Increasing ursodeoxycholic acid in the enterohepatic circulation of pigs through the administration of living bacteria

Pascale Lepercq¹, Dominique Hermier², Olivier David³, Rachel Michelín¹, Clotilde Gibard¹, Fabienne Beguet¹, Purification Relano⁴, Chantal Cayuela⁴ and Catherine Juste¹*⁵

¹Unité d’Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France
²Laboratoire d’Endocrinologie de la Nutrition, Université Paris XI, 91405 Orsay Cedex, France
³Unité Mathématiques et Informatique Appliquées, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France
⁴Danone Vitapole, Nutrivaleur, Groupe Probioctiques et Fonctions Digestives, Route Départementale 128, 91767 Palaiseau Cedex, France

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We investigated the feasibility of increasing ursodeoxycholic acid (UDCA) in the enterohepatic circulation of pigs by administering living bacteria capable of epimerising endogenous amidated chenodeoxycholic acid (CDCA) to UDCA. We first demonstrated that combining Bifidobacterium animalis DN-173 010, as a bile salt-hydrolysing bacterium, and Clostridium absonum ATCC 27555, as a CDCA to UDCA epimerising bacterium, led to the efficient epimerisation of glyco- and tauro-CDCA in vitro, with respective UDCA yields of 55·8 (SE 2·8) and 36·6 (SE 1·5)%. This strain combination was then administered to hypercholesterolaemic pigs over a 3-week period, as two daily preprandial doses of either viable (six experimental pigs) or heat-inactivated bacteria. In bile, UDCA did not increase significantly, but the increase in biliary lithocholic acid with time in the controls was not observed in the experimental pigs (P = 0·035) and UDCA (P < 0·0001) absorbed into the portal vein, which increased 1·6–1·7- and 3·5–7·5-fold, respectively, under administration of living compared with inactivated bacteria. In bile, UDCA did not increase significantly, but the increase in biliary lithocholic acid with time in the controls was not observed in the experimental pigs (P = 0·035) and UDCA (P < 0·0001) absorbed into the portal vein, which increased 1·6–1·7- and 3·5–7·5-fold, respectively, under administration of living compared with inactivated bacteria. 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Bile acids: Ursodeoxycholic acid: Probiotics: Blood lipids

Ursodeoxycholic acid (UDCA, 3α,7β-dihydroxy-5β-cholan-24-oic acid) is a secondary (i.e. derived from bacterial metabolism in the intestine) bile acid of naturally low occurrence in man and pig, normally representing less than 3–4% of total biliary and faecal bile acids (van Gorkom et al. 2002; Woollett et al. 2003). Administered orally, UDCA has important therapeutic uses related to its ability to solubilise small radio-transparent gallstones (Bachrach & Hofmann, 1982) and to improve liver function in cholestatic diseases (Colombo et al. 1990; Podda et al. 1990; Combes et al. 1995). Moreover, UDCA is generally considered a chemopreventive agent. Indeed, supplemental dietary UDCA significantly reduces the incidence of experimental hepatic (Oyama et al. 2002) and colonic (Earnest et al. 1993; Seraj et al. 1997; Battag et al. 1998; Wali et al. 2002) carcinogenesis in rats. In some cases, UDCA has also been found to decrease intestinal absorption of cholesterol in rats (Raicht et al. 1978), mice (Reynier et al. 1981) and human subjects (Ponz de Leon et al. 1980), and to decrease blood cholesterol in rats (Shimizu et al. 1992) and human subjects (Poupon et al. 1993). UDCA at therapeutic doses (13–15 mg/kg per d) has no demonstrated adverse effect on human health.

UDCA is the bacterial 7β-OH epimer of the primary (i.e. synthesised by the liver) bile acid chenodeoxycholic acid (CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic acid). Epimerisation proceeds in two subsequent steps: oxidation of the 7α-hydroxy group of CDCA by a 7α-hydroxysteroid dehydrogenase (7α-HSDH), giving the intermediate product 7-keto-lithocholic acid (7-KLCA), and stereospecific reduction of the 7-keto functionality by a 7β-HSDH, thus giving UDCA (Fedorowski et al. 1979; Sarva et al. 1980; Hirano et al. 1981). Bacterial hydrolysis of the amide bond of conjugated CDCA, resulting in the release of its unconjugated homologue and the amino acid moiety, is a prerequisite to its epimerisation to UDCA.

In the present work, we hypothesised that conversion of CDCA into UDCA by living ingested bacteria could represent a new probiotic activity, useful in preventing and/or treating bile/liver-linked disease.

Abbreviations: BHI, Brain Heart Infusion medium; CDCA, chenodeoxycholic acid; CFU, colony-forming units; GCDCA, glycochenodeoxycholic acid; 7α-HSDH, 7α-hydroxysteroid dehydrogenase; 7β-HSDH, 7β-hydroxysteroid dehydrogenase; 7-KLCA, 7-keto-lithocholic acid; LCA, lithocholic acid; MRS, Man Rogosa and Sharp broth; TCDCA, taurochenodeoxycholic acid; UDCA, ursodeoxycholic acid; YE, yeast extract.

* Corresponding author: Dr Catherine Juste, fax +33 1 34 65 24 92, email juste@jouy.inra.fr
pathologies, in protecting the intestinal mucosa or even in lowering plasma cholesterol. The expected progressive delivery of UDCA by the bacterial metabolism compared with an exogenous bolus administration might favour its increased passive intestinal absorption, since slowing the arrival of UDCA in the intestinal lumen by enteric-coated formulations has been shown to improve absorption (Simoni et al. 1995). The present study was therefore designed to investigate the feasibility of increasing UDCA in the enterohepatic circulation of pigs and decreasing blood cholesterol by administering living bacteria. Since CDCA is mainly in a conjugated form before it enters the colon (with ratio of glycine:taurine conjugation averaging 3:1 in humans and pigs), its early conversion to UDCA by ingested bacteria requires both hydrolysing and epimerising bacterial activities, which were first assayed in vitro.

