

THE ROLE OF RESISTANCE TO BILE SALTS AND ACID TOLERANCE OF EXOPOLYSACCHARIDES (EPSS) PRODUCED BY YOGURT STARTER BACTERIA

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Abstract - The aim of this study was to investigate a possible relation between EPS production and resistance to bile salts and tolerance to low pH. Eight strains which produced the highest and lowest amount of EPS (16- 211mg/l) were selected among 54 bacteria isolated from yogurt. Additionally, they were tested for resistance to bile salts (0.15, 0.3 %) and tolerance to low pH (2.0-3.0). After treatment with bile salts and acid, viable bacteria (log cfu ml⁻¹) were determined by surface plating. The high EPS producing strains (B3, G12, W22) showed a significant (P<0.05) protective effect against low pH (pH 2.0). All *Streptococcus thermophilus* strains showed a higher tolerance to bile salts than the *Lactobacillus delbrueckii* subsp. *bulgaricus* strains. The high EPS-producing *S. thermophilus* (W22, T12) and *L. bulgaricus* (B3, G2) strains showed a significant (P<0.01) protective effect against bile salts (0.3 %).

Keywords: EPS, *Lactobacillus delbrueckii bulgaricus*, *Streptococcus thermophilus*, bile salt resistance, acid tolerance

UDC 547.93:637.146.3

INTRODUCTION

Probiotics including *Lactobacillus*, *Bifidobacterium* and *Streptococcus* spp. are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favorable effects against disease caused by an imbalance of the gut microflora, several experimental observations have showed a potential protective effect of probiotic bacteria against the development of colon tumors (Dunne et al., 2001; Wollowski et al., 2001).

Several lactic acid bacteria (LAB), including *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* are able to produce extracellular polysaccharides (EPS) that either encapsulate the bacterial or are excreted into the extracellular environment. The use of EPS-producing starter cultures, including *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, is one means of enhancing the viscosity of yogurt during manufacture (Looijesteijn et al., 2001; Ruas-Madiedo et al., 2002). Also, the health benefits of LAB have been attributed to the production of EPS which has anti-tumor, anti-ulcer, immunomodula-

ting and cholesterol-lowering activities (Gill 1998; Kumar and Anand 1998). Consequently, EPS-producing probiotic cultures can contribute to human health by positively impacting the gut microflora. On the other hand, they probably have a protective function in the natural environment, e.g. against desiccation, phagocytosis and predation by phage attack, antibiotics or toxic compounds and osmotic stress (Looijesteijn et al., 2001; Ruas-Madiedo et al., 2002). Another physiological benefit is that EPS is retained longer in the gastrointestinal tract, so that colonization by probiotic bacteria can be enhanced (Looijesteijn et al., 2001; Kumar and Anand 1998). The viability and survival of probiotic bacteria are the most important parameters for providing therapeutic functions. Several factors have been claimed to affect the viability of probiotic bacteria in dairy foods such as yogurt and fermented milks, including low pH and bile salts. In order to be used as potential probiotics, dairy lactic acid bacteria (LAB) strains need to be screened for their capacity of transit tolerance to the upper gastrointestinal tract conditions (Chou and Weimer 1999). The low pH is known to provide an effective barrier against the

entry of bacteria into the intestinal tract. The pH of the stomach generally ranges from pH 2.5 to pH 3.5 (Holzapfel et al., 1998).

Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, the major components of which are lipids and fatty acids; these modifications may affect not only the cell permeability and viability, but also the interactions between the membrane and the environment (Succi et al., 2005). Resistance to bile salts is considered an important parameter for selecting probiotic strains. A concentration of 0.15-0.3 % of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin BR and Gorbach 1992).

Most studies have focused on the structure and characterization of the EPS. Very few have studied the physiological benefit of EPS (Goldin BR and Gorbach 1992). Although, resistance to bile salts and low pH are considered an important parameter to select probiotic strains, there are no data about the relation between EPS production and resistance to bile salts or tolerance low pH.

