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Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk

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ABSTRACT

Antimicrobial activity of reuterin in combination with different bacteriocins from lactic acid bacteria against food-borne pathogens in milk was investigated. A strong synergistic effect of reuterin in combination with nisin, lacticin 481 or enterocin AS-48 on *Listeria monocytogenes* was observed. Only nisin increased the antimicrobial activity of reuterin against *Staphylococcus aureus*. Bactericidal activity of reuterin towards *Escherichia coli* O157:H7, *Salmonella enterica, Yersinia enterocolitica, Aeromonas hydrophila* and *Campylobacter jejuni* was not enhanced significantly by the addition of any of the bacteriocins investigated. The synergism of reuterin and nisin against *L. monocytogenes* and *S. aureus* was also found at refrigeration temperatures, where the pathogens were completely inactivated. Refrigerated milk treated with both natural antimicrobials would mean a feasible system to control pathogenic contaminants.

1. Introduction

Reuterin (β-hydroxypropionaldehyde) is a molecule with antimicrobial activity towards a broad spectrum of food-borne pathogens and spoilage organisms. Reuterin is soluble in water, resistant to heat, proteolytic and lipolytic enzymes, and stable over a wide range of pH values (Axelsson, Chung, Dobrogosz, & Lindgren, 1989; Vollenweider, Grassi, König, & Puhan, 2003). The use of reuterin to control Gram-positive and Gram-negative pathogens has been investigated in milk and dairy products (Arqués, Rodríguez, Nuñez, & Medina, 2008; El-Ziney & Debevere, 1998) and in meat products (El-Ziney, van den Tempel, Debevere, & Jakobsen, 1999). In all studies, reuterin has been shown to have a higher antimicrobial activity on Gram-negative than on Gram-positive pathogenic bacteria.

Bacteriocins of lactic acid bacteria (LAB) are small peptides that show a narrow or broad antimicrobial activity spectrum against Gram-positive bacteria. The use of bacteriocins to control food-borne pathogens and spoilage bacteria has been reported in a variety of foods including milk and dairy products (Rodríguez, Arqués, Gaya Nuñez, & Medina, 2001; Zottola, Yezzi, Ajao, & Roberts, 1994). A number of bacteriocins with industrial potential have been purified and characterized. LAB-bacteriocins have a common mechanism of action on sensitive cells by the formation of transitory poration complexes or ionic channels in the cytoplasmic membrane that causes total or major dissipation of the proton motive force (Bruno & Montville, 1993). Their action is generally inactive against Gram-negative bacteria because of the presence of the outer membrane. Nisin, the only bacteriocin authorized as a food preservative in over 50 countries wordwide, is produced by some *Lactoccocus lactis* and it is active against undesirable Gram-positive bacteria associated with food. The combination of bacteriocins with other antimicrobials in order to reduce the selection for resistance to bacteriocins in target strains or to extend its inhibitory activity to Gram-negative bacteria has been reported (Helander, von Wright, & Mattila-Sandholm, 1997; Stevens, Sheldom, Klapes, & Klaenhammer, 1991).

The use of natural antimicrobials in the food industry can help to reduce the addition of chemical preservatives, offering an alternative to satisfy the increasing consumer demand for safe, fresh-tasting, ready-to-eat, minimally-processed foods and also to develop novel food products. Nevertheless, the chemical and physical properties of a food such as pH, enzymes, fat and additives can limit the antimicrobial activity of natural compounds. Research into synergistic effects of the combined action of natural preservatives to increase microbial lethality could achieve an improved level of product safety according to the hurdle concept of food preservation that would benefit both consumers and producers.

Here we sought combinations of reuterin with LAB-bacteriocins in order to enhance individual antimicrobial activity against food-borne pathogens in milk. Subsequently, the inhibitory potential of combining reuterin and nisin on *Listeria monocytogenes* and *Staphylococcus aureus* in milk stored at two refrigeration temperatures has also been investigated.

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2. Materials and methods

2.1. Microorganisms and culture conditions

Escherichia coli O157:H7 ATCC 43894, *Salmonella enterica* subsp. *enterica* CECT 409, *Campylobacter jejuni* LMG 6629, *Yersinia enterocolitica* CECT 559, *Aeromonas hydrophila* subsp. *hydrophila* CECT 839, *L. monocytogenes* Ohio serotype 4b (from R.G. Crawford, Food and Drug Administration, Cincinnati, OH 45226, USA) and *S. aureus* CECT 4013 were used as test microorganisms. The strains were propagated in Tryptic Soy Broth (TSB, Biolife, Milano, Italy) at 37 °C (at 30 °C in the case of *A. hydrophila*) for 18 h. *C. jejuni* was grown in basal medium with the addition of 5% (v/v) lysed horse blood and incubated in a microaerophilic atmosphere of 5% oxygen and 10% carbon dioxide generated by Gas Generating Kit (Oxoid, Unipath Ltd., Basingstoke, UK). Test bacteria were subcultured in sterile reconstituted skim milk supplemented with 0.3% yeast extract before use in milk assays. Lacticin 481-producing *L. lactis* subsp. *cremoris* TAB 24, enterocin I-producing *Enterococcus faecalis* TAB 52 and enterocin AS-48-producing *E. faecalis* TAB 70 from the INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain) culture collection were propagated in MRS broth (Biolife) at 30 °C for 18 h. All the strains were maintained in a frozen stock at −80 °C with 15% glycerol and propagated twice before used in experiments.

