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Antagonistic activity of dairy lactobacilli against gram-foodborne pathogens

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ABSTRACT. Thirty-five strains of lactic acid bacteria were isolated from artisanal raw milk cheese, presumptively identified and tested against one dairy *Escherichia coli* strain. Six lactobacilli, exhibiting antagonistic activity, were identified at the species level and their action was evaluated against four strains of Gram-foodborne pathogens (*Escherichia coli* O26, *Escherichia coli* O157:H7, *Salmonella* spp. 1023, and *Salmonella* Typhimurium) and the control strain *Escherichia coli* ATCC 45922. The antagonistic activity was determined by spot method and the inhibition zones were measured by Autodesk AutoCAD 2007. Three strains, all *Lactobacillus paracasei*, were active against all the pathogens; the other strains, all *Lactobacillus plantarum*, showed antagonistic activity against some pathogens. This study highlights the intense and different antagonistic activity induced by lactobacilli against various foodborne pathogens thus demonstrating that using selected lactic acid bacteria strains as adjunct cultures could be an effective strategy to prevent the development of foodborne pathogens in artisanal raw milk cheeses, and thus improving their safety.

Keywords: food safety, lactic acid bacteria, raw milk cheese.

Atividade antagônica dos lactobacilos lácteos contra bactérias patógenas Gram-transmitidas por alimentos

RESUMO. Trinta e cinco cepas de bactérias lácticas foram isoladas de queijo artesanal de leite cru, presuntivamente identificados e testados contra um coe láctico de *Escherichia coli*. Seis lactobacilos, exibindo atividade antagônica, foram identificados ao nível da espécie e sua ação foi avaliada contra quatro cepas de bactérias patógenos Gram-transmitidas por alimentos (*Escherichia coli* O26, *Escherichia coli* O157:H7, *Salmonella* spp. 1023 e *Salmonella* Typhimurium) e a coe de controlo *Escherichia coli* ATCC 45922. A actividade antagonista foi determinada pelo método de mancha e as zonas de inibição foram medidos por Autodesk AutoCAD 2007. Três cepas, todos *Lactobacillus paracasei*, foram ativos contra todas as patógenos; as outras cepas, todas *Lactobacillus plantarum*, apresentaram atividade antagônica contra alguns patógenos. Este estudo destaca a intensa e diferente atividade antagônica induzida por lactobacilos contra vários agentes patogénicos de origem alimentar. Isto demonstra que o uso de cepas de bactérias lácticas seleccionadas, como culturas adjuntas pode ser de grande ajuda e representa uma estratégia eficaz para evitar o desenvolvimento de agentes patogénicos de origem alimentar em queijos artesanais de leite cru, de modo a melhorar a sua segurança.

Palavras-chave: segurança alimentar, bactérias produtoras de ácido láctico, queijo de leite cru.

Introduction

Foodborne infections are, nowadays, one of the most important challenges for public health, causing illness, sometimes fatal, and also resulting in high economic loss (KAFERSTEIN, 2003). Small ruminants are reported as important reservoirs of foodborne pathogens - among which *Salmonella* and verocytotoxin-producing *Escherichia coli* (VTEC) - resulting in a possible contamination of products from these animals (COLAK et al., 2007; ESSID et al., 2009; OGUNBANWO et al., 2004). Strategies based on the use of antagonistic starter cultures allow the production of foods with improved levels of safety (SPELHAUG; HARLANDER, 1989). Antagonistic activities of lactic acid bacteria (LAB) against microbial pathogens may contribute, among other factors, to their inhibition (SERVIN, 2004). It is actually an important way to expand the range of healthful foods, especially in the dairy industry (SIMOVA et al., 2009).

Artisanal raw milk cheeses - made without the addition of LAB starters - are consumer attractive, with

an intense flavour, due to the action of endogenous microflora (CARIDI, 2002, 2003). However, they could imply hygiene and health concerns; evidence supports the transmission of foodborne pathogens by contaminated raw milk cheeses (MILLER; PAIGE, 1998). Furthermore, some of the above mentioned foodborne pathogens may survive or multiply during the production process of raw milk cheeses (LITTLE et al., 2008). These cheeses are highly prized and, especially in summer when the food-borne risk increases, may reach a large number of consumers.

The aim of the present work was to study influence and antagonistic action of dairy LAB on several foodborne pathogens and to select pathogen-inhibiting strains among the endogenous LAB of the artisanal Calabrian raw milk cheese *Pecorino del Poro*.

Material and methods

Cheese sampling

Seven *Pecorino del Poro* ewes' cheeses were sampled from dairy farms in the region of Calabria, southern Italy. Following traditional procedures, cheeses were made using raw milk without the addition of selected LAB.

