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Research Article

Investigation of Microencapsulated BSH Active *Lactobacillus* in the Simulated Human GI Tract

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This study investigated the use of microencapsulated bile salt hydrolase (BSH) overproducing *Lactobacillus plantarum* 80 cells for oral delivery applications using a dynamic computer-controlled model simulating the human gastrointestinal (GI) tract. Bile salt deconjugation rates for microencapsulated BSH overproducing cells were $4.87 \pm 0.28\,\mu$mol/g microcapsule/h towards glycoconjugates and $0.79 \pm 0.15\,\mu$mol/g microcapsule/h towards tauroconjugates in the simulated intestine, a significant ($P < .05$) increase over microencapsulated wild-type cells. Microcapsules protected the encased cells in the simulated stomach prior to intestinal release, maintaining cell viability above 10^9 cfu/mL at pH 2.5 and 3.0 and above 10^6 cfu/mL at pH 2.0 after 2-hour residence times. In the simulated intestine, encased cell viability was maintained above 10^{10} cfu/mL after 3, 6, and 12-hour residence times in bile concentrations up to 1.0%. Results show that microencapsulation has potential in the oral delivery of live BSH active bacterial cells. However, *in vivo* testing is required.

1. INTRODUCTION

The genus lactobacillus is currently the focus of much scientific and commercial interest due to a myriad of health-promoting effects in the gastrointestinal tract (GI) [1, 2]. In recent years, various lactobacilli strains have proven to lower total or low-density lipoprotein cholesterol (LDL-C) in humans [3, 4] and animals [5, 6]. This effect can at least partially be attributed to the assimilation of cholesterol by bacterial cells and the enzymatic deconjugation of bile salts [5, 7]. Bile salt hydrolase (BSH), the enzyme responsible for bile salt deconjugation in the intestine, has been detected and characterized in several intestinal lactobacillus species [8–10]. Furthermore, increasing bile salt deconjugation in the intestinal lumen through oral delivery of lactobacilli has garnered attention in recent years [8, 11–13]. It has also been suggested that BSH activity is a requirement in the selection of cholesterol lowering microorganisms, as nondeconjugating organisms do not appear to have any significant cholesterol removal ability in culture medium [14]. However, insufficient survival when passing from the mouth to the intestine has limited the potential of many probiotics from clinical or commercial use [15, 16].

Artificial cell microencapsulation, a concept in which biologically active materials are encapsulated in specialized ultrathin semipermeable polymer membranes, has been proposed as a means to improve cell viability in the GI [17–20]. Earlier studies show that microencapsulated BSH overproducing *Lactobacillus plantarum* 80 cells has potential in oral delivery applications [21]. However, no study has determined its actual utility and functionality in oral administration. In this research, towards the goal of oral delivery applications, we investigate the potentials of microencapsulated live BSH active cells in a computer-controlled dynamic human GI model. The computer-controlled GI model simulates the complex GI environment at each stage of transit.

Specifically, the apparatus, consisting of five bioreactor vessels arranged in series, mimics the gradual transit of ingested food products and therapeutics through the human digestive tract in which *in vivo* conditions with regard to pH, temperature, bacteria, enzyme types and activities, volume, agitation, and food particles are closely simulated. Investigation of oral formulations in the GI environment is crucial before further testing in animal models to understand the potentials and limitations of delivery formulations. With this aim, the current study therefore investigates the performance of microencapsulated BSH active lactobacillus in the simulated GI tract.

2. MATERIALS AND METHODS

2.1. Media and chemicals

Lactobacilli de Man, Rogosa, Sharpe (MRS) broth and agar were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Alginate (low-viscosity) and poly-L-lysine (hydrobromide, MW 21,320) were obtained from Sigma (Saint Louis, Mo, USA). The sodium salts of glycocholic acid, taurodeoxycholic acid, and glycodeoxycholic acid and methanol (HPLC-grade) were obtained from Sigma. Sodium acetate was purchased from Fisher Scientific. All food composition materials were purchased from Sigma; pancreatin was obtained from Acros; and oxgall was obtained from Difco. Unless otherwise specified, all other chemicals were of analytical grade and not purified any further prior to use.

2.2. Bacteria and culture conditions

The bacterial strains used in this study were *L. plantarum* 80 BSH overproducing strain (BSH^+) and *L. plantarum* 80 wild type (wt). *L. plantarum* 80 BSH^+ carries the multicopy plasmid pCBH1 containing the *L. plantarum* 80 chromosomal BSH gene and an erythromycin resistance gene. Overproduction of the BSH enzyme in *L. plantarum* 80 BSH^+ was obtained as described by Christiaens et al. [11]. Stock cultures of both strains were stored in 50% glycerol at −80°C. The bacteria were cultivated in sterile de Man, Rogosa, Sharpe (MRS) broth (1% inoculum) at 37°C for 20 hours. Erythromycin was supplemented (100 μg/mL) for plasmid selection. Prior to experimental use, three subcultures were performed in the appropriate medium.

