International Journal of Immunopathology & Pharmacology

Letter to the editor

Labeling quality and molecular characterization studies of products containing *Lactobacillus* spp. strains.

International Journal of Immunopathology and Pharmacology
2016, Vol. 29(1) 121–128

DOI: 10.1177/0394632015600534
iji.sagepub.com
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Abstract

The objective of the study was to characterize at species level by phenotypic and different molecular methods the strains of *Lactobacillus* spp. used as constituents of five oral and four vaginal products. Susceptibilities to representative antibiotics were evaluated. In addition, total viable counts at mid and 3 months to deadline of shelf life, in the different formulations and the presence of eventual contaminant microorganisms were investigated.

In all oral products the molecular characterization at species level of the strains of *Lactobacillus* spp. confirmed the strains stated on the label, except for one strain cited on the label as *Lactobacillus casei*, that our study characterized as *Lactobacillus paracasei*. In oral products total viable cell content complied with content claimed on the label. In three out four vaginal products (one product claimed "bacillo di Döderlein"), molecular characterization complied with the bacterial name stated on the label. Two vaginal products reported viable counts on the label that were confirmed by our study. The other vaginal products, which did not report bacterial counts on the label, showed a similar decrease of viable counts at different dates to deadline compared to the others. From all the tested products, contaminant microorganisms and acquired resistance to representative antibiotics by the probiotic strains were not detected.

Keywords

label quality, *Lactobacillus* spp., molecular characterization, oral and vaginal products

Date received: 5 May 2015; accepted: 23 July 2015

Introduction

In the past, several authors have found discrepancies between information stated on the label and actual contents in food and dietary supplements for both human and veterinary use containing probiotic microorganisms. Deficiencies in labeling included frequent misidentification of the strains or incorrect name in bacteriological terms. Moreover, a reduced number of viable cells, extraneous strains, and/or strains not specified in the label could be detected.[1–7]

In recent years there has been increased attention to the quality and labeling of products with microorganisms as constituent.[8–10] Moreover, European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA)[11] pursuant to Regulation of European Commission N°1924/2006[12] considers in previous opinions that microorganisms which are the subject of health claims (including the term "probiotic") must be sufficiently characterized at species and strain level by different internationally accepted genetic typing molecular methods.

The objective of this study was a microbiological analysis of oral and vaginal products, chosen

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among those most frequently used in Italy and containing only probiotic strains of *Lactobacillus* belonging to different species, and to evaluate whether the quality of available Italian products has been improved.

The study characterizes the strains of *Lactobacillus* used as constituents at species level by phenotypic and different molecular methods, to confirm the species identity of the bacterial cultures. The individual susceptibility to representative antibiotics of these strains was also evaluated. In addition, the viable organisms, at two different dates and in different formulations, and the presence of contaminant microorganisms were examined.

Materials and methods

Probiotics products

Five oral (I–V) and four vaginal (VI–IX) commercial products (Table 1) with different formulations, claiming to contain only probiotic strains of *Lactobacillus* species and marketed in Italian pharmacies, were selected for this study. Each product gave the following information: formulation of the product, probiotic strains, number of viable cells (Colony Forming Units [CFU]/dose). The study did not take into account prebiotic constituents and health claims of the products.

Bacterial isolation

Two samples of different batches of the different formulations of each product were dissolved in 10 mL of physiological solution. All products were examined using a set of different isolation media under standardized cultivation conditions. For the isolation of *Lactobacillus* strains, De Man Rogosa and Sharp Agar (MRSA) (Oxoid) and Rogosa Agar (Oxoid) were used. The plates were incubated for 48 h at 37°C in aerobic and anaerobic atmosphere, and in micro-aerobic conditions (3.5% CO_2, 5% O_2, 7.5% H_2, 84% NH_2). To test eventual contaminations 5% sheep blood agar plates were seeded and then incubated for 48 h at 37°C in CO_2 enriched atmosphere for streptococci and enterococci. Müller-Hinton agar plates (bioMérieux) were seeded and incubated for 48 h at 30–35°C in aerobic and anaerobic conditions for spore forming bacteria. Therefore, MacConkey-agar plates (bioMérieux) were seeded and then incubated for 24–48 h at 37°C in aerobic conditions to investigate for *E. coli* and related bacteria. The presence of yeasts and molds were investigated using Sabouraud-dextrose agar incubated for 24–48 h at 30–35°C.

