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Growth of *Lactobacillus reuteri* NCIMB 30242 during yogurt fermentation and bile salt hydrolysis activity in the product

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Abstract The synthesis of bile salt hydrolase has been linked to the health benefit of *Lactobacillus reuteri* toward lowering blood cholesterol. The aim of this study was to examine the growth and bile salt hydrolysis activity (BSHA) of *L. reuteri* NCIMB 30242 during milk fermentation with a yogurt starter. There was little growth of *L. reuteri* during a 4-h co-fermentation with a yogurt culture, and an inoculation of 4.5×10^7 CFU.mL⁻¹ was needed to obtain the 10^8 CFU.mL⁻¹ target in the product. Enrichment of milk with sugars, minerals, or peptone-based ingredients did not improve growth of *L. reuteri*. Viable counts of *L. reuteri* above 1.5×10^8 CFU.mL⁻¹ generated texture defects. Free and microencapsulated (ME) cultures were tested for BSHA in the yogurt drinks. *L. reuteri* cells which grew during the 4-h lactic fermentation had 40% less BSHA than *L. reuteri* added directly via the commercial culture. The BSHA of free cells was apparently three times higher than in the ME culture. This study adds data showing that the yogurt production process could affect the functionality of probiotic bacteria.

Keywords Probiotic · Inoculation level · Enzyme · Microencapsulation · Fermented milk · Dairy beverage

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1 Introduction

As scientific evidence of the benefits of probiotics continues to build, health claims associated with fermented milks are now being recognized. Thus, the European Food Safety Authority (EFSA) has granted a health claim on lactose digestion for yogurt (EFSA 2010) while Switzerland's "Office Fédéral de la Sécurité Alimentaire et des Affaires Vétérinaires" allows claims on gastrointestinal regulation of transit time for specific fermented milks (OSAV 2014). To our knowledge, no health claims are currently granted for the effects of probiotics on cardiovascular diseases. Thus, products need to be developed and tested for the prevention of heart diseases.

A recognized link to cardiovascular health is the level of blood cholesterol. It is considered that certain forms of cholesterol are detrimental to health and that lowering their level in blood is desirable (Jacobson 2000). Fermented milks are recognized as being very good matrices for functional ingredients (Shiby and Mishra 2012). *Lactobacillus reuteri* NCIMB 30242 has been shown to be clinically efficacious on reducing blood cholesterol (Jones et al. 2012a, 2012d).

However, *L. reuteri* has received much less attention than bifidobacteria, *Lactobacillus acidophilus*, or *Lactobacillus rhamnosus* as potential probiotics for the development of functional fermented milk products. Some data show limited growth in milk and low stability during storage (Hekmat et al. 2009; Liu and Tsao 2009). One of the reasons probiotic bacteria can influence the blood cholesterol level is by producing bile salt hydrolase (Patel et al. 2010a, b; Jones et al. 2012a). However, there are no data on the production of bile salt hydrolase by probiotic lactobacilli and the resulting BSHA during milk fermentation in association with a lactic starter.

The aim of this study was therefore to examine the growth and BSHA of *L. reuteri* NCIMB 30242 during milk fermentation with a yogurt starter.

2 Materials and methods

2.1 Strains

The starter culture type II from Biena (St-Hyacinthe, QC, Canada) was chosen for the yogurt production. It is composed of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains.

L. reuteri NCIMB 30242 (LRC™) is now available at UAS Labs (Madison, WI, USA). It was selected for this study based on its documented health effects (Jones et al. 2012a, d, 2013a and b) as well as its safety (Jones et al. 2012b, c). Although details of the manufacturing process are proprietary to Micropharma (Montréal, QC, Canada) and UAS Labs, the following information can be given: growth in MRS in anaerobic conditions, centrifugation at 3300×g for 20 min, cell pellet re-suspending in a maltodextrin/cysteine solution, and flash freezing in liquid nitrogen. Microencapsulation in alginate and poly-L lysine gel beads was carried out as described by Martoni et al. (2011). The resulting frozen droplets of free and microencapsulated (ME) *L. reuteri* NCIMB 30242 were stored at -80 °C until used. The frozen culture of free cells provided by Micropharma had a 4×10^{10} CFU.mL⁻¹ (10.6 Log), while the ME culture had 3.2×10^9 CFU.mL⁻¹ (9.5 Log).

