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# Cholesterol-lowering effects of dietary blue lupin (*Lupinus angustifolius* L.) in intact and ileorectal anastomosed pigs

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**Abstract** The present study was undertaken to investigate the effect of cholesterol-enriched casein (CAS) and blue lupin seed (BL) diets on the cholesterol metabolism of intact (INT) and ileorectal anastomosed (IRA) pigs. For 3 weeks, four groups of six pigs were allocated to the treatments (CAS-INT, CAS-IRA, BL-INT, and BL-IRA). Diet-induced hypercholesterolemia was inhibited by the BL through a substantial decrease in plasma LDL-cholesterol. The BL also reduced liver esterified and total cholesterol, increased hepatic LDL receptor synthesis and HMG-CoA reductase activity, and stimulated intestinal bile acid reabsorption. The neutral sterol output was higher in BL- than in CAS-fed pigs. The bile acid output was lower in IRA than in INT pigs. Surgery also prevented steroid microbial transformation, but it did not influence plasma cholesterol levels. These results suggest that the hypocholesterolemic effect of the BL, compared with the CAS, is attributable to impaired intestinal cholesterol absorption, probably involving increased bile acid reabsorption and higher contents of dietary phytosterols, both factors that reduce the micellar solubilization of cholesterol. Furthermore, according to our data, the contribution of the large intestine to cholesterol metabolism is very weak.—Martins, J. M., M. Riottot, M. C. de Abreu, A. M. Viegas-Crespo, M. J. Lança, J. A. Almeida, J. B. Freire, and O. P. Bento. Cholesterol-lowering effects of dietary blue lupin (*Lupinus angustifolius* L.) in intact and ileorectal anastomosed pigs. *J. Lipid Res.* 2005. 46: 1539–1547.

**Supplementary key words** pulses • phytosterols • dietary fiber • hepatic enzymes • steroid output • steroid reabsorption

Hypercholesterolemia and its implications for cardiovascular diseases is a major problem in human health, and much attention has been paid to dietary intervention as a tool for its prevention and treatment (1). Legumes have

shown hypocholesterolemic effects in human and animal models (2–4), but these studies have mainly been done with soybean or its components. Therefore, studies involving other legumes, such as lupins, may clarify the mechanism by which plasma cholesterol is reduced and lead to the identification of new functional foods and/or components.

Seeds of several species of lupins have been used as food for >3,000 years in the Mediterranean area (5). These bitter seeds had to be soaked in water before consumption, to remove most of their alkaloid content (6). From the second half of the 20th century onward, low-alkaloid varieties of white lupin (*Lupinus albus*), yellow lupin (*Lupinus luteus*), and blue lupin (*Lupinus angustifolius*) have been domesticated and selected (7). In 2004, sweet varieties of these three species were mainly cultivated in several parts of Australia, Europe, and South America (8) and used for feed and food applications. Blue lupin seeds have higher nonstarch polysaccharide (6) and protein contents than soybean, with a similar amino acid profile (9). Their use in the food industry is being developed, and lupins are beginning to replace soybean in products such as tempe, miso, fermented sauces, and cooked snack foods (6). Lupin-based fiber supplements, cookies, bread, and spaghetti, with a high sensory quality, are also reported (10).

No studies have been undertaken to test the effect of blue lupin seeds on cholesterol metabolism in the pig, an animal model with a plasma lipid profile similar to that of human, which responds markedly to hyperlipidemic diets (11). Moreover, although the role of the small intestine in cholesterol metabolism is well documented (12, 13), the role of the large intestine and its microflora is still unclear.

Abbreviations: BL, blue lupin seed diet; CAS, casein diet; CYP7A1, cholesterol 7 $\alpha$ -hydroxylase; CYP27A1, sterol 27-hydroxylase; GLC, gas-liquid chromatography; INT, intact; IRA, ileorectal anastomosed.

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The absorption of free bile acids by the colon was demonstrated (14), but its contribution to the enterohepatic circulation is poorly understood (15). Furthermore, the microbial transformation of primary into secondary bile acids in the hindgut (16) could affect cholesterol metabolism, because the absorption of hydrophobic secondary bile acids modulates hepatic cholesterol and bile acid synthesis (17). Therefore, bypass of the cecum-colon section was used as a tool to obtain more information concerning the effect of the hindgut in cholesterol metabolism and steroid output.

In this study, we examined the effects of feeding whole blue lupin seeds and the role of cecum-colon bypass on the cholesterol metabolism and neutral and acidic steroid output of growing pigs fed cholesterol-rich diets. Analyses of sterols in the plasma, liver, bile, and feces were carried out.

## MATERIALS AND METHODS

### Chemicals and isotopes

Chemicals and solvents of the highest purity available were purchased from Merck (Darmstadt, Germany), Prolabo (Paris, France), and Sigma-Aldrich (St. Louis, MO). Enzymatic assay kits were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Wako Chemicals GmbH (Neuss, Germany). Hydroxypropyl- $\beta$ -cyclodextrin was a kind gift of Societ  Roquette Fr res (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 [14C]taurocholate sodium salt were purchased from DuPont-NEN Products (Les Ulis, France). 25-Hydroxycholesterol was provided by Roussel-Uclaf (Romainville, France), and 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol were synthesized as described previously (18). Emulsifier-Safe was acquired from Packard Instruments Co. (Meriden, CA). A polyclonal antibody raised against the bovine adrenal cortex LDL receptor was kindly provided by Paul Roach (University of Adelaide, Adelaide, Australia). The anti-rabbit IgG horseradish peroxidase-linked F(ab') $_2$  fragment (from donkey) and the enhanced chemiluminescence reagent were purchased from Amersham Pharmacia Biotech (Les Ulis, France).

