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Selection of *Escherichia coli*-inhibiting strains of *Lactobacillus paracasei* subsp. *paracasei*

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The aim of this study was to select *Escherichia coli*-inhibiting strains among lactic acid bacteria. On the basis of phenotypical and technological characteristics, 20 strains of lactic acid bacteria were screened from a total of 225 isolates, obtained from nine samples of artisanal Caprino d'Aspromonte cheese, made from raw goats' milk. The antagonistic activity of these 20 strains was detected in plates against three different strains of *E. coli*. Two strains of *Lactobacillus paracasei* subsp. *paracasei* showed a marked anti-*E. coli* activity against all three strains tested; the other lactic acid bacteria did not exhibit inhibiting activity. The *E. coli* inhibition can be ascribed to production of bacteriocin-like compounds. The use of *L. paracasei* subsp. *paracasei* strains to increase the safety of the cheeses made from raw milk is recommended because these cultures strongly inhibit *E. coli*, without foreseeable adverse sensory changes.
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Introduction

In several countries, the progressive introduction of pasteurization in the manufacture of artisanal cheeses is accompanied by a concomitant loss of the strong flavors developed by cheeses made from raw milk. This is because pasteurization lowers the levels of certain volatile components, especially alcohols, aldehydes, and ketones in cheeses [39]. In addition, changes induced by pasteurization may kill the thermolabile members of the indigenous nonstarter lactic acid bacteria, which, in some measure, positively contribute to flavor development [15,49].

In other countries, despite the hygienic risks, most traditional cheese consumers continue to be attracted by intensely flavored cheeses made from raw milk without the addition of lactic starters. However, there is new evidence that supports the transmission of several emerging or reemerging pathogens *via* the food chain, especially through contaminated raw milk and cheese [28]. To increase the sanitary state of artisanal cheese making, it seems preferable to improve hygienic conditions of milking, milk storage, and cheese making, and to control the lactic acid fermentation of raw milk using selected endogenous lactic starters rather than to pasteurize the milk. Indeed, artisanal cheeses made from raw milk with a starter culture have received the most favorable scores from a tasting panel, compared to cheeses produced from pasteurized milk with the same starter culture or from raw milk without the addition of a starter culture [37].

Members of the Enterobacteriaceae, a critical group in terms of health hazards, are always present in cheeses made from raw milk throughout ripening. The season significantly affected the counts of Enterobacteriaceae ($P<0.01$) of Cameros, a Spanish cheese made from raw goats' milk [36]. Orinotyri, a Greek cheese from raw ewes' milk, had, after 10 days, mean log counts of 7.94 for Enterobacteriaceae and 7.41 for coliforms; these values were reduced by only 3.02 and 2.76 log units/g, respectively, in 3-month-old cheese [43]. Kashkaval, a Bulgarian cheese from raw ewes' milk, had a coliform content of 10–120 million/g fresh curd [2]. Batzos, a Greek cheese from raw ewes' milk, was monitored during ripening: Enterobacteriaceae dominated throughout [32]. After 7 days of ripening, coliforms constituted the predominant group in Serra, a Portuguese cheese from raw ewes' milk [26]; furthermore, many species of Enterobacteriaceae were greatly affected by dairy-to-dairy variation [51]. Pichtogalo Chanion, a cheese from Crete, produced using a mixture of raw ewes' and goats' milk, contained, after 3–6 days of ripening, high populations of coliforms and, in 88.7% of the samples, *Escherichia coli* yielded levels from 1.32 to 5.66 log units/g [40]. Picante, another Portuguese cheese obtained using a mixture of raw ewes' and goats' milk, contained, after 7 days of ripening, relatively high numbers of Enterobacteriaceae (10^6–10^8/g); these values decreased and virtually disappeared after 80–100 days of ripening [17]. In other cheeses, such as the Cendrat del Montsec, made from goats' milk, Enterobacteriaceae and faecal coliforms disappeared after about 9 weeks [30]. The bacteriological quality of 198 ripened soft or semisoft cheeses from goats' milk was investigated: cheeses from raw milk without starter culture made up the most unsatisfactory group from a food hygiene point of view [54]. Variations in the counts and the species of Enterobacteriaceae during manufacturing and ripening of Armada-Sobado, a hard Spanish cheese from raw goats' milk, were described: Enterobacteriaceae yielded, in fresh curd, levels from 5.16 to 6.63 mean log counts and afterwards decreased until they disappeared after 2–4 weeks of ripening; *E. coli*, which was not isolated from curd or 1-week-old cheese, was the predominant organism in 2-week-old cheese (57.8% of isolates) [55]. This confirmed the findings of other authors who had shown that *E. coli* is one of the most resistant species in

