



Original article

Polysorbate 80 improves the adhesion and survival of yogurt starters with cholesterol uptake abilities

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ABSTRACT

The goal of this study is to improve the adhesion and survival of yogurt bacteria with probiotic traits by using polysorbate 80, a food additive emulsifier commonly found in milk derivative products. Polysorbate 80 was used at 1% (w/v), and its effects on yogurt bacteria's survival under simulated digestive conditions, cholesterol uptake activities, bile salt hydrolase (BSH) activity, and adhesion to HT-29 culture were studied. In the presence of 1% polysorbate 80, both starters demonstrated better cholesterol uptake and BSH activities, as well as higher bacterial survival at pH 2.5, particularly in associated cultures. In the presence of 0.3 % bile or cholic acid, polysorbate 80 reduced the drop in *L. bulgaricus*'s survival load. However, the carbon source had a greater impact on *S. thermophilus* bile tolerance than the food additive emulsifier. Oleic acid was incorporated into both bacterial membranes when grown in the presence of bile and polysorbate 80, resulting in a higher unsaturated/saturated fatty acid ratio. In the presence of polysorbate 80, *S. thermophilus* adhered to HT-29 cells 2.3-fold better, while *L. bulgaricus*'s adhesion remained unchanged. We suggest that polysorbate 80 may have a protective effect on cell survival under simulated digestive stress as well as a role in yogurt bacteria adhesion to the intestines, giving these bacteria more opportunities to exert their purported cholesterol-removal activities.

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1. Introduction

Many clinical scientific studies have been conducted to support the health benefits of yogurt bacteria in improving lactose digestion (Fassio et al., 2018), modulating intestinal transit time (Mofid et al., 2020), and stimulating the gut immune system (Kinoshita et al., 2019). Their most important beneficial effect, however, is the reduction of serum cholesterol levels (Pourrajab et al., 2020). For the first time, Gilliland et al. (1985) described a significant relationship between cholesterol assimilation by lactobacilli and their degree of bile deconjugation. As a result, the activity of bile salt hydrolase (BSH), the enzyme responsible for bacterial bile salt deconjugation, should be considered when selecting bacteria with cholesterol-lowering abilities. Another

strategy would be to boost the bioconversion of cholesterol in the gut lumen to its bacterial metabolite coprostanol (Juste and Gérard, 2021).

Bacteria with beneficial effects on consumers must survive digestive challenges in order to be effective. The majority of bacteria are naturally sensitive to acidity and/or bile. While most studies have attempted to improve the survival of strains from the *Lactobacillus* and *Bifidobacterium* genera during intestinal transit, few studies have focused on the survival of yogurt starters. Indeed, due to their nonintestinal origin, yogurt starters are unable to survive during this transit, according to some researchers (Morelli, 2003; Mater et al., 2005). In contrast, Elli et al. (2006) and Garca-Albiach et al. (2008) found yogurt bacteria, particularly *L. delbrueckii* subsp. *bulgaricus*, in the feces of healthy subjects after only a short period of administration.

Even though the starter strains are not the only ones who have this inconvenient criterion, it would be interesting to propose new approaches to protect these bacteria that have positive health effects. We focused on increasing the survival of yogurt starters with polysorbate 80 because it contains oleic acid, a fatty acid that has been shown to protect lactobacilli strains under hostile culture conditions (Ananta et al., 2004; Yamaguchi et al., 2019). To the best

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of our knowledge, no information is available on polysorbate 80's effect on *S. thermophilus* viability, nor on its effects on BSH, cholesterol uptake, or adhesion abilities of yogurt starters. Moreover, polysorbate 80 is a permitted emulsifier and, like all other approved food additives, has been evaluated by risk assessors and approved as GRAS (Generally Recognized As Safe). It is used as a surfactant in dairy foods, particularly ice cream, and is frequently combined with other emulsifiers (EFSA, 2018).

The current study aimed to improve the survival of yogurt bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340 and *Streptococcus thermophilus* TA040 under digestive-like hostilities, as well as their adhesion to intestinal mucosa in vitro by adding polysorbate 80. We hypothesize that polysorbate 80 directly affects bacterial resistance to hostile digestive conditions and biofilm formation. Then, we look into their cholesterol uptake capacities, bile salt hydrolase (BSH) activities, and survival after being exposed to acid pH, bile, or cholic acid. The effects of polysorbate 80 on the fatty acid profiles of bacterial cell membranes in the presence of bile, as well as bacterial adhesion to HT-29 cells, were also investigated.

2. Materials and methods

2.1. Bacteria, culture conditions and materials

Lactobacillus delbrueckii subsp. *bulgaricus* LB 340 and *Streptococcus thermophilus* TA 040 yogurt starters were purchased from Danisco (France). Stock cultures were kept at -70°C using Microbank cryovials (Pro-Lab Diagnostics, UK). Before using bacteria in experiments, they were serially propagated three times in the appropriate medium (MRS for LB340 and M17 for TA040). A 1% inoculum was used, and the incubation period was 24 h at 37°C in anaerobic conditions. Unless otherwise stated, cultures of each strain were taken at the end of the exponential phase of growth at cell densities of 5×10^6 CFU/mL.