### Animals and diets

Twenty-four 12 week old crossbred male pigs [Duroc boars  $\times$  (Large White  $\times$  Landrace sows)] from Universidade de  vora ( vora, Portugal) with an initial body weight of 30.3  $\pm$  0.5 kg (mean  $\pm$  SEM) were individually penned in metabolism cages (60  $\times$  160 cm). All procedures were approved by the Portuguese Animal Nutrition and Welfare Commission (Lisboa, Portugal).

Two experimental cholesterol-enriched diets, a semipurified casein diet (CAS) and a raw blue lupin seed diet (BL), were formulated to have similar amounts of crude protein, essential amino acids (lysine, methionine, and tryptophan), and gross energy. In the BL,  $\sim$ 60% of the protein supplied by casein in the CAS was replaced by protein from finely ground whole blue lupin dry seeds (Table 1). Cholesterol was included in both diets at a rate of 2.8 g/kg, after solubilization in soybean oil. Pigs were fed at a weekly adjusted daily rate of 50 g/kg body weight in two equal meals (8:30 AM and 6:00 PM) and had free access to water throughout the experimental period.

### Experimental design and sampling procedures

After a postweaning period consuming a commercial diet (S801; Ra oes Ver ssimo, Leiria, Portugal), the pigs (n = 24) were made

TABLE 1. Ingredients and average composition of the experimental diets

Ingredients and Composition	CAS	BL
	<i>g/kg</i>	
Ingredients		
Cornstarch	464.0	342.3
Sucrose	100.0	100.0
Wheat straw	100.0	100.0
Soybean oil	100.0	85.0
Acid hydrolyzed casein	180.0	72.0
Blue lupin whole seeds	—	244.0
L-Lysine	—	1.5
DL-Methionine	1.0	2.0
L-Tryptophan	—	0.5
Calcium carbonate	16.2	18.4
Dicalcium phosphate	30.8	26.3
Sodium chloride	5.0	5.0
Vitamin and mineral mixture <sup>a</sup>	3.0	3.0
Cholesterol	2.8	2.8
	<i>Dry matter g/kg</i>	
Nutrient content		
Dry matter (g/kg)	911.9	928.6
Ashes	74.9	79.0
Crude protein (N $\times$ 6.25)	163.7	155.1
Crude fat	96.5	93.4
Gross energy (MJ/kg DM)	17.8	17.6
Total dietary fiber	84.0	150.0
Soluble dietary fiber	1.0	5.0
Insoluble dietary fiber	83.0	145.0
Cholesterol (g/kg)	2.8	2.7
Phytosterols (g/kg)	0.3 <sup>b</sup>	0.9 <sup>c</sup>

BL, blue lupin seed diet; CAS, casein diet.

<sup>a</sup> Supplied (mg/kg diet): retinol, 30; cholecalciferol, 5.4; DL- $\alpha$ -tocopherol, 22.5; thiamine, 0.75; riboflavin, 4.5; cyanocobalamin, 0.023; biotin, 0.15; menadione, 1.5; folic acid, 0.75; nicotinic acid, 30; pantothenic acid, 15; potassium iodide, 1.62; manganese oxide, 99; zinc oxide, 198; sodium selenite, 0.39; cobalt sulfate  $\cdot$  7H $_2$ O, 0.72; copper sulfate  $\cdot$  5H $_2$ O, 63; iron carbonate, 342; butylated hydroxytoluene, 0.375.

<sup>b</sup> Composed of 0.2 g of  $\beta$ -sitosterol, 0.05 g of campesterol, and 0.05 g/kg stigmaterol.

<sup>c</sup> Composed of 0.3 g of  $\beta$ -sitosterol, 0.2 g of campesterol, 0.1 g of stigmaterol, and 0.3 g/kg other phytosterols.

hypercholesterolemic by feeding the CAS for 2 weeks. At the beginning of week 3, 12 pigs underwent an end-to-side ileorectal anastomosis procedure (19)  $\sim$ 40 mm before the ileocecal valve and without removing the hindgut. The ileorectal anastomosed (IRA) pigs were supplemented daily with sodium chloride (20 g) and sodium bicarbonate (20 g) to prevent low mineral absorption. From week 3 onward, six intact (INT) and six IRA pigs were fed the BL, whereas six INT and six IRA pigs continued to consume the CAS.

During week 5, all of the pigs were subjected to 5 d total feces, ileal digesta, and urine collection. Feces were collected twice per day, and ileal digesta was collected at 3 h intervals. Urine was collected daily in plastic containers with sulfuric acid to prevent nitrogen loss. Individual samples of diet refusals, feces, ileal digesta, and urine were stored ( $-20^\circ\text{C}$ ) until analyses.