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Table 1 Tests used, together with Gram reaction, catalase, and phase contrast microscopy, in presumptive identification of the isolates

Shape	Heterofermentation	Growth in NaCl containing			Growth at		Identification
		20 g/l	40 g/l	65 g/l	10°C	45°C	
Cocci	–[a]	+[b]	±[c]	–	+	–	*Lactococcus*
Cocci	+	+	±	–	+	–	*Leuconostoc*
Cocci	–	+	+	+	+	+	*Enterococcus*
Cocci	–	+	–	–	–	+	*Streptococcus*
Rods	–	ND[d]	ND	ND	ND	ND	Homolactic *Lactobacillus*
Rods	+	ND	ND	ND	ND	ND	Heterolactic *Lactobacillus*

[a]Negative.
[b]Positive.
[c]Positive or negative.
[d]Not determined.

ripening cheeses. The variation in the number of coliforms during ripening of Italian cheese made from goats' milk was described: the fresh curd had 5.15 log units/g, the cheese after 7 days of ripening had 4.91 log units/g, and the cheese after 40 days had 4.52 log units/g [9]. Tenerife cheese from raw goats' milk was studied by analyzing both the interior and the surface of the cheese. In the interior, Enterobacteriaceae counts were 7.12 log units/g after 2 days, 4.89 log units/g after 30 days, and 3.90 log units/g after 60 days; similarly, coliform counts were 6.98–3.55–3.31 log units/g and faecal coliforms 4.59–1.91–<1 log units/g. On the surface, the following values were observed: 6.66–7.70–5.78 log units/g (Enterobacteriaceae), 6.27–7.60–5.26 log units/g (coliforms), and 5.59–5.67–1.87 log units/g (faecal coliforms) [58].

Lactobacilli produce many antimicrobial substances, including organic acids, hydrogen peroxide, and bacteriocins. The following species of lactobacilli are able to produce bacteriocins effective against *E. coli*: *Lactobacillus acidophilus* [6,21,57], *L. curvatus* [19,23], *L. delbrueckii* [29], *L. paracasei* [46], *L. plantarum* [48], *L. reuteri* [13], *L. rhamnosus* [6,21,46], and *L. sakei* [7]. These bacteriocins are also able to inhibit the growth of *Bacillus cereus* [46,48], *Candida albicans* [46], *Clostridium* [6], *E. coli* O157:H7 [7,13,18,21,23], *Helicobacter pylori* [56], *Listeria monocytogenes* [3,7,13,23,29,42], *Pasteurella* [6], *Salmonella* [6,19,56,57], *Sarcina lutea* [46], *Staphylococcus aureus* [29,46], and *Yersinia* [29].

Caprino d'Aspromonte is an artisanal Calabrian cheese made from raw goats' milk without the addition of lactic starters. Therefore, cheese making and the 3–4 weeks of ripening involve the intervention of a naturally occurring microflora. To improve the sanitary state of this cheese, maintaining the high organoleptic standards achieved by the cheesemakers, it is necessary to employ microbial starters exclusively from the endogenous microflora.

The aim of this study was to select *E. coli*-inhibiting strains among the many endogenous lactic acid bacteria isolated from different samples of the artisanal cheese Caprino d'Aspromonte. This was to provide strains that could improve the hygienic quality of this or other raw milk cheeses.

Materials and methods

The endogenous lactic acid bacteria used throughout this work were isolated from nine artisanal cheeses made without the addition of starters. In spring 2000, three independent batches of Caprino d'Aspromonte were simultaneously manufactured according to traditional procedures in three dairy farms located in the province of Reggio Calabria and representative of the full production area. Each batch was from 80–120 l of goats' milk. Cheeses were sampled after 0, 14, and 28 days of ripening, i.e., at the beginning, middle, and end of its usual ripening time. Cheese samples (10 g) were homogenized in 90 ml of sterile physiological saline for 2 min in a blade homogenizer. After filtering the homogenates through sterile gauze, 1-ml aliquots were taken, decimally diluted in physiological sterile saline, and plated on the specific media required for the different microbial groups to be examined. Lactobacilli were isolated on Man–Rogosa–Sharpe (MRS) agar [12] acidified to pH 5.4 with acetic acid and incubated anaerobically (using Oxoid gas pack catalysts) at 22°C for 96 h and at 45°C for 48 h. Coccal-shaped lactic acid bacteria were isolated on M17 agar [53] incubated anaerobically at 22°C for 96 h and at 45°C for 48 h. Enterococci were isolated on Slanetz–Bartley agar (SBA) incubated aerobically at 45°C for 48 h [33]. For each batch, five colonies were taken randomly from MRS–22°C, MRS–45°C, M17–22°C, M17–45°C, and SBA plates. The isolates were purified using the same media and stored as frozen stocks at −20°C in MRS broth (rods) or M17 broth (cocci) containing 25% (vol/vol)

