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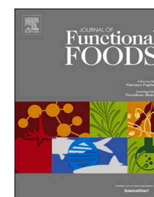

Bile salt hydrolase and lipase inhibitory activity in reconstituted skim milk fermented with lactic acid bacteria

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ABSTRACT

Obesity is the main cause of metabolic syndrome, a condition of which includes hypercholesterolemia. Reduced dietary fat absorption through inhibition of pancreatic lipase and/or hydrolysis of bile salts may provoke weight loss and cholesterol reduction. In this study, the potential anti-obesity properties of milk fermented with lactic acid bacteria was assessed by measuring the expression of bile salt hydrolase (BSH) after milk fermentation and the ability of fermentates to inhibit pancreatic lipase *in vitro*. Thirty BSH positive strains were identified, with 17 strains expressing this enzyme during milk fermentation. Apart from BSH activity, the milks fermented with *L. plantarum* SC70 and SC80 also displayed the capacity to inhibit pancreatic lipase by >35%. As expression of BSH and inhibition of pancreatic lipase are proposed synergistic activities to reduce fat absorption, fermentates produced with these strains are good candidates for use as functional foods for the treatment of obesity and hypercholesterolemia.

1. Introduction

Obesity is one of the main causes of metabolic syndrome, a group of inter-related metabolic conditions that greatly increase the risk of developing cardiovascular disease (Jung & Choi, 2014; Klop, Elte, & Cabezas, 2013). These conditions include hypertension, insulin resistance (and type 2 diabetes mellitus), non-alcoholic steatohepatitis and dyslipidaemia, among others (Chen, He, & Huang, 2014; Martin, Mani, & Mani, 2015; Park, Cho, Kim, & Lim, 2014).

One of the characteristics of obesity-derived dyslipidaemia is a slight increase in low density lipoproteins (LDL, particularly small dense LDL) and a reduction of cholesterol esters linked to high density lipoproteins (HDL-C), as well as a decrease in circulating HDL particles (Jung & Choi, 2014; Klop et al., 2013). These changes in the serum lipid profile lead to a reduction in the transport of cholesterol to the liver and a subsequent increase in cholesterol levels in blood (Klop et al., 2013), namely hypercholesterolemia, which in turn has been associated with adipocyte hypertrophy and disruptions in the secretion of hormones involved in processes such as appetite regulation and satiety (Aguilar & Fernandez, 2014; Klop et al., 2013). In addition, hypertrophied adipocytes exhibit a plasma membrane expansion that dilutes the cholesterol present in this structure, which is detected by the cell as cholesterol depletion and triggers the endogenous synthesis of cholesterol (Aguilar & Fernandez, 2014).

The main cause of obesity is an imbalance between energy intake and expenditure (de la Garza, Milagro, Boque, Campión, & Martínez, 2011; Jung & Choi, 2014), particularly associated with overconsumption of fats and refined sugars. In order to be absorbed, dietary fats need to be hydrolysed into smaller molecules by lipases (Tucci, Boyland, & Halford, 2010), pancreatic lipase being the most important of these enzymes. Therefore the inhibition of pancreatic lipase is an interesting approach to reduce fat absorption from the diet (de la Garza et al., 2011).

The activity of pancreatic lipase requires the formation of fat micelles in the intestinal lumen, as this enzyme exerts its activity at oil-water interfaces (Ogawa, Kobayashi, Sakai, Kadooka, & Kawasaki, 2015). Already in the mouth, fats start to form an emulsion that develops as it transits through the stomach and is stabilised upon reaching the intestine by bile salts and other molecules secreted with the gastrointestinal fluids (Dawson & Karpen, 2015; del Castillo-Santaella et al., 2015). Bile salts are amphipathic molecules that play an essential role in lipid emulsification and solubilisation (Begley, Hill, & Gahan, 2006; Bi, Liu, Du, & Chen, 2016). They are produced in the liver using cholesterol as a precursor, and most are reabsorbed in the intestinal ileum to be recycled and reused (Begley et al., 2006; Dawson & Karpen, 2015; Kumar et al., 2012). A reduction in dietary fat absorption could thus be mediated by two mechanisms: (i) direct inhibition of pancreatic lipase

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and (ii) changes in the fat emulsion properties to increase fat droplet size and subsequently decrease the oil-water interface surface area (del Castillo-Santaella et al., 2015; Ogawa et al., 2015).

Bile salt hydrolase (BSH; choloylglycine hydrolase, EC3.5.1.24) catalyses the hydrolysis of conjugated bile salts into free bile salts and amino acid residues (Liong & Shah, 2005). This enzyme has been identified in the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium* and *Bacteroides* (Sedláčková, Horáčková, Shi, Kosová, & Plocková, 2015), and some strains of *Pediococcus* (Abriouel et al., 2012; Turpin, Humblot, & Guyot, 2011) and *Lactococcus lactis* (Ishimwe, Daliri, Lee, Fang, & Du, 2015; Shehata, El Sohaimy, El-Sahn, & Youssef, 2016), as well as in intestinal archaea (Jones, Begley, Hill, Gahan, & Marchesi, 2008). The expression of BSH in the intestinal lumen decreases the available amount of conjugated bile salts while increasing the amount of free bile salts, which are less soluble and less efficiently absorbed than their conjugated counterparts. As a consequence, the reabsorption and recycling of bile salts is diminished, thus increasing the *de novo* synthesis of bile salts from cholesterol in the liver. This synthesis requires the migration of cholesterol from blood into the liver which, in consequence, decreases the levels of total serum cholesterol. Additionally, the lipid emulsifying capacity in the small intestine is limited, thus increasing the excretion of dietary fat and cholesterol with faeces (Begley et al., 2006; Kumar et al., 2012).

