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Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice

B. M. Corcoran,[1,2] C. Stanton,[1,3] G. F. Fitzgerald[2,3] and R. P. Ross[1,3]

[1]Teagasc, Moorepark Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]Department of Microbiology, University College, Cork, Ireland

[3]Alimentary Pharmabiotic Centre, Cork, Ireland

Correspondence
R. P. Ross
Paul.Ross@teagasc.ie

The effect of inclusion of various C18 fatty acids with 0–2 double bonds in either *cis* or *trans* configuration on *Lactobacillus rhamnosus* GG survival was analysed in simulated gastric juice at pH 2.5. The incorporation of Tween 80 (1 g l^{-1}) in the growth media enhanced subsequent survival of stationary-phase cultures up to 1000-fold following 90 min acid exposure compared with controls grown without Tween 80. There was a significant ($P<0.05$) increase in bacterial content of oleic acid [C18 : 1 (9c), up to 55-fold] after growth of bacteria in MRS supplemented with Tween 80. The inclusion of various C18 fatty acids in the growth media revealed that only oleic and vaccenic acids [C18 : 1 (11t)] had protective effects on the survival of *Lb. rhamnosus* GG when exposed to the acidic environment. Comparative analysis with other lactobacilli indicated that all strains exhibited increased survival when grown in the presence of Tween 80. Further work with a neomycin-resistant mutant with 48 % of the F_0F_1-ATPase activity of the parent indicated that the Tween 80 effect was independent of the complex. The mechanisms behind the effect of fatty acid protection were investigated and proton permeability assays showed that cultures grown in the presence of Tween 80 had higher extracellular pH than controls. Furthermore, there was a significant reduction of oleic acid and a significant increase in stearic acid (C18 : 0) ($P<0.05$) content of bacterial cells following exposure of Tween 80-supplemented cultures to simulated gastric juice. Overall, the data suggest that probiotic lactobacilli can use an exogenous oleic acid source to increase their acid survival and the underlying mechanism most likely involves the ability of increased membrane oleic acid to be reduced by H^+ to stearic acid.

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INTRODUCTION

There are increasing clinical scientific data to support the association of lactic acid bacteria (LAB) with health benefits, including ability to reduce symptoms associated with diarrhoea (Isolauri *et al.*, 1991), irritable bowel syndrome (O'Mahony *et al.*, 2005) and infant asthma (Kalliomaki *et al.*, 2001), as well as reducing the duration of the common cold (de Vrese *et al.*, 2005). Probiotics are defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* are the main genera considered for human probiotic food use and *Lactobacillus rhamnosus* GG is one of the more researched probiotic bacteria (Corcoran *et al.*, 2004; Guandalini *et al.*, 2000; Isolauri *et al.*, 1991; Kalliomaki *et al.*, 2001; Lavermicocca *et al.*, 2005). It is recommended that probiotic bacteria are delivered in high numbers in food products (at least 10^7 c.f.u. ml^{-1} or g^{-1}) for efficacy (Ishibashi & Shimamura, 1993), although this number depends on a number of factors, such as the particular strain used, the food-processing conditions and gastric transit survival. In this regard, acid tolerance is an important probiotic trait for survival during gastric transit and in fermented food products (Holzapfel & Schillinger, 2002). A number of approaches have been investigated for enhancing survival of probiotic bacteria in acid conditions, including physical protection via the choice of food system (Conway *et al.*, 1987; Gardiner *et al.*, 1999), encapsulation (Desmond *et al.*, 2002; O'Riordan *et al.*, 2001) and incorporation of fermentable substrates in the acid environment (Charalampopoulos *et al.*, 2003; Corcoran *et al.*, 2005). *Lb. rhamnosus* GG has been shown to survive passage through the adult human gastrointestinal tract (Saxelin *et al.*, 1995) and could survive in conditions as low as pH 2.5 (Jacobsen *et al.*, 1999).

The bacterial membranes of lactobacilli are typically composed of straight-chain saturated, unsaturated and cyclopropane fatty acids (CFAs; Johnsson *et al.*, 1995). The

Abbreviations: CFA, cyclopropane fatty acid; CLA, conjugated linoleic acid; LAB, lactic acid bacteria.

fatty acid composition of the bacterial membrane depends upon a number of factors, including growth temperature (Suutari & Laakso, 1992), pH (Fozo & Quivey, 2004b), growth phase (Veerkamp, 1971), cultivation medium composition (Johnsson *et al.*, 1995) and NaCl concentration (Machado *et al.*, 2004). When LAB were grown in a medium supplemented with Tween 80 [polyoxyethylenesorbitan mono-oleate, which consists of up to 90 % oleic acid; Partanen *et al.*, 2001], oleic acid was incorporated into the membranes (Johnsson *et al.*, 1995). Tween 80 is routinely included in synthetic media for cultivation of lactobacilli, where it improves aerobic growth rates (Jacques *et al.*, 1980), glucosyltransferase secretion (Jacques *et al.*, 1985) and glycine-betaine accumulation in LAB (Guillot *et al.*, 2000). In addition, it has been reported that both lactobacilli and bifidobacteria are able to form dihydrosterculic acid [*cis*-9,10-methyleneoctadecanoic acid; cyc 19 : 0(9c)] via methylation (Johnsson *et al.*, 1995; Veerkamp, 1971) and these fatty acids, also called CFAs, have been associated with enhanced bacterial acid resistance (Budin-Verneuil *et al.*, 2005; Chang & Cronan, 1999).