2.2 Drinkable yogurt product

Commercial milk (1 and 2% milk fat) (Québon, QC, Canada) were analyzed for protein, fat, and lactose composition by Fourier transform infrared spectroscopy (FTIR) using a Milko Scan FT-120 unit (Foss Electric, Hillerød, Denmark). From the data obtained by FTIR, a milk blend having 1.25% fat and 3.75% protein was prepared by mixing 1% fat milk and 2% fat milk in a 77:23 ratio, to which was added 12.7 g.L⁻¹ of skimmed milk powder (Crino, Agropur, Granby, QC, Canada), as well as 5 g.L⁻¹ corn starch (President's Choice, Loblaws, Toronto, Canada) and 8.3 g.L⁻¹ of Splenda® (Johnson & Johnson, Markham Canada). After 30 min of hydration, the mixture was boiled and cooled to 42 °C. Unless otherwise stated, 2 g.L⁻¹ of pure vanilla extract (President's Choice) was added. The milk blend was inoculated at 1 g.L⁻¹ with the dried starter culture, as per recommended by the supplier. In order to obtain a product with a good texture and cells in a similar physiological state, the inoculated milk samples were incubated at 42 °C until a final pH of 4.5 was reached.

2.3 Inoculation level determination in co-fermentation of *L. reuteri* in yogurt

After inoculation with the dried starter culture, the milk mixture was separated into lots and inoculated with frozen free-cell suspensions of *L. reuteri* at concentrations of 6.75, 7.05, 7.43, 7.65, 7.95, and 8.25 Log CFU.g⁻¹. The products were incubated for 4 h at 42 °C. In some assays, the ME culture was added to finished yogurt in order to compare BSHA of free and ME cells.

2.4 Effect of milk supplementation on the growth of *L. reuteri* NCIMB 30242

The milk blend standardized to 1.25% fat and 3.75% protein described above was used for the “control,” with the exception that no Splenda or vanilla extract were added (unless otherwise stated). The following supplements were separately added to this control blend prior to heating: Casitone (Difco, Detroit MI, USA) at 5 g.L⁻¹, sucrose (LabMat, Beauport, Québec Canada) at 10 g.L⁻¹, Splenda® at 8.3 g.L⁻¹, Lab Lemco meat extract (Oxoid, Nepean, Ontario, Canada) at 10 g.L⁻¹, yeast extract (Difco) at 5 g.L⁻¹, glucose (LabMat) at 10 g.L⁻¹, dextrose (Fisher, Ottawa, Ontario, Canada) at 20 g.L⁻¹, Tween at 1 g.L⁻¹, sodium acetate (Bioshop, Burlington, Ontario, Canada) at 5 g.L⁻¹, ammonium citrate (Bioshop) at 2 g.L⁻¹ and potassium phosphate dibasic (Bioshop) at 2 g.L⁻¹, magnesium sulfate (LabMat) at 0.1 g.L⁻¹, and manganese sulfate (Bioshop) at 0.05 g.L⁻¹. In some samples, 2 g.L⁻¹ of pure vanilla extract (President's Choice) was then added to obtain “vanilla” and a “Splenda®-Vanilla” media. A neutralized pasteurized yogurt (NPY) medium was produced by raising the pH of control yogurt (described before) with KOH (5 N) from pH 4.5 to pH 7.0, in order to obtain pH 6.6 after boiling and cooling. Medium MRS (Difco) that was used has laboratory reference. All media were adjusted to 42 °C and, unless otherwise stated, inoculated with the thawed free-cell suspension of *L. reuteri* to obtain an initial population of 3.4 × 10⁷ CFU.mL⁻¹. After addition of the yogurt starter (1 g.L⁻¹), the milk-based matrices were incubated up to 6 h at 42 °C until the pH reached 4.5 (between 4 and 6 h).

