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ORIGINAL ARTICLE

Comparison of bacteriocins production from *Enterococcus faecium* strains in cheese whey and optimised commercial MRS medium

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Abstract The production of bacteriocins from cheap substrates could be useful for many food industrial applications. This study aimed at determining the conditions needed for optimal production of enterocins SD1, SD2, SD3 and SD4 secreted by *Enterococcus faecium* strains SD1, SD2, SD3 and SD4, respectively. To our knowledge, this is the first use of cheese whey—a low-cost milk by-product—as a substrate for bacteriocin production by *E. faecium*; skimmed milk and MRS broths were used as reference media. This cheese manufacturing residue proved to be a promising substrate for the production of bacteriocins. However, the levels of secreted antimicrobial compounds were lower than those achieved by *E. faecium* strains in MRS broth. Bacteriocin production was affected strongly by physical and chemical factors such as growth temperature, time of incubation, pH, and the chemical composition of the culture medium. The optimal temperature and time of incubation supporting the highest bacteriocin production was determined for each strain. Different types, sources and amounts of organic nitrogen, sugar, and inorganic salts played an essential role in bacteriocin secretion. *E. faecium* strains SD1 and SD2—producing high bacteriocin levels both in cheese whey and skimmed milk—could be of great interest for potential applications in cheese-making.

Keywords Cheese whey · Bacteriocin · *Enterococcus faecium* · *Listeria monocytogenes* · Goat's milk

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Introduction

Lactic acid bacteria (LAB), besides playing important roles in food fermentation and showing a variety of positive, healthy and nutritional effects, are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides (De Vuyst and Vandamme 1994; Todorov 2009). Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (Keymanesh et al. 2009). They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanisms vary and include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis (De Vuyst and Vandamme 1994).

Bacteriocins have been detected in conjunction with *Enterococcus faecium* isolates, but these strains have been

isolated from cheese, boza (cereal based fermented beverage), chicken, nuka (Japanese rice-bran pate), dry sausages and olives (Audisto et al. 2001; Herranz et al. 2001; Javed et al. 2011; Losteinkit et al. 2001; De Vuyst et al. 2003; Moreno et al. 2003; Saavedra et al. 2003; Todorov 2010; Todorov et al. 2012). So far, very few bacteriocin-producing *E. faecium* strains have been isolated from goat's milk or goats' milk cheese (De Vuyst et al. 2003; Achemchem et al. 2005), and optimization of bacteriocin production by *E. faecium* strains isolated from goat's milk has not yet been reported.

The cost of the carbon source for growth of bacteriocin-producing microbes and bacteriocin purification methods are the factors most strongly influencing the commercial price of bacteriocins, greatly affecting their large-scale application in the food industry. Inexpensive and abundant starchy and lignocellulosic residues have been investigated as promising renewable biomass for conversion into fuels, polymers, enzymes and bulk chemicals (Scott et al. 2007; Favaro et al. 2012a, b, 2013; Rødsrud et al. 2012). The use of cheap carbon sources, especially those generated from industrial or agricultural processes, can therefore provide a way to reduce bacteriocin production costs. Cheese whey—a by-product of the dairy industry—is available in large amounts as co-product stream of cheese manufacturing and is rich in lactose, lipids and soluble proteins. Whey is not only a cheap raw material but, in the European Union (EU), also represents a surplus product causing expensive disposal problems. Cheese whey is a promising feedstock for the biotechnological production of polyhydroxyalcanoates (PHAs), bioethanol and other added-value compounds (Povolo et al. 2010; Cota-Navarro et al. 2011; Dragone et al. 2011; Prazeres et al. 2012). However, only a few studies describing the use of this industrial by-product as a substrate for bacteriocin production have been reported (Cladera-Olivera et al. 2004; Leães et al. 2011) and, to the best of our knowledge, no *Enterococcus* spp. strains with the ability to both grow and produce bacteriocins on cheese whey without nutrient supplementation have been reported.

Recent studies (Leroy and De Vuyst 2003; Motta and Brandelli 2003) have examined specific environmental conditions, including those found in food products, to determine their effects on the production of bacteriocins, as optimum production may require a specific combination of environmental parameters. Little is known about the interactions these factors have on the production of bacteriocins, especially in a complex food environment. For example, pH and growth temperature have been shown to affect the amount of pediocin AcH (Biswas et al. 1991) and pediocin PD-1 (Nel et al. 2001) secreted by *Pediococcus* spp., as well as the amounts of a bacteriocin produced by *E. faecium* RZS C5 (Leroy et al. 2003) and enterocin P from an *Enterococcus* spp. (Herranz et al. 2001). However, despite studies on the effects of nitrogen and carbon sources on production of enterocin P by *Enterococcus* spp. (Herranz et al. 2001) and production of a bacteriocin by *E. faecium* CRL 1385 (Audisto et al. 2001), there is much room for investigation into the growth conditions necessary for optimal bacteriocin production by *E. faecium*. In previous research (Todorov and Dicks 2005a), the effect of MRS medium components on bacteriocin production by *E. faecium* ST311LD was determined. In some specific cases, higher bacteriocin production levels have been recorded under sub-optimal growth conditions (Aasen et al. 2000; Todorov et al. 2000; Audisto et al. 2001).

