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Important Features of Probiotic Microorganisms in Pharmaceutical and Dairy Products

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Background: Probiotic products are matrices for delivery of beneficial live bacteria to the host. The viable bacteria are being incorporated into dairy products as well as supplements.
Objectives: The aim of the present study was evaluation and validation of probiotic contents in commercial products to select the optimum matrix for protection of viability and functionality of probiotic bacteria.
Materials and Methods: A total of forty six lactic acid bacteria were isolated from ten pharmaceutical and ten dairy products. Their probiotic properties such as acid, salt and bile tolerance, antibiotic susceptibility tests, adherence to cell line, stability under refrigeration conditions and antagonistic activity against nine bacterial strains were assayed.
Results: Results showed that the viable bacterial count of solid products were lower than stated number on their package. No difference was noticed between strains isolated from dairy and non-dairy products regarding antibiotic susceptibility and adherence properties. Pharmaceutical isolates were more potent against pathogens than dairy isolates.
Conclusions: In conclusion, dairy products are better matrices for delivering bifidobacteria than non-dairy products. But, probiotic isolates from non-dairy products, showed better properties such as pathogen exclusion than dairy isolates.

Keywords: Probiotics; Dairy Products; Databases, Pharmaceutical; Microbial Viability

1. Background

Probiotics are "live microorganisms that, when administrated in adequate amounts, confer health benefits to the host" (1). Probiotics have been developed and commercialized over the past few decades as a means of increasing lactobacilli and bifidobacteria within the gastrointestinal tract (2). The normal flora limits colonization of the gut by potentially pathogenic microorganisms. Conservation and fortification of this flora may be achieved through the use of oral supplements of bacteria (3). Today a growing industry has developed around the sale of probiotics as foods, dietary supplements and pharmaceutical formats for human use. From the public health point of view, it is important to evaluate the safety and efficacy of products, for personal use. Survival and activity of probiotic bacteria in the product may influence by several factors such as: the physiologica condition of probiotics (bile and acid resistance), the physicochemical characteristics of the product (nutrient availability, humidity, oxygen content, temperature and pH), and the interaction of the probiotics with the other microorganisms which are present in the products (antagonisms and synergism) (4, 5). The criteria for probiotic strain have been listed comprehensively by several authors (6). The selected strains should be a member of GRAS (Generally Regarded as Safe) group and preferably of human origin. Resistance to acid, bile and pancreatic enzymes is necessary for probiotic to survive passage through the gastrointestinal tract. The ability of probiotic bacteria to adhere to intestinal cells or mucous is one of the main selection criteria, also possessing ideal antibiotic resistance patterns and antagonistic activity against pathogenic microorganisms are important (7).

2. Objectives

As long-term industrial processing may influence some functional properties and viability of probiotics (8), the present study was carried out to compare isolates from

Implication for health policy/practice/research/medical education:
Probiotics are a group of microorganisms that beneficially affect the host and probiotic products are matrices for delivery of beneficial live bacteria to the host. The viable bacteria are being incorporated into dairy products as well as supplements. The aim of the present study was evaluation and validation of probiotic contents in commercial products to select the optimum matrix for protection of viability and functionality of probiotic bacteria.

ten pharmaceutical probiotic products and ten dairy products, with respect to features that might be important for their value as probiotics. Finally this study may help us to select the best matrices for delivery of each probiotic strain.

3. Materials and Methods

3.1. Probiotic Products and Bacterial Strains

Twenty products being marketed as probiotics, in capsule or tablet form ("C"), or dairy products ("D") were investigated. Details of these products are given in Table 1. All dairy products were obtained from UK and tested in the Biomedical Sciences Department labs of University of Bradford. Eight dairy products labeled as 'live' or 'bio' yoghurts and two as fermented milks. Pharmaceutical products were bought from UK (7 products) and one each from Sweden, Germany and Saudi Arabia. All C products were bought from retail pharmacies or health food shops. Products were stored in the appropriate conditions as recommended by manufacturer and analyzed before their expiry or sell-by date. All tests were carried out in duplicate on three different occasions. Three *lactobacilli* : *L. acidophilus* (701748) L. casei rhamnosus (8010), *L. casei* (11970) and *two bifidobacteria* : *B. bifidum* (702715), *B. longum* (702259) NCIMB type strains were used as control.

Table 1. List of Product's Codes and the Bacterial Content of Products (cfu/Capsule or Tablet) Obtained in the Present Study and Total Recovered Bacterial Content of Each Product.