2.5 Enumeration methods

Yogurt (10 g) was blended with 90 mL of a dissolution medium (1 g.L⁻¹ tryptone, 8.5 g.L⁻¹ sodium chloride, and 10 g.L⁻¹ sodium citrate dehydrate) in a Stomacher™ bag and treated 1 min in the Stomacher at “normal” speed (230 rpm). After a 10-min incubation period at room temperature, 1 mL of the homogenate was transferred to 9 mL of 1 g.L⁻¹ peptone water (Difco) and homogenized 30 s at 27,000 rpm (Omni THQ). In line with the recommendation of Champagne et al. (2011) for the enumeration of probiotics in foods, specific steps (citrate and high-shear homogenization) were taken to allow complete dissolution of the APA ME cultures. Serial dilutions in 1 g.L⁻¹ peptone water were then pour plated in MRS-T agar (tetracycline 0.9 mg.mL⁻¹, Sigma, St. Louis, MO, USA) for enumeration of *L. reuteri* (Hekmat et al. 2009) and in M17 agar for *S. thermophilus* counts. The MRS-T plates were then incubated in anaerobic conditions (*L. reuteri*) (5% CO₂, 10% H₂, and 85% N₂) at 37 °C for 48 h, while M17 plates were incubated aerobically (*S. thermophilus*) (ISO/IDH 2003). No selective plating for *L. bulgaricus* was carried out.

2.6 Enzymatic analysis

Although bile salt hydrolase is mainly produced in the cytosol and analysis can be carried out in cell-free extracts (Liong and Shah 2005), there is activity on bacterial cell membranes (Patel et al. 2010a, b). In this methodology, the inocula and fermented milk contained viable cells. Thus, the reduction of bile salts in the testing solution could be the result of (1) bile salt hydrolase in the yogurt resulting from cell autolysis, as well as (2) assimilation and hydrolysis by the viable cells. Therefore, this test is technically a “bile salt hydrolysis” analysis from multiple bile salt hydrolase sources rather than a “bile salt hydrolase” test of a cell-free enzyme extract. The bile salt hydrolysis activity (BSHA) was carried out as described by Martoni et al. (2007) with modifications. Activity tests were carried out on samples having approximately 10⁸ CFU.mL⁻¹. If dilution of the samples was needed, they were made in MRS before addition to the testing medium. Various cell-free MRS or milk controls were performed. For the analysis, 2 mL of each sample was added to 18 mL of the testing media, which was composed of MRS supplemented with 5 mM of sodium glycodeoxycholate (GDCA, from Sigma) and 5 mM of sodium taurodeoxycholate (TDCA), and incubated at 37 °C with agitation at 100 rpm. Samples were analyzed at incubation times of 15 and 30 min. For each sample, 7 µL of 6 N HCl was added to 700 µL of sample (in Eppendorf tubes) to stop the reaction, vortexed 1 min, and centrifuged for 3 min at 10,000×g at 4 °C. An internal standard (500 µL of GCA (Sigma)) was added to 505 µL of the supernatant, vortexed 10 min, and centrifuged at 1000×g for 15 min at 4 °C. This was followed by filtration of the supernatant (0.45 µ) and analysis by HPLC. The HPLC analysis was carried out as described in Martoni et al. (2007).

3 Results and discussion

3.1 Growth of the cultures in milk

When inoculated alone at 1.8×10⁷ CFU.mL⁻¹, *Lactobacillus reuteri* NCIMB 30242 did not acidify milk very much (Fig. 1) and a viable count of 4.3×10⁷ CFU.mL⁻¹

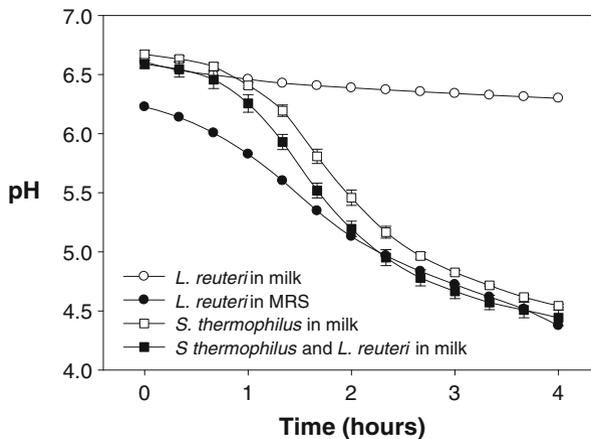


Fig. 1 Effect of the bacterial culture(s) and of the growth medium on the evolution of pH during fermentation at 42 °C. *Streptococcus thermophilus* ST8 was inoculated at 4.9×10^7 CFU.mL⁻¹ (7.69 Log CFU.g⁻¹), while *Lactobacillus reuteri* NCIMB 30242 was added at 4.6×10^7 CFU.mL⁻¹ (7.65 Log CFU.g⁻¹). Error bars represent standard error of the means of three independent assays

