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Commercial lactic acid bacteria and probiotic strains- tolerance to bile, pepsin and antibiotics

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Abstract

Screening and characterization of probiotic strains is crucial for achieving expected health benefits. In the current study, seventeen lactic acid bacteria (LAB) and probiotic strains were screened for survival in simulated gastric juice (pH 3 and 2) and bile (0.5% or 2.0%) for 3 and 12h, and antibiotic tolerance pattern using Etest® and Kirby Bauer Disc diffusion method. All tested strains exhibited survival during simulated gastric transit at pH 3 for 3 h. *Lactobacillus reuteri*, *L. rhamnosus* G5435, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *Streptococcus thermophilus* 1342, *Bifidobacterium lactis* BB12 and *S. thermophilus* M5 were found intrinsically tolerant to gastric and small intestinal transit and most tolerant strains among tested LAB (% survival ≥ 55). All strains were susceptible to ampicillin and erythromycin. Vancomycin and streptomycin tolerances were most common among species whereas tolerances for gentamicin, clindamycin and tetracycline were rare. The tolerances could provide additional benefit to strains in colonizing and replenishing gut microbiota after antibiotic therapy. The results obtained in the study confirm that strain viability in gastric and bile solution and antibiotic susceptibility are important attributes in the selection of potentially probiotic bacteria.

Introduction

Over the past decade, probiotics have received overwhelming attention in promoting better health and well-being. In this regard, lactic acid bacteria (LAB) including *Lactobacillus* and *Bifidobacterium* species are predominantly used (Holzapfel and Schillinger, 2002). The currently accepted definition of probiotics is 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al*., 2014). Probiotic bacteria are commonly incorporated in dairy products such as yoghurt, fermented milk drinks, cheeses, health supplements and other functional foods. It is generally recommended for a given strain to satisfy a number of requirements in order to achieve 'probiotic' status. Consecutively, the probiotic products must fulfil the legislative requirements with respect to labelling, safety and strain integrity of probiotic bacteria (Charteris *et al.*, 1997; Holzapfel and Schillinger, 2002; Balamurugan *et al*., 2014). It is important that the ingested strains survive through gastric transit and reach the colon in quantities large enough to facilitate colonization and confer beneficial effects on host (Weber and Polanco, 2012). Generally accepted minimum number of each viable probiotic strain is $\geq 10^6$ viable cells/g of product at the end of shelf life (Ashraf and Shah, 2011; Champagne *et al*., 2011). Even if bacterial numbers are sustained during shelf life, viability may be compromised after consumption challenged by unfavourable physiological conditions of the gastro-intestinal tract (GIT) including gastric acid present in the stomach and bile in the duodenum (Salminen *et al*., 1998). Survivability of bacteria is also challenged by possible presence of antibiotics after antibiotic therapy. Sensitivity and tolerance of probiotics to these challenges presents a key parameter for their application in different foods (Vasiljevic and Shah, 2008; Ruiz *et al.*, 2011). Thus screening and selection of an appropriate probiotic strain is crucial for achieving expected health benefits and necessitates scrupulous investigation of strain differences (Dunne *et al*., 2001; FAO/WHO, 2002). Therefore, the present study was focused on assessing 'strain variability' of seventeen LAB including commercial probiotic strains in terms of survival to simulated gastric juice, bile solution and antibiotics; under conditions that may mimic human GI environment.

Like other stresses, bacterial strains have evolved

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approaches to overcome antibiotic stress, which had dramatically affected the marked therapeutic successes of antibiotics. These strategies fall into two categories i.e. 'resistance' and 'tolerance'. 'Resistance' allows a microorganism to grow in the constant presence of the antibiotic, given that the concentration of the antibiotic is not too high and 'tolerance' enables a microorganism to survive antibiotic treatment, even at high antibiotic concentrations, provided the duration of the treatment is limited (Fridman *et al*., 2014). Phenotypic tolerance can be elicited by environmental factors (such as nutrient deprivation and pH changes) that result into antibiotic-induced killing, whereas genotypic tolerance can arise from specific genetic changes within the tolerant bacteria (Bayles, 2007). Unfortunately, little is known about the molecular mechanisms for antibiotic tolerance in bacteria and the evolution of 'tolerance' is much neglected than its counterpart. Also, the set back of antimicrobial treatment is greatly blamed for 'resistance' (Fridman *et al*., 2014), so here we followed the term 'tolerance' to characterise the survival of LAB in the presence of antibiotics.

The knowledge of antibiotic tolerance could be deciphered into controlling or treating cases of antibiotic-associated diarrhoea or cases of gastrointestinal disorders through concomitant antibiotic therapy (Salminen *et al.*, 1998; Mackay et al., 1999; Salvana and Frank, 2006; Tommasi *et al*., 2008). In our previous studies (Ashraf *et al*., 2014ab), commercial probiotic and LAB were investigated for their immuno-modulatory responses, in particular to their *in-vitro* cytokine production and induction of regulatory T cell responses. The present study involved the screening these probiotic and LAB for metabolic attributes including assessment of survival in simulated gastric conditions, and antibiotic susceptibilities in order to bring about a rational selection of strains for specific uses and assessing their stability for future immunological studies.