2.3. Preparation of APA microcapsules containing L. plantarum 80 strains

Alginate-poly-L-lysine-alginate (APA) microcapsules containing *L. plantarum* 80 BSH^+ cells were prepared as follows. Grown cultures were isolated after 20 hours by centrifugation at 8000 g for 15 minutes at 15°C. The cell isolates were suspended in sterile saline (0.85% NaCl) and slowly added to a gently stirred 1.5% (w/v) sodium alginate solution. In a sterile environment, the bacterial alginate suspension was passed through an Inotech Encapsulator IER-20 (Inotech Biosystems International Inc., Rockville, Md, USA) with an internal nozzle diameter of 300 μm. After extrusion, the droplets were allowed to gel for 5 minutes in a gently stirred sterile 0.1 M $CaCl_2$ solution. The Ca-alginate beads were coated with 0.1% (w/v) poly-L-lysine and 0.1% (w/v) Na-alginate for 10 minutes and 5 minutes, respectively. Microcapsules were washed in sterile saline between each coat and after the encapsulation procedure. Microcapsules containing *L. plantarum* 80 wild-type and empty microcapsules (control) were prepared as above. All batches of microcapsules were stored in minimal solution (90% saline, 10% MRS broth) at 4°C.

2.4. Investigation of BSH activity of microcapsules in simulated GI model

BSH activity was measured in real time utilizing a simulated human GI model (Figure 1) consisting of vessels representing the stomach, small intestine, ascending colon, transverse colon, and descending colon. Microcapsules containing *L. plantarum* 80 BSH^+ cells were first exposed to the stomach compartment consisting of a food suspension adjusted to pH 2.0 at 37°C and 100 RPM agitation. The food component contained starch 3.0 g/L; pectin 2.0 g/L; mucin 4.0 g/L; arabinogalactan 1.0 g/L; xylan 1.0 g/L; yeast extract 3.0 g/L; peptone 1.0 g/L; glucose 0.4 g/L, and cysteine 0.5 g/L. After 1 hour, microcapsules were transferred to the small intestine compartment consisting of the acidified food suspension readjusted to pH 6.5 and supplemented with 5 mM glycodeoxycholic acid (GDCA), 5 mM taurodeoxycholic acid (TDCA), pancreatin 0.18 g/L, and sodium bicarbonate 2.4 g/L. The total bile salt concentration was approximately 0.5% (w/v). Microcapsules were incubated in the simulated small intestine for 10 hours. Supernatant was sampled at intervals of 1 hour and processed to determine bile salt concentrations in the reaction vessels. Viable cell count was monitored pre-stomach exposure, pre-small intestine exposure, and post-small intestine exposure. Microcapsules containing *L. plantarum* 80 wild type were tested as above and empty microcapsules were used as controls. Microcapsules were visualized microscopically to determine the effect of simulated GI transit on microcapsule integrity. The unpaired student t-test was used to determine which means differed significantly ($P < .05$).

2.5. Analysis of bile salt concentrations using HPLC

Supernatant samples were prepared for HPLC analysis using the procedure described by Jones et al. [21]with some modifications. Briefly, 250 μl samples were acidified with 2.5 μl of 6N HCl and supplemented with 250 μl of Methanol containing 4 mM glycocholic acid (GCA) as internal standard. The samples were vortexed, shaken at 225 RPM for 10 minutes, and centrifuged at 1000 g for 20 minutes at 10°C. The supernatant was filtered through a 0.22 μm PVDF 4 filter (Millipore) and analyzed directly. Standards for calibration containing 0, 1, 2, 3, 4, 5, 6 mM GDCA and TDCA were treated as above.

A modification of the HPLC procedures described by Scalia [22] was used to determine bile salt concentrations. Analyses were performed on a reverse-phase C-18 column: LiChrosorb RP-18 (250 mm × 4.6 mm, 5 μm) from