Danisco (Algiers, Algeria) kindly provided the food additive emulsifier polysorbate 80, which was used in all experiments at a final concentration of 1% (w/v).

2.2. Cholesterol uptake abilities of bacteria

The ability of cultures to uptake cholesterol was determined using the method described by Ziar et al. (2014) in MRS–THIO broth (2% sodium thioglycolate, w/v), with (5 g/L) or without glucose and containing or not polysorbate 80, supplemented with bile (0.3%, w/v) and 100 mg/L of cholesterol (water-soluble cholesterol from sheep, C1145, cholesterol–PEG 600; Sigma). The bacterial culture (single cultures) was added at a 1% (v/v) inoculum size to tubes containing 9 mL aliquots of the test medium. The cultures were statically fermented for 24 h at 37°C under anaerobic conditions with pH monitoring. Following incubation, bacterial cells were removed by centrifugation ($2000 \times g$, 10 min, 4°C), and pellets, spent, or uninoculated control broths were tested for cholesterol content.

2.3. Determination of the bile salt hydrolase (BSH) activity

The cultures' qualitative BSH activity was assessed using the procedure described by du Toit et al. (1998). Sterile filter disks were impregnated in an overnight culture of the assayed strains (with or without polysorbate 80 supplementation) and placed on MRS agar (for LB340) or ST agar (*Streptococcus thermophilus* agar, for TA040) plates supplemented with 0.5% (w/v) bile or cholic acid sodium salt, and 0.37 g/L CaCl_2 . At 37°C , the plates were incubated anaerobically. The diameters of the precipitation zones were mea-

sured after 72 h. Plates with no supplements served as controls. The experiment was repeated three times because each strain was tested in triplicate.

2.4. Acid tolerance

Overnight cultures (16 h) in 25 mL MRS or M17 medium, sub-cultured with 1% (v/v) inocula, were centrifuged at $7000 \times g$ at 4°C for 15 min, washed once in an equal volume of cold isotonic solution, and centrifuged again ($7000 \times g$ at 4°C for 15 min). Pellets were then resuspended in an equal volume of MRS or M17 broth containing 125 mM NaCl, 7 mM KCl, 45 mM NaHCO_3 and 0.3% pepsin, which had previously been adjusted to pH 2.5 and supplemented or not with polysorbate 80. Acidified broths were incubated at 37°C in anaerobic conditions under constant stirring. One mL samples were taken at various times (0, 15, 30, 45, 60, and 120 min), and plated in triplicate onto acidified MRS (pH 5.4, ac-MRS) agar and M17 agar for lactobacilli and streptococci viability enumeration, respectively (plate incubation: 37°C for 48–72 h under anaerobic conditions) (Dave and Shah, 1996).

The inoculum for their associated culture's acid tolerance was set at 1% (1:1) to create a symbiotic relationship (Riazi and Ziar, 2008). Lactobacilli and streptococci viable loads were determined on ac-MRS agar and ST agar, respectively (plate incubation: 37°C for 24–48 h under aerobic conditions) (Dave and Shah, 1996).

2.5. Bile or cholic acid tolerance

The Kimoto et al. (2000) protocol, with some modifications, was used: MRS, M17, and *Streptococcus Thermophilus* (ST) broths were used as culture media, with 3 g/L glucose, 5 g/L lactose, or 20 g/L sucrose, respectively. In 0.1 M PBS buffer, a sterilized bile (0.3% v/v) solution was prepared (pH 7.5). Each broth was inoculated with 0.02 mL of fresh overnight cultures, supplemented or not with polysorbate 80, and incubated at 37°C . Bacterial growth was measured after 24 h using the pourplating method described above.

In another experiment, bile was replaced with cholic acid, and the entire procedure was repeated.

2.6. Fatty acid composition of the cells grown in 0.3% bile containing media

First, 100 mL of MRS or M17 broth with or without polysorbate 80 were prepared and autoclaved at 121°C for 15 min. Broths were supplemented with 0.3% bile and inoculated with overnight cultures at a concentration of 1% before being incubated for 24 h at 37°C in anaerobic conditions. Following that, the cultures ($\text{OD}_{620} \sim 3.0$) were centrifuged at 4°C for 20 min at $1840 \times g$. Pellets were recovered, washed three times in an isotonic solution, and freeze-dried. The lyophilisates were then stored in a nitrogen gas atmosphere.