Code	Name of Product	Stated Organisms	Stated Number / Product[a]	Total Recovered cfu
1C	Advanced Acidophilus Plus	*L. acidophilus, B. lactis*	250 million	6×10^2
2C	Quest Digestive Aids	*Acidophilus, L. casei casei L. rhamnosus*	2 billion	6.3×10^{10}
3C1	Acidophilus Aids	*Lactobacillus acidophilus*	100 million	None, (50)
3C2	Non-Dairy Acidophilus complex	*L. acidophilus, L. rhamnosus L. bifidum*	2.4 billion	3×10^{10}
4C	Multibionta	*L. acidophilus PA 16/8 B. bifidum MF 20/5 B. longum sp. 07/3*	10 million	3.6×10^5
5C	Health Aid acidophilus	*L. acidophilus Acidophilus bifidus*	50 million	1.8×10^6
6C	Children chewy Acidophilus / Chewy Bears and friends	*L. acidophilus, L. rhamnosus, L. plantarum, L. sporogenes, B. longum*	>10 million	3.3×10^8
8C	Probio Omage	*L. Plantarum 299v*	10 billion	3×10^{10}
9C	Probiomax Maghalsa	*L. Reuteri Protectis*	100 billion	5×10^8
10C	Protexin Protect	*L. Casei, L. Acidophilus, L. Rhamnosus, L. Bulgaricus, B. Breve, Strep. Thermophilus*	2 billion	1×10^8
1D	Betta Buy Low fat fruit flavor yoghurt	--	--	$> 10^9$
2D	Low fat natural yoghurt	--	--	$> 10^9$
3D	Yeo Valley organic natural yoghurt	--	--	$> 10^9$
4D	Actimel drinking yogurt	*L. casei Imunitass*	--	$> 10^9$
5D	Natural milk	--	--	$> 10^9$
6D	fermented milk	*L. casei Shirota*	--	$> 10^9$
7D	Activa low fat yogurt	*Bifidus ESSENSIS*	--	$> 10^9$
8D	Vitality probiotic yogurt	*Bifidobacterium Bb-12*	--	$> 10^9$
9D	Probiotic low fat yogurt	*Bifidobacterium Bb-12*	--	$> 10^9$
10D	Flora ProActive	--	--	$> 10^9$

[a] Total colony count per each capsule or tablet for all species included as stated on the label

3.2. Enumeration, Isolation and Identification of Bacterial Strains

Bacterial content of capsules, tablets and probiotic dairy products were obtained by preparation of duplicate serial dilutions of product content for pharmaceutical products (i.e. a tablet was crushed in a sterile mortar, a capsule was opened), or one gram of dairy products in Ringers (Sigma, 96724) and plating out onto the MRS agar (Oxoid, CM361), MRS-bile agar (bovine bile concentration 0.05% w/w -Sigma, B3883) and trypticase phytone yeast (TPY) medium (Sigma, Z699195) plates. Plates were incu-

bated either aerobically or anaerobically using the Genbox system (BioMerieux, 96127) at 37 °C for 72 hours. Colonies were enumerated and colony forming units (cfu) per gram and per capsule or tablet were calculated for each sample (9). For colony selection, representative colonies showing different morphology were selected, purified and coded for further study. All Gram-positive rods which were catalase negative were retained. Colonies showing different morphology isolated from each product were sequentially numbered after the products code.

Isolates from products, which would not grow aerobically, were tested for fructose 6 phosphate ketokinase (F6PKK) which is proposed as the definitive test for *bifidobacteria* (9). Other isolates were identified using biochemical method including: oxidase, easculin test, Ammonium production from arginine, growth under aerobic conditions, gas production from glucose, growth at 15 °C and 40 °C and sugar fermentation test (10).

3.3. Probiotic Properties

3.3.1. Acid, Salt and Bile Tolerance Tests

For acid tolerance, acetic acid or citric acid was added to MRS agar to adjust the pH to 2.5 and for salt tolerance, Sodium chloride (Sigma) was added to MRS agar to adjust the salt concentration to either 4 or 7 % respectively (11). The medium was then sterilized by autoclaving at 121 °C for 15 min. For Bile tolerance test, plates of MRS agar were prepared containing bovine (Sigma) or porcine bile (Sigma) at 0.5, 1, 2, 4, 6 and 8 % concentrations. Twenty-five mL of human bile (kindly supplied by Scarborough General Hospital and kept in a refrigerator) was obtained from the gall bladder of one patient with cholelithiasis. The sterility of human bile was checked then appropriate amounts were added to warm MRS agar to achieve 0, 0.1, 0.25, 0.5, 1, 2, 4, 6 and 8 % concentrations before plates poured. MRS plates were used as control. Using the multipoint inoculator, plates were inoculated with isolated strains. Plates were incubated anaerobically at 37 °C for 48 hours. Good growth was reported as positive, slight growth as +/- and no growth as negative (12).