**Materials and methods**

**Bacterial strains and media**

The strain *Clostridium absonum* ATCC 27555, used in the present study, was originally isolated from soil (Hayase et al. 1974) and was kindly donated by Dr J. P. Carlier (Institut Pasteur, Paris, France). It is known to efficiently epimerise unconjugated CDCA to UDCA in vitro (Macdonald et al. 1981), whereas no safe food micro-organism is hitherto known to carry out this activity (Lepercq et al. 2004b). *C. absonum* ATCC 27555 was preferred to eleven other epimerising strains isolated from human faeces (Lepercq et al. 2004a,b) for its high and reproduceable activity and high biomass yield in a conventional culture medium. Its activity on conjugated CDCA forms remains unknown and was therefore evaluated in the present work.

The strain *Bifidobacterium animalis* DN-173 010 was from the stock culture collection of Danone Vitapole (Palaiseau, France) and was originally isolated from fermented milk. It was chosen from thirty-eight previously tested strains (lactobacilli and bifidobacteria, results not shown) for its effectiveness in deconjugating glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDDA) in culture and its extensive use in commercially available fermented milks. Moreover, we recently demonstrated its ability to hydrolyse bile salts in the intestine of pigs (Lepercq et al. 2004c).

Both micro-organisms were cultured under anaerobic atmosphere according to the Hungate (1969) technique. *B. animalis* was cultured in Man Rogosa and Sharp (MRS) broth supplemented with 0·5 g L-cysteine/l at pH 6·2 and 37°C. *C. absonum* was cultured in Brain Heart Infusion (BHI) medium supplemented with 5 g yeast extract (YE)/l, 0·5 g L-cysteine/l and 0·01 g hemine/l at pH 7·4 and 37°C.

**In vitro assays**

All bile acids used as substrates or standards were from Steraloids (Newport, RI, USA). Bacterial cells were collected in the exponential growth phase, at a time when both micro-organisms were highly active. This corresponded to 7 h of culture in MRS broth for *B. animalis* (Lepercq et al. 2004c) and 4·5 h of culture in BHI–YE medium supplemented with sodium salt of CDCA (final concentration 10⁻⁴ M) as an inductor of 7α- and 7β-HSDH for *C. absonum* (Macdonald et al. 1981). For each micro-organism, cells were harvested by centrifugation (6000g for 10 min at 10°C) from 5 ml culture, and re-suspended in 2 ml sterile anaerobic saline solution. An aliquot of each cell suspension (500 µl) was transferred to a tube containing 1 ml sterile saline solution supplemented with sodium salts of either CDCA, GCDCA or TCDDA (Steraloids) at a final concentration of 10⁻⁴ M. Control assay mixtures were prepared in the same way with cell suspensions that had been autoclaved for 20 min at 121°C. The tubes were flushed with O₂-free N₂ and incubated at 37°C for 3 h without stirring. The reaction was then stopped by the addition of 1 M-HCl until pH 2·0 was reached, and bile acids were extracted three times with 5 ml diethyl ether. The combined solvent fractions were evaporated to dryness and the bile acid extract was reconstituted with 2 ml methanol and stored at −20°C until analysis.

**In vivo assays**

*Bacterial inocula.* Every single dose of bacteria for animal treatment was prepared as follows: 450 ml MRS broth was inoculated with an 8-h-old culture of *B. animalis* (50ml), and 450 ml 10⁻⁴ M–CDCA–BHI–YE broth was inoculated with an 8-h-old culture of *C. absonum* (50 ml). After 7 and 4·5 h of cultivation, respectively, bacteria were harvested by centrifugation (6000g for 10 min at 10°C). The cell pellets, corresponding to ≈3·5 × 10¹¹ colony-forming units (CFU) for *B. animalis* and ≈2·2 × 10¹¹ CFU for *C. absonum*, were then rapidly re-suspended in 20 ml fresh anaerobic MRS and CDCA-devoid BHI–YE broth, respectively. Bacterial suspensions were aspirated into sterile syringes and immediately administered to the pigs. For control experiments, the cultures were killed by autoclaving for 20 min at 121°C before centrifugation.

*Animals.* Twelve healthy castrated Large White pigs (41·3 (SEM 2·0) kg) were obtained from the SCEA Stock Farm (Société Commune d’Exploitation Agricole, Antran, France) and fed a semi-purified diet (10·00 % butter, 2·00 % rapeseed oil, 0·17 % cholesterol, 18·00 % hydrochloric casein, 42·60 % maize starch, 16·13 % sucrose, 7·00 % cellulose, 3·00 % mineral mix, 1·00 % vitamin mix and 0·10 % antioxidant in percentage of fresh matter; 18·547 kJ (4433 kcal)/kg diet) for 15 d before surgery. Antibiotics and other growth-promoting agents were excluded from the diet. This diet, in powdered form, was mixed with water (food:water 1:1) and provided throughout the experimental period in two equal meals of 700 g each at 08.30 and 15.30 hours. After a 24 h fast and under general anaesthesia and aseptic conditions, the pigs (51·8 (SEM 2·0) kg) were obtained from the SCEA Stock Farm (Société Commune d’Exploitation Agricole, Antran, France) and fed a semi-purified diet (10·00 % butter, 2·00 % rapeseed oil, 0·17 % cholesterol, 18·00 % hydrochloric casein, 42·60 % maize starch, 16·13 % sucrose, 7·00 % cellulose, 3·00 % mineral mix, 1·00 % vitamin mix and 0·10 % antioxidant in percentage of fresh matter; 18·547 kJ (4433 kcal/kg diet) for 15 d before surgery. Antibiotics and other growth-promoting agents were excluded from the diet. This diet, in powdered form, was mixed with water (food:water 1:1) and provided throughout the experimental period in two equal meals of 700 g each at 08.30 and 15.30 hours. After a 24 h fast and under general anaesthesia and aseptic conditions, the pigs (51·8 (SEM 2·0) kg) were surgically fitted with two blood catheters, one in the portal vein and another one in the external jugular vein, as well as with a lower choledo- cal and an upper duodenal catheter in order to exteriorise the enterohepatic circulation (Legrand-Defretin et al. 1986). Continuous 0·3 % bile collection was ensured by an automatic apparatus (Juste et al. 1983) throughout the experiment. After surgery, the pigs were housed in individual, metabolism-type restraining cages. They recovered completely from the operation with no complication and resumed a normal feeding pattern over the four postoperative days. The duodenal and portal vein catheters were kept patent by gently rinsing them daily with sterile water and a sterile dilute solution of heparin (10 IU/ml isotonic saline), respectively. All pigs received humane care in compliance with the Principles for Biomedical Research Involving Animals developed by the Council for International Organisations of Medical Sciences. The experimental protocol was approved by