Materials and Methods

Bacterial strains and culture conditions

Seventeen LAB and probiotic strains previously described (Ashraf *et al.*, 2014a) including *Lactobacillus paracasei* 292, *L. salivarius* 5248, *L. reuteri, Lactococcus lactis, L. rhamnosus* G5435, *L. acidophilus* 2401, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *Streptococcus thermophilus* 1342, *L. casei* 290, *Bifidobacterium breve* BB99, *B. animalis* subsp. *lactis* BB12, *B. longum* 1941, *Lc. lactis* R704, *L. plantarum* 276, *L. rhamnosus* 5434 and *S. thermophilus* M5 were used in the current study. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were obtained from Deakin University Culture Collection (Burwood, Victoria, Australia) and used as quality control strains and maintained in prepared sheep blood agar (Microbiology Media Preparation Unit, The University of Melbourne, Parkville, Victoria, Australia) and tripticase soy agar (Sigma-Aldrich Pty Ltd. NSW Australia). The parent stock cultures were kept at -80°C in phosphate buffered saline (PBS; Oxoid, Melbourne Australia) containing 40% glycerol whereas lyophilized cultures were stored at -20°C freezer. Propagation of cultures was carried out twice successively in de Man Rogosa and Sharpe (MRS) broth (Oxoid) at 37°C for 18 h and samples were removed for gram stain to check for purity and bacterial morphology. Bacteria were further grown on MRS agar (1.5% w/v agar) as reference medium to observe the colonial characteristics. For the propagation of bifidobacteria, filter-sterilized L-cysteine-HCl (0.05% w/v) was also added to the medium.

Media preparation

Rehydrated MRS broth was prepared according to the manufacturer instructions. The pH-modified MRS agar was obtained by adjusting the pH of the broth to 7.0 using 1.0 M HCl. In order to facilitate the growth of anaerobic bifidobacteria, filter-sterilized L-cysteine-HCl (0.05% final concentration) was added to the medium. Bacteriological peptone solution was used as diluent and it was prepared by dissolving 0.15% (w/v) bacteriological peptone (Oxoid, West Heidelberg, Australia) in mili-Q water. The pH of diluents was adjusted to 7.0 ± 0.2, and it was dispensed in McCartney bottles in 9ml aliquots. All media were sterilized by autoclaving at 121°C for 15 min.

Viability of probiotic and LAB strains in simulated gastric juice and bile solutions

Preparation of washed cell suspension

The propagated cultures were subjected to low speed centrifugation (Beckman J2/HS centrifuge, JA-14 rotor, Palo Alto, CA, USA) at 4000 g for 10 min at 4ºC to concentrate cells. Cells were harvested and washed three times in phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4) and finally resuspended in PBS. Prior to assay of bacterial tolerance to simulated gastric juice and bile solutions, the total viable count of the washed cell suspension was determined using a pour plate technique as previously described (Ashraf

Table 1: Antibiotic susceptibility profile of LAB and probiotic strains tested by disc diffusion method[a]

culture	TE30	AMP10	CN2	S10	VA30	E15	P5	IPM10	AMC30	DA2
E. coli 25922	60.0±0.0	68.0±2.0	22.0±2.0	18.3±1.0	30.0±0.0	34.0±0.0	68.0±0.0	54.0±1.0	60.0±0.0	21.3±0.6
S. aureus 25923	48.0±1.0	50.7±0.6	23.5±0.6	21.0±3.3	26.5±0.6	27.5±0.6	55.5±0.6	52.0±0.0	52.5±0.6	56.0±0.0
LP292	39.7±3.2	34.4±2.5	14.0±0.0	**0.0±0.0***	**0.0±0.0***	33.3±1.0	32.0±1.8	24.0±1.0	35.8±1.3	**14.0±0.6***
LS5248	37.0±2.0	35.5±2.4	17.5±0.6	14.0±4.4	**0.0±0.0***	30.0±1.0	38.0±0.0	23.5±0.6	36.5±0.6	26.7±1.5
L. reuteri	34.7±7.6	34.5±1.9	13.5±0.6	**11.0±1.0***	**0.0±0.0***	26.5±0.6	32.0±0.0	23.0±0.0	28.3±6.5	32.3±1.5
Lc. lactis	35.5±0.6	33.7±5.5	17.0±1.2	**10.8±1.0***	**0.0±0.0***	32.0±5.4	41.0±0.0	22.5±0.6	41.0±3.1	**13.7±0.6***
LG5434	25.5±1.5	48.0±2.8	**12.0±0.0***	**0.0±0.0***	**0.0±0.0***	29.7±0.6	29.5±0.6	44.0±4.0	52.0±0.0	26.0±1.0
LA2401	37.7±2.1	36.3±4.3	16.5±0.6	13.7±2.1	**0.0±0.0***	33.0±1.7	35.0±0.0	22.5±0.6	35.0±0.6	32.7±1.5
LA388	26.7±3.8	36.3±0.5	14.5±0.6	**10.0±1.0***	**0.0±0.0***	31.3±1.2	35.0±0.6	24.5±0.6	40.0±0.6	35.0±1.0
LB11842	22.5±0.6	37.3±2.3	**10.3±2.1***	**0.0±0.0***	**0.0±0.0***	27.3±3.2	28.0±0.0	25.0±0.0	42.0±0.0	**12.0±1.5***
ST1342	25.5±0.6	36.0±6.3	13.7±2.9	**0.0±0.0***	**0.0±0.0***	31.0±8.7	42.0±0.6	23.5±0.6	45.0±0.6	37.0±1.0
LC290	40.7±8.1	37.5±4.1	17.5±0.6	**10.3±0.6***	**0.0±0.0***	29.7±0.6	41.0±0.6	24.0±1.5	44.0±0.6	29.0±1.0
BB99	31.0±1.0	33.7±6.4	16.7±1.5	11.3±1.2	**0.0±0.0***	32.3±7.6	43.0±0.6	23.5±1.2	49.0±0.0	30.0±0.0
BB12	34.5±1.5	33.0±2.6	16.0±1.7	11.7±1.5	**0.0±0.0***	31.7±2.9	34.0±0.0	24.0±1.7	37.0±0.6	**13.0±0.0***
BL1941	33.5±1.5	32.0±6.1	15.3±1.2	12.0±1.0	**0.0±0.0***	33.0±5.2	40.0±0.0	22.7±1.2	45.0±0.6	**13.3±0.6***
LCR704	37.3±1.5	34.0±3.4	16.6±1.9	13.4±3.0	**0.0±0.0***	32.0±4.8	38.0±0.6	24.3±1.0	42.0±0.6	32.0±1.0
LP276	34.5±1.5	32.3±2.3	14.7±2.3	11.3±1.2	**0.0±0.0***	29.2±2.8	33.0±0.6	24.3±1.2	35.0±0.6	30.0±1.0
LR5434	39.0±1.0	38.0±0.0	16.5±0.6	**9.0±1.0***	**0.0±0.0***	32.3±0.6	31.0±0.0	24.0±1.0	40.0±0.0	28.3±0.6
STM5	24.5±0.5	36.3±0.5	**11.3±0.5***	**0.0±0.0***	**0.0±0.0***	27.3±0.9	34.0±0.0	24.3±0.9	42.0±0.0	32.0±0.6

[a] Data expressed as mean inhibition zone diameter (mm) ± SE

* Tolerant

et al., 2014a).

