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International Journal of Food Microbiology 81 (2002) 1–10

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Identification and antibiotic susceptibility of bacterial isolates from probiotic products

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Received 20 May 2001; received in revised form 27 December 2001; accepted 16 April 2002

Abstract

In the present study, a total of 55 European probiotic products were evaluated with regard to the identity and the antibiotic resistance of the bacterial isolates recovered from these products. Bacterial isolation from 30 dried food supplements and 25 dairy products, yielded a total of 268 bacterial isolates selected from several selective media. Counts of food supplements showed bacterial recovery in 19 (63%) of the dried food supplements ranging from 10^3 to 10^6 CFU/g, whereas all dairy products yielded growth in the range of 10^5–10^9 CFU/ml. After identification of the isolates using whole-cell protein profiling, mislabeling was noted in 47% of the food supplements and 40% of the dairy products. In six food supplements, *Enterococcus faecium* was isolated whereas only two of those products claim this species on their label. Using the disc diffusion method, antibiotic resistance among 187 isolates was detected against kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillinG (23%), erythromycin (16%) and chloramphenicol (11%). Overall, 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistances. Initially, 38% of the isolated enterococci was classified as vancomycin resistant using the disc diffusion method, whereas additional broth dilution and PCR assays clearly showed that all *E. faecium* isolates were in fact vancomycin susceptible.

Keywords: Probiotics; Label correctness; Identification; Antibiotic susceptibility testing

1. Introduction

The past 5 years have witnessed a strong expansion of the probiotic market and, in parallel, a rise in the number of research projects addressing fundamental and applied aspects of probiotics. New research technologies have supported earlier suggestions of health promoting properties of probiotic lactic acid bacteria (LAB) as reviewed by Naidu et al. (1999) including stabilisation of the intestinal microflora by competition against pathogens (Gibson et al., 1997), reduction of lactose intolerance (de Vrese et al., 2001), prevention of antibiotic-induced diarrhea (Pochapin, 2000), prevention of colon cancer (Wollowski et al., 2001), and stimulation of the immune system (Isolauri et al., 2001). Bringing a probiotic to the market involves a step-wise process that needs to be carefully monitored in order to obtain a correctly labeled, functional, and safe product (Sanders and Huis in't Veld, 1999;

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0168-1605/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0168-1605(02)00162-9

Saarela et al., 2000). If a product is not labeled correctly, safety and functionality cannot be guaranteed due to lack of documentation of the product components. However, as many manufacturers rely on the widely acknowledged but occasionally debated GRAS ('generally regarded as safe') status of lactobacilli and bifidobacteria (Salminen et al., 1998), characterization of probiotic LAB strains with regard to taxonomic status, antibiotic resistance, and virulence may sometimes be neglected.

Microbial analyses of probiotic dairy products have demonstrated that the identity and the number of recovered species do not always correspond to the information stated on the product label (Reuter, 1997; Holzapfel et al., 1998; Hamilton-Miller et al., 1999). However, it should be noted, that each of the cited studies was rather limited in number and type of products or was mainly restricted to national products. Various opinions exist as to whether it might be desirable that some probiotic strains show resistance to specific antibiotics that are, for instance, involved in antibiotic-induced diarrhea (Charteris et al., 1998). On the other hand, the commercial introduction of probiotics containing antibiotic resistant strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens (Curragh and Collins, 1992).

In the current paper, an extensive study is presented to verify the label correctness of a range of European probiotic food supplements and dairy products, together with the antibiotic susceptibility testing of the product isolates. For each of these products, the label information was checked through taxonomic characterisation of the recoverable bacterial strains using whole-cell protein profiling. In addition, individual susceptibilities were determined for a selection of six antimicrobial agents.

