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Antibiotic Susceptibility Profile of Bifidobacteria as Affected by Oxgall, Acid, and Hydrogen Peroxide Stress▽

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The effects of acid, oxgall, and H_2O_2 on susceptibilities to antibiotics and nisin were examined for 13 strains of bifidobacteria. Susceptibilities to ampicillin, cloxacillin, penicillin, vancomycin, kanamycin, neomycin, paramomycin, streptomycin, chloramphenicol, erythromycin, tetracycline, and nisin A were assayed by a microdilution broth method. Acid-, oxgall- and H_2O_2-stressed variants were produced and assayed. Exposure to a pH of 2.0 for 60 min reduced susceptibilities to cloxacillin and nisin A but increased susceptibilities to ampicillin, vancomycin, aminoglycosides, chloramphenicol, and erythromycin in a strain-dependent manner. Exposure to oxgall (0.3%) for 90 min increased susceptibilities to cell wall-directed antibiotics and aminoglycosides but increased resistances to tetracycline and nisin A. Oxidative stress increased the susceptibilities of 70% of the strains to ampicillin and chloramphenicol, of 50% of the strains to cloxacillin and tetracycline, and of 40% of the strains to erythromycin but did not affect susceptibilities to vancomycin, kanamycin, and nisin A. This study shows that exposure of bifidobacteria to stressful conditions resembling those in the gastrointestinal tract may substantially modify their susceptibilities to antibiotics and may thus affect their probiotic capacities, especially when they are used for the management of intestinal infections and antibiotic-associated diarrhea.

The discovery of bifidobacteria dates back to the beginning of the last century, when they were first isolated from the feces of breast-fed infants by Tissier (30) and originally named *Bacillus bifidus communis*. Bifidobacteria were later placed in the genus *Lactobacillus* as *Lactobacillus bifidum* and became an independent genus in the 1960s. Today, the genus *Bifidobacterium* is classified in the family *Actinomycetaceae* and includes 30 species (18). It is the predominant bacterial group in the normal intestinal flora of healthy breast-fed newborns, in which it constitutes more than 95% of the total population (32). However, the population of these organisms gradually decreases in number from the time of weaning and may account for 25% of the total intestinal flora in healthy adults (23). Bifidobacteria are nonpathogenic (except for *Bifidobacterium dentium*) and are considered promising probiotic organisms because of their potential role in promoting certain health benefits in the intestinal tracts of humans (17). Several studies have clearly indicated that bifidobacteria can be effective at preventing and alleviating antibiotic-associated or rotavirus-induced diarrhea in infants (4, 12). This has led to their increased use in the production of fermented-milk products and pharmaceutical microbial supplements.

Antibiotic therapy can significantly affect the microbial balance in the intestine and especially can reduce the viability of indigenous bifidobacteria (1). Restoring the microbial balance and reestablishing bifidobacteria is considered necessary in order to prevent increases in the numbers of intestinal pathogens and resulting diarrhea. Black et al. (4) reported that administering *B. longum* reduced the incidence of ampicillin-associated diarrhea and the time required for recolonization of the intestine. Feeding yogurt containing *B. longum* during erythromycin treatment has been reported to reduce the time required to recover from rotavirus diarrhea (11). Successful use of bifidobacteria as a prophylactic against intestinal disorders in general depends on their ability to survive under gastrointestinal conditions, tolerate antibiotic treatment, and compete with, suppress, and eliminate intestinal pathogens.

During their passage through the gastrointestinal tract, bifidobacteria encounter several stressful conditions, any of which may, besides reducing their viability, modify their physiological activities and antimicrobial susceptibilities. Any changes likely to occur in their antimicrobial susceptibilities during gastrointestinal passage should be considered when one is selecting them for prophylactic applications where specific antimicrobial agents are involved. In the present study, we examined changes in susceptibilities to 12 antimicrobial agents among 13 bifidobacteria due to stresses caused by acid, oxgall, and H_2O_2.