Four *E. faecium* SD1, SD2, SD3 and SD4 strains have been characterized previously for the production of bacteriocins and aspects of bacteriocin mode of action. Bacteriocins SD1, SD2, SD3 and SD4 showed a strong and wide spectrum of antimicrobial activity, inhibiting the growth of many food spoilage bacteria and food-borne pathogens, such as 21 *Listeria monocytogenes* and, surprisingly, 6 *Salmonella* spp. strains (Schirru et al. 2012). Activity against Gram-negative bacteria should be considered unusual since it has been reported for only a few bacteriocins, e.g. enterocins produced by *E. faecalis* strains (Sparo et al. 2006), enterocin 012 of *E. gallinarum* (Jennes et al. 2000), and bacteriocins secreted by *E. mundtii* (Knoetze et al. 2008; Todorov et al. 2005). For this reason, *E. faecium* SD1, SD2, SD3 and SD4 strains could be considered as potential bio-preservative cultures for fermented food production, or their purified bacteriocins could ultimately be efficiently used to improve foodstuff hygiene and safety. This study is also the first account describing the conditions needed for optimal production of bacteriocins by *E. faecium* strains isolated from goat's milk. Both the production of bacteriocins from cheese whey and the optimisation of medium components are crucial to the cost-effective production of high bacteriocins levels for use in the food processing industry.

Materials and methods

Bacterial strains and growth conditions

Enterococcus faecium strains SD1, SD2, SD3 and SD4, previously isolated from goat's milk produced in Sardinia, Italy, were kindly provided by the STAA (Scienze e Tecnologie Ambientali ed Alimentari) laboratory of the Dipartimento di Agraria, Università degli Studi di Sassari, Italy.

The four bacteriocin-producing strains were cultured in MRS medium (De Man et al. 1960), at 30 °C, and stored at −80 °C in MRS broth supplemented with 15 % (v/v) glycerol. *Listeria monocytogenes* 211 (collection of Department of food Science and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil), grown overnight at 37 °C, in brain-heart infusion (BHI) medium, was used as indicator strain.

Bacteriocin bioassay

Bacteriocin screening was performed using the agar-spot-test method described by Todorov and Dicks (2005a). Antimicrobial activity was expressed as arbitrary units (AU/mL), calculated as $a^b \times 100$, where "a" represents the dilution factor and "b" the dilution that produces an inhibition zone of at least 2 mm in diameter. Activity was expressed per millilitre by multiplying by the appropriate conversion factor. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of *L. monocytogenes* 211. All experiments were done in triplicate.

Bacteriocin production in different growth media, at different temperatures and initial growth pH

Bacteriocin production was monitored on different media: MRS broth (Oxoid, Milan, Italy), BHI broth with pH adjusted to 6.5 (Oxoid), skimmed milk from Oxoid (5 % or 10 %, w/v), cheese whey from cow (5 or 10 %, w/v). Dry cheese whey powder contained approximately 69.5 % lactose, 12.8 % soluble protein, 1.5 % fat, 2.1 % total nitrogen and 0.9 % total phosphorous, on a dry weight basis.

An 18-h-old culture of *E. faecium* strains SD1, SD2, SD3 and SD4 was inoculated (2 %, v/v) separately into four flasks containing 300 mL MRS medium. Incubation, where not otherwise stated, was carried out at 21 °C, 30 °C and 37 °C, respectively, without agitation, for 36 h. Samples were taken every hour and examined for bacterial growth (OD 600 nm), while bacteriocins activity (AU/mL) was determined after 12, 24 and 36 h. All experiments were done in triplicate.

In a separate experiment, the effect of initial medium pH on the production of bacteriocins SD1, SD2, SD3 and SD4 was studied. Volumes of 300 mL MRS broth were adjusted to pH 3, 4, 5, 6 and 7, respectively, with 6 M HCl or 6 M NaOH and then autoclaved. After autoclaving, the pH was corrected, if needed, by sterile 1 M HCl or 1 M NaOH. Each flask was inoculated with 2 % (v/v) of an 18-h-old culture of *E. faecium* strains SD1, SD2, SD3 and SD4, respectively, and incubated at 30 °C for 24 h without agitation. Changes in culture pH and AU/mL of bacteriocins SD1, SD2, SD3 and SD4 were determined every hour as described elsewhere against *L. monocytogenes* 211. All experiments were done in triplicate.

