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

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ABSTRACT

Reuterin (β -hydroxypropionaldehyde) 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 -hydroxypropionaldehyde; 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.

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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 mM glucose, 50 mM 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 μ L 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 (μ ; expressed in h⁻¹) and lag time (λ , 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 mM, 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*.

Figure 1. Inhibitory activity of *Lactobacillus reuteri* INIA P572 (left dot) and INIA P579 (right dot) on the growth of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes*. Assays were performed on agar plates containing 50 mM glycerol at different diacetyl concentrations (0–50 mg/kg) and pH conditions (7.0 and 5.0). Plates without glycerol were used as negative controls.

Counts of the 3 pathogenic strains tested in milk were significantly ($P < 0.001$) influenced by reuterin, diacetyl, and pH treatments, their combination, and incubation time ($P < 0.001$), according to ANOVA results. Results in Table 2 showed that, at normal milk pH, the counts of *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* were significantly ($P < 0.01$) influenced by diacetyl only at 250 mg/kg, with

counts 7.11, 2.54, and 3.80 log units lower than those in control milk, respectively, after 24 h. After 6 h, reuterin had a bacteriostatic effect on *E. coli* and *Salmonella* Enteritidis, because the initial bacterial levels did not decrease, whereas reuterin led to *L. monocytogenes* counts 0.43 log units lower than in control milk. After 24 h, growth resumed in all cases and *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* reached

Table 1. Estimated maximum growth rate (μ) and lag time (λ) of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* in the presence of different combinations of reuterin (activity units, AU) and diacetyl

Strain	Parameter	Diacetyl (mg/kg)	Reuterin (AU/mL)		
			0	1	2
<i>E. coli</i>	μ (h^{-1})	0	0.171 ^{ab}	0.137 ^b	0.000 ^d
		10	0.164 ^{ab}	0.140 ^b	0.000 ^d
		25	0.187 ^a	0.142 ^b	0.000 ^d
		50	0.126 ^b	0.145 ^b	0.000 ^d
		100	0.070 ^c	0.064 ^c	0.000 ^d
	λ (h)	0	1.11 ^a	7.51 ^b	—
		10	1.73 ^a	9.60 ^b	—
		25	1.68 ^a	10.57 ^b	—
		50	1.35 ^a	9.50 ^b	—
		100	1.47 ^a	12.72 ^c	—
<i>Salmonella</i> Enteritidis	μ (h^{-1})	0	0.110 ^{ab}	0.102 ^{ab}	0.000 ^c
		10	0.119 ^{ab}	0.055 ^{bc}	0.000 ^c
		25	0.075 ^b	0.117 ^{ab}	0.000 ^c
		50	0.148 ^a	0.059 ^{bc}	0.000 ^c
		100	0.097 ^{ab}	0.090 ^{ab}	0.000 ^c
	λ (h)	0	1.74 ^a	12.01 ^b	—
		10	1.70 ^a	13.37 ^b	—
		25	1.99 ^a	14.10 ^b	—
		50	2.36 ^a	14.79 ^b	—
		100	4.43 ^a	19.38 ^c	—
<i>L. monocytogenes</i>	μ (h^{-1})	0	0.147 ^a	0.116 ^{abc}	0.070 ^d
		10	0.114 ^{abcd}	0.104 ^{abcd}	0.002 ^e
		25	0.126 ^{ab}	0.093 ^{bed}	0.007 ^e
		50	0.117 ^{abc}	0.104 ^{abcd}	0.003 ^e
		100	0.111 ^{abcd}	0.102 ^{abcd}	0.077 ^{cd}
	λ (h)	0	5.17 ^a	7.60 ^{cd}	20.42 ^f
		10	6.25 ^{abc}	7.93 ^{de}	21.69 ^f
		25	5.92 ^{ab}	8.42 ^{de}	21.67 ^f
		50	6.30 ^{abc}	8.19 ^{de}	20.63 ^f
		100	6.70 ^{bcd}	9.15 ^e	20.30 ^f

