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Cocoa Powder as Delivery Medium for Probiotic *Lactobacillus* Strains

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Abstract

Three *Lactobacillus* strains previously isolated from artisanal Italian cheeses and identified by species-specific PCR as *L. helveticus*, *L. paracasei and L. rhamnosus*, were evaluated for the presence of functional traits, such as acidifying activity, cell surface hydrophobicity, antibiotic resistance, survival in low pH and in presence of bile salts, in comparison with two commercially available probiotic strains (*Lactobacillus acidophilus* La-5 and *L. rhamnosus* GG). Subsequently, with the aim to develop a new non-dairy functional product, cocoa powder was used as a medium for incorporating freeze-dried cultures of each tested strain and survival at different time/temperature conditions was investigated. The results obtained demonstrated that artisanal dairy products are interesting sources of new probiotic strains; in particular, the dairy origin strain *L. rhamnosus* showed a good probiotic performance and the highest level of survival during storage. Finally, we showed that cocoa powder represents a good delivery medium for lactobacilli: it could be considered a novel functional food exhibiting high antioxidant power and presenting probiotic potential.

Keywords: Lactobacilli, Probiotics, Cocoa Powder

1. Introduction

In recent times, there has been an increased interest to adapt healthy diets and as a consequence, the selection of new probiotic strains and the development of new functional foods has gained much importance [1,2]. Milk and dairy fermented products can be considered the most common and traditional functional foods. The health benefits are the high amounts of specific live probiotic bacteria, mainly Lactic Acid Bacteria (LAB), naturally present or selectively added [3]. However, the increase in the consumer vegetarianism and the allergy to dairy products that affects some persons determine a demand for new products and new preparations [4,5]. In this context, the selection of new bacterial strains with characteristic and differentiated functional traits is important. In particular, in addition to well known ideal properties of the probiotic strains (Generally Regarded As Safe status - GRAS, resistance to acids and bile, colonization of the human intestine, production of antimicrobial substances) [6], other desirable characteristics must also be considered, such as viability during the processing and storage, facility of the application in the products, resistance to the technological processing of the food.

In this study we tested three *Lactobacillus* strains, previously isolated from artisanal dairy products. A comparison of the novel isolates with respect to probiotic strains from commercial products allowed an evaluation of the probiotic potential. Furthermore, with the aim of creating a new functional food, cocoa powder was used as a medium for incorporating lactobacilli, and survival of the cultures during freeze-drying, and during storage of the final product in different time/temperature conditions was studied. Cocoa and chocolate have been suggested as a good medium for the functional health ingredients, because they are rich sources of flavan-3-ols (flavanols) that have the ability to act as in vivo antioxidants [7]. Numerous dietary intervention studies in humans and animals indicate that flavanol-rich foods and beverages might exert cardioprotective effects with respect to vascular function and platelet reactivity [8,9]. Interestingly, cocoa powder has been shown to exhibit greater antioxidant capacity than many other flavanol-rich foods, such as green and black tea, red wine and fruits and vegetables [10].

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Two commercially available probiotic strains, *Lactobacillus acidophilus* La-5 and *L. rhamnosus* GG, were studied in comparison with three *Lactobacillus* strains, previously isolated from artisanal Italian cheeses. Their correct taxonomic position was determined by species-specific PCR according to protocols shown in **Table 1**. The strains were cultivated in MRS (Difco, Becton Dickinson, Sparks, MD) agar or broth at 37˚C under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany) and maintained by weekly transfers. For long-term, cultures were stored at –80˚C in MRS broth containing Bacto glycerol (Difco). The cell concentration of individual strains was evaluated by checking the optical density value at 600 nm (OD_{600}) and then by plating diluted suspensions on MRS agar plates.

2.2. Acidifying Activity

Fresh milk cultures of each strain were inoculated at 1% in 100 ml sterile reconstituted skimmed milk (10% w/v, Difco) pre-warmed at 37˚C. The pH was measured and recorded automatically, throughout the 48 h incubation period. ΔpH values after 6, 10, 24 and 48 h were used to compare the acidifying activity of the strains.

2.3. Hydrophobicity Studies

The cell surface hydrophobicity of the strains was determined as described by Rosenberg *et al.* [11] with some modifications. Briefly, cells were harvested (late log phase from MRS medium), washed twice in PBS buffer and resuspended in 0.1 M KNO_3 (pH 6.2) to give a cell suspension with an OD_{600} of 0.5 - 0.6 (A_0). Three ml of cell suspension were mixed with 1 ml of xylene. After a 10 min of preincubation at room temperature, the two-phase system was mixed by vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage of bacterial adhesion to solvent was calculated as $(1 - A_1/A_0) \times 100$.