3.3.2. Stability Under Refrigeration Conditions

Diluted culture was prepared by addition of 50 μL of overnight bacterial culture to 5 mL Ringer solution. Fifty μL of the diluted culture of each strain was added to bijou bottles containing ultra-high temperature (UHT) milk (Morrisons, UK), MRS broth or skimmed milk. After incubation anaerobically at 37 °C for 48 hours, the bottles were stored in +4 °C. One loop-full of bottle content was streaked on to the surface of MRS agar after 3, 6, 10 and 20 days and incubated anaerobically at 37oC for 48 hours. Colony forming was considered as viability of strains (13).

3.3.3. Antagonistic Activity of Isolates Against Pathogens and other Isolates

Most of the isolates tested in the presented work, are marketed as multi-strains with up to five strains in one preparation. Antagonistic activity of selected isolates was tested against other isolates to evaluate this strategy. Also pathogen antagonistic activity of isolates was tested using spot agar test. The modified method of Jacobsen et al. (14) was used to determine the ability of *lactobacillus* and *bifidobacterium* strains to inhibit growth of other bacteria. Bacterial strains used were *E. coli* (NCTC104818), *Salmonella typhymurium* (ATCC14028), *Pseudomonas aeruginosa* (NCTC6749), and *Salmonella enterica* (S76), *Listeria monocytogenes*, from University of Bradford teaching collection, plus 4 strains of *lactobacillus* isolates subsequently identified as: *L. rhamnosus*, *L. acidophilus*, *L. plantarum* and *L. plantarumarabinosis*. Using the multi-point inoculator, MRS agar plates were inoculated with all isolates. Then the plates were incubated anaerobically at 37 °C for 48 hours. The other bacteria listed above were cultured in nutrient broth, incubated aerobically at 37 °C for 24 hours. Seven ml of overnight culture was added to top agar (for lactobacillus 0.7% agar in MRS broth, for non-*lactobacillus* 0.7% agar in nutrient broth was used). The inoculated top agar was layered on the previously multipoint inoculated and incubated plates. Plates were then incubated according to the conditions required by the overlay culture. Zones of inhibition were recorded. Growth free zones of less than 20 mm were reported as resistant, 20-29 mm recorded as intermediate and more than 30mm was recorded as sensitive (15).

3.3.4. Antibiotic Sensitivity Tests

The antibiotic sensitivity profiles for *lactobacillus* strains were determined using the disc diffusion method (16). Overnight cultures of all strains on agar plates and the Oxford strain of *Staphylococcus aureus* (as a sensitivity control), were suspended in Ringer to provide an inoculum. This was then applied to the surface of MRS agar (for *lactobacilli* and *bifidobacteria* strains) or nutrient agar (for *S. aureus*), using a plate-rotator and a sterile swab. Antibiotic discs used were: *Tetracycline* 5μg, *Chloramphenico l* 30μg, *Ciprofloxacin* 5μg, *Ampicilline* 2 μg, *Penicillin* 1.5 unit, *Erythromycin* 10 μg, *Vamcomycin* 30 μg, *Gentamicin* 10 μg and *Kanamycin* 30 μg (all from Oxoid).

Antibiotic impregnated discs were applied to the inoculated agar surface. Strains were incubated anaerobically at 37 °C for 48 hours, and *S. aureus* was incubated aerobically at 37 °C for 24 hours. The diameter of the zone of inhibition around each disc was recorded. According to NCCLS standard and Verdenelli et al. (17) a growth free zone around antibiotic disks of less than 19 mm was reported as resistant, 19-30 mm intermediate and more than 30 mm as sensitive.