There are several pharmacological therapies to treat obesity and hypercholesterolemia through (i) the reduction of the absorption of dietary fat (e.g., bile acid sequestrants (Begley et al., 2006; Kumar et al., 2012) and inhibitors of pancreatic lipase such as orlistat (Sumithran & Proietto, 2014)) and (ii) the inhibition of the endogenous synthesis of cholesterol (e.g., statins) (Begley et al., 2006). As these treatments are associated with adverse effects, an alternative approach to induce weight loss and decrease blood cholesterol levels would be to partially inhibit pancreatic lipase (Buchholz & Melzig, 2016; Tucci et al., 2010) or slow down its activity (del Castillo-Santaella et al., 2015). Targeting more than one of the mechanisms involved in lipid digestion and absorption may also be advantageous to achieve the desired weight loss with minimal side effects through synergistic activities (Martinussen, Bojsen-Moller, Svane, Dejgaard, & Madsbad, 2017).

The use of natural products or functional foods (such as fermented dairy products) to reduce fat absorption could avoid the administration of drugs or dietary supplements (Buchholz & Melzig, 2016; Ishimwe et al., 2015; Marrelli, Loizzo, Nicoletti, Menichini, & Conforti, 2014). The anti-obesogenic effect of multiple edible plants, fruits and plant extracts has been reported in recent years, and the main mechanisms of action have been identified as an increase in energy expenditure, appetite suppression, lipase inhibition and regulation of lipid metabolism and adipocyte differentiation (Sun, Wu, & Chau, 2016). For instance, black tea and its polyphenols showed a positive effect in reducing body weight via several mechanisms, among them the inhibition of lipid and disaccharide digestion (Pan, Gao, & Tu, 2016). This effect was reported to be due to an increase in fat micelle size and inhibition of pancreatic lipase (Kobayashi et al., 2009). In addition, the anti-obesogenic effect of other edible plants and plant extracts, like pomegranate seed oil, black and red pepper, grapes, onions, chickpeas and other legumes, has been linked to the inhibition of peroxisome proliferator-activated receptor γ (PPARγ) or modulation of its upstream regulators (Feng, Reuss, & Wang, 2016).

The role of probiotics and milk supplemented or fermented with probiotic strains in preventing and reducing obesity has also been widely studied in animal models and humans, and various mechanisms of action have been proposed (Barengolts, 2016; Mazloom, Siddiqi, & Covasa, 2019). However, to the best of our knowledge, the potential synergistic effect of pancreatic lipase inhibition and hydrolysis of bile salts have not been studied in fermented milk with a view to development of functional foods to combat obesity and hypercholesterolemia.

In this study we investigated the potential anti-obesity and anti-hypercholesterolemic effects of 71 strains of lactic acid bacteria (LAB) and their milk fermented products by studying the expression of BSH after milk fermentation and the ability of these products to inhibit pancreatic lipase *in vitro*.

2. Materials and methods

2.1. Chemicals

Culture media for microbial growth (De Man, Rogosa, Sharpe [MRS] and M17) were obtained from BD (Oxford, UK). Skim milk powder was supplied by Carbery Ireland (Ballineen, Ireland). Anaerocult A for generation of anaerobic atmosphere was obtained from OCON Chemicals (Cork, Ireland). All other chemicals were purchased from Sigma Aldrich (Arklow, Ireland).

2.2. Microbial strains and culture conditions

All the strains used in this study belong to species with QPS (qualified presumption of safety) status and were obtained from the DPC culture collection (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland). A total of 71 strains of LAB encompassed in the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* were used (Table 1). In addition, the strain *Lactobacillus reuteri* NCIMB 30242, commercialised as a cholesterol-lowering probiotic, was included for comparison, as this strain is reported to have BSH activity (Jones, Martoni, Parent, & Prakash, 2012; Martoni, Labbé, Ganopolsky, Prakash, & Jones, 2015).

The strains of *Lactobacillus*, *Leuconostoc* and *Pediococcus* were grown in modified MRS (mMRS, i.e., MRS + 0.5 g/L L-cystine) and incubated under anaerobic conditions, whereas *Lactococcus* strains were propagated in LM17 (i.e., M17 + 10 g/L α-lactose). Incubation temperatures were 37 °C for *Lactobacillus* and *Pediococcus* strains and 30 °C for *Lactococcus* and *Leuconostoc* strains.

The strains were preserved at −20 °C in mMRS or LM17 containing 20% (v/v) glycerol. Strains were prepared and grown for assay as follows: (i) the strain was removed from −20 °C and grown overnight in the appropriate broth and temperature as described above, (ii) the culture was streaked onto an appropriate agar plate and incubated for 48 h, (iii) from this plate a single colony was transferred onto a fresh plate and incubated for 48 h, (iv) from which a single colony was transferred into a tube with fresh broth and incubated for 24 h and (v) from this fresh liquid culture a second broth was inoculated (1%, v/v) and incubated for 24 h.

2.3. Preparation of culture media with ox bile

The culture media were prepared as described above and

Table 1
Number of strains per species from the DPC culture collection used in this study. In addition, *Lactobacillus reuteri* NCIMB 30242 was included for comparison as BSH positive.