In aim of this study was to determine the bile salts resistance and low pH tolerance of the low and high EPS-producing strains and to also investigate a possible relation between EPS producing and resistance to bile salts or tolerance to low pH of strains. This evaluation can be performed as an initial step in establishing rational criteria for the screening and selection of food microorganisms with human probiotic properties.

MATERIAL AND METHODS

Bacterial strains and growth conditions

All strains of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains were isolated from traditional hand-made yogurts. The carbohydrate fermentation characteristics of all strains were determined by using the API 50 CHL identification system (BioMerieux SA, France). However, the isolates were identified by DNA sequencing of 16S rRNA

polymerase chain reaction (PCR) products (Ventura et al., 2000; Roy et al., 2001). *L. bulgaricus* and *S. thermophilus* strains were grown in de Man, Rogosa and Sharpe medium (MRS; Oxoid) and Elliker broth (Oxoid), respectively, at 40 °C for 18-20 h (late exponential growth phase). The isolates were stored at -80°C in a medium containing 30% glycerol (Merck) and regenerated twice before use in the manipulations.

Isolation and quantification of EPS

After inoculation, the cultures were incubated at 40 °C for 18 h. The samples were boiled at 100°C for 10 min. Then they were maintained for 10 min at room temperature (25°C), treated with 17 % (v/v) of 85 % trichloroacetic acid (Merck) solution and centrifuged at 13,000 rpm (Frenova et al., 2000). After removal of the cells and protein by centrifugation, the EPS was precipitated with ethanol (90 %). The EPS was recovered by centrifugation at 4 °C at 14,000 rpm for 20 min. Total EPS (expressed as mg/l) was estimated in each sample by phenol-sulphuric method (Dubois et al., 1956) using glucose (Merck) as standard (Torino et al., 2001).

The effect of pH

A modified method was applied in this study (Erkkila and Petaja 2000; Haller et al., 2001). Bacteria were harvested by centrifugation (3000 × g, 10 min) at the late exponential growth phase, and washed three times with phosphate buffered saline (PBS; NaCl 9 g/l, Na₂HPO₄.2H₂O 9 g/l, KH₂PO₄ 1,5 g/l, pH 6.2). Cells were resuspended in PBS and the suspension was incubated at 40°C for 2 h. A 100 µl aliquot of bacterial suspension was inoculated into 10 ml of sterile phosphate-buffered saline. One experimental series contained saline tubes with the following pH values: 2.0, 2.5, 3.0 and 6.2 (adjusted using 8 M HCl). pH 6.2 was used as a control. Cell counts, performed in triplicate, were calculated from the colonies on MRS or Elliker agar after 24 h incubation at 40°C ±1 and

Table 1. EPS production by strains of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.

Strains	EPS (mg/l)*
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> B3	211±1.0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> G12	175±0.0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> A13	27±0.0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> 22	21±4.5
<i>S. thermophilus</i> W22	114±0.0
<i>S. thermophilus</i> T12	100±0.0
<i>S. thermophilus</i> H21	17±3.0
<i>S. thermophilus</i> 24.6	16±0.0

*Each value in the table is the mean ± standard deviation of three trials.

thus expressed as log 10 values of colony-forming units per ml (cfu/ml). The survival percentage was calculated as follows:

$$\% \text{ survival} = \text{final (cfu/ml)} / \text{control (cfu/ml)} \times 100$$

The effect of bile salts

After incubation, bacteria were harvested by centrifugation (10,000 x g, 10 min) at the late exponential growth phase, and washed three times and resuspended in PBS. This suspension was incubated at 40°C for 2 h. A 100 µl aliquot of bacterial suspension was inoculated into 5 ml of sterile MRS broth. 0.15% or 0.30% standardized mixture of the salts of bile acids prepared from ox bile (Sigma) and incubated at 40°C for 2 h and survival cell counts were determined by plating as described previously.