3.3.5. Adherence Assay

Type strains and test isolates were tested in adherence assay cording to our previous study (18). Briefly, monolayers of *Caco*-2 cells (University of Bradford stock) were grown in Minimum Essential Medium (Sigma) contained Non-essential amino acid solution (Sigma) & L-glutamine (GIBCO, UK) and fetal calf serum (Lablech 4-101-500), cultured at a density of 5×10^3 cells / mL into 12-well plates (Corning / Costar 3513) with a coverslip (16 mm diameter, BDH, 406 / 89 / 22) at the bottom of each well and incubated at 37 °C, 5% CO_2. The Cells were ready for use after 14 days. The monolayers were washed twice with PBS. The cells were then incubated with approx. 1.5×10^7 cfu mL -1 of the strain to be tested. After 3 hours 37 °C in a 5% CO_2 atmosphere, the media and unattached bacteria were aspirated and the wells, still containing the cover slips, were washed, fixed and Gram stained using a standard Gram stain method (19).

The stained coverslips were dehydrated and attached on a microscope slide. At least ten different fields or 100 cells from each slide were observed under the microscope (Nikon, Japan) (1000× magnification). The number of cells in each field and the number of bacteria attached to each of those cells were counted (14). The experiments were repeated at three different passage levels in duplicate on each occasion. Images were captured using a light microscope associated camera (Nikon, Eclipse 80i, Japan, 2004) and ACT-2U software (Japan, 2004). The level of adherence was categorized as poor, moderate and good that had respectively < totally 20, totally 21-50 and > totally 51 bacteria per 100 cells. The pattern of adherence was called diffuse if bacteria were spread randomly around the cultured cell line or localized when groups of bacteria were attached to the cells.

4. Results

4.1. Bacterial Content Enumeration and Identification

Our results showed that the bacterial content of all dairy products was higher than 10^{10} cfu/g. The number of bacteria present in the dairy products was not stated. In only 3 out of ten non-dairy probiotic products the number of colonies was in accordance with that declared on the label. Initial attempts to isolate organism from 3C1 failed, so five capsules were used instead of a single one and from these five capsules, only 50 colonies were produced. Another pot of apparently the same product from the manufacturer (but different batch number) was purchased and examined. On opening the pot it was noticed that the capsule shape and content was different compared to the initial purchase which was a gelatinous capsule containing an oil. The count from the new formulation was in accordance with the declared number on the label. The bacterial count of 1C, 2C, 4C and 5C was lower than what was stated on the label. These four products plus 6C were pharmaceutical products which are supposed to contain *bifidobacteria*. So this smaller number may be because of a lack or decrease in the number of *bifidobacteria*.

All Gram-positive and catalase negative rods were retained for further study. All *lactobacilli* were identified at species level and *bifidobacteria* were identified at genus level. Results of product content are shown in Table 1.

4.2. Tolerance Tests

All dairy isolates were resistant to acid and bile and were alive after twenty days refrigeration. All pharmaceutical isolates were able to grow at pH 2.5 and the 0.3 % bile concentration. The exceptions were 1C5 (*L. acidophilus*) that was sensitive to acidity and the type cultures of *B. bifidum* and *B. Longum* that were sensitive to bile, acidity and 7% NaCl (Table 2).

Viable bacteria were isolated from all non-dairy isolates after 20 days refrigeration with the exception of 1C5 (*L. acidophilus*) and 4C2 (*L. acidophilus*) (Table 2).

4.3. Antibiotic Susceptibility and Antagonistic Activity

Almost all strains were resistant to *ciprofloxacin*, *vancomycin*, *gentamicin*, *streptomycin* and *kanamycin* and all isolates were sensitive to a *mpicillin* and *erythromycin*. Antibiotic sensitivity tests and antagonistic activity results are shown in Table 3. There was not any relation between antibiotic sensitivity and source of isolates. Almost all isolates showed antagonistic activity against tested isolates (Table 3). The results showed that isolated strains from multi strain products had inhibitory effects against other *lactobacilli* strains (Table 3).

4.4. Adherence Patterns of Lactobacilli and Bifidobacteria to HEp-2 Cells

The lactobacilli and bifidobacteria showed different levels and patterns of adherence to culture cells. The number of bacteria attached to each cell of the cell line was recorded for 100 culture cells. Adherence patterns were consistent for an isolate between replicates. Nine out of ten (90%) *L. acidophilus* and 4 out of 5 (80%) *L. rhamnosus* isolates gave the diffuse pattern, whilst 5 out of 6 (83.3%) *L. plantarum* showed the localised pattern (Table 3). For *bifidobacteria*, all dairy isolates showed localize adherence, while type strains *B. longum* and *B. bifidum* showed diffuse adherence. The adherence levels of all dairy *bifidobacteria* isolates were good. Type strain *B. longum* showed poor adherence and *B. bifidum* gave moderate adherence.