Sample preparation and strain isolation

Representative cheese samples (10 g) were homogenized in 90 mL of sterile physiological saline for 2 minutes in a blade homogenizer 890-48H (Oster®/Sunbeam®, McMinnville, TN, USA). After filtering through sterile gauze, aliquots of 1 mL were diluted 10-fold in physiological sterile saline to enumerate LAB and *E. coli* and to isolate different microbial groups. Rod-shaped LAB were isolated on Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, England, UK) and incubated anaerobically by Gas Pack catalysts AnaeroGen (Oxoid, Basingstoke, England, UK) at 30°C for 3 days. Coccal-shaped LAB were isolated on M17 agar (Oxoid, Basingstoke, England, UK) and incubated anaerobically at 30°C for 3 days. LAB cultures were presumptively identified at the genus level. Dairy *E. coli* strains were isolated on Petrifilm 3M (Microbiology Products, St Paul, MN, USA), incubated aerobically at 37°C for 24h, and identified at the species level using standard methods. Working cultures were grown on broth media MRS, M17, and lactose broth (Oxoid, Basingstoke, England, UK) for lactobacilli, lactococci, and *E. coli* strains, respectively.

Food-borne bacterial strains

One strain of each of the following Gram- foodborne pathogens was used for the evaluation of the antagonistic activity of LAB: VTEC O26 (VT1 producer), VTEC O157:H7 (VT1 + VT2 producer), *Salmonella* spp. 1023, and *S.* Typhimurium. *E. coli* ATCC 45922, not a pathogenic strain, was used as a control strain. With the exception of the ATCC strain, all the strains used had been previously isolated from dairy products, using standard methods.

Antagonistic activity trials

The isolated LAB were screened on plate against one dairy *E. coli* strain for antagonistic activity, under conditions that eliminated the inhibitory effects of lactic acid and hydrogen peroxide. The active LAB strains were then identified at the species level using standard methods and tested against the above-mentioned foodborne pathogens, using the spot method (SPELHAUG; HARLANDER, 1989), in addition to the control strain *E. coli* ATCC 45922. A 24h culture of LAB grown in appropriate broth media was diluted 10-fold in 10 mmol L^{-1} Tris-HCl (pH 7.0) and 1 µl aliquots were spotted in triplicate onto MRS agar. The spot method uses the Tris-HCl (pH 7.0) solution to neutralize the organic acid produced by LAB in the broth media thus excluding possible inhibiting effects either on dairy *E. coli* or on foodborne pathogens (CARIDI, 2002). Plates were incubated anaerobically for 24h to eliminate inhibition due to hydrogen peroxide production (MESSI et al., 2001), and then overlaid either with Lactose soft agar (0.7% agar) or Triptic Soy soft agar (0.7% agar) inoculated, respectively, with 0.1 mL of overnight cultures of *E. coli* strains and the other foodborne pathogens. Plates were incubated for additional 18h and then checked for clear inhibition zones around spots of the presumed bacteriocin producers. The inhibition zones, measured using AutoCAD 2007 by Autodesk, Inc. (San Francisco, CA, USA), were expressed in mm and referred to the difference between the outer and the inner circles (Figure 1).

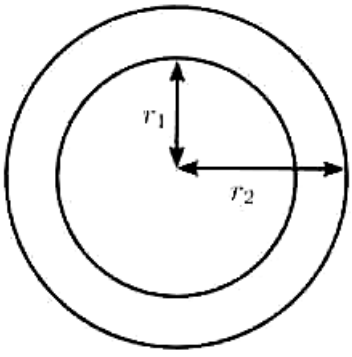

Figure 1. The inhibition halo size is expressed in mm and is referred to the difference between the outer (r_2) and the inner (r_1) radius.

Results and discussion

The evaluation of the amount of LAB and *E. coli* in the artisanal Calabrian raw milk cheese in current research is quite interesting. In the seven cheeses, LAB ranged from 4 x 10^7 cfu g^{-1} to 3 x 10^{10} cfu g^{-1} in the cheeses considered, whereas the amount of *E. coli* varied from zero to 1.4 x 10^6 cfu g^{-1}. With only one cheese over the limit imposed by the 2005/2073 European Commission Regulation (ECR, 2005), these results could be positively considered from a health and safety point of view. Although low values of *E. coli* could be due to the good hygienic quality of the milk or the good hygienic conditions during cheese manufacturing, in our opinion they are also due to the presence of antagonistic LAB.