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our institute in agreement with the US Department of Health and Human Services (identification no. #A5105-01), and conditions of housing were approved by the French Direction of Veterinary Services (Ministry of Agriculture).

Experimental design. Pigs were experimented upon in pairs, one pig from each pair being randomly assigned to treatment with living bacteria (experimental group, \( n = 6 \)) and the other to treatment with heat-inactivated bacteria (control group, \( n = 6 \)). They were housed in two separate experimental units to avoid cross-contamination. Assignment of the experimental and control pigs to the experimental units was permuted in the six trials, following aseptic clean out. From day 15 to 35 after surgery, the pigs received two daily doses of living or inactivated \( B. \) \textit{animalis} \((= 3.5 \times 10^3 \text{ CFU/dose}) + C. \) \textit{absonum} \((= 2.2 \times 10^3 \text{ CFU/dose})\) given intraduodenally immediately prior to each meal.

On the day before initiation of the bacterial treatment (day 14), then at weekly intervals during the 3 weeks of bacterial treatment (days 21, 28 and 35) and finally 1 week following cessation of treatment (day 42), 4 ml of portal blood was drawn into glass tubes at regular intervals of 2 h from 08.30 to 14.30 hours for the analysis of bile acids reabsorbed into the portal vein. This 6 h collection period covered the serum bile acid response to the first daily meal (Legrand-Defretin \textit{et al.} 1986). Blood samples were left to clot overnight at room temperature. After centrifugation \((1500 \text{ g} \) for 15 min at \( 15^\circ \text{C} \)), the separated sera were pooled to give a representative sample of postprandial portal serum for each pig and for each observation day. On the same days (days 14, 21, 28, 35 and 42), continuous 0.3 % bile collections were separated into two daily fractions corresponding to the same postprandial period as blood sampling and the remaining portion of the day–night cycle (i.e. from 14.30 to 08.30 hours the next morning), respectively. At weekly intervals, from the day before initiation of the experiment (day –15) to the end of the experiment (day 42), 7 ml of fasting portal blood was drawn from the jugular catheter into \( \text{Na}_2\text{EDTA} \) as an anticoagulant. Plasma was immediately separated by centrifugation at \( 4^\circ \text{C} \), then divided for lipid and lipoprotein analyses, and stored at \(-20^\circ \text{C}\) until analysis. Finally, stools were collected daily, weighed and frozen under vacuum throughout the entire experiment from day 8 to 42. They were pooled for the analyses to give a weekly representative sample for each pig.

Analytical methods

Bile acids from the resting cell assays. Bile acids were analysed by GLC without previous deconjugation, so that only unconjugated bile acids were volatilised and analysed at the chromatograph temperature. Bile acid extract \((50 \mu \text{l})\) was transferred to a tube and supplemented with of the external standard \(5a\)-cholestan \((5 \mu \text{l}; 0.5 \text{ g/l in hexane})\). Methylation was achieved by adding \(200 \mu \text{l} \text{diazomethane; tubes were stopped and kept overnight at room temperature. After treatment with dryness, the samples were silylated by adding \( \text{N,O-bis(trimethylsilyl)tri} \text{fluoroacetamide--trimethylchlorosilane} \) \((99-1; \text{v/v}) \) \(\text{Supelco, Saint Quentin Fallavier, France} \)) and \( \text{trimethylsilylimidazole--pyridine} \) \((1:4; \text{v/v}) \) \(\text{Supelco, France} \)). Trimethylsilyl derivatives were analysed using a Peri \(2000 \text{ gas chromatograph (Perichrom, Saulx-les-Chartreux, France))} \) equipped with a \( \text{R} \) \( \text{D} \) \( \text{I} \) \text{injector, a flame ionisation detector and an OV-1701 column, 30 m x 0.32 mm x 0.25 m} \) \((\text{Perichrom}) \). The chromatograph was used under isothermal conditions at \(250^\circ \text{C}\). He gas was used as the carrier. Individual GLC peaks were identified by relative retention time in relation to the external standard \(5a\)-cholestan. A pool of standards at different concentrations was chromatographed in parallel for bile acid identification and quantification. The percentage of \( \text{CDCA} \) and \( \text{TCDCA} \) epimerisation was calculated on the basis of the recovery of \( \text{CDCA} \), \( \text{UDCA} \) and \( \text{7-KLCA} \) from the resting cell assays with \( \text{CDCA} \), \( \text{GCDCA} \) and \( \text{TCDCA} \).