2.4. Antibiotic Resistance

The Minimal Inhibitory Concentration (MIC) of the antibiotics, vancomycin, chloramphenicol, tetracycline and streptomycin (Sigma, St Louis, Mo) was determined by the broth dilution method [12], after growth in MRS broth at 37˚C, using 10^5 cells/ml as the initial inoculum.

2.5. Acid and Bile Tolerance

Harvested bacterial cells from overnight cultures were washed twice with PBS buffer (pH 7.2) and then resuspended in a medium containing peptone (1 g/l, Difco), KCl 0.1 M, and pepsin (500 U/ml, Difco), adjusted to pH 2 and pH 3 using 1 M HCl. Samples were incubated for 2 h at 37˚C. The residual viable population was determined by plate counting on MRS agar after 48 - 72 h of incubation under anaerobic conditions. Tolerance to bile salts was tested at 37˚C by inoculation of fresh cultures in MRS broth adjusted to pH 6.3 and enriched with 0.3% Oxgall (Oxoid, Wesel, Germany). Resistance was assessed in terms of viable count, enumerated after incubation for 0 and 2 h.

2.6. Freeze-Drying of Cell Cultures

The recovered strains were used as 1% inoculum for the preparation of 100 ml of culture in MRS broth incubated at 37˚C. The cells were collected from the exponential growth phases (OD_{600} of 1.6), centrifuged (6000 g for 10 min at 4˚C) and washed twice with sterile saline solution (0.9% NaCl in distilled water). After centrifugation, the washed cells were resuspended in sterile 4% bovine

Table 1. PCR primers and conditions used for species-specific gene amplification.

Species	Primer pair (5' to 3')	Amplicon (bp)	Thermal conditions
L. paracasei [15]	Fw: CCCACTGCTGCCTCCCGTAGGAG Rev: CACCGAGATTCAACATGG	290	94˚C × 2 min 54˚C × 1 min × 35 72˚C × 1min
L. rhamnosus [15]	Fw: CCCACTGCTGCCTCCCGTAGGAG Rev: TGCATCTTGATTTAATTTTG	290	94˚C × 45 s 54˚C × 1 min × 35 72˚C × 1 min
L. helveticus [16]	Fw: CTGTTTTCAATGTTGCAAGTC Rev: TTTGCCAGCATTAACAAGTCT	524	94˚C × 2 min 58˚C × 1 min × 35 72˚C × 1 min
	Fw: CGCTGATTCTAAGTCAAGCT Rev: CGACTAAGAAGTGGAACATTA	726	
	Fw: TCTTATTACGCAATGGACCAA Rev: AATACCGTTCTTGAGGTTAGA	918	

serum albumin and desiccated under vacuum in a freeze-drier at room temperature. Freeze-dried cells were stored in hermetically closed containers at 4˚C. For evaluation of the viable counts, the samples were rehydrated to the original volume with sterile deionized water and suitable dilutions were then plated on MRS agar.

2.7. Cocoa Powder Samples and Inoculum of Probiotic Strains

Commercially available cocoa powder was selected from the most common cocoa powder products marketed in Italy. This cocoa sample was checked for the absence of microbial population (sterility), by plating serially diluted suspensions on potato dextrose agar (PDA, Difco), plate count agar (PCA, Difco), MRS and M-17 (Difco).

Freeze-dried cultures of each tested strain were added to the cocoa powder to attain approximately 10^{10} cfu/gr. Viable cell numbers were calculated on MRS agar as described elsewhere. After storage in different time/temperature conditions, the number of cells surviving the treatment was determined.

2.8. Sensory Tests

Sensory properties of the cocoa powder enriched with probiotic strains was measured by untrained panelists (n = 20) recruited from the staff and students of the University of Milan. The commercial cocoa powder as it is (control) and enriched with probiotic strains (sample) were mixed with milk. The subjects received 40 ml of each product in 100 ml glasses at room temperature, in individual booths. They were asked to compare the two formulations and to indicate whether they differed from a sensory point of view.

3. Results and Discussion

The three lactobacilli tested, previously phenotypically characterized, were identified to the species level by species-specific PCR. Strains were designated *L. helveticus* ACH, *L. paracasei* ACP and *L. rhamnosus* ACR.