At the end of week 5 and after a  $\sim$ 10 h food deprivation period, the pigs were killed by electronarcosis and bleeding. Blood samples were taken by cardiac puncture. Plasma was obtained by immediate centrifugation (20 min at  $4^\circ\text{C}$  and 1,500 g) (J-6B; Beckman, Buckinghamshire, UK) and frozen ( $-80^\circ\text{C}$ ) (UF460; Heto, Brondby, Denmark) until analysis. Pigs were eviscerated immediately after slaughter, and their organs were washed with physiologic saline and weighed. The liver was apportioned for the preparation of fresh cellular fractions (1 g) or for storage

( $\pm 20$  g at  $-80^{\circ}\text{C}$ ) under vacuum until analysis. Gallbladders and their contents were stored using similar procedures.

### Diet, feces, and ileal digesta analyses

The compositions of the experimental diets are shown in Table 1. Total, soluble, and insoluble dietary fibers were analyzed according to Association of Official Analytical Chemists Official Method 985.29 (20). Total alkaloids from the blue lupin seeds were determined according to European Commission directive 71/250/EEC (21). Diet total cholesterol and phytosterols were determined by gas-liquid chromatography (GLC) as described below for fecal and ileal digesta sterols.

Lyophilized feces and ileal digesta samples (2 g) homogenized in distilled water (20 ml) were extracted with ethanol for 48 h in a Soxhlet apparatus before the addition of known amounts (20  $\mu\text{l}$ ) of [ $^{14}\text{C}$ ]taurocholate sodium salt. Lipid fractions were saponified for 2 h in boiling ethanolic potassium hydroxide (2 mol/l). Neutral sterols were extracted with petroleum ether. Bile acids in the aqueous phase were deconjugated (22) and extracted with diethyl ether, and  $^{14}\text{C}$  radioactivity was measured by liquid scintillation in a Tri-carb analyzer (Packard, Rungis, France) to account for procedure losses. The neutral sterols and free bile acids were prepared for analysis by GLC (23), using cholestane as an external standard. The assays were done in a Carlo-Erba HRGC 5160 chromatograph (Thermoquest, Les Ulis, France), equipped with a standard fused silica WCOT capillary column (length, 25 m; film thickness, 0.2  $\mu\text{m}$ ) cross-linked with OV101 (Spiral, Dijon, France) for sterols and with OV1701 silicone (Spiral) for bile acids, according to the conditions described by Riottot et al. (24). Daily neutral sterol and bile acid outputs were calculated after correction for fecal and ileal flow, on the basis of a theoretical 90% recovery of dietary  $\beta$ -sitosterol, a reliable marker in pigs (25).

### Plasma and lipoprotein analyses

The determination of plasma levels of triacylglycerols and phospholipids was made with enzymatic kits and an automatic analyzer (704; Hitachi, Tokyo, Japan). Free and total cholesterol were measured with enzymatic kits in an ultraviolet/visible spectrophotometer (DU-530; Beckman, Fullerton, CA) and a Hitachi 917 automatic analyzer, respectively. Plasma LDL-cholesterol (26) and HDL-cholesterol (27) concentrations were measured with direct enzymatic kits using a Hitachi 917 analyzer.

### Liver analyses

**Liver lipids.** Total liver lipids were extracted from frozen samples (0.5 g) (23), and free and total cholesterol were measured in propanol-2 extracts, as described above. Esterified cholesterol was calculated as the difference between total and free cholesterol. Triacylglycerols and phospholipids were also determined in a Hitachi 917 analyzer, as described above.

**Liver cellular fraction and enzymatic assays.** Mitochondrial and microsomal fractions were prepared from fresh liver samples (1 g) (28). The microsomal fraction for the assay of HMG-CoA reductase activity was suspended in a modified buffer with 10 mmol/l DTT. The protein content from cellular fractions was determined (29) using BSA as a standard.

Microsomal HMG-CoA reductase (EC 1.1.1.34) activity was assayed by the radioisotopic technique of Philipp and Shapiro (30) with minor adjustments in the preincubation time with phosphatase (60 min at  $37^{\circ}\text{C}$ ) and in the incubation time after the addition of [ $^{14}\text{C}$ ]HMG-CoA and NADPH (30 min at  $37^{\circ}\text{C}$ ). The microsomal cholesterol  $7\alpha$ -hydroxylase (CYP7A1; EC 1.14.13.17) and mitochondrial sterol  $27$ -hydroxylase (CYP27A1; EC 1.14.13.15) activities were assayed according to Souidi, Parquet, and Lutton (31) and Souidi et al. (28), respectively.

### Immunoassays

Total membranes were prepared from frozen ( $-80^{\circ}\text{C}$ ) liver samples (1 g) according to Kovanen, Brown, and Goldstein (32). Membrane proteins, solubilized in a buffer containing Triton X-100 (2%) (33), were assayed (29) using BSA as a standard. The immunodetection of LDL receptors was done as described previously (34). Relative LDL receptor contents were expressed in arbitrary units per milligram of protein and arbitrary units per organ. The linearity of the response as a function of the protein quantity spotted was verified. Specific antibodies raised against the LDL receptor gave a unique band on Western blots with apparent molecular masses of  $\sim 130$  kDa.

### Gallbladder bile analyses

Bile total lipids were extracted into propanol-2 according to Férézou et al. (23), and bile total cholesterol and phospholipids were measured using enzymatic kits and a Beckman DU-530 spectrophotometer.

Bile samples were diluted (1:1) into propanol-2, and the total bile acid concentrations were determined by the method of Turley and Dietschy (35) in a Uvicon 930 ultraviolet/visible spectrophotometer (Kontron Instruments, Ltd., Watford, Hertfordshire, UK). The individual bile acid concentrations were assayed by GLC as described previously (24). The lithogenic indices were calculated according to Carey (36).

