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ScienceDirect
International Journal of Food Microbiology 115 (2007) 35–42
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products

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Received 3 January 2006; received in revised form 6 July 2006; accepted 3 October 2006

Abstract

The outlines of antibiotic resistance of some probiotic microorganisms were studied. This study was conducted with the double purpose of verifying their ability to survive if they are taken simultaneously with an antibiotic therapy and to increase the selective properties of suitable media for the isolation of samples containing mixed bacterial populations. We isolated from commercial dairy and pharmaceutical products, 34 strains declared as probiotics, belonging to the genera *Bifidobacterium* and *Lactobacillus*, and 21 strains of starter culture bacteria. All the microorganisms have been compared by electrophoresis of the soluble proteins for the purpose of identifying them. A Multiplex-PCR with genus- and species-specific primers was used to detect for *Bifidobacterium animalis* subsp. *lactis* presence. All bifidobacteria were *B. animalis* subsp. *lactis* except one *Bifidobacterium longum*. Sometimes the identification showed that the used strain was not the one indicated on the label. The lactobacilli were *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The streptococci were all *Streptococcus thermophilus*. The minimal inhibitory concentration (MIC) of 24 common antibiotic substances has been valued by the broth microdilution method.

All tested strains were susceptible to ampicillin, bacitracin, clindamycin, dicloxacillin, erytromycin, novobiocin, penicillin G, rifampicin (MIC_{90} ranging from 0.01 to 4 μg/ml); resistant to aztreonam, cycloserin, kanamycin, nalidixic acid, polymyxin B and spectinomycin (MIC_{90} ranging from 64 to >1000 μg/ml). The susceptibility to cephalothin, chloramphenicol, gentamicin, lincomycin, metronidazole, neomycin, paromomycin, streptomycin, tetracycline and vancomycin was variable and depending on the species.
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Keywords: *Bifidobacterium* spp.; *Lactobacillus* spp.; Antibiotic resistance; Probiotics

1. Introduction

The balance and composition of the intestinal microbiota is important for the well-being and the ability of our organism to resist the invasion of pathogens. To increase the natural resistance of the host to infections, probiotic microorganisms such as lactobacilli and bifidobacteria can be consumed.

The safety of these probiotic strains is essential, as is the impossibility to transfer to other bacteria their potential resistance to antimicrobial agents, since antibiotic resistance is an emerging issue.

Lactobacillus and *Bifidobacterium* species constitute a significant portion of probiotic cultures used in developed countries (Fuller, 1992). The actual safety criteria for successful probiotics have been defined in several reviews (Salminen et al., 1998; Adams, 1999; Saarela et al., 2000). These criteria are the following: strains for human use need to have a human origin and be isolated from healthy human gastrointestinal tract, they need to have a non-pathogenic history, not associated with diseases and do not carry transmissible antibiotic resistance genes.

Lactobacilli and bifidobacteria have a long history of safe use as microbial adjunct nutrition (Salminen et al., 1998). Therefore the characterization of these bacteria, particularly in regard to antimicrobial resistance, is often neglected. This practice could become a problem, considering the strong expansion of the probiotic market as well as the increasing microbial drug-resistance. In fact, since probiotic bacteria are added to different kinds of products, they represent a potential source for the spread of antibiotic resistance genes.

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0168-1605/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2006.10.003

Lactobacilli are an important part of the indigenous microbiota of man and animals. Although they have rarely been implicated in cases of infection (Bessis et al., 1995; Coronado et al., 1995), they are normally used technologically as food-associated because they are generally recognized as safe (GRAS) (Salminen et al., 1998).

Bifidobacteria are one of the common bacteria of the intestinal tract (Scardovi, 1986). They are helpful in maintaining a proper balance in the human intestinal flora, playing a protective role against potential pathogens and putrefactive bacteria, hence they have been included in the probiotics group (Biavati et al., 2000). Although they may rarely be implicated in human gastrointestinal and extraintestinal infections in the presence of predisposing factors (Charteris et al., 1997), only one species is considered pathogenic to man: *B. dentium* that causes dental caries (Sgorbati et al., 1995). This study has been carried out to make a contribution to enlarge knowledge about probiotic and starter culture bacteria added to commercial dairy and pharmaceutical products.

2. Materials and methods

2.1. Isolation of bacteria

The bacteria were isolated from 21 food samples derived from yogurts (n=17), yogurt-type fermented milk (n=1) and pharmaceutical products (n=3).