A Folch et al. (1957) method was used to extract fatty acids from lyophilisates in the presence of heptadecanoic acid (C17:0) as an internal standard, and a chloroformic phase was recovered. Fatty acid methyl esters were prepared in hexane using the Stoffel et al. (1959) method with methanolic HCl (1v:1v) at 85°C for one hour. The resulting supernatant (a lipid-containing hexane layer) was transferred to a glass tube, and the hexane was dried under sodium anhydride sulfate.

Methyl esters were separated on a gas chromatograph (column BPX 70, 30 m, 0.32 mm) equipped with a flame ionization detector (Hewlett Packard model 5890; FID, USA).

The carrier gas was helium (37 p.s.i.; 255 kPa). After sample injection, the column oven was programmed to increase at a rate of 10°C per minute up to a final temperature of 180°C , after which it was increased at a rate of 2°C per minute up to a final temper-

ature of 220 °C. The injector and the detector temperatures were kept isothermally constant at 240 °C and 230 °C, respectively. By comparing their retention times to known standards, the fatty acid methyl esters were identified. Data was collected and analyzed on a Minichrom PC system (VG Data System, USA). The composition of each fatty acid was expressed as the weight percentage.

All experiments were carried out in triplicate three times. Unless otherwise specified, all microbiological components were purchased from Merck and chemicals from Sigma (France), unless otherwise specified.

2.7. Adhesion to HT-29 cells

The American Type Culture Collection (ATCC, Rockville, MD) provided the HT-29 cells, which were grown in RPMI supplemented with 50 g/mL streptomycin, 2 mM L-glutamine, 50 IU/mL penicillin, and 10% heat-inactivated foetal calf serum in a humidified 5 % CO₂ atmosphere at 37 °C. HT-29 cells were prepared and incubated until confluent (1×10^7 CFU/mL). Each starter strain was washed twice with PBS buffer (8000 rpm for 8 min) and resuspended (1×10^8 CFU/mL) in RPMI medium after growing on MRS or M17 with or without polysorbate 80. The bacterial suspension was added to the HT-29 cells in a 10:1 ratio, and the plates were incubated at 37 °C for 2 h. Following incubation, the procedure described by Inturri et al. (2016) was followed. Adherent bacteria were counted in 20 different microscopic fields. Yogurt bacteria were classified as non-adhesive if <40 bacteria were found in 20 fields, adhesive if 41–100 bacteria were found in 20 fields, and strongly adhesive if >100 bacteria were found in 20 fields.

The experiment was carried out twice in triplicate.

2.8. Statistical analysis

Analysis of variance was done using SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA) and used to determine whether significant ($p < 0.05$) variation occurred among means in each experiment. The Bonferroni *t* test (least significant difference value) was used to determine which means differ significantly.

3. Results

3.1. Polysorbate 80 improves the cholesterol uptake activity of yogurt bacteria

Fig. 1 depicts the cholesterol uptake activities of yogurt bacteria grown on MRS-THIO broth supplemented with 0.3 % bile and in the presence or absence of polysorbate 80. The results are expressed in milligrams of assimilated cholesterol per gram of dry cells. Both strains' cholesterol uptake activities differed significantly ($p < 0.05$) in the presence of 0.3 % bile and in MRS-THIO broth without glucose (MRS-THIO - G). Both bacteria assimilated cholesterol after 24 h in the presence of polysorbate 80, with *S. thermophilus* displaying the highest value ($p < 0.05$) of around 8.67 mg/g (Fig. 1), followed by *L. bulgaricus* (3.47 mg/g). When polysorbate 80 is removed from control cultures, the ability to uptake cholesterol decreases significantly ($p < 0.05$). As a result, this decrease was approximately –79 % in *S. thermophilus* and –89 % in *L. bulgaricus* (Fig. 1).

In the presence of polysorbate 80 and MRS-THIO broth containing glucose (MRS-THIO + G), *S. thermophilus* had a cholesterol uptake capacity of 8.87 mg/g. This value did not differ significantly ($p > 0.05$) from that obtained in MRS without glucose (Fig. 1). In contrast to *S. thermophilus* and in the presence of glucose, *L. bulgaricus* assimilated more cholesterol ($p < 0.05$), 40.45 mg/g. As a result, our findings show that the food additive polysorbate 80 and glu-