(7.63 Log CFU.g⁻¹) was reached after 4 h of incubation. This limited acidification was not due to a low inoculation rate nor to a lack of acidifying ability of this strain, since strong growth (8.88 Log CFU.g⁻¹) and acidification of MRS occurred following the same inoculation level (Fig. 1). One concern was that the inocula had not been produced in conditions that would optimize its subsequent growth in milk. Indeed, the concentrates were produced on glucose and in a peptide-rich medium which would not induce high β -galactosidase or proteolytic activities. Thus, cells might need to adapt at the beginning of the 4 to 6-h yogurt production period. However, such a need for adaptation to milk can probably be said of most probiotic cultures on the market which are produced on MRS-like media (Champagne and Møllgaard 2008). Thus, the culture used in this study can be considered as being “typical” for probiotics. Further data are needed to assess the effect of the production conditions of concentrated probiotics on their subsequent ability to grow during yogurt fermentation.

In light of this limited growth of *L. reuteri* in milk during the 4 to 6-h incubation period, a series of fermentations were carried out in order to evaluate the inoculation level (between 6.75 and 8.25 Log CFU.g⁻¹) required to obtain at least 8.0 Log CFU.g⁻¹ of *L. reuteri* in the fermented product. It was found that at least 7.65 Log CFU.g⁻¹ was required to achieve our goal.

Probiotics are generally only adjunct cultures, with CFUs typically at 10^7 CFU.mL⁻¹. However, when CFUs attain levels above 10^8 CFU.mL⁻¹ and affect sensory properties (Champagne et al. 2005), they can “become” part of the starter culture. As Fig. 1 shows, milk acidification was enhanced when at least 7.65 Log CFU.g⁻¹ of *L. reuteri* was inoculated in conjunction with the yogurt starter. As a result, the time required to obtain a pH of 4.5 decreased (Fig. 2). Hekmat et al. (2009) had not seen any effect of *L. reuteri* on milk acidification, when also combined with a yogurt starter, but this is presumably because the probiotic’s viable count remained below 10^6 CFU.mL⁻¹. It was also noted in this study that the higher the *L. reuteri* inoculation level was, the less was its increase in CFU (Fig. 2). This might partially be linked to the shorter incubation time to reach pH 4.5.

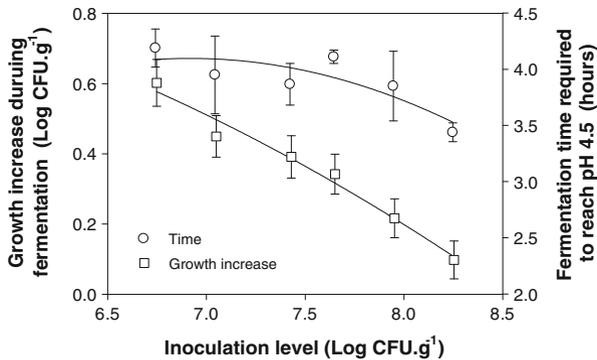


Fig. 2 Effect of the inoculation level of *Lactobacillus reuteri* NCIMB 30242 on the time required to reach a pH of 4.5 and on the increase in CFU during the fermentation period. Error bars represent standard error of the mean

The viable count of *S. thermophilus* was affected by the *L. reuteri* inoculation level. Above an inoculation of 7.95 Log CFU.g⁻¹ of *L. reuteri*, there was a 40% reduction in growth of the streptococci from 8.88 to 8.67 Log CFU.g⁻¹. It can be hypothesized that this was linked to the shorter fermentation time which resulted from the high inoculation level of *L. reuteri* (Fig. 2), which became two times higher than *S. thermophilus*. Since we were unable to selectively enumerate *L. bulgaricus* cells, this study shows that the classic methodologies involving CFUs by selective media have limits. This raises the interest of newer techniques such as PMA-qPCR (Desfossés-Foucault et al. 2012) and flow cytometry (Geng et al. 2014), which enable both “total” and “viable” counts of given species and even strains.

3.2 Effect of *L. reuteri* growth on appearance of the fermented milk

When a population above 8.17 Log CFU.g⁻¹ of *L. reuteri* was attained, sensory problems were noted. Gas bubbles and syneresis became visible. *L. reuteri* has a heterofermentative metabolism, and this resulted in the production of CO₂ and, presumably, ethanol as well as acetic acid (van Niel et al. 2012).