2. Materials and methods

2.1. Bacterial isolation

A total of 55 probiotic products, collected in eight European countries, comprised 30 dried food supplements (Table 1) and 25 dairy products (Table 2). Dairy products were collected using a refrigerated box. None of the 55 products had exceeded their expiry date. All products were examined using a set of four isolation media under standardized cultivation conditions. For the isolation of *Lactobacillus* and *Lactococcus* strains, De Man Rogosa and Sharpe Agar (MRSA) medium (CM361, Oxoid, Basingstoke, UK) was used, whereas streptococci and enterococci were isolated on M17 medium (CM785, Oxoid) and on Kanamycine Aesculine Azide Agar Base (KAAAB) (CM591, Oxoid), respectively. For the isolation of bifidobacteria, Transgalacto-Oligosaccharides (TOS) medium (Matsuki et al., 1999) was used with the following composition: 10 g Trypticase Soy Broth (81-1768-0, Becton Dickinson, Sparks, USA), 1 g Yeast Extract (L21, Oxoid), 3 g KH_2PO_4 (1627, Vel, Leuven, Belgium), 4.8 g K_2HPO_4 (1628, Vel), 3 g $(NH_4)_2SO_4$ (1.01217.1000, Merck, Darmstadt, Germany), 0.2 g $MgSO_4 \cdot 7\ H_2O$ (1433, Vel), 0.5 g L-cystein hydrochloride (C4820, Sigma, Bornem, Belgium), 15 g Na-propionate (P1880, Sigma), 10 g Transgalacto-OligoSaccharides (TOS, Honsha, Tokyo, Japan) and 15 g agar (L11, Oxoid) dissolved in 1000 ml of distilled water. Products were sampled by preparing 10-fold dilutions of 100 μl of the dairy products or 100 mg of the food supplements in 10 ml pepton-physiological solution [PPS, 0.1% (w/v) Pepton (Oxoid, L37) and 0.85% (w/v) NaCl in distilled water]. A total of 50 μl of each dilution was plated in triplicate on all media, using the Whitley Automatic Spiral Plater (WASP™; Led Techno, Eksel, Belgium). All plates were incubated at 37 °C under aerobic conditions, except for TOS plates that were incubated anaerobically (80% N_2, 10% H_2 and 10% CO_2) using an anaerobic chamber. After incubation for 48 h, colony counts were performed and three to five colonies were picked based on different colony morphologies. Selected colonies were further purified on MRSA medium except that those recovered from TOS medium were cultured on Modified Columbia Agar (MCA) comprising 23 g special pepton (L72, Oxoid), 1 g soluble starch (1.01252.0250, Merck), 5 g NaCl (1.06404.1000, Merck), 0.3 g cystein–$HCl \cdot H_2O$, 5 g glucose (500520-887, Vel) and 15 g agar dissolved in 1000 ml of distilled water. The latter medium was also used in a second screening round of products that claimed bifidobacteria on their labels, but did not produce any *Bifidobacterium* strain on the TOS medium during the first isolation round. Products that did not yield any isolates were again

Table 1
Probiotic food supplements: comparison of label claims with identification results of isolates from the products

Product name (type)[a]	Producer (country)	Species claimed on the product label	Isolates from the product
40 + Acidophilus (C)	Solgar Laboratories (The Netherlands)	*Lb. acidophilus, Lb. bulgaricus, B. bifidum, B. longum*	*P. acidilactici, Lb. plantarum, B. lactis*
ABCdophilus powder (P)	Solgar Laboratories (The Netherlands)	*B. bifidum, B. infantis, S. thermophilus*	No identification possible
Acidophilus bifidus (C)	Blackmores (UK)	*Lb. acidophilus, B. bifidum*	No strains could be isolated
Acidophilus Plus (C)	Quest Vitamins (UK)	*Lb. acidophilus, Lb. rhamnosus, Lb. bifidum*[b]	*Lb. paracasei* ssp. *paracasei*
Acidophilus plus bifidus (C)	Kudos Vitamins and Herbals (UK)	*Lb. acidophilus, B. longum, B. infantis, B. casei*[b]	No strains could be isolated
Aciforce (P)	Biohorma (The Netherlands)	*Lb. acidophilus, Lc. lactis, E. faecium, B. bifidum*	*E. faecium, Lc. lactis* ssp. *lactis*
Bacilac (C)	THT (Belgium)	*Lb. acidophilus, Lb. rhamnosus*	No strains could be isolated
Bactisubtil (C)	Synthelabo Belgium (Belgium)	*Bacillus* IP5832	*Bacillus cereus*
Benefact (T)	Unknown	*Lb. bulgaricus*	No strains could be isolated
Beneflora (P)	ORTIS (Belgium)	*Lb. acidophilus, Lb. casei, B. longum, Lb. bulgaricus, S. thermophilus*	*Lb. acidophilus, B. longum, S. thermophilus*
Bifidus complex (C)	Biover (Belgium)	*Lb. acidophilus, Bifidobacterium, Saccharomyces cerevisiae*	*E. faecium*
Colon Clean Naturel (P)	Pharmafood (Belgium)	*Lb. acidophilus*	No strains could be isolated
Colon Clean Plus (P)	Pharmafood (Belgium)	*Lb. acidophilus*	No strains could be isolated
Colon Maintenance (C)	Holland and Barrett (UK)	*Lb. acidophilus*	No strains could be isolated
Culturelle (C)	CAG Functional Foods (UK)	*Lb. GG*	*Lb. rhamnosus*
Diedam (P)	Almond Laboratorios (Spain)	*Lb. acidophilus, Lb. casei, Lb. bulgaricus, B. infantis, S. thermophilus*	No strains could be isolated
Effidigest (P)	Aca Pharma (Belgium)	N/A	*Lb. plantarum*
Lactéol (C)	Menarini Benelux (Belgium)	*Lb. acidophilus*	*Lb. rhamnosus*
Lactimum (C)	Biorès (Belgium)	*Lb. acidophilus, Lb. rhamnosus*	*E. faecium*
Lactobacillus Acidophilus (T)	Blackmores (UK)	*Lb. acidophilus*	No strains could be isolated
Life Top Straw (PC)	BioGaia Biologics (Sweden)	*Lb. reuteri*	*Lb. reuteri*
Milk Free Acidophilus (C)	Holland and Barrett (UK)	*Lb. acidophilus, Lb. bulgaricus, B. bifidum*	No strains could be isolated
Multi-billion dophilus (C)	Solgar Laboratories (The Netherlands)	*Lb. acidophilus, Lb. bulgaricus, S. thermophilus, B. bifidum*	*P. acidilactici*
Novaflora (C)	Pharmafood (Belgium)	*Lb. rhamnosus, Lb. lactis, E. faecium, Bifidobacterium*	*E. faecium*
Prévite acidophilus (C)	Unknown	*Lb. acidophilus*	*E. faecium*
Probiosan (T)	Nutrisan (Belgium)	*Lb. acidophilus, Lb. rhamnosus*	*Lb. crispatus, Lb. rhamnosus*
Proflora (C)	Chefaro (Belgium)	*Lb. acidophilus, Bifidobacterium, Lb. bulgaricus, S. thermophilus*	*Lb. acidophilus, B. lactis, S. thermophilus*
Psyllium actif (P)	Biover (Belgium)	*Lb. acidophilus, Lb. bifidum*[b]	*E. faecium*
Superior Probiotics (T)	BioGaia Biologics (Sweden)	*Lb. reuteri*	*Lb. reuteri*
Vivaflora (T)	Laboratoires Super Diet (France)	*Lb. acidophilus, B. bifidum*	No strains could be isolated