The presence of Tween 80 in the cultivation media led to enhanced survival of *Oenococcus oeni* in wine and resulted in enhanced concentrations of oleic and dihydrosterculic acids, at the expense of palmitic (C16 : 0), vaccenic and lactobacillic acids (CFAs derived from vaccenic acid) in the cells (Guerrini *et al.*, 2002). However, the mechanism behind the change in bacterial fatty acid composition and associated tolerance to wine was not studied. Few studies have addressed the importance of the fatty acid fraction in the bacterial cell membrane in acid tolerance, and those that have been done focused mainly on the activity of CFA synthase (Budin-Verneuil *et al.*, 2005; Chang & Cronan, 1999) or the synthesis of monounsaturated fatty acids to enhance acid tolerance (Fozo *et al.*, 2004; Fozo & Quivey, 2004a, b). The aim of this study was to investigate the effect of inclusion of C18 fatty acids on survival of *Lb. rhamnosus* GG upon exposure to acidic conditions and to investigate the mechanism involved in the protective effect.

METHODS

Bacterial strains. The probiotic strains *Lactobacillus rhamnosus* VTT E-97800 (*Lb. rhamnosus* E800, VTT Biotechnology), *Lb. rhamnosus* VTT E-94522 (*Lb. rhamnosus* ATCC 53103, *Lb. rhamnosus* GG, Valio Ltd), *Lactobacillus salivarius* VTT E-01878 (*Lb. salivarius* UCC 500, University College, Cork, Ireland) and *Lactobacillus paracasei* subsp. *paracasei* NFBC 338 (Teagasc, Moorepark Food Research Centre, Cork, Ireland) were used in this study. *Bifidobacterium animalis* subsp. *lactis* Bb12 (abbreviated to Bb12) was received from Chr. Hansen and *Lactococcus lactis* subsp. *lactis* IL1403 (*Lc. lactis* IL1403) was obtained from University College, Cork (Bolotin *et al.*, 2001). Harvested cells of these strains were stored as stock solutions in 50 % (v/v) aqueous glycerol at −20 °C.

Culture conditions. *Lactobacillus* strains were cultured at 1 % (v/v) in MRS (deMan *et al.*, 1960) Oxoid medium containing Tween 80 (Oxoid) for ~16 h at 37 °C under anaerobic conditions. Bb12 was cultured in MRS medium containing cysteine hydrochloride (0.05 % w/v, Sigma) for 20 h under anaerobic conditions. *Lc. lactis* IL1403, a plasmid-free derivative of a lactococcal starter strain, was subcultured in M17 medium containing glucose (0.5 % w/v) at 37 °C under aerobic conditions for 16 h. All inocula used in this study were centrifuged and washed in quarter-strength Ringer's solution prior to inoculation in order to remove any residual medium. For the enumeration of viable micro-organisms in acid-tolerance studies, samples were serially diluted in maximum recovery diluent (MRD; 10 % w/v; Oxoid) and pour plated onto the appropriate medium in independent triplicate experiments.

Cultivation of *Lb. rhamnosus* GG in media supplemented with fatty acids and Tween 80. *Lb. rhamnosus* GG was grown overnight from 1 % (v/v) inocula (grown in MRS medium and washed in Ringer's solution) to the stationary phase (16 h) in 25 ml MRS medium or general edible medium (GEM) (Saarela *et al.*, 2004) prepared from first principles, both containing Tween 80 (1 g l^{-1}), with controls prepared without Tween 80. In order to assess the effect of different concentrations of Tween 80 on probiotic survival, *Lb. rhamnosus* GG was grown in different concentrations of Tween 80 (0–2 g l^{-1}) and survival analysed in simulated gastric juice at pH 2.5. In a further experiment, cultures were grown with or without Tween 20 (1 g l^{-1}, a fatty acid derivative composed principally of lauric acid) and assessed for acid tolerance, pH 2.5.

Lb. rhamnosus GG was also grown from 1 % (v/v) inocula (grown in MRS medium and washed in Ringer's solution) to the stationary phase in 25 ml GEM in the presence of the following free fatty acids: stearic acid (10 μg ml^{-1}, Sigma), oleic acid (5 μg ml^{-1}, Sigma), elaidic acid [C18 : 1 (9t), 5 μg ml^{-1}, Sigma], vaccenic acid (5 μg ml^{-1}, Sigma), linoleic acid [C18 : 2 (9,12cc), 1 μg ml^{-1}, Sigma], conjugated linoleic acid [CLA, C18 : 2 (9,11ct), 1 μg ml^{-1}, Matreya] or CLA [C18 : 2 (10,12tc), 1 μg ml^{-1}, Matreya] were included in the media. For incorporation into the media, individual free fatty acids were dissolved in ethanol (80 % v/v) and 3 M KOH. This was mixed with a volume of Tergitol NP-40 (20 % v/v), a fatty acid derivative that bacteria cannot metabolize, to a final NP-40 concentration of 0.1 % (v/v) (Chang & Cronan, 1999), as other methods used to solubilize fatty acids required Tween 80 (Coakley *et al.*, 2003). Stock solutions contained 10 mg solubilized fatty acids ml^{-1}. The concentrations of the individual free fatty acids were determined prior to the acid-tolerance experiment based on optimal growth of *Lb. rhamnosus* GG using optical density (OD_{600}, data not shown). The cultures were prepared and analysed for acid tolerance as described below.

