

Deconjugation of bile acids with immobilized genetically engineered *Lactobacillus plantarum* 80 (pCBH1)

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M. L. Jones, C. Martoni, H. Chen, W. Ouyang, T. Metz
and S. Prakash

Biomedical Technology and Cell Therapy Research Laboratory, Department of Biomedical Engineering and Artificial Cells and Organs Research Centre, Faculty of Medicine, McGill University, 3775 University Street, Montreal, Quebec H3A 2B4, Canada

Abstract: Bile acids are important to normal human physiology. However, bile acids can be toxic when produced in pathologically high concentrations in hepatobiliary and other diseases. This study shows that immobilized genetically engineered *Lactobacillus plantarum* 80 (pCBH1) (LP80 (pCBH1)) can efficiently hydrolyze bile acids and establishes a basis for their use. Results show that immobilized LP80 (pCBH1) is able to effectively break down the conjugated bile acids into glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) with bile salt hydrolase (BSH) activities of 0.17 and 0.07 $\mu\text{mol DCA/mg CDW/h}$, respectively. The deconjugation product, deoxycholic acid (DCA), was diminished by LP80 (pCBH1) within 4 h of initial BSH activity. This *in-vitro* study suggests that immobilized genetically engineered bacterial cells have important potential for deconjugation of bile acids for lowering of high levels of bile acids for therapy.

Key words: Immobilization, bile acid, bile salt hydrolase, glycodeoxycholic acid, taurodeoxycholic acid, genetically engineered *Lactobacillus plantarum* cells.

INTRODUCTION

Bile acids are important physiological agents that are required for the disposal of cholesterol and the absorption of dietary lipids and lipid soluble vitamins. Bile salts are the water-soluble end products of cholesterol and are synthesized in the liver. During normal enterohepatic circulation (EHC), the average bile salt pool of 4.0 g is secreted into the duodenum twice during each meal, or an average of 6–8 times per day (Henry 2001, Hofmann 1989). During intestinal transit, 90–95% of secreted bile salts are absorbed in the terminal ileum and are returned to the liver via the portal vein (Hofmann 1989). The bile salt pool is held approximately constant and is replenished by hepatic synthesis of new bile from serum cholesterol.

Daily bile acid secretions approach 20–30 g (Henry 2001, Hofmann 1989) or 20–60 mmol (Bongaerts et al 2000) sustaining an intestinal bile salt concentration of 5–20 mM (Hofmann 1989). During intestinal transit, 90–95% of secreted bile salts are reabsorbed in the terminal ileum and are returned to the liver via the portal vein (Hofmann 1989). About 75% of the bile acid secretion is reabsorbed in the conjugated form in an active sodium-dependent way (Hofmann 1989). The remaining 25% is hydrolyzed during intestinal transit and only 15% is reabsorbed in a passive way (Hofmann 1989). Thus, only 60% of deconjugated bile acids are reabsorbed and 1 mmol of deconjugated bile salt is responsible for a 0.4 mmol faecal excretion (Hofmann 1989).

Although bile acids are important to normal human physiology, they can be cytotoxic agents when produced in pathologically high concentrations. Increased blood bile acid levels are a known symptom of hepatobiliary disease and increased blood levels can be measured in the urine by sulfated bile acid concentrations (Kobayashi et al 2002, Rozga et al 1993, Schafer and Shaw 1989). As well, when ileal transport of bile acids is defective due to a congenital defect, resection of the ileum, or disease,

Corresponding Author:

Dr S. Prakash

Department of Biomedical Engineering

Faculty of Medicine, McGill University, Montreal, Quebec H3A 2B4, Canada

Tel: +1 514 398 3676, Fax: +1 514 298 3671

Email: satya.prakash@mcgill.ca

a compensatory increase in bile biosynthesis occurs and an increased amount of bile acid passes into the colon (Hofmann 1999). An elevated intraluminal concentration of bile acids induces the secretion of electrolytes and water causing diarrhea, loss of electrolytes, and dehydration. Furthermore, deconjugated bile acids are believed to be associated with diet related colon carcinogenesis (De Boever and Verstraete 1999, De Boever et al 2000, Oumi and Yamamoto 2000).