Statistical analysis

Statistical analysis was performed by SPSS (Ver. 11.0). Pearson's correlation was used to determine any significant differences between EPS production amount and bile salts resistance of the strains and

Table 2. Viability of *L. delbrueckii* subsp. *bulgaricus* strains at different pHs.

	Log cfu ml ⁻¹ * (viable percentage ^a)			
	B3	G12	A13	22
Control ^b	8.6 (100%)	8.1 (100%)	6.4 (100%)	9.1 (100%)
pH 3.0	8.0 (93%)	7.8 (96%)	4.7 (73%)	6.6 (72%)
pH 2.5	5.0 (58%)	3.5 (43%)	3.7 (57%)	0.0 (0%)
pH 2.0	4.2 (48%)	2.3 (28%)	0.0 (0%)	0.0 (0%)

*Values are the means of triplicate measurements

^aData are expressed as % survival

^bControl pH: 6.2

between the EPS production amount and low pH of the strains.

RESULTS

Eight *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains were selected according to their capacity of EPS producing among 54 isolates (data not shown) in this study. *L. delbrueckii* subsp. *bulgaricus* B3, G12 and *S. thermophilus* W22, T12 strains which produce high levels (211, 175, 114, 100 mg/l, respectively) of extracellular polysaccharides (EPSs) and *L. delbrueckii* subsp. *bulgaricus* A13, 22 and *S. thermophilus* H21, 24.6 strains which produce low pH EPS levels (27, 21, 17, 16 mg/l, respectively) were selected for this study. These results are shown in Table 1.

The effect of pH on *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains was tested and the number of viable cells and survival percentage at each pH were determined (Table 2 and 3). The viability of all strains was significantly reduced at pH ≤ 3.0 compared with that at control pH (6.2). The low EPS-producing strains (A13, 33, H21, 24.6) showed more sensitivity to acid compared to the high EPS-producing strains. The results showed these bile salts at both concentrations (0.15 and 0.3%) affected the viability of strains. On the other hand, after exposure to bile salts (0.15% and 0.3%),

Table 3. Viability of *S. thermophilus* strains at different pHs.

	Log cfu ml ^{-1b} (viable percentage ^a)			
	W22	T12	H21	24.6
Control ^b	12.2 (100%)	100 (100%)	100 (100%)	8.6 (100%)
pH 3.0	8.0 (65%)	8.4 (81%)	0.0 (0%)	6.4 (74%)
pH 2.5	7.6 (62%)	3.7 (36%)	0.0 (0%)	0.0 (0%)
pH 2.0	5.5 (45%)	3.1 (30%)	0.0 (0%)	0.0 (0%)

*Values are the means of triplicate measurements

^aData are expressed as % survival

^bControl pH: 6.2

the highest viability was determined in the highest EPS-producing strains. The results are given in Tables 4 and 5. In general, all *S. thermophilus* strains obtained had a higher tolerance to bile salts than the *L. delbrueckii* subsp. *bulgaricus* strains. *S. thermophilus* strains (W22 and T12) and *L. delbrueckii* subsp. *bulgaricus* (B3 and G12) strains showed viability (71 and 71 %; 36 and 33 % survival, respectively) when bile salts were 0.3 %. On the other hand, the low EPS-producing *L. delbrueckii* subsp. *bulgaricus* strains (A13 and 22) and *S. thermophilus* strains (H21 and 24.6) showed a very marked inhibitory effect on the survival of cells (9 and 3 %; 4 and 2 % survival, respectively) when bile salt was 0.3 %.

DISCUSSION

Exocellular polysaccharides (EPS) produced by lactic acid bacteria (LAB) have been the subject of much research in recent years because of their immunogenic properties, their role in the texture of fermented dairy products and their potential use as thickening and gelling agents in place of exopolysaccharide produced by non food-grade organisms (Ricciardi et al., 1997). There are several possible benefits these cultures of bacteria intended for use as a probiotic might derive from production of an EPS (Roberts et al., 1995). The important characteristic of a probiotic is its survival at low pH

Table 4. Viability of *L. delbrueckii* subsp. *bulgaricus* strains on different bile salts' concentrations.