Preparation of simulated gastric juice. Simulated gastric juice was prepared as previously described (Beumer *et al.*, 1992) with modifications. Proteose-peptone was omitted from the formulation to avoid providing cultures with a possible source of free amino acids, such as L-glutamate, which, if present in the simulated gastric juice, might have been used by the bacteria to extrude protons, thus potentially enhancing bacterial tolerance (Cotter *et al.*, 2001). Glucose was also omitted, as it is known to increase the acid tolerance of lactobacilli (Charalampopoulos *et al.*, 2003; Corcoran *et al.*, 2005). Simulated gastric juice was formulated using NaCl (2.05 g l^{-1}), KH_2PO_4 (0.60 g l^{-1}), $CaCl_2$ (0.11 g l^{-1}) and KCl (0.37 g l^{-1}), adjusted to pH 2.0 using 1 M HCl and autoclaved at 121 °C for 15 min. Porcine bile (0.05 g l^{-1}), lysozyme (0.1 g l^{-1}) and pepsin (0.0133 g l^{-1}) were added as stock solutions prior to use. Components were obtained from Sigma, BDH Chemicals and Orthana (Orthana Kemisk Fabrik).

Analysis of acid tolerance. Following overnight growth, the cultures were centrifuged at 7000 ***g*** at 4 °C for 15 min, washed once in an equal volume of cold quarter-strength Ringer's solution and recentrifuged (7000 ***g***). Pellets were then resuspended in an equal volume of simulated gastric juice, pH 2.5, at 37 °C and incubated for 90 min with constant stirring. Samples were taken at 0, 30, 60

and 90 min, serially diluted in MRD, plated on MRS and incubated at 37 °C for 72 h. For comparative survival of probiotic lactobacilli grown to stationary phase in MRS medium containing Tween 80 (1 g l^{-1}), two strains of *Lb. rhamnosus*, *Lb. paracasei* NFBC 338 and *Lb. salivarius* UCC 500 were exposed to simulated gastric juice at pH 2.0–3.0 for 90 min and survivors enumerated on MRS plates as described above. In addition, Bb12 and *Lc. lactis* IL1403 were grown in media containing Tween 80 (1 g l^{-1}) and survivors were enumerated following 90 min exposure to gastric juice, pH 2.5 and 3.0, respectively.

Fatty acid composition. Following growth or acid exposure, 10 ml bacterial culture ($\sim 10^9$ c.f.u. g^{-1} and OD_{600} ~ 3.0) was centrifuged and washed in 1 ml saline solution (137 mM NaCl, 7.0 mM K_2HPO_4 and 2.5 mM KH_2PO_4). The cells were vortex mixed and centrifuged at 3632 ***g*** for 30 min and the washing step repeated. The pellet was resuspended in 1 ml saline solution and tridecanoic acid (C13 : 0, an internal standard used for bacterial membrane lipid analysis; Coakley *et al.*, 2003) was added to give a final concentration of 0.75 mg internal standard per sample. 2-Propanol (2 ml; 99 % purity; Alkem Chemicals) was added and the samples vortexed for 30 s and hexane (1.5 ml added initially and vortex mixed before adding a further 3 ml hexane, 99 % purity; LabScan) was added to this mixture, vortex mixed and centrifuged at 2197 ***g*** for 5 min. The resultant supernatant (hexane layer containing lipids) was removed to a glass tube and the hexane dried off under a stream of nitrogen at 45 °C. The extracted lipids were stored at −20 °C prior to preparation of fatty acid methyl esters (FAMEs) for GLC analysis. FAMEs were prepared by acid-catalysed methylation [12 % methanolic HCl (Supelco) at 60 °C for 20 min] as described previously (Stanton *et al.*, 1997). The FAMEs were separated on a CP Sil 88 column (100 m, 0.25 mm i.d., 0.20 μm film thickness; Chrompack) using a gas liquid chromatograph (3400; Varian) fitted with a flame-ionization detector. Helium (37 p.s.i.; 255 kPa) was used as the carrier gas. The injector temperature was held isothermally at 225 °C for 10 min and the detector temperature was 250 °C. The column oven was held at an initial temperature of 140 °C for 8 min and then programmed to increase at a rate of 8.5 °C min^{-1} to a final temperature of 200 °C, which was held for 41 min. Collected data were recorded and analysed on a Minichrom PC system (VG Data System) and expressed as percentages of total fatty acids isolated from bacterial cells.

Effect of Tween 80 supplementation on survival of *Lb. rhamnosus* GG and a mutant with reduced F_0F_1-ATPase activity and proton permeability. A parent strain of *Lb. rhamnosus* GG and a neomycin-resistant mutant strain of *Lb. rhamnosus* GG with reduced F_0F_1-ATPase activity (Corcoran *et al.*, 2005) were grown to the stationary phase in MRS medium with or without Tween 80. The cultures were then exposed to simulated gastric juice pH 2.5, as described above.

Proton permeability of *Lb. rhamnosus* GG was determined by a previously established protocol (Bender *et al.*, 1986; Ma & Marquis, 1997). Fifty millilitres of *Lb. rhamnosus* GG culture supplemented with or without Tween 80 (1 g l^{-1}) were grown overnight in MRS broth and were harvested by centrifugation for 10 min at 9000 ***g*** at 4 °C. The cells were then washed once with 50 ml 5 mM $MgCl_2$. Cells were resuspended in 20 mM potassium phosphate buffer (pH 7.2)/ 50 mM KCl/1 mM $MgCl_2$ to a cell concentration of 5 mg ml^{-1} and incubated anaerobically for 2 h at 37 °C. Cells were then harvested by centrifugation following this starvation period, resuspended to 20 mg ml^{-1} and assessed for proton permeability as described by Fozo & Quivey (2004a).

Statistical analysis. Data were analysed using Student's *t*-test (2-tail paired with significance measured at a probability level of $P \leqslant 0.05$) to determine the probability that the mean of the Tween 80-supplemented experimental replicates was significantly different from the mean of the control. Statistical software used was Graphpad Prism version 3.03.