The 8.17 Log CFU.g⁻¹ threshold for absence of visible gas is very close to the 8.0 Log CFU.g⁻¹ target CFU. This would be difficult to manage in a set-style yogurt production. However, since the strategy is toward a drink, the product will be pumped and blended. It is unknown if these operations would enable expulsion of gas and repair the phase separation problem. Further studies are needed to establish the maximum viable count of *L. reuteri* that does not result in sensory defects during storage of a processed product.

3.3 Effect of milk supplementation or of pre-fermentation on *L. reuteri* growth

Attempts were made to find a supplement which could potentially promote *L. reuteri* growth in milk. When compared to the milk control, none of the supplements tested improved growth by *L. reuteri* (Fig. 3). Multiplication in MRS was at least 15 times higher than in milk (Fig. 3). However, even if differences in CFUs were small, some growth levels were found to be statistically different. CFUs in milk supplemented by a

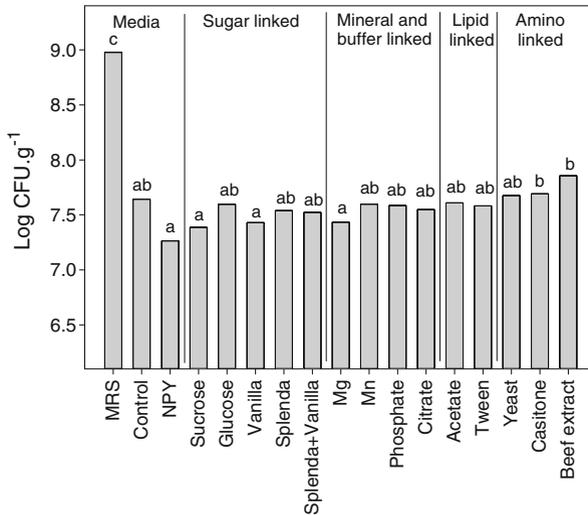


Fig. 3 Effect of milk supplementation on the growth of NCIMB 30242 after a 4-h incubation at 42 °C. MRS (Difco) represents a “positive control.” “Control” represents the milk formulation. NPY neutralized pasteurized yogurt, *abc* bars that have the same letter were not judged to be significantly different ($P>0.05$)

beef extract or by casitone were higher than those obtained in the NPY. None of the individual ingredients significantly improved growth in milk, which suggests that low growth might be linked to multiple limiting factors (Moroshita et al. 1981). The supplements which had the best results were nitrogen-based (mainly casitone and beef extract) (Fig. 3) although it could be argued that yeast extracts and beef extracts could also provide vitamins, nucleotides, and fatty acids.

In the hope of obtaining a symbiosis (Champagne et al. 2009), assays were conducted in which *L. reuteri* was added to the milk blend which had previously experienced growth of the yogurt starter (NPY treatment). This did not occur with the NPY treatment used in the study, which raised the possibility of antagonism between the yogurt and *L. reuteri* cultures.

The possibility that the presence of oxygen was the problem, rather than a nutritional deficiency, was also considered. However, good multiplication (Fig. 3) and activity (Fig. 1) in MRS under the same atmospheric conditions tend to negate this hypothesis. Further studies are required to find means of enhancing the growth of *L. reuteri* NCIMB 30242 in milk and during fermentation with a yogurt culture.

3.4 Analysis of bile salt hydrolase in fermented milk

There was a strong relationship between bile salt hydrolysis activities using TDCA and GDCA substrates. The correlation coefficient (r) between the two sets was 0.87, and the relationship was considered extremely significant (P value is <0.0001). Therefore, although only the data on GDCA is presented, it must be kept in mind that the conclusions also apply to the metabolism of TDCA.