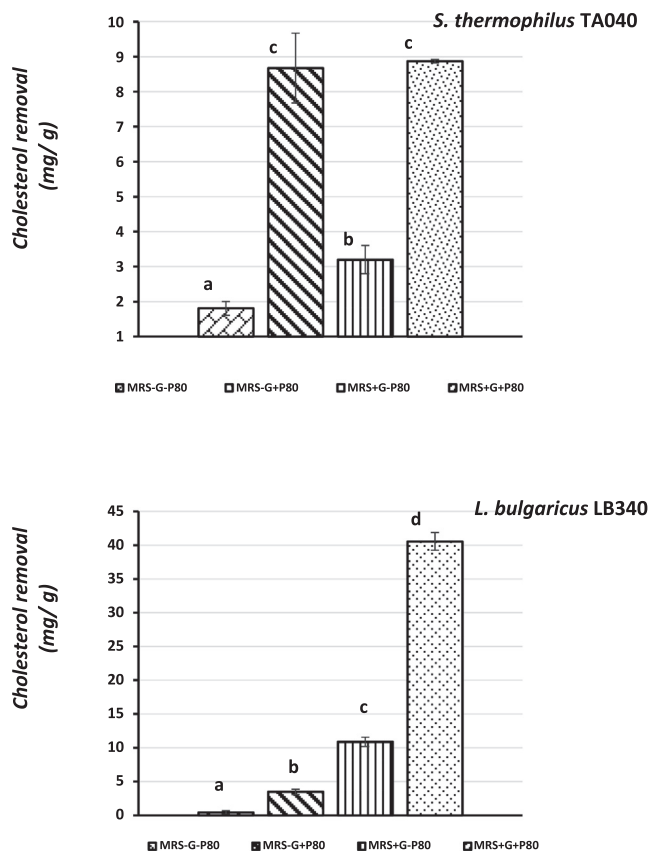


Fig. 1. Cholesterol uptake abilities (mg/g bacterial dry weight) of yogurt starters by the addition of polysorbate 80 “P80”. ^{a-d}: Means with different letters are significantly different ($p < 0.05$).

cose increased Lactobacilli starter's cholesterol uptake ability by 3.7- and 11.65-fold, respectively (Fig. 1).

3.2. Polysorbate 80 increases the BSH activity of yogurt bacteria

Table 1 shows the effect of polysorbate 80 on BSH activity in cell extracts of *L. bulgaricus* LB 340 and *S. thermophilus* TA040 in the presence of bile or cholic acid. The presence of polysorbate 80 and bile in *S. thermophilus* cultures increased BSH synthesis by 32% ($p < 0.05$), and by 70% ($p < 0.05$) in the presence of cholic acid, when compared to control cultures without polysorbate 80 addition. *L. bulgaricus*, on the other hand, exhibited a slightly different defensive response. Cholic acid inhibited BSH 25-fold more than

Table 1

Polysorbate 80's effect on the Bile Salt Hydrolase BSH activities of yogurt starters when exposed to 0.5% bile or cholic acid.

	Culture medium	Precipitation zone diameter (mm)
<i>S. thermophilus</i> TA040	Bile (–P 80)	2.5 ± 0.71d
	Cholic Acid (–P 80)	3 ± 0.45d
	Bile (+ P 80)	3.3 ± 0.5de
	Cholic Acid (+ P 80)	5.1 ± 0.3f
<i>L. bulgaricus</i> LB 340	Bile (–P 80)	1.25 ± 1.6c
	Cholic Acid (–P 80)	0.05 ± 0.05a
	Bile (+ P 80)	3.4 ± 0.35e
	Cholic Acid (+ P 80)	0.1 ± 0.11b

^{a-f}: Means with different letters are significantly different ($p < 0.05$). Results are indicated as means ± SDs (n = 9).

bile ($p < 0.05$). However, in the presence of polysorbate 80 and bile, *L. bulgaricus* cultures increased BSH synthesis by a factor of two (Table 1).

3.3. Yogurt bacteria tolerate gastric like-conditions better when polysorbate 80 is present

Table 2 shows the effects of gastric acidity on yogurt bacteria viability (single or associated cultures), grown in the presence or absence of polysorbate 80. In 15 min of exposure at pH 2.5, *L. bulgaricus* was more acid sensitive than *S. thermophilus* in single cultures. The viability decrease in the associated culture was moderate ($p > 0.05$) (-0.2 Log cycles on average) (Table 2).

Furthermore, no significant ($p > 0.05$) improvement in bacteria viability was observed until 15 min of exposure. However, after 45 min and the addition of this food additive, the decrease in bacteria viability was not statistically significant ($p > 0.05$) (Table 2).

After 120 min at pH 2.5, polysorbate 80 improved cell viability by +2 and +2.5 Log cycles for *L. bulgaricus*, and +0.34 and +1 Log cycles for *S. thermophilus* after 120 min at pH 2.5, as compared to control cultures, in single and associated cultures, respectively (Table 2).

3.4. Polysorbate 80 improves the tolerance of yogurt starters to bile or cholic acid

Table 3 shows the effect of 0.3 % bile or cholic acid on the growth of yogurt bacteria grown with or without polysorbate 80, as measured by viable Log CFU/mL.