Among the thirty-five LAB isolated, six out the rod-shaped LAB possessed antagonistic activity against the dairy *E. coli* strain, whereas none of the coccal-shaped LAB did. These six strains were identified as *Lactobacillus paracasei* (three strains) and *Lactobacillus plantarum* (three strains); subsequently, the six lactobacilli were tested for their antagonistic activity against foodborne pathogens.

Table 1 highlights the antagonistic activity of the six lactobacilli against the four Gram-pathogens and the control, based on their inhibition zones expressed in mm.

Table 1. Antagonistic activity of the six *Lactobacillus* strains and the control strain of *L. paracasei* subsp. *paracasei* L356 against the four Gram-pathogens and the control strain of *E. coli* ATCC 45922.

Strains	*E. coli* ATCC 45922		*E. coli* O26		*E. coli* O157:H7		*Salmonella* spp. 1023		*S. Typhimurium*	
	M [a]	SD [b]	M	SD	M	SD	M	SD	M	SD
Lb06 [c]	9.01	0.23	4.73	0.09	5.90	0.78	9.17	0.43	11.74	0.18
Lb07 [c]	9.66	1.49	5.03	0.18	4.78	0.43	10.77	0.81	12.47	0.42
Lb17 [d]	6.25	1.15	6.43	0.06	- [e]	-	-	-	7.45	0.23
Lb20 [d]	-	-	-	-	5.49	0.28	9.77	1.95	9.74	2.47
Lb21 [d]	9.25	0.82	5.42	0.85	4.46	0.14	-	-	6.13	0.40
Lb23 [c]	6.56	0.91	8.08	0.54	7.06	0.14	6.68	0.57	5.22	0.57

[a]Mean of the inhibition zones, expressed in mm, measured around the three spots; [b]Standard Deviation; [c]*Lactobacillus paracasei*; [d]*Lactobacillus plantarum*; [e]Absence of inhibition zone.

Five of the six *Lactobacillus* strains showed antagonistic activity against *E. coli* ATCC 45922; means of inhibition zones ranged from 6.25 mm for strain Lb17 to 9.66 mm for strain Lb07. Five of the six *Lactobacillus* strains showed antagonistic activity against VTEC O26; means of inhibition zones ranged from 4.73 mm for strain Lb06 to 8.08 mm for strain Lb23. Five of the six *Lactobacillus* strains showed antagonistic activity against VTEC O157:H7; means of inhibition zones ranged from 4.46 mm for strain Lb21 to 7.06 mm for strain Lb23. Four of the six *Lactobacillus* strains showed antagonistic activity against *Salmonella* spp. 1023; mean of inhibition zones ranged from 6.68 mm for strain Lb23 to 10.77 mm for strain Lb07. All the *Lactobacillus* strains showed antagonistic activity against *S. Typhimurium*; means of inhibition zones ranged from 5.22 mm for strain Lb23 to 12.47 mm for strain Lb07.

The three strains of *L. plantarum* selected in current research exhibit antagonistic activity against some of the tested pathogens; the antagonistic activity of other strains of *L. plantarum* had been previously studied.

It was reported that *L. plantarum* NCIM2084 produced a bacteriocin (plantaricin LP84) resistant to heat and catalase and was able to inhibit *E. coli* D21 (SUMA et al., 1998). Agar spot and well diffusion assay tests were applied to determine the antagonistic activity of 19 strains of *L. plantarum* against *E. coli* NRRL B-3704. The vast majority of the strains were able to inhibit the strain in agar spot, conducted by placing 0.5 mL of an overnight LAB culture onto MRS agar containing 0.2% glucose and incubated for 24h at 25°C under anaerobic condition. However, no strain was able to inhibit *E. coli* NRRL B-3704 by well diffusion assay test, using cell-free supernatants adjusted to pH 6.5 and sterilized by filtering through a 0.22 mm pore size cellulose acetate filter (ÇON; GÖKALP, 2000).

The crude filtrate supernatant of *L. plantarum* 35d, isolated from sausages, was able to inhibit *Salmonella* IM300 and IM301, and *E. coli* ATCC13762 and ATTC25922 (MESSI et al., 2001).

Supernatants of two dairy strains, Z11L and Z10, of *L. plantarum* produced strong inhibition against *E. coli* ATCC25922; however, when the supernatants were treated with catalase to eliminate the possible presence of hydrogen peroxide or neutralized to pH 6.5-7.0, only the supernatant of the strain Z10 produced weak inhibition (ASLIM et al., 2005).

L. plantarum ATCC8014 was tested for production of antimicrobial compounds; the study demonstrated that the cell-free supernatant of the strain was effective in inhibiting the growth of *E. coli* 15-5065 and *S. Typhimurium* 15-5351A (LASH et al., 2005).