Table 2. Tolerance Tests of Isolates.

Strains[a]	Refrigeration Stability[b]				NaCl 7%	NaCl 4%	Growth at pH 4.5[c]	Inhibitory Concentration %		
	3 days	6 days	10 days	20 days				Bovine bile	Porcine bile	Human bile
1C1 (*L. pl.arabinosis*)	+	+	+	+	+	+	+	>8	>8	>8
1C2 (*L. acidophilus*)	+	+	+	+	-	-	+	2	1	>8
1C3 (*L. acidophilus*)	+	+	+	+	-	-	+	2	1	>8
2C1 (*L. acidophilus*)	+	+	+	+	+	+	+	6	4	>8
2C2 (*L. casei rhamnosus*)	+	+	+	+	-	+	+	6	4	6
2C3 (*L. acidophilus*)	+	+	+	+	-	-	+	0.5	0.5	2
2C4 (*L. casei casei*)	+	+	+	+	-	-	+	0.5	0.5	2
3C12 (*L. plantarum*)	+	+	+	+	+	+	+	6	2	2
3C14 (*L. plantarum*)	+	+	+	+	+	+	+	>8	>8	>8
3C15 (*L. casei rhamnosus*)	+	+	+	+	+	+	+	>8	>8	>8
3C21 (*L. casei casei*)	+	+	+	+	+	+	+	6	6	6
3C22 (*L. casei casei*)	+	+	+	+	+	+	+	6	4	8
3C23 (*L. casei rhamnosus*)	+	+	+	+	+	+	+	6	2	6
4C1 (*L. acidophilus*)	+	+	+	+	-	w	+	6	4	6
4C2 (*L. acidophilus*)	+	-	-	-	-	w	+	6	N	6
4C3 (*L. casei rhamnosus*)	+	+	+	+	+	+	+	4	0.5	4
5C1 (*L. acidophilus*)	+	+	+	+	+	+	+	8	N	4
5C2 (*L. acidophilus*)	+	+	+	+	+	+	w	6	2	6
6C1 (*L. plantarum*)	+	+	+	+	+	+	+	>8	>8	>8
6C2 (*L. casei rhamnosus*)	+	+	+	+	+	+	+	N	2	6
6C3 (*L. plantarum*)	+	+	+	+	+	+	+	>8	>8	>8
6C4 (*L. plantarum*)	+	+	+	+	+	+	+	>8	>8	>8
6C5 (*L. plantarum*)	+	+	+	+	+	+	+	>8	>8	>8
6C6 (*L. acidophilus*)	+	+	+	+	+	+	+	>8	>8	>8
8C1 (*L. plantarum*)	+	+	+	+	+	+	+	2	2	4
9C1 (*L. reuteri*)	+	+	+	+	-	+	+	2	2	2
10C1 (*L. casei*)	+	+	+	+	+	+	+	4	4	4
10C2 (*L. acidophilus*)	+	+	+	+	-	+	+	4	4	6
1D1 (*L. plantarum*)	+	+	+	+	+	+	+	>8	>8	>8
1D2 (*L. brevis*)	+	+	+	+	+	+	+	>8	>8	>8
2D3 (*L.sanfrancisco*)	+	+	+	+	-	+	w	1	>8	>8
3D1 (*L. lactis*)	+	+	+	+	+	+	+	<0.1	<0.1	4
3D2 (*L. lactis*)	+	+	+	+	+	+	+	0.5	4	>8
4D1 (*L. casei Immunitass*)	+	+	+	+	+	+	+	8	4	>8
4D2 (*L. casei Immunitass*)	+	+	+	+	-	w	+	8	>8	6
5D1 (*L. delbrueckii*)	+	-	-	-	+	+	w	>8	>8	>8
5D2 (*Non-identidified*)	+	+	+	+	-	w	+	<0.1	1	1
6D1 (*L. casei Shirota*)	+	+	+	+	-	+	+	4	4	6
6D2 (*L. casei Shirota*)	+	+	+	+	-	+	+	4	4	4
7D1 (*Bifidobacterium*sp.)	+	+	+	+	-	w	w	0.5	0.5	1
8D1 (*Bifidobacterium*sp.)	+	+	+	+	-	w	w	0.5	0.5	1