Genus	Species	No. strains
Lactobacillus	*Lactobacillus acidophilus*	4
	Lactobacillus brevis	6
	Lactobacillus casei	3
	Lactobacillus curvatus	2
	Lactobacillus delbrueckii ssp bulgaricus	2
	Lactobacillus helveticus	13
	Lactobacillus paracasei	3
	Lactobacillus plantarum	4
	Lactobacillus rhamnosus	5
	Lactobacillus salivarius	1
Lactococcus	*Lactococcus lactis*	12
Leuconostoc	*Leuconostoc lactis*	2
Pediococcus	*Pediococcus acidilactici*	1
	Pediococcus pentosaceus	13

supplemented with calcium chloride ($CaCl_2$), as calcium helps precipitation of bile salts due to the formation of insoluble calcium salts (Hofmann & Mysels, 1992). After addition of 0.375 g/L $CaCl_2$, the media were sterilised in an autoclave (121 °C, 15 min) and allowed to cool to 50 °C. The ox bile was then incorporated into the medium by aseptically adding an aliquot of a filter-sterilised stock solution of 12% (w/v) ox bile dissolved in either mMRS or LM17 (with $CaCl_2$) to reach the desired final concentration of 0.3% (w/v). The solid media for the plate precipitation assay were prepared by adding bacteriological agar at 1% (w/v).

2.4. Ability to grow in presence of 0.3% (w/v) ox bile

The ability to grow in presence of 0.3% ox bile was assessed following the method by Wang and co-workers (Wang et al., 2012) with modifications. Briefly, after propagation as described above, each strain was inoculated (2%, v/v) into two different liquid media: (i) non-supplemented culture medium (mMRS or LM17) and (ii) culture medium supplemented with $CaCl_2$ and ox bile, and incubated for 24 h at 37 °C under anaerobic conditions. All strains, regardless of species, were incubated at 37 °C for this assay to mimic more closely actual human physiological conditions. Aliquots of each liquid culture were diluted ¼ with distilled water and absorbance measured at 560 nm. Fresh media diluted ¼ were used as blanks.

The ability of each strain to grow in presence of ox bile was calculated as percentage of growth in medium with bile with respect to the absorbance of the culture in non-supplemented medium (100%). The following formula was used:

$$\%\ Growth = \frac{AbsB}{AbsC} \times 100$$

where AbsB is the absorbance of the culture in presence of ox bile and AbsC is the absorbance of the culture of the same strain in non-supplemented culture medium.

2.5. Bile salt hydrolase – plate precipitation assay

Bile salt hydrolase is an intracellular enzyme and, in consequence, the detection of BSH activity is dependent on the presence of viable cells, as the enzyme is not released into the medium (Ruiz, Margolles, & Sánchez, 2013; Shehata et al., 2016). Furthermore, it is necessary to decrease the pH of the medium to induce the precipitation of the deconjugated bile salts released by BSH. The pKa of deconjugated bile salts is approximately 5.0, whereas the pKa values of conjugated bile salts are lower (1.9 for tauro-conjugated bile salts and 3.9 for glyco-conjugated bile salts). Consequently; deconjugated bile salts precipitate while conjugated bile salts remain soluble when the pH of the medium is decreased by fermentation (Dashkevicz & Feighner, 1989). Therefore, only the strains with growth >5% in presence of ox bile were included in this screening.

To assess the ability of the different strains to produce BSH, a plate precipitation assay following the protocols by Sedláčková et al. (2015) and Shehata et al. (2016) was used with modifications. A series of wells were bored into agar plates containing soft mMRS or LM17 agar supplemented with $CaCl_2$ and 0.3% ox bile as previously described. Each of these wells was inoculated with 25 µL of a fresh overnight liquid culture and incubated at 37 °C to mimic human physiological conditions under anaerobic conditions for 72 h. As some authors suggest that BSH is only expressed after stimulation with bile and use the non-stimulated strains as negative controls (Sedláčková et al., 2015), each strain was tested for BSH activity after growth in media with and without ox bile. Positive results were observed as precipitation halos around the colonies.

The strain *Lactobacillus reuteri* NCIMB 30242, was used as positive control on each plate. Each liquid culture was inoculated in duplicates in two different plates and experiments were repeated three times.

2.6. Preparation of reconstituted skim milk and fermentation conditions

Reconstituted Skim Milk (RSM) for fermentation was prepared at 10% (w/v) by dissolving skim milk powder (54.5% carbohydrate, 34% protein, 1.2% fat) in distilled water. After slowly adding the powder to the water, the mixture was left stirring for 2 h to facilitate rehydration of the powder. The milk was subsequently heat-treated to inactivate indigenous microorganism (121 °C, 5 min, with 5 min exposure time being selected as this helps to reduce Maillard reactions occurring in the reconstituted milk as a consequence of heating), cooled and stored overnight at 4 °C.

Before inoculation, the milk was heated to 37 °C for 1 h. In order to start the fermentation, an aliquot of a fresh overnight culture was added to the pre-heated milk (1%, v/v) and incubated at 37 °C for 24 h without agitation. Fermentation temperature was set at 37 °C to imitate the physiological temperature. The pH of the fermented milks was measured after fermentation.

2.7. Bile salt hydrolase after milk fermentation

In order to assess whether BSH positive strains expressed this enzyme after milk fermentation, the BSH positive strains were selected and used to produce fermented milk products that were subsequently tested using the plate precipitation assay as described above with some modifications. In this case, the wells were inoculated with 50 µL of milk fermented with each BSH positive strain and incubated 96 h under the conditions described above.