L. plantarum ST26MS and ST28MS, isolated from molasses, produced bacteriocins active against *E. coli* 8 (TODOROV; DICKS, 2005). Strains ST194BZ, ST414BZ and ST664BZ of *L. plantarum*, isolated from boza, produced bacteriocins active against *E. coli* 8; the bacteriocin-containing supernatants were filter-sterilized and adjusted to pH 6.0 before testing their antimicrobial activity (TODOROV; DICKS, 2006a and b). The bacteriocin produced by *L. plantarum* ST69BZ, also

isolated from boza, inhibited the growth of *E. coli* P40 (TODOROV, 2010).

A number of strains of *L. plantarum* were tested for the production of bacteriocins which were able to inhibit the main foodborne pathogens; the best selected strains were effective in inhibiting the growth of *E. coli* (OMAR et al., 2006, 2008).

Agar spot and well diffusion assay tests were applied to determine the antagonistic activity of 17 strains of *L. plantarum*, isolated from a traditional Tunisian salted meat, against *E. coli* DH5a, IPT. By agar spot 16 strains still displayed inhibition zones against *E. coli*. However, using cell-free supernatants treated with catalase to eliminate the possible presence of hydrogen peroxide and neutralized, no strain produced bacteriocin and, consequently, no strain was able to inhibit *E. coli* (ESSID et al., 2009).

A bacteriocin produced by *L. plantarum* KLDS1.0391, isolated from a traditional fermented cream from Inner Mongolia in China, was described; the bacteriocin showed a broad inhibitory activity, including *E. coli* ATCC25923, enteropathogenic *E. coli* CMCC44706, enterotoxigenic *E. coli* CMCC44247, enteroinvasive *E. coli* CMCC44350, and *S.* Typhimurium ATCC14028 (GONG et al., 2010).

Supernatant of *L. plantarum* TN635 strain inhibited the growth of all used indicator microorganisms, among which *E. coli* ATCC8739 (SMAOUI et al., 2010).

L. plantarum LB-B1, isolated from koumiss, produced a bacteriocin active against *E. coli* ATCC80739, AS1.90, 85993 and DH-5 (XIE et al., 2011).

L. plantarum LE27 showed antimicrobial activity against *E. coli* ATCC25922; however, this was obtained only after fructo-oligosaccharides supplementation. Consequently, these results show the feasibility of increasing the antimicrobial activity of bacteriocins by supplementing the growth medium with fructo-oligosaccharides (MUÑOZ et al., 2012).

The three strains of *L. paracasei* selected in current research exhibit antagonistic activity against all the six tested pathogens; the antagonistic activity of other strains of *L. paracasei* had been previously studied.

The dairy strains of *L. paracasei* subsp. *paracasei* L126, L134, L252, L269, L342, L343, L355, L356, L402, and L410 showed, both in plate and in milk, a marked anti-*E. coli* activity (CARIDI, 2002, 2003).

Supernatants of four strains of *L. paracasei* subsp. *paracasei* isolated from new-born infant faeces produced inhibition against *E. coli* ATCC25922; however, when the supernatants were neutralized to pH 6.5 and treated with catalase to eliminate the possible presence of hydrogen peroxide, no supernatant was able to inhibit *E. coli* ATCC25922 (ARICI et al., 2004).

Of the neutralized (pH 6.5) cell-free supernatants of three dairy strains of *L. paracasei* subsp. *paracasei*, one produced inhibition against *E. coli* HB101; the activities of all the bacteriocin-like substances from studied LAB were stable over a pH range between 3 and 11 (GULAHMADOV et al., 2006).

L. paracasei ST242BZ, isolated from boza, produced a bacteriocin active against *E. coli* 8; another strain, *L. paracasei* ST284BZ, produced a bacteriocin active against the two strains of *E. coli* 8 and 40; the bacteriocin-containing supernatants were filter-sterilized and adjusted to pH 6.0 before testing their antimicrobial activity (TODOROV; DICKS, 2006b).

L. paracasei subsp. *paracasei* BMK2005, isolated from healthy infant faeces, showed a remarkable antibacterial activity; the antibacterial activity of sterile cell-free supernatant was described against four strains of *E. coli* (BENDJEDDOU et al., 2012).

Conclusion

This study highlights the intense and different antagonistic activity induced by lactobacilli against various food-borne pathogens, presumably due to production of bacteriocin-like compounds. Further studies are required to best elucidate the mechanism of the bacterial inhibition since it is not attributable to any single antagonist factor (AGUILAR; KLOTZ, 2010). Also the potential production and the effect of bacteriocins in a complex substrate such as cheese remains to be examined. The selected LAB strains as adjunct cultures could be an interesting and effective strategy to improve the safety of artisanal raw milk cheeses.

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