Bile acid sequestrants (BAS) have been shown to lower elevated intraluminal concentrations of bile acids and can provide some symptomatic benefit (Hofmann 1999). BAS bind bile acids in the intestine and form insoluble complexes that are excreted in the feces. It has been well demonstrated over the last 20 years that BAS alone can diminish intraluminal levels of bile acids, and through interruption of the EHC even reduce blood serum cholesterol levels (Lipid Research Clinics Coronary Primary Prevention Trial 1984, Levine et al 1995). However, the common BAS Cholestyramine resin (Locholest, Questran), Colesevelam (WelChol), and Colestipol (Colestid) are well documented to exhibit major adverse effects such as nausea, bloating, constipation, and flatulence (Bristol-Myers Squibb 1995). Furthermore, there have been problems with gaining over-the-counter (OTC) status for BAS, as it uses adsorbents and binders which are found to be unacceptable for this classification by the U.S. Food and Drug Administration (FDA). In fact, in 1995 Bristol-Myers Squibb's application to change Questran, the company's BAS cholesterol-lowering medication to OTC status was denied by the FDA (Bristol-Myers Squibb 1995).

Removal of bile salts from the gastrointestinal tract, through the enzymatic action of the bile salt hydrolase (BSH) enzyme, has been proposed and it has been shown that *Lactobacilli* bacteria have the ability to hydrolyze bile salts in the intestinal tract (Anderson and Gilliland 1999, De Boever and Verstraete 1999, De Smet et al 1994). However, much work needs to be done to establish that lowering luminal levels of bile salts by this approach can be both effective and safe.

Immobilization is a technique used to surround and immobilize biologically active materials in specialized polymer beads (Chang and Prakash 1997, Jankovsky and Vasakova 1996, Simmonds et al 1967). The polymer bead can protect biological materials from harsh external environments, while at the same time allowing for the metabolism of selected solutes capable of passing into and out of the polymer surrounding. In this manner, the enclosed material (in this case live bacteria) can be retained inside and be separated from the external environment, making immobilization particularly useful for biomedical, clinical, and industrial applications (Lim and Sun 1980, Chang 1999, Sefton et al 2000, Jankovsky and Vasakova 1996, Simmonds et al 1967). Studies show that the immobilization of cells can be used for both cell implantation and oral administration of live genetically engineered cells that

can be useful for therapeutic functions (Mullen et al 2000, Prakash and Chang 1996, 2000, Tatarkiewicz et al 2001). In this article we study the potential of immobilized genetically engineered *Lactobacillus plantarum* 80 (pCBH1) for assimilating a complex mixture of human bile acids *in-vitro* to develop a method for the deconjugation of bile acids and potentially an effective therapy method to lower pathologically high levels of bile acids in clinical conditions.

MATERIALS AND METHODS

Media and chemicals

The sodium salts of glycocholic acid (GCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), and deoxycholic acid (DCA) were supplied by SIGMA (St. Louis, MO, USA). De Man-Rogosa-Sharpe (MRS) broth was obtained from Difco (Sparks, MD, USA). The water was purified with an EASYpure™ RO Reverse Osmosis System and a NANOpure® Diamond™ Life Science (UV/UF) ultrapure water system from Barnstead/Thermoline (Dubuque, IA, USA). Methanol was HPLC-gradient from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade.

Bacterial strains and growth conditions

The bacterial strain used in this study was the bile salt hydrolytic (BSH) isogenic *Lactobacillus plantarum* 80 (pCBH1) strain. Overproduction of the BSH enzyme in LP80 (pCBH1) was obtained as described by Christiaens et al (1992). The BSH overproducing LP80 (pCBH1) strain carries the multicopy plasmid pCBH1 carrying the LP80 (pCBH1) chromosomal *bsh* gene and an erythromycin resistance gene. The *Lactobacillus* strains were grown in MRS broth at 37°C in a Sanyo MIR-162 bench top incubator. The MRS broth was supplemented with 100 µg/ml erythromycin from Fisher Biotech (Fair Lawn, NJ, USA) to select for bacteria carrying the multicopy plasmid pCBH1.