### Calculations and data analyses

Results are presented as means  $\pm$  SEM. Statistical analysis was performed by two-way ANOVA for diet and IRA effects using the software package Statview 5.0 (SAS Institute, Inc., Cary, NC).

## RESULTS

### Physiological and organ weight data

Pigs remained in good health throughout the experimental period. Daily food intake was higher ( $P < 0.05$ ) in BL- than in CAS-fed pigs, but it did not influence the daily weight gain and the final body weight of pigs (Table 2). Total dietary fiber intake was also higher ( $P < 0.001$ ) in BL- than in CAS-fed pigs, but cholesterol intake was not different between treatments. Surgery had no significant effect on these parameters. Finally, relative liver weights were lighter ( $P < 0.05$ ) in BL- than in CAS-fed pigs.

### Fasting plasma lipids and lipoproteins

Plasma total cholesterol concentration was decreased ( $P < 0.01$ ) in BL- compared with CAS-fed pigs because of a lower ( $P < 0.01$ ) LDL-cholesterol concentration (Table 3). The LDL- to HDL-cholesterol ratio was also lower ( $P < 0.05$ ) in BL-fed pigs. A tendency to lower LDL-cholesterol ( $P = 0.07$ ) in IRA compared with INT pigs was also observed. All other lipid parameters were unaffected by the treatments (Table 3).

### Liver lipids, enzymatic activities, and LDL receptor abundance

The BL tended to decrease ( $P = 0.07$ ) hepatic free cholesterol and decreased ( $P < 0.001$ ) esterified and total cholesterol levels ( $-56\%$  and  $-20\%$ , respectively) compared with the CAS, but surgery did not affect the liver lipids (Table 4). HMG-CoA reductase specific and total activities

TABLE 2. Physiological data and relative organ weights in INT and IRT pigs fed cholesterol-rich CAS or BL for 3 weeks

Weight and Intake	CAS		BL		ANOVA	
	INT	IRA	INT	IRA	Diet	IRA
Final weight (kg)	41.8 ± 3.4	38.0 ± 3.0	43.7 ± 3.0	42.3 ± 1.1	NS	NS
Daily weight gain (g/day) <sup>a</sup>	537.3 ± 127.7	390.7 ± 76.8	606.5 ± 111.5	458.8 ± 98.7	NS	NS
Daily food intake (g DM/day/kg BW) <sup>a</sup>	37.0 ± 3.1	37.9 ± 1.8	43.6 ± 2.6	42.2 ± 1.6	*	NS
Total dietary fiber intake (g/day/kg BW) <sup>a</sup>	3.39 ± 0.19	3.31 ± 0.21	6.55 ± 0.40	6.65 ± 0.31	***	NS
Cholesterol intake (g/day/kg BW) <sup>a</sup>	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	NS	NS
Liver (g/kg BW)	21.7 ± 0.6	21.5 ± 1.0	19.6 ± 1.3	18.5 ± 0.9	*	NS
Gallbladder (g/kg BW)	0.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	NS	NS

BW, body weight; INT, intact; IRA, ileorectal anastomosed. Values shown are means ± SEM (n = 6, except for CAS-IRA, where n = 5). Significance: \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ . No significant interactions between diet and IRA effects were recorded.

<sup>a</sup>Values reported to the 5 day collection period.

were 16- and 20-fold higher, respectively ( $P < 0.001$ ), in BL- than in CAS-fed pigs. CYP7A1 activities were not affected by diet and surgery, but CYP27A1 total activity was lower ( $P = 0.05$ ) in IRA than in INT pigs. Finally, LDL receptor level per liver was ~2-fold higher ( $P < 0.001$ ) in BL- than in CAS-fed pigs.

### Biliary lipids and bile acids

Diet and surgery had no effect on gallbladder bile total cholesterol and phospholipid concentrations. The biliary bile acid concentration tended to be increased ( $P = 0.07$ ) by the BL and to be decreased ( $P = 0.08$ ) by surgery (Table 5). The bile acid content of gallbladder bile was increased significantly ( $P < 0.01$ ) by the diet but was not affected by the surgery (CAS-INT, 1,960 ± 419 mg; CAS-IRA, 2,277 ± 319 mg; BL-INT, 4,824 ± 988 mg; BL-IRA, 3,898 ± 910 mg). The cholesterol content of gallbladder bile was not affected by the treatments (CAS-INT, 30.5 ± 4.8 mg; CAS-IRA, 46.4 ± 10.1 mg; BL-INT, 52.3 ± 11.2 mg; BL-IRA, 55.2 ± 14.1 mg).

As usual in pigs, the cholic acid synthesis pathway (cholic plus deoxycholic acids) was not effective, and the chenodeoxycholic pathway represented ~99% of total bile acids in all treatments. Diet and surgery modified the biliary bile acid composition. Primary bile acids were increased ( $P < 0.05$ ) in BL- compared with CAS-fed pigs

through an increase in the major primary bile acid, hydrocholic acid. The cecum-colon bypass markedly reduced ( $P < 0.001$ ) the secondary bile acids, mostly hyodeoxycholic acid, leading to an increase ( $P < 0.01$ ) in the primary-to-secondary bile acid ratio (Table 5). Hydrophobic bile acids (chenodeoxycholic, deoxycholic, lithocholic, 3 $\alpha$ -hydroxy-6-oxo-5 $\beta$ -cholanoic, and 7-oxo-lithocholic acids and other ketones) were not significantly affected by the treatments and represented 36, 32, 31, and 26% of total bile acids in CAS-INT, CAS-IRA, BL-INT, and BL-IRA pigs, respectively.

