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Full Length Research Paper

Effect of acid and oxgall on anti-microbial susceptibility of Probiotic Lactobacilli

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The present study was conducted to determine the susceptibility of 13 *Lactobacillus* strains to 14 antibiotics and to evaluate the impact of some gastrointestinal stressful conditions, particularly acid and bile stress, as well as acid adaptation on their antibiogram profiles. The strains tested were 2 of *Lactobacillus acidophilus*, 1 *Lb. delbrueckii* subsp. *bulgaricus*, 2 *Lb. casei*, 1 *Lb. casei paracasei* subsp. *paracasei*, 1 *Lb. delbrueckii* subsp. *lactis*, 4 *Lb. plantarum* and 2 *Lb. rhamnosus*. In control trails, the majority of the strains tested were susceptible to ampicillin, penicillin, chloramphenicol, erythromycin, novobiocin and nisin A, but resistant to vancomycin, kanamycin, neomycin, paromomycin, streptomycin and nalidixic acid. Lactobacilli strains showed variable susceptibility to cloxacilline and tetracycline. Acid-adaptation (strains adapted to grow at pH 4.0) resulted in increased resistance to cloxacilline, erythromycin and tetracycline, in strain dependent manner. Acid- stressed (exposure to pH 2 for 90 min at 37˚C) lactobacilli appeared to be more resistant to ampicillin, cloxacilline, chloramphenicol and tetracycline compared with un-stressed strains. In the presence of 0.3% (w/v) oxgall, lactobacilli became more susceptible to aminoglycosides and slightly resistant to cell wall-targeted antibiotics. However, oxgall stress (exposure to 0.3% (w/v) oxgall for 90 min at 37˚C) slightly modified antibiogram profile depending on the strain tested. Results reported in this study showed that acid and oxgall stresses could substantially modify antibiotic susceptibility/resistance profile of lactobacilli, which may thus affect their probiotic capacity especially when used along with antibiotics.

Key Words: Probiotics, lactobacilli, antibiotic susceptibility, acid, oxgall.

INTRODUCTION

Lactobacilli have been used for long time in the production of foods that require lactic acid fermentation and are considered as GRAS (generally recognized as safe) organisms and can be safely used for medical and veterinary applications (Fuller, 1989). To date, 80 species of lactobacilli are recognized and characterized (Satokari, et al., 2003) . These organisms are strictly fermentative, aerotolerant or anaerobic and have a complex nutritional requirements. Using glucose as carbon source, lacto-bacilli may be either homo- fermentative (producing more than 85% of fermentative products as lactic acid) or heterofermentative (producing lactic acid, carbon dioxide, ethanol and/or acetic acid) (Tannock, 2004). In the dairy industry, lactobacilli are extensively used for the production of wide variety of fermented milks and cheeses either as starter or adjunct cultures. Lactobacilli can significantly contribute to flavor and texture develop-ment in yogurt and cheeses through their acid-producing capacity and ability to produce several proteinases, pepti-dases and esterases (El Abboudi et al., 1991; El Soda et al., 2000).

From health point of view, ingestion of live cells of certain species and strains, probiotic concept, of lactobacilli in adequate amounts is believed to confer several beneficial physiological effects on the host (reviewed by Tannock, 2004). Indeed, there is several definition for probiotic, among them is that given by Fuller (1989) who described a probiotic as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance. Maintaining a healthy and equilibrated intestinal microbiota and reducing inci-dence of intestinal infection is one of major health benefits

fits ascribed for lactobacilli (Gardiner et al., 2002). Different probiotic preparations, containing lactobacilli, are recommended frequently to prevent disturbance in intestinal microflora and antibiotic-associated diarrhoea. Many of commercial probiotic products contain mainly members of genus *Lactobacillus* (Reuter, 1997). *Lactobacillus* species from which probiotic strains have been isolated include *Lactobacillus acidophilus* (Bernet et al., 1994), *Lb. rhamnosus* (Salminen et al., 1993), *Lb. casei* (Azo and Akazan, 1992), *Lb. gasseri* (Pedrosa et al., 1995) and *Lb. delbrueckii* (Fernàndez et al., 2005).

Indeed, the gastrointestinal microbial ecosystem is relatively stable but quantitative and qualitative disturbances are seen after oral administration of antibiotics. The normal and equilibrated flora limits the concentration of potentially pathogenic microorganisms, which can reach high numbers of connection with intake of antimicrobial agents (Vollaard and Clasener, 1994). On the other hand, increased antibiotic resistance is considered to be the most common complication of antimicrobial therapy. Antimicrobial-resistant genes have been shown to be transferable between bacteria of different origin (Kruse and Sorum, 1994). Thus, the performance of antibiotic susceptibility testing is regarded as both a necessary criterion for probiotic selection and an effective guide for specific antimicrobial therapy (Peterson and Shanholtzer, 1992). Meanwhile, the exposure of probiotic organism to stressful conditions, usually encountered in the gastrointestinal tract such as acid and oxgall stress, may affect its antibiotic susceptibility profile. Thus, the present study aimed to evaluate the susceptibility of 13 probiotic lactobacilli strains to 14 antibiotics and to evaluate changes in their susceptibility due to stresses caused by hydrochloric acid, oxgall and adaptation to lactic acid.

MATERIALS AND METHODS

Bacterial strains and growth media

Lactobacillus acidophilus R052, *Lb. casei* R0256, *Lb. casei* R0215, *Lb. delbrueckii* subsp. *lactis* R0187, *Lb. plantarum* R1096, *Lb. plantarum* R1078, *Lb. plantarum* R0202, *Lb. rhamnosus* R0011, and *Lb. rhamnosus* R1039 were obtained from Rosell Institute Inc. (Montreal, PQ, Canada). *Lb. acidophilus* P/N 601379, *Lb. bulgaricus* P/N 601383, *Lb. casei paracasei* subsp. *paracasei* P/N 601385, and *Lb. plantarum* P/N 601387 were obtained from Chr. Hansen Ltd. (Barrie, ON, Canada).