In the presence of 0.3% bile, the survival of *S. thermophilus* in M17 (7.6 Log CFU/mL) and ST (7.8 Log CFU/mL) broths was very similar ($p > 0.05$) and 1 % polysorbate 80 had no effect on this strain's survival (Table 3). However, in M17 broth supplemented with 0.3 % cholic acid (Table 3), our results showed that cholic acid had a negative effect ($p < 0.05$) on *S. thermophilus* survival when compared to bile ($p > 0.05$), and that the protective effect of polysorbate 80 was significantly ($p < 0.05$) appreciated (5.67 and 7.08 Log CFU/mL, respectively, survived in M17 without and with polysorbate 80). In ST broth containing cholic acid, and when compared to M17 broth, *S. thermophilus* growth was significantly higher ($p < 0.05$) in the absence of polysorbate 80 than in its presence.

In the case of *L. bulgaricus*, our findings revealed a higher ($p < 0.05$) survival rate in the presence of bile and polysorbate 80 (7.45 Log CFU/mL) than in the absence (4.56 Log CFU/mL) (Table 3). However, in the presence of cholic acid (0.3 %), the addition of polysorbate 80 to the culture medium appears to be required for the survival of *L. bulgaricus* ($p < 0.05$) (7.64 vs 2.14 Log CFU/mL) (Table 3).

Table 2

Effect of polysorbate 80 (P 80) on yogurt starters' survival under simulated gastric acidity (pH 2.5).

Number of viable cells (Log CFU/mL)		t0	t15	t45	t60	t120
Time (min)						
Strain						
TA040 single culture	-P 80	6.80 ± 0.81Da	6.32 ± 1.00Ca	6.16 ± 0.07Ba	5.74 ± 0.05Bd	5.28 ± 1.56Ae
	+ P 80	6.70 ± 0.21Ca	6.27 ± 0.30Ba	6.23 ± 0.24Bb	6.12 ± 0.34ABe	5.62 ± 1.16Af
LB340 single culture	-P 80	6.79 ± 1.14Da	6.18 ± 0.12Ca	6.05 ± 0.17Ca	4.39 ± 0.20Ba	2.25 ± 0.20Aa
	+ P 80	6.82 ± 0.81Da	6.24 ± 1.00Ca	6.20 ± 0.81Cb	5.08 ± 1.56Bb	4.32 ± 0.11Ac
TA040 in Associated culture	-P 80	6.95 ± 0.81Ba	6.74 ± 0.81Bb	6.71 ± 0.14Bc	5.12 ± 0.08Ab	5.01 ± 0.46Ad
	+ P 80	6.95 ± 0.81Ba	6.78 ± 1.00Bb	6.79 ± 0.41Bd	6.18 ± 0.07Ae	6.04 ± 0.31Ag
LB340 in Associated culture	-P 80	6.83 ± 0.11Da	6.61 ± 0.51CDb	6.19 ± 0.46Cb	5.22 ± 0.46Bc	3.13 ± 0.81Ab
	+ P 80	6.83 ± 0.11Ca	6.68 ± 0.20Cb	6.66 ± 0.31Cc	6.02 ± 0.25Be	5.72 ± 0.21Af

A-D: Means ± SD (n = 9) with different capital letters in the same row are significantly different ($p < 0.05$).

a-f: Means ± SD (n = 9) with different lowercase letters in the same column are significantly different ($p < 0.05$).

3.5. Polysorbate 80 increases the unsaturated/saturated membrane fatty acid ratio

Tables 4 and 5 show the results of an analysis of methyl esters of fatty acids prepared from lipid fractions of yogurt bacteria membranes grown with or without polysorbate 80.

The major fatty acids in the membrane of *L. bulgaricus* grown in MRS medium with polysorbate 80 were C18:0, C18:1, and C19:0 ($p < 0.05$). Because of their high concentrations, the strain appears to be synthesizing cyclopropane fatty acids (C19:0 and C19:1) via palmitoleic, stearic, linoleic, and arachidic acid conversion (Table 4). However, after polysorbate 80 supplementation, the oleic acid concentration in that strain increased 6-fold ($p < 0.05$). When compared to the control, the overall unsaturated/saturated fatty acid ratio was significantly ($p < 0.05$) higher (1.02 vs 0.42).

The fatty acid composition of *S. thermophilus* bacterial membrane was also examined after growth in M17 with polysorbate 80, and the results (Table 5) revealed a shift towards C14:0, C16:1, C18:1, and C20:0 fatty acids that increased in concentrations ($p > 0.05$) compared to the control (Table 5). In *S. thermophilus*, however, almost no significant ($p > 0.05$) changes in the proportion of oleic acid were observed, with registered values shifting from 2.73 to 3.74 % in the absence and presence of polysorbate 80, respectively. Furthermore, the obtained data revealed an increase ($p < 0.05$) in the proportion of cyclopropane fatty acids C19:1 at the expense of C18:0, C18:2, C18:3, and C19:0.

In *S. thermophilus* cultures grown in the presence of polysorbate 80, the unsaturated/saturated fatty acid ratio was significantly ($p < 0.05$) higher than in the control (1.84 vs 0.56) (Table 5).