MATERIALS AND METHODS

Bacterial strains and growth media. *Bifidobacterium animalis* ATCC 27536, *B. bifidum* ATCC 15696, *B. breve* ATCC 15700, *B. infantis* ATCC 15697, *B. longum* ATCC 15707, *B. longum* ATCC 15708, *B. pseudolongum* ATCC 25562, and *B. thermophilum* ATCC 25866 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). *B. bifidum* R0071, *B. breve* R0070, and *B. longum* R0175 were obtained from Rosell Lallemand Institute Inc. (Montreal, PQ, Canada). *B. bifidum* BB12 and *B. longum* P/N 601377 were obtained from

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TABLE 1. Viable counts of bifidobacteria before and after acid, oxgall, or hydrogen peroxide challenge in MRS broth

Organism	Viable count of bifidobacteria (log_{10} CFU/ml on MRS agar) upon challenge with:					
	Acid[a]		Oxgall[b]		H_2O_2[c]	
	0	60 min	0	90 min	0	90 min
B. animalis ATCC 27536	8.73 ± 0.07	7.37 ± 0.18	8.16 ± 0.11	7.94 ± 0.06	8.59 ± 0.05	8.21 ± 0.24
B. bifidum R 0071	8.54 ± 0.06	3.70 ± 0.02	8.17 ± 0.08	8.14 ± 0.05	8.15 ± 0.15	7.17 ± 0.18
B. bifidum ATCC 15696	7.25 ± 0.14	<2.00	6.95 ± 0.02	6.84 ± 0.09	7.45 ± 0.26	<2.00
B. bifidum BB12	8.25 ± 0.19	6.07 ± 0.07	8.39 ± 0.08	8.13 ± 0.04	8.17 ± 0.07	8.19 ± 0.06
B. breve ATCC 15700	8.25 ± 0.13	3.79 ± 0.10	8.79 ± 0.13	8.52 ± 0.15	8.62 ± 0.12	8.63 ± 0.04
B. breve R0070	8.10 ± 0.17	3.69 ± 0.08	8.61 ± 0.15	8.36 ± 0.14	8.16 ± 0.11	7.63 ± 0.19
B. infantis ATCC 15697	8.48 ± 0.28	3.99 ± 0.01	8.33 ± 0.35	7.46 ± 0.15	8.37 ± 0.18	<2.00
B. longum ATCC 15707	8.65 ± 0.24	3.40 ± 0.01	8.47 ± 0.07	8.11 ± 0.10	8.19 ± 0.08	8.00 ± 0.06
B. longum ATCC 15708	8.09 ± 0.35	<2.00	8.32 ± 0.20	8.19 ± 0.19	8.64 ± 0.07	8.01 ± 0.07
B. longum R0175	8.39 ± 0.05	3.49 ± 0.04	8.92 ± 0.08	8.89 ± 0.10	8.70 ± 0.12	8.53 ± 0.23
B. longum P/N 601377	8.05 ± 0.10	3.69 ± 0.04	8.33 ± 0.35	8.15 ± 0.18	8.30 ± 0.30	8.15 ± 0.03
B. pseudolongum ATCC 25562	8.46 ± 0.15	7.78 ± 0.11	7.88 ± 0.08	7.46 ± 0.15	8.60 ± 0.11	<2.00
B. thermophilum ATCC 25866	8.37 ± 0.18	3.69 ± 0.08	8.09 ± 0.06	7.83 ± 0.07	8.27 ± 0.11	8.15 ± 0.06

[a] MRS broth at pH 2.0, incubated anaerobically.
[b] MRS broth plus 0.3% (wt/vol) oxgall, at pH 6.5, incubated anaerobically.
[c] MRS broth plus H_2O_2 at the MIC, at pH 6.5, incubated anaerobically.

