 M Tris-HCl, pH 6.8 5.0ml

10% SDS 0.5g

Sucrose 5.0g

β-mercaptoethanol 0.25ml

Bromophenol blue (0.5% stock) 5.0ml

Make up to 50ml with distilled water.

- **Electrophoresis buffer:** Tris (12g), glycine (57.6g), and SDS (2.0g). Make up to 2l with water. No pH adjustment is necessary.

3. **Protein Stain:** 0.1% Coomassie brilliant blue R250 in 50% methanol, 10%glacial acetic acid. Dissolve the dye in the methanol and water componentfirst, and then add the acetic acid. Filter the solution through Whatman filter paper. (Note: Coomassie brilliant blue is harmful by inhalation or ingestion. Wear appropriate gloves & safety glasses while handling).
4. **Distaining solution:** 10% methanol, 7% glacial acetic acid.
5. **Protein sample.**
6. **Standard Protein molecular weight markers (Bio-Rad brand).**

1) Study Design

The InterLab (University of Murcia, Spain) and the Laboratório de Fisiologia Animal Aplicada, that is part of the Mediterranean Institute for Agriculture, Environment and Development (MED, University of Évora, Portugal), collaborated to carry out the current cross-sectional study between the months of September 2022 and January 2023. According to the European Council Directives for the protection of animals used for experimental purposes, the study protocol was authorised by the Bioethical Commission of the University of Murcia (CEEA 563/2019). All farmers who participated did so voluntarily and with full knowledge. Clinical information and saliva samples were collected on farms in the Murcia region of Spain, close to the university. Proteinseparation and image analysis were carried out in the laboratories of the University of Évora (Laboratório de Fisiologia Animal Aplicada), and mass spectrometry analysis wascarried out at the University of Murcia [286].

2) Pigs' population

This study included two groups of Large White post-weaning pigs weighing 13–15 kg and aged 6–9 weeks. The first was a group of pigs with *Escherichia coli*-related diarrhoea (n = 10, 50% males, 50% females), and the second was a group of clinically healthy pigs (n = 10, 50% males, 50% females). The affected animals showed clinical signs compatible with this disease (diarrhoeal syndrome) and rectal swabs taken according to protocol revealed the presence of *Escherichia coli* [286].

3) Saliva collection

Saliva was collected using a sponge that was fastened to a 10 cm long, flexible, thin metal rod. The sponge was given to the pigs to chew on until it was completely moist. As soon as that was done, the sponges were taken out of the pigs' mouths and put in Salivette tubes (Sarstedt, Aktiengesellschaft & Co., D-51588 Nümbrecht, Germany). Upon arrival at the lab, all samples were kept chilled until the Salivette tubes were centrifuged at 3000 g and 4 °C for 10 min., respectively, to obtain saliva supernatant. After that, the aliquots were put into Eppendorf tubes and kept there at 80 ºC till the analysis was done [286].

4) Concentration of total proteins

The BCA assay, which uses a microplate method, was used to measure the total protein concentration in the samples (Thermo Scientific, Product No. 15041, Rockford, IL, USA) [286].

The working reagent (WR) to sample ratio was 1:8. A microplate well was first filled with 25 L of each standard or unidentified sample in triplicate. Each well received 200 L of the WR, which was then added and well mixed on a plate shaker for 30 seconds. The plate was then covered and incubated for 30 minutes at 37°C. The absorbance was measured at 562 nm after the plate had been cooled to room temperature. Each microplate was subjected to a Bovine Serum Albumin (BSA) curve with standards ranging from 20 to 2000 g/mL. As a control, ultrapure water was used. The protein concentration of the samples was determined using the generated curve equation after a standard curve was created using the blank and standards [286].

5) SDS-PAGE (1-DE)

All young animals' saliva samples were sorted into distinct proteins by SDS PAGE gel electrophoresis on 12% acrylamide gels (mini-protean - Bio-Rad). The acrylamide gels were made by the following steps or procedures. To start, the physical system for gel casting was set-up. The ammonium persulphate (APS) solution was prepared at a final concentration of 1%. The resolution gel was the first to be prepared (10 mL of Tris-HCl 1.5M pH 8.8, 400 µL 10% SDS, 13.n6 mL distilled water, 16 mL of 30% of Acrylamide/Bisacrylamide, 300µl APS and 20µL TEMED). These reagents are going to make the solution solid in the final. In the end thesome drops of distilled water were added to guarantee the polymerization in the absenceof oxygen. After this, all the distilled water were removed from the glasses with the helpof normal paper. The concentration gel was prepared (4 mL of Tris-HCl 0.5M pH 6.8, 160 µL 10% SDS, 9.72 mL distilled water, 2.12 mL of 30% of Acrylamide/Bisacrylamide, 96µl APS and 17.6 µL TEMED.). At the end the combs were put in contact with this last gel [286].

The running system was built, creating upper and lower chambers, which were both filled with running buffer [286].

For the sake of reducing technical faults, samples were ran twice. In a nutshell, each lane received a total of 9 g of protein from each saliva sample. Previously, the final concentration of the sample buffer, which was 6x concentrated and stored, was diluted with ultrapure water. Each freeze-dried sample received a volume of 40 µl of sample buffer (3 ml glycerol, 1 ml of 1.5M Tris-HCl pH 6.8, 0.6 g SDS, 0.4626 g DTT, and bromophenol blue). After that, the samples were put on ice and heated for five minutes at 98 degrees Celsius to denature the proteins. They were then placed back on ice following this procedure. A 1x running buffer was then set up in the Bio-Rad electrophoresis tank system (100 mL of 10x running buffer and 900 mL of distilled water). The final step involved applying 20 µl of sample to each lane (in triplicate) and running the electrophoresis at a constant voltage of 150 V for roughly 1 hour and 20 minutes or until the dye front reached the end of the gel. The gels were fixed in 40% methanol and 10% acetic acid for one hour, then stained with Coomassie Brilliant Blue R-250 for an additional hour before being repeatedly stained with a distaining solution of 10% acetic acid to remove the background staining. Finally, scanned pictures were obtained using Lab Scan software and processed with Image Lab (Bio-Rad) software [286].

6) Two-Dimensional Electrophoresis, Computational Image, and Mass Tandem Spectrometry

Each saliva sample (volume corresponding to 275 g of total protein) was lyophilized and kept at -28°C for the 2-DE method. 250 L of solubilization buffer [7M urea, 2M thiourea, 4% (w/v) 3-(3-cholamidopropyl) dimethylammonium propane sulfonate (CHAPS), 2% (v/v) ampholyte mixture (IPG buffer, GE healthcare), and 40mM dithiothreitol (DTT)] were used to reconstitute the solid material. The mixture was first incubated for one hour at room temperature before being centrifuged for ten minutes at 10,000 rpm. Then, to run each sample in duplicate, the supernatant from each sample was divided into two volumes of 125 L and applied in two different slots of the strip holder of the Multiphor II system (GE Healthcare). The commercial gel strips [7 cm pH gradient 3-11 NL (IPG strips, GE healthcare)] were placed in contact with the sample as the final stage in strip rehydration, and they were passively rehydrated overnight at room temperature while being covered with mineral oil (Dry strip cover fluid, GE healthcare)

[286]. The following programmes (gradient) were used to focus in a Multiphor II (GE, Healthcare) at 12 °C: (1) 0-150 V for 15 min; (2) 150-300 V for 15 min; 300 V for 0.5 h; 300-3500 V for 4 h; and 3500 for 3.5 h. With the addition of 0.2g DTT and 0.5g iodoacetamide in the first and second phases, respectively, focused strips were equilibrated in two steps of 15 min each with equilibration buffer [50 mM Tris-HCl, pH **7.** 8; 6M urea; 30% (v/v) glycerol and 2% (w/v) sodium dodecyl sulphate (SDS)]. The strips were placed on top of a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) over a 12% acrylamide gel after equilibration, and the mini-protein system (Bio-Rad) was operated at 150 V constant voltage. As with SDS-PAGE gels, CBB-R250 was used to dye the gels. Gel scan software and a gel scanner were usedto capture gel images. The "same spots" programme was used to analyse the gel [286].

The following conventional process was used to digest the samples. Selected bands were separated into around 2 mm-wide segments after electrophoresis and image analysis and stained. After that, bands were washed twice in 25 mM ammonium bicarbonate buffer pH 8.5 in 50% acetonitrile over the course of 30 minutes at 37 °C. Bands were removed from the supernatant, dried for 15 minutes using an Eppendorf 5301vacuum evaporator, and then incubated for 20 minutes at 56 degrees Celsius in 100 µl of 25 mM ammonium bicarbonate buffer pH 8.5 with 10 mM DTT. The samples were alkylated by adding 100 µl of 25 mM ammonium bicarbonate buffer pH 8.5 with 25 mM iodoacetamide over the course of 30 min at room temperature in the dark after the supernatant was removed. The supernatant was once more removed, and the spots were then washed twice for 15 minutes each time at 37 degrees Celsius: once with 25 mM ammonium bicarbonate buffer pH 8.5 and once with 25 mM ammonium bicarbonate buffer pH 8.5 in 50% acetonitrile. Spots were washed, dried once more, and thenincubated for 10 minutes at 4 degrees Celsius in 50 µl of 25 mM ammonium bicarbonate buffer containing 0.5 g of Trypsin Gold Proteomics Grade (Promega Corporation, Madison, MI, U.S.A.) and 0.01% ProteaseMax surfactant. This surfactant improves the digestion of trypsin. Finally, materials were put through a 16-hour digestion process at 37°C. The supernatant was collected in a fresh tube, and the spots were then washed twice for 30 minutes each time with 100 µl of an acetonitrile solution that was 50% acetonitrile and 0.5% TFA. Both supernatants after washing were collected in the same tube and driedusing a vacuum evaporator. These washes improved the extraction of digested particles from the gel spots [286].

7) Protein identification through HPLC-MS/MS analysis.

The separation and analysis of the tryptic digests of the samples were performed with an HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an Automated Multisampler module and a High Speed BinaryPump, and connected to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00), experimental settings for HPLC and Q-TOF were specified [286].

Dry trypsin digested samples were resuspended in 20 µl of buffer A, which is composed of water, acetonitrile, and formic acid (94.9:5:0.1). A sample was injected at a flow rate of 0.4 ml/min onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 m, 100 x 2.1 mm, Agilent Technologies). The isolation and analysis of peptides can be done using this column. The digested peptides were eluted using a linear gradient of 0-40% B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) for 40 min, followed by a linear gradient of 40-95% B for 8 min, after the column had been washed with buffer A for 3 min after the injection. For three minutes, 95%B was maintained. Before each injection, the column was lastly equilibrated in the original conditions for 6 minutes [286].

The positive mode was used to run the mass spectrometer. The drying gas flow was set to 14 µl/min at a temperature of 300 ºC, the sheath gas flow to 11 µl/min at a temperature of 250 ºC, and the nebulizer gas pressure was set to 35 psi. The voltages for the capillary spray, nozzle, fragmentor, and octopole RF Vpp were, respectively, 3500 V, 100 V, 360 V, and 750 V. For all MS and MS/MS scans, profile data were collected at 4 GHz in extended dynamic range mode. The mass range for MS and MS/MS was 50-1700 m/z, and the scan rates for MS were 8 spectra/sec and 3 spectra/sec, respectively. Precursors were chosen by abundance and a maximum of 20 could be chosen per cycle in auto MS/MS mode. The collision energy was ramped, with a slope and offset of 3.68 and -4.28, respectively. Upon receiving two successive spectra, the same ion was rejected [286].

Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA) was used to analyse and analyse the data. In a nutshell, default criteria were used to retrieve the raw data as follows: Finding 12C signals requires unmodified or carbamidomethylated cysteines, [MH]+50–10,000 m/z, a maximum precursor charge of +5, and a minimum signal-to-noise MS (S/N) of 25 [286].

The suitable and updated protein database was used for the MS/MS search, and the following criteria were used: Tryptic digestion with a maximum of five missed cleavages, an ESI-Q-TOF instrument, a minimum matched peak intensity of 50%, a maximum ambiguous precursor charge of +5, monoisotopic masses, peptide precursor mass tolerance of 20 ppm, product ion mass tolerance of 50 ppm, and calculation of reversed database scores are all included in the variable modifications search mode. Usingauto thresholds, peptide and protein data were validated [286].