Serum bile acids. Portal serum \((200 \mu \text{l})\) was added drop by drop to \(20 \text{ volumes of ethanol, held in an ultrasonic bath. The resulting mixture was kept overnight at} \(-20^\circ \text{C}\), centrifuged \((1500 \text{ g} \) for 15 min at \(4^\circ \text{C})\), and then the deproteinised supernatant was diluted with an equal volume of water \((4 \text{ ml})\), adjusted to \( \text{pH} 100 \text{ with} \) \(1 \text{ mNaOH} \) and delipidated using \(3 \times 16 \text{ ml petroleum ether. The delipidated sample was evaporated to near dryness, the residue was re-dissolved in} \) \(8 \text{ ml sodium 0.1 M-acetate buffer (pH 5.0)} \) in an ultrasonic bath, and the final \( \text{pH} \) was adjusted to \(5.0\). The sample was then divided into two equal parts for analysis of total and unconjugated bile acids. For total or unconjugated bile acids analysis, \(4 \text{ ml acetate buffer containing or not containing cholyglycine hydrolyase} \) \((=4 \text{U/assay; Sigma, Saint Quentin Fallavier, France})\) and \( \beta \)-glucuronidase \((\text{type HP-2 from Sigma;} \) \(1 \mu \text{l/assay corresponding to} \geq 100 \text{ U} \beta \)-glucuronidase \(\text{and} \geq 7.5 \text{ U sulfatase}) \) was added, respectively. Enzymatic hydrolysis was carried out for \(72 \text{ h at} \) \(37^\circ \text{C} \). All samples were then acidified to \( \text{pH} 3.0 \) with \(1 \text{ M-HCl} \) and passed through a Lipidex™ \(1000 \text{ column (Packard Bioscience, Groningen, The Netherlands; bed size} 4.0 \text{ cm x} 1.1 \text{ cm})\), which had been prepared in methanol and pre-washed with \(0.01 \text{ M-HCl (pH 3.2)} \). After washing with \(0.01 \text{ M-HCl} \) and water, bile acids were eluted with methanol. The methanolic extract was analysed by GLC as described earlier, taking \(5a\)-cholestan as external standard.

Plasma lipids and lipoproteins. Plasma lipids were measured on an Abbott Diagnostics Spectrum CCX bichromatic analyser \((\text{Abbott-VP, Rungis, France})\), using specific enzymatic commercial kits \((\text{BioMérieux, Marcy-l’Etoile, France})\); Cholesterol RTU, Phospholipides Enzymatiques PAP 150 and Triglycérides Enzymatiques PAP 150 for total cholesterol, phospholipids and triacylglycerols, respectively \((\text{Richmond, 1973; Takayama} \text{ \textit{et al.} 1977; Fossati \& Prencipe, 1982})\). Free cholesterol was measured manually on \(20 \mu \text{aliquots using a commercial kit (CHOD-Method without prior hydrosis; Boehringer Mannheim, Meylan, France})\).

In preliminary assays where lipoprotein classes were separated by ultra-centrifugation in a density gradient, we found that \( \text{VLDL-cholesterol could be modified over the experiment whereas triacylglycerols were rather stable. Therefore, using the Friedewald formula (where \text{VLDL-cholesterol is estimated by} \text{triacylglycerols/5) to calculate} \text{LDL-cholesterol would have been hazardous and we decided to separate the different lipoprotein classes accurately by ultra-centrifuging each plasma sample. The gradient used was adapted from that of Ferezou \text{ \textit{et al.} (1997) by laying an additional 1/24-density NaBr solution (0.8 ml) at the bottom of the tube. As in the original method, 2 ml plasma aliquots were adjusted to} \text{d = 1.21 with solid KBr (0.325 g/ml). After a 24 h run at 200 000 g and} 15^\circ \text{C (L-60 centrifuge equipped with an SW41 rotor; Beckman, Palo Alto, CA, USA)}\), twenty-four 0.5 ml fractions were collected from the top of the tube and analysed for their total cholesterol content as described above. For each sample, the partition of cholesterol between \( \text{VLDL}, \text{LDL} \) and \( \text{HDL} \) was determined on the basis of the individual cholesterol profile in the gradient. Indeed, we observed intra-individual
variations in the limit between LDL and HDL, so that fixed volumes could not be assigned to these lipoprotein classes.

**Biliary lipids.** Total bile acids, phospholipids and cholesterol in bile were measured enzymatically using 3α-HSDH (EC 1.1.1.50; Sigma; Turley & Dietschy, 1978), the commercial Enzymatic Color Test (Labo Express Service, Vitry-Chatillon, France) and the CHOD-PAP kit (Boehringer Mannheim), respectively. For the determination of individual molecular species of bile acids, 100 μl of a 1:100 bile dilution in water was hydrolysed in 8 ml 0·1 M-acetate buffer (pH 5·0) containing cholyglycine hydrolase and β-glucuronidase/sulfatase, as described above for serum bile acids. The reactive mixture was then acidified and the unconjugated bile acids were recovered from a Lipidex™ 1000 column as before. The methanolic extract was analysed by GLC, taking 5α-cholestan as external standard.

**Faecal bile acids.** For each pig, faeces collected over 7d were thawed under vacuum, pooled and homogenised. Continuous extraction of 4 g aliquots of homogenised faeces, thoroughly mixed with a predetermined amount of deoxycholic acid as internal standard, was carried out for 48 h using 100 ml of boiling ethanol in a Soxhlet apparatus (Riottot et al. 1993). A 4 ml aliquot of the ethanolic extract was hydrolysed by adding 1 ml 10M-NaOH and heating the alkaline mixture to 120°C for 3 h under pressure. The hydrolysate was then cooled to room temperature, diluted with an equal volume of water (5 ml), and lipids were extracted out with 3×10 ml petroleum ether. The delipidated aqueous phase was then placed on ice, acidified to pH 2·0 with concentrated HCl, and bile acids were extracted with 3×10 ml ethylic ether. The combined solvent fractions were evaporated to dryness and the sample was reconstituted in 2 ml of methanol and analysed by GLC as above, taking 5α-cholestan as external standard. Daily faecal output of bile acids was calculated after correction for faecal flow, on the basis of a theoretical 90% recovery of dietary β-sitostanol, taking into account that this phytosterol is a reliable marker in swine (Marsh et al. 1972).

**Expression of results and statistical analyses**

The data for all blood, bile and faeces variables were analysed by the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). The model (with treatment and observation time arranged as a 2×5 factorial) included treatment, time and interaction between treatment and time in the fixed effects, and the interaction between treatment and pair in the random effects. The pair effects were not kept in the model because they were not significant. The variance components were estimated by REML. F tests were used for fixed effects. When a significant effect of treatment was detected (P<0.05), means were compared using t tests. Because of missing observations, the method of Kenward & Roger (1997) was used for all data. All results are reported as adjusted means with their standard error.