All bacterial strains used in this study were maintained in 20% glycerol stock at 80˚C. They were re-cultured in de Man, Rogosa and Sharpe (MRS) broth (de Man et al., 1960) at 37˚C under aerobic condition. Prior to beginning the experiments, each bacterial strain was sub-cultured at least three times (1%, v/v) at 24 h intervals.

Antibiotics

Erythromycin, penicillin G sodium salt, tetracycline hydrochloride, streptomycin sulfate, chloramphenicol, nalidixic acid, vancomycin, kanamycin, neomycin sulfate and paromomycin sulfate were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cloxacilline sodium salt was obtained from Fluka Chemical Corp. (Ronkonkoma, NY, USA). Novobiocin and ampicillin, both as sodium salt, were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Nisin A was provided by Alpin & Barrett Ltd. (Beaminster, United Kingdom). Stock solutions of test antibiotics were prepared freshly, in water or ethanol (70%, v/v) according to their solubility index, at an initial concentration of 1 mg/mL filtered through 0.22-μ m pore size filter (Cameo 25 N, MSI, Westboro, MA, USA) and kept at 4˚C for a maximum of two days.

Oxgall tolerance

The tolerance of lactobacilli strains to oxgall was tested using sterile flat-bottom 96-well microtiter plates (Falcon, Becton Dickinson and Company, Frankin Lakes, NJ, USA) as described by Gagnon et al. (2004) . MRS broth prepared with (0.1, 0.2, 0.3, 0.4 and 0.5% w/v) oxgall (Sigma) and briefly, 150 μL were added to each well and inoculated with 30 μL of lactobacilli overnight culture previously diluted 1/1000 in the same broth. Microplates were incubated aerobically at 37°C for 24 h. Optical densities were read at 650 nm using a Thermomax microplate reader (Molecular Devices, Opti-Resources, Charny, PQ, Canada). Results expressed as the lowest concentration of oxgall that completely inhibited the tested organism (OD equal to that of un-inoculated broth).

Acid and oxgall challenge

Ten milliliters of mid-log-phase MRS cultures of each lactobacilli were harvested by centrifugation at 6,000 x *g* for 15 min at 4˚C, re-suspended in an equal volume of (i) MRS broth adjusted to pH 2.0 using 1M HCl (for acid challenge) or (ii) MRS broth (initial pH 6.5) containing 0.3% (w/v) oxgall. The suspended cells were incubated aerobically at 37˚C for 90 min. Viable counts were determined before and after incubation by diluting samples in peptone water (0.15%, w/v) and plating appropriate dilutions onto MRS agar. Plates were incubated aerobically at 37˚C for 48 h.

At the end of challenge experiments, acid and oxgall-stressed cells were sub-cultured in MRS broth (pH 6.5), using inoculation level of 1% (v/v), incubated aerobically at 37˚C for 18 h prior to testing their post-challenge sensitivity to antibiotics.

Adaptation to acidic pH

Each lactobacilli strain was adapted to growth in MRS broth adjusted with DL- lactic acid (Sigma) to an initial pH of 4.0 by sub-culturing at least five times. Cultures were transferred at 1% (v/v) at 24 h intervals and incubated aerobically at 37˚C.

Testing sensitivity to antibiotics and nisin A

Sensitivity of bifidobacteria to different antibiotics and nisin A was determined in term of minimum inhibitory concentration (MIC). The MIC values of each tested antibiotic and nisin A were determined by microplate assay following the method described previously (Mota-Meira et al., 2000). Bacteria were grown to mid log phase in MRS broth. The OD_{650} of the culture was adjusted to 0.1 with fresh MRS broth using a Spectronic 20 spectrophotometer (Bausch & Lomb. Inc., Rochester, NY). The number of viable cells in the OD adjusted inoculum was determined by plating appropriate dilutions of 10 -fold diluted culture in peptone water (0.15%, w/v) onto MRS agar and incubating aerobically at 37˚C for 48 h. The viable count was found to range from 5×10^5 to 1×10^6 cfu/mL.

A serial two-fold dilution of 125 μL of tested antibiotic or nisin A was done in a 96-well polystyrene microplate (Becton Dickinson

Table 1. Viable counts (log_{10} cfu/mL) of different lactobacilli at the beginning and the end of acid and oxgall stress experiments. Data are mean values ± standard deviation.

Strains	Acid stress[a]		Oxgall stress[b]	
	0	90 min	0	90 min
Lb. acidophilus R052	9.47±0.10	8.30±0.12	9.43±0.20	9.27±0.32
Lb. acidophilus P/N 601379	8.00±0.19	6.83±0.07	7.90±0.15	7.74±0.23
Lb. bulgaricus P/N 601383	9.15±0.13	4.00±0.05	8.69±0.22	8.47±0.29
Lb. casei R0256	9.6±0.22	5.56±0.12	9.04±0.30	8.65±0.19
Lb. casei R0215	8.69±0.19	5.54±0.21	9.30±0.30	8.69±0.17
Lb. casei paracasei subsp. *paracasei* P/N 601385	9.17±0.24	<2.00	8.40±0.29	7.00±0.22
Lb. delbrueckii spp *lactis* R0187	8.69±0.26	3.00±0.04	9.23±0.17	9.25±0.21
Lb. plantarum R1096	9.00±0.30	8.69±0.22	9.00±0.10	8.69±0.18
Lb. plantarum R1078	9.47±0.32	8.84±0.30	9.32±0.12	9.17±0.16
Lb. plantarum R0202	9.17±0.28	8.78±0.25	9.69±0.19	9.00±0.21
Lb. plantarum P/N 601387	8.93±0.21	2.47±0.03	9.60±0.26	9.40±0.23
Lb. rhamnosus R0011	9.30±0.19	7.08±0.09	9.60±0.23	9.11±0.20
Lb. rhamnosus R1039	9.78±0.30	5.67±0.08	9.47±0.32	9.20±0.20

[a]Cells kept for 90 min in MRS broth adjusted to pH 2.0 at 37˚C.

