Lactic Acid Bacteria in Pharmaceutical Formulations: Presence and Viability of "Healthy Microorganisms"

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Abstract: Many probiotic formulations are available in the market and are advertised for several preventive or curative roles. The aim of this study was the identification of microorganisms composing different lactic acid bacteria based pharmaceutical formulations and the ascertainment of their ability to survive gastro-intestinal (GI) stresses, the main requisite to produce beneficial effects. For this purpose, viable bacteria were enumerated by plate counts in different media. Denaturing Gradient Gel Electrophoresis-Polymerase Chain Reaction (PCR-DGGE) analysis was applied on pure isolates and on crude formulations to confirm the composition in species. Also, crude formulations were subjected to stresses characteristic of the GI tract (GIT) to assess cell survival. Results highlighted concentrations lower than those reported in the labels in almost all the formulations. Moreover, some discrepancies were observed between reported species and those ascertained through the identification, and the use of an erroneous nomenclature was highlighted. The GI stress test revealed that bacteria are strongly injured, and this fact was evidenced by a marked reduction in viable counts after the stress. In conclusion, a widespread number of lactic acid bacteria based formulations are sold as probiotics, but their probiotic requisites are not adequately observed.

Keywords: Probiotic, PCR-DGGE, gastro-intestinal stress, survival, dietary supplement, antidiarrheal drug, lactic acid bacteria.

1. INTRODUCTION

Probiotic microorganisms are available in the market in different forms: probiotic foods, essentially milk-based fermented beverages [1], and dried/lyophilized mono- or mixed cultures of probiotic microorganisms, in the form of powders, pills or tablets. If the former can be considered as "functional foods" (Regulation EC 178/2002) [2], the latter represent a separate group, since tablets, pills or powders are not included in the technical definition of functional foods. As a consequence, the European market offers a widespread variety of probiotic formulations which can be considered as "border products", i.e. formulations that are neither food supplements, nor drugs, nor dietary supplements, because of the lack of an appropriate regulatory system [3].

To be effective, probiotic strains must retain their functional health characteristics for which they were originally selected, including the ability to survive transit through the stomach and small intestine [4]. Also, the Joint FAO/WHO Expert Consultation [5] recommended that proper *in vitro* studies should be carried out to establish the potential health benefits of probiotics prior to undertaking *in vivo* trials. This matter was investigated with conflicting results; in fact, many Authors reported, on the one hand, the ability of some probiotic strains to survive stresses encountered during the passage through the GI tract (GIT), on the other hand, their low survival [6-9]. Numerous techniques were assayed to evaluate the resistance to gastric juices and the ability to grow in the presence of bile under conditions in the intestine. *In vitro* protocols are commonly adopted [9-12], and tests are generally performed on rehydrated strains, sub-cultured in optimal conditions (appropriate medium, optimal temperature and O_2 concentration, etc.). In our opinion, the approach described above is not adequate for lyophilized formulations, since their ingestion, and subsequent passage from the mouth to the gut of the consumer, is not preceded by rehydration/activation steps. On these bases, in the present work pharmaceutical formulations, consisting of mono- or mixed cultures of lactic acid bacteria (LAB), were assayed to verify an important probiotic requisite, such as the resistance to those stresses which are typical of the GIT, without a preliminary rehydration step. The GI stress test was preceded by the enumeration of viable LAB in each pharmaceutical formulation and by the ascertainment of the identity in species, comparing results with those reported on the labels. These goals

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were reached through a multiple approach, based on the application of both culture-dependent and culture-independent methods.

2. MATERIALS AND METHODS

2.1. Probiotic Formulations

Nine commercial formulations, consisting of mono- or mixed cultures of *Lactobacillus* and *Streptococcus* species, were bought as probiotics in chemist's shops. Formulations were stored at 4°C and analysed before the "use by" date expiry. The main characteristics of the products are reported in Table **1**.

2.2. Presumptive Enumeration and Isolation of Viable Bacteria

The dried powder inside one sachet (dose) of each pharmaceutical formulation was aseptically weighed and 1:10 diluted in physiological sterile solution (9 g/L NaCl). Formulation J, in form of tablet (see Table **1**), was roughly crumbled directly inside the blister to avoid contamination and then 1:10 diluted in physiological sterile solution. Samples were homogenized in a Stomacher 400 Lab Blender (PBI International, Milan, Italy) until their complete dissolution. Serial decimal dilutions were plated in appropriate culture media and optimal incubation conditions as follows:

Lactobacillus bulgaricus and *Lb. casei* (formulation A) were differently enumerated on MRS agar (Oxoid, Milan, Italy) at pH 5.2 (MRS pH 5.2) incubated at 43°C [13] and on MRS with 0.01% vancomycin (MRS-V) incubated at 37°C [14], respectively. Those formulations containing *Lb. rhamnosus* (B and C), *Lb. casei* (E), *Lb. paracasei* (F), and *Lb. reuteri* (J) as single species were plated on MRS agar (Oxoid) incubated at 37°C (MRS 37°). *Lb. paracasei* and *Lb. salivarius* (formulation D) were differently enumerated on Homofermentative-Heterofermentative Differential (HHD) agar [14] incubated at 37°C. Tryptone Soy Agar plates incubated at 37°C (TSA 37°), specific for *Bacillus coagulans*, were used to enumerate *Lb. sporogenes* from formulation G [15], since commercial products containing *Bacillus coagulans* use the invalid name "*Lactobacillus sporogenes*" on the label. *Lb. acidophilus* and *Lb. bulgaricus* from formulations G and H were differently counted on BA-maltose agar (BA-M) incubated at 37°C or in MRS pH 5.2 incubated at 43°C, respectively, both prepared as described by Tharmaraj and Shah [13]. *Streptococcus thermophilus*, also reported in G and H, was counted on M17 (M17 37°) agar (Oxoid) and in ST (ST 37°) agar, incubated at 37°C for 72h or for 24h, respectively, as described by Dave and Shah [16]. Incubation was performed in anaerobiosis (Anaerogen, Oxoid, Milan, Italy), except for *B. coagulans* (*Lb. sporogenes*) and *S. thermophilus*,

Table 1: Features Listed on the Labels of 9 Commercial Pharmaceutical Formulations

Product abbreviation	Form	Category	Microorganisms reported on the label
A	Lyophilized powder[a]	Antidiarrheal drug	*Lactobacillus bulgaricus* *Lactobacillus casei*
B	Lyophilized powder (2,46 g)	Dietary supplement	*Lactobacillus rhamnosus* XX[b]
C	Lyophilized powder (2,46 g)	Dietary supplement	*Lactobacillus rhamnosus* XX[b]
D	Lyophilized powder (2 g)	Dietary supplement	*Lactobacillus paracasei* XX[b] and *Lactobacillus salivarius* XX[b] (12:1)
E	Lyophilized powder (3 g)	Dietary supplement	*Lactobacillus casei* sub. *casei* XX[b]
F	Lyophilized powder (2,5 g)	Dietary supplement	*Lactobacillus paracasei* sub. *paracasei* XX[b]
G	Lyophilized powder (4 g)	Dietary supplement	*Lactobacillus sporogenes* *Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Streptococcus thermophilus*
H	Lyophilized powder (4 g)	Antidiarrheal drug	*Lactobacillus acidophilus* XX[b] *Lactobacillus delbrueckii* XX[b] *Streptococcus thermophilus* XX[b]
J	Tablet (0,45 g)	Dietary supplement	*Lactobacillus reuteri* XX[b]

[a]Amount not available.
[b]Reference strain number reported on the label.

which required aerobic conditions. MRS agar (Oxoid) incubated at 37°C was regularly used to confirm lactobacilli counts and, if needed, to isolate colonies.

Counts were performed in duplicate on 2 different doses belonging to the same probiotic package.

Five to ten colonies of various forms and colour were randomly picked from agar plates containing between 30 and 300 colonies, purified by streaking in fresh media and maintained frozen at -80°C in microbanks (Pro-Lab Diagnostics, Richmond Hill, Canada) [17].

2.3. Survival After Gastro-Intestinal Stresses

As before, the experiment was performed in duplicate on 2 different doses belonging to the same package, following the protocols described by de Palencia *et al.* [11] and by Ritter *et al.* [9] with some modifications. In detail, one dose (lyophilized powder or tablet) of each crude pharmaceutical formulation was transferred into a sterile 100 mL Drechsel bottle (Carlo Erba, Milan, Italy) coupled to a nitrogen cylinder. Forty-five milliliters of sterile water was added in each bottle. To simulate the *in vivo* dilution of saliva, 10 mL of a sterile electrolyte solution (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$ and 1.2 g/L $NaHCO_3$) and then lysozyme (Sigma-Aldrich) to give a final concentration of 0.01% were added. To simulate the gastric environment, 10 mL of electrolyte solution containing 0.3% pepsin (final concentration, Sigma-Aldrich) was added and the pH was decreased to 2.0 by adding 1.0 mol/L HCl. Suspensions were incubated for 1 h in aerobic conditions. To simulate the intestinal stress, the oxygen was replaced by nitrogen to obtain an anaerobic atmosphere and pH was adjusted to 5.0 with a sodium bicarbonate saturated solution (8 g sodium bicarbonate in 100 mL distilled water, sterilized at 121°C for 15 min). Eight milliliters of a sterile electrolyte solution containing 0.45% bile salts and 0.1% pancreatin (final concentration, both from Sigma-Aldrich) were added. Then, pH was adjusted to 6.3 and slowly increased to 7.5 until the end of the assay (7 hours in all). The gastric and gastro-intestinal solutions were prepared fresh daily and the entire trial was performed at 37°C. Finally, formulations were analysed for cell survival by plate counts as follows:

a) MRS 37° in anaerobiosis for lactobacilli;

b) M17 37° in aerobiosis for *S. thermophilus*;

c) TSA 37°in aerobiosis for spore-forming bacteria.