3.6. Polysorbate 80 alleviates *S. Thermophilus* adhesion to HT-29 cells

Only *S. thermophilus* yogurt starter was able to adhere to the HT-29 monolayer (Fig. 2). When compared to their respective control cultures without polysorbate 80, the percentage of adhesion was very low and statistically not different ($p > 0.05$) for *L. bulgaricus* (30 vs 20), and approximately 2.3-fold higher ($p < 0.05$) for *S. thermophilus* (470 vs 200).

4. Discussion

Yogurt has been consumed by humans for hundreds of years. As a nutritious product, eating it on a regular basis may improve several aspects of health caused by yogurt starters, which are bacteria used to ferment milk. The growing number of studies on selecting yogurt starters with a high survival rate from acidic niches has captivated scientific interest and continues to do so (Reuben et al., 2020; Tarique et al., 2022). Tween 80 is a non-ionic surfactant and emulsifier that is commonly used in foods, cosmetics, and parenteral medications. It is also known as polysorbate 80 or by its

Table 3

Effect of polysorbate 80 (P 80) on the viability (Log CFU/mL) of yogurt starters upon exposure to bile or cholic acid.

<i>Streptococcus thermophilus</i> TA040	+ Bile		+ Cholic acid	
	+ P 80	-P 80	+ P 80	-P 80
M17	7.67 ± 0.04c	7.64 ± 0.01c	7.08 ± 0.03b	5.67 ± 0.01a
ST	7.87 ± 0.01b	7.88 ± 0.02b	7.23 ± 0.02a	7.94 ± 0.02c
<i>Lactobacillus bulgaricus</i> LB340				
MRS*	7.45 ± 0.37c	4.56 ± 0.01b	7.64 ± 0.16d	2.14 ± 0.10a

a-c: Means with different letters are significantly different ($p < 0.05$). Comparisons are made in the same row.

*: MRS without tween 80 addition.

ST: *Streptococcus Thermophilus* broth.**Table 4***L. bulgaricus* LB340 fatty acid composition in the presence of bile and with or without polysorbate 80 supplementation.

Fatty acid*	LB340	
	-POLYSORBATE 80	+ POLYSORBATE 80
C14: 0	5.11	2.33
C16: 1	16.51	4.13
C18: 0	44.88	25.33
C18: 1	6.31	37.46
C18: 2	13.17	3.89
C19: 0	/	20.27
C19: 1	/	3.94
C20: 0	10.48	2.08
C20: 1	2.91	1.50
UFA/SFA	0.42	1.02

*: Total fatty acid contents were approximately 0.015 mg/ mL.

#: MRS containing 0.3 % (w/v) bile and without tween 80.

Table 5Fatty acid composition of *S. thermophilus* TA040 cultivated in the presence of bile and with or without polysorbate 80 supplementation.

Fatty acid *	TA040	
	- POLYSORBATE 80	+ POLYSORBATE 80
C14 : 0	4.59	6.59
C16 : 0	20.7	11.28
C16 : 1	2.25	4.44
C18 : 0	6.23	4.47
C18 : 1	2.73	3.74
C18 : 2	6.63	5.38
C18 : 3	10.17	9.8
C19 : 0	25.19	17.11
C19 : 1	5.18	37.19
C20 : 0	6.01	8.57
C20 : 1	10.32	/
UFA/SFA	0.56	1.84

*: Total fatty acid contents were approximately 0.011 mg/ mL.

#: M17 containing 0.3 % (w/v) bile.

registered trade name, Tween® 80. Furthermore, polysorbate 80 is widely used as an additive in microbiological growth media, providing bacterial cells with exogenous oleic acid (e.g., *Lactobacillus* sp). Oleic acid is a common fatty acid found in a variety of foods. As a result, during enrichment in cultivation media or in an actual food product, yogurt bacteria are likely to come into contact with polysorbate 80 or its fatty acid group. In this study, the food additive polysorbate 80 was chosen to remedy the survival problem for *L. bulgaricus* LB340 and *S. thermophilus* TA040, which had previously been assigned cholesterol uptake abilities as probiotic traits (Ziar et al., 2014).

