Effects of gassericins A and T, bacteriocins produced by *Lactobacillus gasseri*, with glycine on custard cream preservation

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ABSTRACT

Lactobacillus gasseri LA39 and LA158 isolated from human-infant feces produce bacteriocins named gassericins A and T, respectively. Both gassericins have high heat stability (121°C, 10 min), good pH tolerance (pH 2–11), and strong bactericidal activity against many gram-positive bacteria, especially lactic acid bacteria, and thus are expected to be effective food preservatives. A microwell plate assay against 12 strains of custard cream spoilage bacteria showed that the gassericins had broader antibacterial spectra than nisin A. Although the gassericins allowed gram-negative isolates to grow, they successfully inhibited the growth of all tested bacterial strains in microwells with the addition of glycine. Glycine was bacteriostatic against many strains except lactic acid bacteria. For practical use, gassericin A was efficiently produced by cultivation in a food-grade medium improved using cheese whey, nourishing proteose peptone, and surfactant yolk lecithin. The practical preservative effect of gassericin A and glycine was verified from the viability of 4 isolated strains, *Bacillus cereus*, *Lactococcus lactis* ssp. *lactis*, *Achromobacter denitrificans*, and *Pseudomonas fluorescens*, in custard creams. Custard cream containing 123 arbitrary units of gassericin A per milliliter entirely growth-inhibited the 2 gram-positive strains. In custard cream containing an insufficient amount of gassericin A (49 arbitrary units/mL), the gram-positive strains gradually grew but were completely inhibited by the addition of 0.5% (wt/wt) glycine. The 2 gram-negative strains did not multiply even in the additive-free custard cream, probably because of the unsuitable growth environment. This is the first report showing the combined effect of bacteriocin and glycine and their application for food preservation, which may be helpful for future use in the food industry.

Key words: bacteriocin, glycine, food preservation, custard cream

INTRODUCTION

Highly nutritious foods such as custard creams easily spoil with undesirable bacterial growth. Bacterial contamination not only causes deterioration in taste and flavor but also leads to food poisoning (e.g., *Staphylococcus aureus*). To avoid this, custard cream producers have to control bacterial growth by thermal processing with food additives. However, many consumers do not want artificial additives, because these artifacts negatively affect flavor and may impair health. Therefore, producers manage the control of bacterial growth using as few additives as possible and biopreservatives such as bacteriocins that are primarily produced by lactic acid bacteria (LAB) (Cotter et al., 2005).

Bacteriocins are natural antimicrobial peptides or proteins ribosomally biosynthesized by various groups of bacteria. Many broad-spectrum and listericidal LAB bacteriocins with potential for food preservation have been studied, in which their molecular composition and structure, mode of action, and genetic determinants have been examined. However, there are comparatively few reports on their application to food preservation, and these reports are limited to major bacteriocins such as nisin (Benkerroum and Sandine, 1988; Maisnier-Patin et al., 1992; Jenson et al., 1994; Beuchat et al., 1997), enterocin AS-48 (Molinos et al., 2005; Grande et al., 2006), lactacin 3147 (Cotter et al., 2005; O'Sullivan et al., 2006), and pediocin (Rodriguez et al., 2002; Drider et al., 2006).

In our laboratory, more than 400 strains of lactobacilli isolated from human feces were screened to identify bacteriocinogenic strains. We found that *Lactobacillus gasseri* LA39 and SBT2055/LA158 produce the highly active broad-spectrum bacteriocins gassericins A (GA) and T (GT), respectively (Toba et al., 1991a; Kawai et al., 1994, 1997, 2000, 2006; Itoh et al., 1995). Gassericin A is a cyclic peptide consisting of 58 amino acid residues (5,654 Da) and is encoded on the *gaa* operon

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around the structural gene *gaaA* (Kawai et al., 1998a, b). Gassericin T is a 2-component bacteriocin comprising GatA and GatX, and of the lactacin-F family; it is considered to be the primary bacteriocin produced by *Lb. gasseri* strains (Kawai et al., 2000, 2006). Both gassericins are heat-stable (121°C, 10 min), pH-tolerant (pH 2–11), and bactericidal against several food poisoning gram-positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, and *Staph. aureus* (Itoh et al., 1995; Kawai et al., 1997), suggesting that the 2 gassericins are good candidate biopreservatives.