9D1 (*Bifidobacterium*sp.)	+	+	+	+	-	w	w	0.5	0.5	1
10D1 (*L. casei*)	+	+	+	+	+	+	+	2	2	4
***B. bifidus*T**	+	+	+	+	-	+	-	-	<0.1	0.5
***B. longum*T**	+	+	-	-	-	+	-	-	<0.1	0.5
***L. acidophilus*T**	+	+	+	+	+	+	+	-	<0.1	0.5
***L. casei casei*T**	+	+	+	+	+	+	+	+	>8	>8
***L. rhamnosus*T**	+	+	+	+	+	+	+	+	0.1	0.5

[a] Abbreviations: N, not determined; + , positive growth; -, no growth; w, poor growth.
[b] There were no differences between UHT milk, skimmed milk broth and MRS broth
[c] There was no difference between citric acid and acetic acid

Table 3. Antibiotic Sensitivity Test, Antagonistic Activity and Adherence Properties of Isolates

Isolates[a]	Antibiotic Sensitivity									Antagonistic Activity									Adherence	
	Penicillin 1.5 Units	Ampicillin 2 mcg	Tetracycline 5 mcg	Erythromycin 10 mcg	Ciprofloxacin 5 mcg	Chloramphenicol 30mcg	Kanamycin30 mcg	Vancomycin 30 mcg	Gentamycin10 mcg	*L. monocytogenes*	*S. typhimurium*	*Salmonella enterica*	*P. aeruginosa*	*E. coli* G24	*L. pl.arabinosis* (1C1)	*L. rhamnosus* (3C23)	*L. plantarum* (6C3)	*L. acidophilus* (2C1)	Pattern	Level
1C1 (L. pl.arabinosis)	R	S	R	I	R	R	R	R	R	+	+	+	+	+	+	+	+	+	D	G
1C2 L. acidophilus)	S	I	S	S	R	S	R	R	R	+	+	+	+	+	+	-	+	+	L	G
1C3 (L. acidophilus)	S	I	S	S	R	S	R	R	R	+	+	+	+	+	+	-	+	+	D	G
2C1 (L. acidophilus)	S	I	I	S	R	S	R	R	R	+	+	+	+	+	+	+	+	+	D	G
2C2 (L. casei rhamnosus)	S	I	I	S	R	S	R	R	R	+	+	+	-	+	+	+	+	+	D	M
2C3 (L. acidophilus)	S	S	S	S	R	S	R	R	R	+	+	+	+	+	+	+	+	+	D	G
2C4 (L. casei casei)	S	S	S	S	R	S	R	R	R	+	+	+	+	+	+	+	+	+	D	G
3C12 (L. plantarum)	I	I	I	I	R	I	R	R	R	+	+	+	+	+	+	+	+	+	L	P
3C14 (L. plantarum)	R	S	R	I	R	S	R	R	R	+	+	+	+	+	+	+	+	+	L	G
3C15 (L. casei rhamnosus)	R	S	R	I	R	S	R	R	R	-	-	-	+	+	+	+	-	+	D	P
3C21 (L. casei casei)	I	S	I	I	R	I	R	R	R	+	+	+	+	+	+	+	+	+	L	P
3C22 (L. casei casei)	I	I	I	I	R	I	R	R	R	+	+	+	+	+	+	+	+	+	D	P
3C23 (L. casei rhamnosus)	I	I	I	I	R	I	R	R	R	+	+	+	+	+	+	+	+	+	D	G
4C1 (L. acidophilus)	S	S	S	S	R	S	I	I	I	+	+	-	+	+	+	-	+	+	D	M
4C2 (L. acidophilus)	I	I	I	S	I	S	R	R	R	+	+	-	+	+	+	+	+	+	D	G
4C3 (L. casei rhamnosus)	I	R	I	I	R	I	R	R	R	+	+	-	+	+	+	+	+	+	D	M
5C1 (L. acidophilus)	S	I	I	S	I	S	R	R	R	+	+	-	-	+	+	+	+	+	D	M
5C2 (L. acidophilus)	I	S	R	S	R	S	R	R	R	+	+	-	+	+	+	+	+	+	L	M
6C1 (L. plantarum)	I	I	I	S	I	I	R	R	R	+	+	-	+	+	+	+	+	+	L	M
6C2 (L. casei rhamnosus)	I	S	R	I	R	S	R	R	R	+	+	-	+	+	+	+	+	+	L	G
6C3 (L. plantarum)	R	S	R	I	R	S	R	R	R	+	+	-	+	+	+	+	+	+	D	G
6C4 (L. plantarum)	I	S	R	I	R	S	R	R	R	-	-	-	+	+	+	+	+	+	L	G
6C5 (L. plantarum)	I	S	R	I	R	S	R	R	R	-	-	-	+	+	+	+	+	+	D	G
6C6 (L. acidophilus)	R	I	R	I	R	I	I	R	R	+	+	-	+	+	+	+	+	+	D	G
8C1(L. plantarum)	S	S	I	S	R	R	R	I	R	+	+	-	+	+	+	+	+	+	L	G
9C1(L. reuteri)	S	S	R	R	R	S	R	R	R	+	+	-	-	+	+	+	+	+	D	G
10C1 (L. casei)	I	I	I	R	I	S	R	R	R	+	+	-	+	+	+	+	+	+	D	M
10C2 (L. acidophilus)	S	I	R	I	R	S	R	R	R	+	+	-	+	+	+	+	+	+	D	M
1D1 (L. plantarum)	R	I	R	I	R	I	R	R	R	+	-	-	+	+	+	+	+	+	L	G
1D2 (L. brevis)	I	S	R	I	R	S	R	R	R	+	+	-	+	+	+	+	+	+	L	G
2D3 (L. sanfrancisco)	S	S	S	S	R	S	R	R	R	+	-	-	+	+	+	+	-	-	L	G
3D1 (L. lactis)	S	S	S	S	R	S	R	R	R	+	-	-	+	+	+	+	-	-	D	G
3D2 (L. lactis)	S	S	S	S	R	S	R	R	R	+	-	-	+	+	+	+	-	-	D	G
4D1 (L. casei Immunitass)	S	I	I	S	R	S	I	R	R	+	+	-	-	+	+	+	+	+	D	P