2.4. DNA Extraction from Pure Cultures and PCR-DGGE Conditions

Pure frozen cultures isolated from each pharmaceutical formulation were revitalized in appropriate media. Two milliliters of each overnight culture was than centrifuged at 14 000 ***g*** for 10 min at 4°C (Centrifuge 5415 R; Eppendorf, Hamburg, Germany) to pellet the cells and the pellet was subjected to DNA extraction according to Querol *et al.* [18], with the addition of lysozyme (25 mg/mL, Sigma) and mutanolysin (10 U/mL, Sigma) for bacterial cell-wall digestion. The DNA from each strain was than prepared for DGGE by amplifying the V1 region of 16S rRNA using the following primers: P1V1 (5'-GCG GCG TGC CTA ATA CAT GC-3') and P2V1 (5'-TTC CCC ACG CGT TAC TCA CC-3') [19]. A GC clamp (5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') [20] was attached to the 5' end of the P1V1 primer. Negative controls without DNA template were included in parallel. PCR and gel processing were performed as described by Reale *et al.* [21].

2.5. Sequence Analysis

One representative strain from each cluster obtained by DGGE analysis was amplified with primers P1 and P4, as described by Klijn *et al.* [19], targeting 700 bp of the V1–V3 region of the 16S rRNA gene. After purification (QIAquick PCR purification kit, QIAGEN GmbH, Hilden, Germany), products were sent to a commercial facility for sequencing (Eurofins MWG Biotech Company, Ebersberg, Germany). Sequences were aligned with those in GenBank with the Blast program to determine the closest known relatives, based on the partial 16S rRNA gene homology.

2.6. Direct DNA Extraction from Crude Formulations and PCR-DGGE Conditions

The direct extraction of nucleic acids from pharmaceutical formulations was performed according to Iacumin *et al.* [22]. The DNA from each formulation was then prepared for PCR-DGGE as described above. The DNA of 12 strains, previously isolated from pharmaceutical formulations and identified by sequencing, was re-subjected to DGGE analysis for comparative purposes. Gels were then normalized with the pattern analysis software package, Gel Compare II Version 2.0 (Applied Maths).

3. RESULTS

3.1. Viable Counts Before and After GI Stresses

Microbial concentrations reported on the label of the pharmaceutical formulations were compared with

Table 2: Comparison between Viable Bacterial Counts Reported in 9 Pharmaceutical Formulations and those Detected before and after GI Stresses

		CFU dose^{-1}		CFU dose^{-1a}	
Sample	**Species**	**Reported**	**Medium[b]**	**Detected**	**Detected after GI stresses**
A	*Lactobacillus bulgaricus*	4×10^9	MRS pH 5.2	ND	
	Lactobacillus casei	1.6×10^{10}	MRS-V	4.6×10^8 (±0.2)	
			MRS 37°[c]	4.9×10^8 (±0.2)	3.8×10^1 (±0.4)
B	*Lactobacillus rhamnosus* XX	6×10^9	MRS 37°	4.4×10^8 (±0.2)	5.8×10^2 (±0.3)
C	*Lactobacillus rhamnosus* XX	6×10^9	MRS 37°	3.5×10^8 (±0.4)	3.5×10^2 (±0.4)
D	*Lactobacillus paracasei* XX and	0.1-10 x 10^{9d}	HHD	1.4×10^7 (±0.3)	
	Lactobacillus salivarius XX (12:1)		HHD	2.0×10^7 (±0.3)	
			MRS 37°[c]	3.6×10^7 (±0.2)	1.2×10^3 (±0.3)
E	*Lactobacillus casei* sub. *casei* XX	8×10^9	MRS 37°	3.8×10^7 (±0.2)	1.3×10^3 (±0.2)
F	*Lactobacillus paracasei* sub. *paracasei* XX	1.25×10^{10}	MRS 37°	7.1×10^8 (±0.2)	2.4×10^3 (±0.2)
G	*Lactobacillus sporogenes*	1.5 x 10^{9d}	TSA 37°	1.0×10^7 (±0.1)	3.6×10^5 (±0.3)
	Lactobacillus acidophilus		BA-M	2.2×10^8 (±0.2)	
	Lactobacillus bulgaricus		MRS pH 5.2	ND	
			MRS 37°[c]	3.6×10^8 (±0.2)	4.5×10^6 (±0.2)
	Streptococcus thermophilus		M17 37°	2.0×10^7 (±0.2)	6.4×10^4 (±0.4)
			ST 37°	ND	
H	*Lactobacillus acidophilus* XX	1×10^7	BA-M	ND	
	Lactobacillus delbrueckii XX	5×10^6	MRS pH 5.2	ND	
			MRS 37°[c]	8.0×10^7 (±0.3)	6.0×10^2 (±0.2)
	Streptococcus thermophilus XX	4×10^9	M17 37°	2.7×10^9 (±0.1)	5.3×10^2 (±0.3)
			ST 37°	ND	
J	*Lactobacillus reuteri* XX	1×10^8	MRS 37°	8.3×10^8 (±0.2)	7.7×10^3 (±0.3)