Since the CFU target in yogurt was about 1.4×10^8 CFU.mL⁻¹, BSHA calculations were subsequently done on that cell density basis (Table 1). Attention must be given, when reading Table 1, of the method used to calculate the BSHA readings. As data will

Table 1 Bile salt hydrolysis activity (BSHA) ($\text{U}\cdot\text{mL}^{-1}$) of *Lactobacillus reuteri* NCIMB 30242 in free cell format added to yogurt before or at the end of fermentation with different reaction times

Sample	Reaction time (min)	Apparent BSHA	Corrected BSHA
Free cells in MRS ^a	0–15	35 (2)	35 (2)
	15–30	23 (1)	23 (1)
	0–30	29 (1)	29 (1)
ME cells in MRS ^a	0–15	12 (1)	12 (1)
	15–30	10 (4)	10 (4)
	0–30	11 (2)	11 (2)
Milk only, no starter or <i>L. reuteri</i> cells	0–15	68 (3)	–
	15–30	2 (1)	–
	0–30	35 (1)	–
Yogurt only, no <i>L. reuteri</i> cells	0–15	86 (3)	–
	15–30	0 (0)	–
	0–30	43 (1)	–
Yogurt with <i>L. reuteri</i> grown during fermentation ^b	0–15	100 (6)	14 (4)
	15–30	9 (2)	9 (2)
	0–30	54 (4)	11 (3)
Yogurt with <i>L. reuteri</i> added at the end of the fermentation ^b	0–15	111 (6)	25 (3)
	15–30	10 (1)	10 (1)
	0–30	60 (3)	18 (2)

Values presented on a basis of 1.4×10^8 CFU $\cdot\text{mL}^{-1}$ of *L. reuteri* NCIMB 30242

Values in brackets represent SEM

^a The data of “corrected BSHA” are identical to those of “apparent BSHA” because there is no interference of milk ingredients with the readings

^b The data in these “corrected BSHA” column were calculated by deducting the “apparent BSHA” values of the “yogurt only no *L. reuteri* cells” treatment from the “apparent BSHA” values of the “yogurt with *L. reuteri* added ...” treatments

show, many factors influence the apparent BSHA readings of yogurt samples which were not diluted for analysis. Thus, when establishing the BSHA activity in yogurt samples having *L. reuteri* cells, the “apparent BSHA” of probiotic-free yogurt itself must be considered.

The free-cell concentrate provided by Micropharma used for inoculation appeared to have almost three times more activity than the ME culture (Table 1). It must be mentioned however that the ME culture did not dissolve in the GDCA/TDCA-MRS medium during the 30-min assay test. Since microencapsulation in alginate lowers mass transfer of compounds (Tanaka et al. 1984), it was not surprising that the ME culture “apparently” had a lower specific BSHA than free cells. This is the first report of microencapsulation potentially reducing the BSHA activity prior to dissolution. The ME product would presumably have a higher BSHA in the duodenum since alginate beads rapidly dissolve in that environment (Iyer et al. 2004). One must keep in mind, as well, that ME could provide protection in the yogurt itself during storage (Champagne et al. 2005).

It was first examined if the reduction of the GDCA was constant throughout the 30-min incubation. Thus, activities were calculated after 15 and 30 min of incubation. With MRS-grown cultures, slightly higher activities were noted in the first 15 min of incubation, but a significant activity level was noted between 15 and 30 min (Table 1). This suggested that GDCA reduction was linked to enzyme activity rather than simple adsorption to the bacterial cells.

The BSHA assays on the concentrated free or ME cultures required dilution of the concentrates in MRS broth, since cell suspensions having about 10^8 CFU.mL⁻¹ work well in this method. Thus, the reaction medium essentially contained the cells within a MRS medium. However, the milk and fermented milk sampled contained 1.4×10^8 CFU.mL⁻¹, and the yogurt samples could not be diluted prior to addition to the GDCA/TDCA-MRS reaction medium. It was therefore examined if the milk matrix or the yogurt product itself could interfere with the readings. Adding cell-free milk blend to the GDCA/TDCA-MRS produced an apparent BSHA of 35 U.mL⁻¹ over the 30-min incubation period (Table 1). However, the reduction of GDCA almost only occurred during the first 15 min of incubation. This suggested that GDCA adsorbed to milk ingredients and that BSHA was only apparent. Since a portion of the bile molecule is hydrophobic, it was hypothesized that the bile compound was adsorbed by the fat in the milk. A sample of cell-free skim milk was therefore tested. The result was also an apparent BSHA of 35 U.mL⁻¹, identical to that of the 1.25% fat milk. This suggests that fat in our yogurt mix was not responsible for the reduction of the GDCA from the medium. It remains to be determined if binding to proteins or whey salts (phosphates) could be responsible for the phenomenon.