Chr. Hansen Ltd. (Barrie, Ontario, Canada). All strains were kept in 20% glycerol at −80°C. All bacteria were activated, cultured, and tested in the medium of de Man, Rogosa, and Sharpe (MRS), obtained from Rosell-Lallemand Institute Inc. (Montréal, PQ, Canada), supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO). Organisms were incubated anaerobically in jars using an atmosphere generation system (Oxoid AnaeroGenTM; Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C. Prior to the beginning of the experiments, each bacterial strain was subcultured at least three times (1%, vol/vol) at 24-h intervals.

Antibiotics. Erythromycin, penicillin G sodium salt, tetracycline hydrochloride, streptomycin sulfate, chloramphenicol, vancomycin, kanamycin, neomycin sulfate, and paromomycin sulfate were all obtained from Sigma Chemical Company. Cloxacillin sodium salt was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Ampicillin, as a sodium salt, was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Nisin A was provided by Alpin & Barrett Ltd. (Beaminster, United Kingdom). Stock solutions of each test antibiotic were prepared freshly in water or ethanol (70%, vol/vol) according to the solubility index at an initial concentration of 1 mg/ml, filtered through 0.22-μm-pore-size membranes (Cameo 25 N; MSI, Westboro, MA), and kept at 4°C for a maximum of 2 days.

Oxgall and H_2O_2 tolerance. The tolerance of bifidobacteria to oxgall and H_2O_2 was tested using sterile flat-bottom 96-well microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ) as described previously (13). Results were expressed as the lowest concentration of oxgall or H_2O_2 that completely inhibited the tested organism (giving an optical density [OD] equal to that of uninoculated broth).

Acid, oxgall, and H_2O_2 challenge. Approximately 10 ml of mid-log-phase MRS cultures of each bifidobacterial strain was centrifuged at 6,000 × *g* for 15 min at 4°C. Cell pellets were resuspended in an equal volume of either (i) MRS broth adjusted to pH 2.0 using 1 M HCl (for acid challenge), (ii) MRS broth (initial pH 6.5) containing 0.3% (wt/vol) oxgall, or (iii) MRS broth (initial pH 6.5) containing H_2O_2 at the MIC for each strain. Bacterial suspensions were incubated anaerobically at 37°C for 60 min for acid challenge and 90 min for oxgall and H_2O_2 challenges. Viable counts were determined before and after incubation by diluting samples in peptone water (0.1%, wt/vol) and plating onto MRS agar. Plates were incubated anaerobically at 37°C for 48 h. Bacteria were subcultured in MRS broth (initial pH 6.5) immediately after challenge and were incubated anaerobically at 37°C for 24 h prior to testing of their postchallenge susceptibilities to antibiotics.

Susceptibilities to antibiotics and nisin A. The susceptibilities of bifidobacteria to antimicrobial agents were measured as MICs by a microplate assay described previously (24). Bacteria were grown to the mid-log phase in MRS broth containing 0.05% L-cysteine–HCl. The OD at 650 nm (OD_{650}) of the culture was adjusted to 0.1 with fresh MRS broth by using a Spectronic 20 spectrophotometer (Bausch & Lomb. Inc., Rochester, NY). Viable cells in the OD-adjusted inoculum were enumerated by plating 10-fold dilutions (in peptone water, 0.1% [wt/vol]) on MRS agar (incubated anaerobically at 37°C for 48 h). The viable count was thus found to range from 5×10^5 to 1×10^6 CFU/ml.