Identify confirmation at species level of the strains of Lactobacillus

The phenotypic identification of *Lactobacillus* was performed with microbiological methods on the basis of the Gram stain, colony morphology and biochemical reactions provided by the kit API 50 CH strips (bioMérieux), using API 50 CHL Medium. The data were elaborated by bioMérieux software.[13] The two strains of *L. acidophilus* used in the product VIII (Table 1) were differentiated on the basis of bacterial morphology shapes, colonies morphology and biochemical profiles.

Genotypic identification at species level was carried out after extraction of the total DNA, through PCR/Restriction Fragment Length Polymorphism (RFLP) of 16S rDNA described by Randazzo et al.[14] Rapid and reliable two-step multiplex polymerase chain reaction (PCR) assays as described by Song et al.[15] were established to identify *L. acidophilus*, *L. crispatus*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. jensenii*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius*. Primers used were designed from nucleotide sequences of the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA gene of members of the genus *Lactobacillus* proposed by Song et al.[15] For the specific detection of *L. paracasei*, *L. casei*, and *L. rhamnosus* the strains of *Lactobacillus* spp. isolated were discriminated by *tuf* gene amplification described by Ventura et al.[16] A detailed description of these procedures was recently reported by two of us.[17,18]

Total viable counts

The total number of lactobacilli present in each formulation was determined by the viable count technique. Total viable count was examined during storage at mid and 3 months to deadline of shelf life. Two different batches of each formulation were tested. One dose of each sample, suitably processed if necessary, was dissolved in 10 mL of sterile saline (0.9% NaCl), stirred with vortex and allowed to set for 20 min. Duplicate amounts of decimal dilutions of 0.1 mL were inoculated on the

Table 1. Phenotypic and genotypic identification at species level of the *Lactobacillus* spp. strains used in oral (I–V) and vaginal (VI–IX) products.

Product	Taxon name as reported by depositor	Identification techniques as reported by depositor for EFSA	Taxon name obtained in this study	Identification methods used in this study
I	*Lactobacillus rhamnosus* GG ATCC 53103	API 50 CHL RAPD genotyping Ribotyping PFGE	*Lactobacillus rhamnosus*	API 50 CHL 16S rDNA RFLP Multiplex-PCR *tuf* gene amplification
II	*Lactobacillus rhamnosus* GG ATCC 53103	API 50 CHL RAPD genotyping Ribotyping PFGE	*Lactobacillus rhamnosus*	API 50 CHL 16S rDNA RFLP Multiplex-PCR *tuf* gene amplification
III	*Lactobacillus casei* DG CNCM I-1572	Phenotypic (cell morphology, carbohydrate fermentation pattern) Genotypic (16S/23S rRNA intergenic spacer region sequence analysis and ribotyping)	*Lactobacillus paracasei* *Lactobacillus casei/paracasei* *Lactobacillus paracasei*	API 50 CHL 16S rDNA RFLP Multiplex-PCR *tuf* gene amplification
IV	*Lactobacillus reuteri* DSM 17938	PCR	*Lactobacillus fermentum* *Lactobacillus reuteri*	API 50 CHL 16S rDNA RFLP Multiplex-PCR
V	*Lactobacillus reuteri* DSM 17938	PCR	*Lactobacillus fermentum* *Lactobacillus reuteri*	API 50 CHL 16S rDNA RFLP Multiplex-PCR
VI	*Lactobacillus plantarum* P 17630	Phenotypic (carbohydrate fermentation profile, antibiotic resistance pattern, PAGE) Genotypic (16S rRNA gene sequence analyses, ARDRA, Rep - PCR, PFGE, genome sequencing)	*Lactobacillus plantarum*	API 50 CHL 16S rDNA RFLP Multiplex-PCR
VII	*Lactobacillus plantarum* P 17630	Phenotypic (carbohydrate fermentation profile, antibiotic resistance pattern, PAGE) Genotypic (16S rRNA gene sequence analyses, ARDRA, Rep - PCR, PFGE, genome sequencing)	*Lactobacillus plantarum*	API 50 CHL 16S rDNA RFLP Multiplex-PCR
VIII	*Lactobacillus acidophilus* CH-2 *Lactobacillus acidophilus* CH-5	N/A	*Lactobacillus acidophilus* *for both strains*	API 50 CHL 16S rDNA RFLP Multiplex-PCR *tuf* gene amplification
IX	bacillo di Döderlein	N/A	*Lactobacillus rhamnosus*	API 50 CHL 16S rDNA RFLP Multiplex-PCR *tuf* gene amplification

N/A = Not applicable.

De Man Rogosa and Scharpe agar plates (MRS agar-OXOID). Plates were incubated at 37°C for 48 h in 10% CO_2 enriched atmosphere.