Bacteriocins for food preservation should be used together with other antimicrobial agents. This strategy is based on the “hurdle technology” proposed by Leistner and Gorris (1995). Using multiple preservatives sets “hurdles” that can give broader antimicrobial spectra against food-spoilage bacteria and may be useful in controlling the appearance of strains resistant to the added preservatives. There are some reports showing that the reagents destabilizing the outer membrane of the gram-negative bacteria such as EDTA (Stevens et al., 1991; Schved et al., 1994), citric acid (Bozaris and Adams, 1999; Phillips, 1999; Helander and Mattila-Sandholm, 2000), and lactoferrin (Branen and Davidson, 2004; Murdock et al., 2007) are effective antimicrobial agents in combination with LAB bacteriocins, because most LAB bacteriocins are ineffective against gram-negative bacteria. Glycine, which is a well-known food preservative that inhibits the biosynthesis of bacterial cell-wall peptidoglycan (Hammes et al., 1973), may also be a good candidate for use with LAB bacteriocin. In this study, the combined effect of bacteriocins (gassericins) and glycine was tested not only in broth media but also in custard cream for application to food preservation; the combined effect of gassericins and glycine on the control of food spoilage by gram-positive and gram-negative bacterial growth was described.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Lactobacillus gasseri LA39 [JCM11657; Japan Collection of Microorganisms (JCM), Riken, Wako, Japan], a GA producer, and *Lb. gasseri* LA158 (JCM11064), a GT producer, were isolated from the feces of a 4-month-old male infant and a 6-month-old female infant in our laboratory, respectively (Toba et al., 1991a; Takeda et al., 1997). *Lactobacillus delbrueckii* ssp. *bulgaricus* JCM1002^T, used as the indicator in the antibacterial activity assay, was purchased from JCM. The following spoilage bacterial strains found in commercial custard creams were isolated in our laboratory: *Bacillus amyloliquefaciens* AK1106, *B. cereus* AK1124, *Bacillus sub-*

tilis ssp. *subtilis* AK1107, *Staphylococcus saprophyticus* ssp. *saprophyticus* AK1121, *Staphylococcus warneri* AK1014, *Enterococcus faecalis* AK1048, *Enterococcus faecium* AK1157, *Lactobacillus paracasei* ssp. *paracasei* AK1165, *Lactococcus lactis* ssp. *lactis* AK1155, *Leuconostoc mesenteroides* ssp. *mesenteroides* AK1163, *Achromobacter denitrificans* AK1113, and *Pseudomonas fluorescens* AK1195 (Arakawa et al., 2008a). All strains were propagated 3 times in each suitable broth with a 2% (vol/vol) inoculum for 18 to 24 h. *Lactobacillus* and *Leuconostoc* strains were grown in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Detroit, MI) at 37 and 26°C, respectively. *Enterococcus* strains and *Lactococcus* strain were grown in trypticase soy broth (Difco) at 37 and 30°C, respectively. *Bacillus*, *Staphylococcus*, and gram-negative strains were grown in nutrient broth (Oxoid, Hampshire, UK) at 30, 37, and 26°C, respectively.

Agar-Well Diffusion Assay

Bacteriocin activity was determined using agar-well diffusion assay (Mayr-Harting et al., 1972). The MRS agar plates (9 cm × 4 mm, 15 mL) were overlaid with a 10-mL MRS soft-agar lawn prepared from a 1/10-diluted (250 µL) overnight culture of the indicator strain *Lb. delbrueckii* ssp. *bulgaricus* JCM1002^T. Wells 6 mm in diameter were filled with 65 µL of the samples; the overnight culture supernatants of *Lb. gasseri* LA39 (GA fraction) and LA158 (GT fraction), 1.0% (wt/vol) glycine solution (the commonly used preservative in our custard cream), 0.15% (wt/vol) sodium acetate solution (our other commonly used preservative), which were neutralized to pH 6.8 with 2 M NaOH and sterile-filtered through a 0.20-µm-pore-size membrane (Toyo Roshi Kaisha, Tokyo, Japan). Nisin A (Sigma-Aldrich, St. Louis, MO) was used as a bacteriocin control. The plates were incubated for 18 h at 37°C. A clear zone without cell growth around the well indicated the presence of a bacteriocin. To determine the bacteriocin titer, samples were 2-fold serially diluted using sterile physiological saline [0.85% NaCl (wt/vol)]. The unit of bacteriocin activity (arbitrary unit, AU) was defined as the reciprocal of the highest dilution inhibiting the growth of the indicator strain. Results given are mean values of 3 independent determinations.