Lactobacillus delbrueckii subsp. *bulgaricus* (n=11), *Bifidobacterium* spp. (n=22) and *Streptococcus thermophilus* (n=10) were isolated on both non-selective media "de Man Rogosa Sharpe" (MRS) and "Trypticase–Phytone–Yeast Extract" (TPY) (Biavati and Mattarelli, 2001). The TPY contained (per liter) trypticase (10 g; BBL), phytone (5 g; BBL), glucose (15 g), yeast extract (2.5 g; Difco), tween 80 (1 ml; Merck), cysteine hydrochloride (0.5 g; Merck), di-potassium hydrogen phosphate (2 g; Merck), magnesiumchloride-hexahydrate (0.5 g; Merck) and agar-agar (15 g; Merck). *Lactobacillus acidophilus* (n=6) and *L. casei* (n=6) were obtained with the selective medium MRS–sorbitol agar (Shah, 2000). The MRS–sorbitol agar consisted of "de Man Rogosa Sharpe" (MRS) as basal medium, in which the dextrose was replaced by sorbitol. 10 ml of a filter sterilized 20% sorbitol solution was added to 90 ml (2% final concentration) of this modified MRS basal medium. The plates were anaerobically (BBL GasPak System) incubated for 72 h at 37 °C.

2.2. Identification of bacteria

All isolated bacteria were re-identified after their isolation from the food samples. The species designation was determined by colonial appearance, Gram stain, cell morphology and electrophoresis of the soluble proteins (Biavati et al., 1982). The genus *Bifidobacterium* was also confirmed by fructose-6-phosphate-phosphoketolase assay of cell-free extracts (Scardovi, 1986; Orban and Patterson, 2000). The bifidobacterial cultures were cultivated overnight at 37 °C in 10 ml of TPY broth. The cultures were washed twice (10,000 ×g, 4 °C, 15 min) with a phosphate buffer consisting of potassium di-hydrogen phosphate (0.05 M) and cysteine·hydrochloride (500 mg/l) solution (1:1, v/v) in water, adjusted to pH 6.5 with NaOH. The washed bacteria were resuspended with 1 ml of phosphate buffer. Prior to the phosphoketolase assay, the cells were incubated for 5 min with 0.4 ml of a cetrimonium bromide (CTAB, hexadecyl-trimethylammonium bromide) stock solution (450 µg/ml) for cell disruption (Orban and Patterson, 2000). After this, 0.25 ml of a sodium fluoride (NaF, 3 mg/l) and sodium iodoacetate (5 mg/ml) mixture in water was added. Also 0.25 ml of sodium fructose-6-phosphate solution (80 mg/ml in water) was added. Consequently the obtained solution was incubated at 37 °C for 30 min. Subsequently, 1.5 ml of hydroxylamine·HCl solution (13 g/100 ml) was added and the mix was incubated for 10 min at room temperature. Finally, 1 ml of trichloroacetic acid (TCA, 15 g/100 ml), 1 ml of 4 N HCl and 1 ml of ferric chloride ($FeCl·6H_2O$, 5 g/100 ml in 0.1 N HCl) were added. At this point the color change was evaluated: the development of a yellow color indicated the absence of a phosphoketolase activity while the immediate formation of a red-violet color indicated its presence and consequently a bifidobacterial presence.

After defining bifidobacteria at species level by using the electrophoresis of the soluble proteins method, a Multiplex-PCR approach as proposed by Ventura and Zink (2002) was used in order to allocate the resulting *Bifidobacterium animalis* at the subspecies level: *B. animalis* subsp. *lactis* or *B. animalis* subsp. *animalis* (Ventura and Zink, 2002).

The DNA extraction was conducted with the methodology of Rossi et al. (2000). The bacteria identified as *B. animalis*, were cultivated (37 °C, overnight) in 20 ml of TPY broth. The bacteria were collected by centrifugation (8000 ×g× 15 min) and washed with 3 ml of TE buffer (10 mM Tris–HCl, 1 Mm EDTA, pH 7.6). 1 ml of TE containing 15 mg of lysozyme (Sigma, L-7651, St. Louis, Mo., USA) was added to the pellet obtained after centrifugation. The resuspended pellet was incubated overnight at 37 °C. After this step, 3 ml of a lysis buffer (100 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2), 220 µl of sodium dodecyl sulfate (SDS, 10%, w/v) and 150 µl of a lysozyme stock solution (20 mg/ml in water) was added and the solution was incubated at 60 °C for 2 h. 1 ml of a NaCl-saturated solution wad added and the sample was gently inverted for 5 min. After a centrifugation step (6000 ×g× 15 min, room temperature), the clear surnatant was transferred to a new tube. The DNA was precipitated with cold ethanol (2.5 volumes) and the DNA was taken out with glass rod. The DNA was washed with ethanol 70% (w/v) and, after it has been dried, it was diluted in 300 µl of TE. All the DNA samples were stored at −20 °C.