Antibiotic susceptibility

The antibiotic susceptibility of the isolates of *Lactobacillus* spp. were tested by determination of Minimum Inhibitory Concentration (MIC) using the microtiter broth dilution performed with Cation-adjusted Mueller-Hinton broth (bioMérieux) to 2.5% lyses horse blood (Oxoid), and an inoculum equivalent to 0.5 MacFarland as suggested by Clinical and Laboratory Standards Institute.[19] The antibiotics tested were: ampicillin (Sigma-Aldrich), erythromycin (Sigma-Aldrich), clindamycin (Sigma-Aldrich), vancomycin (Sigma-Aldrich), and gentamycin (Sigma-Aldrich). Each drug was tested for a dilution range of 32–0.25 mg/L. *Streptococcus pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as control strains.[19] The MICs obtained were evaluated using interpretative criteria suggested by the CLSI[19] and EFSA.[20]

Statistical analysis

Each experiment was performed in duplicate, and repeated three times on different days, to ensure results' reproducibility.

Statistical analysis was perfomed by OriginPro 9.0.0 (OriginLab Corporation©) using analysis of variance (ANOVA).

Results

Table 1 shows the results of phenotypic and molecular identification at species level of *Lactobacillus* spp. strains used as constituent of the products in examination. Comparison of viable cell numbers stated on the labels of the probiotic products with the total viable counts determined at different times are shown in Table 2.

Oral products

The identification at species level of the *Lactobacillus* strains using biochemical phenotypic and molecular methods confirmed the identification of *L. rhamnosus* as cited on the label of the products I and II. The strain cited as *L. casei* on the label of the product III was identified as *L. casei* by phenotypic method and as *L. paracasei* by multiplex-PCR; *tuf* gene amplification confirmed *L. paracasei*. RFLP was not able to discriminate *L. casei* from *L. paracasei*. The strain cited as *L. reuteri* on the label of the products IV and V was confirmed using only genotypic methods of identification.

The total viable counts of lactobacilli present at different dates from the deadline gave similar values to those stated on the label. Moreover, some formulations of the products I and the products IV and V at the half-shelf life showed values slightly higher than those stated on the label. For all tested products no substantial difference was observed in the total viable counts for the two different batches of the same formulations (data not shown). None of the products tested showed contaminant microorganisms.

Vaginal products

The identification at species level of the *Lactobacillus* strains using biochemical phenotypic and molecular methods confirmed the identification of *L. plantarum* in the products VI and VII and of the two strains of *L. acidophilus* used in the product VIII, as cited on the label. Product IX claimed to contain "bacillo di Döderlein", name that describes and not identifies vaginal strains of *Lactobacillus* spp. at the specie level. Phenotypic and molecular methods identified the strain as *L. rhamnosus*.

Regarding total viable counts, carried out at different times from the deadline, products VI and VII gave equal or higher values than those reported on the label. The labels for products VIII and IX did not report bacterial count. However our test of the viable counts showed a great number of viable cells (from 1×10^7 to 4.6×10^7), nevertheless lower than those found in products VI and VII.

For all tested products no substantial difference was observed in the total viable counts for the two different batches (data not shown). None of the products tested showed contaminant microorganisms.

Antibiotic susceptibility

On the basis of CLSI[19] and EFSA[20] criteria used showed in Table 3, the strains of *Lactobacillus* spp. tested were susceptible to ampicillin (MIC $\leqslant$1 mg/L), to erythromycin (MIC $\leqslant$0.5 mg/L) and to

Table 2. Total viable counts at different times of oral (I–V) and vaginal (VI–IX) products containing *Lactobacillus* spp. strains during storage.

Products	Oral products											Vaginal products			
	I		II		III		IV		V			VI	VII	VIII	IX
Dosage form*	Sachet 1†	Sachet 2†	Capsule 1†	Capsule 2†	Drops	Sachet	Sachet 1†	Capsule	Tablet	Tablet	Drops	Tablet	Drops	Vaginal capsule	Vaginal capsule	Vaginal capsule	Vaginal capsule
T_1 CFU/dose	2.7×10^{9a}	6.2×10^{9}	2.9×10^{9}	6.4×10^{9}	5.2×10^{9}	5.2×10^{9a}	7.8×10^{9}	22.1×10^{9a}	22.3×10^{9}	23.5×10^{9}	3.0×10^{8c}	2.7×10^{8c}	3.1×10^{8c}	3.5×10^{8c}	7.5×10^{8c}	6.8×10^{8c}	1.5×10^{7}	4.6×10^{7}
T_2 CFU/dose	2.5×10^{9a}	5.7×10^{9}	2.8×10^{9}	5.9×10^{9}	4.8×10^{9}	4.5×10^{9b}	7.7×10^{9}	20.0×10^{9b}	20.3×10^{9b}	21.0×10^{9a}	2.5×10^{8c}	2.3×10^{c}	2.8×10^{8c}	3.0×10^{8c}	6.5×10^{8c}	6.0×10^{8c}	1.0×10^{7}	4.0×10^{7}
Product label CFU/dose	3.0×10^{9}	6.0×10^{9}	3.0×10^{9}	6.0×10^{9}	5.0×10^{9}	6.0×10^{9}	$\geqslant8.0\times10^{9}$	$\geqslant24.0\times10^{9}$	$\geqslant24.0\times10^{9}$	$\geqslant24.0\times10^{9}$	1.0×10^{8}	1.0×10^{8}	1.0×10^{8}	1.0×10^{8}	1.0×10^{8}	1.0×10^{8}	N/A	N/A