Table 2 Bacteria isolated from different media during the ripening of goats' cheese

Categories	Total microorganisms		0 day					14 days					28 days				
	n	%	MRS	M17	SBA	Total		MRS	M17	SBA	Total		MRS	M17	SBA	Total	
			n	*n*	*n*	*n*	%	*n*	*n*	*n*	*n*	%	*n*	*n*	*n*	*n*	%
Enterococcus	143	63.6	16	29	15	60	80.0	15	15	15	45	60.0	12	11	15	38	50.7
Leuconostoc	47	20.9	11	–	–	11	14.7	9	15	–	24	32.0	5	7	–	12	16.0
Homolactic *Lactobacillus*	18	8.0	–	–	–	0	0	6	–	–	6	8.0	12	–	–	12	16.0
Streptococcus	14	6.2	–	1	–	1	1.3	–	–	–	0	0	1	12	–	13	17.3
Lactococcus	3	1.3	3	–	–	3	4.0	–	–	–	0	0	–	–	–	0	0
Total	225	100	30	30	15	75	100	30	30	15	75	100	30	30	15	75	100

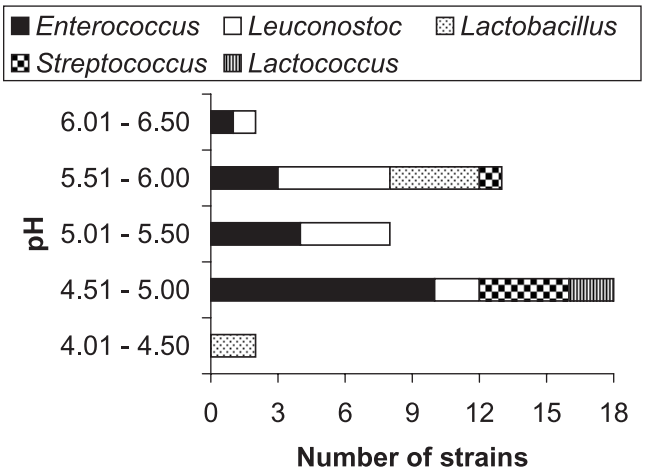

Figure 1 pH in skim milk fermented by lactic acid bacteria at 30°C for 24 h.

glycerol. Working cultures were propagated in appropriate (MRS or M17) broth media. All isolates were tested for their Gram reaction, catalase using 3 g/l H_2O_2, shape by phase contrast microscopy of overnight cultures, and heterofermentation or homofermentation of sugars [1]. Growth of the coccal-shaped bacteria was examined in M17 broth after incubation at 10°C for 7 days, at 45°C for 2 days, and in M17 broth containing 20, 40, and 65 g/l NaCl after incubation at 30°C for 4 days. Based on these results, all Gram-positive and catalase-negative isolates were presumptively classified, to genus level as shown in Table 1, according to Cogan *et al* [10]. A maximum of two copies of the lactic acid bacteria with identical phenotypic characters and isolated from the same cheese were maintained; the other isolates were eliminated from further consideration.

Lactic acid production by the screened strains of lactic acid bacteria was determined by measuring the pH in heat-treated (5 min at 121°C) reconstituted skim milk (100 g/l). One percent of a 24-h preculture was added. The pH was measured after incubation at 30°C for 24 h. On the basis of the drop in pH of the reconstituted skim milk, the lactic acid bacteria selection was carried out by eliminating those strains producing a pH above 5.0. The remaining strains were further identified and characterized by API 50CHL and/or API 20Strep systems (Biomérieux, RCS Lyon, Marcy-l'Etoile, France).

