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Effects of ileo-rectal anastomosis on cholesterol metabolism in pigs fed either casein or extruded soya beans

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The importance of legume proteins in cholesterol metabolism has been recognised, but the hindgut contribution is still unclear. The present work was undertaken to address the role of the caecum–colon in cholesterol metabolism in intact (I) and ileo-rectal anastomosed (IRA) pigs fed with casein or extruded soyabean (ES) diets. Four groups of six growing pigs were assigned to the treatments (casein–I, casein–IRA, ES–I, ES–IRA) for 3 weeks. Plasma total cholesterol, LDL- and HDL-cholesterol were not modified by surgery or diet. In the liver, the ES diet significantly depressed non-esterified, esterified and total cholesterol. The treatments did not affect hepatic 3-hydroxy-3-methylglutaryl CoA reductase, cholesterol 7α-hydroxylase or sterol 27-hydroxylase activities. In the gallbladder bile of ES-fed pigs, total cholesterol was depressed while total bile acid concentration was increased. IRA and the ES diet markedly decreased the biliary bile acid microbial metabolites (namely hyodeoxycholic acid) and increased the primary bile acids (mainly hyocholic acid). The concentration of bile hydrophobic acids was decreased only by the ES diet. Faecal neutral sterol output was increased in ES-fed pigs, but the bile acid and the sum of neutral and acidic steroid outputs were not. Microbial transformation of neutral and acidic steroids was markedly reduced by IRA, especially in the ES-fed pigs. Thus, surgery and ES modulated the steroid profile but the caecum–colon did not seem to play a crucial role in determining cholesterolaemia in pigs.

Ileo-rectal anastomosis: Extruded soya beans: Cholesterol metabolism: Steroid output: Pigs

The role of the small intestine in cholesterol metabolism is well documented (Dietschy & Wilson, 1970; Chevallier & Lutton, 1973; Turley & Dietschy, 2003) whereas that of the hindgut is still unclear. The absorption of cholesterol and conjugated bile acids by the colonic epithelium is almost negligible (Roy *et al.* 1978; Aigueperse *et al.* 1981) but that of free bile acids has been demonstrated (Schiff *et al.* 1972). However, the proportion of these bile acids coming from microbial transformation to the enterohepatic circulation is not well known (Hoffman & Hofmann, 1977). The luminal content and the microflora could also play an important but poorly understood role through the fermentation products or through the production of bioactive compounds such as amines (Strandberg *et al.* 1966) and bile acids (Kellogg, 1971). The decrease in plasma cholesterol is often related to propionate resulting from fibre fermentation (Nishimura *et al.* 1993; Hara *et al.* 1999), a hypothesis not yet confirmed. In fact, hypocholesterolaemia was observed in germ-free rats fed with fibre diets (Sacquet *et al.* 1983; Alvarez-Leite *et al.* 1994) and was absent in conventional rats fed with a propionate-supplemented diet (Levrat *et al.* 1994). The microbial transformation of primary into secondary bile acids (Kellogg, 1971) could also modify cholesterol metabolism. Absorbed hydrophobic secondary bile acids decrease hepatic 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase and cholesterol 7α-hydroxylase (CYP7A1) activities, modulating cholesterol and bile acid syntheses (Pandak *et al.* 1994). These findings suggest that the role of the hindgut in cholesterol metabolism deserves to be further investigated.

Previous studies with human and animal models consuming soyabean diets with (Beynen *et al.* 1990; Potter, 1995) or without (Terpstra *et al.* 1991; Morita *et al.*

Abbreviations: CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; ES, extruded soya beans; HMG CoA, 3-hydroxy-3-methylglutaryl CoA; I, intact; IRA, ileo-rectal anastomosis.

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1997) added cholesterol suggest a hypocholesterolaemic effect of the legume. This effect could be due to the modulation of the hindgut function, i.e., the enhancement of neutral and acidic faecal steroid output (Fumagalli *et al.* 1978; Kim *et al.* 1980; Beynen *et al.* 1990) leading to a hepatic cholesterol metabolism shift to provide cholesterol for bile acid synthesis. Soyabean bioactive components such as fibre, saponins or phytosterols may be implicated (Potter, 1995; Lichtenstein, 1998; Carr *et al.* 2002). The colonic mechanism by which dietary soya beans influence cholesterol metabolism is still unclear.

The aim of the present work was to determine the role of the caecum–colon in cholesterol metabolism in ileo-rectal anastomosed soyabean-fed pigs. Like man, swine develop atherosclerotic lesions linked to dietary conditions and cholesterol metabolism (Vitic & Stevanovic, 1993). An extruded batch of soyabean seeds was used, to prevent the action of other bioactive components (Anderson & Wolf, 1995; Friedman & Brandon, 2001). Fasting plasma lipid levels were measured and lipoprotein profiles analysed. The main hepatic processes involved in cholesterol homeostasis were studied: HMG CoA reductase, CYP7A1, sterol 27-hydroxylase (CYP27A1) and hepatic LDL-receptor activities. The composition of lipids in the liver, bile and faeces and ileal digesta output were also determined.