Preparation of simulated gastric juice and bile solutions

The simulated gastric juice was prepared by suspending 0.3% (w/v) pepsin from porcine gastric mucosa (Sigma) in saline (0.5%, v/v) solution. The pH of gastric suspension was adjusted to 2.0 and 3.0 with 1M HCl and filter-sterilizing using 0.45-µm pore size filter (Merck Millipore, Bayswater Vic, Australia). The bile solutions were prepared by suspending bovine oxgall powder (Sigma) in distilled water to obtain 0.5% (w/v) and 2.0% (w/v) final concentrations respectively, followed by autoclaving at 121ºC for 15 min.

Survival of bacteria in simulated gastric juice and bile solutions

The gastric and acidic tolerance of seventeen probiotic and LAB strains was determined using the method described by Charteris *et al.* (1998a). Briefly, the washed cell suspension (1.0 ml) was added to 9.0 ml simulated gastric juice (pH 3.0 and 2.0) or bile solution (0.5% or 2.0%) and was vortexed for 15 s for complete dispersion of cells. Samples (0 h) were taken immediately after mixing of suspensions and viable counts were determined. The mixtures were then incubated at 37ºC in shaking incubator at 150 rpm (InnovaTM 4230, New Brunswick Scientific, USA) and samples were removed periodically after 3 or 12 h to determine the viable counts of probiotic and LAB strains (Fig. 1). Enumeration of bacterial cell number was carried out as previously described (Ashraf *et al*., 2014a). The survival percent was calculated by dividing the final viable population (cfu/ml) of the test organism inoculated to simulated gastric juice and bile solutions with their initial viable count (cfu/ml) before treatment.

Antibiotic susceptibility pattern of probiotic and LAB strains

Inoculum preparation

The inoculum was prepared by making a direct saline suspension of isolated colonies selected from cultures grown on MRS agar plate for 48 h. The bacterial cell density of suspensions was adjusted to match McFarland turbidity standard 0.5 (≈ 1.5 × 108) using saline, a vortex mixer and spectrophotometer.

Kirby-Bauer disc diffusion test

The bacterial suspensions were swabbed evenly onto MRS agar plates with a sterile cotton swab. The plates were left ajar in laminar flow for 10-15 min to dry and to allow the absorption of excess moisture. Antibiotic discs (Oxoid, Australia) of penicillin G (P5U), imipenem (IPM 10µg), vancomycin (VA 30µg), amoxycillin/ clavulanic acid (AMC 30µg), ampicillin (AMP 10µg), gentamicin (CN 10µg), tetracycline (TE 30µg), streptomycin (S 10µg), erythromycin (E 10µg), clindamycin (DA 10µg) were applied onto the surfaces of inoculated agar using disc dispenser (Oxoid, Australia). The plates were incubated under anaerobic conditions for 48 h at 37°C with the exception of plates for *Lactococcus* strains, *Streptococcus* strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, which were incubated aerobically at 37°C for 24 h. Inhibition zone diameters were measured including the diameter of the discs (in mm) and results were interpreted according to the cut-off levels (Charteris *et al*., 1998a; Tang *et al.*, 2007) and are presented in Table 1. The assays were repeated on three independent occasions

Table 2: Multiple tolerances phenotype and MIC values for LAB determined by Etest® method

Culture	MIC values (µg/mL)							Multiple tolerances
	AM	SM	TC	CM	VA	GM	EM	
LP292	0.064	48	0.19	4	>256	4	0.25	SM^{adeg}, CM^{bei}, VA^{adeg}, EM^{i}
LS5248	0.25	12	0.25	0.5	>256	3	0.25	VA^{aeg}
L. reuteri	0.19	128	3	1	>256	24	0.38	$SM^{abcdegi}$, CM^{b}, VA^{adeg}, GM^{abceg}, EM^{i}
Lc. lactis	0.125	128	0.38	8	>256	32	0.25	SM^{abcefg}, CM^{beg}, VA^{abefg},GM^{abceg},
LG5434	0.023	128	12	0.25	>256	8	0.38	SM^{abcegi}, $TC^{abceghi}$, VA^{aeg}, GM^{aegi}, EM^{i}
LA2401	0.19	96	1	0.75	>256	6	0.19	SM^{abcegi}, CM^{i}, VA^{abeghi}, GM^{i}, EM^{i}
LA388	0.064	192	3	0.094	>256	6	0.125	SM^{abcegi}, TC^{i}, VA^{abeghi}, GM^{i}
LB11842	0.094	192	3	12	>256	8	0.38	SM^{abcegi}, TC^{i}, CM^{abg}, VA^{abgi}, GM^{aegi}, EM^{i}
ST1342	0.064	128	2	0.75	>256	6	0.38	SM^{abceg}, VA^{abeg}
LC290	0.19	32	0.125	0.38	>256	8	0.38	SM^{aeg}, VA^{abe}, GM^{aeg}
BB99	0.19	8	0.38	0.5	>256	2	0.19	SM^{g}, CM^{b}, VA^{abg}
BB12	0.125	16	0.25	8	>256	3	0.25	SM^{ag}, CM^{bg}, VA^{abg}
BL1941	0.19	16	0.38	8	>256	3	0.19	SM^{ag}, CM^{bg}, VA^{abg}
LCR704	0.19	12	0.25	0.125	>256	4	0.19	VA^{abefg}
LP276	0.25	12	0.25	0.5	>256	3	0.19	CM^{i}, VA^{adeg}
LR5434	0.19	12	0.25	0.38	>256	3	0.25	VA^{aeg}, EM^{i}
STM5	0.125	256	12	1	>256	32	0.38	SM^{abcegh}, TC^{abceg}, VA^{abeg}, GM^{abceg}
E. coli ATCC 25923	0.094	24	0.25	0.125	0.75	1	0.38	SM^{b*}
S. aureus ATCC 25923	<.016	48	0.023	<.016	1	2	0.75	-