Effect of medium composition on bacteriocin production

Enterococcus faecium strains SD1, SD2, SD3 and SD4 were grown separately in 100 mL MRS broth (Oxoid) for 18 h at 30 °C, the cells harvested by centrifugation (8,000 *g*, 10 min, 4 °C), and the pellet re-suspended in 100 mL sterile peptone water. In order to determine the effect of the different medium components on bacteriocin production by *E. faecium* SD1, SD2, SD3 and SD4 strains, 4 mL of the cell suspension were used to inoculate 200 mL of the following media: (1) MRS broth as described by de Man et al. (1960); (2) several MRS broths prepared without organic nutrients and supplemented with only one of the following protein sources: tryptone (20 g/L), meat extract (20 g/L), yeast extract (20 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10 g/L) plus yeast extract (10 g/L), or a combination of tryptone (10 g/L), meat extract (5 g/L) and yeast extract (5 g/L), respectively; (3) several MRS broths in which D-glucose was replaced with 20 g/l of only one of the following sugar sources: D-mannitol, mannose, lactose and rhamnose; (4) MRS supplemented with 5 to 50 g/L glucose or lactose as sole carbon source; (5) MRS with K_2HPO_4 from 2 to 10 g/L, or KH_2PO_4 from 2 to 10 g/L, or combination of 2 g/L K_2HPO_4 and 2 g/L KH_2PO_4; (6) MRS supplemented with 2 to 20 g/L glycerol; (7) MRS without $MgSO_4$; (8) MRS without $MnSO_4$; (9) MRS without or supplemented with 10 g/L sodium acetate; (10) MRS without or supplemented with 4 g/L tri-ammonium citrate, and (11) MRS supplemented with 2 or 5 g/L Tween 80.

All cultures were incubated at 30 °C for 24 h. Activity levels of bacteriocins SD1, SD2, SD3 and SD4 were determined as described elsewhere against *L. monocytogenes* 211. All experiments were done in triplicate.

Results and discussion

All data represent the average of three repeats. The values recorded in each experiment did not vary by more than 5 % and standard deviation values are not presented. Identical levels of bacteriocin production (AU/mL) were detected for all three repeats.

Strong antimicrobial action of bacteriocins SD1, SD2, SD3 and SD4 against *L. monocytogenes* and *Salmonella* spp. has been reported previously (Schirru et al. 2012). This activity may have a positive effect on food safety if these bacteriocin-producing strains are applied as starter cultures in cheese production. Furthermore, it is important to underline that all four bacteriocins exhibit no significant inhibitory effect against other LAB; this is a crucial point in order to not inhibit the growth of the starter cultures used in cheese-making.

Microbial bacteriocin production and growth in cheese whey

To investigate cheap and suitable substrates for SD1, SD2, SD3 and SD4 bacteriocin production, cheese whey was

evaluated as a medium for growth of *E. faecium* strains. Interestingly, this cheese making by-product was able to support bacterial growth (data not shown) and antimicrobial production by all the tested strains at 30 °C (Fig. 1). However, the microbes secreted different amounts of bacteriocins, with *E. faecium* SD1 and SD2 capable of producing the highest level (25,600 AU/mL) after 24 and 36 h incubation in cheese whey 10 %, respectively. As reported in Fig. 1, cheese whey concentration influenced bacteriocin production. Once grown in cheese whey 5 %, the *E. faecium* strains, with the exception of *E. faecium* SD4, produced lower levels of antimicrobial activity against *L. monocytogenes* 211. Moreover, after 12 h incubation, only strains SD1 and SD2 exhibited detectable bacteriocin activity, while the other two *E. faecium* isolates secreted antimicrobial peptides only after 24 h.

Although cheese whey has been explored previously as an alternative substrate for bacteriocin production (Mauriello et al. 1999; Cladera-Olivera et al. 2004; Leães et al. 2011), to the best of our knowledge this is the first account reporting efficient bacteriocin production from whey by *E. faecium* strains. Mirhosseini and Emtiazi (2011) detected enterocin A secretion in cheese whey only after supplementing it with 10 g/L yeast extract. Considering also that the *E. faecium* strains tested by Mirhosseini and Emtiazi (2011) were isolated from dairy products (cow cheese, milk and yogurt), the promising ability of the four *E. faecium* SD1, SD2, SD3 and SD4 strains, isolated from goat's milk, is interesting, and probably linked to their efficient ability to use cheese whey both as carbon and nitrogen source.