When calculated by the method of Carey (36), the lithogenic index was ~36% lower ( $P < 0.05$ ) in BL- than in CAS-fed pigs (CAS-INT, 0.52 ± 0.10; CAS-IRA, 0.71 ± 0.15; BL-INT, 0.33 ± 0.03; BL-IRA, 0.46 ± 0.08).

### Fecal and ileal digesta steroid output

The daily neutral sterol output was higher ( $P < 0.001$ ) in BL- than in CAS-fed pigs. The bulk of this output consisted of cholesterol, and its microbial transformation was markedly reduced ( $P < 0.01$ ) by surgery (Table 6). Similar results were obtained in the microbial transformation of dietary  $\beta$ -sitosterol into  $\beta$ -coprosterol, which reached 27% and 28% of total  $\beta$ -sitosterol in CAS-INT and BL-INT pigs, respectively, and was undetectable in IRA pigs. The daily bile acid output was not affected by

TABLE 3. Plasma and lipoprotein lipid concentrations in INT and IRA pigs fed cholesterol-rich CAS or BL for 3 weeks

Lipid	CAS		BL		ANOVA	
	INT	IRA	INT	IRA	Diet	IRA
	<i>mmol/l</i>					
Triacylglycerols	0.65 ± 0.04	0.62 ± 0.05	0.63 ± 0.12	0.77 ± 0.11	NS	NS
Phospholipids	1.86 ± 0.19	1.80 ± 0.19	1.80 ± 0.18	1.77 ± 0.16	NS	NS
Free cholesterol	0.73 ± 0.06	0.64 ± 0.05	0.70 ± 0.10	0.72 ± 0.09	NS	NS
Total cholesterol	4.52 ± 0.22	3.74 ± 0.27	3.20 ± 0.38	3.10 ± 0.36	**	NS
LDL-cholesterol	2.69 ± 0.16	1.95 ± 0.28	1.67 ± 0.20	1.62 ± 0.14	**	0.07
HDL-cholesterol	1.36 ± 0.09	1.33 ± 0.14	1.18 ± 0.16	1.22 ± 0.20	NS	NS
	<i>ratio</i>					
LDL/HDL <sup>a</sup>	2.01 ± 0.14	1.63 ± 0.29	1.45 ± 0.08	1.42 ± 0.14	*	NS

Values shown are means ± SEM (n = 6, except for CAS-IRA, where n = 5). Significance: \*  $P < 0.05$ ; \*\*  $P < 0.01$ . No significant interactions between diet and IRA effects were recorded.

<sup>a</sup>LDL- to HDL-cholesterol ratio.

TABLE 4. Liver lipid concentrations, enzymatic activities, and LDL receptor abundance in INT and IRA pigs fed cholesterol-rich CAS or BL for 3 weeks

Parameter	CAS		BL		ANOVA	
	INT	IRA	INT	IRA	Diet	IRA
	<i>mg/g</i>					
Free cholesterol	4.13 ± 0.22	4.06 ± 0.10	3.69 ± 0.20	3.75 ± 0.24	0.07	NS
Esterified cholesterol	1.16 ± 0.07	1.07 ± 0.09	0.47 ± 0.08	0.51 ± 0.02	***	NS
Total cholesterol	5.29 ± 0.25	5.13 ± 0.14	4.16 ± 0.20	4.26 ± 0.24	***	NS
Triacylglycerols	10.75 ± 1.58	9.96 ± 0.60	11.35 ± 1.08	10.79 ± 1.92	NS	NS
Phospholipids	20.99 ± 0.70	20.78 ± 0.24	20.28 ± 0.37	20.83 ± 0.69	NS	NS
	<i>pmol/min/mg protein</i>					
HMG-CoA reductase	2.05 ± 0.37	1.80 ± 0.39	40.74 ± 10.30	20.55 ± 4.93	***	NS
	10,736 ± 1,336 <sup>a</sup>	11,422 ± 2,645 <sup>a</sup>	278,890 ± 61,656 <sup>a</sup>	176,149 ± 47,378 <sup>a</sup>	***	NS
Cholesterol 7 $\alpha$ -hydroxylase	21.50 ± 4.87	20.48 ± 4.88	19.82 ± 0.50	18.50 ± 1.73	NS	NS
	126,085 ± 34,948 <sup>a</sup>	115,789 ± 39,376 <sup>a</sup>	146,755 ± 27,192 <sup>a</sup>	116,101 ± 16,830 <sup>a</sup>	NS	NS
Sterol 27-hydroxylase	59.68 ± 7.40	33.12 ± 7.08	45.10 ± 11.66	39.36 ± 5.46	NS	0.06
	285,436 ± 53,312 <sup>a</sup>	195,098 ± 39,812 <sup>a</sup>	292,795 ± 41,923 <sup>a</sup>	192,932 ± 37,202 <sup>a</sup>	NS	0.05
	<i>arbitrary units/g protein</i>					
LDL receptors	1.98 ± 0.23	2.45 ± 0.38	4.26 ± 0.49	5.38 ± 0.78	***	NS
	10,968 ± 2,284 <sup>b</sup>	11,631 ± 1,011 <sup>b</sup>	20,458 ± 2,815 <sup>b</sup>	22,839 ± 3,534 <sup>b</sup>	***	NS

Values shown are means ± SEM (n = 6, except for CAS-IRA, where n = 5). Significance: \*\*\*  $P < 0.001$ . No significant interactions between diet and IRA effects were recorded.