Materials and methods

Chemicals and isotopes

Chemicals of the highest purity were bought from Prolabo (Rhône-Poulenc Ltd, Paris, France) and Sigma (St Louis, MO, USA). Enzymic kits were purchased from Roche Diagnostics (Mannheim, Germany) and Wako Chemicals (Neuss, Germany). Hydroxypropyl-β-cyclodextrin was a gift from Roquette Frères (Lestrem, France). Anion exchange AG1-X8 resin was purchased from Bio-Rad (Ivry-Seine, France) and L-3-[glutaryl-3-^{14}C]hydroxymethylglutaryl CoA, [5-^{3}H]mevalonolactone, [4-^{14}C]cholesterol, 25-[26,27-$^{3}H_2$]hydroxycholesterol and [^{14}C]taurocholate were purchased from DuPont-NEN (Les Ulis, France). 25-Hydroxycholesterol was a kind gift from Roussel-Uclaf (Romainville, France) and 7α- and 7β-hydroxycholesterol were synthesised (Yamasmita *et al.* 1989). Emulsifier-Safe was purchased from Packard Instruments Company (Meriden, CA, USA). A polyclonal antibody raised against the bovine adrenal cortex LDL receptor was kindly provided by Paul Roach (Adelaide, Australia). An anti-rabbit IgG, horseradish peroxidase-linked F(ab')2 fragment (from donkey) and the enhanced chemiluminescense reagent were bought from Amersham Pharmacia Biotech (Les Ulis, France).

Animals, housing, diets and feeding

Twenty-four 12-week-old crossbred male pigs (Duroc (male) × (Large White × Landrace) (female)) with an initial mean body weight of 30·1 (SEM 0·8) kg were obtained from Universidade de Évora (Évora, Portugal). Pigs were individually penned in metabolism cages (0·60 × 1·60 m). Animal care and experimental procedures were in accordance with the regulations and ethical guidelines of the Portuguese Animal Nutrition and Welfare Commission.

Two experimental diets enriched in cholesterol, a semi-purified casein diet and an extruded soyabean (ES) diet, were formulated in order to have similar amounts of crude protein and gross energy. About 60 % of the protein supplied by casein in the casein diet was replaced in the ES diet by protein from extruded whole-seed soya beans (Reagro Lda, Salvaterra de Magos, Portugal) (Table 1). Cholesterol, included in the diets at the rate of 3 g/kg, was solubilised in soyabean oil before addition. Pigs were fed equal amounts twice daily (08.30 and 18.00 hours), at a weekly-adjusted daily rate of 50 g/kg body weight. Animals had free access to water throughout the experimental period.

Experimental procedures

After a post-weaning period on a commercial diet (S801; Rações Veríssimo, Leiria, Portugal), the pigs (*n* 24) were fed the casein diet for 2 weeks to induce hypercholesterolaemia. At the beginning of the third week, twelve pigs were submitted to an end-to-side ileo-rectal anastomosis (IRA) (Green, 1988). This surgery was carried out approximately 40 mm anterior to the ileo-caecal valve and the hindgut was not removed. After surgery, the pigs were fasted for 10–12 h and then encouraged to eat increasing amounts of the experimental diets. The IRA pigs were daily supplemented with sodium chloride and sodium

Table 1. Ingredients and average composition of the experimental diets

	Casein diet	Extruded soyabean diet
Ingredients (g/kg)		
Maize starch	464·0	365·7
Sucrose	100·0	100·0
Wheat straw	100·0	100·0
Soyabean oil	100·0	70·0
Acid-hydrolysed casein	180·0	72·0
Extruded whole-seed soya beans	–	246·7
DL-Methionine	1·0	1·0
Calcium carbonate	16·2	12·3
Dicalcium phosphate	30·8	24·3
Sodium chloride	5·0	5·0
Vitamin and mineral mixture*	3·0	3·0
Cholesterol	3·0	3·0
Nutrient content (g/kg DM)		
DM (g/kg)	910·6	917·4
Ashes	73·8	109·7
Crude protein (N × 6·25)	159·8	153·5
Crude fat	95·2	102·8
Gross energy (kJ/kg DM)	17 805·0	17 883·0
Neutral-detergent fibre	82·1	114·1
Acid-detergent fibre	51·2	74·1
Cholesterol (g/kg)	3·06	3·18
Phytosterols (g/kg)	0·30	0·54

* Supplied per kg diet fed: retinol, 4·4 mg; cholecalciferol, 0·0675 mg; vitamin E, 22·5 mg; thiamin, 0·75 mg; riboflavin, 4·5 mg; cyanocobalamin, 0·023 mg; biotin, 0·15 mg; vitamin K_3, 1·5 mg; folic acid, 0·75 mg; nicotinic acid, 30·0 mg; pantothenic acid, 15·0 mg; I, 1·13 mg; Mn, 60·0 mg; Zn, 150·0 mg; Se, 0·18 mg; Co, 0·15 mg; Cu, 15·0 mg; Fe, 135·0 mg; butylated hydroxytoluene, 0·375 mg.

bicarbonate (20 g each), in order to prevent low mineral absorption. After surgery, six intact (I) and six IRA pigs were fed the ES diet and the remaining two groups were fed the casein diet.