[a]Data in the table are mean and ±SD obtained from two repeated assays.
[b]Incubation was performed at 37°C except for MRS at pH 5.2, which required 43°C.
[c]Total amount of lactobacilli was confirmed in MRS for multiple-strain formulations.
[d]Total amount reported.

those obtained by plate counts (Table **2**). In formulation A microbial counts resulted comparable in MRS 37° (about 8 logs) and in MRS-V, used for the selective enumeration of *Lb. casei* in presence of *Lb. bulgaricus* [14]; the sole presence of *Lb. casei* seemed to be confirmed by counts performed in MRS pH 5.2 used for the selective enumeration of *Lb. bulgaricus* [13], which evidenced the absence of colonies. Overall, in this product the load of lactobacilli resulted about 1-1.5 log lower than that reported. The microbial load registered in the different media used to enumerate lactobacilli of formulation D was about 7 logs, i.e. 2 logs lower than that reported. In detail, selective counts of *Lb. paracasei* and *Lb. salivarius* in HDD were obtained considering white colonies for the former and blue-green colonies for the latter. These results were confirmed by counts performed in MRS 37°. In the multi-strain formulation G no colony was detected in MRS pH 5.2, whereas counts of about 8 logs were detected in BA-M, used for the selective enumeration of *Lb. acidophilus*, and in MRS 37°; this fact let us suppose the sole presence of *Lb. acidophilus*. In this formulation, counts of about 7 logs were evidenced in TSA 37° and in M17 37° media, used for the selective enumeration of spore forming bacteria and *S. thermophilus*, respectively, whereas no colony was detected in ST 37°, also used for the selective enumeration of *S. thermophilus*, as suggested by Dave and Shah [16].

In the multiple-strain formulation H, MRS 37° was the sole medium that allowed the growth of lactobacilli, which resulted present with counts of about 7 logs, in accordance with data reported in the label. No colony

was evidenced in BA-M or in MRS pH 5.2. Once again, M17 37° showed the best ability to detect *S. thermophilus*, whose amount confirmed the reported load (about 9 logs), whereas no colony was evidenced in ST 37° after 24 h incubation.

As for single-strain formulations, the microbial load resulted about 1 log lower than that declared in B and C, and about 2 logs lower in E and F. The sole formulation J showed viable counts about 1 log higher than that reported.

Overall, counts registered in appropriate media highlighted the presence of high numbers of viable bacteria for all assayed formulations. The situation after GI stresses indicated a very high injury for almost all bacteria (Table **2**). The highest damage was highlighted for lactobacilli of formulation A and *S. thermophilus* of H, which evidenced a reduction in counts of 7.1 and 6.7 logs, respectively, after GI stresses. Also in formulations B and C a drastic reduction of viable lactobacilli was highlighted, as evidenced by counts of 5.9 and 6 logs lower after GI stresses. On the other hand, microorganisms contained in G showed a good ability to survive harsh conditions, as demonstrated by counts after GI stresses of about 1.4-2.5 logs lower than those registered prior the assay. All the other pharmaceutical formulations (D, E, F, H and J) showed counts of lactobacilli from 4.5 to 5.5 logs lower after GI stresses.