8) Statistical and Bioinformatics Analysis

The Shapiro-Wilk test was used to determine whether the data had a normal distribution. Those variables that showed a normal distribution were handled as-is (BANDS C1, D, E, G, I, J, K, L1, M, M1), whereas those that did not were (log) transformed (BANDS B, C, H). T-Student for independent samples was used to compare the means. Non-parametric tests (Mann-Whitney) were employed if the transformation did not produce a normal distribution (BANDS C2, F, AND L) [286].

Statistical analysis was performed SPSS (IBM SPSS Statistics 28.0) [286].

9) Future perspectives

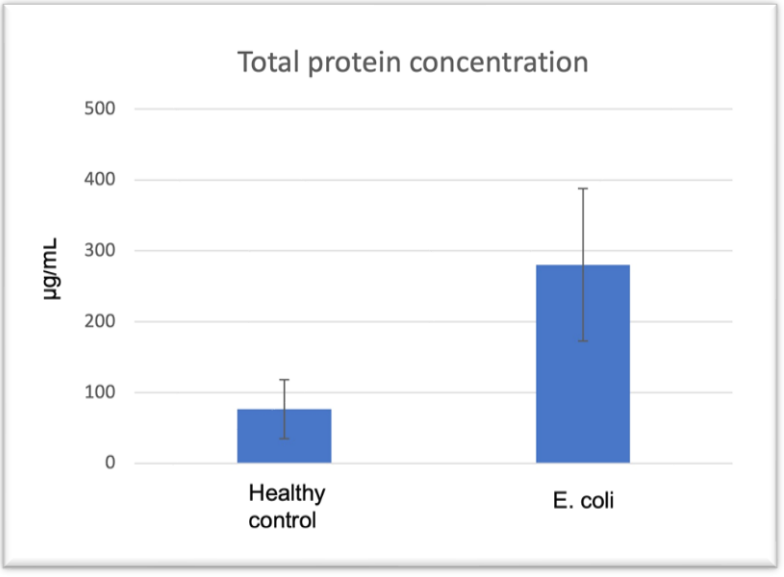
For future perspectives, some studies, reveal that is advisable the use of combination of liquid chromatography and mass spectrometry for the study of metabolites in saliva [289, 290]. There are some studies regarding stress in pigs [291], but none regarding this specific pathology (diarrhoea by *Escherichia coli*) in the use of this combination technique.

PART IV

RESULTS

1) Concentration of total proteins

In comparison to healthy animals, it was shown that the total protein concentration of saliva samples was significantly higher in *E. coli*-infected animals (Graph 1). The mean total protein levels in *E. coli* animals are nearly three times greater than those in healthy animals (76.441.8 vs. 280.5107.7 g/mL, for the healthy and *E. coli* groups, respectively; P=0.001) [286].



Graph 1. Total protein concentration in healthy and *E. coli* diseased pigs (mean values ± standard deviation) [286].

2) SDS-PAGE profile

Salivary SDS-PAGE protein profiles enabled for the continuous visualisation of 21 clearly distinguishable protein bands with molecular weights ranging from 10 to 200 kDa, whose levels were compared between groups (Figure 2) [286].

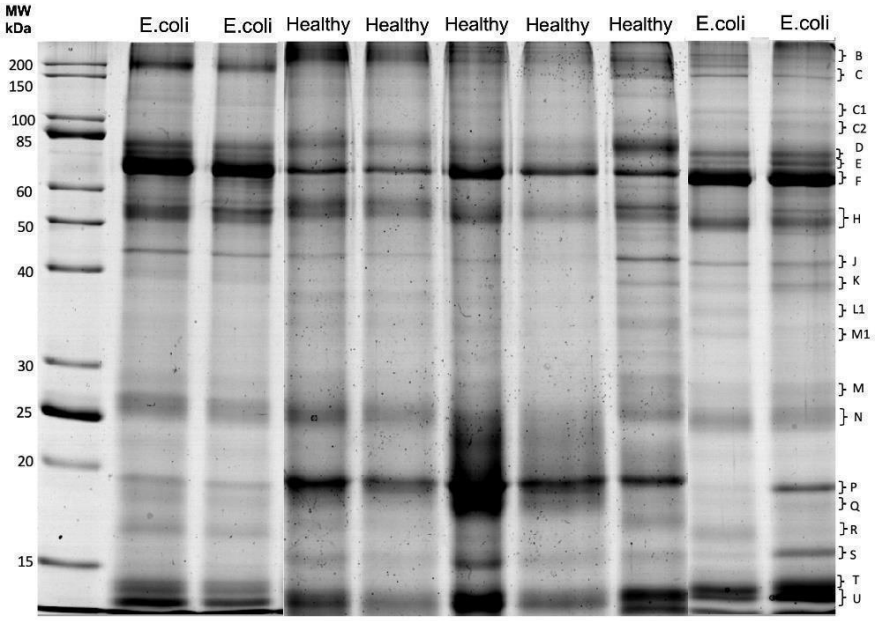


Figure 2. Representative salivary protein profiles (SDS-PAGE) of pigs (control and E. coli diseased animals). Each letter represents the bands used for comparison between groups [286].

Eight protein bands were found to express themselves differentially in healthy and sick animals. Only the *E. coli* group showed evidence of the weak band C1, which was not recognised by mass spectrometry. Despite being present in animals from both groups, the other 7 bands showed statistically significant variations, with bands P and T declining in ill animals while bands B, H, M, N, and R increased. The Table 1 lists the variations between the groups as well as mass spectrometry identifications of the proteins found in those bands [286].

Band	Healthy	*E. coli*	*p*-Value	UNIPROT Protein Accession number	Protein (Entry Name)	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
B	1.62 ± 0.80	5.36 ± 3.06	0.001	018758	Submaxillary apomucin	1.3	238.4	1184.1	>200 kDa
C1	-				ni				120
H	3.74 ± 0.59	9.77 ± 2.91	0.0005	A0A287B626	IgA constant region	39.3	209.6	44.2	54
M#	1.35 ± 1.09	2.94 ± 0.75	0.015	A0A0A0MY58 and F1SN92	Immunoglobulin heavy constant mu and Salivary lipocalin	28.5 and 25.1	75.1 and 43.5	32.7 and 21.6	28.5
N	6.88 ± 2.44	10.20 ± 1.43	0.009	F1SN92	Salivary lipocalin	54.9	152.5	21.6	26
P	17.51 ± 4.27	3.40 ± 2.10	0.0005	P81245	Odorant-binding protein	75.1	199.5	17.7	18
R	1.22 ± 1.63	4.00 ± 2.47	0.033	A0A4X1TU02	Salivary lipocalin	57.5	143.4	21.6	16.5
T #	14.15 ± 4.91	8.33 ± 4.70	0.043	A0A286ZRW6 and A0A287ASS4	Double-headed protease inhibitor, submandibular gland-like and Prolactin inducible protein	29.4 and 36	58.31 and 56.35	13.3 and 12.4	13

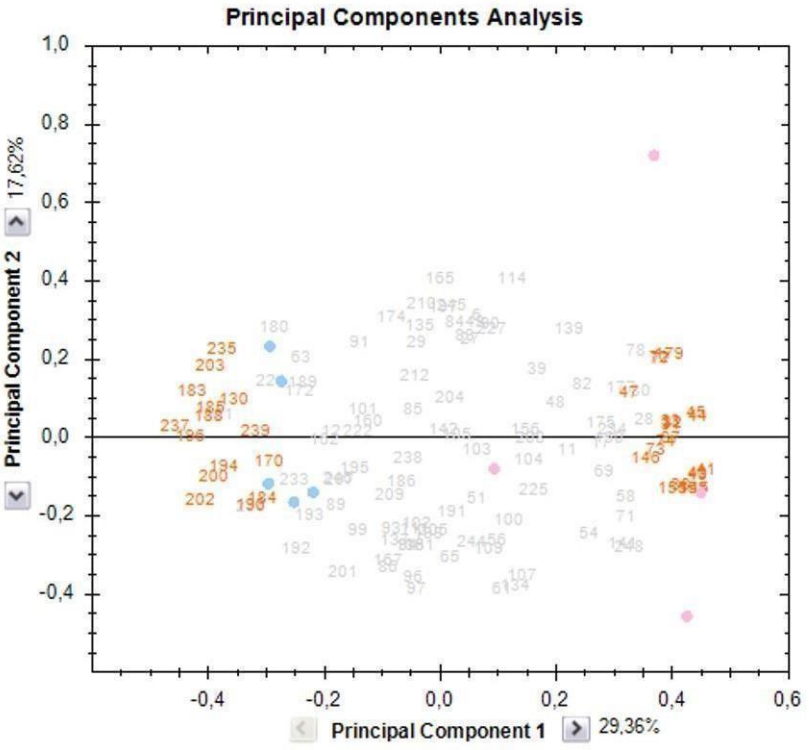
Table 1. Differences in protein bands expression levels (mean standard deviation of %Vol) between E. coli diseased and healthy pigs and correspondent protein identification and MS [286].

ni – protein that could not be identified by MS; in the tryptic mixture, peptides corresponding to multiple proteins were seen in the spectra, showing that the band contained multiple proteins [286].

From 1DE analysis, *E. coli*-infected pigs have higher levels of salivary lipocalin and IgA bands, whereas these animals have lower levels of bands containing proteins like odorant-binding protein, a protease inhibitor from the submandibular origin, and/or prolactin inducible protein [286].

3) Two-Dimensional Protein Profile (2-DE)

Following gel analysis, 127 protein spots that were consistently present in the various pool samples could be compared between healthy and *E. coli* sample pools. Using Principal Component Analysis to examine the feasibility of separating the two groups, it is possible to show that the two components account for 46.98% of the data variability (Graph 2) [286].



Graph 2. Distribution of sample pools among the two first components obtained by Principal Component Analysis (pink – healthy controls; blue – E. coli; orange – spots whose levels (%Vol) were significantly different between groups) [286].

A total of 35 protein locations were found to have a statistically significant difference (P below 0.05) using the between-subjects test (independent T-test) (Figure 3). In *E. coli* animals, 15 of these protein sites were increased whereas 20 were decreased. Table 2 lists the discovered salivary proteins as well as the degree of variance [286].

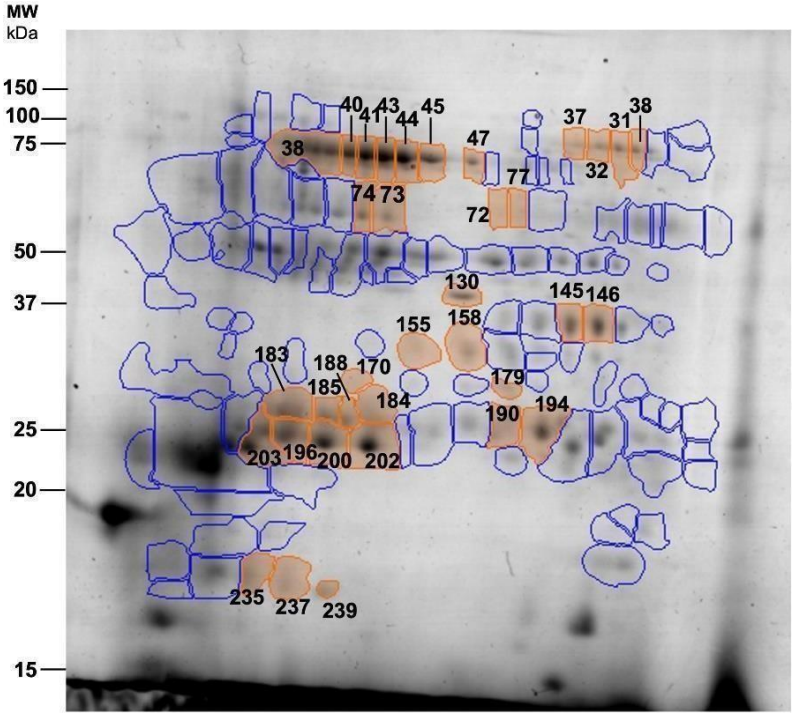


Figure 3. Protein spots differently expressed between healthy and diseased animals (orange) [286].