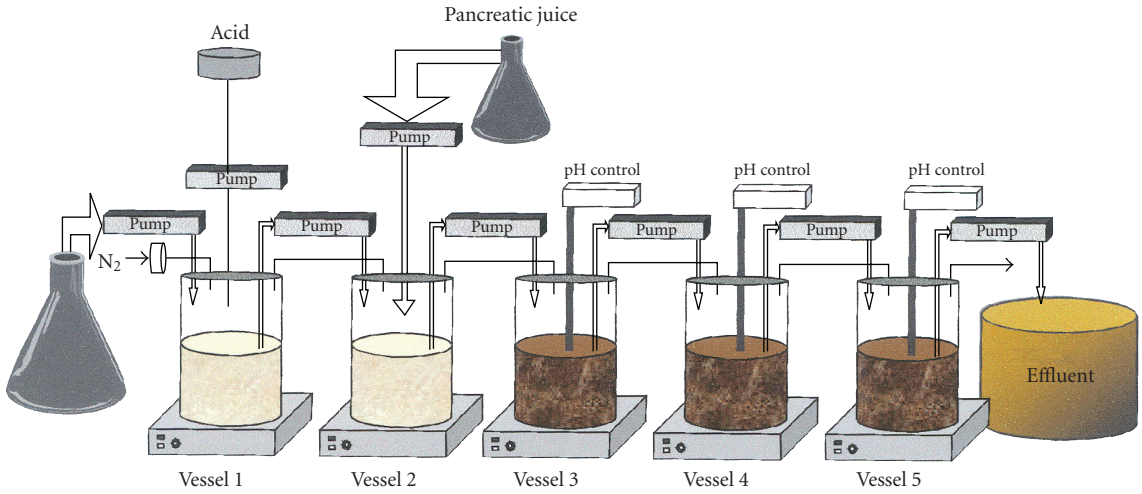

Figure 1: Computer-controlled dynamic human GI model. Bioreactors in series representing stomach (Vessel 1), small intestine (Vessel 2), ascending colon (Vessel 3), transverse colon (Vessel 4), and descending colon (Vessel 5).

HiChrom (Novato, Calif, USA). The solvents used were .05 M sodium acetate buffer adjusted to pH 4.3 with o-phosphoric acid and filtered through a 0.22 μm filter (Nalgene) (Solvent A) and HPLC-grade Methanol (Solvent B). An isocratic elution was applied, consisting of 30 percent Solvent A and 70 percent Solvent B at a flow rate of 1.0 mL/min. An injection loop of 20 μl was used and sample detection occurred at 210 nm.

2.6. Survival of microencapsulated L. plantarum 80 BSH$^+$ in simulated stomach

Gastric survival of microencapsulated *L. plantarum* 80 BSH$^+$ cells was examined by exposing the microcapsules to the simulated stomach compartment of the simulated human GI model adjusted to pH 1.5, 2.0, 2.5, and 3.0 with 1.0 M HCl. Incubation was performed at 37°C and 100 RPM agitation for 4 hours. For viability studies, aliquots were removed after 0, 30, 60, 90, 120, and 240 minutes. At each time point, microcapsules were mechanically ruptured to release the encased bacteria and serial dilutions were performed. Aliquots were plated on MRS agar, and the plates were incubated at 37°C for 72 hours.

2.7. Survival of microencapsulated L. plantarum 80 BSH$^+$ in simulated small intestine

Small intestinal survival of microencapsulated *L. plantarum* 80 BSH$^+$ cells was measured by exposing the microcapsules to the small intestine compartment of the simulated human GI model. The effect of bile concentration on viability was measured by supplementing oxgall at concentrations of 0, 0.25, 0.5, and 1.0%. Pancreatin and sodium bicarbonate were supplemented at physiological levels of 0.18 g/L and 2.4 g/L, respectively. Incubation was performed at 37°C and 100 RPM agitation for 72 hours. Cell viability studies were performed after 0, 3, 6, 12, 24, 36, 48, and 72 hours. As mentioned above, aliquots were plated on MRS agar, and the plates were incubated at 37°C for 72 hours.

3. RESULTS

3.1. Microencapsulation of L. plantarum 80 BSH$^+$ strains

APA microcapsules containing *L. plantarum* 80 cells were prepared using optimized encapsulation procedures. 5 g of bacterial cell isolate were used per 100 g of microcapsules, resulting in a bacterial enumeration of at least 10^9 cfu/mL microcapsule. No significant differences in size ($P > .05$) were noted between empty APA microcapsules and *L. plantarum* 80 loaded APA microcapsules. The resultant diameter of all batches of APA microcapsules was 608 ± 36 μm.

3.2. Bile salt hydrolyzing activity of microencapsulated L. plantarum 80 BSH$^+$ strains in simulated GI model

APA microcapsules containing *L. plantarum* 80 strains were exposed to stomach conditions for 60 minutes prior to intestinal conditions for 10 hours. Figure 2 shows bile salt deconjugation of taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA) over time by gastric stressed Lp80 BSH$^+$ microcapsules in comparison with Lp80 wild type microcapsules. Lp80 BSH$^+$ microcapsules deconjugated both GDCA and TDCA at a significantly greater rate ($P < .05$) than Lp80 wild type microcapsules. Furthermore, both encapsulated Lp80 strains showed a preference for GDCA over TDCA ($P < .05$). As seen in Figure 3, the average BSH activity towards glycoconjugates as a function of time was 4.87 ± 0.28 μmol/g microcapsule/h for Lp80 BSH$^+$ microcapsules and 0.65 ± 0.12 μmol/g microcapsule/h for Lp80 wild type microcapsules. Towards tauroconjugates, the average BSH activity over time was 0.79 ± 0.15 μmol/g microcapsule/h for Lp80 BSH$^+$ microcapsules and 0.35 ± .07 μmol

Table 1: Survival of microencapsulated *L. plantarum* 80 wild-type (control) cells and microencapsulated *L. plantarum* 80 BSH$^+$ (test) cells after simulated stomach (60 minutes) and intestinal (10 hours) transit during bile salt hydrolase assay.