Effect of temperature and pH on bacteriocin production

Bacteriocin production by the tested *E. faecium* strains was influenced greatly by growth temperature (Fig. 2). For *E. faecium* SD1, optimal production was recorded at 37 °C after 24 or 36 h of incubation in MRS broth (51,200 AU/mL). However, although the strain was able to grow well at 21 °C (data not shown), bacteriocin activity after 24 or 36 h was only 6,400 AU/mL. The effect of temperature was even stronger for *E. faecium* SD2: after 36 h of growth in MRS at 21, 30 and 37 °C, bacteriocin SD2 activity was 12,800, 51,200, and 102,400 AU/mL, respectively. No bacteriocin production was recorded for *E. faecium* SD3 when cultured in MRS at 21 °C. However, at 30 °C in MRS broth the strain produced lower bacteriocins levels after 36 h incubation. A similar finding was observed for *E. faecium* SD4 when cultured in MRS at 21 °C. No significant differences in activity levels for bacteriocin SD4 were recorded when *E. faecium* SD4 was grown at 30 or 37 °C after 24 or 36 h. Overall, the results obtained in this study clearly indicate that growth temperature and bacteriocin production are frequently correlated, as has been observed previously for lactocin S (Mørtvedt-Abildgaard et al. 1995), amylovorin 1471 (De Vuyst et al. 1996), nisin Z (Matsusaki et al. 1996), enterocin MC13 (Kanmani et al. 2011).

We next evaluated the effect of pH on bacteriocin production. Optimal bacteriocin SD1 production was recorded in MRS at a pH adjusted to 6 or 7 (25,600 AU/mL, Table 1). For bacteriocin SD2, an activity level of 25,600 AU/mL was achieved in MRS broth adjusted to pH 6. When the MRS pH

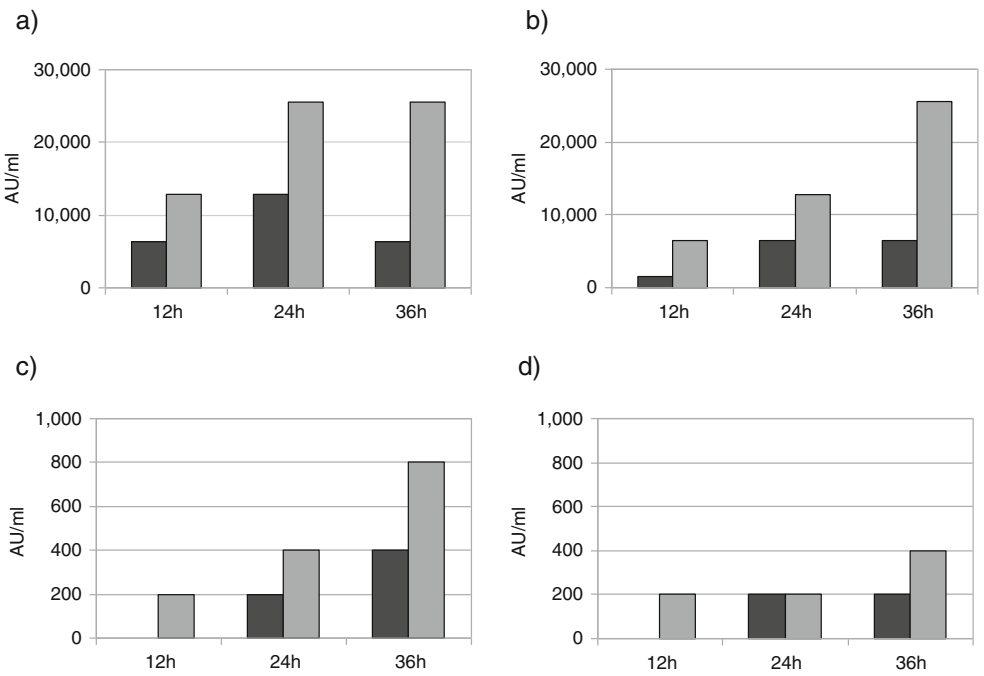

Fig. 1 Levels of bacteriocins SD1 (**a**), SD2 (**b**), SD3 (**c**) and SD4 (**d**) produced against *Listeria monocytogenes* 211 after 12, 24 and 36 h of incubation at 30 °C in cheese whey at 5 % w/v (*black histograms*) and 10 % w/v (*grey histograms*)