3.2. DGGE Analysis

Migration profiles obtained by DGGE analysis of pure cultures allowed the individuation of 18 clusters with 80% similarity level, arbitrarily chosen for defining species (Figure **1**). One strain belonging to each cluster was than subjected to sequencing for identification purposes. On the basis of the results of sequencing (Table **3**) and cluster analysis (Figure **1**) it was possible to correctly identify 10 strains as *S. thermophilus* (clusters 1 and 3), 9 strains as *Bacillus coagulans* (clusters 2, 6 and 7), 5 strains as *Lb. delbrueckii* ssp. *bulgaricus* (clusters 4 and 5), 5 strains as *Lb. reuteri* (cluster 8), 9 strains as *Lb. paracasei* (clusters 9 and 12), 10 strains as *Lb. rhamnosus* (clusters 10, 11 and 13), 6 strains as *Lb. salivarius* (cluster 14), 6 strains as *Lb. acidophilus* (cluster 15), and 15 strains as *Lb. casei* (clusters 16, 17 and 18). Comparing these results with the species reported on the labels of formulations (Table **1**), we found a full correspondence for 6 products (B, C, D, E, F and J) and a partial one for the remaining 3 (A, G and H). In detail, in preparation A, 5 isolates were obtained from plates of MRS-V and 5 from MRS 37°, since MRS pH 5.2 did not allow the isolation of colonies (see Table **2**). All the isolates were

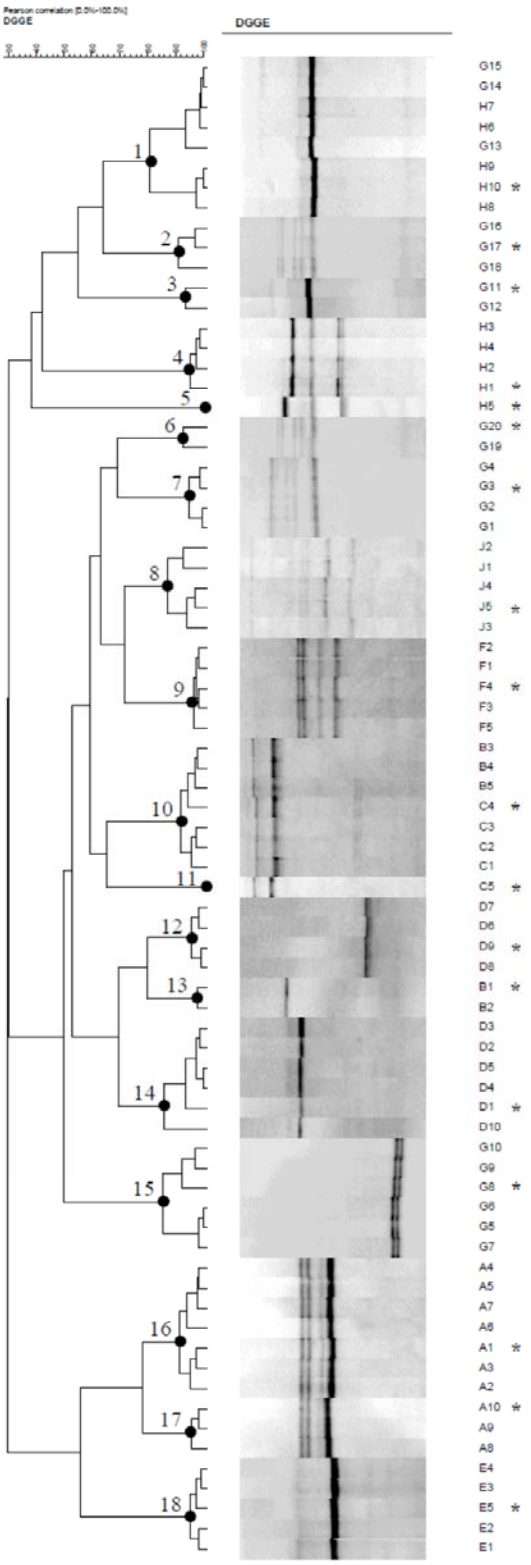

Figure 1: Dendrogram obtained after elaboration of DGGE profiles (Gel Compare II Version 2.0, Applied Maths) of DNA extracted from microorganisms isolated from 9 pharmaceutical formulations. Asterisks (*) indicate those isolates identified by sequencing.

identified as *Lb. casei* (clusters 16 and 17, Figure **1**), whereas *Lb. bulgaricus*, also reported in the label, was not found out. This datum confirmed results obtained by plate counts, i.e. the absence of colonies in MRS pH 5.2 incubated at 43°C.

Ten colonies were isolated from MRS 37° plates used for microbial counts of formulations B and C and were grouped into clusters 10, 11 and 13 (Figure **1**), confirming the sole presence of *Lb. rhamnosus* in those formulations. Six out of 10 isolates from D were identified as *Lb. salivarius* (Figure **1**, cluster 14), while the remaining 4 isolates were identified as *Lb. paracasei* (cluster 12), confirming the microbial composition reported on the label.

All the colonies isolated from the single-strain formulation E were grouped into the cluster 18 and identified as *Lb. casei*. Analogously, all the strains of *Lb. paracasei* or *Lb. reuteri* from single-strain formulations F or J, respectively, were grouped into clusters 9 or 8 (Figure **1**), confirming data reported on labels.