2.8. Lipase inhibitory activity test

The potential of the milk fermentates to inhibit pancreatic lipase was assessed using the spectrophotometric method based on the hydrolysis of 4-nitrophenyl octanoate (NPC) described by our group (Gil-Rodríguez & Beresford, 2019, 2020) Briefly, 500 µL of sample were mixed with 2 mL of Tris-HCl buffer and 50 µL of NPC (5 mM in DMSO). In order to start the enzymatic reaction, 50 µL of pancreatic lipase (Type II, from porcine pancreas, 5 mg/mL in Tris-HCl buffer) were added. The mixture was agitated by vortex for 2 min and incubated at 37 °C for 30 min. In order to stop the reaction, 1 mL of Clarifying Reagent for Dairy Products was added. After 3 min incubation at 37 °C, absorbance at 412 nm (A_{412}) was measured using a Jenway 6300 Spectrophotometer (Cole-Parmer Ltd, Staffordshire, UK).

A blank was included for each sample by substituting the sample with Tris-HCl buffer. Lipase inhibition was calculated as percentage with respect to a 100% activity reference control without sample.

2.9. Statistical analysis

Values are given as average ± standard deviation. The ability of each strain to grow in bile was assessed in three independent experiments, where each sample was analysed in triplicates. The fermentates were produced in triplicates and each of them was analysed for lipase inhibitory activity twice in duplicates.

Pearson's chi-squared test was used to identify significant differences. Differences are considered statistically significant with P values < 0.01.

3. Results

3.1. Ability to grow in 0.3% (w/v) ox bile

In total, 49 strains (69.0% of the total) exhibited growth levels of >5% in presence of 0.3% ox bile (32 of them with growth >25%), while 22 strains were inhibited by this bile concentration with growth levels below 5% (Fig. 1).

In general, ability to grow in presence of ox bile seemed a species-

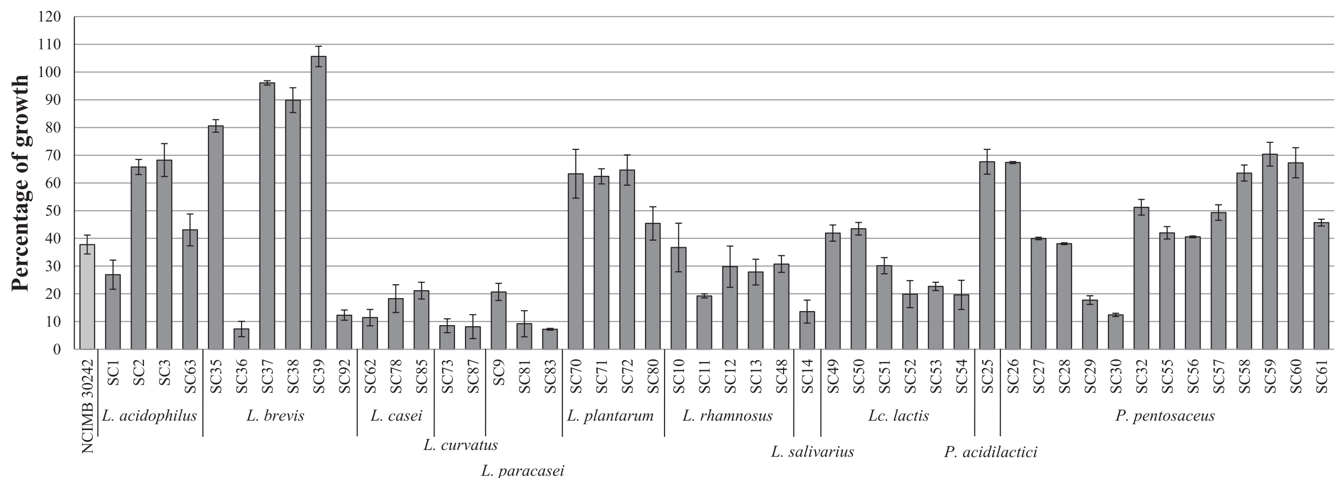

Fig. 1. Percentage of growth in culture medium with 0.3% ox bile. Only the strains with percentages of growth >5% are displayed.

specific trait, as all strains from certain species exhibited ability to grow in bile at varying degrees (i.e., *L. acidophilus*, *L. brevis*, *L. casei*, *L. curvatus*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. salivarius*, *P. acidilactici* and *P. pentosaceus*), whereas all strains of other species were inhibited by ox bile (i.e., *L. helveticus, Ln. lactis* and *L. delbrueckii* ssp *bulgaricus*). Only *Lc. lactis* had a mixed response to bile, with six strains growing and six strains being inhibited.

Under these conditions, the control strain *L. reuteri* NCIMB 30242 exhibited growth of 37.8 ± 3.4% in presence of ox bile with respect to the culture in mMRS. Of the strains examined here, some displayed higher levels of tolerance to bile, with 11 strains (15.5% of all strains tested) exhibiting growth between 50 and 75% and 4 strains (5.6%) with growth levels in excess of 75% in presence of ox bile (Fig. 1). The strains with highest levels of growth in medium with bile were all *L. brevis* strains (i.e., SC39, SC37, SC38 and SC35 with growth of 105.6 ± 3.7%; 96.1 ± 0.8%; 89.9 ± 4.5%; and 80.6 ± 2.3%, respectively), followed by strain *P. pentosaceus* SC59 with growth of 70.4 ± 4.3%. Interestingly, 9 of the strains with growth >50% in presence of bile were of dairy origin, isolated from cheese, yoghurt, curd and whey (Table 2).