Lb. = *Lactobacillus*, *B.* = *Bifidobacterium*, *S.* = *Streptococcus*, *E.* = *Enterococcus*, *Lc.* = *Lactococcus*, *P.* = *Pediococcus*.
Lb. bulgaricus = corresponds to *Lactobacillus delbrueckii* ssp. *bulgaricus*.
[a] Type of product: P = Powder, C = Capsule, T = Tablet, PC = Powder as Coating.
[b] Indistinct or invalid name.

Table 2
Probiotic dairy products: comparison of label claims with identification results of isolates from the products

Product name	Producer (country)	Species claimed on the product label	Isolates from the product
Actimel	Danone (France)	*Lb. casei Immunitas*, living yoghurt cultures	*Lb. paracasei* ssp. *paracasei*
Actimel Orange	Danone (France)	*Lb. casei Immunitas*, living yoghurt cultures	*Lb. paracasei* ssp. *paracasei*
Almighurt	Almighurt (Germany)	Living yoghurt cultures	*Lb. bulgaricus, S. thermophilus*
B′A fruits	B′A (France)	*Bifidobacterium*[a]	*S. thermophilus*
B′A vanille	B′A (France)	*Bifidobacterium*[a]	*S. thermophilus*
Benecol	McNeil Consumer Nutritionals (UK)	*Bifidobacterium*[a]	*Lb. acidophilus, S. thermophilus*
BI′AC	TMA (Germany)	*Lb. acidophilus, Lb. casei*	*Lb. acidophilus, S. thermophilus, Lb. paracasei* ssp. *paracasei*
BIO abricot	Danone (France)	*Bifidobacterium*, living yoghurt cultures	*Lc. lactis* ssp. *lactis*
BIO framboise	Danone (France)	*Bifidobacterium*, living yoghurt cultures	*S. thermophilus, Lc. lactis* ssp. *lactis*
Biogarde halfvol Naturel	Strothmann (Germany)	*Lb. acidophilus, Bifidobacterium, S. thermophilus*	*Lb. acidophilus, S. thermophilus*
Biogarde plus (naturel)	Almhof (The Netherlands)	*Lb. acidophilus, Lb. casei, Bifidobacterium*	*Lb. acidophilus, S. thermophilus*
Biomild Drink	Mona (The Netherlands)	*Lb. acidophilus*, *B. longum, S. thermophilus*	*Lb. johnsonii, S. thermophilus, B. lactis*
Bio Snac'	Danone (France)	*Bifidobacterium,* living yoghurt cultures	*Lc. lactis* ssp. *lactis*
Fitness Quark	Onken (Germany)	*Lb. acidophilus* OCA5, *Bifidobacterium* OCB111	*Lb. johnsonii, S. thermophilus*
Fysiq	Mona (The Netherlands)	*Lb. acidophilus Gilliland,* living yoghurt cultures	*Lb. crispatus, S. thermophilus*
Gefilus	Valio (Finland)	*Lb. GG,* living yoghurt cultures	*Lb. rhamnosus*
Joghurt Mild Gartenfrucht	Bremerland (Germany)	*Lb. acidophilus* LA55, *Bifidobacterium* CB111	*Lb. johnsonii, B. lactis, S. thermophilus*
Kinderyoghurt mild	J. Bauer KG (Germany)	*Lb. acidophilus, Lb. bifidus*[b]	*Lb. acidophilus, Lb. johnsonii, S. thermophilus*
Lactus Nature	Carrefour (France)	*Lb. casei* ssp. *rhamnosus,* living yoghurt cultures	*Lb. rhamnosus*
Lc1	Nestlé (Germany)	*Lb. johnsonii*, living yoghurt cultures	*Lb. johnsonii, S. thermophilus*
Natumild	Natuur Hoeve (The Netherlands)	*Lb. acidophilus, Lb. bifidus*[b], *S. thermophilus*	*S. thermophilus*
Procult Drink	Alois Müller (Germany)	*B. longum* BB536, living yoghurt cultures	*Lb. acidophilus, S. thermophilus*
Vifit Drink	Mona (The Netherlands)	*Lb. casei GG*[b], *Lb. acidophilus, B. bifidum*	*Lb. rhamnosus, Lb. acidophilus*
Weight Watchers Bifidus	Senoble (France)	*Bifidobacterium*[a]	*S. thermophilus*
Yakult	Yakult (The Netherlands)	*Lb. casei Shirota*	*Lb. paracasei* ssp. *paracasei*