As for formulations G and H, some discrepancies were underlined between data obtained in our work and what reported in the labels. In fact, *Lb. bulgaricus* was not found in formulation G; moreover, in the same formulation the species reported as *Lb. sporogenes* was correctly identified as *B. coagulans* by sequence analysis (Table **3** and Figure **1**, clusters 2, 6 and 7). As previously reported for A, also in G the absence of *Lb. bulgaricus* seemed to be confirmed by the absence of colonies in MRS pH 5.2 incubated at 43°C. To confirm results obtained in the media used for lactobacilli counts, MRS agar was used to isolate 5 additional colonies, but all the isolates resulted ascribable to *Lb. acidophilus* or *B. coagulans*.

With regard to formulation H, no growth was detected in MRS pH 5.2 nor in BA-M; once again, MRS agar was used to isolate colonies of presumptive lactobacilli. All of them were identified as *Lb. bulgaricus* (Figure **1**, clusters 4 and 5) whereas *Lb. acidophilus*, also reported in the label, was not found. *S. thermophilus* was isolated from M17 plates, in confirmation of the presence of this species in formulation H.

DGGE analysis performed on the DNA directly extracted from crude formulations partially confirmed the results reported earlier. In particular, the profile A

Table 3: Identification, Based on BLAST Comparison in GenBank, of the Strains Obtained by PCR-DGGE Analysis Performed Using Universal Primers for Bacteria

Cluster	Strain	Size	Closest relative	% Identity	Source[a]
1	H10	621	*S. thermophilus*	99%	HM059005.1
2	G17	617	*B. coagulans*	99%	GU904695.1
3	G11	622	*S. thermophilus*	100%	HM462405.1
4	H1	626	*Lb. delbrueckii* ssp. *bulgaricus*	99%	HM058598.1
5	H5	601	*Lb. delbrueckii* ssp. *bulgaricus*	99%	HM007590.1
6	G20	617	*B. coagulans*	99%	GU904695.1
7	G3	613	*B. coagulans*	99%	GU904695.1
8	J5	647	*Lb. reuteri*	98%	AB494732.1
9	F4	638	*Lb. paracasei*	99%	HM067019.1
10	C4	622	*Lb. rhamnosus*	99%	GU550100.1
11	C5	621	*Lb. rhamnosus*	99%	FM179322.1
12	D9	612	*Lb. paracasei*	99%	HM462420.1
13	B1	626	*Lb. rhamnosus*	99%	GU550100.1
14	D1	618	*Lb. salivarius*	99%	EU099039.1
15	G8	582	*Lb. acidophilus*	98%	HM162411.1
16	A1	640	*Lb. casei*	99%	HQ111078.1
17	A10	610	*Lb. casei*	98%	HQ111078.1
18	E5	641	*Lb. casei*	98%	HQ111078.1

[a]Accession number of the sequence of the closest relative found by BLAST search.