**Results**

**Activity of bacteria in vitro**

Results are summarised in Table 1. Resting cells of C. absonum alone essentially transformed free CDCA to UDCA, with a small yield of the intermediate 7-KLCA. C. absonum alone had no activity on GCDCA but efficiently deconjugated TCDCA. Table 1. Percentages of bile acids recovered from resting cell assays of Clostridium absonum ATCC 27555, Bifidobacterium animalis DN-173 010 and the combination of both strains, with chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA) or taurochenodeoxycholic acid (TCDCA) as the substrate (Values are means and standard errors of experiments done in triplicate).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CDCA</th>
<th>UDCA</th>
<th>7-KLCA</th>
<th>GCDCA</th>
<th>CDCA</th>
<th>UDCA</th>
<th>7-KLCA</th>
<th>TCDCA</th>
<th>CDCA</th>
<th>UDCA</th>
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<td>Mean</td>
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<td>4·2</td>
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<td>6·7</td>
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</tr>
<tr>
<td>C. absonum + B. animalis</td>
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<td>5·9</td>
<td>9·4</td>
<td>2·2</td>
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</table>

UDCA, ursodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); 7-KLCA, 7-keto-lithocholic acid (3α,7b-dihydroxy-5β-cholan-24-oic acid).
and partially epimerised the released unconjugated homologue to UDCA. As expected, resting cells of B. animalis alone revealed a very efficient bile salt-hydrolysing activity against both GCDDA and TCDCA. Conversely, this strain was unable to epimerise either substrate. When resting cells of C. absonum and B. animalis were combined, epimerisation of unconjugated CDCA was as efficient as in the presence of C. absonum alone. With this strain combination, GCDDA was totally deconjugated and the respective yields of UDCA and 7-KLCA were similar to those measured when starting from free CDCA. Finally, combining C. absonum and B. animalis increased deconjugation and epimerisation of TCDCA as compared with C. absonum alone. No transformation product could be detected in the control mixtures containing autoclaved cell suspensions (results not shown).

We therefore decided to combine both micro-organisms, for their respective bile salt-hydrolysing and epimerising activity, in the following in vivo assays.

Activity of bacteria in vivo

Surgery and treatment with living or heat-inactivated bacteria were well tolerated by all the animals. Missing values on day 28, 35 or 42 after surgery were due to rejection of bile and/or blood catheters in those growing pigs. Faeces data were, however, taken into account provided that collections could be completed over the first 4 d of the current experimental week, under normal conditions of the enterohepatic circulation.

Portal serum bile acids. Results are summarised in Table 2. Concentration of total and unconjugated bile acids and the respective proportions of the individual bile acid species in postprandial portal sera did not significantly differ between both groups of pigs before starting the bacterial treatments (day 14). Compared with inactivated bacteria, living bacteria significantly increased the proportion of unconjugated bile acid sodium of 1.6–1.7-fold. This was significant after 1 (day 21, \( P=0.027 \)), 2 (day 28, \( P=0.027 \)) and 3 (day 35, \( P=0.046 \)) weeks of treatment, but was no longer significant 1 week after cessation of treatment (day 42, \( P=0.276 \)). The main effect of treatment (\( P<0.0001 \)) on the distribution of the different bile acid species in portal blood was on UDCA, which increased 3.5–7.5-fold under administration of living compared with inactivated bacteria (\( P=0.016, 0.036 \) and 0.005 at day 21, 28 and 35, respectively). This increase was no longer significant 1 week after cessation of treatment (\( P=0.080 \) at day 42). A weak effect of treatment (\( P=0.042 \)) was also observed on hydroxydeoxycholic acid, which was slightly lower under treatment with living compared with inactivated bacteria. All other serum bile acids remained unaffected by treatment. For both groups of pigs, we observed a decrease in the proportion of hyocholic acid with time.

Biliary lipids. We separately analysed the two daily fractions of bile, collected over the same postprandial period as blood sampling and the remaining portion of the day–night cycle (from 14:30 to 08:30 hours the next morning), respectively. Statistical analyses led to the same conclusions whatever the period considered (results not shown). We therefore express the results per d, and summarise them in Fig. 1 and Table 3. Daily flows of bile and biliary lipids (bile acids, phospholipids and cholesterol) did not differ between both groups of pigs before initiation of treatments and remained unaffected by treatment throughout the experiment (Fig. 1). Nor was the biliary lipid equilibrium affected by treatment, as inferred from the steadiness of the respective molar percentages of bile acids, phospholipids and cholesterol (results not shown). Otherwise, we observed a highly significant (\( P<0.0001 \)) increase in bile flow and bile acid secretion rates over time in both groups of pigs (Fig. 1). When considering the distribution of the different bile acid species in bile (Table 3), the only significant effect of treatment was on lithocholic acid (LCA), which was clearly lower on days 35 (\( P=0.005 \)) and 42 (\( P=0.002 \)) in the experimental pigs. There was also a significant effect of time and a significant interaction between treatment and time on biliary LCA, which means that the increase of biliary LCA with time in the control group was not observed in the experimental group (Table 3).

Faecal bile acids. Excretion of total bile acids and distribution of bile acid species in faeces were not affected by treatment, although isolated differences could be detected: the increase in faecal LCA observed with time in the control group was not observed when living bacteria were administered (Table 4).

Plasma lipids and lipoproteins. The experimental diet was formulated in order to induce hypercholesterolaemia, which was the prerequisite to then demonstrate an eventual cholesterol-lowering effect of treatment. As expected, total cholesterol effectively increased after 15 d on the experimental diet in both groups of pigs, and this was essentially due to increased esterified cholesterol, as inferred from the moderate increase in the free cholesterol fraction (Fig. 2). At the same time, fasting values of plasma phospholipids, but not of triacylglycerols, increased to the same extent in both groups of pigs. Although surgery transiently depleted total cholesterol and phospholipid values, total cholesterol returned to pre-surgery levels and did not differ between both groups of pigs on day 14 after surgery, i.e. on the day before starting bacterial treatments. Thereafter, treatment did not modify the time course of plasma lipid levels, which remained the same up to day 42, whether living or inactivated bacteria were administered (Fig. 2).