[b]Cells exposed to 0.3% (w/v) oxgall in MRS broth for 90 min at pH 6.5 at 37˚C.

Labware, Lincoln Park, NJ, USA) containing 125 µL/well of MRS or MRS broth containing 0.3 (w/v) oxgall. Standardized bacterial suspension (50 µL) was then added to each well. This volume corresponded to approximately 2.5-5.0 x 10^4 cfu/well which is within the range recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1991) as standard inoculum density for the determination of antibiotic MIC by the microdilution method. The microplates were incubated at 37°C for 24 h and the OD was read at 650 nm using a Thermomax microplate reader. Control (wells inoculated with the tested culture without added inhibitor) and blank (wells containing un-inoculated broth medium with added antibiotic or nisin A) were run on each microplate. The MIC was the lowest concentration of tested inhibitor giving complete inhibition of growth (OD equal to OD of blank) (Karakoc and Gerceker, 2001). The microplate assay was repeated four times and the MIC values were presented as median of the four repetitions.

RESULTS AND DISCUSSION

Oxgall tolerance

Prior to oxgall challenge experiments, it was necessary to determine oxgall inhibitory concentration for each strain in order to determine the oxgall concentration that would be challenged by tested lactobacilli. The sensitivity of lactobacilli to oxgall determined by microdilution method did not differ widely among tested strains, since all strains grew in the presence of 0.5% oxgall, except for both *Lb. acidophilus* R052 and P/N 601379 strains that were inhibited at 0.4%. Consequently, oxgall concentration of 0.3% (w/v) was chosen for oxgall challenge experiment. Generally, lactobacilli have been shown to exhibit strain variation in oxgall tolerance (Gilliland et al., 1984; Château et al., 1994). Dunne et al. (2001) evaluated the growth of different lactobacilli and bifidobacteria on MRS agar supplemented with bovine or porcine bile to final concentrations between 0.3% and 7.5%. The authors reported that lactobacilli showed variable sensitivity to such bile and porcine bile was more inhibitory to both lactobacilli and bifidobacteria. A strain of *Lb. acidophilus* 1748 could tolerate bovine bile concentration of 0.5%, while *Lb. paracasei* 2123 tolerate concentrations up to 7.5%.

Acid and oxgall challenge

Table 1 shows the survival of tested lactobacilli after exposing to acid and oxgall challenge. HCl was more harmful to lactobacilli than oxgall. Exposing to acidic condition (pH 2.0 for 90 min) resulted in reduced viability of different lactobacilli by 0.3 to > 6.0 log cfu/mL depending on strain tested. The reason for 90 min of incubation time during acid challenge experiment is that the time elapsed from entrance to release form the stomach (Jin et al., 1998). Indeed, acid tolerance varied widely among tested strains and appeared to be strain dependent rather than species characteristic. Similar observation has been reported previously by Berrada et al. (1991) for *Bifidobacterium* strains that showed high variability in their tolerance to gastric conditions. In this study, *Lb. bulgaricus* P/N 601383, *Lb. casei paracasei* subsp. *paracasei* P/N 601385, *Lb. delbrueckii* spp *lactis* R0187 and *Lb. plantarum* P/N 601387 were the most acid sensitive strains and their survival reduced by 5-7 log cycles after 90 min of acid exposure. While strains belonging to *Lb. plantarum* were the most tolerant to acid and slight reductions in viability ranged from 0.3 to 0.6 log cycle were observed for strains R1096, R1078 and R0202. On contrary, *Lb. plantarum* P/N 601387 showed

Table 2. Minimum inhibitory concentrations[a] (μg/mL) of antibiotics against probiotic strains of lactobacilli.

Strains	Antibiotics[b]													
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nal	Nov	Nis
Lb. acidophilus R052	0.98	0.98	0.98	0.98	125	62.5	62.5	7.8	0.98	0.98	0.98	250	0.98	0.98
Lb. acidophilus P/N 601379	0.98	0.98	0.98	0.98	250	62.5	62.5	7.8	0.98	0.98	0.98	125	0.98	0.98
Lb. bulgaricus P/N 601383	0.98	62.5	3.9	>500	500	62.5	125	250	3.9	3.9	62.5	>500	0.98	0.98
Lb. casei R0256	1.9	15.6	0.98	>500	500	250	250	250	3.9	0.98	7.8	>500	0.98	0.98
Lb. casei R0215	0.98	15.6	0.98	>500	500	250	125	125	3.9	0.98	0.98	>500	0.98	3.9
Lb. casei paracasei subsp. *paracasei* P/N 601385	0.98	3.9	0.98	>500	250	62.5	125	125	1.9	0.98	0.98	>500	0.98	0.98
Lb. delbrueckii subsp. *lactis* R0187	1.9	3.9	0.98	>500	500	250	250	250	3.9	0.98	7.8	>500	0.98	0.98
Lb. plantarum R1096	1.9	31.2	1.9	>500	500	250	250	250	3.9	0.98	7.8	>500	0.98	0.98
Lb. plantarum R1078	1.9	31.2	1.9	>500	500	500	250	250	3.9	0.98	7.8	>500	0.98	0.98
Lb. plantarum R0202	0.98	31.2	1.9	>500	500	250	125	125	3.9	0.98	7.8	>500	0.98	0.98
Lb. plantarum P/N 601387	0.98	31.2	1.9	>500	500	250	125	250	3.9	1.9	31.2	>500	0.98	0.98
Lb. rhamnosus R0011	3.9	7.8	0.98	>500	500	250	250	250	3.9	0.98	7.8	>500	0.98	0.98
Lb. rhamnosus R1039	1.9	62.5	1.9	>500	500	250	250	250	3.9	7.8	7.8	>500	0.98	0.98

Values without parentheses are MIC and those in parentheses are MBC. [a]Medians of 4 repetitions.