Microwell Plate Assay

The antibacterial activities of the bacteriocins, glycine, and sodium acetate against the 12 strains of spoilage bacteria isolated from putrid custard cream were assayed using the microwell plate method described by Toba et al. (1991b) and Kawai et al. (1997). An appropriate culture broth (140 µL) and an antibacterial

solution (50 μ L) were added to a well of a 96-well plate, which was then inoculated with 10 μ L of a target strain suspension (10^3 cfu/mL). The overnight culture supernatants of *Lb. gasseri* LA39 and LA158, 1.0% (wt/vol) glycine solution, and 0.15% (wt/vol) sodium acetate solution were neutralized, sterile-filtered, and individually or jointly used as antibacterial substances. Nisin A was used as a bacteriocin control. Antibacterial activity was evaluated with 4 levels (A, B, C, and no effect) by visual comparison to preservative-free wells after 6, 12, 18, 24, and 48 h of incubation at the strain's optimum temperature (26, 30, or 37°C). Level A (strong and durable inhibition) means complete inhibition of the indicator's growth, which is judged from clarity of the solution in the well for 48 h. The levels B (durable inhibition) and C (weak inhibition) mean delay of the indicator's growth after the long (24 h) and short (6–18 h) incubation, respectively, compared with the preservative-free control. Results given as A, B, C, and no effect (–) are the most frequent evaluations among 5 independent determinations.

Effect of Food-Grade Surfactants on Bacteriocin Activity in Milk-Based Media

Reconstituted skim milk [RSM; 8% (wt/vol) solution of skim milk powder; Morinaga Milk Industry Co. Ltd., Tokyo, Japan], RSM containing 0.5% (wt/vol) proteose peptone (PP-RSM), reconstituted cheese whey [RCW; 8% (wt/vol) solution of demineralized cheese whey powder; Yotsuba Inc., Tokyo, Japan], and RCW containing 0.5% (wt/vol) proteose peptone (PP-RCW) were prepared as food-grade media for the cultivation of *Lb. gasseri* LA39 and LA158. Gassericin production was improved by supplementation with a food-grade surfactant aimed at the separation of hydrophobic gassericin molecules. The following food-grade surfactants including oleic acid (an essential and optimal fatty acid for lactobacilli cell growth; Nikkila et al., 1995; Partanen et al., 2001) were individually added to PP-RSM and PP-RCW: 0.1% (wt/vol) Tween 80 (TW; polyoxyethylene sorbitan monooleate; MP Biomedicals, Irvine, CA), glyceryl monooleate (GO; Taiyo Kagaku, Mie, Japan), sorbitan monooleate (SO; Riken Vitamin, Tokyo, Japan), and hen egg yolk lecithin (YL; Q. P. Corporation, Tokyo, Japan). The efficiency of supplemented surfactants was evaluated using the agar-well diffusion assay of the culture supernatant of *Lb. gasseri* LA39.

Manufacture of Custard Cream

Custard cream (basal components: powdered milk, saccharides, oils and fats, eggs, and starch) containing 0.5% (wt/wt) glycine, 10% or 25% (wt/wt) cul-

ture supernatant from *Lb. gasseri* LA39 cultivated in PP-RCW with 0.1% (wt/vol) YL (YL-PP-RCW), or 0.5% (wt/wt) glycine and 10% (wt/wt) or 25% (wt/wt) culture supernatant was prepared in units of 10 kg each. Additive-free custard cream was prepared as a control. In the custard cream containing GA, the LA39 culture supernatant was added after the neutralization to pH 6.8 with sodium carbonate powder. Each custard cream sealed in a plastic bag was pasteurized by heating at 115°C for 30 s.

Antibacterial Effects of GA and Glycine in Custard Creams

Our manufactured custard creams were individually inoculated with the selected spoilage bacterial strains at 10^3 cfu/g: *B. cereus* AK1124, *Lc. lactis* ssp. *lactis* AK1155, *A. denitrificans* AK1113, and *P. fluorescens* AK1195; they were then incubated for 30 d at 30°C. The antibacterial effect of GA and glycine were evaluated after 0, 1, 2, 5, 15, and 30 d of incubation by counting viable bacterial cells in the custard creams using nutrient agar and trypticase soy agar. Results given are mean values of 3 independent determinations.