The Multiplex-PCR was performed by using bifidobacterial genus-specific primers (primer Lm3 and primer Lm26) (Kaufmann et al., 1997) and *B. lactis* (Ventura et al., 2001) or *B. animalis* (Ventura and Zink, 2002) species-specific primers. PCR was conducted in a total volume of 50 µl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP (Fermentas), 10 pmol of Lm3 (5′-CGGGTGCTICCCACTTT-CATG-3′) (MWG Oligo Synthesis, Germany) and 10 pmol of Lm26 (5′-GATTCTGGCTAGGATGAACG-3′) (MWG Oligo Synthesis, Germany), 50 pmol of primer Ban2 (5′-CATATTG-GATCACGGTCG-3′) (MWG Oligo Synthesis, Germany)

specific for *B. animalis*, 50 pmol of 23Si (5′-CATTCGGA-CACCCTGGGATC-3′) (MWG Oligo Synthesis, Germany), 2. 5 U of Taq DNA polymerase (Fermentas) and 25 ng of template DNA. To detect the presence of *B. lactis*, we used *B. lactis*-specific primers in place of primer Ban2 and primer 23Si. The *B. lactis* Bflact2 (5′-GTGGAGACACGGTTTCCC-3′) and Bflact5 (5′-CACACCACACAATCCAATAC-3′) primers (MWG Oligo Synthesis, Germany), were used at the same conditions and quantity as the *B. animalis* primers. The amplification program consisted of 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 54 °C for 1 min, 72 °C for 1.5 min and 1 cycle of 72 °C for 7 min. Amplifications were carried out with a DNA thermocycler (BIOMETRA, T-Gradient 96). The amplification products were then separated by electrophoresis in 1.5% (w/v) agarose gel and ethidium bromide (0.5 μg/ml) staining was utilized to study the amplicons under UV transillumination.

The identified bacteria were stored at −135 °C (SANYO, ultra low temperature freezer, MDF-2136ATN) in 10% sterile skimmed milk (Biolife) and serially anaerobically transferred in "Trypticase–Phytone–Yeast Extract" (TPY) broth at 37 °C for 48 h three times prior to assay.

2.3. Antibiotics

The following 24 antimicrobial agents were used: ampicillin (A-9518), aztreonam (A-6848), bacitracin (B-0125), cephalotin (C-4520), D-cycloserin (30020), dicloxacillin (D-9016), penicillin G (P-3032), vancomycin (V-2002) as inhibitors of cell wall synthesis; clindamycin hydrochloride (C-5269), chloramphenicol (C-0378), erythromycin (E-6376), gentamicin sulfate (48760), kanamycin sulfate (K-4000), lincomycin (L-6004), neomycin sulfate (N-1876), paromomycin sulfate (P-9297), spectinomycin (S-9007), streptomycin sulfate (S-6501), tetracycline (T-3383) as inhibitors of protein synthesis; metronidazole (M-3761), nalidixic acid (N-8878), novobiocin (N-1628), rifampicin (R-3501) as inhibitors of nucleic acid synthesis and polymyxin B sulfate (P-1004) as inhibitors of cytoplasmic functions. All the antibiotic powders were obtained from Sigma (St. Louis, Mo., USA) except D-cycloserin and gentamicin sulfate that were obtained from Fluka (Buchs SG, Switzerland).

Each of the antibiotic powders was carefully weighed, dissolved, diluted in appropriate diluents and filter sterilized prior to addition to TPY medium. Serial dilutions of antibiotics ranging from 256 to 0.5 mg/l were prepared. The following concentrations were also tested: 1000, 500, 0.1, 0.01 mg/l.

2.4. Minimal inhibitory concentration determination

Minimal inhibitory concentration (MIC) values for all bacterial isolates were determined by the broth microdilution procedure (Phillips et al., 1991). The liquid medium used was TPY broth, since it supported the best growth of all bacteria used in this study. The TPY broth containing antibiotics at different concentrations was used to prepare each well of a micro-well plate. The inoculum was adjusted to a turbidity equivalent to 0.5 McFarland standard ($\approx 5\times10^5$ cfu/ml). The inoculum was derived from a broth culture which was incubated for 18 h at 37 °C and 10 μl of the inoculum was used to inoculate each well. The trays were covered and placed in plastic bags and anaerobically incubated at 37 °C for 18–20 h. An anaerocult P (Merck) was inserted in each plastic bags in order to generate an anaerobic atmosphere. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. The experiments were replicated at least three times to verify the methodology reproducibility when using the above-mentioned conditions.