Immobilization of *Lactobacillus plantarum* 80 (pCBH1)

Low viscosity alginate, 1.5%, 50 ml (Kelco, Chicago, IL), solution was prepared and filtered through a 0.22 µm Sterivex-GS filter (Millipore, Bedford, MA) into a sterile 60 ml syringe. LP80 (pCBH1) was grown at 37°C in MRS broth and prepared as a concentrated microorganism suspension by resuspension of microorganism in 10 ml of sterilized physiologic solution. The 10 ml concentrated microorganism suspension was added to the 50 ml low viscosity alginate solution and mixed well. The alginate/microorganism mixture was immobilized, through a 300 µm nozzle, into a filtered solution of CaCl₂ with an Inotech Encapsulator IER-20 (Inotech Biosystems International Inc., Rockville, MD). This procedure was performed in a Microzone Biological Containment Hood (Microzone Corporation, ON, Canada) to assure sterility. The immobilized LP80 (pCBH1) was stored in 1.0 l minimal solution (10% MRS and 90% Physiologic Solution) at 4 °C.

BSH activity of immobilized *Lactobacillus plantarum* 80 (pCBH1) alginate beads

To investigate the BSH activity of the immobilized BSH overproducing LP80 (pCBH1), batch experiments were performed. Five grams cell dry weight (CDW) of immobilized LP80 (pCBH1) was added to fresh MRS broth to which 10.0 mM GDCA and 5.0 mM TDCA were added. Samples were taken at regular time intervals during the 24 h incubation to determine the bile salt concentration in the reaction vessels. The experiment was performed in triplicate.

Bile salt hydrolase assay

A modification of the HPLC procedure described by Scalia (1988) was used to determine BSH activity. Traditionally, *in-vitro* bile acid experimentation has involved the use of HPLC to determine the quantity of various tauro- and glycol-bile acids in complex mixtures of added bile acids in complex aqueous media (Cantafora et al 1987, Coca et al 1994, Scalia 1988). Methods of separating such mixtures have required a lengthy workup involving a (1:4; v:v) sample:isopropanol extraction followed by evaporation and resuspension steps (Cantafora et al 1987, Coca et al 1994, Scalia 1988). While this method can produce accurate results, we eliminated the time-consuming and labor-intensive workup step of evaporation, allowing for an efficient workup while preserving the quality of bile acid separation and quantification.

Analyses were performed on a reversed-phase C-18 column: LiChrosorb RP-18, 5 μ m, 250 \times 4.6 mm from HiChrom (Novato, CA, USA). The HPLC system was made up of two ProStar 210/215 solvent delivery modules—a ProStar 320 UV/Vis detector and a ProStar 410 AutoSampler—and Star LC Workstation Version 6.0 software was used. The solvents used were HPLC-grade methanol (solvent A), and solvent B, which was acetate buffer prepared daily with 0.5 M sodium acetate, adjusted to pH 4.3 with *o*-phosphoric acid, and filtered through a 0.22 μ m filter (Whatman®, England). An isocratic elution of 70% solvent A and 30% solvent B was used at a flow rate of 1.0 ml/min at room temperature. An injection loop of 20 μ l was used, and the detection occurred at 205 nm within 25 min after injection of the bile salt extract.

Quarter milliliter samples to be analyzed were acidified by the addition of 2.5 μ l of 6 N HCl to stop any further enzymatic activity. A modification of the extraction procedure described by Cantafora was used (Cantafora et al 1987, De Smet et al 1994). From the 0.25 ml sample, bile salts were extracted using a solution of methanol (1:1; v:v). GCA was added as an internal standard at 4.0 mM. The samples were mixed vigorously for 10 min and centrifuged at 1000 *g* for 15 min. The supernatant was then filtered through a 0.22 μ l syringe driven HPLC filter (Millipore, Japan) and the samples were analyzed directly after filtration.