Both yogurt starters in this study were able to uptake cholesterol from the culture medium. Because the study was carried out under pH control, our findings could be attributed to direct assimilating activity or cholesterol molecule binding to the bacterial membrane. In addition, any remaining cholesterol in the spent

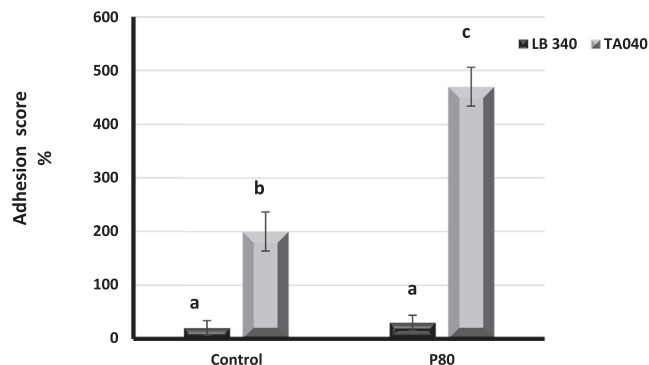


Fig. 2. Adhesion scores (%) to carcinoma HT-29 cells calculated for yogurt starters by the addition of polysorbate 80 "P80". a-c: Means with different letters are significantly different ($p < 0.05$).

broth was excluded. Lactic acid bacteria have been shown in vitro to bind cholesterol and/or incorporate it into the cell wall or cytoplasmic membrane (Ziar et al., 2014; Tarique et al., 2022). The current findings indicate that glucose had no effect on *S. thermophilus* cholesterol binding. This result was almost predictable given that the starter was a non-glucose fermenter. In contrast, *L. bulgaricus* is capable of fermenting glucose (Riazi and Ziar, 2008; Ziar et al., 2014) and removing more cholesterol. In fact, polysorbate 80 improved both starters' ability to bind cholesterol. We advocate that the culture medium composition (polysorbate 80 as a carbon source) influences the cholesterol uptake abilities of lactic acid bacteria by producing the required ATP.

Second, the BSH activities of yogurt starters in broth containing bile or cholic acid, with or without the addition of polysorbate 80, were determined. According to Pereira et al. (2003), bile salt hydrolyase (BSH) activity is required for the selection of probiotic organisms with cholesterol uptake abilities. Despite the fact that BSH activity has been studied in several studies on intestinal genera, such as *Lactobacillus* sp. and *Bifidobacterium* sp. (Elkins and Savage, 1998; Hernández-Gómez et al., 2021), few studies on non-intestinal lactic acid bacteria have been conducted (Ziar et al., 2014; Ru et al., 2019; Tarique et al., 2022).

Both strains produced BSH in the present study due to the presence of bile or cholic acid in the medium. *S. thermophilus* TA040, a bile-tolerant strain (Ziar et al., 2014), produced comparable amounts of BSH in response to bile or cholic acid toxicity and was unaffected by polysorbate 80 addition. *L. bulgaricus* LB340, a moderate bile-tolerant strain (Ziar et al., 2014), displayed higher BSH activity in the presence of polysorbate 80. This is consistent with previous findings (Ziar et al., 2014; Ru et al., 2019), indicating that deconjugation is a strain-dependent defensive behaviour.

In the current study, the survival of yogurt starters, both single and associated cultures, was also monitored under gastrointestinal-like conditions and in the presence of polysorbate 80. Most bacteria can not survive in low pH environments. As a result,

it has been suggested that microbial cultures intended for use as probiotics should be tested for acidity resistance. Polysorbate 80 was revealed to contribute to higher viable loads of yogurt starters after 2 h under gastric-like conditions (pH of 2.5), especially at associated culture. Indeed, Riazi and Ziar (2008) demonstrated that both strains had a very good symbiotic relationship and were more likely to survive in acidified milk. [Soni et al. \(2020\)](#) stated that the yogurt bacteria NCDC-253 and –199 were the most acid sensitive, surviving 3 h at pH 3 or 4.

Under colonic-like conditions, *S. thermophilus*'s resistance to cholic acid appears to be influenced by the carbon source present in the medium rather than polysorbate 80 supplementation. According to some authors, the presence of an easily metabolized carbon source (here, saccharose in ST broth) may alleviate lactobacilli, bifidobacteria, and streptococci resistance to bile ([Patel et al., 2004](#), [Ziar et al., 2014](#)). Our findings revealed that *S. thermophilus*'s response to the toxic effects of bile and cholic acid varied depending on the availability of nutrients from carbon or nitrogen sources in the media, but not to polysorbate 80. Different media were used in this study with the hypothesis that the negative effects of bile or cholic acid would be reversed by the addition of polysorbate 80 singly or together with (synergic effect) the preferred carbon source to the broth. The general composition of the medium may influence bacterial cholic acid resistance, which most likely has no synergy with polysorbate 80, explaining why the latter's protective effect was not observed.