<sup>a</sup>These values are in pmol/min/organ.

<sup>b</sup>These values are in arbitrary units/organ.

diet but was 3.1-fold lower ( $P < 0.01$ ) in IRA than in INT pigs. With the exception of chenodeoxycholic acid, which was higher in BL- than in CAS-fed pigs, the outputs of primary and secondary bile acids were not affected by diet. The bulk of bile acid output was composed of secondary bile acids (mainly hyodeoxycholic), which represented 87% and 85% of total bile acids in BL- and CAS-fed pigs, respectively. As expected, the microbial transformation of bile acids was markedly reduced by surgery. This effect was mainly attributable to a 5.7-fold lower ( $P < 0.01$ ) hyodeoxycholic acid output. As a result, the primary-to-secondary

bile acid output ratio was higher ( $P < 0.01$ ) in IRA than in INT pigs. Finally, the total daily neutral and acidic steroid output was 55% higher ( $P < 0.01$ ) in the BL group than in the CAS group (Table 6).

## DISCUSSION

The present study examined for the first time the effects of a diet containing raw blue lupin seeds on the cholesterol metabolism and steroid output of INT and IRA

TABLE 5. Biliary lipid and total and individual bile acid concentrations in INT and IRA pigs fed cholesterol-rich CAS or BL for 3 weeks

Parameter	CAS		BL		ANOVA	
	INT	IRA	INT	IRA	Diet	IRA
	<i>mmol/l</i>					
Total cholesterol	4.01 ± 0.78	5.04 ± 1.22	3.06 ± 0.29	3.73 ± 0.55	NS	NS
Phospholipids	10.07 ± 2.55	9.73 ± 1.29	8.81 ± 1.40	6.02 ± 1.07	NS	NS
Bile acids	189.37 ± 22.56	143.80 ± 9.08	224.59 ± 27.63	189.64 ± 21.91	0.07	0.08
Cholic acid	0.44 ± 0.34	0.20 ± 0.15	1.40 ± 0.73	0.82 ± 0.24	NS	NS
Chenodeoxycholic acid	38.00 ± 7.98	34.66 ± 8.48	48.14 ± 7.92	33.87 ± 4.04	NS	NS
Hyocholeic acid	73.45 ± 30.45	95.04 ± 8.19	119.63 ± 25.25	138.08 ± 21.65	0.06	NS
Deoxycholic acid	1.59 ± 0.36	0.93 ± 0.12	1.09 ± 0.11	0.71 ± 0.17	0.08	*
Lithocholic acid	8.91 ± 3.72	7.04 ± 2.21	6.29 ± 0.97	5.49 ± 0.42	NS	NS
Hyodeoxycholic acid	50.45 ± 15.39	1.86 ± 0.44	34.80 ± 10.16	3.51 ± 0.81	NS	***
3 $\alpha$ -Hydroxy-6-oxo-5 $\beta$ -cholanoic acid	2.84 ± 2.07	0.00 ± 0.00	3.57 ± 3.02	0.00 ± 0.00	NS	NS
7-Oxo-lithocholic acid	6.51 ± 0.46	1.08 ± 0.32	4.75 ± 1.14	2.64 ± 0.96	NS	***
Other ketones	7.18 ± 1.96	2.99 ± 0.43	4.92 ± 0.77	4.52 ± 0.95	NS	*
	<i>ratio</i>					
Primary-secondary bile acids <sup>a</sup>	2.16 ± 0.93	10.69 ± 1.55	4.20 ± 1.28	12.70 ± 3.77	NS	**

Values shown are means ± SEM (n = 6, except for CAS-IRA, where n = 5). Significance: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ . No significant interactions between diet and IRA effects were recorded.

<sup>a</sup> Primary-to-secondary gallbladder bile acid ratio, in which primary acids are cholic, chenodeoxycholic, and hyocholic acids.

TABLE 6. Fecal and ileal digesta neutral and acidic steroid output in INT and IRA pigs fed cholesterol-rich CAS or BL for 3 weeks