When considering the distribution of cholesterol between the different lipoprotein classes (Fig. 3 (A)), LDL-cholesterol increased most strikingly with the experimental diet (+120 % day 0 v. day −15, \( P<0.0001 \)), with a smaller effect on HDL-cholesterol (+51 %, \( P=0.0593 \) and +59 %, \( P=0.0036 \) in experimental and control pigs, respectively) and VLDL-cholesterol (+39 %, \( P=0.0382 \) and +46 %, \( P=0.0371 \), respectively). Surgery depleted cholesterol in all lipoprotein fractions, but the effect on LDL-cholesterol was low so that it remained well above values observed before the experimental diet. On day 14, pre-treatment values of VLDL-, LDL- and HDL-cholesterol did not differ between both groups of pigs. Bacterial treatment with either living or inactivated bacteria did not significantly affect these values, or the LDL-cholesterol:HDL-cholesterol ratio (Fig. 3 (B)).

Discussion

We first studied hydrolysis and epimerisation of GCDDA and TCDCA by resting rather than growing cells of B. animalis and C. absonum in vitro, since exogenous bacteria are not considered to multiply during their transit in the intestine (Drouault et al. 2002). As expected, B. animalis alone was very efficient in hydrolysing both GCDDA and TCDCA. It was, however, unable to further transform free CDCA, which is in accordance with our previous results demonstrating that none of twenty-five growing...
Table 2. Effect of administering living (experimental pigs) or inactivated (control pigs) bacteria on bile acids reabsorbed into the portal vein

<table>
<thead>
<tr>
<th>Bile acids (µM)</th>
<th>% deconjugation</th>
<th>% UDCA</th>
<th>% 7-KLCA</th>
<th>% CDCA</th>
<th>% HCA</th>
<th>% HDCA</th>
<th>% LCA</th>
<th>% 6-KLCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
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<tr>
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<td>1.4</td>
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<td>2.0</td>
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<tr>
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<td>0.252</td>
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<td>0.801</td>
<td>0.673</td>
<td>0.640</td>
<td>0.905</td>
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</table>

UDCA, ursodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); 7-KLCA, 7-keto-lithocholic acid (3α-hydroxy-7-keto-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); HCA, hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid); HDCA, hydeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid); 6-KLCA, 6-keto-lithocholic acid (3α-hydroxy-6-keto-5β-cholan-24-oic acid).

a,bMean values with unlike superscript letters were significantly different (P < 0.05) over time within the same group, when an overall time effect was detected (P < 0.05).

c,dMean values with unlike superscript letters were significantly different (P < 0.05) between control and experimental groups at a given observation time, when an overall treatment effect was detected (P < 0.05).
Bile salt metabolism by bacteria in transit

Bifidobacterium strains could epimerise CDCA to UDCA (Lepercq et al. 2004b). Conversely, C. absonum alone efficiently epimerised free CDCA, whereas it was completely inefficient in transforming GCDCa, the main conjugated form of CDCA in human and pig bile. With TCDCA as the substrate, the UDCA yield was less than 30%. These results, originally obtained from resting cells of C. absonum in the present study, coincide with our previous assays on growing cells of the same strain or of other clostridia isolated from human intestinal microbiota (Lepercq et al. 2004a). The present study also clearly demonstrates that combining resting cells of B. animalis as a bile salt-hydrolysing bacterium, and C. absonum as a CDCA to UDCA epimerising bacterium, led to the efficient epimerisation of both GCDCa and TCDCA in vitro, with respective UDCA yields of about 60 and 40%. We therefore decided to test this bacterial strain combination in vivo.

This was directly assessed by the analysis of bile acids at different levels of the enterohepatic circulation of pigs, before and after the animals had been treated with living or heat-inactivated bacteria. Bacteria were administered via the duodenal route rather than per os, in order to avoid uncontrolled loss of viability in the stomach (Varnam & Sutherland, 1994). The main effects of treatment were on unconjugated bile acids and UDCA absorbed into the portal vein, which were both significantly increased following 1, 2 and 3 weeks of treatment with living compared with inactivated bacteria. The present study therefore confirms our previous results supporting the bile salt-hydrolysing activity of living B. animalis in the intestinal tract of pigs (Lepercq et al. 2004c), and further supports the in vivo epimerising activity of living C. absonum. Our measurements of bile acids secreted into the bile and lost in the faeces make it possible to estimate that approximately 100 mmol of bile acids were reabsorbed daily by the intestine and recycled via the portal vein. Of these, approximately 30% were recycled over the 6 h postprandial period of blood sampling, as inferred from the daily percentage of bile acids secreted into the bile over the same period of time (results not shown) and from the close coincidence between kinetics of portal absorption and biliary secretion of bile acids (Legrand-Defretin et al. 1986). From these data and from bile acid distribution in portal blood, bile acids absorbed in their unconjugated form over the 6 h postprandial period could be estimated as 8 mmol at baseline or under treatment with inactivated bacteria, and 14 mmol under treatment with living bacteria, approximately 30% of these being CDCA. The respective figures for UDCA recycling would approximate 0.4 and 2.4 mmol. It is therefore evident that epimerisation of free CDCA to UDCA, rather than hydrolysis of conjugated CDCA, was the rate-limiting step of the UDCA increase in the portal vein of the experimental pigs. The present experiment does not make it possible to determine whether this was due to rapid intestinal absorption and disappearance of the unconjugated CDCA substrate, or to weak activity and/or survival of C. absonum in the digestive tract of pigs. Indeed, survival of bifidobacteria during their transit in the intestine is well documented and seems to be high in man (Pochart et al. 1992; Chen et al. 1999; Fujiwara et al. 2001) and different animal models (Brown et al. 1997; Wang et al. 1999), whereas survival of exogenous clostridia remains unknown. In complementary assays (not reported), we estimated the number of total CDCA to UDCA epimerising bacteria in faeces freshly obtained from the rectum of pigs treated with either living or inactivated bacteria. The most probable number of these epimerising bacteria (without distinguishing between endogenous and exogenous ones) was estimated by the highest faecal dilution showing bacterial growth in CDCA-enriched BHI–YE broth associated with UDCA production, and was found to approximate 10^9 CFU/g of fresh faeces in both groups of pigs. This suggests that the administered clostridia were either unviable or metabolically inactive when excreted in faeces, but does not allow us to come to any conclusion on their survival and activity in the intestine. Both 7α- and 7β-dehydrogenase activities are known to be inducible by substrate or non substrate-specific bile acids (Macdonald & Roach, 1981; Sutherland & Williams, 1985). For this reason, we cultured C. absonum in the presence of the inducer CDCA. It is clear, however, that the discovery of the genes encoding for the 7β-dehydrogenase (four genes encoding for 7α-dehydrogenases have already been described (Baron et al. 1991; Yoshimoto et al. 1991; Coleman et al. 1994; Bennett et al. 2003)) will allow their over-expression in recipient bacteria, which might be used to stimulate the bioavailability of UDCA even more efficiently.