Spot Number	Fold Change	Group with Higher Level	*p*-Value	Protein (Entry Name)	UNIPROT Protein Accession number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
237	4.24	*E. coli*	5.24×10^{-5}	Adenosine deaminase and salivary lipocalin	A0A0B8RW47 and A0A4X1TU02	22.5 and 15.7	39.5 and 23.8	40.9 and 21.6	17.5
33	1.72	Healthy control	0.000222			n.i.			
185	2.30	*E. coli*	0.00063	IgA constant region	A0A287B626	3.8	23.6	44.2	27.5
188	2.41	*E. coli*	0.000733	IgA constant region	A0A287B626	2.6	20.0	44.2	27.5
41	3.29	Healthy control	0.000763	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	74.5
145	1.56	Healthy control	0.000794	Carbonate dehydratase VI	A0A4X1W7S7	15.1	39.5	34.7	36.0
40	2.72	Healthy control	0.000871	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	74.5
202	2.28	*E. coli*	0.000887	Ig-like domain-containing protein	A0A287A4Y3	15.4	41.4	24.7	26.0
44	3.03	Healthy control	0.001118	Lactoperoxidase	A0A480RK36	6.6	48.7	80.3	74.5
196	2.97	*E. coli*	0.001675	Albumin (fragment) and salivary lipocalin	A0A286ZT13 and A0A4X1TU02	13.6 and 23.5	100.5 and 31.5	68.2 and	26.0
200	2.34	*E. coli*	0.002233	Albumin (fragment) and salivary lipocalin	A0A286ZT13 and A0A4X1TU02	13.6 and 23.5	100.5 and 31.5	68.2 and 21.6	26.0
45	2.10	Healthy control	0.002679	Lactoperoxidase	A0A480RK36	6.7	48.8	80.3	74.5
43	3.30	Healthy control	0.003706	Lactoperoxidase and polymeric immunoglobulin receptor	A0A480RK36 and A0A0E3M2Q4	7.5 and 6.5	45.5 and 37.3	80.3 and 67.3	74.5
194	1.71	*E. coli*	0.004477	Albumin (fragment)	A0A286ZT13	7.8	64.3	68.2	26.5
31	2.29	Healthy control	0.005324			*n.i.*			
47	1.66	Healthy control	0.005441	Lactoperoxidase and polymeric immunoglobulin receptor and	A0A0E3M2Q4 and A0A480RK36	12.3 and 4.9	86.1 and 34.7	67.3 and 80.3	74.0
184	1.86	*E. coli*	0.007066			*n.i.*			
203	2.94	*E. coli*	0.007897	Ig-like domain-containing protein	A0A287A4Y3	18.5	34.0	24.7	26.0
38	2.12	Healthy control	0.009251	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	101.0
32	1.50	Healthy control	0.012577			*n.i.*			
37	2.03	Healthy control	0.01381			*n.i.*			

155	1.51	Healthy control	0.015799	Carbonic anhydrase	A0A4X1W9S1	11.0	27.7	36.3	36.0
179	1.96	Healthy control	0.020918	Carbonate dehydratase VI	A0A4X1W7S7	11.5	47.2	34.7	27.5
73	1.78	Healthy control	0.021757	Alpha-amylase	F1S573	30.1	146.0	55.8	58.0
74	1.39	Healthy control	0.026339	Alpha-amylase	F1S573	30.9	123.4	55.8	58.0
235	2.49	*E. coli*	0.030702	Adenosine deaminase and salivary lipocalin	A0A0B8RW47 and A0A4X1TU02	22.5 and 15.7	39.5 and 23.8	40.86 And 21.61	18.0
130	2.26	*E. coli*	0.033046			*n.i.*			
146	1.38	Healthy control	0.037883	Carbonate dehydratase VI	A0A4X1W7S7	9.8	25.6	34.7	36.0
77	1.67	Healthy control	0.039092			*n.i.*			
190	1.75	*E. coli*	0.040238	Albumin (fragment)	A0A286ZT13	9.2	61.0	68.2	26.0
239	2.94	*E. coli*	0.042094	Salivary lipocalin	F1SN92	4.4	24.51	21.6	17.5
170	1.36	*E. coli*	0.046073			*n.i.*			
72	2.01	Healthy control	0.046326			*n.i.*			

Table 2. Protein spots differently expressed between healthy and E. coli diseased pigs [286].

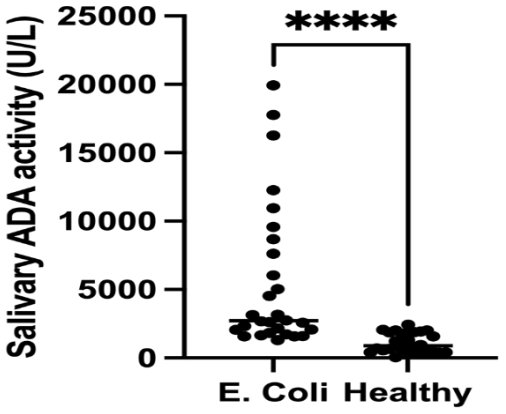
Note – *n.i.* means spots that were not identified [286].

Adenosine deaminase, IgA, and albumin peptides were also discovered as being more expressed in *E. coli* pools when 2-DE data were taken into consideration. However, in pools from the ill animals, the presence of spots containing alpha-amylase, carbonic anhydrase, carbonate dehydratase VI, and the whole albumin was diminished [286].

Compared to SDS-PAGE, 2-DE electrophoresis has a better resolution of proteins, which allows them to be quantified more individually (glycosylated proteins, phosphorylated proteins, proteins with modified amino acids, etc) [292].

4) Validation

Pigs with diarrhoea brought on by *E. coli* had considerably greater levels of salivary ADA activity measured (median 2712 U/L, minimum-to-maximum range 1293-19936 U/L) than healthy pigs (median 1750 U/L, minimum-to-maximum range 60.8-13280 U/L) ($p < 0.001$) (Graph 3) [286].



Graph 3. Comparison of the salivary adenosine deaminase activity (ADA) in pigs with diarrhoea caused by E. coli and healthy pigs. The plot shows the individual values of each group. ***P < 0.001 [286].

PART V

DISCUSSION

In the present study, with the help of 1-DE and 2-DE proteomic techniques, it was possible to observe changes in the proteins of the saliva of pigs diagnosed with *Escherichia coli* diarrhoea. The 1-DE technique allows the separation of proteins according to their molecular mass (as well as identifying a wide variety of proteins in the gel) whereas, the 2-DE technique separates proteins according to their isoelectric point and molecular mass. The latter, is not able to separate proteins with extreme isoelectric points or with higher hydrophobicity. Due to the lower total protein requirement, capability of the technique allowed to evaluate the samples on an individual level. The acquisition of a more complete protein profile is thanks to the 2-DE technique, as proteins are separated in electrical charge and mass. Both the individual 1-DE samples as well as the 2-DE sample sets were done in duplicate to minimise the impact of technical glitches inherent in the approaches [286].

In the salivary bands of *E. coli* infected pigs, there is an increase in salivary lipocalin and IgA bands while there was a decrease in salivary bands that include proteins such as odorant binding protein and/or prolactin-inducible protein [286].

Lipocalins (expressed in a variety of organs) are small proteins (18-40kDa) that are involved in various physiological processes such as inflammation, detoxification and immune activity. In addition, they transport hydrophobic substances such as steroids, retinoids or lipids [260, 286]. These proteins play key roles in physiological processes as they bind and transport hydrophobic molecules in plasma and other body fluids [261, 286]. There are acute phase proteins that show an increase in inflammation. As an example, we have lipocalin-2 also referred to as neutrophil gelatinase-associated lipocalin (a member of this family of proteins) [262, 286]. It has been studied that when humans are diagnosed with inflammatory bowel disease, lipocalin-2 levels are elevated in the blood serum and this increase is related to disease activity [263,264, 286]. In addition, it has been shown that siderophores (from other harmful bacteria) are taken up by *Escherichia coli*. Also, animals deficient in lipocalin-2 are more likely to suffer from infections and sepsis [265, 286]. LCN decreased in the saliva of pigs infected with *Streptococcus suis* (this information relates to a previous article), although it increased in the present study [4, 286]. Further studies should

be carried out to clarify the mechanisms involved in the alteration of LCN, since in some circumstances, as in the case of *Streptococcus suis* infection, the decrease in lipocalin may suggest a high vulnerability to severe sepsis [4, 286].

The odorant binding proteins are small, soluble olfactory proteins (it is a chemical binder of the odorant that can be discovered in the nasal epithelium) that are responsible for olfaction and protection against oxidative damage. In addition, they help odour molecules to reach the odorant receptors on the dendritic membrane of olfactory sensory neurons (also called olfactory receptor neurons) [266, 286]. After treatment with LPS, there is a reduction of this (inflammation-associated) protein in bovine lungs. Thedecrease in the levels of these proteins can be considered as a complementary method bywhich inflammatory mediators in the lung (and possibly other tissues) trigger a recruitment of neutrophils as well as oxidative burst [267, 286].

Prolactin is a key protein in physiological processes in pigs such as lactogenesis and lactation [268, 286]. Expressed in various parts of the human body, including the lungs, prostate, muscle, lacrimal gland, trachea and mammary glands, there is a small (17kDa) single polypeptide chain protein called prolactin-inducible protein (PIP) [269, 286]. Prolactin and oestrogens increase its expression while androgens decrease it. Furthermore, this protein can prevent the growth of certain bacterial species, also contributing to an immune response [270, 286]. Associated with inflammation in pigs, the decrease in PIP, observed in our study, may be linked to the decrease in prolactin [271, 286] and humans with sepsis [272, 286].

Regarding the 2-DE technique, the stains for carbonic anhydrase, carbonic dehydratase VI, alpha-amylase and total albumin showed a decrease in the pools of sick animals. Lipocalin, adenosine deaminase (ADA), IgA and albumin peptides spots increased in these same sick animals. When DNA is broken, a molecule called deoxyadenosine is released. The function of adenosine deaminase is precisely to eliminate this molecule. Deoxyadenosine is transformed into deoxyinosine (a harmless molecule) by this enzyme. Deoxyadenosine is dangerous for lymphocytes [273, 286]. Adenosine deaminase was used for validation of the proteome data. This was due to the availability of an automated assay that was approved for use in this animal species (pigs) [274,286]. The development of inflammation and sepsis is due to ADA, which is present in the saliva of pigs [4,275, 286].

Corroborating the increase in ADA found in this research is related to the higher amounts of this protein in saliva in this pathology (presumably reflecting the activation of inflammation and the immune system) [286]. This increase was demonstrated in a higher percentage of infected pig population compared to healthy pigs [286]. Since the pig IgA system has been shown to be the same as that observed in other species, it has been hypothesised that the primary biological function of this immunoglobulin is linked to local defence of mucosal surfaces [276, 286]. In the present thesis, IgA is produced by the immune system to prevent invasion by harmful bacteria and is increased in mucosal secretions of the gastrointestinal system and in saliva. This may be compatible with some earlier studies showing that after an *E. coli* infection, there is an elevation of IgA in mucosal secretions [277, 286].

The reaction, which is catalysed by carbonic anhydrases (CAs), is determined by the bidirectional conversion of water (H2O) and carbon dioxide into bicarbonate (HCO3-) and protons (H+) [278, 286]. The lower water absorption may result from inhibition of the activity of carbonic anhydrases in the colonic mucosa, since these control the transfer of electrolytes in the organ [263,264, 286]. Changes in colonic acid-base balance are mediated by carbonic anhydrase activity. Human studies have found that human patients, compared to healthy patients, had significantly lower total CA activity, CA isoenzyme I mRNA and CA protein in the inflamed mucosa [279, 286]. Consequently, it was hypothesised that the decrease in AC (shown in the results) results from lesions of the intestinal mucosa. For the same reason, demonstrated above, it is possible to mention that in this investigation, the isoenzyme of AC, known as carbonic dehydratase VI was decreased [286].

In sick animals, it was also possible to observe a reduction in areas containing alpha-amylase. The activity of this protein in the saliva of pigs often increases when the animals are stressed or sick [280, 286]. The effect of amylase action on starch digestion in the pig stomach includes degradation by bacterial enzymes resistant to this organ (stomach) and hydrolysis by the animal's saliva [281, 286]. It is possible that the decrease in amylase quantity observed in our study is different from the increases in activity described in other diseases, as there may be differences between the quantity of an enzyme and its activity (notably in the case of alpha-amylase) [282, 286]. The 2-DE spots show the relative amounts of the protein types. These may not be the most important for enzyme activity [286].