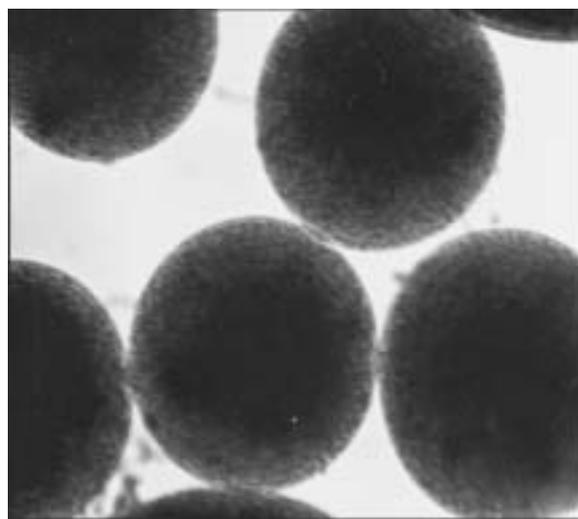


Figure 1 Photomicrograph of alginate beads containing immobilized *Lactobacillus plantarum* 80 (pCBH1) cells at 175 \times magnifications.

RESULTS

Preparation of alginate beads containing immobilized genetically engineered *Lactobacillus plantarum* 80 (pCBH1) cells

Alginate beads containing immobilized genetically engineered *Lactobacillus plantarum* 80 (pCBH1) cells (Figure 1) were prepared using the methods described above and stored at 4 °C for use in experiments. Sterile conditions and procedures were strictly adhered to during the process of immobilization.

Determination of bile acids by HPLC

We prepared a calibration curve to quantify the HPLC sample results. Known quantities of GDCA and TDCA were added to MRS broth and 0.25 ml samples and analyzed using the modified HPLC bile salt hydrolase assay outlined above. Figure 2 shows the calibration curves for TDCA and GDCA with a 4.0 mM GCA internal standard and correlation of determinants factors (R^2) of 0.997829 and 0.993246, respectively. Results show (Figure 2) that this modified method allows accurate identification and quantitative measurements of various bile acids.

BSH activity of alginate beads containing immobilized *Lactobacillus plantarum* 80 (pCBH1)

To investigate the BSH activity of alginate beads containing immobilized LP80 (pCBH1), previously stored at 4 °C, 5 g CDW of immobilized LP80 (pCBH1) was incubated in MRS broth supplemented with 10.0 mM GDCA and 5.0 mM TDCA. The concentration of bile acids was monitored by analyzing media samples at regular intervals over 24 h. Figure 3 shows superimposed HPLC chromatograms of bile acids in reaction media taken from one of the experiments at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. The internal standard was GCA and was the first peak eluted.