N/A, not applicable; T_1, half shelf life; T_2, 3 months to deadline.
* Dosage of different formulations= one sachet (powder), one capsule, one tablet, one vaginal capsule, five drops.
† Same formulation with different CFU/dose.
Statistically significant difference between T_1 and label and between T_2 and label: $^{a}P \leqslant 0.05$; $^{b}P \leqslant 0.001$; $^{c}P \leqslant 0.0001$.
Not statistically significant values are not indicated. For the products VIII and IX ANOVA test cannot be applied.

clindamycin (MIC ⩽0.5 mg/L). The strains were resistant to gentamicin (MIC >16 mg/L). All strains, except *L. acidophilus* strains (MIC = 1 mg/L), were resistant to vancomycin (MIC >32 mg/L) (Table 3).

Discussion

Products containing probiotic strains are of considerable and growing economic importance.[10,21–24] Moreover probiotic trade name in food and dietary supplements is becoming more popular. International and European organizations[8,9,12,25] have given guidelines for probiotic food and supplements, but products containing microorganisms can still show deficiencies, including the identity of the strain and low bacterial counts, in comparison to the label claims.[23]

Current genus and species designations should be used on labels.[9,10,12]

L. casei group is an example of the state of flux for lactobacilli nomenclature; in fact, *L. casei, L. paracasei, L rhamnosus*, and *L. zeae* are phylogenetically correlated and appear as distinct cluster by other *Lactobacillus* included in *L. casei* group.[10,26] Our study characterized the strains of *Lactobacillus* present in the probiotic products at species level by phenotypic and different molecular methods. The probiotic strain used in product III is indicated by depositor as *L. casei* DG (CNCM I-1572) and is considered sufficiently characterized by EFSA Panel on Dietic Products, Nutrition and Allergies (NDA).[27] However, our study, using multiplex PCR[15] and *tuf* gene amplification,[16] not used by depositor, identified the strain as *L. paracasei*. In fact, *tuf* gene is an important molecular marker to distinguish so related taxa as *L. casei* group.[16–18] The strains of *L. rhamnosus* GC, *L. reuteri* DSM 17938, and *L. plantarum* P 17630 were identified as the species indicated on the label, even if genotypic techniques different from our methods were used by depositor. EFSA Panel on NDA retains *L. rhamnosus* GG sufficiently characterized at species level,[28] but not *L. reuteri* DSM 17938.[29] Regarding the species identification of the *Lactobacillus* strains used in vaginal products, phenotypic and genotypic methods of our study confirmed the identification cited by depositors for *L. plantarum* P 17630 used in products VI and VII, and for the two strains of *L. acidophilus* used in product VIII. EFSA Panel on NDA, that was asked to provide a scientific opinion on *L. plantarum*

Table 3. Susceptibilities of the *Lactobacillus* spp. to ampicillin, erythromycin, clindamycin, vancomycin, and gentamycin, in oral (I–V) and vaginal (VI–IX) products.

Product	Taxon name obtained in this study	Ampicillin			Erythromycin			Clindamycin			Vancomycin			Gentamycin		
		MIC mg/L	Break point		MIC mg/L	Breakpoint		MIC mg/L	Breakpoint		MIC mg/L	Breakpoint		MIC mg/L	Breakpoint	
			CLSI[a]	EFSA[b]		CLSI[a]	EFSA[b]		CLSI[a]	EFSA[b]		CLSI[a]	EFSA[b]		CLSI[a]	EFSA[b]
I	*L. rhamnosus*	⩽0.25	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R
II	*L. rhamnosus*	⩽0.25	S	S	0.5	S	S	0.5	S	S	>32	R	NA	>32	R	R
III	*L. paracasei*	⩽0.25	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R
IV	*L. reuteri*	⩽0.25	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R
V	*L. reuteri*	I	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R
VI	*L. plantarum*	⩽0.25	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R
VII	*L. plantarum*	⩽0.25	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R
VIII	*L. acidophilus - A*	0.5	S	S	⩽0.25	S	S	⩽0.25	S	S	I	S	S	>32	R	R
	L. acidophilus - B	0.5	S	S	⩽0.25	S	S	⩽0.25	S	S	I	S	S	>32	R	R
IX	*L. rhamnosus*	⩽0.25	S	S	⩽0.25	S	S	⩽0.25	S	S	>32	R	NA	>32	R	R