All dairy products were fermented drinks or yoghurt based products.
Lb. = *Lactobacillus, B.* = *Bifidobacterium, S.* = *Streptococcus, Lc.* = *Lactococcus*.
Lb. bulgaricus = corresponds to *Lactobacillus delbreuckii* ssp. *bulgaricus*.
[a] Indicated on the product label as 'active bifidus'.
[b] Indistinct or invalid name.

screened, now using anaerobic and micro-aerophilic (3,5% CO_2, 5% O_2, 7,5% H_2, 84% N_2) cultivation conditions. In addition, these products were subjected to an enrichment step in MRS broth (CM359, Oxoid) using the same aerobic and anaerobic incubation conditions.

2.2. Identification of recovered isolates

Isolates were identified by Sodium Dodecyl Sulphate–Polyacrylamide Gelelectrophoresis (SDS–PAGE) analysis of whole-cell proteins, using standardized cultivation conditions for comparison with the

available protein pattern database of lactic acid bacteria (Pot and Janssens, 1993). Extraction of cellular proteins was performed according to the method described by Pot et al. (1994) for Gram-positive bacteria. Extracts were separated using SDS–PAGE with a 5% total acrylamide stacking gel (12 mm long) and a 12% total acrylamide separation gel (126 mm long). Gels were stained using Coomassie Blue R-250. The patterns were then densitometrically digitized using an LKB 2202 Ultrascan Laser Densitometer (LKB, Bromma, Sweden). Subsequently, these digital protein patterns were normalized using GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) so it became possible to identify the isolates by comparison of their protein patterns with the SDS–PAGE protein pattern database available at the laboratory. Upon repeated analyses, the inter-gel and intra-gel reproducibility was found to be 90.3% and 97.1%, respectively.

2.3. Antibiotic susceptibility testing

At least one isolate per identified species recovered from a given product was included for antibiotic susceptibility testing, resulting in 187 isolates screened for possible resistance against a selection of six antibiotics, including kanamycin (30 μg), vancomycin (30 μg), erythromycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg) and penicillinG (10 μg) using a slightly modified version of the agar disc diffusion method (Kirby et al., 1966). Strains were grown in MRS broth (Oxoid, CM 359) (MC Broth for bifidobacteria) for 48 h at 37 °C. Following the preparation of a 10-fold dilution in PPS, freshly poured MRSA plates (MCA for bifidobacteria) were equally inoculated with this dilution. Antibiotic discs (Oxoid) were placed on the inoculated plates using the Oxoid Disc Dispenser. Following a 24-h incubation at 37 °C, inhibition zones around the discs were measured using a digital callipers (Mauser, Switzerland). Results were interpreted according to the cut-off levels proposed by Charteris et al. (1998) with strains considered resistant if inhibition zone diameters were equal to or smaller than 19 mm for penicillinG, 14 mm for vancomycin and tetracycline, and 13 mm for kanamycin, chloramphenicol and erythromycin.

In addition to the agar disc-diffusion method, two other methods were used to confirm the presence of vancomycin resistance in enterococci. First, by growing the enterococci in a series of Trypticase Soy Broth (L21, Oxoid) Yeast Extract (211768, Becton Dickinson) (TSYE) tubes containing different concentrations of vancomycin or teicoplanin, the Minimal Inhibitory Concentration (MIC) for these antibiotics was determined according the protocol as described by Arthur and Courvalin (1993). The combination of the MIC value obtained for both antibiotics is considered indicative for the preliminary classification as to what extent a strain is vancomycin resistance. In a second approach, the presumptive presence of vancomycin resistance was assessed, using a PCR protocol according to Dutka-Malen et al. (1995) with primer pairs (A1, A2 and B1, B2) specific for the vancomycin resistance genes *vanA* and *vanB*.