Interestingly, other bacterial metabolites of CDCA in the portal blood, i.e. 7-KLCA and LCA, remained unaffected by treatment. Otherwise, we found a weak effect of treatment on portal hydroxocholic acid, which was slightly lower under treatment with living compared with inactivated bacteria. This might be due to early hydrolysis of hydroxocholic acid by living bacteria in the small intestine, followed by its proximal absorption by passive
Table 3. Effect of administering living (experimental pigs) or inactivated (control pigs) bacteria on total bile acid concentration and molar percentages of individual bile acids in bile

(Values are means and standard errors)

<table>
<thead>
<tr>
<th>Bile acids (mM)</th>
<th>% UDCA</th>
<th>% 7-KLCA</th>
<th>% CDCA</th>
<th>% HCA</th>
<th>% HDCA</th>
<th>% LCA</th>
<th>% 6-KLCA</th>
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<tbody>
<tr>
<td>Day 14</td>
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<tr>
<td>Control (n 6)</td>
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<td>Experimental (n 6)</td>
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<tr>
<td>Control (n 2)</td>
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<td>26.7</td>
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<td>Experimental (n 2)</td>
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<td>Observation time</td>
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<td>0.079</td>
<td>0.798</td>
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UDCA, ursodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); 7-KLCA, 7-keto-lithocholic acid (3α-hydroxy-7-keto-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); HCA, hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid); HDCA, hydeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid); 6-KLCA, 6-keto-lithocholic acid (3α-hydroxy-6-keto-5β-cholan-24-oic acid).

a,b,cMean values with unlike superscript letters were significantly different (P < 0.05) over time within the same group, when an overall time effect was detected (P < 0.05).

x,yMean values with unlike superscript letters were significantly different (P < 0.05) between control and experimental groups at a given observation time, when an overall treatment effect was detected (P < 0.05).
diffusion (Aldini et al. 1996), so that this bile acid would be less exposed to 7α-dehydroxylation by the endogenous colonic microbiota.

Our estimations of UDCA flows in the portal vein indicated that an additional 2 mmol UDCA was presented to the liver under treatment with living bacteria over the 6 h postprandial period. This corresponds to the daily dose administered to patients under UDCA therapy (Fischer et al. 1993; Lindor et al. 1998; Invernizzi et al. 1999). However, contrary to what is generally observed in those patients, the additional UDCA entering the liver of pigs under treatment with living bacteria was not recovered in bile. This discrepancy could be attributable to differences in duration of treatment, hepatic extraction of UDCA and/or hepatic metabolism of UDCA between man and pig, or endogenously formed v. daily added exogenous UDCA. An additional assay (not reported) was therefore carried out in one pig, where

### Table 4. Effect of administering living (experimental pigs) and inactivated (control pigs) bacteria on total bile acid excretion and molar percentages of individual bile acids in faeces

(Values are means and standard errors)

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<td>3.2 ± 0.8</td>
<td>62.0 ± 6.5</td>
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<td>37.5 ± 6.4</td>
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<td></td>
<td>Control (n 6)</td>
<td>1.4 ± 0.4</td>
<td>1.7 ± 1.6</td>
<td>3.6 ± 0.5</td>
<td>2.2 ± 0.8</td>
<td>47.9 ± 6.5</td>
<td>42.8 ± 6.4</td>
<td>1.8 ± 0.5</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Experimental (n 6)</td>
<td>2.0 ± 0.4</td>
<td>1.4 ± 1.6</td>
<td>2.9 ± 0.5</td>
<td>4.4 ± 0.8</td>
<td>62.0 ± 6.5</td>
<td>27.7 ± 6.4</td>
<td>1.6 ± 0.5</td>
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<tr>
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<td>Control (n 5)</td>
<td>1.9 ± 0.4</td>
<td>1.3 ± 1.7</td>
<td>2.9 ± 0.6</td>
<td>1.8 ± 0.9</td>
<td>50.0 ± 7.0</td>
<td>42.5 ± 6.9</td>
<td>1.5 ± 0.5</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Experimental (n 6)</td>
<td>1.3 ± 0.4</td>
<td>6.0 ± 1.6</td>
<td>2.8 ± 0.5</td>
<td>2.2 ± 0.8</td>
<td>57.7 ± 6.5</td>
<td>29.0 ± 6.4</td>
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<td>0.5 ± 0.0</td>
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<td></td>
<td>Control (n 5)</td>
<td>1.5 ± 0.4</td>
<td>1.9 ± 1.7</td>
<td>3.3 ± 0.6</td>
<td>2.1 ± 0.9</td>
<td>51.5 ± 7.0</td>
<td>39.6 ± 6.9</td>
<td>1.6 ± 0.5</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Experimental (n 5)</td>
<td>1.1 ± 0.4</td>
<td>1.3 ± 1.7</td>
<td>2.9 ± 0.6</td>
<td>2.4 ± 0.9</td>
<td>55.7 ± 7.0</td>
<td>36.7 ± 6.9</td>
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<tr>
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<td>Control (n 4)</td>
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<td>1.2 ± 1.9</td>
<td>3.4 ± 0.6</td>
<td>1.6 ± 0.9</td>
<td>48.8 ± 7.7</td>
<td>43.6 ± 7.5</td>
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<td>Experimental (n 3)</td>
<td>1.8 ± 0.5</td>
<td>4.1 ± 2.2</td>
<td>2.1 ± 0.7</td>
<td>2.8 ± 1.1</td>
<td>46.3 ± 8.8</td>
<td>44.7 ± 8.5</td>
<td>0.0 ± 0.6</td>
<td>0.5 ± 0.0</td>
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P values
- Treatment: 0.673
- Observation time: 0.179
- Treatment * Observation time: 0.317