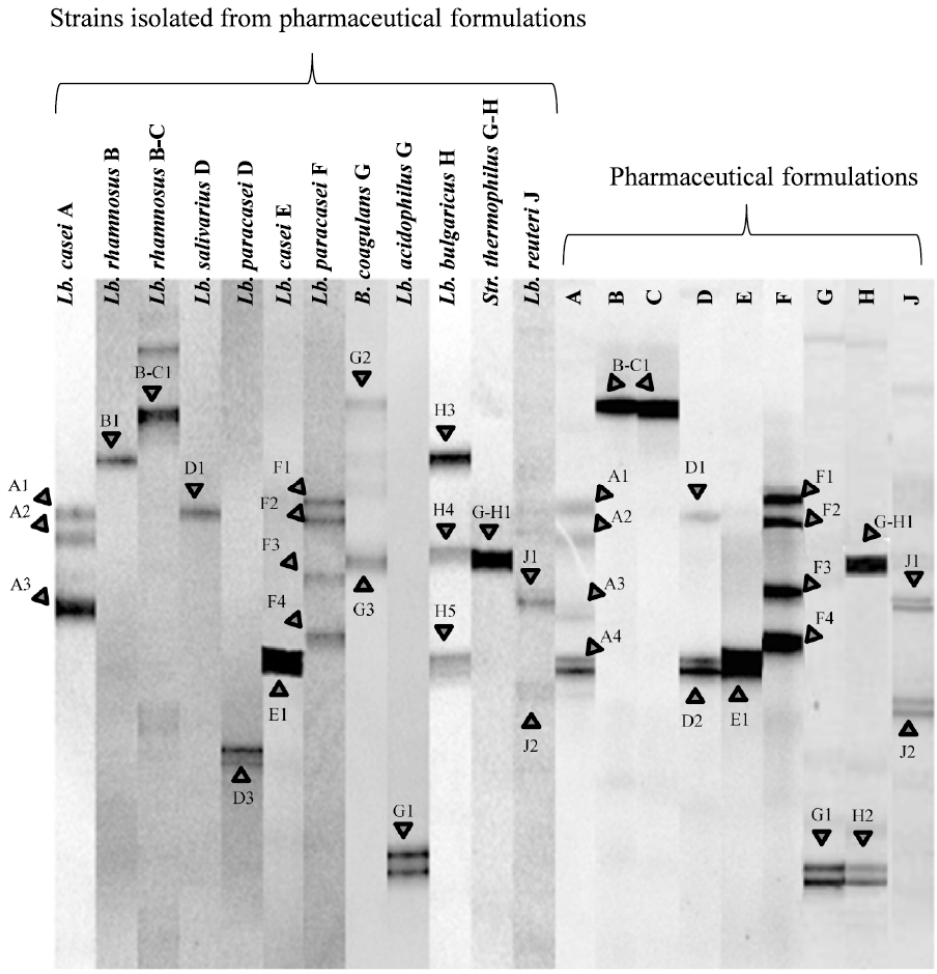

Figure 2: Normalized DGGE profiles (Gel Compare II Version 2.0, Applied Maths) of 12 isolates from pharmaceutical formulations, identified by DGGE and sequencing and used as reference strains, and 9 pharmaceutical formulations. Lane 1, *Lactobacillus casei* isolated from formulation A; Lane 2, *Lb. rhamnosus* isolated from formulation B; Lane 3, *Lb. rhamnosus* isolated from formulations B and C; Lane 4, *Lb. salivarius* isolated from formulation D; Lane 5, *Lb. paracasei* isolated from formulation D; Lane 6, *Lb. casei* isolated from formulation E; Lane 7, *Lb. paracasei* isolated from formulation F; Lane 8, *Bacillus coagulans* isolated from formulation G; Lane 9, *Lb. acidophilus* isolated from formulation G; Lane 10, *Lb. delbrueckii* ssp. *bulgaricus* isolated from formulation H; Lane 11, *Streptococcus thermophilus* isolated from formulations G and H; Lane 12, *Lb. reuteri* isolated from formulation J. Lanes 13-21, pharmaceutical formulations A-J.

(Figure **2**) showed 3 bands (A1, A2 and A3) corresponding to those of *Lb. casei* A, the sole species isolated from this formulation. The lane A also showed the presence of one other band, marked as A4, which was absent in the lane of *Lb. casei* A and which could be imputable to *Lb. delbrueckii* ssp. *bulgaricus*, the second species indicated in the label of formulation A, and whose presence was not confirmed by the culture-dependent method used in this study.

Profiles of both formulations B and C (Figure **2**) evidenced the presence of only one band, indicated as B-C1, corresponding to that of *Lb. rhamnosus* B and C, isolated from both formulations B and C and grouped in the same cluster, as reported earlier (Figure **1**). Band B1, characterizing the profile of 2 *Lb. rhamnosus* strains also isolated from formulation B (Figure **1**, cluster 13), was not detectable in the profile B. Lane D evidenced the presence of one band marked as D1, which corresponded to that of *Lb. salivarius* D, whereas the band D2 did not correspond to the band D3 of *Lb. paracasei* D, the second species isolated from this formulation, whose presence was also reported in the label.

The profile of the single-strain formulation E was characterized by the presence of band E1, which corresponded to that of *Lb. casei* E. Four bands (F1–F4) were present in the profile F and all resulted attributable to *Lb. paracasei* ssp. *paracasei* F, isolated from this single-strain formulation.

Lane G showed the presence of one band, marked as G1, which corresponded to that of *Lb. acidophilus*

G. The profile of G did not show any other bands, neither those distinctive of *B. coagulans* G, erroneously labelled as *Lb. sporogenes* (bands G2 and G3), or *S. thermophilus* G (band G-H1), both isolated from this formulation, nor those of *Lb. delbrueckii* ssp. *bulgaricus*, the other species reported in the label, whose presence was not confirmed by culture-dependent methods. The multiple-strain formulation H showed 2 bands, marked as G-H1 and H2. If the former was attributable to *S. thermophilus* H (band G-H1), the latter did not correspond to any bands of *Lb. delbrueckii* ssp. *bulgaricus* H (bands H3-H5), the second species detected through the use of culture-dependent techniques. Interestingly, the band H2 was in the same migration position of band G1 (lane G), referring to *Lb. acidophilus* G, even if this species was not detectable in formulation H by culture-dependent methods. Finally, the profile of formulation J showed at least two bands (J1 and J2) referring to *Lb. reuteri* J, the sole species reported in the label of this pharmaceutical product.