RESULTS

Tween 80 supplementation in growth media protects *Lb. rhamnosus* GG in simulated gastric juice

Lb. rhamnosus GG was grown in GEM, an economic food-grade alternative to MRS medium (Saarela *et al.*, 2004), in the presence of Tween 80 (1 g l^{-1}) for 16 h and subsequently exposed to simulated gastric juice, pH 2.5, for 90 min and survival compared with the control culture, grown without Tween 80. The final pH in the media of *Lb. rhamnosus* GG grown with and without Tween 80 over the 16 h period was similar (pH 3.88, Fig. 1). The presence of Tween 80 in the growth media resulted in $\sim$1000-fold increased survival of *Lb. rhamnosus* GG, when subsequently exposed for 90 min to simulated gastric juice, pH 2.5, compared to controls (Fig. 2). In addition, the effects of Tween 20 supplementation in GEM on *Lb. rhamnosus* GG acid tolerance were determined. However, cultures only grew to 8.14 $\log_{10}$(c.f.u. ml^{-1}) ($\sim$10-fold lower than cultures grown with Tween 80 or cultures grown without Tween 80), and Tween 20 failed to protect the cultures upon exposure to simulated gastric juice, pH 2.5 (data not shown).

MRS medium is commonly used for growth of probiotic lactobacilli; therefore *Lb. rhamnosus* GG was also grown in this medium in the presence and absence of Tween 80, to assess the reproducibility of the protective effect in a different medium. As with growth in GEM, the growth rate and final pH were similar ($\mu = 0.24$ h^{-1} and pH 3.86, respectively) in media with and without Tween 80. When these cultures were exposed to gastric juice as above, Tween 80-supplemented cultures grown in MRS medium

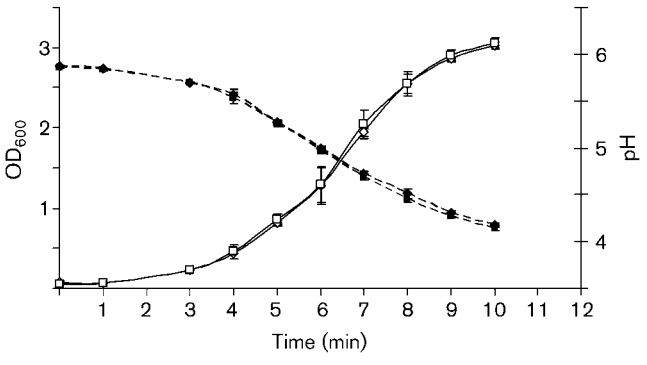

Fig. 1. Growth of *Lb. rhamnosus* GG in MRS in the presence (■, □) and absence (◆, ◇) of oleic acid. Black data points represent the decline in pH over time; white data points represent the optical density data.

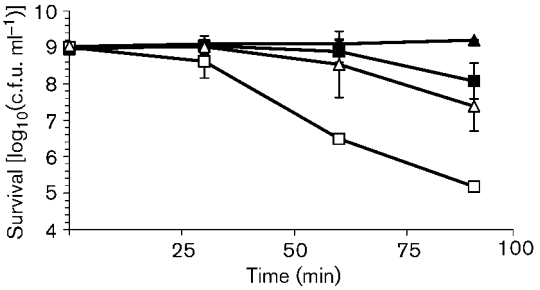

Fig. 2. Survival of *Lb. rhamnosus* GG grown in MRS (▲, △), or GEM (■, □) in simulated gastric juice pH 2.5. Black data points represent cultures supplemented with Tween 80 (1 g l^{-1}); white data points are cultures grown without Tween 80.

experienced ~100-fold enhanced survival (Fig. 2) compared to those grown without Tween 80.

Increased survival in the presence of increasing Tween 80 concentrations and comparative survival of different probiotic lactobacilli in Tween 80-supplemented media

Lb. rhamnosus GG was grown to ~10^9 c.f.u. ml^{-1} in different concentrations of Tween 80 (0–2 g l^{-1}) and analysed for survival in simulated gastric juice, pH 2.5, following 90 min exposure. The results showed that increasing concentrations of Tween 80 steadily increased acid tolerance up to 4000-fold (Fig. 3). The protective effect of Tween 80 was assessed in three probiotic lactobacilli (*Lb. paracasei* NFBC 338, *Lb. rhamnosus* E800 and *Lb. salivarius* UCC 500) and results were compared with *Lb. rhamnosus* GG. MRS was used in preference to GEM, as some strains did not grow to high numbers in this medium (data not shown). Inclusion of Tween 80 in the growth medium led to enhanced survival of all lactobacilli following 90 min, but at different pH values (Table 1). For example, the survival of *Lb. salivarius* UCC 500 grown in the presence of Tween 80

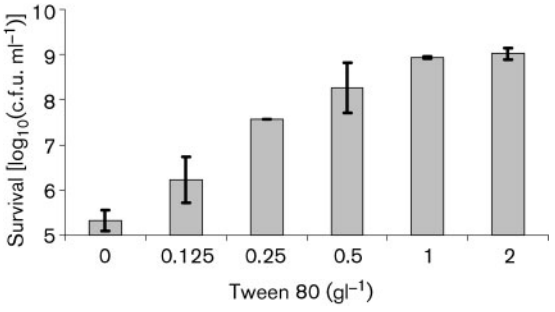

Fig. 3. Survival of *Lb. rhamnosus* GG in simulated gastric juice, pH 2.5, grown in MRS in the presence of different concentrations of Tween 80. Results are the mean of duplicate experiments and error bars represent standard deviation.

Table 1. Survival of LAB cultures [log_{10}(c.f.u. ml^{-1})] grown in MRS with or without Tween 80 and exposed to simulated gastric juice, pH 2.0–3.0, with or without Tween 80 supplementation

The results are the mean of triplicate experiments. Figures in parentheses represent the standard error.