[b]Amp, ampicillin; Clo, Cloxacilline; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nal, nalidixic acid; Nov, novobiocin and Nis, nisin A.

to be very sensitive to acid and its viability reduced by approximately 6.5 log cycle cfu/mL after 90 min of exposure to HCl. Indeed, the ability of probiotic strains to survive acidic conditions varies widely among strains and species (Ross et al., 2005). Hood and Zottola, 1988 showed that no cells of a *Lb. acidophilus* culture were recovered following 45 min exposure to pH 2.0. Olejnik et al. (2005) evaluated the survival of probiotic strains of *Lb. casei, Lb. acidophilus* and *Lb. helveticus* during 3 hours of incubation at pH 3.0 in MRS broth. The authors reported that *Lb. casei* was much more tolerant to acid than either *Lb. acidophilus* or *Lb. helveticus* and the highest reduction in viability of such strain appeared after the first hour of incubation.

The results presented in Table (1) show that the exposure to 0.3% oxgall for 90 min exerted slight inhibitory effect on the viability of 13 tested *Lactobacillus* strains. Reductions in viability ranged from 0.2 to 0.7 log cycle cfu/mL were found for most of strains tested, however *Lb. casei paracasei* subsp. *paracasei* P/N 601385 was the most susceptible to oxgall and reduction of 1.4 log cycle cfu/mL was found after 90 min of exposure to oxgall. Indeed, lactobacilli are generally more resistant to gastrointestinal conditions, especially acid and bile, than other probiotic organisms such as bifidobacteria (Ross et al., 2005). Jacobsen et al. (1999) evaluated the probiotic potential of 47 lactobacilli isolated from fermented maize and human stool samples and reported that all strains could successfully resist oxgall concentration of 0.3%.

Antibiotic susceptibility of lactobacilli

The results of the susceptibility tests of un-stressed lactobacilli to different antibiotics are shown in Table 2. All tested lactobacilli were extremely susceptible to ampicillin, penicillin G, chloramphenicol, erythromycin, novobiocin and nisin A and being inhibited by these antibiotics at concentrations ranged from 0.98 to 3.9 μg/mL. Cloxacilline, streptomycin and tetracycline showed variable MIC values ranging from 0.98 to 62.5, from 7.8 to 250 and from 0.98 to 62.5 μg/mL, receptively. However, they were resistant to kanamycin, neomycin, paromomycin and nalidixic acid with MIC values ranging from 125 to 500, from 62.5 to 250, from 62.5 to 250 and from 125 to >500 μg/mL, respectively.

The resistance to vancomycin was much more evident among tested lactobacilli with MIC values of >500 μg/mL, while *Lb. acidophilus* R052 and P/N 601379 strains were the most susceptible and appeared to be inhibited at a vancomycin concentration of 0.98 μg/mL. Resistance to vancomycin has been reported to be species-specific character and may be helpful in classification of lactobacilli (Hamilton-Miller and Shah, 1998). The authors reported that vancomycin sensitivity was characteristic to strains belonging to *Lb. acidophilus* and *Lb. delbreuckii*, while strains belonging to *Lb. rhamnosus* were resistant.

In accordance to results reported herein, previous studies have shown that lactobacilli, isolated from different origins, were sensitive to -lactams (penicillin, ampicillin and cloxacilline), erythromycin, chloramphenicol and

Table 3. Minimum inhibitory concentrations[a] (μg/mL) of antibiotics for acid-adapted[b] probiotic lactobacilli.

Strains	Antibiotics[c]													
	Amp	**Clo**	**Pen**	**Van**	**Kan**	**Neo**	**Par**	**Str**	**Chl**	**Ery**	**Tet**	**Nal**	**Nov**	**Nis**
Lb. acidophilus R052	0.98	125	0.98	0.98	125	125	62.5	7.8	0.98	125	125	250	0.98	0.98
Lb. acidophilus P/N 601379	**Did not adapt to grow at pH 4.0**													
Lb. bulgaricus P/N 601383	0.98	62.5	3.9	>500	500	125	250	250	3.9	3.9	62.5	500	0.98	0.98
Lb. casei R0256	1.9	62.5	0.98	>500	500	250	250	250	3.9	3.9	125	>500	0.98	0.98
Lb. casei R0215	1.9	62.5	0.98	>500	500	125	250	125	3.9	3.9	62.5	>500	0.98	3.9
Lb. casei paracasei subsp. *paracasei* P/N 601385	1.9	3.9	0.98	500	250	62.5	125	125	0.98	0.98	0.98	>500	0.98	0.98
Lb. delbrueckii subsp. *lactis* R0187	1.9	62.5	3.9	>500	500	125	250	250	3.9	0.98	31.2	>500	0.98	0.98
Lb. plantarum R1096	1.9	62.5	7.8	>500	500	250	250	250	3.9	3.9	62.5	>500	0.98	0.98
Lb. plantarum R1078	1.9	125	1.9	>500	500	125	125	250	1.9	3.9	125	>500	0.98	0.98
Lb. plantarum R0202	0.98	62.5	3.9	>500	500	62.5	125	125	1.9	3.9	125	>500	0.98	0.98
Lb. plantarum P/N 601387	0.98	62.2	1.9	>500	500	250	125	250	1.9	1.9	31.2	500	0.98	0.98
Lb. rhamnosus R0011	3.9	7.8	0.98	>500	500	125	250	250	1.9	0.98	15.6	>500	0.98	0.98
Lb. rhamnosus R1039	1.9	62.5	1.9	>500	500	125	250	250	3.9	7.8	62.5	>500	0.98	0.98

[a]Medians of 4 repetitions.
[b]To grow in MRS broth at pH 4.0
[c]Amp, ampicillin; Clo, Cloxacilline; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nal, nalidixic acid; Nov, novobiocin and Nis, nisin A.

lactobacilli for their susceptibility to 25 antimicrobial agents and reported that 83.8, 55.8 and 53% of tested strains had MIC values of more than 256 μg/mL for kanamycin, vancomycin and streptomycin, respectively.