vancomycin (VA), gentamicin (GM) and erythromycin (EM) according to the breakpoints defined/ suggested by [a] CLSI (2007), [b] EFSA (2008), [b*] Possible interference of growth medium, [c] Dušková and Karpíšková (2013), [d] SCAN (2003), [e] FEEDAP Panel Report (EFSA, 2005), [f] Flórez *et al.* (2007), [g] Anadón *et al.* (2006), [h] Danielsen and Wind (2003), [i] Klare *et al*. (2007) (oxytetracycline was used to present tentative ECOFF values).

and in duplicate each time. MRS agar was used in the assays rather than the standard susceptibility test media including Mueller-Hinton and Iso-Sensitest agar, in order to support the good growth of LAB strains (Danielsen and Wind, 2003). Disc-diffusion assay was repeated using MRS agar pH 7.0, in order to check the influence of pH variation and found insignificant ($p > 0.05$) differences in antimicrobial susceptibility pattern (data not shown).

Etest®

Minimum inhibitory Concentration (MIC) of 7 antibiotics was determined by Etest® method using MRS agar as medium. Etest®- strips (BioMérieux - Australia Pty Ltd.) of ampicillin (AM), clindamycin (CM), erythromycin (EM), gentamicin (GM), tetracycline (TC) and vancomycin (VA) were used in concentration range of 0.016-265 µg/ml while streptomycin (SM) was used in 0.064-1024 µg/ml. MRS agar plates were inoculated with the bacterial suspension as described above. The plates were left ajar in laminar for 10-15 min to allow the absorption of excess moisture. After drying the surfaces of the plates, Etest®- strips of all antibiotics were applied directly onto the surface of agar using Etest®- strip manual applicator (BioMérieux - Australia Pty Ltd). The plates were incubated under anaerobic conditions at 37ºC for 48 h with the exception of plates for *Lactococcus, Streptococcus, Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, which were incubated aerobically at 37ºC for 24 h. MICs were read directly from the test strip according to the manufacturer instructions. Since, there is no definitive and established breakpoint list for lactic acid bacteria, susceptibility to the antibiotics was determined by comparing MIC values to proposed breakpoints from several studies, as presented in Table 2. Strains with MICs equal to or higher than the breakpoints were considered tolerant.

Statistical analysis

All experiments and analyses were repeated twice or otherwise indicated. The Statistical Analysis System (SAS) was used to perform data analysis. Results were analysed using the General Linear Model (GLM) and significance was considered at $p \leq 0.05$ for all analyses. The results for viability assay were presented as logarithmic values for averages of at least two replicates with overall standard error of mean. The results for disc-diffusion assay were presented as averages of three replicates with their standard deviation.

Results and Discussion

Probiotic bacteria are selected for their beneficial health properties as well as their ability to tolerate intestinal conditions (Lee *et al*., 2004). An essential element in their selection is their ability to reach, survive and persist in the environment in which they are proposed to act. Preferential site of colonization for lactobacillus in human gastrointestinal tract (GIT) is the terminal ileum and colon (Charteris *et al*., 1998c), where viability of these cultures is affected

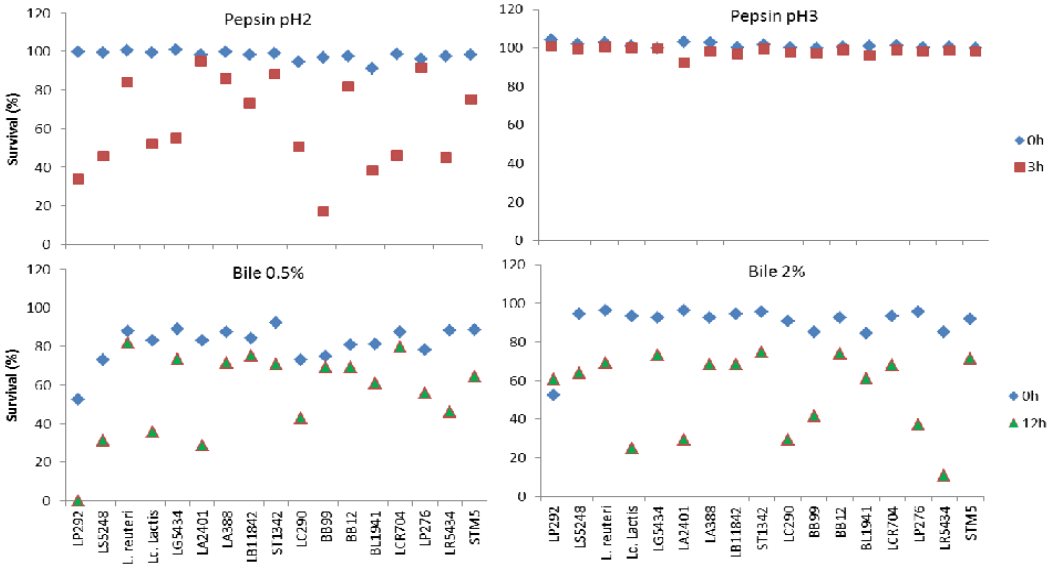

Figure 1: The survival (%) of LAB and probiotic strains in simulated gastric (pepsin) and small intestinal transit (bile).
Results analysed using the General Linear Model (GLM) and significance was considered at $p \leq 0.05$ for all analyses. The standard error of mean (SEM) for viability of LAB and probiotic strains in simulated gastric juice was 0.097179 and in small intestinal transit was 0.130661.

mainly by gastric acid present in the stomach and bile in the duodenum (Lo *et al.*, 2004; Mainville *et al.*, 2005). Approaches for improving the survival and functionality of probiotic bacteria include but not limited to the selection of acid and bile tolerant strains. Also, selection of antibiotic tolerant strains could be advantageous for replenishing or maintaining the gut microbiota after antibiotic treatment (Jose *et al.*, 2015). The present study was conducted to study the tolerances of seventeen LAB strains to bile, pepsin and antibiotics in order to ascertain their stability and select potential probiotic strains.