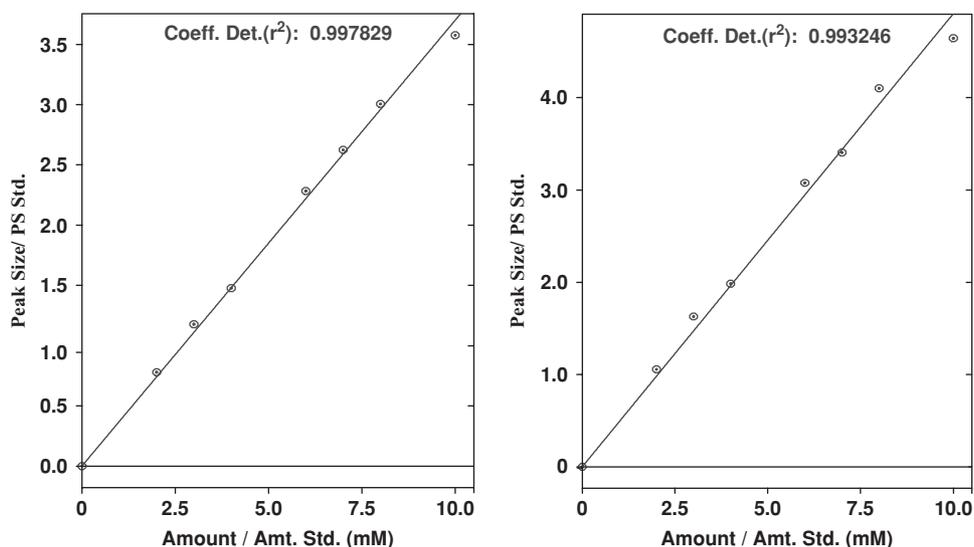


Figure 2 HPLC calibration curves for GDCA and TDCA measurements.

Decreasing peak areas of TDCA and GDCA bile acids indicate BSH activity of alginate beads containing immobilized LP80 (pCBH1). At time zero an unidentified peak was detected, just before the measured TDCA peak, and was diminished totally within 4 h (Figure 3).

The BSH activity of 0.25 g CDW of immobilized LP80 (pCBH1), previously stored at 4 °C, was determined and is shown in Table 1. The BSH activity of 0.26 g CDW of immobilized LP80 (pCBH1) was calculated based on the depletion of 0.2 mmol of GDCA in a 5 h period, and the BSH activity toward TDCA based on the breakdown of 0.1 mmol of TDCA in a 6 h period. This calculation was also based on the *in-vitro* depletion of bile acids with 0.25 g CDW LP80 (pCBH1) in 5.0 g alginate beads in a complex mixture of the bile acids.

Figure 4 shows the BSH activity of immobilized LP80 (pCBH1) in alginate in an *in-vitro* bile acid experiment over

Table 1 Bile salt hydrolase (BSH) activity ($\mu\text{mol DCA}/\text{mg CDW}/\text{h}$) of immobilized *Lactobacillus plantarum* 80 (pCBH1), previously stored at 4 °C, toward glyco- and tauro-bile acids

Strain	BSH activity ($\mu\text{mol DCA}/\text{mg CDW}/\text{h}$) toward		
	GDCA	TDCA	DCA
Immobilized LP80 (pCBH1)	0.17	0.07	0.24

a 12 h period. The concentration of GDCA and TDCA bile acids is shown to decrease over time. Results from Figure 4 show that the BSH activity of immobilized LP80 (pCBH1) began immediately and depleted GDCA at a greater initial rate. While TDCA began to break down

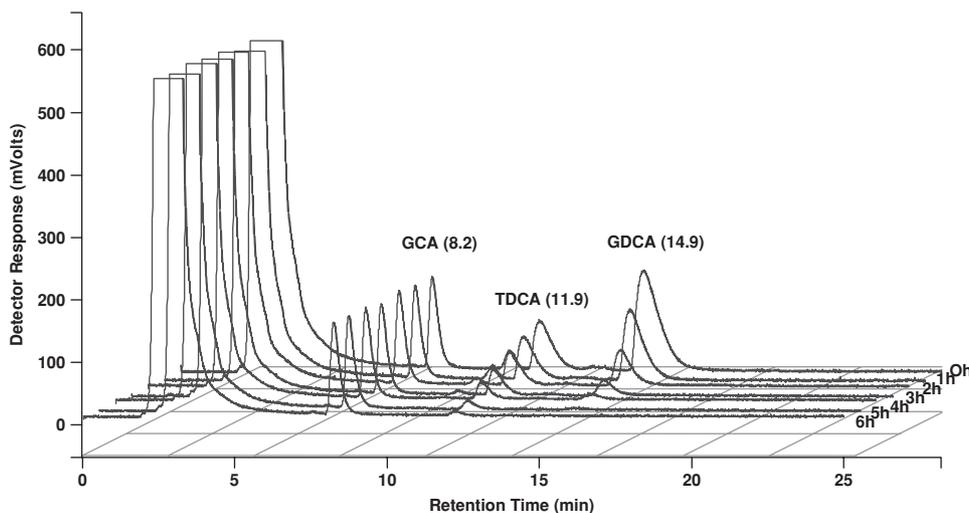


Figure 3 Overlaid HPLC chromatograms of bile acids in reaction media over time (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h). Decreasing peak areas of TDCA and GDCA indicate BSH activity of immobilized *Lactobacillus plantarum* 80 (pCBH1).