3. Results

3.1. Bacterial isolation from probiotic products

Depending on the medium used, colony counts of the 25 investigated dairy products ranged from 10^5 to 10^9 CFU/ml. Among the 30 food supplements tested in this study, counts varied from below 1 to 10^6 CFU/g. During a first isolation round, we were unable to isolate viable bacteria out of 12 (i.e. 40%) of the food supplements. These products were subjected to a second isolation round including an enrichment step in MRS broth and applying anaerobic as well as micro-aerophilic conditions. Only 1 of these 12 products displayed bacterial growth in MRS broth but again not on MRS agar plates. At the end of the two isolation rounds, a total of 323 isolates were obtained. All isolated genera with exception of *Bifidobacterium* grew on all media used. However, it was noted that lactobacilli and enterococci grew best on MRS agar and KAAAB, streptococci grew best on M17 and TOS, and bifidobacteria only grew on TOS.

3.2. Identification of recovered isolates

Identification results are presented in Tables 1 and 2. From a total of 323 isolates, 268 bacteria could be identified at the species level. The remaining 55 isolates were classified as yeasts after microscopical investigation or were lost during purification on MRS

Table 3
Summary of isolation and identification results

Description	Food supplements	Dairy products
Number of products	30	25
* from which no viable strains could be isolated	11 (37%)	0
* containing all claimed species	4/30 (13%)	2 (8%)
* containing other species than those claimed	9/19 (47%)	10 (40%)
* claiming more species than found	22/30 (73%)	16 (64%)
Most frequently claimed species	*Lb. acidophilus*	*Lb. acidophilus*
Most frequently isolated species	*E. faecium, Lb. rhamnosus*	*S. thermophilus, Lb. acidophilus*

agar. Only six products yielded all species indicated on the product label. However, when disregarding the presence of the yoghurt cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, this number of products rises to 13. In 19 products, the isolated species were entirely different from those mentioned on the product label, even after a second isolation round using a new batch of the same products. In Table 3, a brief summary is given of the isolation and identification results. The most frequently recovered species among the food supplements was *Enterococcus faecium* followed by *Lactobacillus rhamnosus*. Of the 6 products in which *E. faecium* was found, only two actually claimed this species on their label. *Lactobacillus acidophilus,* which was claimed on the label of 22 food supplements, could only be isolated out of 2 of these products. Although all 13 food supplements claiming bifidobacteria were screened twice using two different media for the selective isolation of *Bifidobacterium* (TOS and MCA), only 3 of these 13 products produced a bifidobacterial strain. Among the 25 dairy products, *Lb. acidophilus* was claimed, as well as isolated most frequently. As it was the case with the food supplements, only a poor retrieval of bifidobacteria was possible among the dairy products, despite the use of two different media. Only 2 out of 14 dairy products claiming bifidobacteria actually produced a *Bifidobacterium* strain during isolation.

3.3. Antibiotic susceptibility

Of the 268 identified isolates, 187 strains were subjected to antibiotic susceptibility testing using the agar disc diffusion method of which the results are presented in Table 4. It was found that 79% and 65% of the isolates were resistant to kanamycin and vancomycin, respectively. Furthermore, 23% and 21% of

Table 4
Percent of isolates resistant against six tested antibiotics[a] using the disc diffusion method

Taxa (# strains tested)	K (30 μg)	TE (30 μg)	E (10 μg)	P (10 μg)	C (30 μg)	VA (30 μg)
Lactobacillus acidophilus (13)	100	8	8	0	8	69
Lactobacillus rhamnosus (24)	100	21	4	71	0	100
Lactobacillus casei (29)	100	7	10	17	10	100
Lactobacillus johnsonii (17)	100	0	6	0	0	76
Lactobacillus plantarum (6)	0	17	33	66	0	100
Lactobacillus reuteri (6)	100	100	0	100	33	100
Lactobacillus delbreuckii ssp. *bulgaricus* (3)	67	0	0	0	0	67
Lactobacillus crispatus (6)	83	83	33	0	17	66
Streptococcus thermophilus (30)	60	3	0	3	10	40
Enterococcus faecium (29)	90	24	97	41	34	38
Lactococcus lactis ssp. *lactis* (8)	100	63	38	0	0	0
Pediococcus acidilactici (8)	0	38	0	25	38	100
Bifidobacterium longum (4)	100	0	0	0	0	0
Bifidobacterium lactis (4)	100	0	0	0	0	50
Total[b] (187)	79	26	16	23	11	65