Parameter	CAS		BL		ANOVA	
	INT	IRA	INT	IRA	Diet	IRA
	<i>mg/day/kg BW</i>					
Neutral sterols	59.6 ± 8.9	64.0 ± 3.2	100.6 ± 10.3	99.5 ± 12.1	***	NS
Cholesterol	52.1 ± 9.7	61.7 ± 3.3	85.9 ± 11.2	99.5 ± 12.1	**	NS
Microbial sterols	7.5 ± 2.2	2.3 ± 0.4	14.7 ± 5.4	0.0 ± 0.0	NS	**
Coprostanol	6.1 ± 1.9	1.6 ± 0.6	14.4 ± 5.2	0.0 ± 0.0	NS	**
Epicoprostanol	1.0 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NS	NS
Cholestanol	0.4 ± 0.2	0.7 ± 0.3	0.3 ± 0.3	0.0 ± 0.0	0.08	NS
Bile acids	30.1 ± 8.3	9.0 ± 1.4	38.1 ± 8.6	13.2 ± 3.8	NS	**
Chenodeoxycholic acid	0.4 ± 0.1	0.9 ± 0.4	2.1 ± 0.6	1.1 ± 0.4	*	NS
Hyochoolic acid	0.3 ± 0.1	3.3 ± 0.8	1.0 ± 0.3	3.4 ± 1.1	NS	**
Lithocholic acid	9.4 ± 3.4	0.5 ± 0.1	5.1 ± 1.1	2.2 ± 0.9	NS	**
Hyodeoxycholic acid	16.5 ± 5.8	1.3 ± 0.4	22.0 ± 5.4	5.5 ± 2.4	NS	**
3 $\alpha$ -Hydroxy-6-oxo-5 $\beta$ -cholanoic acid	0.9 ± 0.2	0.8 ± 0.2	1.5 ± 0.3	0.4 ± 0.2	NS	*
7-Oxo-lithocholic acid	2.0 ± 1.1	1.7 ± 0.9	5.5 ± 2.2	0.5 ± 0.2	NS	0.07
Other ketones	0.6 ± 0.2	0.5 ± 0.2	0.9 ± 0.2	0.1 ± 0.1	NS	*
	<i>ratio</i>					
Primary-secondary bile acids <sup>a</sup>	0.02 ± 0.01	0.97 ± 0.39	0.08 ± 0.01	0.90 ± 0.32	NS	**
	<i>mg/day/kg BW</i>					
Total neutral and acidic steroids	89.7 ± 11.9	73.0 ± 8.0	138.7 ± 9.4	112.7 ± 14.0	**	0.08

BW, body weight. Values shown are means ± SEM (n = 6, except for CAS-IRA, where n = 5). Significance: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ . No significant interactions between diet and IRA effects were recorded.

<sup>a</sup> Primary-to-secondary bile acid output ratio, in which primary bile acids are chenodeoxycholic and hyochoolic acids.

growing pigs made hypercholesterolemic by diet. The pigs remained in good health throughout the study, and final weight and daily weight gain were not influenced by diet and surgery. The daily food intake, higher in BL- than in CAS-fed pigs, was not affected by the blue lupin seed total alkaloid concentration (0.3 g/kg), which is lower than the concentration shown to depress feed intake in pigs (6).

Plasma total cholesterol levels in pigs fed the cholesterol-rich CAS were 56% higher than those in other Duroc × (Large White × Landrace) pigs fed a diet with no added cholesterol [2.64 ± 0.08 mmol/l (34)] but were similar to those observed by Férézou et al. (23) in pigs fed a cholesterol-rich (3 g/kg) CAS. Compared with the CAS-fed pigs, the BL-fed pigs had lower plasma total cholesterol (−24%) because of a substantial reduction in the LDL-cholesterol fraction (−29%), which also influenced the LDL- to HDL-cholesterol ratio. This hypocholesterolemic effect was reported previously in rats (37) and chickens (38) fed whole blue lupin seed meal and in humans consuming lupin fiber-based products (10). The LDL-cholesterol reduction observed by feeding legumes by feeding legumes or their fractions to normocholesterolemic and hypercholesterolemic subjects (2–4, 39) could result from reduced LDL synthesis and/or increased LDL metabolism (3, 40). The latter mechanism was observed in the present study, where increased LDL receptor abundance was detected in BL-fed pigs.

Dietary cholesterol intake was not different regardless of the treatment. Cholesterol synthesis in CAS-fed pigs, as determined by hepatic HMG-CoA reductase activity, was similar to that observed in pigs fed a cholesterol-rich CAS but lower than that observed in pigs fed a diet with no

added cholesterol (23). However, this synthesis was increased dramatically in BL-fed pigs. These pigs, consuming cholesterol-rich diets, should have had limited endogenous cholesterol synthesis levels. Therefore, the marked increase in hepatic cholesterol synthesis and the reduction in the level of cholesteryl esters observed in the BL-fed pigs suggest that cholesterol intestinal absorption was decreased in these pigs compared with CAS-fed pigs. In agreement with this suggestion, the elimination of neutral sterols from the body of pigs, mainly in the form of cholesterol, was 60% higher in the BL-fed animals. This dietary effect was probably modulated by total phytosterol intake, which was 4.1-fold higher in BL- than in CAS-fed pigs (1.72 vs. 0.42 g/day).

Studies reported by Hicks and Moreau (41), Carr et al. (42), and de Jong, Plat, and Mensink (43) showed that reduced plasma total and LDL-cholesterol in humans and animals consuming phytosterols or phytostanols were observed within 2 weeks and at relatively low dosage consumption [1–1.5 g/day; reviewed by Hicks and Moreau (41)]. These bioactive components interfere with the uptake from the intestinal tract of both dietary and endogenous (biliary) cholesterol, apparently through: *i*) the displacement of cholesterol from mixed micelles as a result of their higher hydrophobicity (44), generally considered the primary mechanism (42); *ii*) modulation of the expression of the intestinal bile acid transporter *ABCA1*, *ABCG5*, and *ABCG8* genes in the enterocyte, with effects in the cholesterol absorption not yet quantified (13, 43); and/or *iii*) reduction in the esterification rate of cholesterol within the enterocyte, thus decreasing the amount of this sterol incorporated into chylomicrons (45). This inhi-

bition of cholesterol intestinal absorption led to a compensatory increase in liver de novo cholesterol synthesis and LDL receptor abundance, as observed previously (46, 47), but the net effect was still a reduction in plasma total cholesterol. Furthermore, the key enzymes involved in bile acid synthesis and bile acid output (an estimation of total bile acid synthesis) were not modified by the diets. This suggests that the BL reduced specifically the cholesterol intestinal absorption and that this was sufficient to decrease cholesterolemia. This effect was also observed in human and hamsters receiving dietary phytosterols (42, 47, 48).