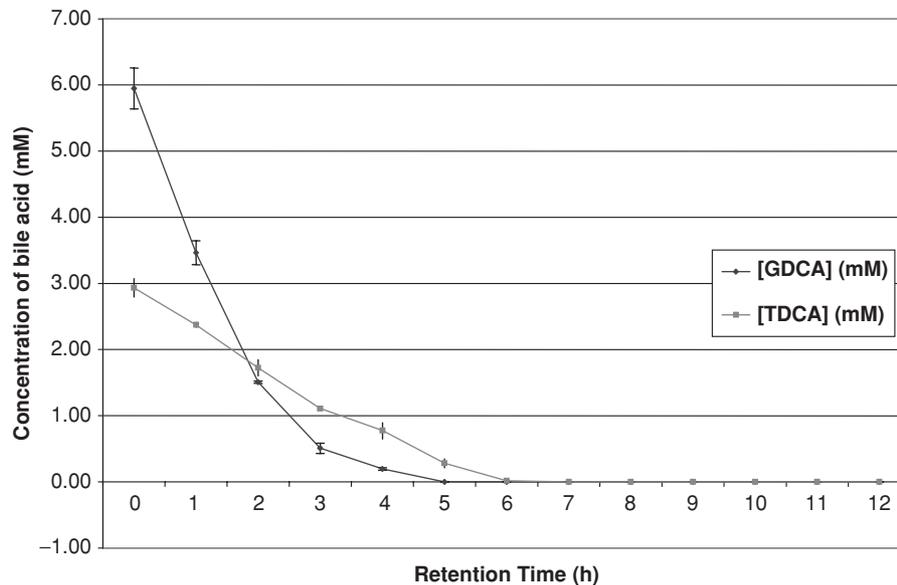


Figure 4 BSH activity and GDCA and TDCA depletion efficiency of immobilized *Lactobacillus plantarum* 80 (pCBH1) in *in-vitro* experiment. The concentration of GDCA and TDCA bile acids is shown over time. The experiment was performed in triplicate: error bars indicate standard deviations.

immediately, it did so at a slower rate than GDCA. The removal of GDCA, however, experienced concentration effects as it depleted and thus the breakdown of GDCA slowed as the experiment progressed (Figure 4).

To investigate the fate of the products of deconjugation, the above experiment was performed with a calibration of increasing concentrations of TDCA, GDCA, and DCA. Figure 5 shows superimposed HPLC chromatograms of bile acids in MRS reaction media taken from one of the experiments at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. Decreasing peak areas of TDCA and GDCA bile acids indicate BSH activity of alginate beads containing immobilized LP80 (pCBH1). The peak detected, just before the measured TDCA peak, was diminished totally within 4 h and corresponds to the calibration peak of DCA. The internal standard was GCA and was the first peak eluted. The experiment was performed in triplicate and the results were averaged.

DISCUSSION

Bile salt hydrolase (BSH) activity is a common phenomenon in the human gut; however, *Lactobacillus plantarum* 80 (LP80) (pCBH1) used in this study has been genetically engineered to overproduce the BSH enzyme. Although it has earlier been shown that the free bacteria was able to break down bile acids *in-vitro* (De Smet et al 1994), we intended on proving the efficacy of immobilized LP80 (pCBH1) at breaking down tauro- and glycol-bile acids, and hence establish a basis for their use in deconjugating pathologically high levels of bile acids.