Essential for the movement of ions, electrically neutral molecules, and for maintaining colloidal osmotic pressure in the blood, albumin is one of the main proteins

present in humans and animals [286]. The ability of albumin to bind to many drugs, nutraceuticals and hazardous chemicals influences both their pharmacokinetics and toxicokinetics [283, 286]. There was an increase in peptides with MW values lower than those of the primary form of albumin, although the amount of albumin decreased. This means that it is possible that albumin may have undergone some proteolysis in the saliva of the diseased pigs. Increased albumin fragments in the blood (because of albumin proteolysis) are associated with several diseases, including kidney failure [284, 286].

The use of 2-DE pools presents some limitations in this present study. However, the results of 1-DE (which were obtained from individual samples) and 2-DE gels were in concordance with the identification of certain proteins such as lipocalin and IgA [286]. Then, using an automated assay (on a larger number of individual samples) it was possibleto check for increases in ADA on 2-DE gels [286]. Further research involving the validationof proteins and a larger number of animals will help to confirm the results of the present study [286]. Finally, doing future research would be interesting as the evaluation of potential protein species and proteoforms help in better understanding the complexity of the salivary proteome of healthy pigs and of pigs diagnosed with *Escherichia coli* diarrhoea [286].

PART VI

CONCLUSIONS

It can be concluded that gel proteomics techniques contributed and allowed for the identification of alterations at the level of the salivary proteome of pigs diagnosed with *Escherichia coli* diarrhoea. Since they are linked to several pathophysiological mechanisms (which are triggered in conditions such as inflammation and immune function) these proteins, present in the study, may serve as biomarkers that help in the detection and monitoring of this pathology in these animals [286].

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ANNEXES

- **1-DE GELS IMAGES (INDIVIDUAL SAMPLES)**

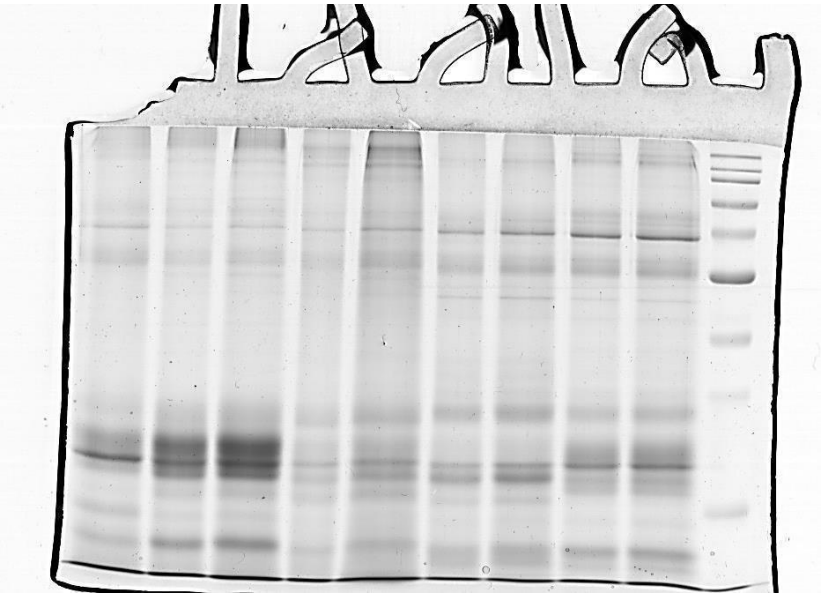

Figure 4. Gel 3 27/09/2022 (control group)

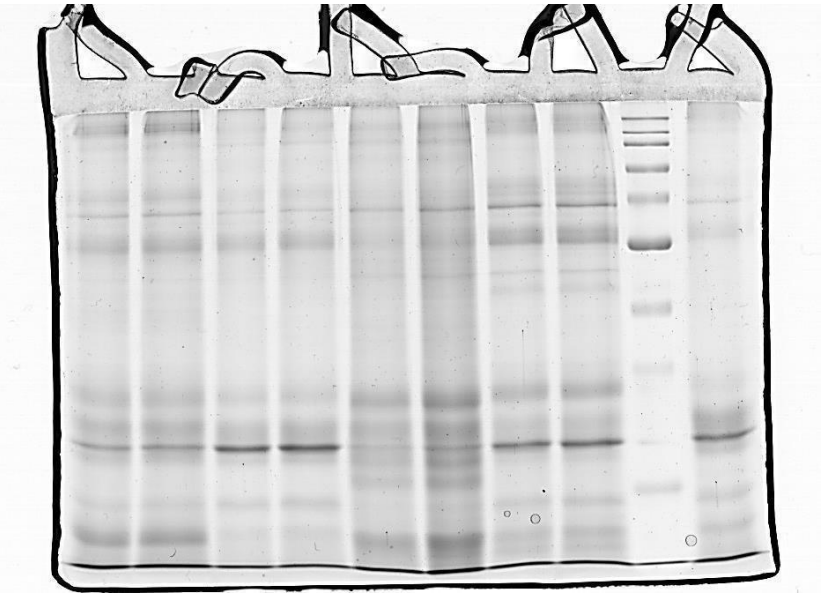

Figure 5. Gel 4 27/09/2022 (control group)

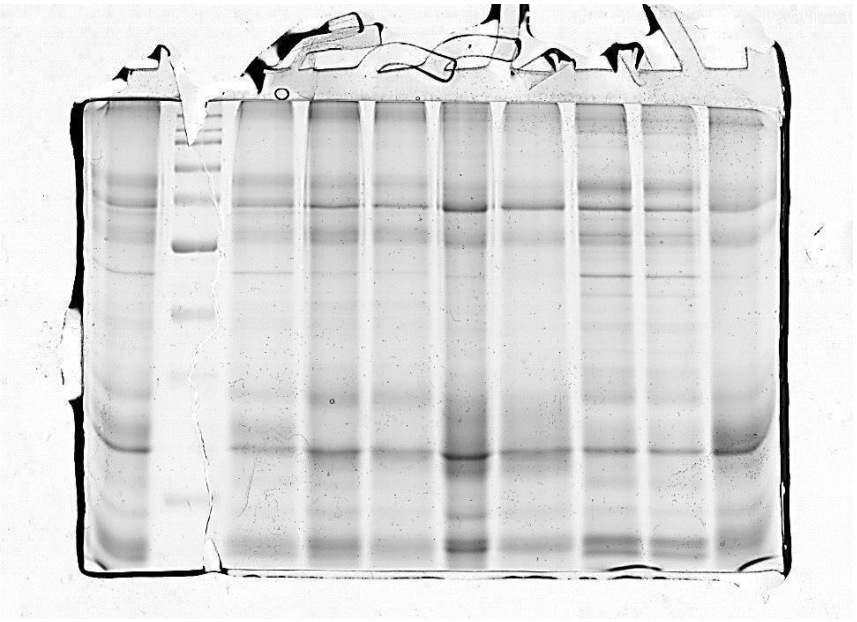

Figure 6. Gel 5 29/09/2022 (*E. coli* group)

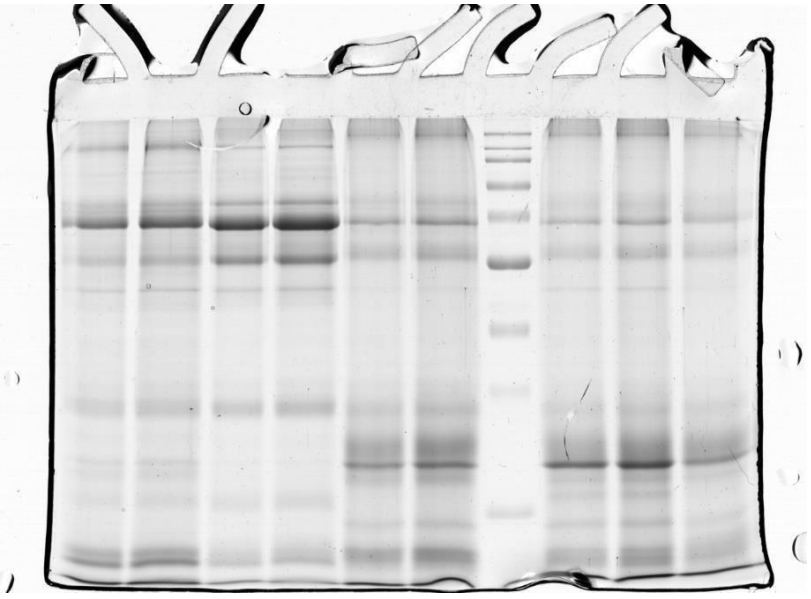

Figure 7. Gel 6 29/09/2022 (*E. coli* group)

- **2-DE GELS IMAGES (POOLS)**

YOUNG HEALTHY PIGS

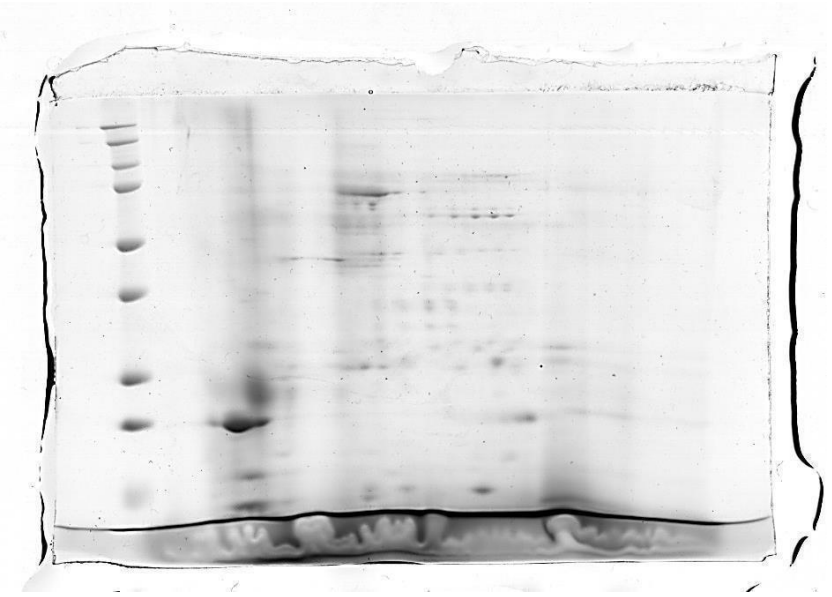

Figure 8. Pool 4 replica 1 28/10/2022 (control group)

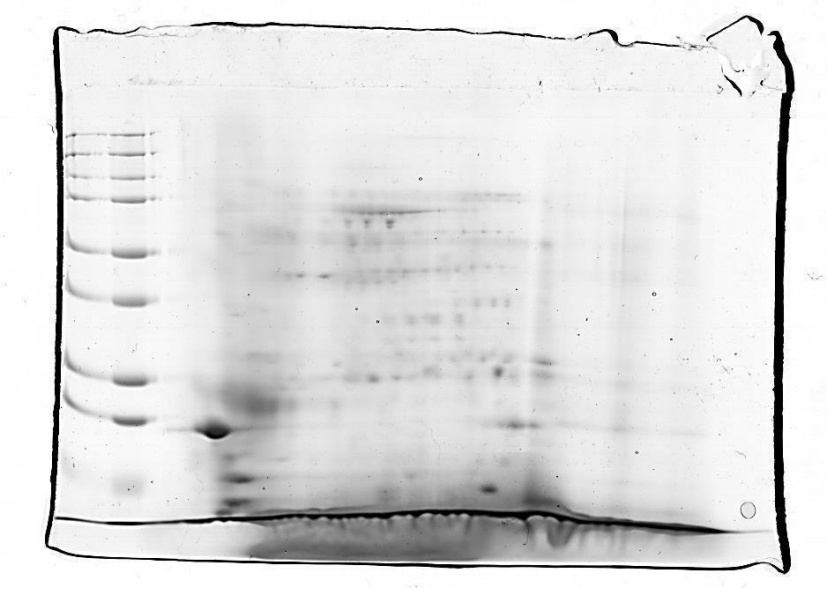

Figure 9. Pool 4 replica 2 27/10/2022 (control group)