Antibiotic susceptibility of acid-adapted lactobacilli

Lactobacilli are known to be acidophilic or aciduric (McLauchlan et al., 1989). Acid-adapted variants from all strains, except for *Lb. acidophilus* P/N 601379, could be developed by extensive propagation in MRS broth adjusted at pH 4.0. This indicates that *Lb. acidophilus* P/N 601379 strain may encounter difficulty to survive in food with acidic environment such as yogurt and thus limit its industrial application as probiotic candidate. However, *Lb. acidophilus* R052 can successfully be adapted to pH 4.0 and may thus have potential applications in acidic foods and yogurt compared with P/N 601379. In general, the viability of *Lb. acidophilus* is known to be affected by low pH environment and a rapid decrease in variability has been observed under acidic conditions, both in vitro and in vivo (Conway et al., 1987; Shah and Jelen, 1990). Indeed, *Lb. acidophilus* is not as acid-tolerant as Lb. *delbrueckii* subsp. *bulgaricus* (Shah et al., 1995) and its growth ceases at pH 4.0 (Playne, 1993).

Adaptation to acidic environment did not appear to increase the sensitivity to -lactams and vancomycin in any of 12 tested acid- adapted variants (Table 3). However, resistance to ampicillin, cloxacilline and penicillin G increased for 2 (17%), 8 (67%) and 3 (25%) of tested variants, respectively. Among variants with increased resistance to -lactams, acid-adapted *Lb. acidophilus* R052 showed the highest resistance to cloxacilline and being inhibited at a concentration of 125 μg/mL, which is approximately 125-fold higher than concentration required to inhibit its un-adapted strain (0.98 μg/mL). Vancomycin MIC values for acid-adapted variants were almost identical to those reported for un-adapted strains.

For aminoglycosides, the MIC values for kanamycin and streptomycin for acid-adapted variants were identical to those reported for un- adapted strains. However, resistance to neomycin and paromomycin were deve-loped against each antibiotic in 2 acid-adapted variants. *Lb. bulgaricus* P/N 601383 was the only strain that can develop resistance against both antibiotics after acid-adaptation and being inhibited at MIC values of 2-fold higher of each antibiotic compared with un-adapted strain. Of 12 acid adapted variants, 6 (50%) and 1 (8.5%) became more susceptible to neomycin and paromomycin, respectively, and appeared to be inhibited at concentration equivalent to 2-fold lower compared with their corresponding un-adapted organisms.

On the other hand, acid adaptation induced the susceptibility to chloramphenicol for 5 (42%) of 12 acid-adapted variants, but did not induce resistance to this antibiotic in any of the tested variants. Resistance to erythromycin and tetracycline was also induced for 6 (50%) and 9 (75%) of tested 12 acid-adapted variants, respectively. Among strains with increased resistance to these antibiotics, *Lb. acidophilus* R052 developed the highest resistance against erythromycin and tetracycline and being inhibited at 125 μg/mL of such antibiotic, while

Table 4. Minimum inhibitory concentrations[a] (μg/mL) of antibiotics for acid-stressed[b] lactobacilli.

Strains	Antibiotics[c]													
	Amp	**Clo**	**Pen**	**Van**	**Kan**	**Neo**	**Par**	**Str**	**Chl**	**Ery**	**Tet**	**Nal**	**Nov**	**Nis**
Lb. acidophilus R052	62.5	0.98	0.98	0.98	7.8	125	250	250	0.98	0.98	0.98	>500	0.98	0.98
Lb. acidophilus P/N 601379	3.9	7.8	0.98	>500	250	250	250	250	3.9	0.98	0.98	>500	0.98	0.98
Lb. bulgaricus P/N 601383	250	125	15.6	>500	500	500	500	500	125	3.9	125	>500	0.98	0.98
Lb. casei R0256	3.9	31.2	0.98	>500	500	250	250	250	7.8	0.98	7.8	>500	0.98	0.98
Lb. casei R0215	3.9	15.6	0.98	>500	250	250	250	250	15.6	0.98	3.9	>500	0.98	0.98
Lb. casei paracasei subsp. *paracasei* P/N 601385	**Could not survive acid stress**													
Lb. delbrueckii subsp. *lactis* R0187	125	7.8	0.98	>500	500	250	250	250	3.9	0.98	125	>500	0.98	0.98
Lb. plantarum R1096	125	250	3.9	>500	500	250	250	250	125	0.98	125	>500	125	7.8
Lb. plantarum R1078	125	250	3.9	>500	500	250	250	250	125	0.98	250	>500	125	7.8
Lb. plantarum R0202	125	250	3.9	>500	500	250	125	125	125	0.98	250	>500	125	7.8
Lb. plantarum P/N 601387	125	125	15.6	>500	500	250	125	250	125	1.9	125	>500	0.98	0.98
Lb. rhamnosus R0011	3.9	7.8	0.98	>500	>500	250	500	250	7.8	0.98	7.8	>500	0.98	0.98
Lb. rhamnosus R1039	1.9	62.5	1.9	>500	500	250	250	250	250	7.8	250	>500	1.9	7.8

[a]Medians of 4 repetitions.

[b]Stressed for 90 min at pH 2.0 at 37˚C and cultured in MRS broth at pH 6.5 (18 h/37˚C) prior to testing their post-challenge susceptibility to antibiotics.

[c]Amp, ampicillin; Clo, Cloxacilline; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nal, nalidixic acid; Nov, novobiocin and Nis, nisin A.

its un-adapted variant was inhibited at 0.98 μg/mL. The sensitivity to nalidixic acid, novobiocin and nisin A did not appear to be affected by acid adaptation. The MIC values for nalidixic acid, novobiocin and nisin A for acid adapted variants were 250->500, 0.98 and 0.98- 3.9 μg/mL, respecttively, which were identical to those values determined for un-adapted strains.