A serial twofold dilution of 150 μl of antimicrobial agent was done in a 96-well polystyrene microplate (Becton Dickinson Labware) containing 150 μl/well of MRS broth. A standardized bacterial suspension (30 μl) was then added to each well. This volume corresponded to approximately 2.5×10^4 to 5.0×10^4 CFU/well, which is within the range recommended by the CLSI (formerly the National Committee for Clinical Laboratory Standards) (25) as a standard inoculum density for the determination of antibiotic MICs by the microdilution method. The microplates were incubated anaerobically at 37°C for 18 h, and the OD_{650} was read using a Thermomax microplate reader (Molecular Devices, Opti-Resources, Charny, PQ, Canada). Controls (wells inoculated with the tested culture without any added antimicrobial agent) and blanks (wells containing uninoculated broth medium with an added antimicrobial agent) were run on each microplate. The MIC was the lowest concentration of the tested agent giving complete inhibition of growth (an OD equal to the OD of the blank). The microplate assay was repeated four times for each antimicrobial-bacterium combination, and the MIC was determined as the median of the four repetitions.

RESULTS

Tolerance of oxgall and H_2O_2. All strains tested in this study survived oxgall concentrations of 0.3%. They showed more variable susceptibility to H_2O_2, with MICs ranging from 0.3 to 38.4 μg/ml. Tolerance to H_2O_2 appears to be more strain dependent than species dependent.

Acid, oxgall, and H_2O_2 challenge. The survivability of bifidobacteria during different chemical stresses is shown in Table 1. Acid appeared to be more damaging than oxgall or H_2O_2. The 60-min exposure to a pH of 2.0 reduced viable counts by less than 1 log cycle or by more than 6 log cycles, depending on the bacterial strain.

The 90-min exposure of bifidobacteria to oxgall (0.3%) reduced viable counts by only 0.2 to 0.5 log cycles. In contrast, the 90-min exposure to H_2O_2 at the MIC for each strain resulted in drastic 5- to 6-log-cycle losses in viable counts of some strains.

Bifidobacterial antibiograms. The antibiograms of the various bifidobacteria are presented in Table 2. The strains tested in this study were resistant to kanamycin and vancomycin up to concentrations of 500 μg/ml and to neomycin and paromomy-

TABLE 2. MICs of antibiotics for bifidobacteria

Organism	MIC (μg/ml)[a] of the following antibiotic[b] for the indicated organism:											
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nis
B. animalis ATCC 27536	15.6	3.9	0.98	500	500	250	250	250	7.8	3.9	3.9	0.98
B. bifidum R0071	1.9	15.6	3.9	500	500	250	250	250	1.9	1.9	31.2	0.98
B. bifidum ATCC 15696	0.98	7.8	0.98	>500	>500	250	250	250	1.9	7.8	31.2	0.98
B. bifidum BB12	31.2	0.98	0.98	>500	>500	250	250	250	1.9	15.6	15.6	0.98
B. breve ATCC 15700	3.9	15.6	0.98	>500	>500	250	250	250	7.8	1.95	31.2	0.98
B. breve R0070	15.6	31.2	1.9	500	500	250	250	15.6	7.8	0.98	62.5	0.98
B. infantis ATCC 15697	7.8	15.6	0.98	>500	>500	250	250	250	62.5	15.6	125	0.98
B. longum ATCC 15707	15.6	15.6	0.98	500	500	250	250	250	15.6	0.98	62.5	0.98
B. longum ATCC 15708	7.8	7.8	0.98	500	500	250	250	250	31.2	3.9	31.2	0.98
B. longum R0175	7.8	62.5	3.9	500	500	250	250	62.5	7.8	0.98	62.5	0.98
B. longum P/N 601377	0.98	31.2	0.98	>500	250	125	250	125	3.9	0.98	15.6	0.98
B. pseudolongum ATCC 25562	3.9	3.9	0.98	>500	>500	250	250	250	7.8	3.9	15.6	0.98
B. thermophilum ATCC 25866	0.98	15.6	0.98	>500	>500	250	250	250	7.8	0.98	62.5	0.98

[a] Median of four repetitions.
[b] Amp, ampicillin; Clo, cloxacillin; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nis, nisin A.

cin up to concentrations of 250 μg/ml. Ten of the 13 strains also resisted up to 250 μg/ml streptomycin. In a strain-dependent manner, bifidobacteria showed variable susceptibility to ampicillin (0.98 to 31.2 μg/ml), cloxacillin (0.98 to 62.5 μg/ml), chloramphenicol (1.9 to 62.5 μg/ml), erythromycin (0.98 to 15.6 μg/ml), and tetracycline (3.9 to 125 μg/ml). Penicillin G and nisin A proved to be the strongest inhibitors of bifidobacteria, with MICs of 0.98 to 3.9 μg/ml for the former and 0.98 μg/ml for the latter.