RESULTS AND DISCUSSION

Antibacterial Activities of Bacteriocins, Glycine, and Sodium Acetate Against Spoilage Bacteria

The potential for food preservation of the 2 LAB bacteriocins, GA and GT, produced by probiotic *Lb. gasseri* LA39 and LA158 was examined using the microwell plate method. Table 1 shows the antibacterial activities of GA, GT, glycine, and sodium acetate against the 12 bacterial strains isolated from putrid custard cream. The neutralized culture supernatants of *Lb. gasseri* LA39 and LA158 cultivated in MRS broth were used as GA and GT solutions, because no other antimicrobial components except GA and GT were previously found through purification steps of the culture supernatants (Kawai et al., 1998a, 2006). In the microwell plate method, GA and GT solutions (985 and 15,750 AU/mL, respectively) had broader antibacterial spectra against the tested gram-positive isolates than nisin A solutions with equivalent activities against *Lb. delbrueckii* ssp. *bulgaricus* JCM1002^T (Table 1). From the point of view that the effects of both gassericins were displayed at neutral pH, they would be superior to nisin A working only at acidic pH. However, gassericins, as well as nisin A, were ineffective for the inhibition of the growth of the gram-negative bacteria (Table 1).

On the other hand, no antibacterial activity of 1.0% (wt/vol) glycine and 0.15% (wt/vol) sodium acetate

against the indicator *Lb. delbrueckii* ssp. *bulgaricus* JCM1002^T was observed using the agar-well diffusion assay; however, 10 strains of the spoilage bacteria were found to be sensitive to them using the microwell plate assay (Table 1). Glycine had a durable antibacterial activity against *Bacillus* spp., *Staphylococcus* spp., and the gram-negative strains, but had a comparatively weak activity against LAB strains, with no particular activity against *Lb. paracasei* ssp. *paracasei* AK1165 and *Lc. lactis* ssp. *lactis* AK1155. Sodium acetate (0.15%, wt/vol) had an antibacterial activity against *Bacillus* spp., *Staphylococcus* spp., and gram-negative strains, but showed weaker activity against gram-positive bacteria than glycine and was ineffective against LAB strains.

Furthermore, in the case where the gasserins and 1.0% (wt/vol) glycine were used in combination, the growth of all the spoilage bacterial isolates was inhibited (Table 1). Generally, LAB bacteriocins have strong activity against closely related LAB strains, but no activity against gram-negative bacteria except for some such as enterocin AS-48 (Abriouel et al., 2003), whereas glycine is generally effective against not only gram-positive species but also gram-negative species at neutral pH, but has a very weak activity against LAB that inhabit an acidic condition (Tsutsumi and Ohotaka, 1985). The results that the mixed solutions of gasserins and glycine inhibited the growth of all the tested bacterial strains as shown in Table 1 indicate their effective combination by compensating for each other's lack of active range. Here, for the first time, we showed the combined effect of LAB bacteriocins and glycine in culture media as a prototypic liquid environment. This combination demonstrated Leistner's hurdle technology to control the overall growth of many food-spoilage bacteria.

Effect of Food-Grade Surfactants on Bacteriocin Activity in Milk-Based Media

Bacteriocin compounds added to food must be of food-grade material origin, so the LAB fermentate should be produced from a natural medium such as milk, not a semisynthetic medium such as MRS broth. However, many *Lb. gasseri* strains isolated from mammalian intestinal tracts and feces do not grow well in unmodified natural milk, RSM, and RCW. Previously, we improved milk-based media using supplementation with proteose peptone (PP-RSM and PP-RCW) and succeeded in growing *Lb. gasseri* strains (Arakawa et al., 2008b). However, the GA activities in PP-RSM and PP-RCW were low—about 20% of that from MRS broth, which is often insufficient for food preservation. It was speculated that this low activity was caused not by poor GA production but by the inactivation of GA

molecules. Because GA is produced proportionally to the exponential growth of *Lb. gasseri* LA39 (Kawai et al., 1994), LA39 growth in PP-RSM and PP-RCM is not markedly different from that in MRS broth (Arakawa et al., 2008b). Furthermore, it was speculated that hydrophobic GA molecules may be inactivated by reversible hydrophobic aggregation with milk proteins and each other in defatted milk-based media; and this inactivation may not occur if surfactants are included (e.g., TW in MRS broth). Therefore, to increase activated GA molecules, *Lb. gasseri* LA39 was cultured in PP-RSM and PP-RCW supplemented with the food-grade oleic surfactants TW, GO, SO, and YL.