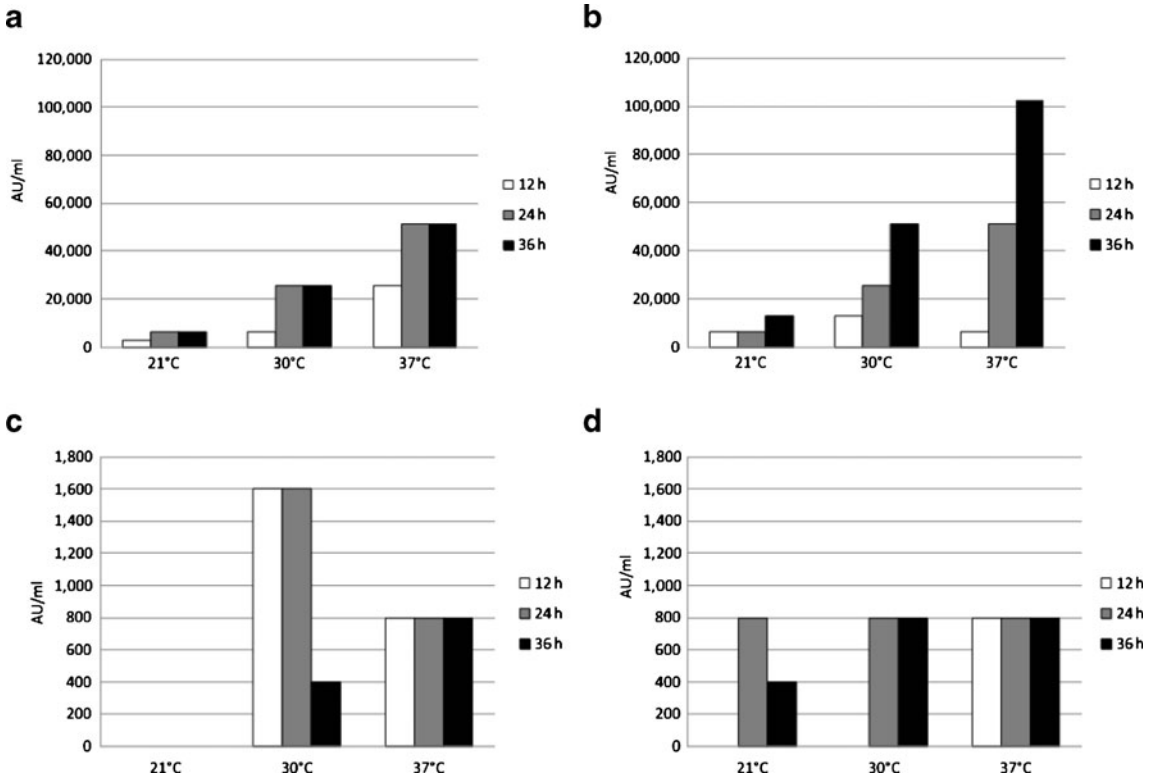

Fig. 2 Levels of bacteriocins SD1 (**a**), SD2 (**b**), SD3 (**c**) and SD4 (**d**) produced against *Listeria monocytogenes* 211 in MRS broth (Oxoid) after 12, 24 and 36 h of incubation at 21, 30, and 37 °C

was adjusted to 7, the activity level of bacteriocin SD2 decreased to 12,800 AU/mL. In the case of bacteriocin SD3, optimal production (1,600 AU/mL) was obtained by growing the strain at pH 6 and 7. *E. faecium* SD4 produced the highest bacteriocin levels (800 AU/mL) at pH values close to 6.

For bacteriocin produced by *E. faecium* ST311LD (Todorov and Dicks 2005a), MRS adjusted to pH values of 5, 5.5 and 6 yielded bacteriocin levels of 6,400 AU/mL. However, if grown at pH 6.5 and 4.5, *E. faecium* ST311LD produced lower antimicrobial activity. Similar findings have been reported for other bacteriocins (Daeschel et al. 1990; Jimenez-Diaz et al. 1993; Todorov et al. 2000). The optimal pH for enterocin P production ranged between pH 5.7 and pH 6, while maximal growth occurred at pH 6.2–7 (Herranz et al. 2001). Optimal levels of bacteriocin production by *E. faecium* RS C5 were obtained at pH 6.5 with temperatures values ranging from 20 to 35 °C. At 35 °C, enterocin RS C5 activity was detected only between pH 5.5 and pH 8 (Leroy et al. 2003). According to the results obtained in this study, and those reported in the literature (Daeschel et al. 1990; Jimenez-Diaz et al. 1993; Todorov et al. 2000, 2012; Todorov and Dicks 2005a), the optimal production of *E. faecium* bacteriocins occurs during the early logarithmic growth phase, usually at a pH value above 4.5.