After 2 weeks, faeces and ileal digesta were collected for 5 d from the I and IRA pigs, respectively. The total amount of faeces was collected twice daily while the ileal digesta was collected at 3 h intervals throughout the day–night cycle. Urine was collected daily in plastic containers containing sulfuric acid to prevent N loss. Individual samples of diet refusals, faeces, ileal digesta and urine were stored at −20°C until analyses.

At the end of the collection period and after an 8 h food-deprivation period, the pigs were killed by electronarcosis and bleeding. Blood samples were taken by cardiac puncture and plasma was immediately separated by centrifugation (20 min at 4°C and 1500 ***g***). Immediately after slaughter, the animals were eviscerated and the organs were washed with physiological saline (9 g NaCl/l) and weighed. The liver was apportioned for preparing cellular fractions (1 g), or for storing (±20 g) under vacuum, at −80°C until analyses. The same storage procedure was used for the entire gallbladder.

Diet, faeces and ileal digesta analyses

The composition of the experimental diets is presented in Table 1. Dietary cholesterol and phytosterols were determined by GLC on saponified lipid extracts, following the method described below for faecal and ileal digesta sterols.

Lyophilised faeces and ileal digesta samples (2 g) were homogenised in distilled water (20 ml) and submitted to an ethanol extraction in a Soxhlet apparatus for 48 h, before the addition of known amounts (20 μl) of [^{14}C]taurocholate sodium salt and saponification in boiling ethanolic potassium hydroxide (2 mol/l) for 2 h. The neutral sterols were extracted with petroleum ether. Bile acids from the aqueous phase were deconjugated (Grundy *et al.* 1965), extracted with diethyl ether, and ^{14}C radioactivity measured by liquid scintillation in a Tri-carb analyser (Packard, Rungis, France) to account for procedure losses. The preparation of the neutral sterols and free bile acids for analysis was done according to Férézou *et al.* (1997). The assays were done by GLC in the presence of cholestane as an external standard, using a Carlo-Erba HRGC 5160 chromatograph (Thermoquest, Les Ulis, France). The chromatograph was equipped with a standard fused silica WCOT capillary column (length 25 m; film thickness 0·2 mm) cross-linked with an OV101 (Spiral, Dijon, France) for sterols, or with an OV1701 (Spiral, Dijon, France) for bile acids and according to the conditions described by Riottot *et al.* (1993). Daily neutral sterol and bile acid outputs were calculated after correction for faecal and ileal digesta flow, based on a theoretical 90 % recovery of dietary β-sitosterol, a reliable marker in pigs (Marsh *et al.* 1972).

Plasma and lipoprotein analyses

Plasma levels of urea, glucose, triacylglycerols and phospholipids were determined by enzymic kits (Roche Diagnostics) in an automatic analyser (Hitachi 704; Hitachi, Tokyo, Japan). Non-esterified cholesterol (Wako Chemicals) was measured in a UV/VIS spectrophotometer (Beckman DU-530; Fullerton, CA, USA) and total cholesterol (Roche Diagnostics) in a Hitachi 917 Automatic Analyser. Plasma LDL-cholesterol (Nakamura *et al.* 1997) and HDL-cholesterol concentrations (Sugiuchi *et al.* 1995) were determined by direct enzymic assays (Roche Diagnostics) in a Hitachi 917 Automatic Analyser.

Liver analyses

Liver lipids. Liver lipids were extracted from frozen samples (0·5 g) according to a method described by Férézou *et al.* (1997). Non-esterified and total cholesterol were measured in isopropanolic extracts, using enzymic kits (Wako Chemicals and Roche Diagnostics) and a Beckman DU-530 UV/VIS spectrophotometer. Esterified cholesterol was calculated as the difference between total and non-esterified cholesterol. Triacylglycerols and phospholipids were determined using enzymic kits (Roche Diagnostics) and a Hitachi 917 Automatic Analyser.

Liver cellular fraction and enzymic assays. Mitochondrial and microsomal fractions were prepared from fresh liver samples (1 g) according to Souidi *et al.* (1999), with the microsomal fraction for the determination of HMG CoA reductase activity being suspended in a modified buffer containing 10 mmol dithiothreitol/l. Cellular fraction protein content was assayed according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

Microsomal HMG CoA reductase (*EC* 1.1.1.34) activity was determined by Philipp & Shapiro's (1979) radioisotopic technique, with slight modifications in the pre-incubation time with phosphatase (60 min at 37°C) and in the incubation time after the addition of [^{14}C]HMG CoA and NADPH (30 min at 37°C). Radioisotopic assays have been described in detail for microsomal CYP7A1 (*EC* 1.14.13.17; Souidi *et al.* 1998) and mitochondrial CYP27A1 (*EC* 1.14.13.15; Souidi *et al.* 1999) activities. All the enzymic activities, calculated in pmol/min per mg microsomal or mitochondrial protein, were also expressed as pmol/min per organ, taking into account the variations in liver weights due to the diet.