The GA activity in the culture supernatants from PP-RSM and PP-RCW supplemented with surfactants was determined using the agar-well diffusion assay (Figure 1). The broth media and surfactant solutions had no antibacterial activity against *Lb. delbrueckii* ssp. *bulgaricus* JCM1002^T. The GA activity of the PP-RSM culture supernatant was 21% (185 AU/mL) of the MRS activity (862 AU/mL); however, it increased to 73% (627 AU/mL) and 48% (410 AU/mL) with cultivation in PP-RSM supplemented with 0.1% (wt/vol) TW and YL, respectively. The activities of the culture supernatants from PP-RSM supplemented with 0.1% (wt/vol) GO and 0.1% (wt/vol) SO were 164 and 287 AU/mL (19 and 33% of the MRS activity), respectively. The PP-RCW culture supernatant of *Lb. gasseri* LA39 had an activity 20% (176 AU/mL) of the MRS broth culture supernatant and its activity increased to 103% (886 AU/mL) with the cultivation of *Lb. gasseri* LA39 in YL-PP-RCW. The activities of the culture supernatants from PP-RCW with 0.1% (wt/vol) TW, GO, and SO were 394, 176, and 149 AU/mL, respectively.

The results of increased GA activity suggest that the added surfactants kept GA separated and activated. Furthermore, it was inferred that the added surfactants facilitated the detachment of GA molecules from bacteriocin-producing cells to promote more activated GA molecules, because the GA activities of the culture supernatants from the presupplemented media with the surfactants before LA39 cultivation were higher than those of the culture supernatants where the surfactants were added after cultivation (data not shown). Otherwise, the surfactants may be precursors to bacteriocin synthesis. An increase in bacteriocin activity using TW was reported with nisin A (Jung et al., 1992) and pediocin AcH (Degnan et al., 1993). However, there are no reports on the increase in bacteriocin activity using other food-grade surfactants such as SO and YL. In this study, we found that adding food-grade surfactants, particularly TW and YL, to the culture broth of a bacteriocin producer increases the bacteriocin activity in the collected fraction.

Table 1. Antimicrobial activity¹ of food preservative and culture supernatants containing gassericsins A and T against custard cream spoilage bacteria, as assayed by the microwell plate method

Preservative and bacteriocin ²	Activity ³ (AU/mL)	Target strain ⁴											
		<i>Bacillus</i> sp.			<i>Staphylococcus</i> sp.		Lactic acid bacteria					Gram-negative bacteria	
		<i>BA</i>	<i>BC</i>	<i>BSS</i>	<i>SSS</i>	<i>SW</i>	<i>EFS</i>	<i>EFM</i>	<i>LPP</i>	<i>LLL</i>	<i>LMM</i>	<i>AD</i>	<i>PF</i>
1.0% Glycine	0	A	B	B	B	B	C	C	—	—	B	B	B
0.15% Sodium acetate	0	C	C	—	C	C	—	—	—	—	—	B	B
1.0% Glycine + 0.15% sodium acetate	0	A	B	B	B	B	C	C	—	—	B	B	B
LA39 MRS sup.	985	C	C	C	C	C	B	B	B	A	B	—	—
1.0% Glycine + LA39 MRS sup.	985	A	B	B	B	B	B	B	B	A	B	B	B
1.0% Glycine + 0.15% sodium acetate + LA39 MRS sup.	985	A	B	B	B	B	B	B	B	A	B	B	B
LA158 MRS sup.	15,750	C	B	C	C	C	A	A	B	A	A	—	—
1.0% Glycine + LA158 MRS sup.	31,500	A	A	A	B	B	A	A	A	A	A	C	B
1.0% Glycine + 0.15% sodium acetate + LA158 MRS sup.	31,500	A	A	A	B	B	A	A	A	A	A	B	B
LA39 MRS sup. + LA158 MRS sup.	15,750	B	B	C	C	C	B	B	B	A	B	—	—
1.0% Glycine + LA39 MRS sup. + LA158 MRS sup.	15,750	A	B	B	C	B	A	A	A	A	A	C	C
1.0% Glycine + 0.15% sodium acetate + LA39 MRS sup. + LA158 MRS sup.	15,750	A	B	B	B	B	A	A	A	A	A	B	C
0.5 µg/mL nisin A	985	B	—	B	—	—	C	C	—	A	A	—	—
2.5 µg/mL nisin A	15,750	B	C	B	C	—	C	C	C	A	A	—	—

¹A, B, and C indicate antibacterial activity: A = strong and durable growth inhibition; B = durable growth inhibition; C = weak inhibition; — = no inhibition.