Strain	pH	Tween 80	0 min	90 min
Lb. rhamnosus GG	2.5	Yes	8.94 (0.14)	8.65 (0.09)
Lb. rhamnosus GG	2.5	No	8.96 (0.19)	6.22 (0.15)
Lb. rhamnosus E800	2.5	Yes	8.94 (0.12)	7.96 (0.83)
Lb. rhamnosus E800	2.5	No	9.09 (0.18)	5.85 (0.46)
Lb. paracasei NFBC 338	2.75	Yes	8.92 (0.12)	8.58 (0.44)
Lb. paracasei NFBC 338	2.75	No	8.97 (0.25)	6.88 (0.08)
Lb. salivarius UCC 500	2.0	Yes	9.60 (0.01)	6.97 (0.15)
Lb. salivarius UCC 500	2.0	No	9.50 (0.04)	5.14 (0.01)
Lc. lactis IL1403	3.0	Yes	9.62 (0.07)	2.34 (0.37)
Lc. lactis IL1403	3.0	No	9.46 (0.19)	4.70 (0.04)
B. animalis subsp. *lactis* Bb12	2.5	Yes	8.99 (0.16)	8.77 (0.25)
B. animalis subsp. *lactis* Bb12	2.5	No	8.90 (0.12)	8.62 (0.32)

was enhanced 70-fold at pH 2.0. In addition, the survival of *Lb. paracasei* NFBC 338 was enhanced 50-fold at pH 2.75 and *Lb. rhamnosus* E800 exhibited ~300-fold enhanced survival at pH 2.5 following Tween 80 supplementation. In contrast, the viability of *Lc. lactis* IL1403 cultures decreased by ~200-fold following exposure to simulated gastric juice, pH 3.0, in the presence of Tween 80 supplementation compared to cultures grown without Tween 80. Furthermore, the absence of Tween 80 did not affect the acid tolerance of Bb12 (Table 1).

Fatty acid supplementation in growth media protects *Lb. rhamnosus* GG in simulated gastric juice

The effect of inclusion of a number of different C18 fatty acids with 0–2 double bonds of varying *cis* and *trans* configuration in the cultivation media on the survival of *Lb. rhamnosus* GG upon exposure to simulated gastric juice, pH 2.5, was investigated. Among oleic, vaccenic, elaidic, linoleic, stearic acid and two CLA isomers, oleic acid led to 1500-fold enhanced survival after 90 min exposure compared to the control, while vaccenic acid enhanced survival 50-fold (Table 2). Some linoleic acid was incorporated into the cell following supplementation with this fatty acid, although it did not enhance survival. In addition, stearic acid, elaidic acid and CLA and C18 : 2 (10,12tc)] did not enhance survival of *Lb. rhamnosus* GG following 90 min exposure to simulated gastric juice when present in the growth media, with viability reductions similar to the control (~5000-fold reduced survival after 90 min).

Table 2. Survival of cultures [$\log_{10}$(c.f.u. ml^{-1})] of *Lb. rhamnosus* GG grown in GEM in the presence of different fatty acid substrates and exposed to simulated gastric juice, pH 2.5

The results are the mean of triplicate experiments. Figures in parentheses represent the standard error. ND, Not detected.

Fatty acid	0 min	90 min	Percentage fatty acid*
Stearic acid	8.92 (0.10)†	4.75 (1.37)	10.43 (6.12)
Oleic acid	8.77 (0.03)	8.67 (0.26)	32.59 (1.97)†
Elaidic acid	8.87 (0.12)	4.75 (0.79)	ND
Linoleic acid	8.72 (0.04)	4.09 (0.76)	6.06 (3.88)‡
Vaccenic acid	8.81 (0.06)	6.08 (0.31)	1.25 (0.07)‡
CLA (9,11ct)	8.82 (0.13)	4.44 (1.58)	1.04 (0.92)
CLA (10,12tc)	8.87 (0.15)	3.89 (1.48)	ND
Control	8.86 (0.05)	5.00 (0.17)	–

*Concentration of particular fatty acid as a percentage of total fatty acid study as analysed via GC analysis. Culture pellets had a total fatty acid content of approximately 0.011 mg ml^{-1}.
†Significant increase in fatty acid ($P<0.01$).
‡Significant increase in fatty acid ($P<0.05$).

Bacterial fatty acid composition changes as a result of fatty acid supplementation

The effects of Tween 80 with and without fatty acid supplementation on cell fatty acid composition of *Lb. rhamnosus* GG were subsequently assessed. The membranes of cells grown in MRS medium containing Tween 80 contained 55-fold higher oleic acid content compared to controls. In addition, CFA concentrations were fivefold higher following Tween 80 supplementation (Fig. 4a). The overall unsaturated/saturated fatty acid ratio was significantly higher ($P<0.05$) compared to cultures grown in the absence of Tween 80. Cultures grown in the presence of Tween 80 also had 3.5-fold lower palmitic acid ($P<0.05$, Fig. 4a).

The fatty acid composition of the bacterial membrane of *Lb. rhamnosus* GG was also analysed following growth in GEM and results followed a similar trend with shifts towards unsaturated fatty acids when Tween 80 was present (Fig. 4b). GEM-grown cells had oleic acid present, which was likely to be due to endogenous levels present in the medium. Oleic acid concentrations were twofold higher ($P<0.05$) and palmitic acid 1.6-fold lower ($P<0.05$) when cultures were grown in the presence of Tween 80. However, approximately 10-fold lower concentrations of CFA (1.7 %) were identified compared to cultures grown in MRS containing Tween 80 (17.5 % of total fatty acids identified, Fig. 4a).

Concentrations of oleic, vaccenic and linoleic acid increased significantly ($P<0.05$) when added as supplements compared to the control culture grown without the addition of fatty acids (Table 2). The content of CLA (9,11ct) and stearic acid were not significantly different, while elaidic acid and CLA (10,12tc) could not be detected in membranes when cultures were grown in the presence of these fatty acids.