[a] K = Kanamycin, TE = Tetracycline, E = Erythromycin, P = PenicillinG, C = Chloramphenicol, VA = Vancomycin.
[b] Total percentage of resistance calculated as the number of isolates (from a total of 187 isolates) resistant against the respective antibiotic.

the isolates were grouped as resistant or intermediately resistant, respectively, to penicillin. Concerning the other antibiotics, the intermediate resistant fraction was never larger than 6.5%. It was also found that 38% of the isolated enterococci were vancomycin resistant according to the disc diffusion method. These resistant enterococci originated from four dried food supplements. However, when using the dilution method and a PCR assay for confirmation, none of the presumptively vancomycin resistant *Enterococcus* strains were found to be resistant against vancomycin (MIC < 2 μg/ml (results not shown)).

4. Discussion

Considering the significant rise in the annual consumption of probiotic products worldwide, it is important that such products are correctly labeled and that the probiotic strains are well-documented regarding safety and functionality (Sanders and Huis in't Veld, 1999). Hitherto, in Europe, there are no widely acknowledged regulations concerning the labeling issues and claims that can be made by the manufacturers of functional foods (Berner and O'Donnell, 1998; Przyrembel, 2001). Our findings clearly indicate the need for such regulations. Counts of viable bacteria were subtantially lower among the 30 food supplements compared to the 25 dairy products. Possibly, higher isolation numbers could have been obtained when food supplements were analysed using anaerobic isolation conditions. However, it can be speculated that the significant difference in relative numbers between the two main types of products will not be affected by incubation under aerobic or anaerobic conditions. Therefore, it is possible that a number of the investigated food supplements may comprise a bacterial concentration below the minimum value required for any probiotic strain to affect the gastro-intestinal tract, and thus, to be able to promote a significant health effect. Using our protocol, a total of 11 food supplements (37%) did not yield any viable bacteria on the four isolation media. It can be speculated that the absence of living bacteria in a dried food supplement is due to damage of the culture caused by the drying and capsulation process, the possibility that some of these food supplements were sterilised for safety reasons or because of a too-long shelf-life period although the products investigated had not yet reached the expiry date at the moment of the isolation procedure. Some immunological activities have been assigned to dead bacteria (Wagner et al., 2000), but many health promoting properties, e.g. competitive exclusion of pathogens, nutrient supplementation for the host, and anti-tumor effects can only be exerted by living bacteria (Ouwehand and Salminen, 1998). Given the present findings, it is therefore more likely for dairy products to exert these probiotic properties than it is for dried food supplements.

Identification of 268 isolates using protein profiling revealed that *E. faecium* was the most frequently recovered species out of the food supplements. This taxon was found in 6 out of the 19 food supplements (32%) containing living bacteria. With the exception of one product, *E. faecium* was the only species isolated out of these food supplements (Table 1). Because of the high isolation numbers (10^4–10^5 CFU/g), it is unlikely that *E. faecium* entered the production process via contamination. The second most frequently recovered species in food supplements was *Lb. rhamnosus*. *Lb. acidophilus* was claimed to be present in 22/30 (73%) products but was only found twice (Table 1). Although a poorer growth was observed for *Lb. acidophilus* on MRSA medium compared to *Lb. rhamnosus, Lb. casei* and *E. faecium*, the relatively low recovery rate cannot be clearly explained. Likewise, the poor retrieval of bifidobacteria could not readily be explained because isolation results were comparable after testing various isolation parameters, e.g. atmosphere, temperature and duration of incubation (data not shown). More likely, it is possible that the nutritional content of the TOS and MCA medium used in this study did not meet the specific growth requirements of a number of probiotic bifidobacterial strains. Therefore, it can be speculated that more products claiming bifidobacteria may have produced these organisms during isolation, when a series of well-defined strain-specific media were used. The need for broad-spectrum isolation media for bifidobacteria is clearly demonstrated by this study and has also been suggested by Roy (2001). Among the tested food supplements, a total of nine products contained species other than those stated on the product label. This mislabeling has also been reported previously by Hoa et al. (2000) for *Bacillus* contain-

ing food supplements and by Hamilton-Miller et al. (1999) for 20 out of 29 tested food supplements.

Since *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* are the main starter cultures of yoghurt, it could be expected that these two species were among the most frequently isolated ones from the dairy products. However, *Lb. delbrueckii* subsp. *bulgaricus* was only found once possibly because this species is rapidly overgrown by other lactobacilli in the dairy products. The fact that *Lb. acidophilus* was more easily isolated from dairy products than from food supplements, could be related to (1) the supporting matrix of the product in which the strains have to survive for the complete shelf-life, (2) the ambient temperature at which the different products are usually maintained, (3) the total shelf-life (average of 30 days for dairy products, average of 24 months for food supplements) or (4) to the individual strain differences with respect to survival in the stationary phase at the given temperature. Fourteen dairy products also claim to contain bifidobacteria, whereas in only two instances, *Bifidobacterium lactis* was recovered instead of the claimed *B. longum*. As outlined above, the low recovery of bifidobacteria might be due to the lack of optimal isolation media for specific *Bifidobacterium* strains. Although to a lesser extent than with food supplements, our results suggest that also quite a number of dairy products suffer from mislabeling, which underscores similar findings of other workers (Reuter, 1997; Holzapfel et al., 1998; Hamilton-Miller et al., 1999).