4. DISCUSSION

It is well known that the ability of probiotic microorganisms to provide health benefits is strictly related to the amount of viable bacteria able to reach the large intestine. With regard to commercial probiotic formulations, this information should be deductible by the label of each product, since it is not possible to provide one minimum dose for all products, because different probiotics are effective at different levels [23]. In our study, this information was reported for all the products under analysis, but in the case of multi-strain formulations the concentration of each microorganism was reported in two cases (A and H), in some others (D and G), only the total number was indicated in the label. Moreover, the reported number of live microorganisms delivered in each serving dose was about 8-9 logs, but we found in almost all the commercial formulations a number of viable bacteria lower than that indicated. In this connection, it is just the case to remember that some Authors supposed a positive role attributable to the administration of dead cells of probiotics or of their DNA [24-26]. Nevertheless, data recorded in our work can be considered reassuring, taking into account that counts were always included in the generally accepted range for probiotic efficacy (between 7 and 9 logs live microorganisms daily/dose).

With regard to the effective presence of species reported on the labels, the concurrent application of both culture-dependent and independent methods gave different results. In detail, it is generally accepted that DGGE analysis can be considered an appropriate method for directly analysing microbial communities when abundance is greater than 5 logs. So, it seemed to be a suitable approach for probiotic preparations, where concentrations reported were about 8-9 logs. However, results obtained in this study showed that the best approach consists in the concurrent application of both culture-dependent methods, based on the isolation and identification of cultivable bacteria, and independent PCR-DGGE, directly applied on crude preparations. In fact, the first method allowed the identification of viable bacteria whereas the latter revealed the presence of the DNA of some species, such as *Lb. bulgaricus* in A and *Lb. acidophilus* in G, which were not detected through the isolation technique. This fact could be imputable to the culture media used in this study. For instance, in our work we ascertained the unsuitability of MRS pH 5.2 to isolate *Lb. delbrueckii* ssp. *bulgaricus*, probably due to the use of an incubation temperature (43°C) too high for lactobacilli selected for probiotic purposes. As for HHD, this medium seems more suitable for the selective enumeration of obligately homo- and hetero-fermentative lactobacilli species than homo- and facultative hetero-fermentative ones [27], since it did not allow the expected discrimination between *Lb. salivarius* and *Lb. paracasei* based on defined colony colours. However, it should be reminded that MRS agar was used to count and isolate lactobacilli when other specific media did not allow the growth of colonies and, in our opinion, MRS represents an excellent medium for the cultivation of lactobacilli, even if it does not allow the discrimination between different species. Also, ST agar, proposed to selectively enumerate *S. thermophilus* at 37°C after 24 h incubation, did not allow the growth of this species nor of other ones.

Apart from this interpretation, we can also assume that several factors influenced the stability and viability of searched LAB, and for these reasons some species were not detectable by culture-dependent methods because damages suffered during the industrial phases of production compromised their viability. This fact is probably imputable to the severity of processing conditions adopted to produce dried cultures, as already reported by Aureli *et al.* [14]. Furthermore, the decay of viable cell count is considered a typical phenomenon of every probiotic formulation [28].

In our work, we also highlighted the use of an inappropriate nomenclature in one product, i.e. *Lb.*

sporogenes instead of *B. coagulans*. In our opinion, the facts reported above have to be taken into serious account, since the consumer has the right to receive a correct information regarding the composition of microbial cultures used for probiotic purposes. Moreover, the inappropriateness of microbial species reported on the labels represents a problem for the researchers. In fact, we did not find the reference strain number for all bacteria composing pharmaceutical preparations under analysis, while the Food and Agriculture Organization, as well as the World Health Organization [5], strongly recommend the deposit of probiotic bacteria in international culture collections in order to facilitate their identification.

Data regarding the resistance of bacterial formulations to GI stresses evidenced a drastic reduction in viable counts. In detail, the approach used in this study was scheduled in order to mime the ingestion of formulations, and the subsequent passage of microorganisms from the mouth to the gut of the consumer, without preparatory rehydration/activation steps, generally used to test the survival of probiotic strains during the passage through the GIT [9, 29, 30]. We observed a high rate of injured/dead cells, with counts up to 7.4 logs lower after GI stresses in several probiotic formulations. *In vivo* food intake probably protects bacteria during gastric passage, and the use of simulated human juices and bile salt formulations could drastically influence the survival of probiotic bacteria. However, it is also well known the fact that these microorganisms are often poorly adapted to conditions encountered in the GIT and their delivery from the mouth to the large intestine remains the major problem for their use in human therapies [31].

In conclusion, this work documented the need to regulate the marketing of probiotic products and to establish routinely and well-defined checks able to assess their real composition and efficacy. Further studies will be carried out in order to verify the viability and resistance to GI stresses of probiotic lactic acid bacteria in probiotic foods.

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