On the other hand, all the strains from species *L. casei*, *L. curvatus*, *L. paracasei* and *L. salivarius*, as well as *L. brevis* SC36 and SC92 and *P. pentosaceus* SC29 and SC30 had lower percentages of growth in presence of bile, not exceeding 22% (Fig. 1). The strains with lowest bile tolerance levels were isolated from cheese, except *L. salivarius* SC14, isolated from saliva, and *L. paracasei* SC9, of unknown origin. The two *L. brevis* strains with markedly lower activities than the other strains (i.e., SC36 and SC92) were isolated from cheese as well (Table 2), and only one of the strains of this species with growth >80% has a dairy origin (SC35, isolated from curd). Contrarily, all strains of *L. plantarum* analysed, with growth >45% in presence of bile, were isolated from cheese, and all *Pediococcus* strains with growth >60% were isolated from food products (yoghurt, curd and sake mash) as well (Table 2).

3.2. Bile salt hydrolase activity after growth in complex culture media

The strains tested for BSH activity were preselected according to their capacity to grow in presence of 0.3% ox bile (growth >5% relative to control), as strains without this ability could not be tested with this assay. A total of 60% of the strains analysed (30 out of 49) yielded positive results in at least one of the conditions examined (i.e., after exposure to bile or without prior exposure to bile) (Table 2). Of the BSH positives, 16 strains were lactobacilli of species *L. acidophilus* (all 4 tested strains), *L. brevis* (4 strains out of 6 tested), *L. casei* (all 3 tested strains), *L. plantarum* (all 4 tested strains) and *L. salivarius* (one strain tested), and 14 strains were pediococci of species *P. acidilactici* (one strain tested) and *P. pentosaceus* (all 13 tested strains). None of the lactococci analysed in this study displayed BSH activity. Similarly, no BSH positives were found among the strains of *L. curvatus*, *L. paracasei* or *L. rhamnosus* tested (Table 2).

The strain *L. casei* SC62 exhibited BSH activity only without prior exposure to bile (i.e., a precipitation halo appeared only around the wells that had been inoculated from a liquid culture not supplemented with bile). However, due to the low percentage of growth of this strain in bile (11.4 ± 3.0%), it is possible that the amount of viable bacterial cells inoculated into the assay was too low to yield a positive result. Contrarily, strains *L. brevis* SC92 and *L. salivarius* SC14 displayed BSH activity only after stimulation with bile, although these strains also exhibited low percentages of growth in bile (12.3 ± 1.8% and 13.6 ± 4.1%, respectively). The other 27 BSH positive strains yielded positive results both after growing in medium with and without ox bile (Table 2). These findings contrast with those of Sedláčková et al. (2015), who analysed the strains grown in culture medium without bile as negative controls, and suggest that in these cases exposure to bile prior to analysis is not necessary to induce expression of BSH.

With regard to their origin, 23 of the 30 BSH positive strains were isolated from dairy products, 10 of them from cheese and nine from yoghurt, but also from curd (two strains), fermented milk (one strain) and whey (one strain) (Table 2). Interestingly, only one of the four strains isolated from an intestinal environment was BSH negative (*L. brevis* SC38, isolated from bovine faeces).

3.3. Bile salt hydrolase activity after milk fermentation

The 30 BSH positive strains representing members of *L. acidophilus, L. brevis, L. casei, L. plantarum, L. salivarius, P. acidilactici* and *P. pentosaceus* in addition to the control strain *L. reuteri* NCIMB 30242 were inoculated into 10% RSM to produce milk fermentates in order to evaluate whether BSH was expressed after milk fermentation as well as under the conditions tested above. Growth, as indicated by final pH, ranged from pH 6.49 to pH 4.18 with 14 strains reducing the pH to <6.00 (the pH of RSM before fermentation was 6.46 ± 0.02) (Table 2). In total, 16 strains in addition to the control strain gave BSH positive results after milk fermentation, of which 8 produced precipitation halos > 10 mm (Table 2).

Each of the four *L. acidophilus* and *L. plantarum* strains tested expressed BSH after milk fermentation with precipitation halos >10 mm in all cases except for *L. acidophilus* SC1 (precipitation halo 4–10 mm). The pH of the milk fermented by this strain is distinctively higher than the pH values of the other strains of *L. acidophilus* (Table 2). This could indicate a lower level of growth for this strain in milk leading to a lower number of viable cells and, consequently, smaller precipitation halo. Interestingly, the strain *L. plantarum* SC80 also exhibits a pH value

Table 2
BSH activity of different strains of LAB. **E:** results of BSH test after exposure to bile. **NE:** results of BSH test without prior exposure to bile. **M:** results of BSH test after milk fermentation without prior exposure to bile. **NT:** not tested. **–**: negative result. +: BSH positive with precipitation halo <4 mm, ++: BSH positive with precipitation halo 4–10 mm, +++: BSH positive with precipitation halo > 10 mm. * Indicates data published as part of a previous study (Gil-Rodríguez and Beresford, 2019).