Using the disc diffusion method, high frequencies of resistance were detected for kanamycin (79%) and vancomycin (65%). Most of the kanamycin resistant isolates belonged to the genera *Lactobacillus* and *Enterococcus*. The latter genus is intrinsically resistant against kanamycin (Franz et al., 1999), but the finding that 81% of the isolated lactobacilli was also resistant against kanamycin somewhat counteracts the specificity of the *Enterococcus*-specific KAAAB medium. Likewise, the relatively high percentage of vancomycin resistance observed among the entire collection of isolates is due to the fact that the majority of the lactobacilli are intrinsically resistant to this glycopeptide (Nelson, 1999). Noteworthy, intraspecies variations were found among the *Lb. johnsonii* and *Lb. acidophilus* isolates, which is in agreement with previous observations of Charteris et al. (1998). Strikingly, 38% of the *E. faecium* isolates also showed to be resistant against vancomycin according to the disc diffusion method. However, these findings could not be confirmed by the dilution method (Arthur and Courvalin, 1993) or by a PCR-based *van* gene detection assay (Dutka-Malen et al., 1995). Collectively, these findings indicate that all enterococci isolated from probiotic products were susceptible to vancomycin, which again highlights the limited reliability of the disc diffusion method to determine the occurrence of vancomycin resistance with enterococci (Swenson et al., 1989). The high frequencies of vancomycin resistance found among other lactic acid bacterial genera do not pose a problem as this type of vancomycin resistance is different from the inducible, transferable mechanism observed in enterococci (Salminen et al., 1998; Klein et al., 2000). The lactobacilli in the present study comprised strains resistant to tetracycline (29.5%), chloramphenicol (8.5%), and erythromycin (12%) and overall, more than 68% of our isolates exhibited resistance to two or more antibiotics (data not shown). With regard to general concerns on biosafety of probiotics, further research should focus on the location and potential transferability of these antibiotic resistance determinants.

In conclusion, it can be stated that quite a number of dried food supplements and—to a lesser extent—dairy products are incorrectly or inadequately labeled with regard to the correct identity of the incorporated probiotic strains. Despite earlier reports concerning mislabeling of probiotic products (Reuter, 1997; Holzapfel et al., 1998; Hamilton-Miller et al., 1999), the new data indicate that this situation has not significantly improved. Although specific antibiotic resistance traits among probiotic strains may be desirable (Charteris et al., 1998), the finding of tetracycline, chloramphenicol and erythromycin resistance among the investigated probiotic isolates indicates that continuous attention should be paid to the selection of probiotic strains free of transferable antibiotic resistance. It is of paramount importance that at a time when consumers become more aware of the importance of good nutrition and health, probiotic products designed especially for their health promoting purposes are safe and well-documented in order to provide consumers with the full benefits of the aspects of probiotics.

Acknowledgements

This research was financially supported by a PhD grant from the Flemish Institute for the Promotion of Innovation by Science and Technology (IWT—Vlaanderen, Brussels, Belgium). Part of this research was supported by Research Programme 3G030901 of the Fund for Scientific Research—Flanders (Belgium) (F.W.O.—Vlaanderen). G.H. is a postdoctoral fellow of the Fund for Scientific Research—Flanders (Belgium) (F.W.O.—Vlaanderen). Also, we would like to thank Dirk Dewettinck, Dirk Gevers and Geert Kindt for their contributions to this research.

References

Arthur, M., Courvalin, P., 1993. Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrobial Agents and Chemotherapy 37, 1563–1571.

Berner, L.A., O'Donnell, J.A., 1998. Functional foods and health claims legislation: applications to dairy foods. International Dairy Journal 8, 355–362.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K., 1998. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. Journal of Food Protection 61, 1636–1643.

Curragh, H.J., Collins, M.A., 1992. High-levels of spontaneous drug-resistance in *Lactobacillus*. Journal of Applied Bacteriology 73, 31–36.

de Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., Schrezenmeir, J., 2001. Probiotics—compensation for lactase insufficiency. American Journal of Clinical Nutrition 73, S421–S429 (suppl.).

Dutka-Malen, S., Evers, S., Courvalin, P., 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by CR. Journal of Clinical Microbiology 33 (1), 24–27.

Franz, C., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroads of food safety? International Journal of Food Microbiology 47, 1–24.