The effect of simulated gastric and small intestinal transit on the survivability (%) of seventeen LAB strains is shown in Fig. 1. All variables (the effect of bile at two different concentrations and pepsin at two different pH and their interactions) changed significantly ($p < 0.05$) during simulated gastric and small intestinal transit. All the tested strains exhibited survival during simulated gastric transit at pH 3 for 3 h; however, viability was affected at pH 2. *L. reuteri, Lc. lactis, L. rhamnosus* G5435, *L. acidophilus* 2401, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *S. thermophilus* 1342, L. casei 290, *B. lactis* BB12, *L. plantarum* 276 and *S. thermophilus* M5 demonstrated survival rate more than 50% and retained viability during 3h exposure to pepsin pH 2 and are considered intrinsically tolerant to gastric transit.

L. reuteri, L. rhamnosus G5435, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *S. thermophilus* 1342, *B. breve* BB99, *B. lactis* BB12, *B. longum* 1941, *Lc. lactis* R704, *L. plantarum* 276 and *S. thermophilus* M5 retained viability after exposure to 0.5% bile for 12 h. Also, these strains except *B. breve* BB99 and *L. plantarum* 276 survived 2% bile for 12 h (1-2 log reduction in count) are intrinsically tolerant to small intestinal transit. *L. paracasei* 292 and *L. salivarius* 5248 showed significant reduction ($p < 0.05$) in viability at 0.5% bile but recovered viability after 12 h at higher bile concentration (2%). For some strains such as *L. rhamnosus* G5435, *L. acidophilus* 388, *S. thermophilus* 1342, *B. lactis* BB12, *B. longum* 1941 and *S. thermophilus* M5, there was almost no variation in behaviour for increasing concentrations of bile. Although viability of all tested strains was influenced by increasing bile concentration (2%), more than 80% of isolates showed tolerance to 2% bile after 18 h exposure (data not shown). Overall, LAB and probiotic strains including *L. reuteri, L. rhamnosus* G5435, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *S. thermophilus* 1342, *B. lactis* BB12 and *S. thermophilus* M5 remained viable at most extreme conditions of bile (2% for 12 h) and pepsin (pH 2 for 3 h). As such, the bile tolerance and low pH survivability make them most tolerant strains among the tested bacterial cultures.

The pH of the stomach generally ranges from pH 2.5 to pH 3.5 (Holzapfel *et al.*, 1998) and the physiological concentrations of human bile range from 0.3 to 0.5% (Dunne *et al.*, 2001). The concentration of bile salts in the small intestine varies

from approximately 0.2% to 2.0% (w/v) in relation to the individual, type and amount of food consumed (Gunn, 2000). While screening for tolerant strains, 0.3% is considered to be critical concentration for bile-tolerance (Gilliland *et al*., 1984; Hyronimus *et al*., 2000; Zhou *et al.,* 2007) and pH 3 is set as standard for acid tolerance (Sahadeva *et al.*, 2011). It can thus be suggested, all tested strains are acid- and bile-tolerant (> 92% survival in pepsin pH 3 for 3 h; > 60% survival to 0.5% bile for 12h) except *L. paracase*i 292.

Tolerance to bile has not been linked to a specific mechanism but rather to a complex regulation of gene expression (Sánchez *et al*., 2005; Sánchez *et al*., 2007; Andriantsoanirina *et al*., 2013; Ruiz *et al*., 2013). The protonated (non-dissociated) form of the bile salts cause dissociation of lipid bilayer and integral protein of cell membranes, resulting in bacterial content leakage and finally cell death (Mandal *et al*., 2006). Bile salt tolerance is related to the activity of the bile salt hydrolase (BSH) which hydrolyses conjugated bile, thus minimizing its bactericidal effect on strains (Moser and Savage, 2001). As such *in vitro* conditions may not be truly reflective of in situ state and other physiological conditions might affect the strain survival (Morelli, 2000). In a probiotic product, the presence of food and food ingredients improve the viability and enhance 'bile tolerance' of the strains in GIT by preventing the bacteria from bile exposure (Huang and Adams, 2004; Begley *et al*., 2006; G. Vinderola *et al.*, 2011). Thus in our study, strains demonstrating low tolerances to bile and pepsin may improve upon survival in gastric and small intestinal transit when consumed in food or encapsulated using different biopolymeric substances (Chávarri *et al*., 2010).

Viability of bifidobacteria at pH of gastric juices is generally low (Charteris *et al*., 1998c; Matsumoto *et al*., 2004; Mättö et al., 2004; Collado *et al.*, 2005). Survival rates of less than 1% (at pH 3 for 2 h) have been reported (Takahashi *et al.*, 2004). In comparison, strains of *L. acidophilus* appear to be more acid tolerant than *Bifidobacterium* spp. (Boylston *et al*., 2004). However this was not reflective for strain BB12 in our study, where viability of the strain was not much affected during simulated gastric transit. Similarly our results for *S. thermophilus* strains differed from the earlier findings reporting poor acid tolerance of some bacterial strains (Conway *et al.*, 1987; Vinderola and Reinheimer, 2003). Strains of *L. acidophilus* (NS1, M23) and *L. casei* (MYB3) appear highly tolerant to 0.3% bile (Song *et al*., 2014), which supports our results for *L. acidophilus* spp. Tolerance to stomach and intestinal conditions is an important trait for probiotic bacteria in terms of their performance to survive, grow and exert action in the gut (Hyronimus *et al*., 2000). The oral administration of *L. acidophilus* NS1 to mice fed on high-fat diet was reported to increase the expression of sterol regulatory element-binding protein 2 (SREBP2) and LDL receptor (LDLR) in the liver, leading to a decrease in plasma cholesterol levels (Song *et al.*, 2014). If proven, plasma cholesterol levels may be lowered using bile tolerant strains; tolerant strains e.g. *L. reuteri, L. rhamnosus* G5435, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *S. thermophilus* 1342, *B. lactis* BB12, *B. longum* 1941, *Lc. lactis* R704 and *S. thermophilus* M5 in our study could be of benefit in the improvement of hyperlipidemia and hepatic lipid metabolism.