Species	SC No.	Origin	Fermented milk pH	BSH activity		
				E	NE	M
Lactobacillus acidophilus	1	Rat faeces	5.47 ± 0.16*	+	+	++
	2	Human faeces	4.83 ± 0.43*	+	+	+++
	3	Human faeces	4.87 ± 0.47*	+	+	+++
	63	Fermented milk	4.83 ± 0.04*	+	+	+++
Lactobacillus brevis	35	Curd	6.40 ± 0.08	+++	+++	+++
	36	Cheese	5.00 ± 0.18	–	–	NT
	37	Silage	6.49 ± 0.07*	+	+	–
	38	Bovine faeces	6.47 ± 0.04	–	–	NT
	39	Udder wash	6.18 ± 0.24	+	+	–
	92	Cheese	6.47 ± 0.06*	++	–	–
Lactobacillus casei	62	Cheese	4.35 ± 0.41	–	+	+
	78	Cheese	4.45 ± 0.06	++	+	–
	85	Cheese	4.18 ± 0.04	++	++	+
Lactobacillus curvatus	73	Cheese	6.09 ± 0.07	–	–	NT
	87	Cheese	6.13 ± 0.06	–	–	NT
Lactobacillus paracasei	9	Unknown	6.06 ± 0.05	–	–	NT
	81	Cheese	4.48 ± 0.17	–	–	NT
	83	Cheese	4.25 ± 0.12	–	–	NT
Lactobacillus plantarum	70	Cheese	4.67 ± 0.04*	++	++	+++
	71	Cheese	4.70 ± 0.07*	++	++	+++
	72	Cheese	4.70 ± 0.06*	++	++	+++
	80	Cheese	5.09 ± 0.12*	++	++	+++
Lactobacillus rhamnosus	10	Unknown	5.01 ± 0.21	–	–	NT
	11	Cheese	4.40 ± 0.25	–	–	NT
	12	Cheese	6.21 ± 0.09	–	–	NT
	13	Cheese	4.35 ± 0.13	–	–	NT
	48	Cheese	6.13 ± 0.06	–	–	NT
Lactobacillus salivarius	14	Saliva	6.14 ± 0.07	++	–	–
Lactococcus lactis	49	Buttermilk plant	4.50 ± 0.14	–	–	NT
	50	Buttermilk plant	4.52 ± 0.15	–	–	NT
	51	Buttermilk plant	4.61 ± 0.24	–	–	NT
	52	Buttermilk plant	4.65 ± 0.22	–	–	NT
	53	Green peas	4.93 ± 0.40	–	–	NT
	54	Corn	4.60 ± 0.14	–	–	NT
Pediococcus acidilactici	25	Curd	6.21 ± 0.06	++	++	+
Pediococcus pentosaceus	26	Sake mash	6.19 ± 0.23	++	++	–
	27	Yoghurt	6.08 ± 0.09	++	++	–
	28	Yoghurt	6.07 ± 0.11	++	++	–
	29	Cheese	5.94 ± 0.16*	++	++	++
	30	Cheese	5.87 ± 0.07*	++	++	++
	32	Whey	5.91 ± 0.09	++	++	–
	55	Yoghurt	6.08 ± 0.10	++	++	–
	56	Yoghurt	6.07 ± 0.06	+	+	+
	57	Yoghurt	6.03 ± 0.01*	+	+	++
	58	Yoghurt	6.12 ± 0.13	+	+	–
	59	Yoghurt	6.11 ± 0.12	++	++	–
	60	Yoghurt	6.12 ± 0.11	++	++	–
	61	Yoghurt	6.10 ± 0.11	++	++	–
Lactobacillus reuteri NCIMB 30242				++	++	++

higher than the pH of the milks fermented with the other strains of the same species, but the precipitation halo in this case is equal in diameter.

Two of the three *L. casei* strains tested displayed BSH activity after milk fermentation (SC62 and SC85). While all the *L. casei* strains grew well in milk reducing the pH to ≤4.45 the two BSH positive strains only produced halos of <4 mm. Only one of the four *L. brevis* strains tested was positive after being incubated in milk (SC35). None of the four strains grew extensively in milk as demonstrated by the final pH values in the range 6.49–6.18. It is interesting to note that the final pH achieved by strain SC35, which displayed BSH activity, was pH 6.40 but that with such relatively poor levels of growth it produced a halo >10 mm.

The single strain of *P. acidilactici* SC25 and four of the 13 strains of *P. pentosaceus* (SC29, SC30, SC56 and SC57) were also BSH positives after milk fermentation. The single strain of *L. salivarius* tested did not demonstrate BSH activity following growth in milk.

Interestingly, nine of the strains (SC1, S22, SC3, SC63, SC70, SC71, SC72, SC80 and SC57) displayed precipitation halos that were bigger in diameter after milk fermentation than after growth in culture medium (both with and without bile). In this regard a recent study reported that BSH activity by *L. reuteri* NCIMB 30242 was lower after milk fermentation than in the lyophilised commercial preparations, due to lack of stimulation with bile (Champagne, Raymond, Guertin, Martoni, & Jones, 2016). However, in these studies NCIMB 30242 was co-cultured with commercial yoghurt starters and this may influence BSH activity. It is also interesting to note that in the current study we observed equivalent levels of BSH activity for NCIMB 30242 whether grown in complex culture medium with or without bile or in milk.

Three of the strains displaying higher BSH activity when grown in milk were isolated from faeces, while six are of dairy origin (fermented milk, cheese and yoghurt). It is also interesting to note that *L. brevis* SC35 displayed high levels of BSH activity regardless of whether they were grown in complex culture media or milk or whether they were exposed to bile.