Antibiogram of acid-stressed bifidobacteria. Of 11 strains that survived acid stressing (pH 2.0 for 60 min), 9, 4, 2, and 3 strains became more susceptible to ampicillin, cloxacillin, penicillin G, and vancomycin, respectively (Table 3). For aminoglycosides, susceptibility to kanamycin, neomycin, paromomycin, and streptomycin increased by acid stressing for four, two, two, and five strains, respectively. Also, susceptibility to chloramphenicol, erythromycin, and tetracycline increased for eight, four, and five strains, respectively.

Antibiogram of oxgall-stressed bifidobacteria. Oxgall stress had a remarkable effect on the susceptibility of bifidobacteria to antibiotics (Table 4). Even within the same antibiotic group, the susceptibility to each antibiotic changed according to the strain tested. For β-lactams, the majority of the strains were more susceptible to ampicillin but more resistant to cloxacillin. Penicillin resistance did not increase for any of the 13 strains, but susceptibility increased slightly for 4 strains.

Susceptibility to kanamycin was hardly affected by oxgall stress, while susceptibility to neomycin, paromomycin, and streptomycin increased for eight, six and five strains, respectively. Oxgall stress increased resistance to neomycin for two strains, to paromomycin for four strains, and to streptomycin for two strains.

Oxgall stress increased the susceptibility of bifidobacteria to chloramphenicol and to some extent to erythromycin but conferred resistance to tetracycline and nisin A. Ten and four strains developed resistance to tetracycline and nisin A, respectively, after the oxgall treatment.

Antibiogram of H_2O_2-stressed bifidobacteria. Oxidative stress by H_2O_2 resulted in noticeable changes in the susceptibilities of bifidobacteria to β-lactams, depending on the anti-

TABLE 3. MICs of antibiotics for acid-stressed[a] bifidobacteria

Organism	MIC (μg/ml)[b] of the following antibiotic[c] for the indicated organism:											
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nis
B. animalis ATCC 27536	0.98	3.9	0.98	>500	500	250	500	125	1.9	1.9	62.5	3.9
B. bifidum R 0071	0.98	15.6	1.9	>500	0.98	250	250	250	7.8	0.98	31.2	1.9
B. bifidum ATCC 15696	Did not survive acid stress											
B. bifidum BB12	0.98	0.98	0.98	>500	250	125	125	125	1.9	3.9	0.98	0.98
B. breve ATCC 15700	0.98	125	0.98	>500	500	125	125	125	62.5	3.9	250	3.9
B. breve R0070	3.9	15.6	1.9	>500	500	250	250	125	3.9	0.98	31.2	3.9
B. infantis ATCC 15697	0.98	125	0.98	0.98	500	250	250	250	0.98	3.9	125	0.98
B. longum ATCC 15707	3.9	15.6	0.98	>500	500	500	500	250	7.8	0.98	62.5	0.98
B. longum ATCC 15708	Did not survive acid stress											
B. longum R0175	0.98	0.98	0.98	0.98	250	250	250	31.2	3.9	0.98	0.98	0.98
B. longum P/N 601377	0.98	0.98	0.98	>500	250	250	250	62.5	1.9	0.98	0.98	0.98
B. pseudolongum ATCC 25562	0.98	0.98	0.98	0.98	250	250	250	250	3.9	3.9	0.98	0.98
B. thermophilum ATCC 25866	3.9	31.2	0.98	>500	500	250	500	250	3.9	0.98	125	0.98

[a] Organisms were stressed for 60 min at pH 2.0, followed by 18 h of anaerobic growth in MRS broth, pH 6.5.
[b] Median of four repetitions.
[c] Amp, ampicillin; Clo, cloxacillin; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nis, nisin A.