3. Results and discussion

3.1. Identification of bacteria

Through the identification of the bacterial species used in the tested products, it has emerged that the lactobacilli were *L. casei* (n=6), *L. acidophilus* (n=6), *L. delbrueckii* subsp. *bulgaricus* (n=11). All the streptococci were *S. thermophilus* (n=10). The bifidobacteria were *B. animalis* subsp. *lactis* (n=21) and *Bifidobacterium longum* (n=1).

When considering the bifidobacterial identification the response was not always in agreement with the indication on the product label. *B. longum* was isolated from 1 pharmaceutical product, in accordance with the product label. *B. animalis* subsp. *lactis* was found in all the investigated samples. At the time during which this study was performed, in 1 pharmaceutical product we found *B. animalis* subsp. *lactis*, although in the product label was declared only the presence of bifidobacteria from human origins such as *B. breve*, *B. infantis* and *B. longum*. On the label of the other products, the presence of *B. animalis* subsp. *lactis* was never fully specified. Thus, even if the indications found (*Bifidobacterium*, *B. lactis*, *Bifidobacterium* Bb12) were correct they remain to be partial.

All the 55 strains isolated and identified have been submitted to the antibiotic susceptibility test.

3.2. Antibiotic susceptibility

The minimal inhibitory concentration (MIC) is the lowest antibiotic concentration that inhibits the visible bacterial growth after overnight incubation (Phillips et al., 1991). We evaluated the lowest antibiotic concentration that inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the tested strains (Moubareck et al., 2005), in order to show MIC distributions.

The results obtained (Table 1) are listed in terms of MIC_{50}, MIC_{90}, MIC range and MIC mode and they are given by species. Due to the observation that bacteria develop antibiotic resistance, it is considered that when MICs are ≥8 μg/ml the bacteria may be considered as "moderately resistant"; when MICs are above 32 μg/ml it may be classified as "clinically resistant" to the antibiotic (Walsh, 2003).

Considering the genus of the bacteria it is seen (Table 1) that for lactobacilli the less active compounds were aztreonam, cycloserin, kanamycin, metronidazole, nalidixic acid, spectinomycin and polymyxin B having a MIC_{90} range from 64 to >1000 μg/ml. All the strains of *L. acidophilus* and *L. casei*