²MRS = de Man, Rogosa, and Sharpe agar; sup. = culture supernatant.

³Activity (arbitrary units per mL) was measured by agar-well diffusion assay against *Lactobacillus delbrueckii* ssp. *bulgaricus* JCM1002^T.

⁴*BA* = *Bacillus amyloliquefaciens* AK1106; *BC* = *Bacillus cereus* AK1124; *BSS* = *Bacillus subtilis* ssp. *subtilis* AK1107; *SSS* = *Staphylococcus saprophyticus* ssp. *saprophyticus* AK1121; *SW* = *Staphylococcus warneri* AK1014; *EFS* = *Enterococcus faecalis* AK1048; *EFM* = *Enterococcus faecium* AK1157; *LPP* = *Lactobacillus paracasei* ssp. *paracasei* AK1165; *LLL* = *Lactococcus lactis* ssp. *lactis* AK1155; *LMM* = *Leuconostoc mesenteroides* ssp. *mesenteroides* AK1163; *AD* = *Achromobacter denitrificans* AK1113; *PF* = *Pseudomonas fluorescens* AK1195.

Figure 1. Effects of food-grade surfactants added to milk-based media for gassericin A activity in the culture supernatants of *Lactobacillus gasseri* LA39. The vertical axis shows the unit of activity (arbitrary units/mL). The milk-based media were 8% (wt/vol) reconstituted skim milk (RSM), RSM containing 0.5% (wt/vol) proteose peptone (PP-RSM), 8% (wt/vol) reconstituted cheese whey (RCW), and RCW containing 0.5% (wt/vol) proteose peptone (PP-RCW). The food-grade surfactants were Tween 80 (TW), glyceryl monooleate (GO), sorbitan monooleate (SO), and yolk lecithin (YL). MRS = de Man, Rogosa, and Sharpe agar. Each bar represents the mean of 3 data points and the error bars are standard deviations.

On the other hand, we detected no antibacterial activity of GT in PP-RSM and PP-RCW, because GT production was inhibited by milk ingredients, especially divalent metal ions (data not shown) as described by Parente and Ricciardi (1999). Therefore, the culture supernatant of *Lb. gasseri* LA158 from the milk-based media (GT solution) was not used for the following experiments to inhibit the spoilage bacteria in custard cream.

Growth Inhibition of the Spoilage Bacteria in Custard Creams Supplemented with GA and Glycine

The practical use of GA and glycine for food preservation was tested by the growth-inhibition of 4 selected strains of cream spoilage bacteria (*B. cereus* AK1124, *Lc. lactis* ssp. *lactis* AK1155, *A. denitrificans* AK1113, and *P. fluorescens* AK1195) in the manufactured custard creams containing the culture supernatant of *Lb. gasseri* LA39 from YL-PP-RCW (GA solution) or glycine or both. The inoculated *B. cereus* AK1124 was used as a representative of the 3 *Bacillus* and 2 *Staphylococcus* strains, and *Lc. lactis* ssp. *lactis* AK1155 was selected from the 5 LAB strains, which were judged from the results of the microwell plate assay (Table 1). The GA activity of the YL-PP-RCW culture supernatant added

to the custard creams was 492 AU/mL. This activity, which is about half of 886 AU/mL in the laboratory scale, might have resulted from the mass cultivation of *Lb. gasseri* LA39.