	Log cfu ml ^{-1*} (viable percentage ^a)			
	B3	G12	A13	22
Control ^b	7.0 (100%)	6.0 (100%)	6.7 (100%)	7.0 (100%)
0.15%	2.7 (39%)	2.2 (37%)	0.6 (9%)	0.3 (4%)
0.3%	2.5 (36%)	2.0 (33%)	0.2 (3%)	0.2 (3%)

*Values are the means of triplicate measurements

^aData are expressed as % survival

^bControl pH: 6.2

Table 5. Viability of *S. thermophilus* strains on different bile salts' concentrations.

	Log cfu ml ^{-1*} (viable percentage ^a)			
	W22	T12	H21	24.6
Control ^b	9.6 (100%)	9.0 (100%)	9.1 (100%)	8.8 (100%)
0,15%	7.5 (78%)	6.9 (75%)	0.6 (7%)	0.4 (5%)
0,3%	6.9 (71%)	6.4 (71%)	0.4 (4%)	0.2 (2%)

*Values are the means of triplicate measurements

^aData are expressed as % survival

^bControl pH: 6.2

and high bile salts (Brink et al., 2006). A protective coating of EPS may allow the bacterium to better withstand stomach acid and bile salts (Roberts et al., 1995). Some of these effects depend on the resistance of EPS during its passage through the intestine (Looijesteijn et al., 2001).

Our results showed that there is a positive correlation ($r = 0.965$ for streptococci strains, $r = 0.970$ for lactobacilli strains) between the EPS production quantity of the strains and tolerance to low pH and their correlation is significant at the 0.05 level. The variability of dairy *Propionibacteria* strains to survive at pH 2 and 3 suggests that the acid tolerance of dairy *Propionibacteria* is strain-specific, and pH values of 2 and 3 could be considered as

critical for the selection of potential probiotic dairy *Propionibacteria* (Zarate et al., 2000). Although pH could be used as a suitable direct measure for the selection of probiotic strains, most probiotics are consumed in food products. The presence of food and food ingredients has been reported to improve the viability of microorganisms during gastric transit (Huang and Adams 2004).

The number of viable cells and survival percentage at different levels of bile salts was determined. The growth of strains in the presence bile salts was slower compared to growth in the absence of bile salts. Concentrations of 0.15-0.3 % of bile salts have been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin and Gorbach 1992). Our results showed that there is a positive correlation ($r = 0.998$ for streptococci strains, $r = 0.992$ for lactobacilli strains) between the EPS-production quantity of the strains and resistance to bile salts and their correlation is significant at the 0.01 level.

Xanthopoulos et al., (2000) reported that *Lactobacillus* spp. isolates (*L. acidophilus*, *L. gasseri*, *L. rhamnosus* and *L. reuteri*) from infant feces were tested for their ability to tolerance low pH (pH 3.0) and bile salt. Survival of the test strains ranged between 0.01%- 68.3% at pH 3.0 and 10.3% - 57.4% at 0.15% bile salts. Another research has shown that all cells of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* lost their viability at pH 2.0 and 0.2 % bile salts (Kim et al., 1999). When we compare our results with these results, our strains have shown a very good performance at low pH and bile salts.

In conclusion, our results demonstrated that EPS was protecting bacteria in gastrointestinal conditions. We have suggested that the bacterial EPSs are thought to play a role in the protection of microbial cells against low pH and bile salts. This investigation showed that high EPS production may be important in the choice of probiotic strains. This has not been investigated so far. The result is very important for the selection of probiotic bacteria and may be important for producing effective probiotic

yogurt. Also, these strains could have a broad application as probiotic strains.

Acknowledgment - This research was supported by TUBITAK through project no: TBAG-2090 (101T129) and FEF Gazi University BAP project no: 05/2002-65.

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