3.4. Development of lipase inhibitory activity during milk fermentation

The milk fermentates produced with the BSH positive strains were also analysed for potential lipase inhibitory activity. The activities observed in all the samples are moderate, with seven out of 16 samples giving inhibition levels significantly higher than that of the non-fermented skim milk (NFSM) control (Table 3). The highest levels of lipase inhibition were obtained for milk samples fermented with strains *L. plantarum* SC70 and SC80 (37.2 ± 4.0 and 35.5 ± 3.6%, respectively). Other milk fermentates that exhibited inhibition levels significantly higher than the control (>30%) were those produced with *L. acidophilus* SC1 and SC63 (32.8 ± 1.4% and 31.9 ± 3.2%, respectively), *L. brevis* SC35 (33.9 ± 0.6%) and *P. pentosaceus* SC30 and SC57 (32.3 ± 1.9% and 31.7 ± 0.6%, respectively). Interestingly, one of the fermented milks tested gave a value of lipase inhibition significantly lower than that of the control (i.e., *L. casei* SC85, with a value of 8.7 ± 0.3%). This could indicate that this strain has certain lipolytic activity that is adding to the lipase present in the assay mixture, thus increasing the hydrolysis of NPC.

Our data suggest that the development of lipase inhibitory activity during milk fermentation is strain dependent, as different strains of the same species give diverse levels of inhibition and no species-specific pattern can be identified.

Table 3
Lipase inhibitory activity of milk fermented with BSH positive strains which express this enzyme after milk fermentation. A control of non-fermented skim milk (NFSM) is included for comparison. *denotes statistically significant differences between the sample and the control.

Species	SC No.	Lipase inhibitory activity (%)
NFSM control	—	22.24 ± 2.51
Lactobacillus acidophilus	1	32.84 ± 1.43*
	2	25.86 ± 0.79
	3	24.15 ± 2.37
	63	31.94 ± 3.20*
Lactobacillus brevis	35	33.87 ± 0.56*
Lactobacillus casei	62	25.43 ± 0.14
	85	8.71 ± 0.32*
Lactobacillus plantarum	70	37.22 ± 4.00*
	71	25.71 ± 2.21
	72	26.02 ± 2.06
	80	35.54 ± 3.62*
Pediococcus acidilactici	25	26.84 ± 0.61
Pediococcus pentosaceus	29	26.88 ± 2.22
	30	32.27 ± 1.87*
	56	28.89 ± 1.77
	57	31.69 ± 0.61*

4. Discussion

In this study, growth in the presence of 0.3% ox bile was found to be a widespread characteristic among the strains of LAB tested but with marked differences between species. Although 31.0% of the strains were inhibited, the remaining strains exhibited growth of >5% in presence of 0.3% ox bile relative to growth in the absence of bile, with 35.2% of the strains analysed displaying levels of growth in bile higher than that of the control strain *L. reuteri* NCIMB 30242. Furthermore, 30 BSH positive strains were identified, of which 24 were isolated from food, mostly dairy products.

It has been proposed that conjugated bile salts cause cell toxicity through a mechanism similar to that of organic acids, i.e., via acidification of the cytoplasm (Begley et al., 2006). The hydrolysis of the conjugated bile salt and the co-transport of the proton with the deconjugated product to the outside of the cell could thus be one of the mechanisms of resistance against this natural barrier, hence resistance to bile salts has been attributed to bile salt hydrolase activity (Bi et al., 2016; Bustos, Saavedra, de Valdez, Raya, & Taranto, 2012; Joyce et al., 2014). In accordance with this hypothesis, most of the strains reported as BSH positives in this study were among those with the highest levels of growth in the presence of bile. However, some strains with high percentages of growth in bile did not display BSH activity under any of the conditions tested (i.e., *L. brevis* SC38 with a level of growth >80% in bile and *Lc. lactis* SC49 and SC50, with growth levels in bile >40%). These data suggest that mechanisms other than BSH may be involved in the tolerance to bile of these strains, such as active transport of bile salts to the outside of the cell and structural changes in both the cell wall and membrane, e.g., increased production of exopolysacharide (Ruiz et al., 2013).

In contrast, some of the BSH positive strains exhibited low levels of growth in bile (<15%, e.g., *L. brevis* SC92, *L. salivarius* SC14 and *P. pentosaceus* SC30). This could imply that, although BSH is present, other mechanisms that contribute to bile tolerance are absent or not being expressed in these strains under these conditions. This could be due to lack of exposure to other intestinal factors (Ruiz et al., 2013).

Many of the BSH positive strains exhibited this activity after being cultured both in presence and absence of bile, with only two strains being positive after exposure to bile and negative without prior exposure (i.e., *L. brevis* SC92 and *L. salivarius* SC14). These findings contradict the hypothesis of Sedláčková et al. (2015) of BSH only being expressed after stimulation with bile salts and suggest a fast adaptation following exposure to bile or a constitutive expression of BSH in these strains, as is the case of bifidobacteria (Ruiz et al., 2013).