Immunoassays. Total membranes from frozen liver samples (1 g) stored at −80°C were prepared according to Kovanen *et al.* (1979). Membrane proteins solubilised in a buffer containing Triton X-100 (2 %) (Schneider *et al.* 1982) were assayed (Lowry *et al.* 1951) using bovine serum albumin as a standard. For the immunodetection of LDL receptors, liver protein membranes were diluted in a dilution buffer and spotted onto a nitrocellulose membrane using a Dot-blot apparatus (Bio-Rad, Richmond, VA, USA) according to the procedure described by Loison *et al.* (2002) with slight modifications. Briefly, the nitrocellulose membranes were incubated overnight at 4°C in a quenching buffer containing 5 % (w/v) fat-free milk. The nitrocellulose membranes were then submitted to a sequential cycle of washes with Tween–Tris-buffered saline (three times) and to a 90 min incubation in the presence of the antibody against LDL receptors diluted 1:2000 in an incubation buffer containing 0·1 % (w/v) fat-free

milk. Then they were washed with Tween–Tris-buffered saline (three times), incubated for 90 min with anti-Ig antibodies conjugated with horseradish peroxidase diluted at 1:5000, washed with Tween–Tris-buffered saline (three times) and finally incubated for 1 min with a chemiluminescense reagent. Sensitive films (Hyperfilm; Amersham Pharmacia Biotech, Les Ulis, France) were exposed, developed and scanned with a laser densitometer Ultroscan 2222 (LKB, Bromma, Sweden). The relative LDL receptor content in each spot was estimated by the scan peak height and results were expressed in arbitrary units/mg protein and arbitrary units/organ. The linearity of the response as a function of the protein quantity spotted was checked. The specific antibodies raised against the LDL receptors gave a unique band in Western blots with apparent molecular weights of about 130 kDa.

Gallbladder bile analyses

Bile lipids. Total lipids were extracted into isopropanol (Férézou *et al.* 1997). Bile total cholesterol and phospholipids were measured using enzymic kits (Roche Diagnostics) and a Beckman DU-530 UV/VIS spectrophotometer.

Bile acids. Bile samples were diluted (1:1) into isopropanol and total bile acid concentrations determined (Turley & Dietschy, 1978) in a Uvicon 930 UV/VIS spectrophotometer (Kontron Instruments Ltd, Watford, Herts, UK). Individual bile acid concentrations were determined by GLC, as described by Riottot *et al.* (1993).

Calculations and data analyses

Statistical analyses were performed by a two-way ANOVA for IRA and diet effects by using the general linear model procedure of the statistical package Statview version 5.0 (SAS Institute Inc., Cary, NC, USA). The Tukey–Kramer multiple comparison was used as a *post hoc* test to compare the IRA effect between the two diets. Differences were considered significant when $P<0{\cdot}05$.

Results

Physiological data and organ weights

The surgical procedure did not affect the measured parameters but ES-fed pigs presented a lower ($P<0{\cdot}05$) daily weight gain than casein-fed ones. Food consumption tended ($P=0{\cdot}06$) to be higher, and the neutral-detergent fibre ($P<0{\cdot}001$) and the calculated cholesterol intake ($P<0{\cdot}05$) were higher in ES-fed than in casein-fed pigs. Surgery and diet did not affect gallbladder weights but livers were lighter ($P<0{\cdot}05$) in ES-fed than in casein-fed pigs (Table 2).

Fasting plasma parameters

Uraemia was lower ($P<0{\cdot}01$) in IRA than in I pigs, but higher ($P<0{\cdot}05$) in pigs fed the ES diet in comparison with those fed the casein diet. LDL-cholesterol tended ($P=0{\cdot}07$) to be lower in IRA than in I pigs. All the other parameters were unaffected by surgery and diet. LDL-cholesterol:HDL-cholesterol and HDL-cholesterol: total cholesterol ratios (data not shown) were also not modified (Table 3).

Liver parameters

The ES diet decreased non-esterified ($P<0{\cdot}05$), esterified and total cholesterol ($P<0{\cdot}001$) and triacylglycerol ($P<0{\cdot}05$) concentrations, and tended ($P=0{\cdot}05$) to reduce phospholipids as compared with the casein diet. HMG CoA reductase, CYP7A1 and CYP27A1 activities were not modified by surgery or diet, but IRA tended ($P=0{\cdot}05$) to increase LDL receptors as compared with I animals (Table 4).

Biliary lipids and bile acids

When compared with the casein diet, the ES diet significantly depressed ($P<0{\cdot}05$) total cholesterol concentration

Table 2. Effects of ileo-rectal anastomosis (IRA) and diet (D) on physiological data and organ weights of pigs†
(Mean values and standard errors of the mean)

	Casein–I		Casein–IRA		ES–I		ES–IRA		ANOVA		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	D	IRA	D × IRA
Final weight (kg)	37·1	4·3	37·0	3·1	34·2	2·0	39·7	2·3	NS	NS	NS
Daily weight gain‡ (g/d)	330·3	42·9	326·8	50·1	181·5	37·7	165·6	96·6	*	NS	NS
Daily food intake‡ (g DM/d per kg BW)	37·73	2·12	38·82	2·47	42·10	1·48	42·39	1·49	*P*=0·06	NS	NS
NDF intake‡ (g/d per kg BW)	3·12	0·29	3·15	0·27	4·56	0·28	4·74	0·27	***	NS	NS
Cholesterol intake (g/d per kg BW)	0·13	0·01	0·13	0·01	0·14	0·01	0·14	0·01	*	NS	NS
N retention‡§ (g/d per kg BW)	0·62	0·05	0·64	0·04	0·63	0·02	0·62	0·06	NS	NS	NS
Metabolisable energy‡§ (kJ/d per kg BW)	648·1	37·2	635·1	42·9	661·3	19·3	644·3	15·1	NS	NS	NS
Liver (g/kg BW)	21·7	0·9	22·4	1·0	19·5	1·3	19·7	1·2	*	NS	NS
Gallbladder (g/kg BW)	1·0	0·2	0·8	0·1	1·1	0·2	0·8	0·2	NS	NS	NS