Table 1 Influence of the pH of MRS broth on production of bacteriocins SD1, SD2, SD3 and SD4 after 24 h incubation at 30 °C. *AU* Arbitrary units

MRS medium pH	Bacteriocin activity (AU/mL)			
	SD1	SD2	SD3	SD4
pH 6.2 (control)	25,600	25,600	1,600	800
pH 3.0	NG[a]	NG	NG	NG
pH 4.0	NG	NG	NG	NG
pH 5.0	800	800	0	0
pH 6.0	25,600	25,600	1,600	800
pH 7.0	25,600	12,800	1,600	400

[a] No growth

Growth of *E. faecium* SD1, SD2 in BHI broth at pH 6.5 produced, after 24 h, bacteriocin levels similar to those detected in MRS broth (Table 2); in contrast, *E. faecium* SD3 and SD4 grown in BHI instead of MRS showed reduced bacteriocin activity. Lower levels of bacteriocins were also achieved in 50 g/L skimmed milk for strains SD1 and SD2, while no bacteriocins were detected for *E. faecium* SD3 and SD4. When 100 g/L skimmed milk were used, 12,800 AU/mL was recorded for bacteriocin SD1 and SD2, whereas strains SD3 and SD4 did not produce any antimicrobial activity.

Moreover, as reported in Table 2, cheese whey was able to support higher bacteriocin production than skimmed

Table 2 Influence of growth medium on production of bacteriocins SD1, SD2, SD3 and SD4 after 24 h incubation at 30 °C. *BHI* Brain-heart infusion medium (reference medium)

Medium	Bacteriocin activity (AU/mL)			
	SD1	SD2	SD3	SD4
BHI (pH 6.5)	25,600	12,800	400	400
Skimmed milk (5 % w/v)	6,400	3,200	0	0
Skimmed milk (10 % w/v)	12,800	12,800	0	0
Cheese whey (5 % w/v)	12,800	6,400	200	200
Cheese whey (10 % w/v)	25,600	12,800	400	200

milk. Compared with BHI, the *E. faecium* strains exhibited similar bacteriocin activity.

Low levels of bacteriocin activity (800 AU/mL) were achieved by *E. faecium* ST311LD when grown in BHI and M17 broths, despite the large cell numbers recorded (Todorov and Dicks 2005a). On the contrary, MRS medium sustained a much higher bacteriocin level production (6,400 AU/mL), while relatively good production of bacteriocin activity (3,200 AU/mL) was detected in the presence of 10 % (w/v) soy milk (Todorov and Dicks 2005a). These results suggest that specific nutrients are required for the production of the bacteriocins SD1, SD2, SD3 and SD4, and other bacteriocins, according to the different *E. faecium* bacteriocinogenic strain used.

Effect of MRS broth components on bacteriocin production

As reported in Table 3, the addition in MRS broth of a combination of tryptone (or peptone, as in the basal MRS medium), meat and yeast extract yielded the highest bacteriocin level (25,600 AU/mL for SD1 and SD2; 1,600 and 800 AU/mL for SD3 and SD4). Even tryptone alone (20 g/L) allowed the highest bacteriocin production to be achieved, except in the case of *E. faecium* SD2. Growing the *E. faecium* strains in the presence of a combination of two nitrogen sources (tryptone and meat extract 1:0.6; tryptone and yeast extract 1:0.6; meat and yeast extracts 1:1) strongly reduced their antimicrobial activity, apart from strain SD1, which was capable of producing 25,600 AU/mL once grown in MRS supplemented with meat and yeast extracts 1:1. Finally, the addition of yeast or meat extract (20 g/L) resulted in sharply reduced bacteriocin production for all strains tested.

The results showed that, at least for the strains SD2, SD3 and SD4, production of bacteriocin was stimulated by using tryptone, but not yeast or meat extract, as nitrogen source. This finding is consistent with that of Todorov and Dicks (2005a), who reported that growth of *E. faecium* ST311LD in the presence of tryptone as a sole nitrogen source resulted in bacteriocin activity of 12,800 AU/mL, which was 2-fold that recorded in the MRS broth supplemented with meat extract, yeast extract and tryptone. Using yeast extract or yeast plus meat extracts, *E. faecium* ST311LD produced lower bacteriocin levels (3,200 AU/mL). However, supplementing MRS with meat extract (20 g/L), or a combination of tryptone and meat extract (1:0.6), the bacteriocin activity was 6,400 AU/mL. Growth in MRS broth supplemented with a combination of tryptone and yeast extract (1:0.6) resulted in an activity level of 12,800 AU/mL.

Similar findings were described previously for plantaricin 423 (Verellen et al. 1998), ST151BR and ST112BR (Todorov and Dicks 2004), bacteriocins ST712BZ (Todorov and Dicks 2007), and AMA-K (Todorov 2008). In the case of plantaricin 423, optimal bacteriocin production was recorded in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract. Stimulation of bacteriocin production by yeast and meat extract has been described also for helveticin J (Joerger and Klaenhammer 1986) and for *E. faecium* MC13 (Kanmani et al. 2011).