Our results for *B. lactis* (BB12) tolerance to gastrointestinal stresses are consistent with the findings of much previous research (Haschke *et al.*, 1998; Vinderola and Reinheimer, 2003; Matsumoto *et al.*, 2004; Vernazza *et al*., 2006; Li *et al*., 2010; Jungersen *et al*., 2014). *B. longum* and *B. breve* harboured the best tolerance to oxygen, bile and acid stresses among the bifidobacteria tested (Andriantsoanirina *et al.*, 2013). This is critically relevant to our results for three *Bifidobacterium* species (breve, lactis and longum) for which survivability to pepsin and bile was >95% and > 60%, respectively. Also, these strains demonstrated excellent recovery (> 80%) under aerobic growth conditions (data not shown). These 'characteristics' are important for the survival of *Bifidobacterium* species in human GIT and could be advantageous for these strains as probiotics in food industry where high viability is warranted in the end product.

This study also set out to assess the antibiotic susceptibilities of LAB strains and the results obtained by disc diffusion and Etest® methods are shown in Tables 1 and 2. Table 3 demonstrates the overall summary for the survivability of these strains in gut correlated with their tolerances and immune cytokine influence (Ashraf *et al.*, 2014a). Results obtained by disc diffusion assay could be ranked for an increased incidence of antibiotic tolerance in tested strains as: vancomycin> streptomycin > clindamycin> gentamicin. All strains appeared tolerant to vancomycin and susceptible to tetracycline, ampicillin, erythromycin, penicillin, imipenem and amoxycillin/clavulanic acid. For streptomycin, strains were either tolerant or moderately sensitive. Also, gentamicin and clindamycin tolerances were profound.

Tolerance of LAB and probiotic strains to some antibiotics varied considerably depending on

Table 3: Survivability of LAB and probiotic strains in human gut with reference to their tolerances and immune responses

	Tolerances[a]			Immune markers[b]								
Culture	Bile[c]	Pepsin[d]	Antibiotics[e]	IL-2	IL-4	IL-10	IL-12	IFN-γ	TNF-α	TGF-β	%CD3$^+$CD4$^+$CD25$^+$ T cells	%CD4$^+$CD25$^+$FoxP3$^+$ T cells
LP292	–	–	CM	↑	↑	↑	↓	↑	↑	↑	↑	
LS5248	+	+		↑	↑	↑	↑	↑	↑	↑	↑	↑
L. reuteri	+	+	SM, CM, GM	↑	↑	↑	↓	↑	↑	↑	↑	
Lc. lactis	+	+	SM, CM, VA, GM	↑	↑	↑	↓	↑	↑	↑	↑	↑
LG5434	+	+	SM, TC	↑	↑	↑	↑	↑	↑	↑	↑	↑
LA2401	+	+	SM, VA	↑	↑	↑	↑	↑	↑	↑	↑	
LA388	+	+	SM, VA	↑	↑	↑	↑	↑	↑	↑	↑	
LB11842	+	+	SM, CM, VA	↓	↑	↑	↓	↑	↑	↑	↑	↑
ST1342	+	+	SM, VA	↑	↑	↑	↑	↑	↑	↑	↑	↑
LC290	+	+	VA	↑	↑	↑	↑	↑	↑	↑	↑	↑
BB99	+	+	CM, VA	↑	↑	↑	↑	↑	↑	↑	↑	
BB12	+	+	CM, VA	↑	↑	↑	↑	↑	↑	↑	↑	↑
BL1941	+	+	CM, VA	↑	↑	↑	↓	↑	↑	↑	↑	
LCR704	+	+	VA	↑	↑	↑	↓	↑	↑	↑	↓	
LP276	+	+		↑	↑	↑	↑	↑	↑	↑	↑	
LR5434	+	+		↑	↑	↑	↓	↑	↑	↑	↑	
STM5	+	+	SM, TC, VA, GM	↑	↑	↑	↓	↑	↑	↑	↑	↑

[a] appeared in the current study; [b] compiled from previous work (Ashraf *et al*., 2014ab); [c] 0.5 % for 12h; [d] pH3 for 3h; [e] according to microbiological breakpoints defined by EFSA (2008)

CM clindamycin, SM streptomycin, GM gentamicin, VA vancomycin, TC tetracycline

– sensitive, + tolerant, ↑ increased and ↓ decreased secretion compared to the control (RPMI) after 72 h stimulation of human peripheral blood mononuclear cell (PBMCs) with live strains. Average percent increase for all immune markers was > 100% and average percent decrease was < 55% for IL-2, IL-12, and < 15% for %CD3+CD4+CD25+ T cells.

breakpoints used for determining the MICs (Table 3). All the tested strains were sensitive to ampicillin and erythromycin, and highly tolerant to vancomycin. Majority were streptomycin tolerant except *L. salivarius* 5248, *B. breve* BB99, *Lc. lactis* R704, *L. plantarum* 276 and *L. rhamnosus* 5434. *L. reuteri, Lc. lactis, L. rhamnosus* G5434, *L. delbrueckii* subsp. *bulgaricus* 11842, L. casei 290 and *S. thermophilus* M5 were tolerant to gentamicin (≥8 µg/ml). Tetracycline tolerance was found in *L. rhamnosus* G5434 and *S. thermophilus* M5 (EFSA, 2005; CLSI, 2007; EFSA, 2008). All the strains were clindamycin sensitive except *L. delbrueckii* subsp. *bulgaricus* 11842 (CLSI, 2007; EFSA, 2008).