Other studies found that adding oleic acid to the growth medium increased the viability of lactic acid bacteria ([Ananta et al., 2004](#)). Regarding bacterial viability, oleic acid can be incorporated into bacterial membranes and can then promote the transportation of nutrients and exterior protons into cells, resulting in an enhanced survival rate. [Kimoto et al. \(2002\)](#) demonstrated that an oleic acid source, such as tween 80, forms micelles that reduce bile adhesion to cells. Tween 20, tween 80, and free oleic acid were found to confer resistance to *L. plantarum* TMW 1.708 against high hydrostatic pressure ([Reitermayer et al., 2018](#)). The role of cyclopropane fatty acids (CFAs) in the membrane of lactic acid bacteria is undisclosed, but they are thought to increase membrane fluidity in a way similar to polyunsaturated fatty acids. According to [Corcoran et al. \(2007\)](#), palmitic acid concentration was lower when lactobacilli cultures were grown in the presence of tween 80. This is consistent with our findings regarding palmitooleic acid content in *L. bulgaricus* grown in polysorbate 80. Its lower concentration may have an effect on the biosynthesis of *de novo* fatty acids. Similarly, [Corcoran et al. \(2007\)](#) found that the membrane of *L. rhamnosus* GG cells grown in MRS medium with tween 80 contained 55-fold more oleic acid than controls. Interestingly, our findings suggest that exogenous oleic acid from the food additive Polysorbate 80 was likely incorporated into bacterial cell membranes, possibly inducing oleate conversion to CFAs.

Several studies have found that the biosynthesis of fatty acids is the primary metabolic pathway used by lactic acid bacteria to express their resistance to adverse storage conditions or their probiotic effects ([Kishino et al., 2013](#); [Balthazar et al., 2016](#); [Ding et al., 2020](#)). Our findings show that polysorbate 80 provides exogenous oleic acid, which reduces the need for bacterial fatty acid synthesis. In fact, CFA synthase was found to convert monounsaturated fatty acids into their corresponding CFA in lactobacilli. Furthermore, CFA content has been linked to stressor resistance ([Chen and Gänzle, 2016](#)).

We also reported the effect of polysorbate 80 on in vitro yogurt starter adhesion in this study. This in vitro test was commonly used to estimate strains' probiotic potential to colonize the intestinal epithelium, allowing the bacteria to exert their beneficial effects. Polysorbate 80, however, increased the adhesion of *S. thermophilus* to HT-29 cells but not that of *L. bulgaricus*. We hypothe-

sized that polysorbate 80 could influence bacterial adhesion by acting as an ionophore and thus transporting divalent cations like magnesium, calcium, and iron for biofilm formation ([Banin et al., 2006](#)). *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVJ1-21, isolated from artisanal sour milk and yogurt, were found to be capable of adhering to Caco-2 cells ([Popovic et al., 2020](#)).

It is important to highlight that some reports have suggested that polysorbate 80 may potentiate colitis in in vitro or ex-vivo models of the gastrointestinal tract ([Malik et al., 2020](#); [Naimi et al., 2021](#)). Interestingly, [Partridge et al. \(2019\)](#) underlined that results from most studies on animals found that mice fed polysorbate 80 developed low-level inflammation and metabolic syndrome compared to those receiving polysorbate 80 in drinking water. In another recent study, the emulsifiers carboxymethylcellulose (CMC) and polysorbate 80 (P80) were compared ([Rousta et al., 2021](#)). They found that CMC induces greater inflammation in humanized mice than does P80. Indeed, P80 also increased the relative abundance of Actinobacteria in the cecal contents compared to CMC and water controls. However, to date, these effects have not been confirmed in humans and current knowledge still favours the safety of using the food additive Polysorbate 80. It has been approved as a safe ingredient by the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), as well as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) ([EFSA, 2018](#)).

Intestinal adhesion is not a necessary criterion for estimating a potential health benefit. *S. thermophilus* can produce lactate, which can strengthen endogenous bacterial populations in the intestines ([Veiga et al., 2010](#)). Indeed, lactate is a potent antimicrobial metabolite that promotes the growth of beneficial bacteria and, as a result, the production of secondary short-chain fatty acids such as propionate and butyrate ([Louis and Flint, 2017](#)). Polysorbate 80, on the other hand, was found to have a completely opposite effect on gram-negative bacteria such as *H. pylori* ([Di Stefano et al., 2019](#)), resulting in less biofilm formation by both *L. monocytogenes* and *P. fluorescens* ([Nielsen et al., 2016](#)).

5. Conclusions

The results show that yogurt starters in the presence of a high oleic acid content, such as the food additive polysorbate 80, were more resistant or tolerant to acid and bile salts. We propose that it plays an important role in reducing acid and bile resistance in yogurt starters by inducing specific changes in their lipid membranes. Polysorbate 80 could be a promising ingredient to include in probiotic mixtures containing yogurt bacteria to improve their survival and adhesion in the digestive tract. Furthermore, these strains are of milk origin, which may be advantageous in their useful incorporation in many milk products. However, these results need confirmation, and in vivo studies aiming at hypercholesterolemia endpoints are necessary.

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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Authors' Contributions

Hasnia Ziar and Ali Riazi contribute equally in study conceptualization, data analysis, data interpretation, writing- original draft, review and editing of the final version of the article. Ali Riazi is the head of the project.

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Availability of data and material

Data sharing not applicable to this article as no datasets were generated during the current study. Please contact author for data requests.

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