4D2 (L. casei Immunitass)	R	R	R	R	R	R	I	R	R	-	-	-	-	-	-	-	-	-	D	P
5D1 (L. delbrueckii)	S	S	I	R	R	S	R	R	I	+	-	-	+	+	+	+	+	+	D	G
5D2 (Non-identidified)	I	I	I	S	R	S	R	R	R	+	+	-	+	+	+	+	+	+	D	P
6D1 (L. casei Shirota)	S	I	I	S	R	S	R	R	R	+	+	-	+	+	+	+	+	+	L	P
6D2 (L. casei Shirota)	S	I	I	S	R	S	R	R	R	+	+	-	+	+	+	+	+	+	L	P
7D1 (Bifidobacterium sp.)	I	S	S	I	R	S	R	R	R	+	-	+	-	+	+	+	+	-	L	G
8D1 (Bifidobacterium sp.)	I	S	S	S	R	I	R	R	R	+	-	+	+	+	+	+	+	-	L	G
9D1 (Bifidobacterium sp.)	I	S	S	I	R	S	R	R	R	+	-	+	-	+	+	+	-	-	L	G
10D1 (L. casei)	R	I	S	S	R	I	R	R	R	+	+	-	+	+	+	-	+	+	L	G
B. bifidus T8010	I	S	I	S	R	S	R	R	R	+	+	-	+	+	+	+	+	+	D	P
B. longum T701748	S	S	S	I	R	S	R	R	R	+	+	+	+	+	+	+	+	+	D	P
L. acidophilus T11970	I	R	S	S	I	I	R	I	R	+	+	+	+	+	+	+	+	+	D	P
L. casei casei T702259	R	S	I	S	R	S	R	R	R	-	-	-	+	+	+	+	-	+	D	G
L. rhamnosus T702715																				

[a] Abbreviations: S, sensitive; R, resistant; I, intermediate; -, no antagonistic activity; +, antagonistic activity; P, Poor; M, Moderate; G, Good; L, Localised; D, Diffuse; T, NCIMB type strains

5. Discussion

The results of the present study were: the smaller numbers of microorganisms in the products especially *bifidobacteria* than what was stated by manufacture on the label, the higher number of viable bacteria in dairy products than pharmaceutical, possessing the important criteria for probiotic bacteria with the exception of adherence ability in which 30% of dairy isolates and 14% of pharmaceuticals were poorly adherent, the higher activity of pharmaceutical isolates against pathogen compared to dairy products and finally inhibitory effects of some probiotic bacteria against each other's in multi-strains products.