The whole blue lupin seed contains high amounts of protein and dietary fiber that could also modulate the intestinal absorption of sterols and thus cholesterolemia. Vegetable proteins have been related to increased fecal output of neutral and acidic steroids (48–50) and in vitro bile acid binding (51). Soluble dietary fiber, which is 5-fold higher in the BL than in the CAS, is also known to increase the fecal output of cholesterol and bile acids by sequestration in gels (52, 53) and of bile acids by binding affinities (54). These bioactive components could play a role in the neutral sterol output in BL-fed pigs, but an effect through a complexation of bile acids seems more unlikely in this study, in which the bile acid output was not affected by diet. Furthermore, the bile acid content of the gallbladder bile was almost 2-fold higher in BL- than in CAS-fed pigs. If one assumes that this bile acid content at slaughter is close to the bile acid pool, its size is related to hepatic bile acid synthesis and to intestinal bile acid reabsorption (55). Because bile acid synthesis, as evaluated by daily fecal excretion and by hepatic enzymatic activities, was not modified by diets and was reduced by surgery, the bile acid content of the gallbladder in BL-INT and BL-IRA pigs was related to intestinal reabsorption. This indicates that BL has stimulated intestinal bile acid reabsorption, even in the IRA pigs. Dietary components such as normal or amylo maize starches are known to markedly stimulate the ileal absorption of taurocholate in the rat (56). Moreover, in BL-fed pigs, the high level of the biliary trihydroxylated hyocholic acid that was poured into the duodenum could stimulate its absorption by bile acid transporters (14, 57). Taken together, these data suggest that in the BL-fed pigs, a specific increase in bile acid intestinal absorption could also prevent cholesterol absorption by modifying its micellar solubilization, as was suggested for the BL phytosterols. Thus, blue lupin seed stimulates the fecal elimination of cholesterol by means of at least two factors, phytosterols and bile acid reabsorption, both acting in the same way.

The IRA did not modify plasma cholesterol and the other cholesterol parameters, with the exception of the microbial transformation of sterols in CAS- and BL-fed pigs. The cecum-colon is a known site of free bile acid absorption (14) and microbial fermentation of undigested food or endogenous secretions. Its bypass should have increased the fecal output of acidic steroids and decreased their contents in the gallbladder. Yet, IRA pigs unexpectedly excreted less bile acids than did INT pigs, and their

gallbladder bile acid content was not significantly affected. As reported previously by Laplace (58), partial resection of the small intestine of pigs can stimulate an overdevelopment of the remaining intestine, which balanced the loss. Thus, surgery may have influenced the reabsorption of bile acids through morphometric changes in the small intestine. Salgado et al. (59), when comparing IRA to INT pigs, observed that IRA pigs presented an increased absorptive area in the mid distal section of the small intestine, a section in which bile acid reabsorption is very important (14, 60). This reabsorption in IRA pigs could have been facilitated by two other factors: *i*) a higher biliary content in the trihydroxylated hyocholic acid, actively absorbed in the small intestine (61); and *ii*) a lower impact of the intestinal microflora on bile acids, reducing their deconjugation and dehydroxylation and thus increasing their absorption by the intestinal bile acid transporter (57).

The microbial transformation of cholesterol and  $\beta$ -sitosterol, as determined in feces, was not affected by the diets. This transformation was low in BL-INT (15% and 28% of total neutral sterols and  $\beta$ -sitosterol, respectively) and CAS-INT (13% and 27%) pigs. This was probably attributable to a deleterious effect in this microbial activity modulated by the high levels of dietary cholesterol in the intestinal lumen, as observed previously in pigs fed cholesterol-rich diets (23, 25). Surgery almost completely prevented the microbial transformation of these sterols but not the transformation of nonabsorbed bile acids. The secondary bile acid output in IRA pigs reached 61% of total bile acids and 9% of biliary bile acids. These results agree with those reported by Martins et al. (34) in IRA pigs and by Hakala et al. (62) in ileoanal anastomosed patients. The content of secondary bile acids in IRA pigs compared with INT pigs suggests a colonization of their distal small intestine by a microbial population more able to deconjugate and dehydroxylate bile acids than to reduce the cholesterol double bond. Thus, the cecum and the colon have no significant effect on cholesterol metabolism in the pig.

In conclusion, feeding whole blue lupin seeds to pigs for 3 weeks exerted a marked hypocholesterolemic effect. This effect was mainly the consequence of a marked decrease in the intestinal absorption of cholesterol, probably modulated by bile acid reabsorption and blue lupin phytosterols. Bile acid metabolism was stimulated by blue lupin consumption. As observed previously with other legumes such as peas (34), ileorectal anastomosis did not modify cholesterol metabolism, which suggests that the cecum and the colon are poorly involved in this metabolism. Thus, being an accepted food item among Mediterranean populations, blue lupin seems to have high potential as a functional food to prevent hypercholesterolemia and cardiovascular diseases. Nevertheless, the relationship between blue lupin components and cholesterol metabolism deserves to be investigated further. ■

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