	Viable cell count [log (cfu/ml)]	
	Lp80 wt (Control)	Lp80 BSH$^+$ (Test)
Pre-stomach transit	9.47 ± 0.18	9.40 ± 0.11
Post-stomach transit	7.88 ± 0.38	8.01 ± 0.27
Post-stomach and intestinal transit	8.75 ± 0.46	8.86 ± 0.41

DCA/g microcapsule/h for Lp80 wild type microcapsules. Lp80 BSH$^+$ microcapsules exhausted the entire GDCA content within 6 hours. Maximal activity was reached in the second and third hours, with deconjugation rates of 7.53 and 9.24 μmol DCA liberated/g microcapsule/h, respectively. There was no significant increase in BSH activity towards TDCA after GDCA was completely displaced in the medium.

Simulated stomach exposure at pH 2.0 for 60 minutes resulted in a viability decrease of 1.39 log cfu/mL for microencapsulated *L. plantarum* 80 BSH$^+$ and 1.59 log cfu/mL for microencapsulated *L. plantarum* 80 wild type (Table 1). There were no significant differences ($P > .05$) between strains prior to release into the intestine, with both batches of microcapsules maintaining viability at approximately 10^8 cfu/mL. The alginate microcapsule core shrank significantly ($P < .05$) in gastric conditions, decreasing from 608 ± 36 μm to 544 ± 40 μm (Figure 5). However, mechanical stability remained intact at pH 2.0 and there was no significant release of bacteria into the surrounding medium (data not shown).

Simulated intestinal exposure for a 10-hour period resulted in a viability increase of 0.9–1.0 log cfu/mL, with no significant differences between microencapsulated strains (Table 1). APA microcapsules swelled in the simulated small intestine, with diameter increasing significantly ($P < .05$) from 544 ± 40 μm post-gastric exposure to 725 ± 55 μm post-intestinal exposure. Both microencapsulated strains lowered batch pH of intestinal contents after 10-hour incubation in comparison to control, with Lp80 BSH$^+$ microcapsules having a significantly greater effect than Lp80 wild type microcapsules (Figure 4).

3.3. *Tolerance of microencapsulated Lp80 BSH$^+$ cells to simulated stomach conditions*

Microcapsules containing Lp80 BSH$^+$ cells were investigated for survival in stomach conditions in the simulated human GI model at pH 1.5, 2.0, 2.5, and 3.0 at 37°C and 100 RPM agitation. In all experiments, pH variation in the medium due to the incubation of microencapsulated live cells was less than 10% during the 4-hour period. Figure 6 shows virtually no loss of cell viability during the estimated stomach residence time at pH 2.5 and 3.0. At pH 3.0, cell viability was retained over the first 90 minutes and then decreased slightly, resulting in a total cell loss of approximately 0.6 log cfu/mL