I, intact; ES, extruded soya beans; BW, body weight; NDF, neutral-detergent fibre.
Significance of effect (ANOVA): *$P<0{\cdot}05$, ***$P<0{\cdot}001$.
† For details of diets and procedures, see Table 1 and p. 690.
‡ Values reported for the 5 d collection period.
§ Calculated as the difference between daily intake and output (through faeces, ileal digesta and urine) per kg BW.

Table 3. Effects of ileo-rectal anastomosis (IRA) and diet (D) on some plasma parameters (mmol/l)†
(Mean values and standard errors of the mean)

	Casein–I		Casein–IRA		ES–I		ES–IRA		ANOVA		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	D	IRA	D × IRA
Micro-packed cell volume (L/L)	0·39	0·01	0·41	0·01	0·41	0·01	0·39	0·01	NS	NS	NS
Urea	4·39	0·40	2·52	0·27	5·12	0·58	4·15	0·51	*	**	NS
Glucose	7·34	0·74	5·99	0·26	7·44	0·71	8·18	0·77	NS	NS	NS
Non-esterifred cholesterol	0·72	0·04	0·61	0·05	0·79	0·08	0·74	0·06	NS	NS	NS
Total cholesterol	4·24	0·19	3·28	0·24	4·15	0·46	4·11	0·53	NS	NS	NS
LDL-cholesterol	2·68	0·23	1·96	0·27	2·21	0·15	2·10	0·18	NS	$P=0·07$	NS
HDL-cholesterol	1·34	0·07	1·25	0·10	1·56	0·25	1·63	0·26	NS	NS	NS
Triacylglycerols	0·61	0·09	0·66	0·03	0·52	0·10	0·64	0·07	NS	NS	NS
Phospholipids	1·94	0·13	1·81	0·14	2·12	0·28	1·87	0·13	NS	NS	NS

I, intact; ES, extruded soya beans.
Significance of effect (ANOVA): *$P<0·05$, **$P<0·01$.
† For details of diets and procedures, see Table 1 and p. 690.

in gallbladder bile, as well as increased ($P<0·05$) total bile acid concentration (Table 5).

Surgery and diet markedly modified gallbladder bile acid composition. As previously observed in pig bile, the cholic acid synthesis pathway (cholic + deoxycholic acids) was almost ineffective. In contrast, the chenodeoxycholic synthesis pathway (the other bile acids) represented more than 98 % of the total bile acids. Hyocholic acid, the trihydroxylated derivative of chenodeoxycholic acid, was the major bile acid and was markedly increased ($P<0·01$) in ES-fed pigs. Hydrophobic bile acids (chenodeoxycholic, deoxycholic, lithocholic, 3α-hydroxi-6-oxo-5β-cholanoic, 7-oxo-lithocholic acids and other ketones) represented 31·0, 29·9, 15·3 and 17·6 % of the total biliary bile acids in casein–I, casein–IRA, ES–I and ES–IRA pigs, respectively. Thus, the ES diet significantly reduced ($P<0·01$) the concentration of hydrophobic acids in the bile of pigs. The caecum–colon bypass markedly reduced the secondary bile acids (mainly hyodeoxycholic acid) but the ES diet also contributed to the prevention of microbial transformation of bile acids. Thus, the primary bile acids:secondary bile acids ratio was increased in IRA as compared with I pigs ($P<0·001$) and in ES-fed as compared with casein-fed pigs ($P<0·01$) (Table 5).

Neutral and acidic steroid output

The total steroid output was not significantly affected by surgery and diet. IRA did not influence total neutral sterol and cholesterol output, but markedly prevented the microbial hydrogenation of the 5–6 double bond of cholesterol into coprostanol. The ES diet also prevented this hydrogenation of cholesterol, stimulating cholesterol and therefore neutral sterol output. Similar effects were observed on β-sitosterol transformation (data not shown). The effects of surgery and the ES diet in the total bile acid output were not similar. IRA markedly decreased ($P<0·05$) bile acid output in casein-fed, but not in ES-fed pigs. The surgery also markedly decreased the microbial transformation of bile acids as shown by the dramatic increase in the primary bile acids:secondary bile acids ratio. In the casein–IRA pigs the primary bile acids increased to 51 % and in the ES–IRA pigs to 67 % of the total bile acids. Microbial metabolites of bile acids represented more than 94 % of total bile acid output in the casein–I pigs and hyodeoxycholic acid was the major bile acid (more than 62 % of the bile acid output). The ES diet markedly modified this pattern and reduced the microbial transformation of bile acids to only 69 % of the total bile acid output in the ES–I pigs. Thus, both surgery and the ES diet prevented the microbial transformation of bile acids in the pigs (Table 6).