As indicated by tentative ECOFF values (Klare *et al*., 2007), *L. reuteri, L. rhamnosus* G5434, *L. acidophilus* 2401, *L. acidophilus* 388 and *L. delbrueckii* subsp. *bulgaricus* 11842 harboured non-wild type (NWT) tolerance to streptomycin and erythromycin (except *L. acidophilus* 388, WT for erythromycin). *L. paracasei* 292 and *L. rhamnosus* 5434 were also tolerant to erythromycin but were rather WT (Table 2). All tested strains were sensitive (WT) for ampicillin. *L. rhamnosus* G5434, *L. acidophilus* 2401, *L. acidophilus* 388 and *L. delbrueckii* subsp. *bulgaricus* 11842 were tolerant to gentamicin (NWT except *L. rhamnosus* G5434). *L. acidophilus* 2401, *L. acidophilus* 388 and *L. delbrueckii* subsp. *bulgaricus* 11842 got NWT tolerances for vancomycin while insufficient evidence was found for rest of the strains to report breakpoints. *L. paracasei* 292, *L. acidophilus* 2401 and *L. plantarum* 276 demonstrated tolerance to clindamycin from available MICs breakpoints, where *L. acidophilus* 2401 appeared NWT. Moreover, *L. rhamnosus* G5434 and *L. delbrueckii* subsp. *bulgaricus* 11842 showed NWT tolerances for tetracycline. According to species-specific MIC breakpoints (Danielsen and Wind, 2003), *L. rhamnosus* G5434 appeared tetracycline tolerant and none of the strains was tolerant to erythromycin.

We identified tolerance to vancomycin and streptomycin common among bile and pepsin tolerant strains. The information is new and suggests there could be a similar mechanism of protection for these traits. Moreover, gentamicin and clindamycin tolerances were considerably frequent among the tested LAB strains. The tolerances to aminoglycosides (gentamicin, streptomycin) and glycopeptides (vancomycin), however, appeared intrinsic to the strains (Klein *et al*., 2000; Katla *et al*., 2001; Danielsen and Wind, 2003; Temmerman *et al*., 2003), it can be lost due to presence of conjugated bile salts (Charteris *et al*., 2000). The observed glycopeptide (vancomycin) tolerances in LAB are consistent with earlier observations (Charteris *et al*., 1998b; Blandino *et al*., 2008; Liu *et al*., 2009; Gueimonde *et al*., 2013; Gad *et al*., 2014). Interestingly, in our study MICs >256 µg/mL for vancomycin were common, which was consistent with earlier research (Salminen *et al*., 2006).Our results contradict with earlier research

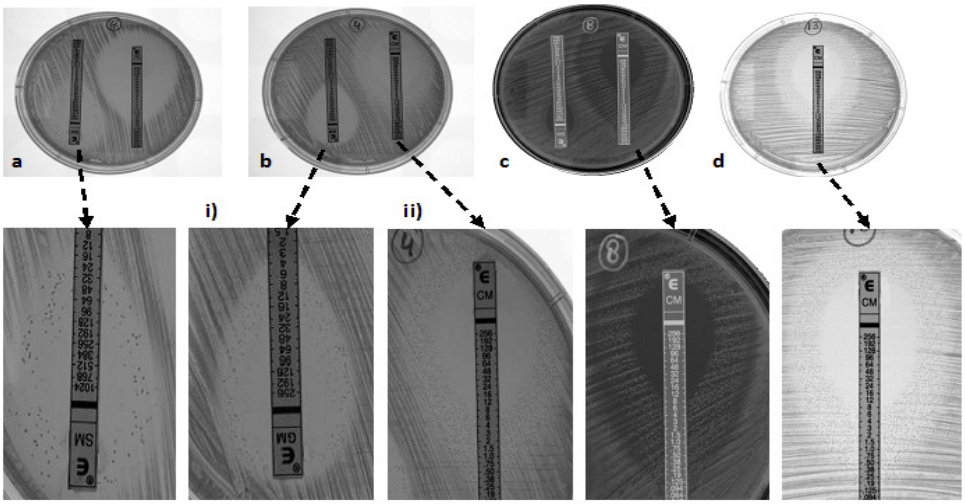

Figure 2: Occurrence of isolated tolerant colonies or lighter zone of tolerant colonies in elliptical inhibition zones when testing the susceptibility of (A) *Lc. lactis* strain to streptomycin (MIC of 128 µg/mL) (B) *Lc. lactis* strain to i) gentamicin (MIC of 32 µg/mL) ii) clindamycin (MIC of 8 µg/mL) (C) *L. delbrueckii* subsp. *bulgaricus* 11842 strain to clindamycin (MIC of 12 µg/mL) (D) *B. longum* 1941 strain to clindamycin (MIC of 8 µg/mL) by Etest®. According to the manufacturer's instructions, the MIC was read as the concentration at total inhibition, including discrete colonies, despite the apparent inhibition ellipse.

(Klare *et al*., 2007) that showed *L. acidophilus* were relatively more susceptible to streptomycin than other *Lactobacillus* species and members of several *Lactobacillus* species (including *L. paracasei* and *L. plantarum* and to some extent also *L. rhamnosus* and *L. fermentum*) appeared less susceptible to streptomycin.