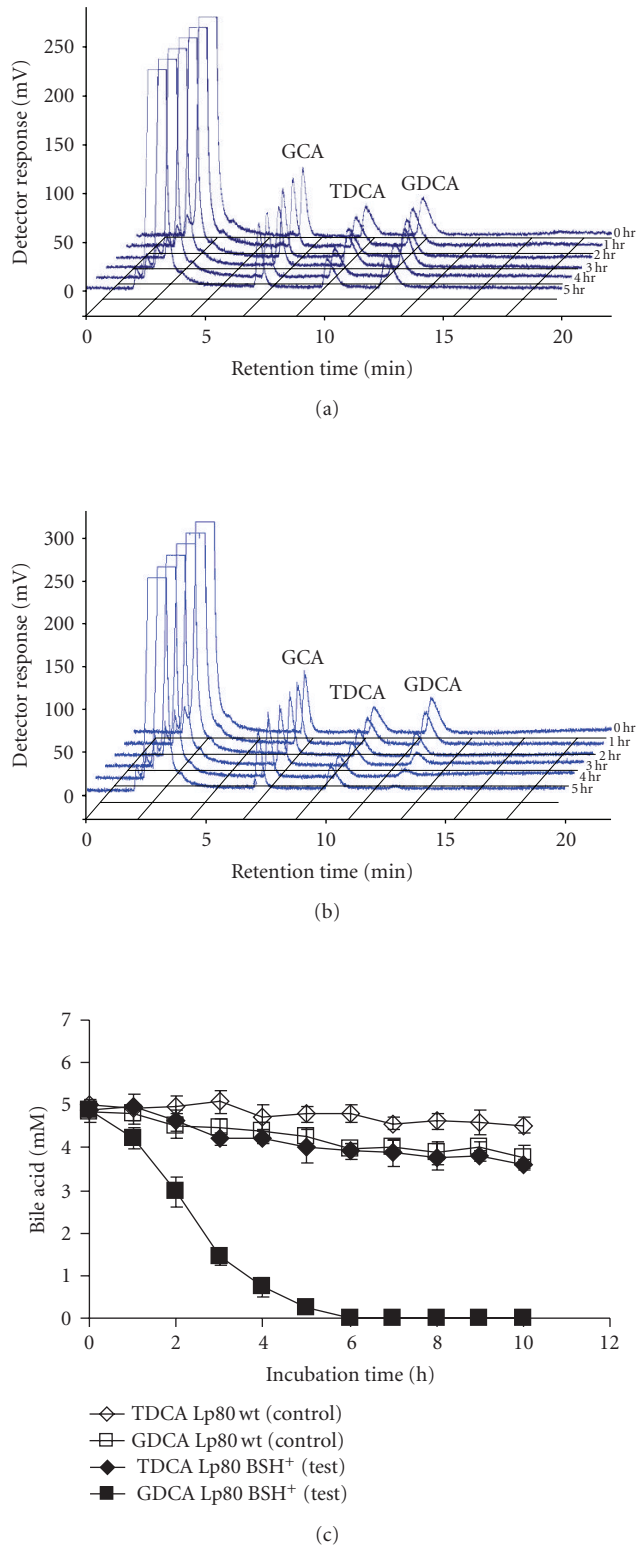

Figure 2: Bile acid deconjugation by microencapsulated *L. plantarum* 80 wild type (control) cells and microencapsulated *L. plantarum* 80 BSH$^+$ (test) cells in simulated intestine following 60-minute exposure time in simulated stomach. Overlaid HPLC chromatograms showing decreasing TDCA and GDCA concentrations over time by microencapsulated (a) Lp80 wild type cells and (b) Lp80 BSH$^+$ cells. (c) Graphical comparison of control and test microcapsules. GCA was used as internal standard.

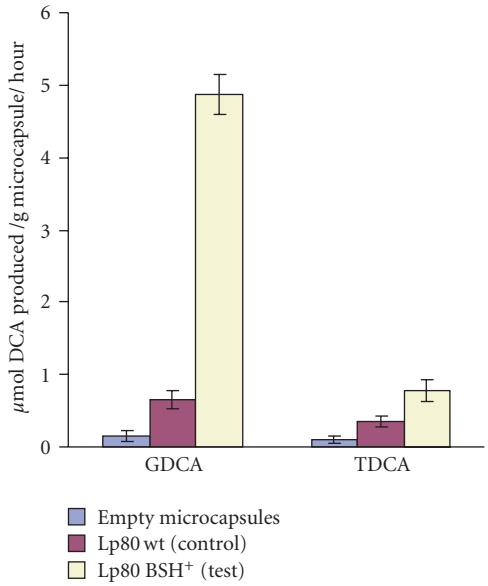

Figure 3: Average BSH activity towards GDCA and TDCA substrates in simulated intestine containing empty microcapsules, microencapsulated *L. plantarum* 80 wild type (control) cells, or microencapsulated *L. plantarum* 80 BSH$^+$ (test) cells.

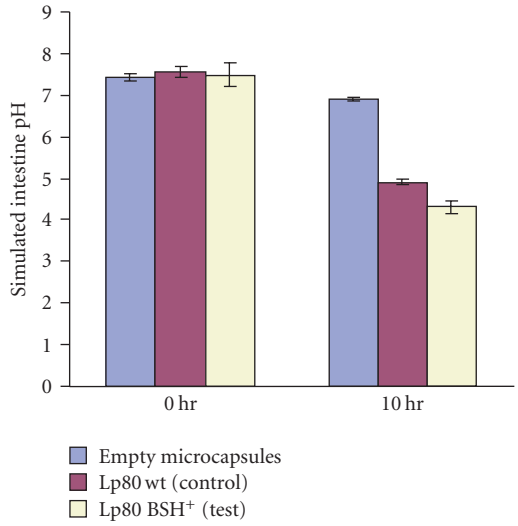

Figure 4: Effect of empty microcapsules, microencapsulated *L. plantarum* 80 wild type (control) cells, and microencapsulated *L. plantarum* 80 BSH$^+$ (test) cells on simulated intestine pH.

after 4 hours. At pH 2.5, microencapsulated cells showed a gradual loss of viability during the first 90 minutes in contrast to pH 3.0. This was followed by a leveling off effect resulting in a total cell loss of approximately 1.09 log cfu/mL after 4 hours. At pH 2.0, viability decreased linearly over time with total cell loss of 0.58, 2.13, 2.63, and 3.64 log cfu/mL after 30, 60, 90, and 120 minutes, respectively. In simulated stomach conditions at pH 1.5, microencapsulated cells were not viable after 30-minute exposure time.