TABLE 4. MICs of antibiotics for bifidobacteria after short-term oxgall stress[a]

Organism	MIC (μg/ml)[b] of the following antibiotic[c] for the indicated organism:											
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nis
B. animalis ATCC 27536	0.98	125	0.98	1.9	500	125	125	125	1.9	1.9	125	0.98
B. bifidum R 0071	0.98	62.5	0.98	>500	500	62.5	125	125	3.9	3.9	62.5	0.98
B. bifidum ATCC 15696	0.98	62.5	0.98	>500	125	250	500	1.9	3.9	3.9	125	0.98
B. bifidum BB12	0.98	1.9	0.98	>500	500	250	250	500	1.9	3.9	3.9	3.9
B. breve ATCC 15700	3.9	3.9	0.98	>500	500	125	250	250	3.9	0.98	31.2	0.98
B. breve R0070	0.98	31.2	0.98	>500	500	62.5	125	125	3.9	0.98	62.5	0.98
B. infantis ATCC 15697	0.98	125	0.98	0.98	>500	500	500	250	0.98	3.9	250	3.9
B. longum ATCC 15707	0.98	125	0.98	>500	250	125	125	62.5	1.9	3.9	125	0.98
B. longum ATCC 15708	0.98	125	0.98	0.98	500	500	500	250	0.98	1.9	125	0.98
B. longum R0175	0.98	125	0.98	>500	500	62.5	125	125	1.9	1.9	125	3.9
B. longum P/N 601377	0.98	62.5	0.98	>500	250	62.5	62.5	125	1.9	3.9	62.5	3.9
B. pseudolongum ATCC 25562	0.98	62.5	0.98	0.98	500	250	500	250	0.98	3.9	125	0.98
B. thermophilum ATCC 25866	1.9	62.5	0.98	>500	500	125	250	250	3.9	3.9	125	0.98

[a] Organisms were exposed to 0.3% (wt/vol) oxgall for 90 min prior to 18 h of anaerobic growth in MRS broth, pH 6.5.
[b] Median of four repetitions.
[c] Amp, ampicillin; Clo, cloxacillin; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nis, nisin A.

biotic and the strain tested (Table 5). Of the 10 strains that survived the challenge, 7, 5, and 3 became more susceptible to ampicillin, cloxacillin and penicillin G, respectively. Meanwhile, one strain developed slight resistance to ampicillin and two developed slight resistance to cloxacillin, but none developed resistance to penicillin G.

The MICs of vancomycin and kanamycin did not change for any strain after H_2O_2 treatment. However, neomycin, paromomycin, and streptomycin susceptibilities increased for three, two, and two strains, respectively. H_2O_2 stress induced resistance to neomycin for one strain and to paromomycin and streptomycin for four strains.

Seven strains became susceptible to chloramphenicol, four to erythromycin, and five to tetracycline, but none to nisin A. None increased their resistance to chloramphenicol, while increased resistance to erythromycin and nisin A was observed for one strain and increased resistance to tetracycline was observed for two strains.

DISCUSSION

Antibiotic susceptibilities of unstressed bifidobacteria. The antimicrobial susceptibility of probiotic candidates, particularly bifidobacteria, is a major criterion for selecting the organism that can best be used as a prophylactic against intestinal infection. Most evaluations of the antimicrobial susceptibility of bifidobacteria have focused on selective enumeration of bifidobacteria in fermented products and/or determining the alteration of antimicrobial susceptibility by extensive antibiotic treatments (3, 22).