The mechanism of acid adaptation of lactobacilli has not been fully elucidated but appears to be associated with the fatty acid composition of the cell membrane. The un-saturation level of cellular membrane lipid has been reported to be the most important response to various stressful conditions such as low pH, temperature and oxidative stress (Booth and Kroll, 1989; Guerzoni et al., 2001). Adaptation to acid has been shown to be associated with a decrease in the ratio of un-saturated to saturated fatty acids, which decrease the fluidity of the membrane and so protect the cell from the low pH (Bodnauk and Golden 1996; Brown et al., 1997) . Indeed, there is no available reposts on the impact of acid adaptation of antibiotic susceptibility of lactobacilli or any other lactic bacteria. However, changes in antibiotic susceptibility of acid-adapted lactobacilli, reported in this study, compared with un-adapted strains may be attributed to changes in the fluidity of cell membrane due to acid habituation process.

Antibiotic susceptibility of acid-challenged lactobacilli

The MIC data of acid-challenged lactobacilli are shown in Table 4. It is obvious that acid challenge (pH 2.0 for 90 min) induced the resistance of tested lactobacilli to -lactam antibiotics. Of 12 acid-challenged variants, 10 (83%), 8 (66%) and 5 (42%) showed more resistance against ampicillin, cloxacilline and penicillin, respectively. Resistance to these antibiotics was more evident among *Lb. plantarum* strains. The MIC values for ampicillin, cloxacilline and penicillin determined for the 4 tested strains of *Lb. plantarum* increased by 62 –125, 4-8 and 2-8 times, respectively, compared with values for un-stressed strains. The susceptibility to vancomycin remainned relatively constant, except for *Lb. acidophilus* P/N 601379 which became resistant to a concentration of 500 μg/mL (its un-stressed variant inhibited at a concentration of 0.98 μg/mL).

On the other hand, the increased resistance to aminoglycosides due to acid challenge condition was less evident compared with - lactams. Acid challenge increased the MIC values for neomycin, paromomycin and streptomycin for 3 (25%), 5 (42%) and 4 (33%) strains, respectively. On contrary, the sensitivity to kanamycin induced in 2 (17%) strains after acid challenge. This was more evident in the case of *Lb. acidophilus* R052 which being inhibited at a concentration of 7.8 μg/mL instead of 125 μg/mL for un-stressed strain.

Acid challenge also increased the resistance to chloramphenicol and tetracycline but not to erythromycin to which the MIC values of all tested variants were identical to those determined for un-stressed strains. The incidentce of chloramphenicol resistance was slightly higher than that for tetracycline. Of 12 acid-challenged variants, 10 (83%) and 8 (66%) showed increased resistance to chloramphenicol and tetracyclinee, respectively. The resis -tance to nalidixic acid was slightly increased for both *Lb. acidophilus* strains but remained constant for other tested lactobacilli. The resistance to novobiocin

Table 5. Minimum inhibitory concentrations[a] (μg/mL) of antibiotics for probiotic strains of lactobacilli in the presence of 0.3% (w/v) oxgall.

Strains	Antibiotics[b]													
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nal	Nov	Nis
Lb. acidophilus R052	0.98	3.9	0.98	0.98	125	15.6	31.2	7.8	3.9	3.9	0.98	500	0.98	0.98
Lb. acidophilus P/N 601379	0.98	125	0.98	0.98	125	15.6	31.2	7.8	125	125	125	500	0.98	0.98
Lb. bulgaricus P/N 601383	1.9	125	15.6	>500	250	31.2	125	125	3.9	3.9	125	>500	0.98	0.98
Lb. casei R0256	1.9	31.2	0.98	>500	250	62.5	125	125	1.9	1.9	62.5	>500	0.98	0.98
Lb. casei R0215	1.9	15.6	0.98	>500	125	31.2	62.5	62.5	3.9	0.98	0.98	>500	0.98	125
Lb. casei paracasei subsp. *paracasei* P/N 601385	0.98	3.9	0.98	125	62.5	15.6	62.5	62.5	3.9	1.9	0.98	>500	0.98	0.98
Lb. delbrueckii subsp. *lactis* R0187	3.9	62.5	7.8	>500	250	62.5	125	125	3.9	1.9	62.5	>500	0.98	0.98
Lb. plantarum R1096	3.9	62.5	7.8	>500	250	62.5	125	250	3.9	1.9	62.5	>500	0.98	0.98
Lb. plantarum R1078	1.9	62.5	1.9	>500	125	62.5	125	125	3.9	1.9	125	>500	0.98	0.98
Lb. plantarum R0202	0.98	62.5	1.9	>500	125	31.2	62.5	62.5	1.9	3.9	125	>500	0.98	0.98
Lb. plantarum P/N 601387	0.98	31.2	0.98	>500	125	31.2	62.5	62.5	1.9	1.9	31.2	>500	0.98	0.98
Lb. rhamnosus R0011	3.9	62.5	0.98	>500	250	62.5	125	125	1.9	3.9	125	>500	0.98	0.98
Lb. rhamnosus R1039	3.9	62.5	3.9	>500	250	62.5	125	125	3.9	15.6	62.5	>500	0.98	0.98

[a]Medians of 4 repetitions.

[b]Amp, ampicillin; Clo, Cloxacilline; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nal, nalidixic acid; Nov, novobiocin and Nis, nisin A.

and nisin A was substantially increased among *Lb. plantarum* strains compared with other tested species of lactobacilli. Of 4 tested *Lb. plantarum* strains, 3 (75%) became inhibited at concentrations of 125 and 7.8 μg/mL of novobiocin and nisin A, respectively, instead of a concentration of 0.98 μg/mL of each substance for un-stressed strains.