The antagonistic effect against *E. coli* growth was detected on plates under conditions that eliminated the inhibitory effects of lactic acid and hydrogen peroxide. The selected lactic acid bacteria were propagated in appropriate (MRS or M17) broth media and incubated at 30°C for 48 h. Three strains of *E. coli*, one from each of the three dairy farms, were isolated from Caprino d'Aspromonte cheese on 3 M Petrifilm for *E. coli* and coliforms [34], incubated aerobically at 37°C for 48 h, identified by API 20 E (Biomérieux), and collected on nutrient lactose agar. The three strains of *E. coli* were propagated in nutrient lactose broth and incubated at 30°C for 24 h. Antagonistic activity was detected against the three strains of *E. coli* using the spot method [47]. A 24-h culture of the lactic acid bacteria grown in appropriate broth medium was diluted 10-fold in 10 mmol/l Tris–HCl (pH 7.0), and 2-μl aliquots were spotted in triplicate onto M17 or MRS agar. The Tris–HCl (pH 7.0) solution was used to exclude the effect of organic acids on *E. coli*. Plates were incubated anaerobically for 24 h, then overlaid with 5 ml of nutrient lactose soft agar (0.7% agar) seeded with 0.1 ml of an overnight culture of the *E. coli* strains. To exclude the effect of hydrogen peroxide, catalase (EC 1.11.1.6; Sigma-Aldrich Chemie, Munich, Germany) was incorporated in the overlay agar to a final concentration of 68 IU/ml [5]. Plates were incubated for an additional 18 h and then checked for clear zones around spots of the presumed producers.

Results

Table 2 shows the identification of 225 isolates of lactic acid bacteria, 207 with coccal-shaped cells and 18 with rod-shaped

Table 3 Characteristics of the 20 selected strains of lactic acid bacteria

Strain	API identification	pH in skim milk	Antagonistic activity against *E. coli*		
			Strain EC3	Strain EC4	Strain EC5
C76	*L. lactis*	4.76	–[a]	–	–
C82	*E. faecium*	4.66	–	–	–
C85	*L. lactis*	4.73	–	–	–
C89	*E. faecium*	4.67	–	–	–
C116	*E. faecium*	4.75	–	–	–
C123	*E. faecium*	4.79	–	–	
L126	*L. paracasei* subsp. *paracasei*	4.46	+++[b]	+++	+++
L134	*L. paracasei* subsp. *paracasei*	4.44	+++	+++	+++
C138	*S. thermophilus*	4.74	–	–	–
C142	*E. faecium*	4.85	–	–	–
C143	*E. faecium*	4.85	–	–	–
C145	*S. thermophilus*	4.75	–	–	–
C162	*E. faecium*	4.75	–	–	–
C171	*E. faecium*	4.75	–	–	–
C178	*Leuconostoc* sp.	4.93	–	–	–
C187	*Leuconostoc* sp.	4.99	–	–	–
C192	*E. faecium*	4.92	–	–	–
C198	*E. faecium*	4.92	–	–	–
C207	*S. thermophilus*	4.90	–	–	–
C219	*S. thermophilus*	4.91	–	–	–

[a]Absent.
[b]Intensive.