Results clearly show that immobilized LP80 (pCBH1) was able to effectively break down the bile acids within 6 h

(Figures 3 and 4). The BSH activity of immobilized LP80 (pCBH1) began immediately and depleted GDCA at a greater initial rate. While TDCA also began to break down immediately, it did so at a slower rate than GDCA. The removal of GDCA, however, experienced concentration effects as it was depleted. Thus, the breakdown of GDCA slowed as the experiment progressed.

The BSH activity results show that 0.25 g CDW of immobilized LP80 can break down 0.2 mmol of GDCA in a 5 h period and 0.1 mmol of TDCA in a 6 h period. This calculation is based on the *in-vitro* depletion of bile acids with 0.25 g CDW LP80 (pCBH1) in 5.0 g of alginate beads.

At time zero an unidentified peak was detected (Figure 5), just before the measured TDCA peak, and was diminished totally within 4 h. As depicted in Figure 6, the deconjugation of TDCA and GDCA leads to the formation of deoxycholic acid (DCA); thus, it was probable that the unidentified peak was a measure of DCA. To confirm this, we designed the experiment using a calibration of increasing concentrations of TDCA, GDCA, and DCA (Figure 5). Results show that indeed the unidentified peak was DCA, and that DCA was diminished totally within 4 h of the onset of the experiment (Figure 5). That raised several questions regarding the fate of DCA, the most important of which was: Where was the DCA going and why was it not doing so immediately? It is our assumption that DCA is being consumed or altered by the LP80 (pCBH1) cells and is either bound to the bacterial membrane or precipitated within the bead. This is based on previous findings that *Lactobacillus* bacteria are able to remove deconjugated bile acids from reaction media and make them less bioavailable (De Boever et al 2000).