^{a-f}Means with different superscripts indicate significant differences ($P < 0.01$) within the same strain and parameter.

counts 1.46, 1.07, and 0.39 log units lower than those of control milk, respectively. A synergistic antimicrobial activity was observed between diacetyl and reuterin on the 3 pathogens tested: the combined effect was greater than the sum of reductions obtained by diacetyl and reuterin individually. The combination of 1 AU/mL reuterin with 50 or 100 mg/kg of diacetyl synergistically decreased the counts of *E. coli* O157:H7 and *Salmonella* Enteritidis. For *L. monocytogenes*, synergistic activity was also detected in milk with 1 AU/mL reuterin in combination with 100 mg/kg diacetyl. When normal milk pH was adjusted to 5.0, an inhibitory effect was detected on *L. monocytogenes* after 24 h, with levels 2.00 log units lower than those in control milk. The combined effect of low pH and reuterin was bactericidal after 6 h in the pathogenic strains tested. After 24 h, *E. coli* and *Salmonella* Enteritidis counts decreased to 1.68 and 0.45 log cfu/mL, respectively, whereas growth of *L. monocytogenes* restarted, with counts reaching 4.73 log cfu/mL. No additive bactericidal effect was obtained in the presence of diacetyl at any concentration, except

for counts of *L. monocytogenes*, which showed levels 1.12 log units lower in acidified milk treated with reuterin and 100 mg/kg diacetyl compared with levels in acidified milk with only reuterin.

These results are in concordance with previous studies that have shown a higher antimicrobial activity of reuterin or diacetyl on gram-negative than on gram-positive pathogenic bacteria (Jay, 1982; Arqués et al., 2011; Stevens et al., 2011). The higher rate of inhibition observed for reuterin in combination with diacetyl, low pH, or both, is an interesting feature because many dairy foods have a low pH due to the formation of lactic acid or diacetyl production during fermentation. A pH of 5.0 or less is common in many cheese varieties and fermented milks, and antimicrobial activity of diacetyl at concentrations ranging between 300 and 10,000 mg/kg against *E. coli*, *Salmonella*, and *L. monocytogenes* has been reported (Jay, 1982; Olasupo et al., 2003; O'Bryan et al., 2009). Here, we were able to detect antimicrobial activity at diacetyl concentrations of 250 mg/kg, and this concentration could be reduced to 100

Table 2. Counts (log cfu/mL) of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* in milk without biopreservatives (–) or containing 1 AU (activity units)/mL reuterin in combination with different diacetyl concentrations (mg/kg) at normal milk pH or acidified to pH 5.0 after 6 and 24 h incubation at 37°C