UDCA, ursodeoxycholic acid (3β,7α,12β-trihydroxy-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (3β,7α,12β-trihydroxy-5β-cholan-24-oic acid); HCA, hyocholic acid (3β,6α,7α-trihydroxy-5β-cholan-24-oic acid); HDCA, hyodeoxycholic acid (3β,6α-hydroxy-5β-cholan-24-oic acid); LCA, lithocholic acid (3β-hydroxy-5β-cholan-24-oic acid); 6-KLCA, 6-keto-lithocholic acid (3β-hydroxy-6-keto-5β-cholan-24-oic acid).

Mean values with unlike superscript letters were significantly different (P < 0.05) over time within the same group, when an overall time effect was detected (P < 0.05).
14C-labelled UDCA was produced in vitro by *C. absonum* from 14C-labelled CDCA, then purified by preparative TLC and injected into the portal vein. At the time of injection, the enterohepatic circulation was interrupted by the complete diversion of bile out of the animal. We found that only 3.3% of the labelling recovered in bile was on UDCA whereas 63.9% and 22.9% were on CDCA and 7-KLCA, respectively. This strongly suggests that the hepatic extraction of UDCA was very efficient in the experimented pigs, as in man under physiological conditions (Crosignani *et al.* 1996), and that hepatic metabolism of the extracted UDCA to CDCA prevented its recovery in bile and its accumulation in the enterohepatic circulation. Although the hepatic metabolism of UDCA is poorly documented in pigs, UDCA was shown to account for only 3.6% of total biliary bile acids following a single oral dose of UDCA as high as 40 mg/kg in this animal model (Nowak *et al.* 2001). Formation of CDCA from UDCA has also been reported in human hepatocytes (Ellis *et al.* 2003), and this could contribute to explain very low recoveries of biliary UDCA occasionally observed in human subjects under UDCA therapy (Lindor *et al.* 1998).

Another result concerns LCA, which increased throughout the experiment in the bile of control pigs unlike in experimental pigs. The same trend was observed in faeces, whereas no effect could be demonstrated for other faecal bile acids, including UDCA. This lowering effect of living bacteria on biliary and faecal LCA could be related to early intestinal absorption of unconjugated CDCA, which would therefore be less exposed to 7α-dehydroxylation by endogenous microbiota in the large intestine. We recently assumed, from bile acid profiles in the portal

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**Fig. 3.** Effect of administering living (experimental group, E) or inactivated (control group, C) bacteria on (A) cholesterol content of the different lipoprotein classes (I, HDL; II, LDL; and III, VLDL) and (B) LDL-cholesterol:HDL-cholesterol ratio in pigs. Values are means with standard errors shown by vertical bars. a,b,c,dMean values (ordered according to increasing values) with unlike superscript letters were significantly different (*P*<0.05) over time within the same group, when an overall time effect was detected (*P*<0.05). All bars without a letter were not significantly different. There were no significant differences at a given observation time between groups treated with either living or inactivated bacteria.
vein, that early deconjugation of bile salts by *B. animalis* during its transit in the intestinal tract of pigs was not accompanied by increased formation of secondary bile acids beyond the hydrolysis reaction (Lepercq et al. 2004c). The figures obtained here in bile and faeces confirm this assumption. This point is of clinical importance, since increased faecal LCA has been suggested to be a risk marker for colon cancer (Latta et al. 1993; Bajjal et al. 1998; Kozeni et al. 2000).

Finally, the biliary lipid equilibrium was not affected by treatment, as inferred from the steadiness of the respective molar percentages of bile acids, phospholipids and cholesterol. Nor was the dietary-induced hypercholesterolaemia improved by the administration of living bacteria. Early deconjugation of bile salts and increase of UDCA in the intestine have been proposed as efficient tools for decreasing micellisation and absorption of luminal cholesterol and, therefore, lowering cholesterolaemia (De Rodas et al. 1996; De Smet et al. 1998; Taranto et al. 1998; Usman & Hosono, 2001). However, as in our study, many nutritional trials using living bifidobacteria or lactobacilli (Roberfroid, 2000; Drouall & Corbier, 2001; Pereira & Gibson, 2002) have failed to provide evidence of their hypocholesterolaemic action.

Since hypocholesterolaemic responses to various dietary manipulations are usually observed in middle- to long-term nutritional trials (Richelsen et al. 1996; Agerholm-Larsen et al. 2000; Xiao et al. 2003), we cannot ascertain that increasing the duration of the bacterial treatment would not have had a beneficial effect.

The present study therefore demonstrated the feasibility of increasing UDCA in the intestine and portal blood of conventional pigs treated with a combination of CDCA-hydrolysing and -epimerising living bacteria. This, together with signs of decreased metabolism of CDCA to LCA, may represent an interesting new probiotic activity, provided that in future it could be expressed by a safe food micro-organism.

Acknowledgements

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