Gibson, G.R., Saveedra, J.M., MacFarlane, S., MacFarlane, G.T., 1997. Probiotics and intestinal infections. In: Fuller, R. (Ed.), Probiotics 2: Applications and Practical Aspects. Chapman & Hall, New York, pp. 10–39.

Hamilton-Miller, J.M.T., Shah, S., Winkler, J.T., 1999. Public health issues arising from microbiological and labelling quality of foods and supplements containing probiotic microorganisms. Public Health Nutrition 2, 223–229.

Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E., Cutting, S.M., 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Applied and Environmental Microbiology 66, 5241–5247.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U., Huis in't Veld, J.H.J., 1998. Overview of gut flora and probiotics. International Journal of Food Microbiology 41, 85–101.

Isolauri, E., Sütas, Y., Kankaanpää, P., Arvilommi, H., Salminen, S., 2001. Probiotics: effects of immunity. American Journal of Clinical Nutrition 73, S444–S450 (suppl.).

Kirby, W.M.M., Bauer, A.W., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology 45, 493–496.

Klein, G., Hallmann, C., Casas, I.A., Abad, J., Louwers, J., Reuter, G., 2000. Exclusion of vanA, vanB and vanC type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by Polymerase Chain Reaction and hybridization methods. Journal of Applied Microbiology 89, 815–824.

Matsuki, T., Watanabe, K., Tanaka, R., Fukuda, M., Oyaizu, H., 1999. Distribution of Bifidobacterial species in human intestinal microflora examined with 16 S rRNA-gene-targeted species-specific primers. Applied and Environmental Microbiology 65, 4506–4512.

Naidu, A.S., Bidlack, W.R., Clemens, R.A., 1999. Probiotic spectra of lactic acid bacteria (LAB). Critical Reviews in Food Science and Nutrition 38 (1), 13–126.

Nelson, R.R.S., 1999. Intrinsically vancomycin-resistant Gram-positive organisms: clinical relevance and implications for infection control. Journal of Hospital Infection 42, 275–282.

Ouwehand, A.C., Salminen, S.J., 1998. The health effects of cultured milk products with viable and non-viable bacteria. International Dairy Journal 8, 749–758.

Pochapin, M., 2000. The effect of probiotics on *Clostridium difficile* diarrhea. American Journal of Gastroenterology 95, S11–S13 (suppl.).

Pot, B., Janssens, D., 1993. The potential role of a culture collection for identification and maintenance of lactic acid bacteria. In: Foo, E.L., Griffin, G., Molby, R., Heden, C.G. (Eds.), The Lactic Acid Bacteria, Proceedings of the First Lactic Acid Bacteria Computer Conference. Horizon Scientific Press, Norfolk, VA, pp. 81–87.

Pot, B., Ludwig, W., Kersters, K., Schleifer, K.-H., 1994. Taxonomy of lactic acid bacteria. In: De Vuyst, L., Vandamma, E.J. (Eds.), Bacteriocins of Lactic Acid Bacteria; Microbiology, Genetics and Applications. Chapman & Hall, London, UK, pp. 13–90.

Przyrembel, H., 2001. Consideration of possible legislation within existing regulatory frameworks. The American Journal of Clinical Nutrition 73, S471–S475 (suppl.).

Reuter, G., 1997. Present and future of probiotics in Germany and in Central Europe. Bioscience and Microflora 16, 43–51.

Roy, D., 2001. Media for the isolation and enumeration of bifidobacteria in dairy products. International Journal of Food Microbiology 69, 167–182.

Saarela, M., Mogensen, G., Fondén, R., Mättö, J., Matilla-Sandholm, T., 2000. Probiotic bacteria: safety, functional and technological properties. Journal of Biotechnology 84, 197–215.

Salminen, S., Von Wright, A., Morelli, L., Marteau, P., Brassart, D., De Vos, W.M., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Mattila-Sandholm, T., 1998. Demonstration

of safety of probiotics—a review. International Journal of Food Microbiology 44, 93–106.

Sanders, M.E., Huis in't Veld, J., 1999. Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 76, 293–315.

Swenson, J.M., Bertha, C.H., Thornsberry, C., 1989. Problems with the disk diffusion test for detection of vancomycin resistance in enterococci. Journal of Clinical Microbiology 27, 2140–2142.

Wagner, R.D., Pierson, C., Warner, T., Dohnalek, M., Hilty, M., Balish, E., 2000. Probiotic effects of feeding heat-killed *Lactobacillus acidophilus* and *Lactobacillus casei* to *Candida albicans*-colonized immunodeficient mice. Journal of Food Protection 63 (5), 638–644.

Wollowski, I., Rechkemmer, G., Pool-Zobel, B.L., 2001. Protective role of probiotics and prebiotics in colon cancer. The American Journal of Clinical Nutrition 73, S451–S455 (suppl.).