Table 1
MIC values for the bacteria investigated given by species

Antibiotics	n[a]	Bacterial species	MIC[b] (μg/ml)			
			90%[c]	50%[c]	Range	Mode
Ampicillin	22	*Bifidobacterium* spp.	2	2	1–2	2
	6	*L. acidophilus*	0.5	0.5	0.5	0.5
	6	*L. casei*	2	2	2	2
	11	*L. bulgaricus*	0.5	0.5	0.5	0.5
	10	*S. thermophilus*	0.5	0.1	0.01–0.5	0.1
Aztreonam	22	*Bifidobacterium* spp.	1000	1000	128–1000	1000
	6	*L. acidophilus*	500	500	500–1000	500
	6	*L. casei*	1000	1000	1000	1000
	11	*L. bulgaricus*	256	128	128–256	128
	10	*S. thermophilus*	1000	500	500–1000	500
Bacitracin	22	*Bifidobacterium* spp.	4	4	1–4	4
	6	*L. acidophilus*	4	4	1–4	4
	6	*L. casei*	4	4	1–4	4
	11	*L. bulgaricus*	1	0.5	0.5–1	1
	10	*S. thermophilus*	1	1	0.5–1	1
Cephalothin	22	*Bifidobacterium* spp.	32	16	8–32	16
	6	*L. acidophilus*	2	2	2	2
	6	*L. casei*	32	32	32	32
	11	*L. bulgaricus*	0.5	0.5	0.5	0.5
	10	*S. thermophilus*	0.5	0.1	0.1–0.5	0.5
Chloramphenicol	22	*Bifidobacterium* spp.	2	2	2–4	2
	6	*L. acidophilus*	8	8	8	8
	6	*L. casei*	16	16	16	16
	11	*L. bulgaricus*	16	16	4–16	16
	10	*S. thermophilus*	8	4	4–8	4
Clindamycin	22	*Bifidobacterium* spp.	0.5	0.1	0.01–0.5	0.1
	6	*L. acidophilus*	4	4	4	4
	6	*L. casei*	0.5	0.5	0.5	0.5
	11	*L. bulgaricus*	0.5	0.5	0.5	0.5
	10	*S. thermophilus*	0.5	0.5	0.1–0.5	0.5
Cycloserin	22	*Bifidobacterium* spp.	128	128	128	128
	6	*L. acidophilus*	128	128	32–128	128
	6	*L. casei*	1000	1000	1000	1000
	11	*L. bulgaricus*	128	128	128	128
	10	*S. thermophilus*	128	128	128	128
Dicloxacillin	22	*Bifidobacterium* spp.	4	4	4–16	4
	6	*L. acidophilus*	0.5	0.5	0.5	0.5
	6	*L. casei*	2	2	1–2	2
	11	*L. bulgaricus*	0.5	0.5	0.1–0.5	0.5
	10	*S. thermophilus*	0.5	0.5	0.1–0.5	0.5
Erytromycin	22	*Bifidobacterium* spp.	0.5	0.1	0.1–0.5	0.1
	6	*L. acidophilus*	0.1	0.1	0.1	0.1
	6	*L. casei*	0.5	0.5	0.5	0.5
	11	*L. bulgaricus*	0.5	0.1	0.1–0.5	0.5
	10	*S. thermophilus*	0.5	0.5	0.5	0.5
Gentamicin	22	*Bifidobacterium* spp.	128	64	64–128	128
	6	*L. acidophilus*	16	2	2–16	2
	6	*L. casei*	16	4	4–16	4
	11	*L. bulgaricus*	32	16	4–32	16
	10	*S. thermophilus*	32	16	16–32	16
Kanamycin	22	*Bifidobacterium* spp.	1000	500	500–1000	500
	6	*L. acidophilus*	64	16	16–64	16
	6	*L. casei*	64	64	64	64
	11	*L. bulgaricus*	64	32	32–64	64
	10	*S. thermophilus*	64	64	64	64
Lincomycin	22	*Bifidobacterium* spp.	1	1	0.5–2	1
	6	*L. acidophilus*	8	8	4–8	8
	6	*L. casei*	0.5	0.5	0.5–1	0.5
	11	*L. bulgaricus*	0.5	0.5	0.5	0.5
	10	*S. thermophilus*	1	1	1	1
Metronidazole	22	*Bifidobacterium* spp.	16	8	2–16	8
	6	*L. acidophilus*	500	500	500	500
	6	*L. casei*	500	500	500	500
Metronidazole	11	*L. bulgaricus*	256	256	256	256
	10	*S. thermophilus*	256	256	256	256
Nalidixic acid	22	*Bifidobacterium* spp.	256	256	256–500	256
	6	*L. acidophilus*	>1000	>1000	>1000	>1000
	6	*L. casei*	>1000	>1000	>1000	>1000
	11	*L. bulgaricus*	500	500	500	500
	10	*S. thermophilus*	500	500	500	500
Neomycin	22	*Bifidobacterium* spp.	500	128	64–500	128
	6	*L. acidophilus*	4	4	4–64	4
	6	*L. casei*	4	4	2–4	4
	11	*L. bulgaricus*	256	64	16–256	64
	10	*S. thermophilus*	256	64	16–500	64
Novobiocin	22	*Bifidobacterium* spp.	4	4	4–16	4
	6	*L. acidophilus*	0.5	0.5	0.5	0.5
	6	*L. casei*	0.5	0.5	0.5–1	0.5
	11	*L. bulgaricus*	4	4	2–4	4
	10	*S. thermophilus*	2	2	0.1–2	2
Paromomycin	22	*Bifidobacterium* spp.	500	500	128–500	500
	6	*L. acidophilus*	64	64	64	64
	6	*L. casei*	16	16	16–32	16
	11	*L. bulgaricus*	128	64	16–128	128
	10	*S. thermophilus*	256	128	64–256	128
Penicillin G	22	*Bifidobacterium* spp.	0.5	0.01	0.01–0.5	0.01
	6	*L. acidophilus*	1	1	0.01–1	1
	6	*L. casei*	1	1	0.01–1	1
	11	*L. bulgaricus*	0.01	0.01	0.01	0.01
	10	*S. thermophilus*	0.1	0.01	0.01–0.5	0.01
Polymyxin B	22	*Bifidobacterium* spp.	1000	500	128–1000	500
	6	*L. acidophilus*	256	256	256–500	256
	6	*L. casei*	1000	1000	1000	1000
	11	*L. bulgaricus*	500	256	64–500	500
	10	*S. thermophilus*	128	32	16–128	128
Rifampicin	22	*Bifidobacterium* spp.	2	1	0.5–2	1
	6	*L. acidophilus*	1	1	1	1
	6	*L. casei*	0.5	0.5	0.5	0.5
	11	*L. bulgaricus*	0.5	0.5	0.1–1	0.5
	10	*S. thermophilus*	0.5	0.5	0.1–1	0.5
Spectinomycin	22	*Bifidobacterium* spp.	128	64	64–128	64
	6	*L. acidophilus*	64	16	16–64	16
	6	*L. casei*	64	64	64	64
	11	*L. bulgaricus*	128	64	64–128	64
	10	*S. thermophilus*	128	128	64–256	128
Streptomycin	22	*Bifidobacterium* spp.	64	64	32–64	64
	6	*L. acidophilus*	4	4	4–32	4
	6	*L. casei*	16	16	16	16
	11	*L. bulgaricus*	64	32	16–64	32
	10	*S. thermophilus*	64	32	16–64	32
Tetracycline	22	*Bifidobacterium* spp.	32	32	16–32	32
	6	*L. acidophilus*	4	4	4–16	4
	6	*L. casei*	16	16	16	16
	11	*L. bulgaricus*	16	16	16	16
	10	*S. thermophilus*	1	0.5	0.5–1	0.5
Vancomycin	22	*Bifidobacterium* spp.	1	1	0.5–1	1
	6	*L. acidophilus*	0.5	0.5	0.5	0.5
	6	*L. casei*	1000	1000	1000	1000
	11	*L. bulgaricus*	0.5	0.5	0.5–1	0.5
	10	*S. thermophilus*	0.5	0.5	0.5–1	0.5