Discussion

The present study examined the effects of two factors: the caecum–colon shortcut from the digestive process and the ingestion of a diet containing ES on cholesterol and bile acid metabolism in growing pigs fed a cholesterol-enriched diet. Physiological data were not affected by surgery, but the ES diet decreased the daily weight gain of the pigs. This decrease could be partly due to the anti-trypsin activity factor contained in ES (7·2 mg trypsin inhibitor activity/g) and to the higher neutral-detergent fibre content of that diet, which is negatively correlated with the efficiency of utilisation of metabolisable energy as net energy by the pig (Noblet *et al.* 1989). Diet effects on liver weights, lighter in ES-fed than in casein-fed pigs, could result from changes in hepatic tissue composition, namely a lower lipid content.

Plasma cholesterol in the pigs receiving the enriched cholesterol casein diet was 50 % higher than that determined in similar growing pigs fed a diet containing no added cholesterol (2·68 (SEM 0·10) mmol/l; JM Martins, OP Bento, MC de Abreu, JPB Freire, JAA Almeida and M Riottot, unpublished results). This level of plasma cholesterol in the casein-fed pigs was identical to that observed in growing Large White pigs receiving a 0·3 % (w/w) cholesterol-enriched diet (Férézou *et al.* 1997). Surgery and the ES diet had no effect on the levels of plasma cholesterol, lipoprotein cholesterol and other lipids measured, but modified the hepatic metabolism of cholesterol and bile acids and markedly affected the action of microflora in the intestinal lumen.

The dietary cholesterol intake (absorbed plus non-absorbed cholesterol) was almost similar in the four groups of pigs. The cholesterol synthesis, as determined by hepatic HMG CoA reductase activity, was not modified,

Table 4. Effects of ileo-rectal anastomosis (IRA) and diet (D) on hepatic parameters (mg/g)†
(Mean values and standard errors of the mean)

	Casein–I		Casein–IRA		ES–I		ES–IRA		ANOVA		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	D	IRA	D × IRA
Non-esterified cholesterol	4·01	0·26	4·18	0·18	3·55	0·16	3·56	0·14	*	NS	NS
Esterified cholesterol	1·19	0·09	1·12	0·08	0·51	0·06	0·60	0·11	***	NS	NS
Total cholesterol	5·20	0·32	5·30	0·19	4·06	0·18	4·16	0·16	***	NS	NS
Triacylglycerols	10·35	1·58	10·75	1·78	7·72	0·22	7·52	0·65	*	NS	NS
Phospholipids	21·72	0·57	20·48	0·06	19·97	0·58	20·31	0·42	$P=0·05$	NS	NS
HMG CoA reductase:											
pmol/min per mg protein	2·14	0·50	6·52	2·77	6·33	0·24	7·49	1·26	NS	NS	NS
pmol/min per organ	11 896	1585	47 090	19 322	35 045	2837	36 518	3635	NS	NS	NS
CYP7A1: pmol/min per mg protein	19·92	2·44	18·93	3·77	14·07	2·23	20·97	3·42	NS	NS	NS
pmol/min per organ	102 917	14 577	101 226	20 195	69 974	14 677	110 958	21 164	NS	NS	NS
CYP27A1: pmol/min per mg protein	61·44	11·70	51·82	15·14	55·86	11·59	54·84	11·69	NS	NS	NS
pmol/min per organ	399 170	48 380	306 444	39 877	332 302	106 860	257 919	40 478	NS	NS	NS
LDL receptors: Arbitrary units/g protein	1·83	0·27	2·39	0·45	1·51	0·24	2·77	0·70	NS	$P=0·05$	NS
Arbitrary units/organ	10 694	2228	11 025	1686	8604	1751	16 321	4012	NS	NS	NS

I, intact; ES, extruded soya beans; HMG CoA, 3-hydroxy-3-methylglutaryl CoA; CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase.
Significance of effect (ANOVA): $*P<0·05$, $***P<0·001$.
† For details of diets and procedures, see Table 1 and p. 690.

Table 5. Effects of ileo-rectal anastomosis (IRA) and diet (D) on biliary lipid and bile acid concentrations (mmol/l)†
(Mean values and standard errors of the mean)

	Casein–I		Casein–IRA		ES–I		ES–IRA		ANOVA		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	D	IRA	D × IRA
Total cholesterol	4·18	0·53	4·77	1·27	2·76	0·30	2·43	0·31	*	NS	NS
Phospholipids	10·98	4·15	9·15	1·58	9·36	1·06	12·17	1·96	NS	NS	NS
Total bile acids	178·10	23·88	135·96	3·90	216·31	15·12	191·96	30·88	*	NS	NS
Cholic acid	0·27	0·19	0·29	0·15	0·34	0·18	0·27	0·17	NS	NS	NS
Chenodeoxycholic acid	32·42	7·03	28·88	9·22	16·19	2·04	21·96	3·41	$P=0·08$	NS	NS
Hyocholic acid	81·44	30·83	92·79	7·44	169·46	18·34	155·01	30·10	**	NS	NS
Deoxycholic acid	1·09	0·39	0·91	0·11	0·73	0·08	0·83	0·10	NS	NS	NS
Lithocholic acid	8·03	2·91	6·91	1·64	4·25	0·14	5·63	1·03	NS	NS	NS
Hyodeoxycholic acid	41·93[a]	12·22	1·75[b]	0·38	14·12[b]	2·83	3·07[b]	1·19	*	***	*
3α-Hydroxi-6-oxo-5β-cholanoic acid	1·59	1·52	0·00	0·00	0·32	0·14	0·00	0·00	NS	NS	NS
7-Oxo-lithocholic acid	4·25	1·12	1·29	0·25	4·25	0·47	0·86	0·21	NS	***	NS
Other ketones	7·08	2·37	3·14	0·39	6·65	1·73	4·33	0·69	NS	*	NS
Primary bile acids:secondary bile acids‡	2·38	0·89	9·24	1·07	6·70	1·26	12·05	1·39	**	***	NS