Lb. rhamnosus GG was grown in MRS medium in the presence and absence of Tween 80 and subjected to simulated gastric juice, pH 2.5, for 90 min and the fatty acid profile was assessed (Fig. 4c). There was 1.5-fold lower oleic acid in cultures grown in the presence of Tween 80 following acid treatment, which was a significantly lower ($P<0.05$) proportion of the total fatty acids compared to cultures before treatment (compare with Fig. 3). In addition, there was 1.6-fold higher stearic acid content in acid-treated cells ($P<0.05$). In contrast, no significant changes ($P>0.05$) occurred in cultures grown in the absence of Tween 80. The data suggest that the enhanced survival observed following Tween 80 supplementation (see Fig. 2) was associated with increased membrane saturated fatty acids following acid exposure.

Tween 80 protects cultures with reduced F_0F_1-ATPase activity

Acid tolerance is mediated via a number of mechanisms, in particular F_0F_1-ATPase. Previously, we isolated a number of spontaneous neomycin-resistant mutants of *Lb. rhamnosus* GG with reduced survival in simulated gastric juice, even in the presence of glucose (Corcoran *et al.*, 2005). In order to determine whether the F_0F_1-ATPase had any involvement in the Tween 80 effect, the wild-type and a mutant of *Lb. rhamnosus* GG were grown in MRS medium with and without Tween 80 and survival was analysed in simulated gastric juice, pH 2.5. Both cultures survived acid exposure well after growth in Tween 80 and survival of the mutant was approximately twofold lower than the wild-type after 90 min (results not shown). Remarkably, the mutant strain had over 1800-fold lower survival in the absence of Tween 80 compared to the mutant grown with Tween 80. In contrast, the wild-type strain declined in viability by less than 100-fold under the same conditions.

Cultures grown in the presence of Tween 80 have higher extracellular pH in simulated gastric juice

The pH of simulated gastric juice containing *Lb. rhamnosus* GG was assessed and the results showed that the Tween 80-supplemented culture was approximately 0.1 pH units higher over 90 min compared with the control (2.90 for Tween 80-supplemented cultures compared to 2.81 for cultures without Tween 80, Fig. 5a). Assessment of proton permeability showed that the cultures grown in the presence of Tween 80 had a higher proton permeability than the non-supplemented strain (Fig. 5b). After 50 min, the pH had increased by 0.70 for Tween 80-supplemented cultures and 0.65 for controls. Following the addition of butanol, the final pH of both cultures was similar.

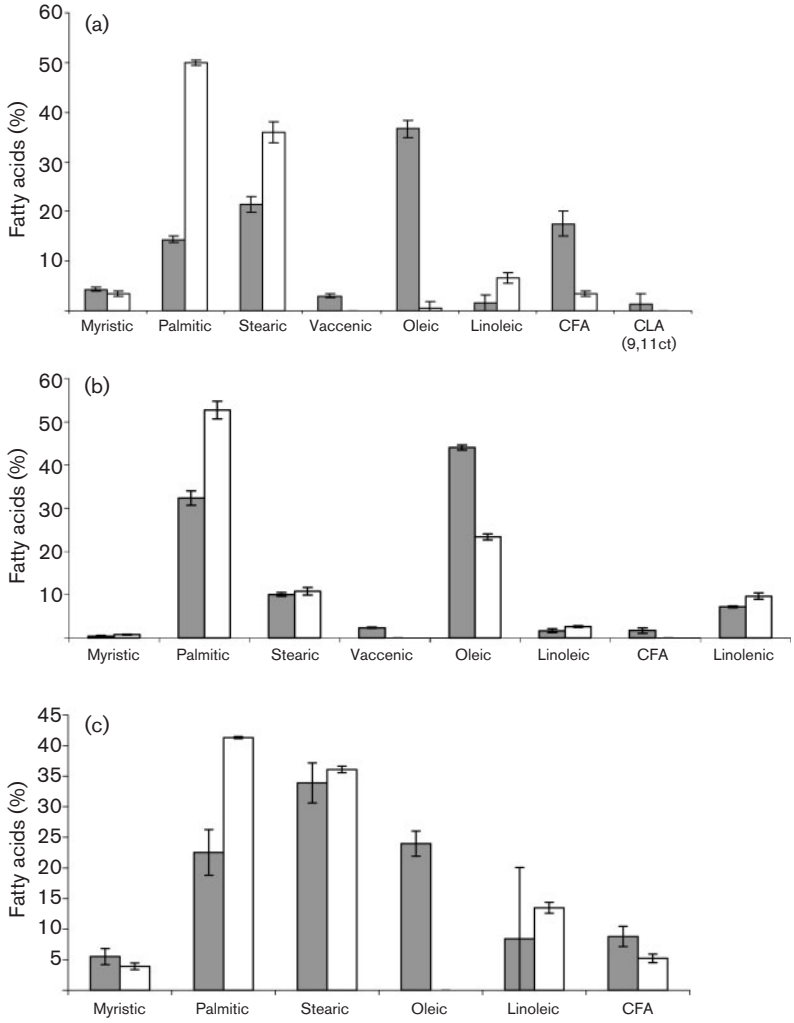

Fig. 4. Fatty acid profiles of *Lb. rhamnosus* GG in media with (grey bars) and without (white bars) Tween 80. (a) Cultures grown in MRS; (b) cultures grown in GEM; (c) cultures grown in MRS and then subjected to simulated gastric juice, pH 2.5, for 90 min. Culture pellets (OD_{600} 3.00) had total fatty acid content of approximately 0.011 mg ml^{-1}. Results are the mean of triplicate experiments and error bars represent standard deviation.