All data reported below, regarding the characteristics of these new isolates in comparison with two commercial probiotic strains, represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%. Single data points are, therefore, presented without standard deviation bars.

3.1. Acidifying Activity

At first, the isolates were screened for acidifying activity. Lactic acid production, together with other low molecular weight metabolites, is an important parameter for probiotic strains, since this primary metabolite shows antagonistic properties against many harmful organisms of the colonic flora. The lactic acid production profiles, after growth in milk at 37˚C, are shown in **Figure 1**. The performance of the newly isolated strains was comparable or superior to those of the probiotic reference strains. Particularly, the commercial *L. rhamnosus* GG strain showed slower rates and extent of acid development; the pH decrease was lower than 0.8 pH units, after 48 h of growth. On the contrary, *L. acidophilus* La-5 and *L. helveticus* ACH could be considered as fast-acidifying strains, with a ΔpH_{24h} higher than 2.5 pH units.

3.2. Cell Surface Hydrophobicity of the Strains

To assess the potential adhesion ability, we studied the cell wall hydrophobicity properties, a bacterial trait that

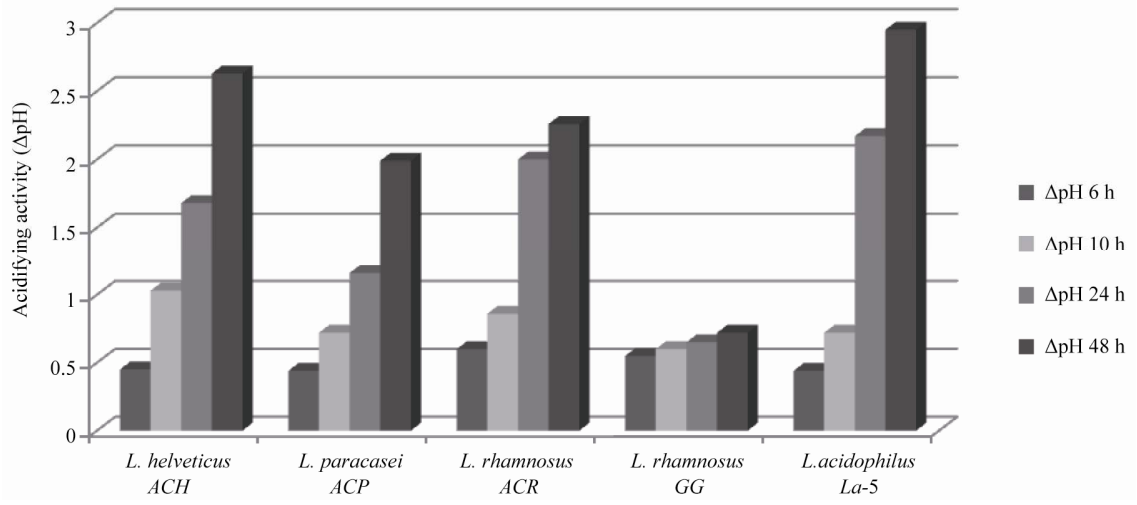


Figure 1. Acidifying activity of *Lactobacillus* strains after growth in skim milk (10% w/v) for different time intervals at 37˚C.

could be indicative of adhesiveness of probiotic bacteria. Among the tested strains, only *L. acidophilus* La-5 showed strong affinity for xylene, demonstrating the hydrophobicity of cell surface (**Table 2**); the *L. rhamnosus* strain isolated from cheese exhibited a moderate hydrophobicity, while the remaining strains showed very poor affinity for the apolar solvent.

3.3. Antibiotic Resistance

Antibiotic susceptibility of the strains is shown in **Table 2**. All strains, with the exception of *L. rhamnosus* ACR, were susceptible to tetracycline (break-point 8 μg/ml), whereas for the chloramphenicol, MICs for all strains were determined only just higher (8 μg/ml) than the break-point level (4 μg/ml). In the case of vancomycin, all strains, with the exception of *L. acidophilus* La-5 were highly resistant (>100 μg/ml). Finally, all strains were found to be resistant to streptomycin, with MICs ranging from 50 to 500 μg/ml. From a safety point of view, this phenotypic result underlines the difficulty to find antibiotic-susceptible strains. Due to the indiscriminate use of antibiotics in human and veterinary medicine and in animal growth promoters, antibiotic resistance has become an increasingly common characteristic in microorganisms [13]. Lactobacilli display a wide range of natural antibiotic resistances [14], but in most cases antibiotic resistance is not of the transmissible type. *Lactobacillus* strains with non-transmissible antibiotic resistances do not usually form a safety concern [1]. However, checking the ability of a proposed probiotic strain to act as a donor of antibiotic resistance genes may be a further prudent precaution.