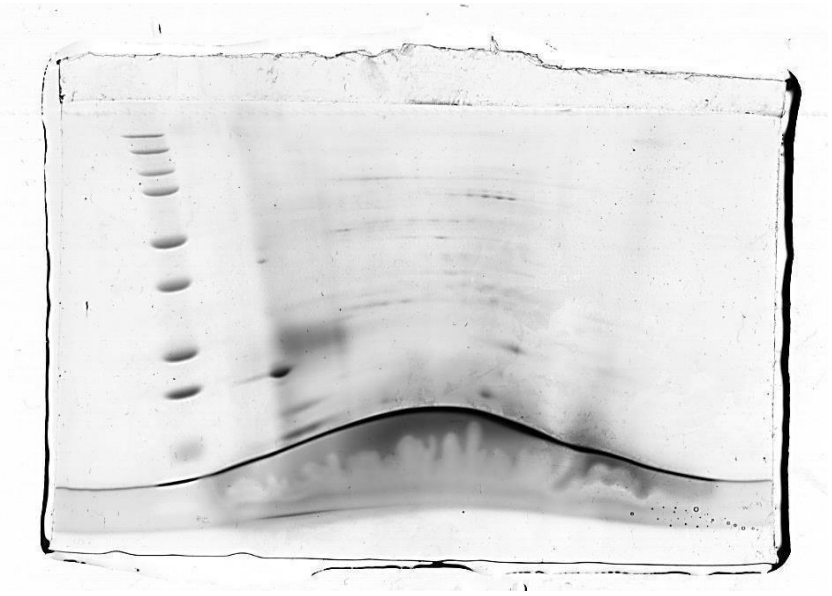

Figure 10. Pool 5 replica 1 28/10/2022 (control group)

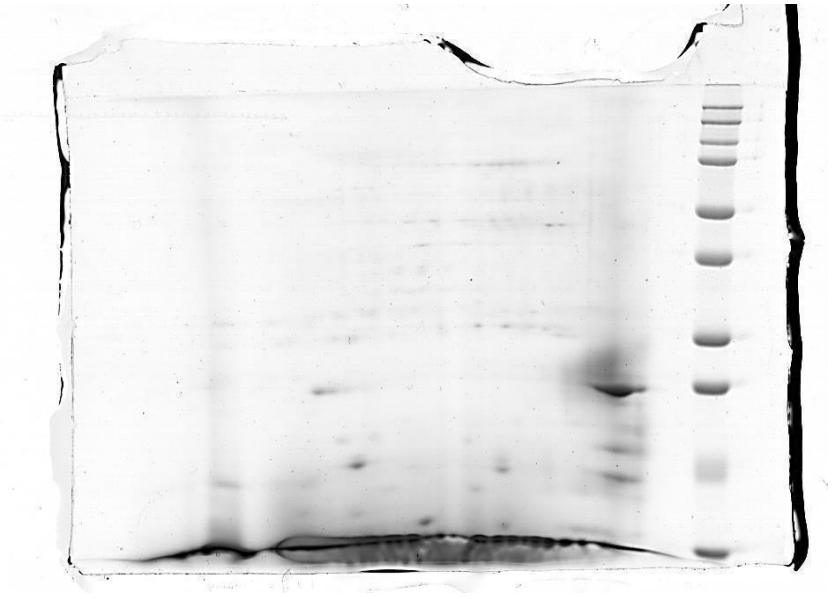

Figure 11. Pool 5 replica 2 27/10/2022 (control group)

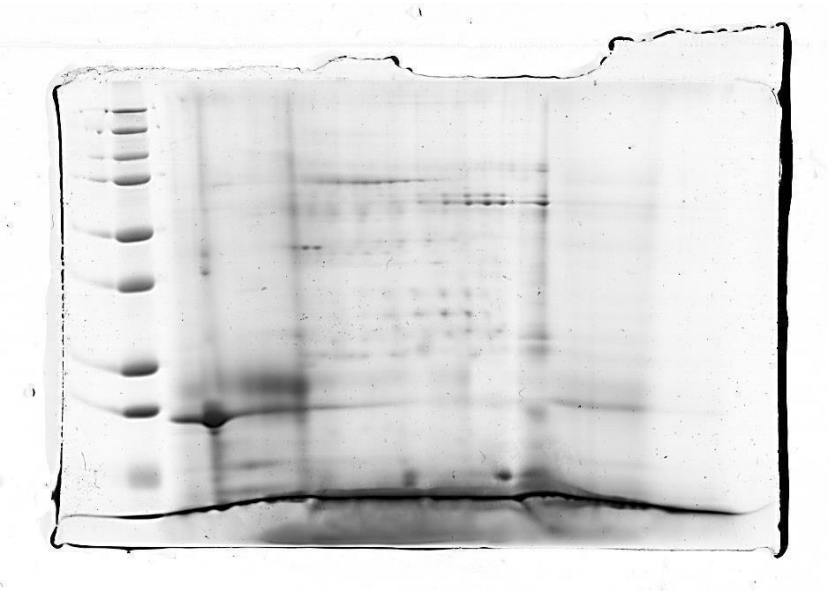

Figure 12. Pool 6 not duplicated 27/10/2022 (control group)

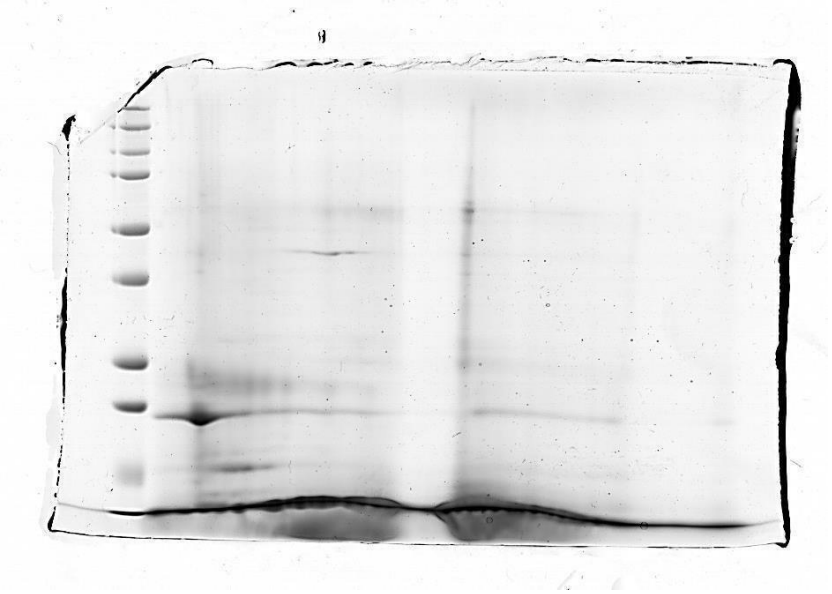

Figure 13. Pool 14 not duplicated 27/10/2022 (control group)

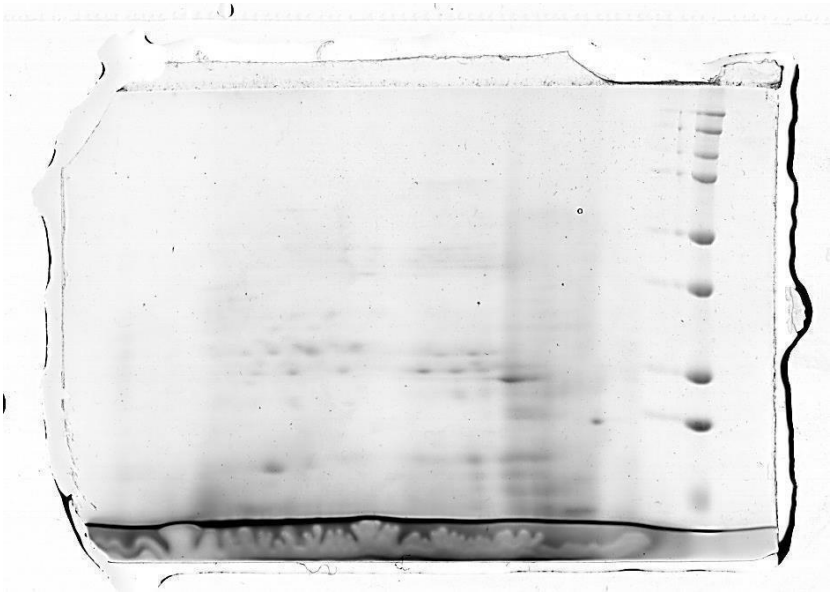

Figure 14. Pool 7 replica 1 28/10/2022 (*E. coli* group)

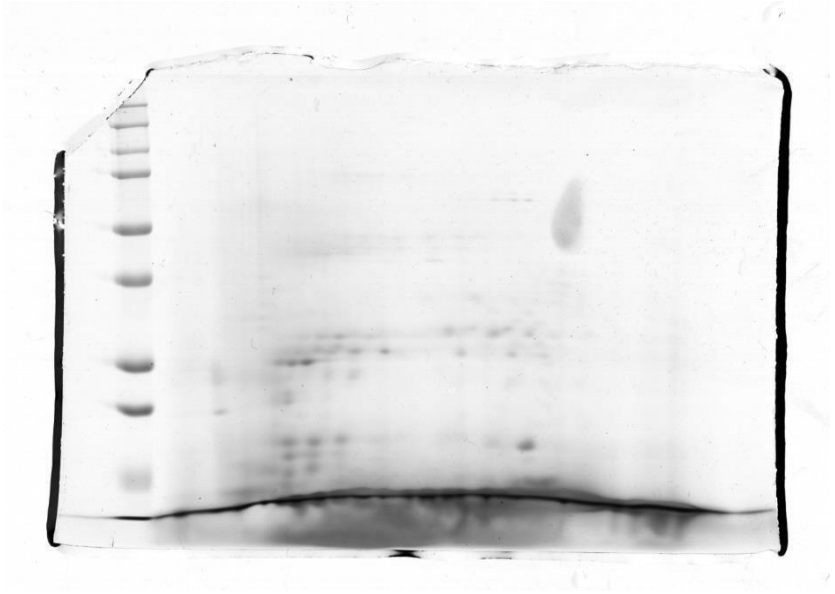

Figure 15. Pool 7 replica 2 27/10/2022 (*E. coli* group)

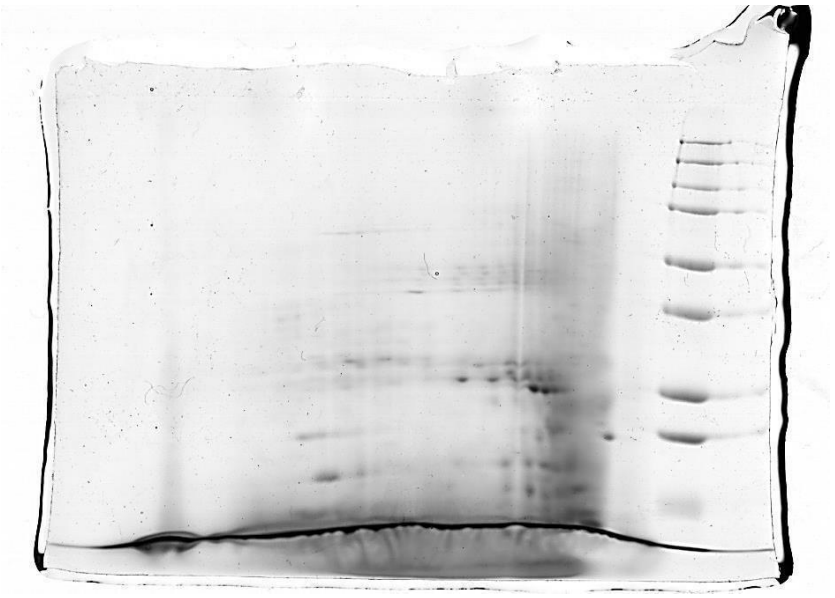

Figure 16. Pool 8 replica 1 27/10/2022 (*E. coli* group)