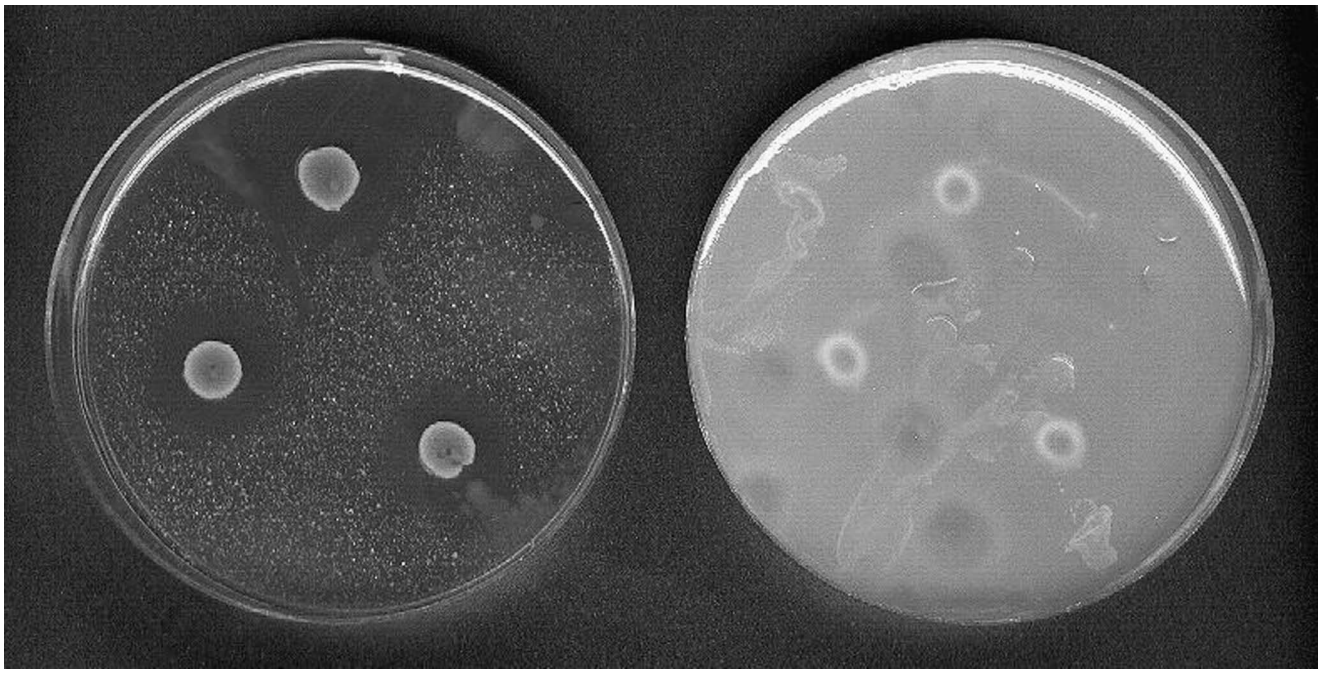

Figure 2 A comparison of *L. paracasei* subsp. *paracasei* L134 (on the left) and *E. faecium* C82 (on the right) in their antagonistic activity against *E. coli* EC4 on plates. The bacteriocin-like activity is shown by inhibition of *E. coli*. Lactic acid bacteria were spotted in triplicate; *E. coli* was spread over the surface (plate 90 mm).

cells. Among the 75 isolates from cheeses at 0 day of ripening, of all coccal-shaped, 60 matched *Enterococcus*, 11 matched *Leuconostoc*, 3 matched *Lactococcus*, and 1 matched *Streptococcus*. Among the 75 isolates from cheeses at 14 days of ripening, the coccal-shaped represented 92% and the rod-shaped 8%; 45 matched *Enterococcus*, 24 matched *Leuconostoc*, and 6 matched homolactic *Lactobacillus*. Among the 75 isolates from cheeses at 28 days of ripening, the coccal-shaped represented 84% and the rod-shaped 16%; 38 matched *Enterococcus*, 13 matched *Streptococcus*, 12 matched *Leuconostoc*, and 12 matched homolactic *Lactobacillus*. Thus, among the 225 lactic acid bacteria isolated, 63.6% was classified as *Enterococcus*, 20.9% as *Leuconostoc*, 8% as homolactic *Lactobacillus*, 6.2% as *Streptococcus*, and only 1.3% as *Lactococcus*. There seems to be considerable variation in types of isolates for cheeses after only 28 days; however, this could be due to the fact that cheeses produced from raw milk usually contain a larger number of more diverse strains than cheeses produced from pasteurized milk [11]. In fact, the same cheese produced in different factories contains many different species and strains of lactic acid bacteria; DNA fingerprinting analysis showed that 75 isolates corresponded to at least 18 distinct nonstarter microorganisms [49]. The preliminary screening, carried out on the basis of the phenotypic characteristics, reduced the 225 isolates to 43: 18 *Enterococcus*, 12 *Leuconostoc*, 6 homolactic lactobacilli, 5 *Streptococcus*, and 2 *Lactococcus*. These 43 strains were selected using skim milk (Figure 1): the strains producing pH above 5.0 were excluded. The most numerous frequency class was that where the pH varied between 4.51 and 5.00. The majority of *Enterococcus* and *Streptococcus* strains and the two *Lactococcus* strains were included in this frequency class. Almost all the *Leuconostoc* strains produced higher levels of pH and, therefore, were excluded. Great differences were found among the six *Lactobacillus* strains; only two out the six passed the test, with a pH between 4.01 and 4.50. Overall, 20 lactic acid bacteria passed the selection; the strains were further identified and characterized by API 50CHL (rods) or API 20Strep (cocci) systems. The 10 *Enterococcus* strains matched *Enterococcus faecium*, the four *Streptococcus* strains matched *Streptococcus thermophilus*, the two *Lactobacillus* strains matched *L. paracasei* subsp. *paracasei*, and the two *Lactococcus* strains matched *Lactococcus lactis*. The two *Leuconostoc* strains were not identified beyond genus level (Table 3).