The antibiogram data reported in this study for unstressed bifidobacterial strains are generally in agreement with the findings of previous studies of bifidobacterial susceptibility to antibiotics (22, 31) and with our previously reported results on the susceptibilities of commercial and infant isolates of bifidobacteria to 14 antibiotics and nisin A (19). In general, bifidobacteria were highly susceptible to penicillin G, erythro-

TABLE 5. MICs of antibiotics for hydrogen peroxide-stressed[a] bifidobacteria

Organism	MIC (μg/ml)[b] of the following antibiotic[c] for the indicated organism:											
	Amp	Clo	Pen	Van	Kan	Neo	Par	Str	Chl	Ery	Tet	Nis
B. animalis ATCC 27536	3.9	3.9	0.98	>500	500	500	500	250	3.9	0.98	62.5	0.98
B. bifidum R 0071	0.98	31.2	0.98	>500	500	125	125	250	1.9	500	62.5	0.98
B. bifidum ATCC 15696	Did not survive H_2O_2 stressing											
B. bifidum BB12	0.98	1.9	0.98	>500	500	250	500	250	1.9	0.98	7.8	0.98
B. breve ATCC 15700	3.9	3.9	0.98	>500	>500	250	500	500	1.9	0.98	31.2	0.98
B. breve R0070	0.98	15.6	0.98	>500	500	250	250	250	1.9	0.98	7.8	0.98
B. infantis ATCC 15697	Did not survive H_2O_2 stressing											
B. longum ATCC 15707	0.98	0.98	0.98	>500	500	250	500	62.5	1.9	0.98	7.8	0.98
B. longum ATCC 15708	3.9	7.8	0.98	>500	500	250	250	500	3.9	0.98	31.2	0.98
B. longum R0175	1.9	31.2	0.98	>500	500	125	250	250	1.9	0.98	15.6	1.9
B. longum P/N 601377	0.98	31.2	0.98	>500	250	125	125	125	1.9	1.9	125	0.98
B. pseudolongum ATCC 25562	Did not survive H_2O_2 stressing											
B. thermophilum ATCC 25866	3.9	7.8	0.98	>500	500	500	500	500	1.9	0.98	15.6	0.98

[a] Organisms were exposed for 90 min to H_2O_2 at the MIC prior to 18 h of anaerobic growth in MRS broth, pH 6.5.
[b] Median of four repetitions.
[c] Amp, ampicillin; Clo, cloxacillin; Pen, penicillin G; Van, vancomycin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Str, streptomycin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline hydrochloride; Nis, nisin A.

mycin, and nisin A, less susceptible to ampicillin, cloxacillin, chloramphenicol, and tetracycline, and resistant to vancomycin and aminoglycosides. Resistance of bifidobacteria to aminoglycosides and vancomycin has been reported previously (7). Resistance to aminoglycosides is common among gram-positive anaerobes and is attributed to the multiple cationic charges characterizing their membranes, making them impermeable to aminoglycosides (10). Resistance to vancomycin has been increasing and is reported frequently for lactic acid bacteria, including lactobacilli, pediococci, *Leuconostoc* spp., and bifidobacteria (7, 8, 16, 17, 28, 29).

All strains tested in this study were inhibited by 0.98 μg/ml of nisin A. Nisin has previously been shown to be a strong inhibitor of bifidobacteria. A concentration of 2 to 3 μg/ml was found to inhibit the growth of *B. thermophilum* ATCC 25866 and lactate production by this strain at less than 1 μg/ml (21).