In the presence of glucose, all the *E. faecium* strains showed the highest bacteriocin production when grown with 20 g/L sugar in the basal MRS medium (25,600 AU/mL for SD1 and SD2; 1,600 and 800 AU/mL for SD3 and SD4). *E. faecium* SD1 reached the same bacteriocin levels even at 30 g/L (Table 3). Decreased bacteriocin production was recorded in the presence of 5 and 10 g/L glucose for all the tested strains, while the lowest production was observed at 50 g/L glucose (1,600, 800, 200 and 0 AU/mL for SD1, SD2, SD3 and SD4, respectively). When glucose was replaced with 10 g/L lactose, all the strains confirmed their ability to use this sugar for growth as described also in cheese whey (Fig. 1), resulting in optimal bacteriocin production (25,600, 51,200, 3,200, and 400 AU/mL for SD1, SD2, SD3, and SD4, respectively). However, bacteriocin levels were different from those achieved in milk by-products (at 30 °C for 24 h), suggesting that each strain may exploit MRS medium components differently. As a result, *E. faecium* SD2 was able to produce 51,200 AU/mL even on 5, 10, 20, and 30 g/L lactose. Supplementing MRS with 20 g/L lactose reduced bacteriocin SD1, SD3 and SD4 production to 50 % while lower activities were recorded at higher (30 and 50 g/L) and at the lowest (5 g/L) lactose concentrations for all the strains. Growth in the presence of mannose (20 g/L) yielded the same activity as that detected using 20 g/L glucose for SD1, SD3 and SD4 strains, or 20 g/L lactose for strain SD2. When rhamnose was added as a carbon source, only *E. faecium* SD2 was able to produce bacteriocins (6,400 AU/mL), while strains SD1 and SD3 did not even grow.

Based on the results reported in Table 3, the production of all these bacteriocins depends strongly on the type of carbohydrate added to the MRS medium. Similar findings were described by Todorov and Dicks (2005a) for strain *E. faecium* ST311LD, where optimal bacteriocin levels (6,400 AU/mL)

Table 3 Influence of each component on the production of bacteriocins SD1, SD2, SD3 and SD4 in modified MRS broths incubated at 30 °C for 24 h

Component[a]	Concentration (g/L)	Bacteriocin activity (AU/mL)			
		SD1	SD2	SD3	SD4
Nitrogen sources					
Control (peptone + meat extract + yeast extract)	10+8+4	25,600	25,600	1,600	800
Tryptone	20	25,600	6,400	1,600	800
Meat extract	20	6,400	6,400	400	400
Yeast extract	20	6,400	1,600	400	400
Tryptone + meat extract	12.5+7.5	12,800	3,200	200	200
Tryptone + yeast extract	12.5+7.5	6,400	6,400	200	200
Meat extract + yeast extract	10+10	25,600	3,200	400	400
Tryptone + meat extract + yeast extract	10+5+5	25,600	25,600	1,600	800
Sugar sources					
Control (glucose)	20	25,600	25,600	1,600	800
Glucose	5	12,800	3,200	200	200
Glucose	10	12,800	6,400	200	200
Glucose	30	25,600	6,400	400	200
Glucose	50	1,600	800	200	0
Lactose	5	25,600	51,200	800	400
Lactose	10	51,200	51,200	3,200	400
Lactose	20	25,600	51,200	1,600	200
Lactose	30	12,800	51,200	400	200
Lactose	50	6,400	25,600	200	200
D-Mannitol	20	NG[b]	NG	NG	NG
Mannose	20	25,600	51,200	1,600	800
Rhamnose	20	NG	6,400	NG	0
Inorganic salts					
Control (di-potassium phosphate + magnesium sulphate + manganese sulphate + tri-ammonium citrate + sodium acetate)	2+0.1+0.05+2+5	25,600	25,600	1,600	800
Di-potassium phosphate + mono-potassium phosphate	2+2	3,200	3,200	0	0
Di-potassium hydrogen phosphate	0	NG	NG	200	200
Di-potassium hydrogen phosphate	5	12,800	1,600	400	200
Di-potassium hydrogen phosphate	10	25,600	800	800	200
Mono-potassium phosphate	0	NG	NG	200	200
Mono-potassium phosphate	2	NG	NG	NG	NG
Mono-potassium phosphate	5	NG	NG	200	NG
Mono-potassium phosphate	10	NG	NG	0	0
Magnesium sulphate	0	6,400	400	200	200
Manganese sulphate	0	3,200	6,400	200	400
Tri-ammonium citrate	0	12,800	6,400	400	400
Tri-ammonium citrate	4	1,600	12,800	0	0
Sodium acetate	0	3,200	1,600	400	400
Sodium acetate	10	1,600	1,600	200	200
Other components					
Control (Tween 80)	1	25,600	25,600	1,600	800
Tween 80	2	3,200	6,400	200	200
Tween 80	5	3,200	6,400	200	200
Glycerol	0	25,600	25,600	1,600	800
Glycerol	2	6,400	6,400	400	400
Glycerol	5	3,200	6,400	400	400