pH	Reuteurin	Diacetyl	<i>E. coli</i>		<i>Salmonella</i> Enteritidis		<i>L. monocytogenes</i>	
			6 h	24 h	6 h	24 h	6 h	24 h
Normal milk pH	–	0	6.36 ^a	8.90 ^a	6.01 ^a	8.77 ^{ab}	5.30 ^a	7.95 ^a
	–	10	6.20 ^{ab}	8.89 ^a	5.94 ^a	8.80 ^a	5.18 ^{ab}	7.90 ^a
	–	25	6.03 ^{ab}	8.89 ^a	5.94 ^a	8.86 ^a	5.13 ^{ab}	7.79 ^{ab}
	–	50	5.64 ^{abc}	8.88 ^a	5.54 ^a	8.75 ^{ab}	4.94 ^{abc}	7.79 ^{ab}
	–	100	4.59 ^{abcd}	8.82 ^a	4.85 ^{ab}	8.79 ^a	4.09 ^c	7.56 ^{abc}
	–	250	2.19 ^g	1.79 ^d	3.16 ^{bc}	6.23 ^{de}	2.85 ^d	4.15 ^{ef}
	+	0	3.41 ^{defg}	7.44 ^a	3.11 ^{bc}	7.70 ^{abc}	4.87 ^{abc}	7.56 ^{abc}
	+	10	3.56 ^{defg}	7.59 ^a	3.16 ^{bc}	7.91 ^{abc}	4.66 ^{abc}	7.49 ^{abc}
	+	25	3.39 ^{defg}	7.23 ^{ab}	3.09 ^{bc}	7.72 ^{abc}	4.59 ^{abc}	7.39 ^{abc}
	+	50	3.27 ^{defg}	6.67 ^{ab}	3.09 ^{bc}	5.41 ^{ef}	4.33 ^{bc}	7.30 ^{abc}
	+	100	3.15 ^{defg}	4.71 ^{bc}	2.93 ^{bed}	4.88 ^f	4.01 ^c	6.88 ^{abcd}
Acidic (pH 5.0)	–	0	4.53 ^{bcde}	8.22 ^a	1.50 ^{cd}	7.70 ^{abc}	2.68 ^d	5.95 ^{bcde}
	–	10	4.08 ^{cdef}	8.07 ^a	2.03 ^{cd}	7.66 ^{abc}	2.63 ^d	5.95 ^{bcde}
	–	25	4.69 ^{abcd}	8.14 ^a	2.18 ^{cd}	7.75 ^{abc}	2.51 ^d	5.98 ^{bcde}
	–	50	4.46 ^{bcde}	8.20 ^a	2.24 ^{cd}	7.42 ^{bcd}	2.52 ^d	5.72 ^{cdef}
	–	100	3.76 ^{defg}	7.50 ^a	1.78 ^{cd}	6.65 ^{cde}	2.40 ^d	5.33 ^{def}
	+	0	2.14 ^g	1.68 ^d	1.81 ^{cd}	0.45 ^g	2.29 ^d	4.73 ^{ef}
	+	10	2.05 ^g	2.16 ^{cd}	0.85 ^d	0.70 ^g	2.32 ^d	4.98 ^{def}
	+	25	2.25 ^g	2.07 ^d	0.70 ^d	0.69 ^g	2.55 ^d	5.16 ^{def}
	+	50	2.70 ^{efg}	2.32 ^{cd}	0.70 ^d	0.50 ^g	2.47 ^d	4.91 ^{ef}
	+	100	2.26 ^{fg}	1.95 ^d	0.70 ^d	0.35 ^g	2.29 ^d	3.61 ^f

^{a–g}Means with different superscripts within the same column indicate significant differences ($P < 0.01$).

mg/kg when diacetyl was used in combination with 1 AU/mL reuterin. The combination of reuterin with diacetyl allows use of both preservatives at reduced amounts that could occur naturally in the food systems. Reuterin concentrations of 4 and 2 AU/mL have been produced in situ by *Lb. reuteri* INIA P572 and INIA P579 in cheese and yogurt, respectively (Langa et al., 2013), whereas different diacetyl concentrations (between 0 and 4,500 mg/kg) have been detected in different cheese varieties and other dairy products (Zeppa et al., 2001). Therefore, those reuterin-producing strains could be used in combination with diacetyl, as a food ingredient, or with diacetyl-producing *Lactococcus lactis* strains for continuous in situ production of both compounds in dairy products over time. The exact mechanism of the synergistic activity of these 2 compounds is not clear. Reuterin antimicrobial activity is mainly attributed to its ability to react with sulfhydryl groups of amino acids, triggering an oxidative stress response (Schaefer et al., 2010; Vollenweider et al., 2010), whereas the antimicrobial activity of diacetyl might be exerted by inactivating arginine utilization through its reaction with the arginine-binding protein (Jay, 2000). The combination of the 2 antimicrobials could prevent the development of resistance.

This work highlights the synergistic effect of reuterin and diacetyl, 2 natural antimicrobial compounds produced by LAB in acidified dairy products. Addition or

in situ production of both antimicrobials by selected LAB added as adjuncts to the starter culture might be an interesting tool that could be exploited to control the growth of pathogens potentially present in dairy products.

ACKNOWLEDGMENTS

This work was supported by the projects RTA 2010-00116-00-00, AGL2010-16600, and RC2010-06925 from the Spanish Ministry of Economy and Competitiveness (Madrid, Spain).

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