When a yogurt control without *L. reuteri* was tested, apparent BSHA was similarly observed, at 43 U.mL⁻¹ (Table 1). However, as was the case for culture-free milk, the reduction of GDCA with the yogurt sample basically appeared in the first minutes of incubation. No activity was noted between 15 and 30 min of incubation. This suggests that the yogurt starter culture itself was devoid of BSHA. It has not been identified if this slight increase in apparent BSHA, as compared to milk, was due to the acidity brought by the yogurt and/or if the presence of the starter culture itself contributed to GDCA adsorption. We were unable to find a method to adequately separate viable starter bacteria from the fermented milk to test this possibility. All these data indicated that, in order to ascertain the BSHA in fermented milk specifically linked to *L. reuteri*, the blank (control) had to be a yogurt product without the probiotic strain (Table 1).

When using yogurt as a blank, the BSHA readings of 1.4×10^8 CFU.mL⁻¹ of *L. reuteri* directly added to yogurt was 33% lower when they were in MRS (Table 1).

3.5 Levels of bile salt hydrolysis activity in fermented yogurt

In yogurt manufacture, the probiotic culture is generally added at the beginning of fermentation, because growth of the probiotic can occur during the incubation (Champagne et al. 2009) and because the probiotic can subsequently be more stable during storage (Hull et al. 1984). When the *L. reuteri* culture was added at the beginning of the fermentation, the overall “corrected BSHA” (0–30 min) was almost 40% lower than when cells were added directly to the yogurt (Table 1). It is noteworthy that the difference is mainly linked to BSHA in the first 15 min (Table 1). The lower level of BSHA in the fermented milk is possibly due to the lack of bile salts during the growth of *L. reuteri*. Indeed, in many cases, the synthesis of bile salt hydrolase is

inducible (Patel et al. 2010a, b) and the commercial culture provided by the supplier had been produced to have a high BSHA. Therefore, in order to have high BSHA in the fresh yogurt, it is advisable to add cells having high BSHA directly to fermented product. It is unknown if this inoculation approach negatively affects the subsequent stability of *L. reuteri* during storage.

The ME culture can only be added to the fermented milk. Indeed, it would not remain homogeneously distributed in milk prior to fermentation and would sediment before coagulation would occur. Data from this study suggest that inoculation of *L. reuteri* in the fermented product is a good alternative for high BSHA levels. Therefore, products with ME cultures could potentially be of commercial interest. It remains to be ascertained, however, if ME could enhance the stability of *L. reuteri* during storage and during passage in the stomach. Further data on BSHA in these two conditions are needed.

4 Conclusion

This study has furthered the previously published data whereas it is possible to produce fermented milk products with *L. reuteri* NCIMB 30242 having BSHA. Thus, this study provided the following eight novel observations to the sector: (1) *L. reuteri* shows limited growth during a 4-h co-fermentation with a yogurt culture; (2) within the 5.6×10^6 and 1.8×10^8 CFU.mL⁻¹ range, the higher the inoculation level of *L. reuteri*, the less there is an increase in the probiotic population during the fermentation; (3) when the yogurt starter is inoculated at 5×10^7 CFU.mL⁻¹, an inoculation level of *L. reuteri* below 1×10^8 CFU.mL⁻¹ does not affect the fermentation rate; (4) when the *L. reuteri* population was $\geq 1.5 \times 10^8$ CFU.mL⁻¹, texture defects (syneresis, gas bubbles) appeared in the fermented product; (5) individual enrichment of milk with various sugars, lipids, minerals, or peptone-based products did not improve the growth of *L. reuteri* over a 4-h incubation period, (6) the BSHA of free cells was apparently three times higher than in the ME culture; (7) milk and yogurt interfere with the BSHA test, as carried out by evaluating GDCA reduction from a MRS broth; (8) *L. reuteri* cells which were grown during a 4-h fermentation has 40% less BSHA than *L. reuteri* added directly via the commercial culture. When comparing the BSHA in different formats, an important consideration is that the ME culture may be superior to free cells in protecting BSHA during storage as well as during passage in the stomach. Further, the BSHA activity of free cells may be related, in part, to a liberation of intracellular content.

As a result, further studies are now underway to establish the stability of free or ME *L. reuteri* NCIMB 30242 cultures during storage of the fermented milk as well as during gastric transit.

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