Indeed, the impact of gastric stress on the antibiotic susceptibility of lactic acid bacteria is generally absent in the literature. Results reported in this study are the first to report on the effect of gastric acid exposure on the susceptibility of lactobacilli to different antibiotics. The antibiogram data of acid-stressed lactobacilli were remarkably different from those for un-stressed organisms. As for acid adaptation, changes in antibiogram profiles of acid-stressed lactobacilli may also be attributed to alterations in membrane lipid composition, structure and fluidity due to acid exposure. Variations in antibiotic susceptibility between acid-stress and un-stressed strains were quite expected, since it has been previously reported that acid- stressed variants of lactobacilli had distinct physiolo-gical properties compared with the parent cultures (Chou and Weimer, 1999).

Antibiotic susceptibility of lactobacilli in the presence of oxgall

The presence of 0.3% of oxgall during growth appeared to substantially alter the antibiotic susceptibility of tested lactobacilli in a strain-dependent manner (Table 5). The presence of oxgall increased the MICs values for ampicillin, cloxacilline and penicillin for 4 (31%), 8 (62%) and 5 (38%) of tested lactobacilli, respectively, but did not increase sensitivity to these - lactams in the remaining strains. Among tested strains, three, *Lb. bulgaricus* P/N 601383, *Lb. delbrueckii* subsp. *lactis* R0187 and *Lb. plantarum* R1096, showed remarkable resistance against the three - lactams. However, the highest resistance developed in the presence of oxgall against - lactams antibiotics was that shown by *Lb. acidophilus* P/N 601379 against cloxacilline. This strain inhibited at a concentration of 125 μg/mL of cloxacilline in the presence of oxgall instead of 0.98 μg/mL in the absence of oxgall.

The MICs for vancomycin did not change for the majority of tested lactobacilli, except for *Lb. casei paracasei* subsp. *paracasei* P/N 601385 which being inhibited at a concentration of 125 μg/mL although it tolerated a concentration of 500 μg/mL in the absence of oxgall.

Sensitivity to aminoglycosides increased in the presence of oxgall for the majority of tested strains. Of 13 strains, 12 (92%), 13 (100%), 12 (92%) and 10 (77%) became, respectively, sensitive to kanamycin, neomycin, paromomycin and streptomycin in the presence of oxgall with MICs values of approximately 2- to 4-fold lower compared with those values determined in the absence of oxgall.

Of 13 tested strains, 3 (23%), 11 (85%) and 9 (69%) developed resistance to chloramphenicol, erythromycin and tetracycline, respectively. While 4 (31%) become sensitive to chloramphenicol but none to erythromycin and tetracycline. Among strains with increased resistance, *Lb. acidophilus* P/N 601379 was the only strain that could develop resistance against the three antibiotics. MICs for chloramphenicol, erythromycin and tetracycline determined for this strain in the presence of oxgall were 125 μg/mL which were approximately 125-fold than

Table 6. Minimum inhibitory concentrations[a] (µg/mL) of antibiotics for oxgall-stressed[b] lactobacilli.

Strains	Antibiotics[c]													
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nal	Nov	Nis
Lb. acidophilus R052	0.98	0.98	0.98	0.98	125	125	62.5	7.8	1.9	0.98	0.98	>500	0.98	0.98
Lb. acidophilus P/N 601379	0.98	0.98	0.98	0.98	250	62.5	125	15.6	0.98	0.98	1.9	>500	0.98	0.98
Lb. bulgaricus P/N 601383	0.98	31.2	7.8	>500	500	125	125	250	3.9	1.9	15.6	>500	0.98	0.98
Lb. casei R0256	3.9	31.2	0.98	>500	500	250	250	250	3.9	0.98	15.6	>500	0.98	0.98
Lb. casei R0215	1.9	62.5	0.98	>500	500	125	250	125	3.9	0.98	15.6	>500	0.98	15.6
Lb. casei paracasei subsp. *paracasei* P/N 601385	1.9	3.9	0.98	>500	250	62.5	125	125	3.9	0.98	0.98	>500	0.98	0.98
Lb. delbrueckii subsp. *lactis* R0187	3.9	31.2	0.98	>500	500	125	250	250	1.9	0.98	31.2	>500	0.98	0.98
Lb. plantarum R1096	1.9	62.5	7.8	>500	500	125	250	250	3.9	0.98	15.6	>500	0.98	0.98
Lb. plantarum R1078	3.9	31.2	1.9	>500	500	250	250	250	1.9	1.9	31.2	>500	0.98	0.98
Lb. plantarum R0202	0.98	7.8	3.9	>500	500	125	125	125	3.9	0.98	7.8	>500	0.98	0.98
Lb. plantarum P/N 601387	0.98	15.6	0.98	>500	500	250	250	250	1.9	0.98	15.6	>500	0.98	0.98
Lb. rhamnosus R0011	3.9	31.2	3.9	>500	500	250	250	250	3.9	0.98	125	>500	0.98	0.98
Lb. rhamnosus R1039	3.9	62.5	15.6	>500	500	500	250	500	3.9	0.98	62.5	>500	0.98	0.98

[a] Medians of 4 repetitions.

[b] Stressed by 0.3% (w/v) oxgall for 90 min at pH 6.5 at 37˚C and cultured in MRS broth at pH 6.5 (18 h/37˚C) prior to testing their post-challenge susceptibility to antibiotics.

[c] Amp, ampicillin; Clo, Cloxacilline; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nal, nalidixic acid; Nov, novobiocin and Nis, nisin A

in the oxgall- free tests. The MIC values for nalidixic acid increased for both *Lb. acidophilus* strains and became 2- to 4-fold higher compared with values determined in the absence of oxgall. While, MIC values for the other 11 strains remained at >500 µg/mL. For novobiocin, all strains inhibited at a concentration of 0.98 µg/mL which is the same required for inhibiting tested strains in the absence of oxgall. On the other hand, the MIC values for nisin A did not change for 12 strains and remained at 0.98 µg/mL. Only *Lb. casei* R0215 developed a substantial resistance against nisin A in the presence of oxgall and being inhibited at a concentration of 125 µg/mL which is 32- fold higher than the value determined for the same strain in the absence of oxgall.