I, intact; ES, extruded soya beans.
[a,b] Mean values within a row, where the effect by ANOVA is significant, with unlike supercript letters are significantly different ($P<0·05$) by the Tukey–Kramer multiple comparison test.
Significance of effect (ANOVA): $*P<0·05$, $**P<0·01$, $***P<0·001$.
† For details of diets and procedures, see Table 1 and p. 690.
‡ Primary bile acid:secondary gallbladder bile acids ratio, where primary acids are cholic, chenodeoxycholic and hyocholic acids.

Table 6. Effects of ileo-rectal anastomosis (IRA) and diet (D) on faecal and ileal digesta neutral sterol and bile acid output (mg/d per kg body weight)†
(Mean values and standard errors of the mean)

	Casein–I		Casein–IRA		ES–I		ES–IRA		ANOVA		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	D	IRA	D × IRA
Total neutral and acidic steroids	81·73	15·42	61·01	10·24	94·73	12·75	97·22	13·14	$P=0·08$	NS	NS
Neutral sterols	52·62	8·56	57·42	9·92	81·31	11·51	81·66	15·33	*	NS	NS
Cholesterol	39·08	9·01	56·67	9·82	77·38	9·69	81·08	15·56	*	NS	NS
Microbial sterols	13·54[a]	3·52	0·75[b]	0·34	3·93[b]	1·90	0·58[b]	0·58	*	**	*
Coprostanol	11·78[a]	3·43	0·19[b]	0·19	2·91[b]	1·40	0·58[b]	0·58	$P=0·06$	**	*
Epicoprostanol	1·36	0·53	0·00	0·00	0·44	0·44	0·00	0·00	NS	*	NS
Cholestanol	0·40	0·13	0·56	0·19	0·58	0·43	0·00	0·00	NS	NS	NS
Bile acids	29·05[a]	11·10	3·59[b]	0·91	13·42[ab]	3·21	15·57[ab]	6·71	NS	$P=0·09$	*
Chenodeoxycholic acid	0·63	0·20	0·43	0·22	0·88	0·20	2·56	0·95	*	NS	NS
Hyocholic acid	0·90	0·53	1·41	0·50	3·26	0·50	7·82	4·67	$P=0·07$	NS	NS
Lithocholic acid	6·23	3·12	0·16	0·03	1·22	0·54	0·59	0·12	NS	*	NS
Hyodeoxycholic acid	18·11[a]	6·71	0·66[b]	0·12	2·94[b]	0·50	1·51[b]	0·34	*	**	*
3α-Hydroxi-6-oxo-5β-cholanoic acid	1·73	1·41	0·35	0·12	0·55	0·10	0·64	0·14	NS	NS	NS
7-Oxo-lithocholic acid	1·07	0·25	0·32	0·06	3·05	1·96	1·84	1·24	NS	NS	NS
Other ketones	0·38	0·18	0·26	0·07	1·52	0·59	0·61	0·13	$P=0·06$	NS	NS
Primary bile acids:secondary bile acids‡	0·06	0·01	0·98	0·29	0·52	0·06	1·69	0·51	$P=0·05$	**	NS

I, intact; ES, extruded soya beans.
[a,b] Mean values within a row, where the effect by ANOVA is significant, with unlike superscript letters are significantly different ($P<0·05$) by the Tukey–Kramer multiple comparison test.
Significance of effect (ANOVA): *$P<0·05$, **$P<0·01$.
† For details of diets and procedures, see Table 1 and p. 690.
‡ Primary bile acids:secondary bile acids output ratio, where primary acids are chenodeoxycholic and hyocholic acids.