3.4. Adaptive response of microencapsulated Lp80 BSH$^+$ to bile stress in simulated small intestine

Microcapsules containing Lp80 BSH$^+$ cells were investigated for survival in simulated intestinal juices in the presence of physiological concentrations of pancreatin (0.18 g/L) and oxgall (0%, 0.25%, 0.50%, and 1.0% w/v) for 72 hours. The initial pH of all solutions was in the range of 7.4 to 7.6. At all concentrations, APA microcapsules remained intact and cells were retained for up to 72 hours with 100 RPM agitation. As seen in Figure 8, encapsulated cells showed significant increases in viability over the first 3-, 6-, and 12-hour periods in the absence of bile and at bile concentrations in intermediate physiological range of 0.25% w/v. In contrast, cell number was retained but not increased at high-oxgall concentrations of 0.50% and 1.0% w/v after 12 hours. Over the subsequent 12 hours, a drop in cell number was observed at all concentrations; however, the cell viability at 0% and 0.25% w/v batches were still above initial levels.

Figure 8 shows change in pH over time at different oxgall concentrations. In solutions containing no oxgall, a pH minimum of approximately 4.5 was reached after 12 hours and was subsequently retained for the remainder of the experiment. With oxgall present in solution, it required a longer time for pH to reach its minimal level. This level was subsequently retained for the remainder of the experiment. The final pH in each flask increased with increasing oxgall concentration.

4. DISCUSSION

The current study investigated the use of microencapsulated bile salt hydrolase overproducing *Lactobacillus plantarum* 80 cells for oral delivery applications using a dynamic computer-controlled model simulating the human gastrointestinal tract. The microcapsules were found to be adept at protecting the encased cells at less acidic stomach conditions of pH 2.5 and 3.0 with cell viabilities remaining above 10^9 cfu/mL after a 4-hour residence time in the simulated stomach (Figure 6). The oral potential of lactobacilli is directly related to their ability to produce acid and capacity to function at low pH. In the average human, 2.5 L of gastric juice is secreted on a daily basis having a pH of 2–2.5 and a salt content of at least 0.5% w/v. Conditions at pH 2.0 began to affect microencapsulated cell survival showing a linear decrease in viability over time. However, even after 2 hours at pH 2.0, the microcapsules with surviving viable entrapped cells satisfied the established criterion of a minimum of 10^6 viable probiotic cells per mL frequently cited to gain a therapeutic benefit in the intestine [23, 24]. In contrast, the threshold value was not reached with nonencapsulated *L. plantarum* 80 cells which suffered viability losses of 2.0 log cfu/mL and 3.6 log cfu/mL after 30 minutes and 60 minutes, respectively, at pH 2.0. Survival enhancements offered by APA include the cross-linked membrane providing physical barriers against the entry of harmful components found in the GI tract. Furthermore, the alginate core may offer a buffering capacity thus limiting the hostile effect induced by the low pH in the stomach. At pH

1.5, the retained cells showed significant cell loss on account of the loss of core cross-linking. Due to this harmful effect at pH 1.5, coupled with heightened survival at pH 2.5 indicates that oral microcapsule therapy would be best accomplished if ingested in a buffered system such as milk, yogurt, or milk-based foodstuffs.

The inner alginate core was mildly susceptible to acid hydrolysis at pH 2.0 in the simulated stomach, as seen in Figure 5, which resulted in a 10% decrease in microcapsule diameter. Alginates have been shown to undergo proton-catalyzed hydrolysis dependent on factors such as pH, time, and temperature [25]. Capsules remained stable, however, with no significant structural breakage. Microcapsules released into the small intestine after one-hour residence time in the stomach experienced a time delay before reaching maximal BSH activity (Figure 2). Re-establishment of cell growth was found to coincide with BSH activity in the simulated intestine. It has been previously shown for free *L. plantarum* 80 cells that a correlation exists between growth and BSH activity [12]. Although some studies indicate that the optimal pH for bile salt deconjugation by lactobacilli is approximately 6.0 [26], others have suggested that the high BSH activity of certain lactobacillus species during stationary phase is attributed to the low pH of the medium at this stage [27]. In the current study, increases in BSH activity coincided with lowering pH due to the production of lactic acid, however, the BSH activity of the encapsulated cells was determined to be more a product of cell growth than of pH conditions in the environment.

BSH activity of microencapsulated BSH overproducing *L. plantarum* 80 cells was found to have a 5-fold preference of glycodeconjugation over taurodeconjugation (Figure 3). This compares with earlier studies showing BSH activity in optimal MRS medium without initial gastric stress to have a ratio of glycodeconjugation to taurodeconjugation of 2.5 : 1 [21]. This bears potential significance in terms of *in vivo* hypocholesterolemic activity, since glycoconjugates outnumber tauroconjugates in human bile with ratios as high as 9 : 1 in some individuals [28]. A myriad of data in the literature suggests that substrates are predominantly recognized at the amino acid moieties and most BSHs are more efficient at hydrolyzing glycoconjugates than tauroconjugates [29–31]. This is despite the fact that GDCA carries a higher toxicity, which is magnified at low pH [14]. The initial gastric stress period therefore appeared to acclimatize the bacteria to the low pH environment, therefore lessening the relative toxicity to glycoconjugates in the intestine. In addition, there was a significant decrease in deconjugation rate towards both GDCA and TDCA in the simulated GI model as compared to optimal MRS media [21]. In comparing the two mediums, simulated intestinal juices were found to be only moderately more inhibitory to encapsulated cell viability and enzymatic activity than MRS-bile media (data not shown), primarily due to the addition of pancreatic enzymes into the medium. The stress and viability losses imposed by gastric transit resulted in an internal acidification of the cells, which in turn likely caused the reduction in activity of the acid sensitive BSH enzyme.

