J. Dairy Sci. 97:6116–6121
http://dx.doi.org/10.3168/jds.2014-8306

Short communication: Combined antimicrobial activity of reuterin and diacetyl against foodborne pathogens

S. Langa,[1] I. Martín-Cabrejas, R. Montiel, J. M. Landete, M. Medina, and J. L. Arqués
Dpto. de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Carretera de La Coruña Km 7, 28040 Madrid, Spain

ABSTRACT

Reuterin (β-hydroxypropionialdehyde) is a broad-spectrum antimicrobial substance produced by some strains of *Lactobacillus reuteri* during anaerobic fermentation of glycerol. Some of these strains are able to survive and produce reuterin in cheese and yogurt when added as adjuncts to the starter. Similarly, in fermented dairy foods, other inhibitory compounds such as lactic acid and diacetyl are produced during fermentation. In this work, we studied the combined effect of reuterin and diacetyl under different pH conditions against *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes*. Results from agar spot assays showed that the antimicrobial activity of reuterin-producing strains against the gram-negative bacteria tested was enhanced as the concentration of diacetyl increased to 50 mg/kg, and was higher under acidic conditions (pH 5.0) for the 3 pathogenic strains. The combination of reuterin and diacetyl had an additive effect against *L. monocytogenes* only at diacetyl concentrations of 50 mg/kg and pH 5.0. In addition, growth kinetics studies showed that the combination of 1 activity unit (AU)/mL of reuterin with 100 mg/kg diacetyl increased the lag time of the 3 pathogens. In milk, synergistic antimicrobial activity was observed with the combination of 1 AU/mL reuterin and 50 or 100 mg/kg of diacetyl on the gram-negative strains tested, and with 1 AU/mL reuterin and 100 mg/kg of diacetyl on *L. monocytogenes*. The greatest inhibition of the 3 pathogens was achieved in acidified milk at pH 5.0 with reuterin (1 AU/mL) and diacetyl (100 mg/kg). Based on these results, the combination of reuterin and diacetyl in acidified dairy products could be a promising strategy to control food pathogens in these products.
Key words: reuterin, diacetyl, bioprotection, foodborne pathogen

Short Communication

Reuterin (β-hydroxypropionialdehyde; β-HPA) is a broad-spectrum antimicrobial substance produced by some strains of *Lactobacillus reuteri* during anaerobic fermentation of glycerol, which shows activity against foodborne pathogens and spoilage microorganisms (Axelson et al., 1989). The use of reuterin to control gram-positive and gram-negative foodborne pathogens has been previously investigated in milk and dairy products (el-Ziney and Debevere, 1998; Arqués et al., 2008a,b). Interestingly, reuterin is soluble in water and resistant to heat and proteolytic and lipolytic enzymes, and it maintains its antimicrobial activity at low pH and high NaCl concentrations (Rasch, 2002; Rasch et al., 2007). Diacetyl (2,3-butanodione) is a volatile compound produced during cheese ripening and yogurt production by different citrate-utilizing lactic acid bacteria (**LAB**) strains (Hugenholtz, 1993). It is responsible for the typical flavor of butter and some cheese varieties and is often used as a flavoring compound of many food products, being considered a generally recognized as safe (GRAS) food ingredient (Jay, 1982). The antimicrobial activity of diacetyl against foodborne pathogens has been previously described (Jay, 1982; Kang and Fung, 1999). *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* are pathogens of major concern to the food industry. Combinations of reuterin or diacetyl with other antimicrobials have shown a synergistic antimicrobial effect against foodborne pathogens (Arqués et al., 2008b, 2011; O'Bryan et al., 2009).

In practical terms, the addition of combinations of different preservatives in small amounts (hurdle technology) is often more effective than the use of only preservative in higher amounts, because different preservatives may act synergistically. The combined antimicrobial activity of reuterin and diacetyl, 2 biopreservatives that can be produced in situ in dairy products by LAB used as starters or adjunct cultures, has never been studied. Hence, the aim of this work was to evaluate the antimicrobial activity of different combinations of reuterin and diacetyl at different pH

Received April 30, 2014.
Accepted June 14, 2014.
[1]Corresponding author: langa.susana@inia.es

conditions against *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes*.

Reuterin-producing (*Lactobacillus reuteri* INIA P569, INIA P570, INIA P572, INIA P577, and INIA P579) and nonproducing (*Lb. reuteri* INIA P581, INIA P582, and INIA P583) strains were selected from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) culture collection (Madrid, Spain). All *Lb. reuteri* strains were grown as previously described (Langa et al., 2013). *Escherichia coli* K12 CECT 433, *E. coli* O157:H7 CECT 4972, *Salmonella enterica* ssp. *enterica* serovar Enteritidis CECT 4300 from the Spanish Type Culture Collection (Valencia, Spain) and *Listeria monocytogenes* OHIO serotype 4b (from R. G. Crawford, Food and Drug Administration, Cincinnati, OH), were grown in tryptic soy broth (Biolife s.r.l., Milan, Italy). Reuterin was purified and determined as described in Montiel et al. (2014).