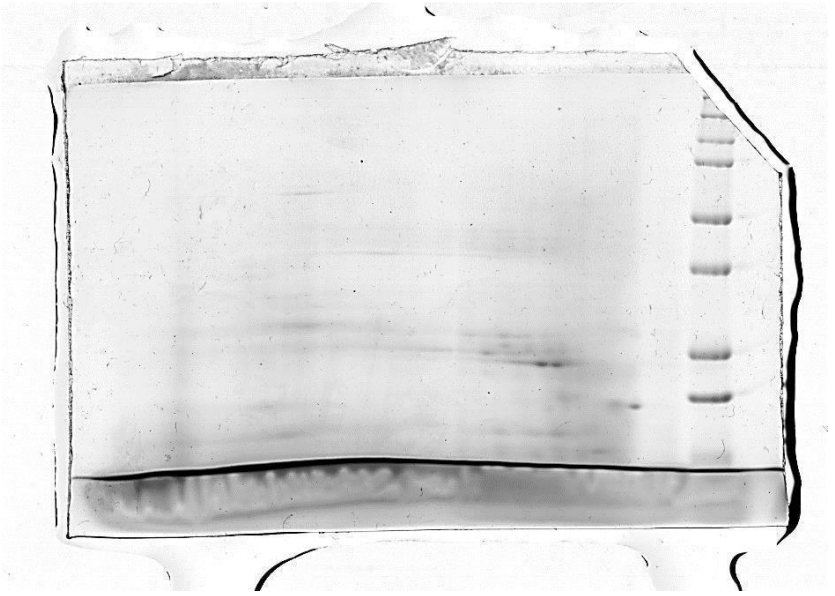

Figure 17. Pool 8 replica 2 28/10/2022 (*E. coli* group)

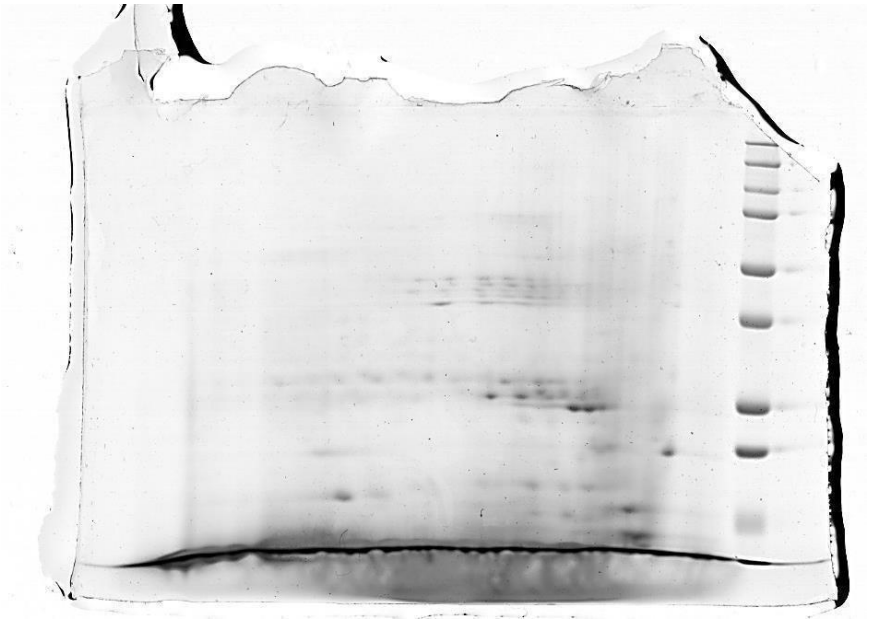

Figure 18. Pool 9 replica 1 27/10/2022 (*E. coli* group)

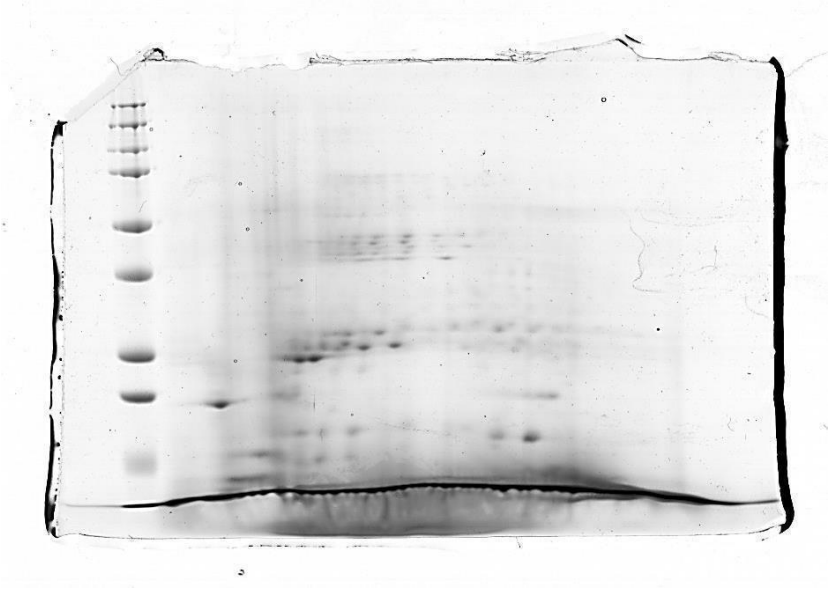

Figure 19. Pool 9 replica 2 27/10/2022 (*E. coli* group)

[Annex I]

proteomes

MDPI

Article

Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by *Escherichia coli*

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Abstract: *Escherichia coli* represents the main cause of diarrhoea in pigs. Saliva can provide information about the pathophysiology of diseases and be a source of biomarkers. We aimed to identify changes in the salivary proteome of pigs with diarrhoea caused by *E. coli*. Saliva samples were collected from 10 pigs with this disease and 10 matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed, and significantly different protein bands and spots were identified by mass spectrometry. For validation, adenosine deaminase (ADA) was measured in 28 healthy and 28 diseased pigs. In 1DE, increases in lipocalin and IgA bands were observed for diseased pigs, whereas bands containing proteins such as odorant-binding protein and/or prolactin-inducible protein presented decreased concentrations. Two-dimensional gel electrophoresis (2DE) results showed that saliva from *E. coli* animals presented higher expression levels of lipocalin, ADA, IgA and albumin peptides, being ADA activity increased in the diseased pigs in the validation study. Spots containing alpha-amylase, carbonic anhydrase VI, and whole albumin were decreased in diseased animals. Overall, pigs with diarrhoea caused by *E. coli* have changes in proteins in their saliva related to various pathophysiological mechanisms such as inflammation and immune function and could potentially be biomarkers of this disease.

Keywords: *E. coli*; salivary proteome; pigs; diarrhoea; lipocalin; ADA; biomarkers

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1. Introduction

Nowadays, saliva is considered an innovative and important source of biomarkers for many diseases in animals and humans. Overall, its composition can change due to stress, inflammation and alterations in the immune system or redox status, which can lead to the use of saliva analytes as biomarkers of pathological conditions [1]. This type of biological sample collection has many advantages, as it is painless and can be obtained by easy and non-invasive methods. In fact, saliva can be sampled without the need for specialized personnel in the field, anytime and anywhere [2]. Saliva is especially valuable in pigs, as in this species the collection of blood is stressful and painful for the animals [2].

It has been observed that saliva can show proteomic changes in sepsis experimentally induced by lipopolysaccharide (LPS) administration in pigs [3]. Aldolase A and serpin 12 were proteins in saliva that were significantly upregulated in sepsis. In addition, the pro- teome of saliva in pigs with *Streptococcus suis* infection has been studied, with the proteins metavinculin (VCL) and desmocollin-2 (DSC2) showing the highest relative abundance [4].

Moreover, proteomic changes have been reported in the saliva of pigs in situations of compromised welfare, with the proteins cornulin, heat shock protein 27, and lactate dehydrogenase (LDH) showing significant increases, and the immunoglobulin J chain showed a significant decrease [5].

Enterotoxigenic Escherichia coli (ETEC) is considered one of the main causes of diarrhoea in piglets [6], having a major economic impact on swine production [7]. ETEC produces several virulence factors, such as colonization factors (adhesins) and/or toxins. Colonization factors promote adherence to the host small intestine, and enterotoxins stimulate the lining of the intestine and induce watery diarrhoea [6], leading to sepsis [8]. Proteomic studies have been made to evaluate the changes in the intestine of pigs with *E. coli* diarrhoea [6,7] but, to our knowledge, no studies have been made in saliva.

The main objective of this study was to evaluate the possible changes in the salivary proteome of pigs with diarrhoea caused by *E. coli*, compared to healthy controls. To this end, SDS-PAGE and 2DE gel electrophoresis were used for the separation of proteins. After profile comparison, the mass spectrometry technique was used for the identification of the proteins differentially expressed between diseased and healthy animals. In addition, one protein showing significant changes in the proteomic study was selected for validation.

2. Materials and Methods

2.1. Population of Animals

For the proteomic studies, two groups of Large White weaning pigs from 6 to 9 weeks old were selected from commercial farms located in Southern Spain. One was a group of pigs diagnosed with diarrhoea caused by *E. coli* (n = 10, half males and half females), and theother were clinically healthy pigs (n = 10, half males and half females). The diseased animalshad clinical signs compatible with this disease (diarrheic syndrome) and were positive for the presence of *E. coli* in rectal swabs following standard analytical procedures [9], being positive for *E. coli* F4 and heat-labile toxin. Additionally, 28 healthy pigs and 28 pigs with diarrhoea caused by *E. coli* from 6 to 9 weeks old were used for the validation study.

2.2. Saliva Collection and Sample Processing

A sponge was used for saliva collection. The pigs were allowed to chew on the sponge until it was thoroughly moist. Then, the sponges were placed in Salivette tubes (Sarstedt, Aktiengesellschaft & Co., D-51588 Nümbrecht, Germany) and kept at 4- 8 °C until arrival at the laboratory, where the Salivette tubes were centrifuged at 3000 × g and 4 °C for 10 min to obtain saliva supernatant. Saliva was transferred into the Eppendorf tubes and stored at −80 °C.

2.3. SDS PAGE

This technique was made according to a previously published procedure [10]. Proteins from individual saliva samples from all young animals (both healthy and diseased) were separated by SDS-PAGE gel electrophoresis on 12% acrylamide gels using Bio-Rad equipment (mini-protean, Bio-Rad, Alges, Portugal). Samples were carried out in duplicate to minimize technical errors. The total protein concentration of the samples was determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Briefly, a total of 9 µg of protein from each saliva sample was lyophilised and reconstituted with 40 µL of sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% DTT and bromophenol blue). Then, the samples were placed on ice and heated for 5 min at 98 °C to denature proteins.The Bio- Rad electrophoresis tank system was set up with running buffer (0.025 M Tris HCl, 0.192 M Glycine, and 0.1% (w/v) SDS; pH 8.3. Twenty µL of the reconstituted sample were applied to each lane (in duplicate), and electrophoresis was run at a constant voltage of 150 V until the dye front reached the end of the gel. The gels were fixed in 40% methanol, and 10% acetic acid for one hour, stained with Coomassie Brilliant Blue R-250 (0.2% in 40% methanol, 10% acetic acid) for another hour, and destained with 10% acetic acid several times until staining background remotion. Finally, LabScan software was used to acquire

scanned images of the gels, and ImageLab software (Bio-Rad, Alges, Portugal) was used for gel analysis.

2.4. Two-Dimensional (2-DE) Gel Electrophoresis

For the 2DE technique, 3 pools of pig saliva samples were prepared from the group of healthy pigs and other 3 pools from the group of pigs with diarrhoea caused by *E. coli*. The volume of each individual corresponded to the same amount of total protein, in order to have a final total volume corresponding to 275 µg of total protein (determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Each pool was lyophilized and stored at − 28 °C. The solid material was reconstituted with 250 µL of solubilization buffer [7 M urea, 2 M thiourea, 4% (*w*/*v*) 3-(3-cholamidopropyl) dimethylammonium propane sulfonate (CHAPS), 2% (*v*/*v*) ampholyte mixture (IPG buffer pH 3-11, GE Healthcare, Chicago, IL, USA), and 40 mM dithiothreitol (DTT)]. The mixture was incubated for 1 h at room temperature and subsequently centrifuged for 10 min at 10,000 rpm at room temperature. After this, the supernatant from each sample was divided into two volumes of 125 µL and applied in two different slots of the strip holder of the Multiphor II system (GE Healthcare, Chicago, IL, USA) to run each sample in duplicate. The last step in strip rehydration was to place the commercial gel strips [7 cm pH gradient 3–11 NL (IPG strips, GE Healthcare, Chicago, IL, USA)]in contact with the sample and leave them in passive rehydration overnight at room temperature, covered with mineral oil. Focusing was performed in a Multiphor II (GE, Healthcare, Chicago, IL, USA) at 12 °C with the following program (gradient): (1) 0–150V for 15 min; (2) 150–300 V for 15 min; 300 V for 0.5 h; 300–3500 V for4 h; 3500 for 3.5 h. Focused strips were equilibrated and applied on top of a sodium dodecyl sulphate- polyacrylamide gelelectrophoresis (SDS-PAGE) on a 12% acrylamide gel and run at 150 V constant voltage on a mini-protein system (Bio-Rad, Alges, Portugal). Stainingwas made withCBB-R250. The image acquisition of the gels was made by a gel scanner (ImageScanner III, GE Healthcare, Chicago, IL, USA) and Lab scan software (GE Healthcare, Chicago, IL, USA), and the analysis was performed using the SameSpots software (v5.1.012, TotalLab, Gosforth,UK).