[a] Number of strains tested.

[b] The minimal inhibitory concentration (MIC) is defined as the lowest antibiotic concentration that inhibits the visible bacterial growth after overnight incubation (Phillips et al., 1991).

[c] MIC values that inhibit 50% and 90% of the strains belonging to the same species.

Table 2
Antibiotic susceptibility of the bacterial isolates

Susceptible (MIC_S < 8 μg/ml)	Moderately resistant ($MIC_S \geq 8$ μg/ml)	Resistant (MIC_S > 32 μg/ml)
Ampicillin		Aztreonam
Bacitracin		Nalidixic acid
Clindamycin		Polymyxin B
Erytromycin		
Penicillin G		
Rifampicin		

Subdivision of the antibiotic in three classes in function of their effect on the bacterial species evaluated by their MIC_S values as indicated by Walsh (2003).

were observed to be highly resistant to nalidixic acid having a MIC_{90} value >1000 μg/ml. *L. casei* was strongly resistant to aztreonam, cycloserin, polymyxin B and vancomycin too, having a MIC_{90}=1000 μg/ml. *S. thermophilus* was resistant to cycloserin, kanamycin, metronidazole, nalidixic acid, neomycin, paromomycin, polymyxin B, spectinomycin and streptomycin (MIC_{90} ranging from 64 to 500 μg/ml). Particularly it results that *S. thermophilus* was highly resistant to aztreonam having a MIC_{90}=1000 μg/ml.

Bifidobacteria were resistant to aminoglycoside (MIC_{90} ranges from 64 to 1000 μg/ml), cycloserin (MIC_{90}=128 μg/ml), nalidixic acid (MIC_{90}=256 μg/ml). Particularly they were strongly resistant to kanamycin, polymyxin B and aztreonam (MIC_{90}=1000 μg/ml).

The screening of antibiotic resistance obtained permits the division of the antimicrobial agents in three classes, considering the MIC_S values of the different species (Table 2). Considering the strains tested it results that they were all susceptible to ampicillin, bacitracin, clindamycin, dicloxacillin, erytromycin, novobiocin, penicillin G, rifampicin (MIC_{90} ranging from 0.01 to 4 μg/ml) and resistant to aztreonam, cycloserin, kanamycin, nalidixic acid, polymyxin B and spectinomycin (MIC_{90} ranging from 64 to >1000 μg/ml). The susceptibility to cephalothin, chloramphenicol, gentamicin, lincomycin, metronidazole, neomycin, paromomycin, streptomycin, tetracycline and vancomycin was variable and depending on the species.