whatever the treatment. This activity was similar to that observed in growing Large White pigs fed a cholesterol-rich diet, but lower than that observed in pigs fed a diet with no added cholesterol (Férézou *et al.* 1997). Thus, though the total synthesis of cholesterol in the body was not determined in the present experiment, the cholesterol input in the experimental groups was probably very close. The neutral sterol output was not affected by surgery, in agreement with the fact that the intestinal absorption of cholesterol occurs mostly in the small intestine. Conversely, the ES diet enhanced neutral sterol output by 48 %. This enhancement suggests a decrease in dietary cholesterol intestinal absorption. This could be due to: (i) the presence of phytosterols (Carr *et al.* 2002), 1·8-fold higher in the ES than in the casein diet; (ii) high intestinal contents of undigested components of soya beans such as fibres (Potter, 1995) and hydrophobic peptides (Sugano *et al.* 1990), known to interfere with steroid intestinal absorption. Surgery reduced the bile acid output in casein-fed pigs, but not in ES-fed ones. It has been suggested that the faecal output of bile acids is balanced by hepatic bile acid synthesis (Lindstedt, 1957). However, in the present experiment bile acid synthesis, as estimated by the activities of CYP7A1 and CYP27A1, did not change whatever the treatment. The observed discrepancy between synthesis and output of neutral and acidic sterols could be related to an unsteady state, as growing mammals need cholesterol to build cell membranes (Spady & Dietschy, 1983). In terms of sterol balance, the difference between the input and output of sterols could represent the amount of cholesterol daily deposited in tissues. However, this difference in the experimental groups was high and probably overestimated due to the theoretical correction based on the β-sitosterol intestinal absorption and to other additional errors on each term of the balance. Moreover, no determination of the cholesterol concentration in tissues was made in the present experiment, except in the liver tissue. In this tissue, the amount of cholesterol determined was 2-fold higher than that observed in pigs receiving a cholesterol-free diet (Férézou *et al.* 1997) but the amount of liver cholesterol can vary markedly without variation in the tissues (Marsh *et al.* 1972). Further investigations could be undertaken to determine cholesterol deposition in the tissues of those pigs. Finally, the daily total neutral and acidic steroid output was not significantly different among the four experimental groups. As determined in pigs (Férézou *et al.* 1997), a 2-fold increase in the faecal total steroid output was necessary to decrease plasma cholesterol by 50 %. Thus, the poor variation of the input and output of cholesterol from the body of our pigs could explain the absence of effects on cholesterolaemia.

The action of intestinal microflora on neutral sterol and bile acid composition was markedly modified by surgery and reinforced by the ES diet. The microbial transformation of cholesterol and β-sitosterol, as determined in faeces, was weak in I pigs fed either the casein or ES diets (26 and 5 % of the total neutral sterols and 33 and 16 % of the total β-sitosterol, respectively). This was most probably due to the use of high levels of dietary cholesterol that overloaded the transformation capacity of the microflora, as previously observed by Férézou *et al.* (1997) and Marsh *et al.* (1972). Surgery and the ES diet prevented almost completely the microbial transformation of cholesterol. This inhibition was not present in animals fed unextruded soyabean protein (Beynen *et al.* 1990; Morita *et al.* 1997), but it was observed in rats, hamsters and pigs fed poorly digestible carbohydrates (Riottot *et al.* 1993; Levrat *et al.* 1994; Khallou *et al.* 1995). In contrast to that observed for cholesterol, the microbial transformation of bile acids was almost complete (95 %) in the casein–I pigs. In this group, secondary bile acids represented up to 36 % of the total gallbladder bile acids. The microbial transformation of non-absorbed bile acids was markedly reduced by surgery; for the casein–IRA and ES–IRA pigs, the secondary bile acids represented respectively 49 and 33 % of the total bile acid output and 9·6 and 7·2 % of the biliary bile acids. Similar results were observed in IRA growing pigs fed baked beans (Costa *et al.* 1994) and in ileoanal anastomosed patients (Hakala *et al.* 1997). The presence of microbial metabolites of bile acids in IRA pigs also suggests that the distal small intestine of these pigs was colonised by a metabolically active microflora, more efficient towards acidic than neutral steroids. The ES diet but not surgery decreased the hydrophobic bile acid concentration in the gallbladder bile. This decrease could also explain the absence of variation of hepatic steroid syntheses, due to the involvement of these acids in their syntheses modulation (Pandak *et al.* 1994). Like surgery, the ES diet markedly increased the primary bile acids:secondary bile acids ratio in bile and faeces. This increased ratio suggests that this diet, containing 25 % ES, played a specific role in the inhibition of the bile acid microbial transformation. To obtain a similar inhibition of microbial bile acids by dietary amylomaize or potato starch in the rat, Andrieux *et al.* (1989) had to use diets with 58 and 32 % of these poorly digestible carbohydrates. The impaired transformation of steroids by diet is quite an interesting topic because these modified molecules are involved in colonic diseases and particularly cancers (Nagengast *et al.* 1995).

The absence of a hypocholesterolaemic effect of soya beans was unexpected. Modifications of the soya beans by the extrusion process could explain this discrepancy. This thermal and pressure treatment is known to modify the physicochemical properties of the legume (Harper, 1978). Soyabean extraction with water and ethanol can also reduce its non-protein components (Anderson & Wolf, 1995) and thus, as reported in Syrian hamsters (Lucas *et al.* 2001), prevent its hypocholesterolaemic effect. Due to the common use of ES in the food industry (Friedman & Brandon, 2001), this suggestion deserves to be further investigated.

The absence of a hypocholesterolaemic effect in IRA pigs, whatever the diet used, suggests that in the present study conditions the large intestine did not play a determining role in the regulation of steroid metabolism. Though surgery reduces the microbial transformation of steroids, only ES were able to reduce the hydrophobicity of biliary bile acids and to reinforce the impaired transformation of steroids. This could be interesting to prevent intestinal diseases. The addition of a dietary factor to a surgical

treatment decreases more efficiently potentially hazardous molecules such as cytotoxic steroids and deserves to be further investigated.

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