[a]CLSI M45-P; Interpretative Criteria for Broth microdilution Susceptibility Testing:[19] Ampicillin: Sensible ⩽8 mg/L; Erythromycin: Sensible ⩽0.5 mg/L, Intermediate 1–4 mg/L, Resistant ⩾8 mg/L; Clindamycin: Sensible ⩽0.5 mg/L, Intermediate 1–2 mg/L, Resistant ⩾4 mg/L; Vancomycin: Sensible ⩽4 mg/L, Intermediate 8–16 mg/L, Resistant ⩾32 mg/L; Gentamycin: Sensible ⩽4 mg/L, Intermediate 8 mg/L, Resistant ⩾16 mg/L.
[b]EFSA Guidance on the assessment of bacterial antimicrobial susceptibility; microbiological cutoff values:[20] Ampicillin: *L. acidophilus* group 1 mg/L, *L. reuteri* 2 mg/L, *L. plantarum/pentosus* 2 mg/L, *L. rhamnosus* 4 mg/L, *L. casei/paracasei* 4 mg/L; Erythromycin: *L. acidophilus* group 1 mg/L, *L. reuteri* 1 mg/L, *L. plantarum/pentosus* 1 mg/L, *L. rhamnosus* 1 mg/L, *L. casei/paracasei* 1 mg/L; Clindamycin: *L. acidophilus* group 1 mg/L, *L. reuteri* 1 mg/L, *L. plantarum/pentosus* 2 mg/L, *L. rhamnosus* 1 mg/L, *L. casei/paracasei* 1 mg/L; Vancomycin: *L. acidophilus* group 2 mg/L, *L. reuteri* n.r., *L. plantarum/pentosus* n.r., *L. rhamnosus* n.r., *L. casei/paracasei* n.r.; Gentamycin: *L. acidophilus* group 16 mg/L, *L. reuteri* 8 mg/L, *L. plantarum/pentosus* 16 mg/L, *L. rhamnosus* 16 mg/L, *L. casei/paracasei* 32 mg/L.

P 17630, consider this strain sufficientlycharacterized.[30] Product IX claimed presence of Döderlein bacillus on the label but our study identified it as *L. rhamnosus*. For this product, without an identification of the microorganisms used, the deposit of the strain in an international culture collection, that should preserve the integrity of the strain and guarantee safety and functionality of the product, is uncertain.

As suggested by Ventura et al.,[16] the low rate of 16S rDNA gene evolution is often responsible for the failure in identification of highly related bacterial species. In this scenario, the polyphasic taxonomy suggested by Vandamme et al.[31] and by other authors,[17,18,32] is the better choice for bacterial identification.

Lactobacillus spp. identification is a valid example of highly related bacterial species that require such approach that provides the polyphasic analysis of two distinct phylogenetic markers: the 16S rDNA gene and the *tuf* gene encoding for the elongation factor Tu (EF-Tu).[16–18,33]

Seven products tested in this study showed levels of viable counts similar to or slightly higher than the label claim until 3 months to deadline. The viable bacteria, recognized in two vaginal products without indication, were acceptable. The number of viable cells greater than that reported on the label, found in some formulations, could account for the possible decline in viability over the course of shelf life.[10]

All strains used in products with microorganisms as constituent must be examined to identify antimicrobial acquired resistance that is considered to have a high potential for lateral spread. Aminoglycoside resistance, observed in this study and also by other investigators,[3,7] could be determined as a possible interference of the growth medium used.[20] Intrinsic resistance to glycopeptides in *Lactobacillus* spp. is species-dependent and probably due to the presence of D-Ala-D-lactate in peptidoglycan, instead of the normal dipeptide D-Ala-D-Ala.[34]

In conclusion, it was encouraging that the majority of the products examined in this study showed adequate description of contents (characterization at species level of the strains and numbers of viable bacteria) compared to the label claims. However, both dose and valid bacterial name were not reported on the label of vaginal product IX.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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