Analysis of the results has also underlined the presence of some MIC ranges that did not overlap, and this implies that the antibiotics related to these MIC ranges are usable as ingredients to obtain selective media. These media allow discrimination among different probiotic bacteria species used in the same product (Table 3). In detail dicloxacillin, gentamicin and kanamycin resulted usable to select *Bifidobacterium* spp., whereas clindamycin and lincomycin to select *L. acidophilus* and cycloserin and vancomycin to enumerate *L. casei*.

3.3. Taxonomic description of probiotics

Considering the increase of the world market of probiotic products, it is of primary importance to carefully document the safety and functionality of the selected strains (Sanders and Huis in't Veld, 1999; Saarela et al., 2000). In doing this, more caution needs to be paid to two important features. These are the exact documentation of the identity of the strains on the product label, and their antibiotic susceptibility.

Previous analyses on probiotic products showed that the identity of the strains used did not always correspond to the information reported on the product label (Biavati et al., 1992a, b; Yaeshima et al., 1996; Mattarelli et al., 2002; Temmerman et al., 2002). *B. animalis* has been recognized as the most frequent in the tested probiotic products but it never appeared on the product label. *B. animalis* (Scardovi and Trovatelli, 1974) colonizes the bowel of some animals such as rats, rabbits, and chickens and shows a typical phase variation in colony appearance and in cellular morphology (Biavati et al., 1992a,b).

B. animalis has useful characteristics for industrial applications such as resistance to freeze-drying (Modesto et al., 2004). Moreover its resistance in acid environments (Mattarelli et al., 2002) allows survival at the pH that occurs during the production and storage of yoghurt. This process, known under the name "post-acidification" (Shah, 2000), is caused by the production of lactic acid by *L. delbrueckii* subsp. *bulgaricus*.

Bifidobacteria have an optimum temperature for growth between 37 °C and 41 °C and depending upon the habitat of origin (Biavati and Mattarelli, 2001). A temperature of 45 °C enables to discriminate between animal and human strains, since most of the animal but not human strains are able to grow at this temperature (Gavini et al., 1991). As *B. animalis* originates from an animal habitat and has a high thermal resistance, it is suitable to be used during yogurt production. Due to its high thermal resistance, a higher temperature can be used during milk fermentation, which results in a shortened production time.

Previous studies also demonstrated that *B. animalis* has the ability to colonize the bowel during periods of food intake, and that it disappears gradually afterwards (Biavati et al., 1995).

However to be considered probiotic, a product has to contain bacteria from human origin for their natural attitude to colonize the human bowel (Samona and Robinson, 1991; Saarela et al., 2000). As *B. animalis* naturally colonizes animal habitats, its use in humans seems to be inappropriate.

Based on taxonomic studies of strains isolated from yoghurt samples, Meile et al. (1997) proposed a new species of bifidobacteria: *B. lactis*.

Before this proposal the most representative species of bifidobacteria isolated from probiotic products was *B. animalis*. Several studies have evaluated the similarity between *B. animalis* and *B. lactis*; Cay et al. (2000) proposed that *B. lactis* is a subjective synonym of *B. animalis*.

Table 3
Selective ranges of antibiotics usable to isolate the bacterial species indicated

Antibiotics	*Bifidobacterium* spp.	*Lactobacillus acidophilus*	*Lactobacillus casei*
Dicloxacillin	$2 \leq sr^{a} < 4$		
Gentamicin	$32 \leq sr < 64$		
Kanamycin	$64 \leq sr < 500$		
Clindamycin		$0.5 \leq sr < 4$	
Lincomycin		$2 \leq sr < 4$	
Cycloserin			$128 \leq sr < 1000$
Vancomycin			$1 \leq sr < 1000$

[a] Selective range (μg/ml).

Subsequently Ventura and Zink (2002, 2003) and Zhu et al. (2003) suggested to split *B. animalis* in two subspecies: *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*.

The habitat of *B. animalis* subsp. *animalis* is rat feces, while the habitat of *B. animalis* subsp. *lactis* is rabbit and chicken feces and it has also been found in sewage, fermented milk and human feces (Ventura and Zink, 2002).

A previous study had already shown the possibility to determine the ecological niche of *B. animalis* throughout the comparison of the electrophoretograms of soluble cellular proteins. There are two different protein profiles: one of strains isolated from rat feces and one from rabbit and chicken feces, sewage and milk fermented products (Mattarelli et al., 1991).

Finally, a recent study of Masco et al. (2004), describes the two subspecies *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*.