Microcapsule swelling upon intestinal release (Figure 5) resulted from among other factors, the affinity of calcium to phosphate in the medium and sodium/calcium exchange. Swelling was enhanced due to pre-treatment in stomach conditions which resulted in slightly weaker core cross-linking. However, over 90% of APA microcapsules maintained structural integrity through both stages of GI transit. The majority of BSH activity was therefore the direct result of retained cells acting on conjugated bile salts diffusing through the microcapsule membrane pores. Since large amounts of deconjugated bile salts may have undesirable effects for the human host, concerns may arise over the safety of administering a BSH-positive probiotic strain. However, it is likely that the deconjugated products are precipitated at the low pH values in the intestine caused by the fermentation products of lactic acid bacteria (Figure 4). Furthermore, this localized phenomenon is potentially increased within the microcapsule membrane, allowing for a greater precipitation of deconjugation products which could then be bound and retained within the microcapsule membrane and excreted with the feces. In the current study, no environmental concentration of DCA was observed in the simulated intestine after 10 hours.

Resistance to bile toxicity is an important criteria used to select for microencapsulation systems and probiotic strains capable of performing effectively in gastrointestinal environments. In a given day, 0.7 L of pancreatic juice is secreted into the proximal small intestine with a pH of 7.5–8.0 and a salt content of at least 0.5% w/v. Daily bile acid secretions approach 20–30 g/day sustaining an intestinal bile salt concentration between 0.15 and 1.0% [32, 33]. According to Charteris et al., the majority of lactobacillus and bifidobacterium strains are intrinsically resistant to simulated pancreatic juice without the addition of bile salts [34]. In contrast, 31 of 47 lactobacillus strains examined by Jacobsen et al. were severely inhibited by bile and did not replicate in broth supplemented with 0.3% oxgall [35]. Furthermore, the protective capability offered by BSH active bacteria in toxic bile environments is not always evident [36]. As seen in Figure 7, microcapsules containing *L. plantarum* 80 BSH^+ cells exposed to the simulated small intestine were shown to be highly resistant to bile, either retaining or increasing viability after 3, 6, and 12-hour transit times at bile concentrations up to 1.0% w/v. It has been reported that survival at a bile concentration of 1000 mg/L is considered optimal bile tolerance for probiotic strains. It is therefore shown here that encapsulated strains do not lose viability after 12 hours in bile concentrations at least 10 times the level proposed. While high-oxgall concentrations of 0.5% and 1.0% were shown to inhibit growth, encapsulated cells maintained initial viability counts of 10^{10} cfu/mL during the first 12 hours. Cell metabolic activity was inhibited at higher concentrations of oxgall as decrease in pH was more limited at higher concentrations. It could be concluded that production of lactic acid was inhibited with increasing bile concentrations. Overall, the protective effect of microencapsulated BSH active bacteria in simulated intestinal transit was evident, particularly at high-bile levels.

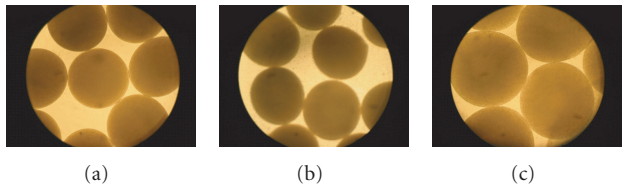

Figure 5: Evaluation of microcapsule integrity and morphological changes during simulated GI transit. (a) Pre-stomach transit. (b) Post-stomach transit (60 minutes). (c) Post-stomach (60 minutes) and intestinal (10-hour) transit. Microcapsule size (a) 608 ± 36 μm (b) 544 ± 40 μm (c) 725 ± 55 μm.

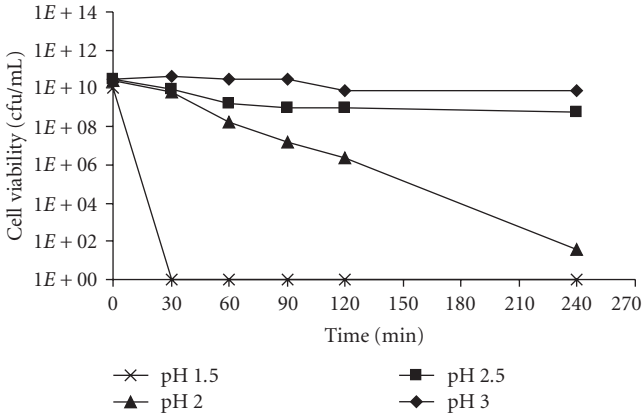

Figure 6: Viability of microencapsulated *L. plantarum* 80 BSH$^+$ cells in simulated stomach at pH 1.5, 2.0, 2.5, and 3.0, 37°C and 100 RPM agitation.