In the current study, lactobacilli and bifidobacteria appeared to be clindamycin tolerant and the observed tolerances seemed to be intrinsic (Delgado *et al*., 2005; Gad *et al*., 2014). Moderate or variable activity of clindamycin against lactobacilli and bifidobacteria has been reported (Danielsen and Wind, 2003; Coppola *et al*., 2005; Masco *et al*., 2006), whereas others (Charteris *et al*., 1998a; Lim *et al*., 2002; Ammor *et a*l., 2007; Botina *et al*., 2011; Gueimonde *et al*., 2013) have evidenced high sensitivity of lactobacilli to lincosamide.

In our study, bifidobacteria demonstrated tolerances to vancomycin, streptomycin and clindamycin that is consistent with others (Charteris *et al*., 1998b; Delgado *et al*., 2005; D'Aimmo *et al*., 2007). Aminoglycosides tolerance could be as a consequence of the lack of cytochrome-mediated drug transport (Mayrhofer *et al*., 2011). Tolerance phenotype for streptomycin in these strains has not been linked to the acquisition of specific antibiotic tolerance genes but rather it has been related to chromosomal mutations on the rpsL gene for ribosomal protein S12 in *B. bifidum* and *B. breve* (Kiwaki and Sato, 2009; Sato and Iino, 2010). Therefore, streptomycin tolerances essentially do not represent a potential risk of transferability in bifidobacteria. Moreover, *Bifidobacterium* species including *B. animalis, B. breve*, and *B. longum*, with QPS status have not been linked to any infective processes in healthy individuals yet (EFSA, 2013). Contrary to our results, tetracycline tolerance has been identified common in bifidobacteria (Scott *et al.*, 2000; Masco *et al*., 2006; Aires *et al*., 2007; Ammor *et al*., 2007; Ammor *et al*., 2008; Gueimonde *et al.*, 2010).

In the current study, few challenges were revealed in testing antimicrobial susceptibilities in LAB including difficulty in interpreting MIC values according to ECOFF for some species. The data on determinants of antibiotic tolerance using ECOFF values in bifidobacteria are relatively scarce, and limited to a fewer antibiotics including tetracycline and macrolide. ECOFF values determined for Lactobacillus group for erythromycin (0.5 or less) and gentamicin (4 or 8) were fairly lower (Klare *et al.*, 2007) than proposed by others (Katla *et al*., 2001; Danielsen and Wind, 2003; EFSA, 2005; CLSI, 2007). Applying MIC breakpoints (Klare *et al*., 2007), erythromycin tolerances were observed in few LAB strains including *L. rhamnosus* G5434, did not coordinated with disc-diffusion results. In *L. rhamnosus* chromosomal mutation has been identified in 23S rRNA gene reducing the affinity of erythromycin for the ribosome, resulting into macrolide tolerance in strains (Flórez *et al*., 2007). 'The transfer risk is considered to be very low for intrinsic, or acquired tolerance due to chromosomal mutation(s)' (Klare *et al.*, 2007; Gueimonde *et al*., 2013). Similarly, MIC breakpoints indicated by

SCAN (2003) and FEEDAP (2005) were lower for some antibiotics than initially proposed (Danielsen and Wind, 2003). The differences in interpretive criteria might be explained by differences in dosages, administration intervals, inoculum size, and test media.

Interesting phenomenon of substantial ingrowth in Etest® elliptical inhibition zones was found at few occasions during experiment (Fig. 2A-D). It appeared most common with clindamycin, in *Lc. lactis, L. delbrueckii* subsp. *bulgaricus* 11842, *B. longum* 1941 and *B. lactis* BB12. *Lc. lactis* showed similar tolerances with streptomycin and gentamicin as well. The present findings are consistent with other study, where *L. gasseri* and *L. johnsonii* showed similar tolerances against clindamycin and erythromycin (Mayrhofer *et al*., 2008). Danielsen and Wind (2003) also observed isolated colonies within the inhibition zone of the Etest® in testing susceptibility of Lactobacillus spp. to imipenem and nitrofurantoin. It could be due to high frequency of spontaneous mutation in antibiotic genes observed fairly common in lactobacilli (Curragh and Collins, 1992; Danielsen and Wind, 2003). Our data in this case is novel illustration of phenomenon of persistence that likely shows regulated cellular heterogeneity. Survival of small fraction of cells after exposure to severe stress of antibiotics has been linked to transient state of slow or arrested growth of cells in the colony, which is different from resistance (Martins and Locke, 2015) and provide an ideal clue for LAB evolution of 'tolerance' to antibiotics. Though yet to prove, we believe that LAB populations have all adapted to the antibiotic regimen through tolerance and not resistance (Fridman *et al*., 2014).

Conclusion

Tolerances to gastric and intestinal transit are exhibited by the dairy-based strains of *Lactobacillus, Bifidobacterium, Streptococcus,* and *Lactococcus* spp. *L. reuteri, L. rhamnosus* G5435, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *S. thermophilus* 1342, *B. lactis* BB12 and *S. thermophilus* M5 appeared highly tolerant to gastrointestinal stresses among the tested strains. Tolerances to tetracycline, penicillins (amoxicillin, ampicillin and penicillin), macrolides (erythromycin) and carbapenems (imipenem) generally did not occur among LAB. Tolerance to tetracycline and erythromycin was less frequent and appeared only in a few cases. In this regard LAB strains can be categorized in order of high to low frequency of tolerance as vancomycin> streptomycin> gentamicin> clindamycin> tetracycline. Although majority of tolerances are believed to be intrinsic, this will need to be further scrutinized to confirm a genetic basis but this was not part of the study. These strains previously demonstrated a substantial contribution in the induction of innate and adaptive defence mechanisms, can survive GIT and antimicrobial stresses, and could be helpful in replenishing gut microbiota after antibiotic therapy in the presence of residual antibiotic in the gut. In conclusion, the study provides a thorough understanding of gastrointestinal tolerances and antibiotic tolerance phenotype of LAB that value-add to their multiple applications in probiotic products. It also provides some support for the conceptual premise for therapeutic approaches such as treatment of Lactobacillus-related bacteraemia or antibiotic resistances in superbugs (e.g. *Clostridium difficile* cases, CDI cases) in hospital settings.

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