2.5. In-Gel Trypsin Digestion

After image analysis, the bands and spots that were observed to differ, in relative amounts, between healthy and *E. coli* individuals in SDS-PAGE and 2DE gels were selected for identification by MS. They were spliced into approximately 2 × 2 mm parts and distained. Then, they were alkylated and incubated with trypsin (Promega Corporation, Madison, MI, USA) and ProteaseMax surfactant (Promega Corporation, Madison, MI, USA) for 10 min at 4 °C. Finally, samples were digested at 37 °C for 16 h.

2.6. Protein Identification through HPLC-MS/MS Analysis

An HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) connected to an Agilent 6550 Q-TOF mass spectrome- ter (Agilent Technologies, Santa Clara, CA, USA) was used in this study. Parameters for the equipment analysis were set in MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00, Santa Clara, CA, USA).

Dry samples from trypsin digestion were resuspended in a buffer with water/acetonitrile/ formic acid and injected onto an Agilent AdvanceBio Peptide Mapping HPLC column, thermostated at 50 °C, at a flow rate of 0.4 mL/min.

The data processing and protein identification was made on Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA). The criteria used for MS/MS search against the appropriate and updated protein database were: variable modifications search mode (carbamidomethylated cysteines, STY phosphorylation, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid); tryptic digestion with 5 maximum missed cleavages; ESI-Q-TOF instrument (Agilent Technologies, Santa Clara, CA, USA); minimum matched peak intensity 50%; maximum ambiguous pre-

cursor charge +5; monoisotopic masses; peptide precursor mass tolerance 20 ppm; product ion mass tolerance 50 ppm; and calculation of reversed database scores.

2.7. Statistical Analysis

The data were evaluated for normal distribution using the Shapiro–Wilk test. Variables (protein concentration, protein bands and spots) for which normal distribution was not observed were transformed (log transformation). When normal distribution was achieved, Student's *t*-test was used for group comparison, whereas non-normally distributed vari- ables were compared using a non-parametric test (Mann–Whitney). Statistical analysis was performed with SPSS (v.28.0, IBM SPSS Statistics, New York, NY, USA). Statistically significant differences were considered when the p-value < 0.05.

2.8. Validation

Among the proteins identified with the relative abundance in saliva showing sig- nificant changes between healthy and diseased pigs, ADA was selected as a biomarker candidate for validation in an additional group of pigs with *E. coli* diarrhoea (n = 28), which was compared with a group of healthy pigs (n = 28). In both groups, half of the animals were male and half female.

The activity of ADA was measured using an automated assay that was previously validated in the saliva of pigs [11].

3. Results

3.1. Total Protein Concentration

The total protein concentration of saliva samples was observed to be significantly higher in *E. coli*-diseased animals compared to the healthy ones. Mean *E. coli* animals have almost 3 times higher values of total protein than healthy animals (76.4 ± 41.8 µg/mL vs. 280.5 ± 107.7 µg/mL, for healthy and *E. coli* groups, respectively; p = 0.001).

3.2. SDS-PAGE Profile

Salivary SDS-PAGE protein profiles allowed the constant visualization of clearly distinct 21 protein bands, with molecular masses between 10 and 200 kDa, whose levels were compared between groups (Figure 1).

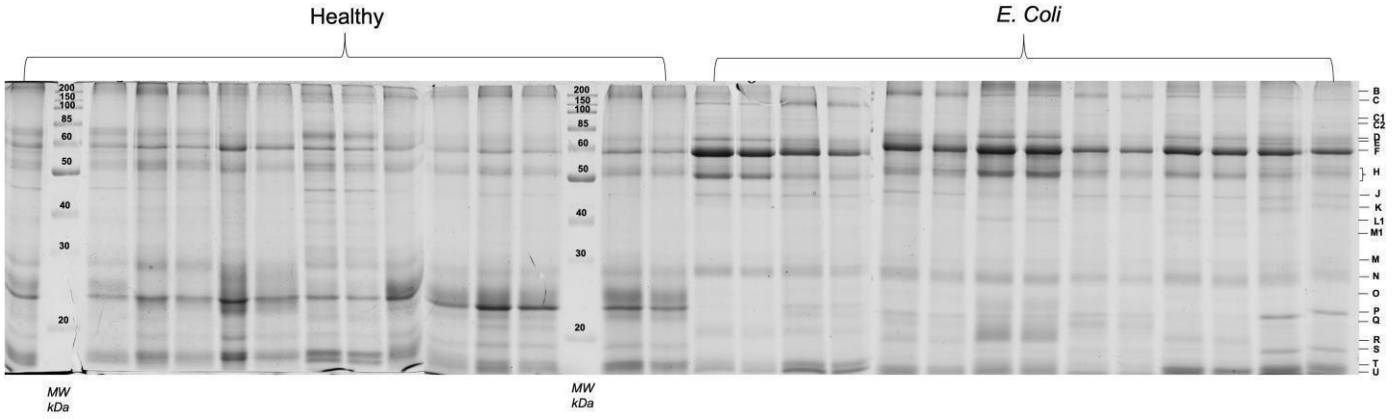

Figure 1. Salivary protein profiles (SDS-PAGE) of all the samples (healthy controls and *E. coli* diseased pigs). Each capital letter, on the right side, represents the bands compared between groups.

Eight protein bands were observed to be differently expressed between healthy and diseased animals. Band C1 was a faint band, not identified through mass spectrometry, which was only observed in the *E. coli* group. The other 7 bands, although observed in animals from both groups, presented statistically significant differences, with bands B, H, M, N, and R increasing in diseased animals and bands P and T decreasing in those. The

differences between groups, as well as mass spectrometry identifications of the proteins present in those bands, are presented in Table 1.

Table 1. Differences in protein band expression levels (mean ± standard deviation of %Vol) between *E. coli* diseased and healthy pigs and correspondent protein identification and MS.

Band	Healthy	*E. coli*	*p*-Value	UNIPROT Protein Accession Number	Protein (Entry Name)	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
B	1.62 ± 0.80	5.36 ± 3.06	0.001	018758	Submaxillary apomucin	1.3	238.4	1184.1	>200 kDa
C1	-				ni				120
H	3.74 ± 0.59	9.77 ± 2.91	0.0005	A0A287B626	IgA constant region	39.3	209.6	44.2	54
M #	1.35 ± 1.09	2.94 ± 0.75	0.015	A0A0A0MY58 and F1SN92	Immunoglobulin heavy constant mu and Salivary lipocalin	28.5 and 25.1	75.1 and 43.5	32.7 and 21.6	28.5
N	6.88 ± 2.44	10.20 ± 1.43	0.009	F1SN92	Salivary lipocalin	54.9	152.5	21.6	26
P	17.51 ± 4.27	3.40 ± 2.10	0.0005	P81245	Odorant-binding protein	75.1	199.5	17.7	18
R	1.22 ± 1.63	4.00 ± 2.47	0.033	A0A4X1TU02	Salivary lipocalin	57.5	143.4	21.6	16.5
T #	14.15 ± 4.91	8.33 ± 4.70	0.043	A0A286ZRW6 and A0A287ASS4	Double-headed protease inhibitor, submandibular gland-like and Prolactin inducible protein	29.4 and 36	58.31 56.35 and	13.3 and 12.4	13

ni—protein failing identification by MS; # in the tryptic mixture, peptides corresponding to more than one protein were observed in the spectra, indicating that more than one protein was present in the band.

From the 1DE analysis, it was evident an increase in salivary lipocalin and IgA bands in *E. coli* diseased pigs, whereas bands containing proteins such as odorant-binding protein, a protease inhibitor from the submandibular origin and/or prolactin inducible protein were present in decreased levels in these animals.

3.3. Two-Dimensional Protein Profile (2-DE)

After gel analysis, it was possible to consider 127 protein spots constantly present in the different pool samples, which were compared between healthy and *E. coli* sample pools. Testing the possibility of separation of the two groups using principal component analysis, it is possible to see that the two components explain 46.98% of data variability (Supplementary Figure S1).

Through the between-subjects test (independent *t*-test), a total of 35 protein spots were observed to present a statistically significant difference ($p < 0.05$) (Figure 2). Among these, 15 protein spots were increased in *E. coli* animals, whereas 20 were decreased. The level of variation, as well as the salivary proteins identified, are presented in Table 2.

Table 2. Protein spots differently expressed between healthy and *E. coli*-diseased pigs.

Spot Number	Fold Change	Group with Higher Level	*p*-Value	Protein (Entry Name)	UNIPROT Protein Accession Number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
237	4.24	*E. coli*	5.24×10^{-5}	Adenosine deaminase and salivary lipocalin	A0A0B8RW47 and A0A4X1TU02	22.5 and 15.7	39.5 and 23.8	40.9 and 21.6	17.5
33	1.72	Healthy control	0.000222	n.i.					
185	2.30	*E. coli*	0.00063	IgA constant region	A0A287B626	3.8	23.6	44.2	27.5
188	2.41	*E. coli*	0.000733	IgA constant region	A0A287B626	2.6	20.0	44.2	27.5
41	3.29	Healthy control	0.000763	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	74.5
145	1.56	Healthy control	0.000794	Carbonate dehydratase VI	A0A4X1W7S7	15.1	39.5	34.7	36.0
40	2.72	Healthy control	0.000871	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	74.5
202	2.28	*E. coli*	0.000887	Ig-like domain-containing protein	A0A287A4Y3	15.4	41.4	24.7	26.0
44	3.03	Healthy control	0.001118	Lactoperoxidase	A0A480RK36	6.6	48.7	80.3	74.5

Table 2. *Cont.*

Spot Number	Fold Change	Group with Higher Level	*p*-Value	Protein (Entry Name)	UNIPROT otein Accession Number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
196	2.97	*E. coli*	0.001675	Albumin (fragment) and salivary lipocalin	A0A286ZT13 and A0A4X1TU02	13.6 and 23.5	100.5 and 31.5	68.2 and 21.6	26.0
200	2.34	*E. coli*	0.002233	Albumin (fragment) and salivary lipocalin	A0A286ZT13 and A0A4X1TU02	13.6 and 23.5	100.5 and 31.5	68.2 and 21.6	26.0
45	2.10	Healthy control	0.002679	Lactoperoxidase	A0A480RK36	6.7	48.8	80.3	74.5
43	3.30	Healthy control	0.003706	Lactoperoxidase and polymeric immunoglobulin receptor	A0A480RK36 and A0A0E3M2Q4	7.5 and 6.5	45.5 and 37.3	80.3 and 67.3	74.5
194	1.71	*E. coli*	0.004477	Albumin (fragment)	A0A286ZT13	7.8	64.3	68.2	26.5
31	2.29	Healthy control	0.005324			n.i.			
47	1.66	Healthy control	0.005441	Lactoperoxidase and polymeric immunoglobulin receptor and	A0A0E3M2Q4 and A0A480RK36	12.3 and 4.9	86.1 and 34.7	67.3 and 80.3	74.0
184	1.86	*E. coli*	0.007066			n.i.			
203	2.94	*E. coli*	0.007897	Ig-like domain-containing protein	A0A287A4Y3	18.5	34.0	24.7	26.0
38	2.12	Healthy control	0.009251	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	101.0
32	1.50	Healthy control	0.012577			n.i.			
37	2.03	Healthy control	0.01381			n.i.			
155	1.51	Healthy control	0.015799	Carbonic anhydrase	A0A4X1W9S1	11.0	27.7	36.3	36.0
179	1.96	Healthy control	0.020918	Carbonate dehydratase VI	A0A4X1W7S7	11.5	47.2	34.7	27.5
73	1.78	Healthy control	0.021757	Alpha-amylase	F1S573	30.1	146.0	55.8	58.0
74	1.39	Healthy control	0.026339	Alpha-amylase	F1S573	30.9	123.4	55.8	58.0
235	2.49	*E. coli*	0.030702	Adenosine deaminase and salivary lipocalin	A0A0B8RW47 and A0A4X1TU02	22.5 and 15.7	39.5 and 23.8	40.86 and 21.61	18.0
130	2.26	*E. coli*	0.033046			n.i.			
146	1.38	Healthy control	0.037883	Carbonate dehydratase VI	A0A4X1W7S7	9.8	25.6	34.7	36.0
77	1.67	Healthy control	0.039092			n.i.			
190	1.75	*E. coli*	0.040238	Albumin (fragment)	A0A286ZT13	9.2	61.0	68.2	26.0
239	2.94	*E. coli*	0.042094	Salivary lipocalin	F1SN92	4.4	24.51	21.6	17.5
170	1.36	*E. coli*	0.046073			n.i.			
72	2.01	Healthy control	0.046326			n.i.			