The presence of the declared probiotic species and number is considered an important requisite for a product to be defined as reliable. The present study showed that some products contained fewer *lactobacilli* or *bifidobacteria* than claimed. *Bifidobacteria* were isolated from dairy products but none from the pharmaceutical products. It may be due to the sensitivity of *bifidobacteria* to oxygen, preparation process or storage conditions (20). The smaller numbers of *lactobacilli* or *bifidobacteria* in probiotics bacteria have been reported by other authors (21, 22). Using resistant cultures and new methods such as encapsulation of bacteria in the starch matrices has been suggested to improve shelf-life of bacteria (23). The present study showed that some products contained two or more species or strains that had inhibitory effects against each other, such as *L. acidophilus* and *L. plantarum* in 6C product. This may be an explanation for the lower number of probiotic bacteria detection from the products.

The viable count of bacteria in dairy products was higher than pharmaceuticals. This was in agreement with Masco et al. (22) that stated the numbers of viable bacteria were generally lower in pharmaceuticals than in dairy products, with no viable bacteria being found in 37% of pharmaceuticals. It may be because of the technological stresses, i.e. freeze-drying or spray drying loss of viability (24).

All tested strains (except *L. acidophilus* isolated from 1C), fulfilled characteristics important for survival through the gastrointestinal tract such as acid and bile tolerance. Resistance to acidic condition (pH 3.5) can be used as a criterion for *lactobacilli* and *bifidobacteria* selection (11). Bile resistance results were in agreement with results reported previously (11). Human bile tolerance of all isolates was more than 1%, greater than human bile concentration in the small intestine (25).

If a strain is to be used as a probiotic, it ought to be verified that they do not contain transferable antibiotic resistance genes that could be transferred to other bacteria especially pathogens (26). Antibiotic resistance profiles of isolated strain were consistent within a species. This is similar to Danielsen and Wind (28) who reported that the level of susceptibility to the antimicrobial agents is species-dependent. The present study showed that all strains were resistance to *vancomycin*, *kanamycin* and *ciprofloxacin*. This was reported previously by Verdenelli (17) as well. Using antibiotic resistant probiotic isolates could be useful to neutralize the side effects of antibiotics, because the probiotics will survive while administrated with antibiotics.

The ability of strains to adhere to culture cells was evaluated, as it is believed to be one of the essential features required for the delivery of their health benefits (28, 29). In this study, adhesion to cell lines was found to be a discriminative parameter, showing variation among the strains independent of species. This variation among *Lactobacillus* sp. has been observed before (14, 30).

We found that 33% probiotic dairy isolates and 14% pharmaceutical probiotic isolates were poorly adherent. The relation between adherence level of isolates and batch product is comparable with the results of Clements and co-workers (32) who noticed significant batch-to-batch variation between freezes dried *lactic acid* bacteria *in vitro* and *in vivo*. The method of bacterial maintenance may alter adherence of probiotics. Elo et al. (33) reported that weekly transfer of bacteria in MRS broth decreased the

adherence of bacteria after 3.5 years.

An important aspect of the function of probiotic bacteria is the protection of the host gastrointestinal microenvironment from invading pathogens. The present study showed that 2 strains isolated from 2C *identified* as *L. casei* and *L. acidophilus* were active against a range of pathogens. None of strains isolated from dairy products had broad-spectrum antibacterial activity and this may be due to technical limitations for selection of the strain for dairy preparation. Most of dairy isolates produce lower acid and hydrogen peroxide and it may affect their antagonistic activity against pathogen (33).

Most of the isolates tested in the presented work, are marketed as multi-strains with up to five strains in one preparation. To test if this is a valuable strategy, antagonistic activity of selected isolates was tested against other isolates. Our results showed antagonistic activity of multi-strain products needs to be tested before marketing.

Novel cultivation techniques should be considered and matrix effects in the probiotic viability and stability must be elucidated. The study of resistant bacteria may be helpful in recognizing survival mechanisms of probiotics and consequently in development of strain. The focal point of additional research should be on probiotic culture stability and viability in relation to conferring health benefits for the consumer.

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Author's Contribution

Dr. khodaii and Dr. Mehrabani discussed original ideas, designed the study protocol, analyzed the data, and wrote the manuscript; Dr. Khodaii is guarantor for the paper. Dr. Ghaderian contributed to the development of the protocol and prepared the manuscript.

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