3.4. Acid and Bile Tolerance

Determination of resistance to upper gastrointestinal transit was obtained by exposing bacterial cells to simulated gastric juice environment, which contains pH-dependent and enzymatic barriers (**Table 2**). All strains tested retained their viability after 2 h of exposure to pH 3 in presence of pepsin. When the strains were subjected to the pepsin solution at pH 2, a loss of viability, ranging between 1.3 and 3.0 log cycles, was found. The performance of the dairy strains was superior to those of the probiotic reference strains: highest survival was observed with *L. paracasei* ACP (1.3 log cycle reduction), while the two commercial strains, *L. acidophilus* La-5 and *L. rhamnosus* GG displayed the highest loss of viability (3 log cycles). In addition, all strains were resistant in the presence of 0.3% bile salts. Bile tolerance is an important characteristic since it enables the probiotic strains to survive, grow, and exert their beneficial effects in the host. These results highlight the potential of the strains of dairy origin to survive under gastrointestinal conditions.

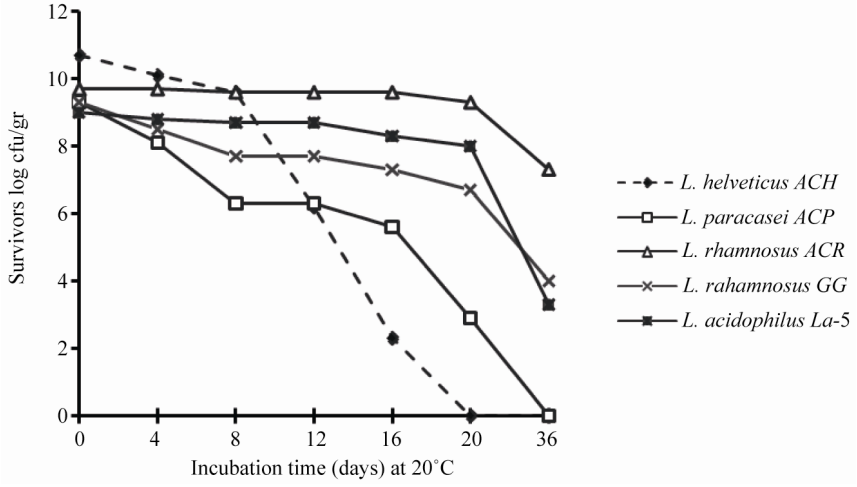
3.5. Development of a Novel Product Enriched with Probiotic Bacterial Strains

As a delivery medium for probiotic *Lactobacillus* strains, we selected cocoa powder, a food product naturally rich in antioxidant compounds and that can be consumed in compatible amounts, with a balanced and diversified normal feeding, as a component of milk, soymilk or non dairy beverages. Namely, we tested the finished food product, into which the probiotic lactobacilli have been directly incorporated as freeze-dried cultures. For this scope, firstly we investigated survival of the tested strains during freeze-drying. All tested strains retained their viability with little (<1 log cycle) or no loss at all. Moreover, all the freeze-dried cultures could be stored for long time at 4˚C without any significant loss of viability. Subsequently, 1 gr of cocoa powder was mixed, in sterile conditions, with an aliquot of freeze-dried samples containing about 10^{10} viable cells of each tested strain, and stored in hermetically closed containers at different time/temperature conditions. The calculated viability of the probiotic strains during storage is reported in **Figure 2**. In refrigerated conditions (4˚C) all strains, with the exception of *L. acidophilus* La-5, showed a high-level of