The changes in antibiotic susceptibility of tested lactobacilli grown in the presence of oxgall may be attributed to modifications in cell membrane permeability caused by oxgall, which is known to enhance cell envelope permeability (Appelbaum and Chatterton, 1978). The presence of bile salts in the culture medium has been reported to affect both the glycolipids and phospholipids fractions of the cell membrane. In response to the presence of bile, *L reuteri* cells increased the amount of low melting-point unsaturated fatty acids, e.g. $C_{18:1}$ and decreased the content in $C_{16:0}$ and C_{19}-cyclic acids (Taranto et al., 2003). The $C_{18:1}$ is known to enhance the fluidity and permea-bility of the cell membrane (Fèrnandez Murga et al., 1999), while $C_{16:0}$ and C_{19}- cyclic fatty acids are involved in its rigidity (Annous et al., 1999). According to Sinensky (1984), all these modifications in the lipid pattern would enable the cells to maintain constant membrane fluidity in harmful environments, which is fundamental for cellular functions.

Since, the presence of oxgall can modify the fluidity of the cell membrane, remarkable changes in the antibiogram of lactobacilli are thus being expected. In accordance to results reported herein, the presence of oxgall (0.5%, w/w) has been reported to increase susceptibility of bifidobacteria and lactobacilli to some antibiotics, especially aminoglycosides, (Charteris et al., 2000). The authors observed that lactobacilli loss their resistance to gentamycin, kanamycin and streptomycin in the presence of oxgall. This was attributed to the increased enhancement of cell envelope permeability caused by oxgall leading to facilitated aminoglycoside penetration.

Antibiotic susceptibility of oxgall-challenged lactobacilli

The antibiogram data of oxgall-stressed lactobacilli are presented in Table 6. Of 13 lactobacilli tested, oxgall stress treatment increased resistance to ampicillin for 6 (46%) and to cloxacilline and penicillin for 5 (38.5%) strains. Oxgall stress did not induce the sensitivity to ampicillin and penicillin in any of tested lactobacilli. While 3 (23%) strains, *Lb. bulgaricus* P/N 601383 and *Lb. plantarum* R0202 and *Lb. plantarum* P/N 601387, became slightly susceptible to cloxacilline after oxgall stress treatment. These strains were inhibited at concentrations respectively 2-, 4- and 2-fold lower than those for their un-stressed equivalent strains.

Oxgall stress did not substantially change the suscepti-

bility of lactobacilli to aminoglycosides compared with trails where it was added to the growth media. Also, oxgall-stressed strains responded differently to each antibiotic belonging to this group. Kanamycin MIC values did not change by oxgall treatment and remained identical to those for un-stressed strains. Five strains became more susceptible to neomycin after the oxgall treatment with MIC values of 2-fold lower compared with un-stressed strains. While, 3, 3 and 2 strains, respectively, appeared to be inhibited at neomycin, paromomycin and streptomycin concentrations equivalent to 2-fold higher compared with those concentrations required to inhibit their un-stressed strains.

Of 13 oxgall-stressed lactobacilli, 3 (23%), 3 (23%) and 2 (15%) became more susceptible to chloramphenicol, erythromycin and tetracycline, respectively. Among these antibiotic, resistance to tetracycline was more evident than for the two other antibiotics. The oxgall stress treatment increased the MIC of tetracycline, chloramphenicol and erythromycin for 8 (61.5%), 2 (15.3%) and 1 (7.7%) strains, respectively.

Oxgall stress did not affect the susceptibility of lactobacilli to vancomycin and novobiocin, Also, susceptibility to nisin A did not change, except for *Lb. casei* R0215 which was inhibited at a concentration equivalent to 4-fold higher than that for its un-stressed strain. Following oxgall stress, *Lb. acidophilus* R052 and *Lb. acidophilus* P/N 601379 did not appear to be inhibited at nalidixic acid concentration of 500 µg/mL, while their equivalent un-stressed strains were inhibited at concentrations of 250 and 125 µg/mL, respectively.

This study showed that the short-term (90 min) exposure to oxgall resulted in less pronounced changes in antibiotic susceptibility of lactobacilli compared with long-term exposure where it was added to the growth media (results presented in Tables 5 and 6). In a previous study, the exposure of *L. reuteri* CRL 1098 to oxgall for 90 min was reported to produce changes in the cellular ultra-structure as visualized by the electron microscopy (Valdez et al., 1997). These changes were evident by the presence of randomly distributed folds and buds on the cell membrane. These modifications may affect not only the cell permeability and viability but also the interactions between the membrane and the environment (Kociubinski et al., 2002). Also, these ultra-structure modifications might be responsible for the changes in antibiotic susceptibility of oxgall-stressed lactobacilli.

Conclusion

This study shows that lactobacilli responded differently to both acid and oxgall stress and tolerance to these substances was strain-dependent. It clearly appears in this study that both acid and oxgall can drastically modify antibiotic susceptibility/resistance profile of lactobacilli depending on stress applied and on the antibiotic and strain tested. Also, changes in antibiotic susceptibility of lactobacilli are substantially different for the same stress agent depending on the time of exposure (e.g. short- or long-term exposure to 0.3% oxgall).

The success of lactobacilli to achieve the desired probiotic effects including maintaining healthy intestinal ecosystem and reducing the incidence of intestinal disorder, for example antibiotic-associated diarrhea, would largely depend on their ability to survive the gastrointestinal stressful conditions along with the given antibiotic. Consequently, the selection of a *Lactobacillus* strain for a probiotic application as prophylactic agent must take into account changes in its susceptibility to antibiotics due to various stressors encountered in the gastrointestinal tract.

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