The reuterin antimicrobial activity assay was a modification of the agar spot method described by Spinler et al. (2008). Two-microliter spots of *Lb. reuteri* strains were grown for 18 h at 37°C under anaerobic conditions and then overlaid with 0.7% brain-heart infusion agar with 20 m*M* glucose, 50 m*M* glycerol, and 6 log cfu/mL of the pathogenic strain. Different diacetyl concentrations (0–100 mg/kg; Sigma-Aldrich, St Louis, MO) and pH reduction to 5.0 with lactic acid were also tested with *Lb. reuteri* INIA P572 and INIA P579. Plates were incubated at 37°C for 3 h under anaerobic conditions and, then, under aerobic conditions for up to 24 h. Plates without glycerol or with non-reuterin-producing *Lb. reuteri* strains were used as negative controls. Inhibition curves were performed in microtiter plates with 200 μL of tryptic soy broth containing 6 log cfu/mL of the pathogenic strain, different combinations of purified reuterin [0, 1, and 2 activity units (**AU**)/mL], and diacetyl (0–100 mg/kg). The kinetic parameters maximum growth rate ($\boldsymbol{\mu}$; expressed in h^{-1}) and lag time ($\boldsymbol{\lambda}$, expressed in h) were studied as in Montiel et al. (2014). Pathogenic strains were inoculated individually at approximately 3 log cfu/mL in reconstituted skim milk supplemented with different concentrations of purified reuterin (0, 1, or 2 AU/mL) and diacetyl (0–250 mg/kg). When necessary, normal milk pH (approximately 6.6) was adjusted to 5.0 with 10% lactic acid. Milk was kept at 37°C and counts of *E. coli*, *Salmonella* Enteritidis, and *L. monocytogenes* were determined on duplicate plates of tryptic soy agar. Two separate experiments were carried out. Data were subjected to ANOVA using the SPSS program 12.0 for Windows (SPSS Inc., Chicago, IL). Significant differences were assessed by Tukey's test at $P < 0.01$ using the same program.

Results from agar spot assays indicated that all reuterin-producing *Lb. reuteri* strains tested were able to inhibit the 3 pathogenic strains. No inhibition was observed in plates that did not contain glycerol or that had the non-reuterin-producing strains (data not shown). Strains *Lb. reuteri* INIA P572 and INIA P579 were selected for additional assays because of their higher reuterin production. The results at pH 7.0 and 5.0 and with different diacetyl concentrations are shown in Figure 1. Results indicated that, at pH 7.0, the zones of inhibition produced by reuterin-producing *Lb. reuteri* colonies in plates inoculated with 2 gram-negative strains enlarged as the concentration of diacetyl increased. However, in the case of *L. monocytogenes*, no increase in the inhibition zones was observed in the presence of any diacetyl concentration tested. The zones of inhibition were larger at pH 5.0 for the 3 pathogenic strains. This increase was particularly marked in the case of *E. coli* O157:H7 as inhibition zones increased drastically compared with those at pH 7.0 at all diacetyl concentrations. The combination of 50 mg/kg of diacetyl and pH 5.0 resulted in the highest inhibitory activity of the 2 reuterin-producing *Lb. reuteri* strains against the 3 pathogens (Figure 1). Previously, el-Ziney et al. (1999) showed that lactic acid enhances the efficacy of reuterin as an antimicrobial.

The reuterin purified stock was used for inhibition curve experiments at 1 and 2 AU/mL reuterin, which correspond to 2.5 and 5.1 m*M*, respectively. Reuterin at 1 AU/mL was not enough to completely inhibit the growth of pathogens. However, this treatment significantly increased ($P < 0.01$) lag time in the 3 pathogens. Its combination with 100 mg/kg diacetyl significantly increased ($P < 0.01$) lag time in all strains compared with 1 AU/mL reuterin alone, although the increase was higher against the gram-negative strains. Maximum growth rate was significantly reduced ($P < 0.01$) by the combination of 1 AU/mL reuterin and 100 mg/kg of diacetyl only in *E. coli* O157:H7. Diacetyl treatment alone at 100 mg/kg was able to significantly inhibit ($P < 0.01$) the maximum growth rate of *E. coli*, whereas growth rates of *Salmonella* Enteritidis or *Listeria* were not influenced by any of the diacetyl concentrations tested. However, for *Listeria*, we did observe a significant increase in the lag time when the diacetyl concentration reached 100 mg/kg (Table 1). Addition of 2 AU/mL reuterin was enough to inhibit completely *E. coli* O157:H7 and *Salmonella* Enteritidis growth during the first 24 h, whereas some bacterial growth was detected after 20 h for *L. monocytogenes*. The combination of 2 AU/mL reuterin with diacetyl did not enhance the antimicrobial effect observed for reuterin in *L. monocytogenes*.