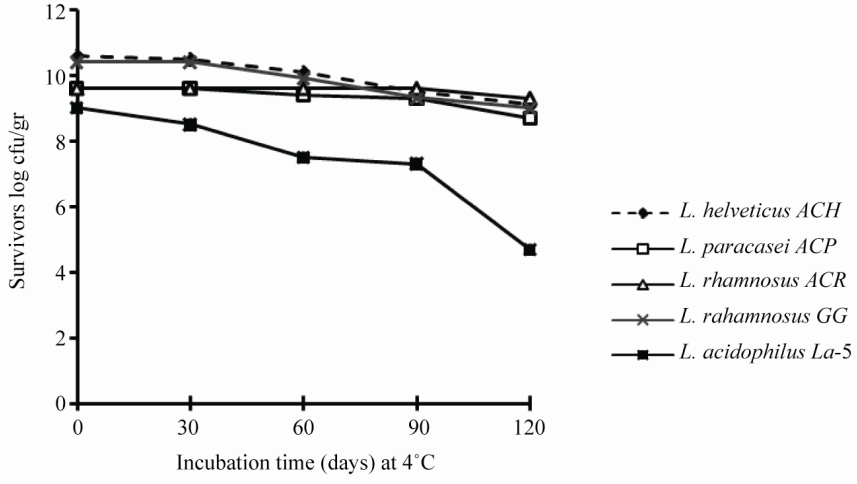
Table 2. Antibiotic resistance and probiotic properties of *Lactobacillus* strains.

Strains	Antibiotic resistance MIC (μg/ml)[a]				Hydrophobicity[b]	Viability of the strains at low pH and in presence of bile salts (log cfu/ml)					
	Cm	Str	Tet	Van		Pepsin at pH 2		Pepsin at pH 3		Oxgall (0.3%)	
						0 h	2 h	0 h	2 h	0 h	2 h
L. helveticus ACH	8	500	5	>100	5	7.0	4.8	6.8	6.7	6.5	6.3
L. paracasei ACP	8	250	5	>100	2	6.7	4.9	7.5	7.0	6.3	6.0
L. rhamnosus ACR	8	500	20	>100	35	7.0	5.3	7.0	6.8	7.7	7.7
L. rhamnosus GG	8	250	5	>100	5	6.7	3.7	6.8	6.5	6.0	6.0
L. acidophilus La-5	8	50	5	<5	90	7.3	4.3	7.8	7.0	6.9	6.8

[a]Abbrevations: Cm, chloramphenicol; Str, streptomycin; Tet: tetraclycline; Van: vancomycin. [b]Determined as xylene adhesion (%).



(a)



(b)

Figure 2. Survival of freeze-dried probiotic bacteria in cocoa powder after storage at room temperature (a), or in refrigerated conditions (b). All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%. Single data points are, therefore, presented without standard deviation bars.

survival, because the viable count remained relatively constant throughout a 120 day-storage period. At room temperature, a loss in viability in the time was observed, however, most strains retained a high level of survival for 10 days. After this time, for *L. helveticus* ACH strain number of viable cells declined from about 10^6 to less than 100 cfu/gr within 14 days of storage, while counts for *L. rhamnosus* ACR fell only to about 10^8 cfu/gr over the same storage period, and remained at about 10^7 cfu/gr after 36 days. Finally, no clear differences could be observed between the sensory characteristics of the novel food and the control, when cocoa powder was added to milk beverage.

The results reported here suggest that cocoa powder is a suitable substrate for delivering probiotic strains: the level of viable cells was more than 10^8 cfu/gr during storage in refrigerated conditions for the commercial *L. rhamnosus* GG and for the three dairy strains *L. helveticus* ACH, *L. paracasei* ACP and *L. rhamnosus* ACR. The latter strain also shows a good survival at room temperature. Considering a minimal cocoa-intake of about 1 - 2 gr per day, an amount of 10^8 cfu of viable probiotic strains could be ingested using preparations stored for 4 months at 4˚C. These amounts are comparable to those of milk-based probiotic products, e.g., bioyogurt, containing about 10^6 cfu of probiotic bacteria per ml at the end of their shelf life, which does not exceed 30 days when stored under refrigeration.

4. Conclusions

Our preliminary results suggest that artisanal dairy pro-

ducts are interesting sources for the isolation of bacterial strains with useful probiotic traits and satisfying technological characteristics. Notably, the isolates characterized in this study, and particularly *L. rhamnosus* ACR, exhibited high tolerance to bile salts and were able to survive in high numbers during storage either in refrigerated conditions or at room temperature.

The results obtained also suggest that cocoa powder represents a simple formulation of a non-dairy functional food in which the probiotic strains, manufactured under industrial conditions, are able to survive and to retain their functionality during storage. Moreover it can be consumed as a component of milk, soymilk or non dairy beverages in amounts compatible with a balanced and diversified normal feeding.

This is the first information on the survival of lactobacilli in cocoa powder: these initial assessments will provide useful and helpful information for continue studying the performance of new isolates and the development of new functional foods.

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