Antibiotic susceptibility of acid-challenged bifidobacteria. Acidity, imposed by HCl in the stomach, is the most detrimental stress condition limiting the probiotic potential of bifidobacteria. In this study, tolerance of gastric pH (2.0) differed widely among species and even among strains belonging to the same species. Pochart et al. (27) noted a rapid decline in the viability of *Bifidobacterium* sp. strain BB at pH 1.0 and no survival after 1 h. Charteris et al. (6) evaluated the transient tolerance of eight bifidobacterial strains to simulated gastric juice (pH 2.0) and noted declines in survivability of 1.3 to 3.5 log CFU/ml after 3 h of acid exposure. Like other adverse conditions, acid stress can confer protection against other stressful conditions and alter physiological activities and susceptibility to antibiotics. However, little is known about the effect of acid stress on the physiological activity and antibiotic susceptibility of bifidobacteria. Our study showed that the antimicrobial susceptibility of acid-stressed variants differed remarkably from that of unstressed organisms. Generally, acid-stressed variants were more susceptible to ampicillin, vancomycin, aminoglycosides, chloramphenicol, and erythromycin but resistant to cloxacillin and nisin compared with unstressed organisms.

Effect of oxgall on the antibiotic susceptibility of bifidobacteria. Since our strains all tolerated 0.3% (wt/vol) oxgall, this concentration was chosen for the challenge. Previous studies have indicated that tolerance of oxgall differs widely among bifidobacteria. Noriega et al. (26), determining susceptibilities to sodium cholate, oxgall, and sodium deoxycholate, found that 17 strains were more susceptible to cholate and deoxycholate than to oxgall, although they were inhibited by oxgall at concentrations ranging from 0.12 to >2.0% (wt/vol).

The 90-min exposure of bifidobacteria to oxgall (0.3%) resulted in remarkable increases in susceptibility to aminoglycosides. Increased susceptibility of bifidobacteria to aminoglycosides in the presence of oxgall (0.5%, wt/wt) has been reported previously by Charteris et al. (7), who observed complete losses of resistance to gentamicin, kanamycin, and streptomycin for most strains. It has been reported previously that aminoglycoside uptake by bacteria can be enhanced in the presence of cell wall-directed antibiotics such as β-lactams, which facilitate aminoglycoside penetration (14). Similar synergistic effects can be provided by oxgall, which is known to enhance cell envelope permeability (2), leading to facilitated aminoglycoside uptake.

Effect of H_2O_2 on the antibiotic susceptibility of bifidobacteria. Tolerance to H_2O_2 is an important criterion for the selection of probiotic bifidobacteria, since H_2O_2 could seriously affect their viability in coculture with H_2O_2-producing organisms (e.g., lactobacilli), either in fermented foods or in the intestinal ecosystem. In yogurt, for example, 17 μg/ml H_2O_2 was found to be detrimental to bifidobacteria (9). H_2O_2 at a concentration of 40 μM was reported to reduce the growth of *B. thermophilum* ATCC 25866 and to abate lactate production at a concentration of 250 to 800 μM (20). The inhibitory action of H_2O_2 is attributed to the formation of highly reactive OH free radicals in the presence of iron or copper (15). These free radicals primarily attack polyunsaturated fatty acids directly in cell membranes and initiate lipid peroxidation, which leads to alterations in membrane properties and fluidity and disrupts membrane-bound proteins (5). This may explain the increased susceptibility of our bifidobacteria to cell wall-directed β-lactams, chloramphenicol, erythromycin, and tetracycline antibiotics after exposure to H_2O_2.

Conclusion. The bifidobacterial strains tested in this study showed variable susceptibility to inhibition by acid, oxgall, and H_2O_2. Exposing bifidobacteria to acid, oxgall, and oxidative stress by H_2O_2 resulted in substantial modifications in their antibiotic susceptibility/resistance patterns, in a strain-dependent manner. These modifications make it very difficult to select a single strain of bifidobacteria to be used prophylactically during antibiotic treatment. Thus, it would be more effective to use a probiotic formula containing several bifidobacterial strains with varied susceptibility/resistance patterns. On the other hand, since probiotic bifidobacteria will encounter several stressful conditions sequentially, a study of the impact of combined stresses on their antibiotic susceptibility would be helpful in selecting strains for prophylactic use.

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