5. CONCLUSIONS

Recent advances in metabolic engineering have enhanced the enzymatic and immunomodulatory effects of probiotics and with time may provide more active therapeutic intervention. For some time now, clinical and epidemiological evidence have established a clear link between elevated serum cholesterol and coronary artery disease (CAD) [37–39]. Lactobacilli with active BSH are suggested to lower cholesterol levels through an interaction with host bile salt metabolism [8, 12]. The main proposed mechanism of cholesterol reduction involves an increased production of deconjugated products in the intestine, thereby increasing the demand for cholesterol as a precursor of bile salt synthesis. Furthermore, deconjugated bile salts do not function as well as their conjugated counterparts in the solubilization of cholesterol and therefore prevent it from being absorbed [40]. The preceding study suggests that microcapsules containing *L. plantarum* 80 with enhanced BSH activity show excellent probiotic potential in the simulated GI model. This study represents a unique biotechnological approach indicating that microencapsulation maintains the enzymatic activity of BSH active bacteria in the simulated GI transit while at the same time avoiding the problems associated with the oral administration of free bacterial cells. In addition to specific bile salt hydrolase oral delivery applications, this study provides knowledge to-

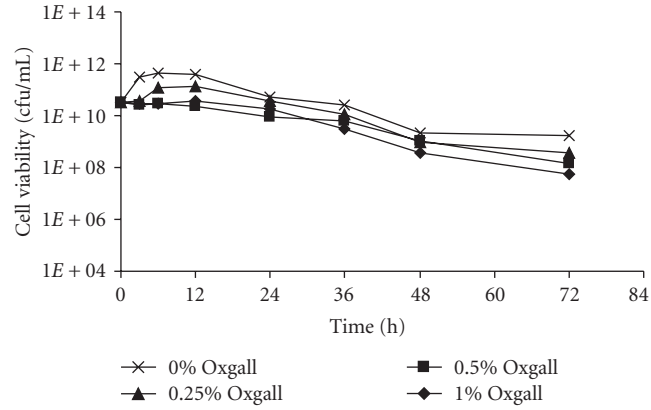

Figure 7: Adaptive response of microencapsulated *L. plantarum* 80 BSH$^+$ cells to bile stress in simulated intestine at bile concentrations of 0%, 0.25%, 0.50%, and 1.0% w/v, 37°C, and 100 RPM agitation. Pancreatin (0.18 g/L) and sodium bicarbonate (2.4 g/L) concentrations were kept constant.

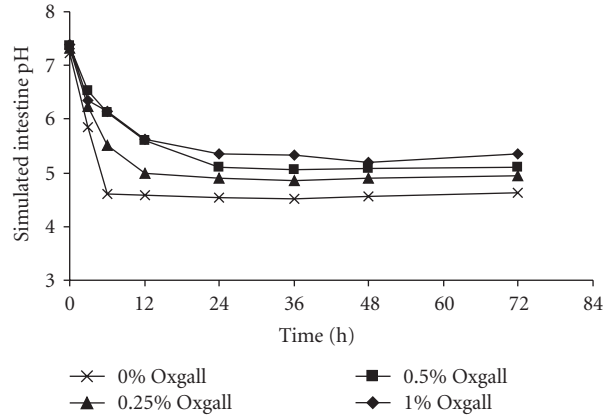

Figure 8: Effect of bile concentration on metabolic activity of microencapsulated *L. plantarum* 80 BSH$^+$ and change in pH in simulated intestine. Triplicate samples were pooled prior to analysis.

wards the potential use of orally administered probiotic cells for therapy.

While the potential of this approach is widespread, current limitations must be addressed in order for this technology to be properly applied. Safety studies have indicated that the use of genetically modified microorganisms pose no greater risk than the original unmodified product [41]. However, there remain public and scientific concerns about the safety of gene manipulation technology. One of the main concerns is the possibility of repeated large doses of novel microorganisms resulting in the transfer of genes to organisms in the environment [42]. Consequently, even though the encased cells are classified as nonpathogenic, regulatory agencies may require exclusively no leaking of engineered cells from ingested microcapsules into the host's GI system. Therefore, the design of an appropriate polymeric microcapsule membrane for such oral delivery applications is an important focus going forward.

Additional research is required to substantiate these results; in particular *in vivo* studies demonstrating the link between increased bile salt deconjugation and cholesterol reduction using microcapsules. Important considerations for *in vivo* studies may include dosage, frequency, and timing of therapeutic administration, composition of microcapsule membrane, and potential side effects of byproducts. While these factors present challenges, we believe that the potential of this approach is to carry heightened benefit while minimizing risk.

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