Figure 2 shows the growth curves of the 2 strains of gram-positive spoilage bacteria in the manufactured custard creams. *Bacillus cereus* AK1124 and *Lc. lactis* ssp. *lactis* AK1155 grew to 10^6 cfu/mL in additive-free custard cream at 2 and 5 d, respectively. In the custard cream with 0.5% (wt/wt) glycine alone, the growth of *B. cereus* AK1124 was markedly inhibited, but *Lc. lactis* ssp. *lactis* AK1155 grew to 10^5 cfu/mL after 15 d. In the custard cream containing 10% (wt/wt) of the YL-PP-RCW culture supernatant of *Lb. gasseri* LA39 alone (49 AU/mL of GA), the growth of *B. cereus* AK1124 and *Lc. lactis* ssp. *lactis* AK1155 was delayed; nevertheless, the numbers of cells of these strains eventually reached the same level as that of the additive-free control. The combined use of 0.5% (wt/wt) glycine and 49 AU/mL GA was effective in inhibiting the growth of *B. cereus* AK1124 and *Lc. lactis* ssp. *lactis* AK1155 over time. Supplementation with 25% (wt/wt) culture supernatant (123 AU/mL GA) was also effective against the 2 gram-positive strains without 0.5% (wt/wt) glycine. Furthermore, 123 AU/mL GA lowered the initial viable cell counts of *B. cereus*

Figure 2. Changes in cell viability of *Bacillus cereus* AK1124 (panel A) and *Lactococcus lactis* ssp. *lactis* AK1155 (panel B) in custard creams containing glycine, the culture supernatant of *Lactobacillus gasseri* LA39 [gassericin A (GA) solution], or both. *Lactobacillus gasseri* LA39 was cultured in 8% (wt/vol) reconstituted cheese whey, 0.5% (wt/vol) proteose peptone, and 0.1% (wt/vol) yolk lecithin medium (YL-PP-RCW). ♦ = preservative-free custard cream; ■ = custard cream containing 0.5% (wt/wt) glycine; ▲ = containing 49 AU(arbitrary units)/mL GA; × = containing 0.5% (wt/wt) glycine and 49 AU/mL GA; ○ = containing 123 AU/mL GA; ● = containing 0.5% (wt/wt) glycine and 123 AU/mL GA. The data show the average values of triplicate determinations and the error bars are standard deviations.

AK1124 and *Lc. lactis* ssp. *lactis* AK1155 in the custard creams (Figure 2, 0 h), indicating that this quick action of GA can reduce the supplemental quantity of slow-acting glycine. These data for the growth of the 2 gram-positive strains in the custard creams (Figure 2) correlated well with those found in the microwell plate assay (Table 1). This suggests that the growth of all gram-positive spoilage bacteria in custard cream can be completely inhibited by the mixed supplementation of GA and glycine, which is impossible by either GA or glycine alone.

The cell populations of the 2 gram-negative isolates inoculated, *A. denitrificans* AK1113 and *P. fluorescens* AK1195, gradually decreased to a level below the detection limit after 5 d of incubation in all the custard creams including the additive-free control (data not shown). The reason for this may be the low water activity of the custard creams tested. The low water activity forms osmotically stressful environments where gram-negative bacteria with a relatively low turgor are unable to grow (Gutierrez et al., 1995; Mellefont et al., 2003; Wijnker et al., 2006). The gram-negative strains used in our study were originally isolated from custard creams in commercial products where the water activity might have been increased by adding other ingredients such as water, milk, eggs, and liqueur to improve taste and flavor. However, even if gram-negative bacteria grow in custard cream, glycine will significantly control their growth as shown in the microwell plate assay (Table 1).

There are some other reports on the application of LAB bacteriocins, but they are limited only against

particular pathogens such as *L. monocytogenes* (Benkerroum and Sandine, 1988; Maisnier-Patin et al., 1992; Molinos et al., 2005; O'Sullivan et al., 2006) and *B. cereus* (Jenson et al., 1994; Beuchat et al., 1997; Grande et al., 2006). In this study, we showed that the combined use of bacteriocin and glycine thoroughly inhibits the growth of food spoilage bacteria isolated from putrid custard cream in vitro and in custard cream formulation. For the first time, we showed the potential of gassericins and thus proposed the combined effect of LAB bacteriocins and glycine for total food preservation. Such a combined effect may enable the control of the growth of most food spoilage bacteria, which is impossible by either compound, in various foods—not only fermented dairy products and meats but also non-fermented neutral foods and ready-to-eat foods. Furthermore, we succeeded in reducing the added glycine concentration by 50% and in discontinuing the addition of sodium acetate in custard cream manufacture. The preservation technique described here may contribute to the future use of bacteriocin for safe long-lasting food biopreservation.

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