The expression of BSH is normally studied as part of the probiotic potential of bacterial strains, thus it has been identified in numerous lactobacilli and bifidobacteria. However, there is growing evidence of the widespread presence of BSH in pediococci and other LAB. Abriouel et al., (2012) reported that 90% of *Pediococcus* strains (species not identified) isolated from fermented manzanilla Aloreña green table olives exhibited the ability to produce BSH. In accordance with these results, all pediococci analysed here were BSH positive. Additionally, other authors have reported strains of *P. pentosaceus* (Damodharan, Lee, Palaniyandi, Yang, & Suh, 2015; Lee et al., 2014) and *P. acidilactici* (Mandal, Sen, & Mandal, 2009; Tsai et al., 2014) as BSH positive. Similarly, Turpin et al. (2011) found the *bsh* gene and other genes of bile resistance in multiple *P. pentosaceus* and *P. acidilactici* strains isolated from fermented pearl millet.

BSH activity is usually found in strains of intestinal origin (Begley et al., 2006; Bustos et al., 2012), but it has also been found in bacteria isolated from other environments, such as food (Archer & Halami, 2015; Shehata et al., 2016). The strains identified as BSH positives in this study had diverse origins, including both faeces and food products. However, as the presence of this enzyme is considered an adaptation of microorganisms to the gastrointestinal environment (Jones et al., 2008), this could indicate an intestinal origin regardless of the substrate where these strains have been isolated from.

The milk products fermented with strains *L. plantarum* SC70 and SC80 displayed the highest levels of lipase inhibition reported here (>35%). In addition, these two strains express BSH after milk fermentation. Strains of *L. plantarum* have already been proposed as probiotics with cholesterol lowering effect due to their high expression levels of BSH (Bosch et al., 2014), but their potential to directly inhibit pancreatic lipase had not been tested.

This level of lipase inhibitory activity in milk fermented with strains *L. plantarum* SC70 and SC80 was lower than levels previously reported for milk fermented with other LAB strains (e.g., *L. helveticus* SC8, SC44 and SC45, with inhibition levels > 49%) (Gil-Rodríguez & Beresford, 2019), and for cell extracts prepared with other LAB strains, e.g., *L. plantarum* Q180, that was reported to inhibit pancreatic lipase by 83.61 ± 2.31% at concentrations of 100 μg/mL (Park et al., 2014). However, the combination of this ability to partially inhibit pancreatic lipase plus the expression of bile salt hydrolase may have a synergistic effect leading to an increased excretion of fat.

Although several studies have shown the ability of LAB cells and cell extracts to inhibit pancreatic lipase, this activity could also be exerted via compounds produced during the milk fermentation process, such as organic acids and bioactive peptides. In a recent work by our group, the lipase inhibitory activity found in milk fermented with strains *L. helveticus* SC8, SC44 and SC45 was present after fractionation in the <3 kDa fraction (Gil-Rodríguez & Beresford, 2019). Similarly, the enzymatic hydrolysis of camel milk and camel milk whey produced fractions rich in peptides that exhibited pancreatic lipase inhibitory activity levels between 25 and 55% (Jafar, Kamal, Mudgil, Hassan, & Maqsood, 2018; Mudgil, Kamal, Yuen, & Maqsood, 2018).

When testing lipase inhibition *in vitro* no bile salts were added and therefore, the effect of bile salt deconjugation on lipase activity has not been evaluated here. Interestingly, BSH activity in *Lactobacillus gasseri* SBT2055 has been proposed as the mechanism responsible for the modification in the fat emulsion properties that lead to a decrease in lipase activity by an enlargement of fat droplet size (Ogawa et al., 2015).

Apart from this direct effect on fat absorption, enhanced BSH expression in the intestine has been demonstrated to reduce weight gain and decrease LDL levels in mice fed both a normal and a high fat diet (Joyce et al., 2014). Similarly, inhibition of pancreatic lipase by orlistat decreases blood triglyceride and LDL levels in humans (Sumithran & Proietto, 2014). It has also been reported that BSH activity in the gut regulates host lipid metabolism by influencing the expression of genes involved in cholesterol transport and lipid transport and synthesis in the duodenum, ileum and liver, resulting in a decrease in weight gain, serum

cholesterol and liver triglyceride levels in mice (Joyce et al., 2014). In addition to a direct reduction of the amount of fat being incorporated to the organism, a decrease in fat absorption would lead to higher amounts of fat reaching the ileum, which has been associated to delayed gastric emptying, extended transit time through the intestine and increased secretion of satiety hormones, particularly cholecystokinin (CCK), consequently reducing food intake (Maljaars et al., 2008).

5. Conclusions

Our results indicate that different strains of LAB have the ability to produce fermented milk with the potential to be beneficial for the control of obesity and hypercholesterolemia by targeting two mechanisms involved in the digestion and absorption of dietary fat, i.e., emulsification by bile salts and hydrolysis by pancreatic lipase. In particular, strains *L. plantarum* SC70 and SC80 have the capacity to produce fermented milks that have both BSH and moderate lipase inhibitory activity. These two activities could be synergistic and help both weight loss and decrease blood cholesterol levels in obese subjects without the side effects provoked by other anti-obesity therapies. However, further research is required to identify the molecules responsible for these observations, to assess whether these activities are retained during gastrointestinal passage and their efficacy validated in appropriate *in vivo* models if these fermentates are to be developed as potential functional foods.

Credit author statement

The authors' responsibilities were as follows—TB acquired the Funding to support the project and was responsible for overall Project Administration, AMGR and TB Conceptualised and designed the study; AMGR established the required Methodology, conducted the Investigation and performed the statistical analyses; AMGR and TB wrote, reviewed edited the manuscript.

Ethics statements

We have read and adhere to the Publishing Ethics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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