DISCUSSION

This study highlights the importance of the growth environment and the fundamental importance of addition of an exogenous source of fatty acid in the growth medium of probiotic lactobacilli and the subsequent implications for enhanced acid tolerance. Acid tolerance is one of the main desirable characteristics on which probiotics have been selected (Holzapfel & Schillinger, 2002); therefore, insights into the nature of acid resistance increase our ability to adapt them for technological applications. Initially, we studied the effect of Tween 80 supplementation on *Lb. rhamnosus* GG in both GEM and MRS media. It was observed that survival in simulated gastric juice was enhanced following Tween 80 supplementation. It has been reported that Tween 80 is important for alleviating the effects of stressful environments of lactobacilli, resulting in higher survival during storage in freeze-dried powders (Ananta *et al.*, 2004) and of *Oenococcus oeni* in wine (Guerrini *et al.*, 2002).

There were significantly higher concentrations of oleic acid in *Lb. rhamnosus* GG membranes grown in both GEM and MRS media containing Tween 80 in contrast to cultures grown without Tween 80. Low concentrations of oleic acid were present in each pellet grown without Tween 80 and analysis of GEM medium revealed the presence of up to 15 μg oleic acid ml^{-1}, while approximately 1 μg oleic acid ml^{-1} was present in MRS prepared without Tween 80 (Guerrini *et al.*, 2002). The high concentration of oleic acid in the GEM medium is probably associated with the soy peptone fraction. Interestingly, *Streptococcus mutans* showed increased monounsaturated fatty acid concentrations (C18 : 1) in response to growth at lower pH (Fozo & Quivey, 2004b), which was vital for optimum acid tolerance (Fozo & Quivey, 2004a). We also found significantly higher CFA levels in Tween 80-supplemented cultures, which are important for bacterial acid tolerance (Budin-Verneuil *et al.*, 2005; Chang & Cronan, 1999; Klaenhammer *et al.*, 2005). Furthermore, CFA increased in *Lactobacillus fermentum* cells grown below 20 °C or above 35 °C and in *Lactobacillus casei* cells cultivated in media containing 1 M NaCl, suggesting that these fatty acids aid resistance to other stressful conditions (Machado *et al.*, 2004; Suutari & Laakso, 1992). The higher levels of CFA in cultures grown in MRS and Tween 80 may explain the greater acid resistance

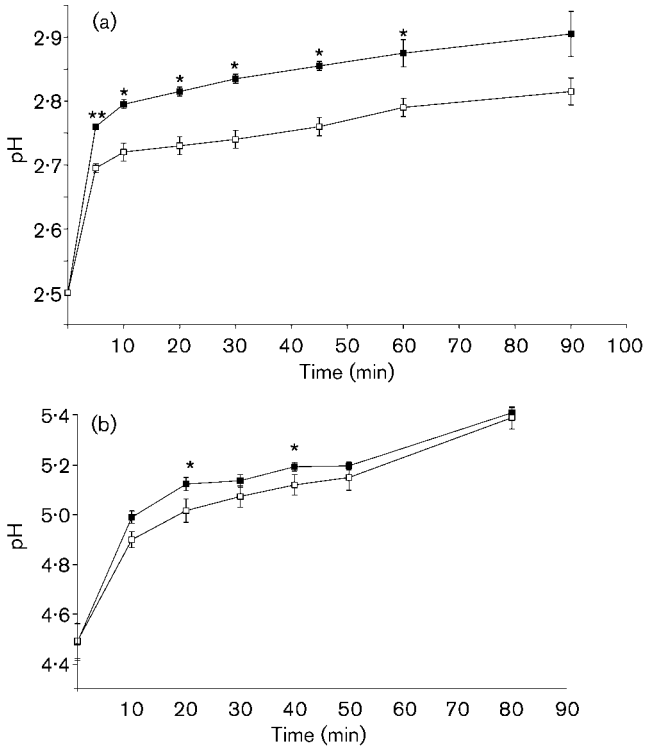

Fig. 5. (a) Change in pH of cultures of *Lb. rhamnosus* GG in simulated gastric juice, pH 2.5, following growth in MRS in the presence (■) and absence (□) of Tween 80; (b) proton permeability assay of *Lb. rhamnosus* GG grown in MRS in the presence (■) and absence (□) of Tween 80. At 50 min, butanol was added to a final concentration of 10 % in order to permeablize the cells and allow for pH to reach equilibrium. Results are the mean of triplicate experiments and error bars represent standard deviation. Asterisks represent significant differences (*$P<0.05$, **$P<0.01$).

compared to those grown in GEM supplemented with Tween 80 (see Fig. 2). The palmitic acid content in the cultures grown without Tween 80 was higher than in the cultures supplemented with Tween 80, which agrees with a previous study (Johnsson *et al.*, 1995). Lower concentrations of palmitic acid in Tween 80-supplemented cultures may suggest an impact upon *de novo* fatty acid biosynthesis. Furthermore, no vaccenic acid was present in place of the oleic acid fraction during growth of *Lb. rhamnosus* GG without Tween 80, suggesting that the strain is unable to synthesize this fatty acid, which is produced by other lactobacilli in the absence of Tween 80 (Johnsson *et al.*, 1995).

We showed that Tween 80 addition to the growth media enhanced the acid tolerance of *Lb. rhamnosus* E800, *Lb. salivarius* UCC 500 and *Lb. paracasei* NFBC 338, but at different pH values. We also found this to be evident in the enhanced acid tolerance of probiotic lactobacilli following glucose addition to simulated gastric juice (Corcoran *et al.*, 2005). In addition, we did not observe any benefit from Tween 80 supplementation of Bb12 and a negative effect was observed for *Lc. lactis* IL1403. Furthermore, we showed a positive correlation between the concentration of Tween 80 in the growth medium and subsequent acid tolerance of *Lb. rhamnosus* GG.