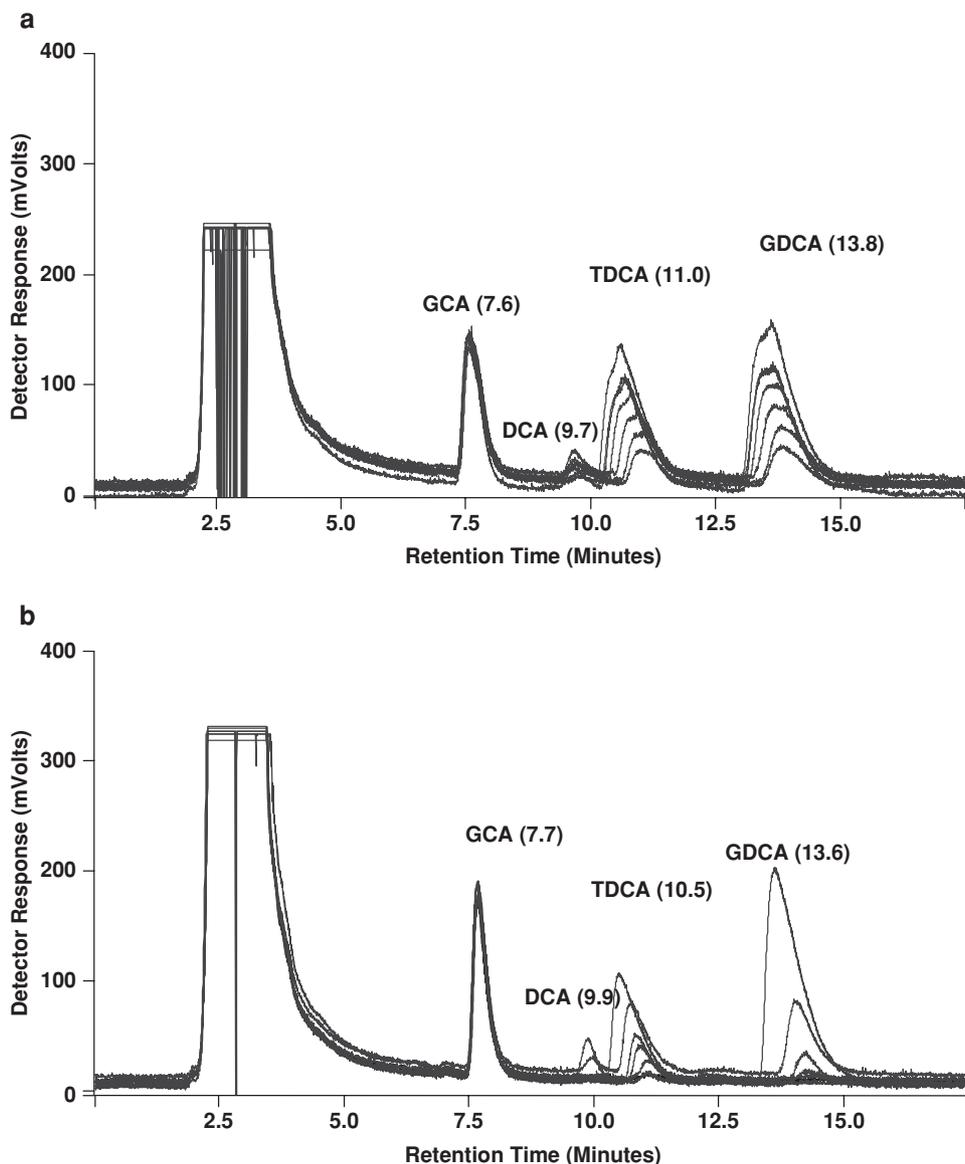


Figure 5 (a) Overlaid HPLC chromatograms of samples (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h) from an experiment in which immobilized LP80 (pCBH1) was used to deconjugate 10 mM GDCA and 5 mM TCDA in MRS reaction media. (b) Overlaid HPLC chromatograms from a calibration with increasing concentrations (2 mM, 3 mM, 4 mM, 6 mM, 7 mM, 8 mM, 10 mM) of TDCA GDCA and DCA. A 4 mM internal standard was used for the preparation of all samples.

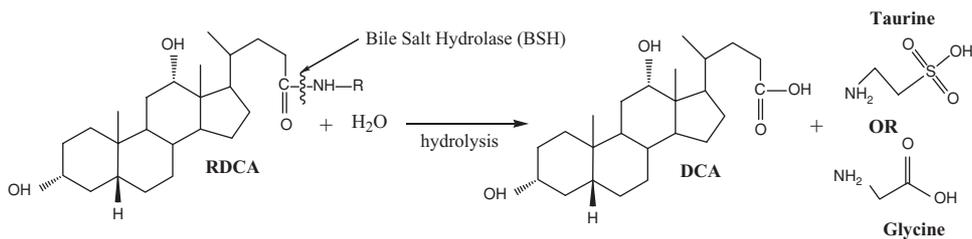


Figure 6 Hydrolysis of conjugated bile salts by the bile salt hydrolase (BSH) enzyme overproduced by genetically engineered *Lactobacillus plantarum* 80 (pCBH1) (10). R indicates the amino acid glycine or taurine. RDCA: glyco- or tauro-deoxycholic acid, DCA: deoxycholic acid.

This *in-vitro* study shows that immobilized *Lactobacillus plantarum* 80 (pCBH1) can actively break down tauro- and glycol-conjugated bile acids in complex mixtures and at similar concentrations to human physiological values. The results illustrate the potential for alginate beads, containing immobilized genetically engineered *Lactobacillus plantarum* 80 (pCBH1), to mitigate pathologically high levels of bile acids. Also, it has given evidence that LP80 (pCBH1) cells may remove deconjugated bile acids once they have been produced through BSH activity. However, it should be stated that this *in-vitro* study is only suggestive of the values for the *in-vivo* deconjugation of bile acids by immobilized LP80 (pCBH1). Even so, this study has established a basis for the investigation of the bile acid deconjugation potential of immobilized LP80 (pCBH1) when transplanted or administered orally allowing for the safe and effective control of bile acid levels. The potential uses of immobilized beads containing LP80 (pCBH1) are many and include the removal of unwanted and pathologically high levels of bile acids, in patients with fulminant hepatic failure (FHF).

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