In the antagonistic test, the two strains of *L. paracasei* subsp. *paracasei* showed a marked anti-*E. coli* activity against all three endogenous strains of *E. coli* tested. Lactobacilli inhibited *E. coli* growth, whereas none of the other strains manifested any inhibiting activity (Figure 2). With the utilization of Tris–HCl (pH 7.0), the possibility that the observed antimicrobial activity was due to organic acid production was excluded. Inhibition was not due to hydrogen peroxide production by *L. paracasei*, since the catalase (EC 1.11.1.6) treatment did not suppress inhibition. This is a necessary verification because several *L. paracasei* subsp. *paracasei* are H_2O_2-producing [35]. Therefore, the observed antagonistic effect against *E. coli* is possibly due to production of bacteriocin-like compounds. Furthermore, the inhibitory substances produced by *L. paracasei* subsp. *paracasei* had a bactericidal mode of action, as highlighted by the clearing around the spot of the producer culture.

Discussion

Only a minority (18 of 90) of the isolates on MRS medium are lactobacilli. This is dependent on a very low degree of selectivity of the medium, which permits growth of different genera of coccal-shaped lactic acid bacteria. In similar studies, MRS agar was used, adjusted to pH 5.4 [27], to pH 5.5 [9], or not acidified [38]; however, the percentage of lactobacilli isolated was not specified.

Recently, several works on goats' and ewes' dairy lactobacilli were performed using *Lactobacillus* selective agar, also known as Rogosa agar [45]. One study reported that 70% of the isolates were lactobacilli [58]; another study, using this medium with the addition of cycloheximide (50 μg/ml), reported that 63% of the isolates were lactobacilli [24]; a third paper did not specify the percentage of lactobacilli isolated on this medium [52]. Thus, the accompanying bacterial flora appears to be more strongly suppressed by Rogosa agar than by MRS agar. All 90 isolates on M17 agar are coccal-shaped lactic acid bacteria; 55 match *Enterococcus*, 22 match *Leuconostoc*, 13 match *Streptococcus*, no isolate matches *Lactococcus*; in fact, the only three *Lactococcus* isolated in this study come from MRS agar and not from M17 agar. M17 agar shows a low selectivity for lactococci; it permits the growth of different coccal-shaped lactic acid bacteria: enterococci, up to 66% of the isolates [16]; lactic streptococci, as *S. thermophilus* [9]; and *Leuconostoc*. SBA agar shows a much higher selectivity for enterococci: all 45 isolates on this medium match *Enterococcus*.

The inhibitory activity of *L. paracasei* subsp. *paracasei* against *E. coli* is interesting because it is possible that these strains inhibit not only *E. coli*, but also different food-borne pathogens, as do other lactobacilli. The so-called *L. paracasei* group includes strains that are among the dominant nonstarter lactic acid bacteria within mature cheese [14]. The role of this adventitious microflora in cheese ripening is essentially related to its autolysis and to its contribution to amino acid fermentation [50]. *L. paracasei* has been introduced in many probiotic foods [22]. It was recently demonstrated that fermented dairy products made with *L. paracasei* enhance immune response; the observed bioactivity suggests that substances of bacterial origin could be released during fermentation by lactic acid bacteria [31]. *L. paracasei*, when used as an adjunct in cheese manufacture, also provides some important aroma and flavor attributes [4,8,20,25]. Therefore, *L. paracasei* may be used in the preparation of milk products, yoghurts, and infant foods [44]. In addition, strains of *L. paracasei* were shown to be the best binders of lactic acid bacteria in a study conducted to assess the binding efficiencies for aflatoxin B1 [41].

Artisanal Calabrian cheeses made from raw goats' milk could be protected from contamination by *E. coli* by utilizing selected strains of *L. paracasei* subsp. *paracasei* — if necessary, together with other lactic starters. The results indicate that this *E. coli* inhibition can be ascribed to the production of bacteriocin-like compounds. Use of *L. paracasei* to increase the safety of cheeses from raw milks is recommended because these cultures strongly inhibit *E. coli*, with the added benefit that no adverse sensory changes were to be observed [4,8,20,25].

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