Subsequently, we analysed the acid tolerance of *Lb. rhamnosus* GG following growth in GEM supplemented with different C18 fatty acids. The results demonstrated that cultures grown in oleic acid had comparable acid tolerance to cultures supplemented with Tween 80. Vaccenic acid (*trans* isomer) also enhanced survival of *Lb. rhamnosus* GG, which has been observed for the *cis* isomer (10 μg ml^{-1}) in *Streptococcus mutans* (Fozo & Quivey, 2004a). No protective effect was observed following the addition of elaidic acid and this fatty acid was not incorporated in the cells. Linoleic acid or CLA (9,11ct) failed to protect *Lb. rhamnosus* GG during acid exposure, even though they have a double bond at position 9 in the *cis* configuration similar to oleic acid and were present in the membrane after growth. Interestingly, CLA (10,12tc) was not present in the cell membrane following supplementation. Different types and concentrations of fatty acids can affect the proton permeability following supplementation into growth media (Ma & Marquis, 1997), which may have affected the acid tolerance of cultures analysed in our study. Furthermore, Fozo & Quivey (2004b) reported that fatty acids with a double bond at position 11 in the *cis* configuration had different effects on the acid tolerance of *S. mutans*. Linoleic acid (10 μg ml^{-1}) has been reported to increase membrane leakage of *Staphylococcus aureus* (Raychowdhury *et al.*, 1985), which may explain the poor survival of *Lb. rhamnosus* GG cultures grown with this fatty acid. From a functional perspective, the incorporation of polyunsaturated acids in the growth media can be disadvantageous, as polyunsaturated acids such as linoleic acid inhibit growth at concentrations of 5 μg ml^{-1} or above as well reducing the adhesion of *Lb. rhamnosus* GG to mucus (Kankaanpää *et al.*, 2001).

The F_0F_1-ATPase generates a proton-motive force, via proton extrusion, and is therefore involved in maintaining intracellular pH (Kullen & Klaenhammer, 1999). In this study, we observed that growth with Tween 80 alleviated the acid sensitivity associated with a mutant with reduced F_0F_1-ATPase activity, as survival of the neomycin-resistant mutant was similar in the presence of Tween 80 to that of the wild-type strain. The F_0F_1-ATPase activity of both parent and mutant were determined in a previous study (Corcoran *et al.*, 2005). Remarkably, the mutant was more acid sensitive than the parent strain following growth without Tween 80, suggesting that Tween 80 can override the acid-sensitive phenotype.

We analysed the proton permeability of *Lb. rhamnosus* GG in the presence and absence of Tween 80 supplementation. Surprisingly, cultures grown in the presence of Tween 80 had higher extracellular pH values during analysis. Interestingly, Fozo & Quivey (2004a) also observed that *Strep. mutans*,

which has an intact vaccenic acid synthesis pathway, was more permeable to protons, while disruption of the pathway reduced permeability. These mutants had approximately twofold higher F_0F_1-ATPase activity compared to cultures synthesizing vaccenic acid (Fozo & Quivey, 2004a). We then assessed the pH change of the simulated gastric juice, pH 2.5, during 90 min, observing that the culture grown in Tween 80 had higher pH by approximately 0.1 during the analysis. Such subtle differences between mutants with different acid-tolerance phenotypes have been previously reported by Fozo & Quivey (2004a). Although the differences are indeed small, they may provide some explanation to the increased survival of cultures observed, i.e. that such a small change in pH may be sufficient to protect cultures. There were significant reductions in the concentration of oleic acid in the membrane compared to cultures analysed before acid treatment. The lower proton permeability of cultures grown without Tween 80 may be a consequence of higher F_0F_1-ATPase activity, which may explain the lower survival of cultures with lower F_0F_1-ATPase activity grown without Tween 80. Furthermore, Ma & Marquis (1997) reported that supplementation of LAB with palmitic acid reduced proton permeability, while oleic acid-supplemented cultures had higher proton permeability. The increased permeability of Tween 80-supplemented cultures to protons may be a consequence of the oleic acid fraction providing a proton sink as suggested by Fozo & Quivey (2004a) and data generated in our study appear to support this proposal. However, an altered physiological cell state, leading to acid sensitivity, cannot be ruled out, as Fozo *et al.* (2004a) reported a change in the rate of glycolysis and phosphotransferase activity in an acid-sensitive mutant unable to synthesize vaccenic acid. A complete genome sequence of *Lb. rhamnosus* GG would furnish greater information on the phenomenon reported here.

CONCLUSIONS

A source of oleic acid such as Tween 80 enhanced subsequent acid survival of probiotic lactobacilli when added to the growth medium, while other C18 fatty acids did not. The addition of Tween 80 was associated with a change in the composition of the bacterial membrane fatty acids, increasing oleic acid and CFA, while reducing palmitic acid concentrations. The incorporation of oleic acid and CFA into the bacterial cell membrane appeared to increase the acid tolerance of neomycin-resistant mutants, thus alleviating the acid-sensitive phenotype observed in this mutant. Tween 80-supplemented cultures had higher proton permeability and increased the extracellular pH relative to controls. Overall, the data suggest that probiotic lactobacilli can use an exogenous oleic acid source to increase their acid survival, while the mechanism most likely involves the ability of increased membrane oleic acid to be reduced by H^+ to stearic acid. The resulting membrane would also be expected to have a more rigid structure, given its increased fatty acid saturation level. This may have further implications on properties such as bile tolerance and adhesion to intestinal epithelia.

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