3.4. Antibiotic susceptibility for probiotics

The other emerging issue is the antibiotic resistance of the strains used. Lactobacilli were resistant to: nalidixic acid, aztreonam, cycloserin, kanamycin, metronidazole, polymyxin B, spectinomycin and susceptible to rifampicin, bacitracin, clindamycin, erytromycin, novobiocin and penicillins. Previous studies document that lactobacilli were highly sensitive to ampicillin, cephalothin and erythromycin (Katla et al., 2001). According to Danielsen and Wind (2003), *L. acidophilus* was not resistant to vancomycin. Among lactobacilli only *L. casei* was resistant to vancomycin (MIC_{90} 1000 mg/l). This resistance was previously documented (Swenson et al., 1990; Charteris et al., 1998a). Until the present date there has been no indication that vancomycin resistant bacteria can transfer this resistance to other bacteria, but this possibility has to be taken into consideration. From the analysis of the MIC_{90}, it resulted that *L. acidophilus* and *L. casei* were resistant to concentrations of nalidixic acid superior to 1000 mg/l, so they can be classified as high level resistance to nalidixic acid.

The finding that bifidobacteria were intrinsically resistant to kanamycin, gentamicin, streptomycin, polymyxin B, nalidixic acid, paromomycin, neomycin and susceptible to penicillin G ($MIC_{90}=0.5$ μg/ml), bacitracin ($MIC_{90}=4$ μg/ml), chloramphenicol ($MIC_{90}=2$ μg/ml), erythromycin ($MIC_{90}=0.5$ μg/ml), lincomycin ($MIC_{90}=1$ μg/ml) and vancomycin ($MIC_{90}=1$ μg/ml), confirms the results of previous studies (Sutter and Finegold, 1976; Matteuzzi et al., 1983; Lim et al., 1993; Temmerman et al., 2002; Moubareck et al., 2005). They were also susceptible to novobiocin, ampicillin, dicloxacillin, rifampicin and clindamycin ($MIC_{90}=4$, 2, 4, 2 and 0.5 μg/ml respectively) and moderately resistant to metronidazole, tetracycline and cephalotin ($MIC_{90}=$ 16, 32 and 32 μg/ml respectively). The resistance to aztreonam has also been found by Charteris et al. (1998b), as the moderate susceptibility to tetracycline. To the authors' knowledge, nobody has studied the genetic mechanisms of bifidobacteria antibiotic resistance. These kinds of studies are necessary to understand the above-mentioned mechanisms.

Although MIC is generally considered a "true" measure, some authors have underlined that it can have low reproducibility between different laboratories (Ericsson and Sherris, 1971; King and Phillips, 1988). It is clear, in fact, that tests conducted in different ways give different results, thus it is necessary to standardize the method. We obtained reproducible results when repeating the experiments and this could be linked to the use of standard conditions, according with the above-mentioned observation. Satisfactory and reproducible results can be achieved keeping in mind the factors that condition the data. Despite some reservation, the MIC is considered the point of reference for the determination and the comparison of the effect of antibiotics on bacteria and in fact their effectiveness is described in terms of MIC (Phillips et al., 1991).

Without doubt, the careless use of antimicrobial agents in human medicines and in farming practice has assisted the spread of resistant organisms. Therefore a much stricter control over the use of these drugs is essential. Many countries have already started research programs leading to a national control of the antibiotic resistance from bacteria occurring in the food industry (Aarestrup et al., 1998; Mevius et al., 1999). These studies demonstrate that genes coding for antibiotic resistance, are transferable among bacteria of different origins (Kruse and Sørum, 1994). Because of the possible impact on human health, probiotic bacteria should not be allowed to have the capacity to transfer antibiotic resistance (Saarela et al., 2000). The ability to transfer genes for antibiotic resistance must be considered as an important parameter for the selection of the probiotic strains.

As the analysis of the results underlined, some MIC ranges did not overlap. This implies that the antibiotics related to these MIC ranges are exploitable as ingredients to obtain selective media. Another research is suggested to determine the best antibiotic concentration, in the MIC range indicated (Table 3), usable to select the bacteria species of interest. These media are usable for qualitative and quantitative analyses of probiotic products. To attribute beneficial effects to these products it is important that every strain used is vital up to the expiry date of the product and present to at least 10^5 cfu/ml (Lankaputra et al., 1996). Due to the actual trend to use more probiotic species in the same product, the enumeration of every bacterial species is difficult (Samona and Robinson, 1991). Therefore it is important to obtain media which enable discrimination among the various species which are used.

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