2.2. Biopreservatives

The reuterin-producing *L. reuteri* INIA PRO 137 was used for reuterin production in a process carried out in two steps, biomass generation and reuterin production as previously described (Arqués et al., 2004). Nisin (Nisaplin®, Danisco, Copenhagen, Denmark) was resuspended in 0.02 N HCl to 10000 IU/ml and stored at −40 °C. The nisin stock was diluted prior to use in experiments. LAB-bacteriocins were obtained from bacteriocinogenic cultures. After cultivation the supernatants obtained by centrifugation (10,000 *g*, 15 min, 4 °C) were filtered (0.22 μm, Millipore Corporation, Bedford, MA, USA) and adjusted to pH 6 with 1 N NaOH. Inhibitory activity of reuterin and bacteriocins (lacticin 481, enterocin I and enterocin AS-48) was determined with the modified assay of Chung, Axelsson, Lindgren, and Dobrogosz (1989) using *E. coli* K12 CECT 433 as indicator strain for reuterin and *L. monocytogenes* Ohio as indicator strain for LAB-bacteriocins. Arbitrary units (AU) were defined as the reciprocal of the highest serial two-fold dilution which did not show inhibition of the indicator strain.

2.3. Inhibitory activity of reuterin in combination with LAB-bacteriocins against pathogens in milk

Bacterial strains were separately inoculated at approximately 10^4 cfu/ml into flasks containing 15 ml of UHT skim milk with 0.04% fat (Pascual, Aranda del Duero, Spain). Biopreservatives were added individually or in combination immediately after inoculation. Reuterin and nisin were added at an estimated final activity of 8 AU/ml and 100 IU/ml, respectively, and lacticin 481, enterocin I and enterocin AS-48 at 170 AU/ml. Milk inoculated with each pathogen with no addition of reuterin or LAB-bacteriocins served as control. Flasks were incubated at 37 °C for 24 h except for *A. hydrophila* which was incubated at 30 °C. Microbiological counts were determined after 4 and 24 h on Tryptic Soy Agar (TSA, Biolife) plates. *C. jejuni* was determined on Blood Agar Base No 2 plates (Oxoid) supplemented with 5% laked horse blood and Campylobacter growth supplement (Oxoid). Two separate experiments were carried out. Microbiological analyses were performed in duplicate.

2.4. Inhibitory activity of reuterin combined with nisin against Gram-positive pathogens in milk at refrigeration temperatures

The milk assays at refrigeration temperatures were performed as described above using *L. monocytogenes* and *S. aureus* as test microorganisms. Flasks were stored at 4 °C and 8 °C for 12 d. Microbiological counts were determined after 8 h and at 1, 3, 5, 7 and 12 d on duplicate plates of TSA. Two separate experiments were carried out.

2.5. Statistical analysis

Data were subjected to ANOVA with treatment and time of refrigeration as main effects on pathogen counts at each temperature using the SPSS program Win version 12.0 (SPSS Inc., Chicago, IL, USA). Significant differences in pathogen counts in milk between different treatments for a given pathogen and time of refrigeration were further analyzed using Tukey's test with a significance level of $\alpha = 0.01$, using the same program.

3. Results

3.1. Inhibitory activity of reuterin combined with LAB-bacteriocins against pathogens in milk

Inhibitory activities of reuterin, LAB-bacteriocins and their combination on Gram-positive and Gram-negative pathogens in milk are shown in Tables 1 and 2, respectively. The counts of the different pathogens in milk were significantly influenced by the treatment ($P < 0.001$) and the incubation time ($P < 0.001$), according to ANOVA. After 4 h, antimicrobial activity of reuterin was bacteriostatic, since the initial bacterial levels did not decrease, or slightly bactericidal on all the pathogens, except on *C. jejuni* where a strong bactericidal activity was observed. Nisin, lacticin

Table 1
Log counts (cfu/ml) of *L. monocytogenes* and *S. aureus* in milk without biopreservatives (C) or with reuterin (R), nisin (N), lacticin 481 (L481), enterocin I (EI), enterocin AS-48 (AS48) or their combination (R + N, R + L481; R + EI; R + AS48) after incubation at 37 °C for 4 and 24 h.

Time (h)	*L. monocytogenes*	*S. aureus*
0		
C	3.91	4.10
4		
C	4.66^{f}	5.89^{d}
R	3.77^{e}	4.19^{c}
N	0.15^{a}	1.21^{a}
L481	3.22^{d}	5.88^{d}
EI	4.40^{f}	5.88^{d}
AS48	2.00^{b}	5.97^{d}
R + N	nd^{a}	3.54^{b}
R + L481	0.15^{a}	4.15^{c}
R + EI	2.70^{c}	4.22^{c}
R + AS48	0.24^{a}	4.18^{c}
24		
C	8.95^{e}	8.89^{d}
R	4.81^{b}	3.46^{b}
N	7.44^{d}	8.52^{c}
L481	5.99^{c}	9.07^{d}
EI	8.61^{e}	9.11^{d}
AS48	7.28^{d}	9.09^{d}
R + N	nd^{a}	2.59^{a}
R + L481	nd^{a}	3.40^{b}
R + EI	4.68^{b}	3.59^{b}
R + AS48	nd^{a}	3.41^{b}

Values with different superscripts indicate statistically significant ($P < 0.01$) differences between treatments for a given organism and time. nd, below detection limit (1 cfu/ml).