Note: n.i. means spots thatwere not identified.

Taking together the 2DE results, it is possible to observe that *E. coli* pools presented higher expression levels of spots identified as lipocalin, adenosine deaminase, IgA, and albumin peptides. On the other hand, spots containing alpha-amylase, carbonic anhy- drase, carbonate dehydratase VI, and whole albumin were decreased in pools from the diseased animals.

3.4. Validation

The measurements of salivary ADA activity showed significantly higher activity levels in pigs with diarrhoea caused by *E. coli* (median 2712 U/L, minimum–maximum range 1293–19936 U/L) compared with healthy pigs (median 881.6 U/L, minimum–maximum range 60.8–2435 U/L) ($p < 0.001$) (Figure 3).

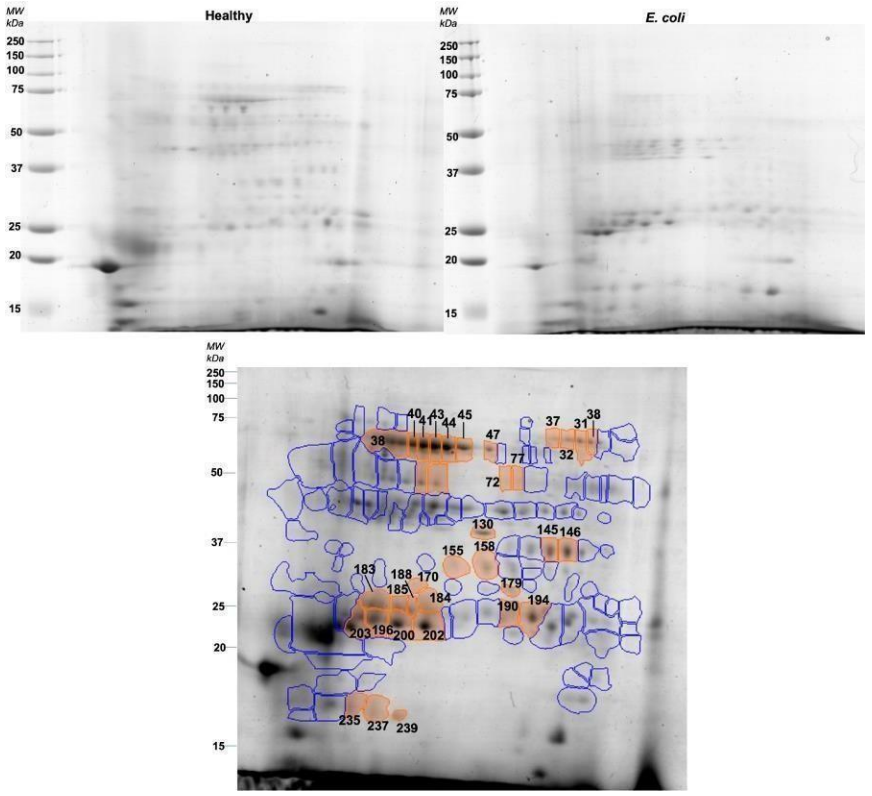

Figure 2. Representative gels of healthy (upper left) and *E. coli* (upper right) pools. The lower image represents the reference gel with protein spots differently expressed between groups (orange) and spots that did not show differences between groups (blue).

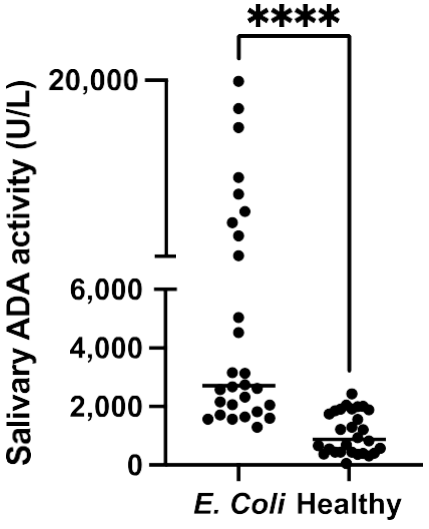

Figure 3. Comparison of the salivary adenosine deaminase activity (ADA) in pigs with diarrhoea caused by *E. coli* and healthy pigs. The plot shows the individual values of each group. **** $p < 0.001$.

4. Discussion

In this report, changes in various proteins in the saliva of pigs with diarrhoea caused by *E. coli* were detected. To the authors' knowledge, this is the first report in which a proteomic analysis of saliva is performed in pigs with diarrhoea due to *E. coli* infection and where changes in salivary proteins in this disease are described. The proteomic approach of this study used 1DE and 2DE gels. 1DE allows the separation of proteins only according to their molecular masses and the entry into the gel of a broad range of proteins, whereas 2DE may

not be able to separate proteins with extreme isoelectric points or higher hydrophobicity. The lower requirement for total protein allowed testing samples at the individual level with this technique. On the other hand, 2DE allows for a more detailed protein profile, obtained after proteins are separated both by their charge and mass. Both 1DE individualsamples and 2DE sample pools were run in duplicate to minimize the effect of technical errors inherent to the techniques.

From the 1DE analysis, it was evident that there was an increase in salivary lipocalin and IgA bands in *E. coli*-diseased pigs, whereas bands containing proteins such as odorant- binding protein and/or prolactin-inducible protein were present in decreased concentra- tions in these animals.

Lipocalin (LCN) family proteins are small proteins (18–40 kDa) expressed in numerous tissues and involved in multiple processes (i.e., inflammation, detoxification, and immune activation) by transporting hydrophobic molecules (e.g., steroids, retinoids, or lipids) to cells [12]. Some members of this family of proteins such as lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin) are considered acute phase proteins showing increases in inflammation [13]. Lipocalin-2 is increased in the serum of humans with inflammatory bowel disease and is correlated with the activity of this disease [14,15]. In addition, it has been described to capture bacterial siderophores produced by pathogenic bacteria, such as *E. coli* and, indeed, Lcn2-deficient mice are prone to infection and sep- sis [16]. Although in our study LCN increased, in a previous report it was observed a decrease of LCN in the saliva of pigs with *Streptococcus suis* infection [4]. Further studies should be undertaken to elucidate the mechanisms involved in the change in LCN since in some cases, such as in the *Streptococcus suis* infection, the decrease of lipocalin could indicate a high susceptibility to worsening sepsis [4].

Odorant binding protein (OBP) is involved in olfaction and defence against oxidative injury. In addition, this protein has been related to inflammation, showing a decrease in lungs in bovine after LPS administration. This decrease in OBP levels may be an additional mechanism to allow inflammatory mediators to stimulate neutrophil recruitment and oxidative burst in the lung and possibly in other tissues [17].

Prolactin-inducible protein (PIP) is a small (17 kDa) single polypeptide chain protein expressed in various human body parts, including the salivary gland, lacrimal gland, trachea, prostate, muscle, mammary glands, and lungs [18]. Its expression is upregulated by prolactin and androgens, and oestrogens downregulate it. It is involved in the immune response and can inhibit the growth of bacterial species [19]. The decrease in PIP found in our study could be related to a decrease in prolactin, which has been described in pigs with inflammation [20]and humans with sepsis [21].

In 2DE, lipocalin, adenosine deaminase (ADA), IgA, and albumin peptides were increased in the saliva of pigs with *E. coli*, whereas spots containing carbonic anhydrase, carbonic dehydratase VI, alpha-amylase, and whole albumin were decreased in pools from the diseased animals. ADA was selected to validate the proteomic results due to the existence of an automated assay validated for pigs [22]. ADA increases inflammation and sepsis in the saliva of pigs [4,11]. The increase in ADA found in our proteomic study was also confirmed in the larger population of pigs with diarrhoea with *E. coli* compared to healthy pigs, corroborating the higher levels of this protein in saliva in this disease, possibly reflecting activation of inflammation and the immune system. In addition, IgA, which is produced by the immune system to prevent the invasion of pathogenic microbes and is found in large amounts in the mucosal secretions of the gastrointestinal tract and saliva, was increased in our study. This could agree with other reports that have described an increase in IgA in mucosal secretions after an *E. coli* infection [23].

Carbonic anhydrase (CA; EC 4.2.1.1) represents a group of enzymes that catalyse the reversible hydration/dehydration of CO_2 and water. It is involved in the regulation of colonic electrolyte transport and inhibition of CA activity in the colonic mucosa can lead to a decrease in water absorption [14,15]. In addition, CA has been suggested to mediate the colonic absorptive response to changes in systemic acid-base balance. In this line, human

patients with mild or moderate ulcerative colitis showed a significant reduction of the CA isoenzyme I mRNA and protein and total CA activity in the inflamed mucosa compared to controls [24]. Therefore, it could be postulated that the decreases in CA found in our report would be related to damage in the intestinal mucosa. Carbonic dehydratase VI, which is considered an isoenzyme of CA, was also decreased in our study, possibly due to the reasons described above.

A decrease in spots containing alpha-amylase was also observed in the diseased animals. Usually, the activity of alpha-amylase in the saliva is increased in situations of stress and disease in pigs [25]. The divergence of the decrease found in the amount of amylase in our study compared with the increases in the activity reported in other diseases could be due to the divergences between the amount of one enzyme and its activity, which can occur especially in the case of alpha-amylase [26]. In fact, the 2DE spots represent the relative amount of the forms of the protein, which may not be the ones most contributing to the enzymatic activity. Regarding the albumin, there was a decrease in whole albuminbut an increase in peptides with MW lower than the MW of the primary form of albumin.This could indicate that albumin could have some proteolysis in the saliva of diseased pigs. Increases in albumin fragments in the blood due to albumin proteolysis have been described in some diseases such as renal failure [27].

Overall, in our report, we found changes in proteins in saliva related to inflammation and the immune system, as have been described in saliva in pigs with sepsis experimentally induced by LPS administration and other infectious diseases such as *S. suis* infection [3,4].

This report has a limitation in the use of pools for 2D, which does not accurately represent the contribution of the different individual samples. However, there was an agreement in proteins such as lipocalin and IgA between the results of 1D (that was made in individual samples) and 2D gels; also, the increases in ADA in 2D gels were later confirmed by an automated assay in a larger number of individual samples. Further studies involving the validation of a larger number of proteins and a larger number of animals should be made to corroborate the results of our report. In this line, although the study of diseased animals on farms provides a real picture of the disease under field conditions, ideally additional studies in which *E. coli* infection is induced in experimental pigs should be performed to confirm the findings of this report. In addition, it would be of interest to perform additional studies to evaluate possible different proteoforms and protein species to better elucidate the proteome complexity in the saliva of healthy pigs and pigs with diarrhoea caused by *E. coli*.

5. Conclusions

It can be concluded that pigs with diarrhoea caused by *E. coli* infection have changes in proteins in their saliva that can be detected by gel proteomics. These proteins are related to various pathophysiological mechanisms activated in diseases such as inflammation and immune function, and could potentially be biomarkers that could help detect and monitor this disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/proteomes11020014/s1, Figure S1: Distribution of sample pools among the two first components obtained by principal component analysis.

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