Table 3 (continued)

Component[a]	Concentration (g/L)	Bacteriocin activity (AU/mL)			
		SD1	SD2	SD3	SD4
Glycerol	8	3,200	12,800	400	400
Glycerol	10	6,400	12,800	400	200
Glycerol	20	6,400	6,400	200	200

[a] Control means normal components of MRS broth

[b] No growth

were obtained in the presence of 20 g/L glucose or maltose, while in the presence of 20 g/L lactose, mannose, or fructose, much lower quantities of bacteriocin ST311LD were produced. The effects of glucose on bacteriocin production have also been reported in conjunction with sakacin P (Aasen et al. 2000) and plantaricin ST31 (Todorov et al. 2000). Maximal bacteriocin activity levels were recorded for *E. faecium* RZS C5 when it was cultured in MRS broth supplemented with lactose 50 g/L (Moreno et al. 2003). Growth of *Lactobacillus plantarum* AMA-K in the presence of glucose (20 and 50 g/L) yielded 12,800 AU/mL bacteriocin AMA-K (Todorov 2008). Increased bacteriocin production (25,600 AU/mL) was detected in the presence of 30 g/L glucose, while lower concentrations of glucose, at 5 g/L and 10 g/L, yielded 3,200 AU/mL and 6,400 AU/mL, respectively. *L. plantarum* AMA-K grown in the presence of maltose (20 g/L) and sucrose (20 g/L) gave the same antimicrobial activity detected with 20 g/L glucose, while mannose (20 g/L) and fructose (20 g/L) reduced bacteriocin production by 75 % (Todorov 2008).

Since little is known regarding the influence of potassium ions on the production of bacteriocins, it seemed interesting to investigate the effect of different K_2HPO_4 or KH_2PO_4 concentrations on bacteriocin production by our *E. faecium* strains (Table 3).

KH_2PO_4 strongly affected the ability of the four *E. faecium* strains to grow and produce bacteriocinogenic activity, while K_2HPO_4 was necessary for growth and bacteriocin production by all strains tested. Moreover, increasing concentrations of K_2HPO_4 positively influenced bacteriocin levels. Optimal activity was recorded in the presence of 2 g/L, and for SD1 strain also at 10 g/L K_2HPO_4. Decreased bacteriocins production was recorded in the presence of K_2HPO_4 concentrations higher than 2 g/L. Supplementing MRS broth with K_2HPO_4 and KH_2PO_4 (2 g/L and 2 g/L) resulted in a strong decrease of bacteriocin production in the case of *E. faecium* SD1 and SD2 whereas strains SD3 and SD4 did not produce antimicrobial activity.

According to these results, the influence of potassium ions on the production of bacteriocins should be considered a strain-related trait. The literature reports several conflicting responses to K_2HPO_4 and KH_2PO_4: no differences in antibacterial activity were recorded when strain *E. faecium* ST311LD was grown in the presence of 2 g/L K_2HPO_4 or 2 g/L KH_2PO_4 (Todorov and Dicks 2005a). The same work reported that K_2HPO_4 concentrations between 0.2 g/L and 10 g/L reduced bacteriocin activity from 6,400 AU/mL at 0.2 g/L to 3,200 AU/mL at 0.5 g/L to 5 g/L, and to 800 AU/mL at 10 g/L. Different concentrations of K_2HPO_4 and KH_2PO_4 did not significantly affect the production of bacteriocin ST712BZ secreted by *Lactobacillus pentosus* ST712BZ (Todorov and Dicks 2007). In the case of bacteriocin ST112BR, higher levels of activity were recorded in medium containing up to 20 g/L KH_2PO_4 (Todorov and Dicks 2004) while the optimal concentration of K_2HPO_4 required for plantaricin ST31 production was between 2 and 5 g/L (Todorov et al. 2000).

Glycerol seemed to negatively affect the bactericidal activity of the *E. faecium* strains since production of bacteriocins SD1, SD2, SD3 and SD4 was the highest (25,600 AU/mL, 25,600 AU/mL, 1,600 AU/mL and 800 AU/mL, respectively) in the absence of glycerol, while concentrations of 5 g/L and higher (up to 20 g/L) led to progressively decreased levels of bacteriocin production (Table 3). Similar results were reported for *E. faecium* ST311LD, in which bacteriocin production was inhibited when glycerol levels of 1 g/L and higher were added to the medium (Todorov and Dicks 2005a). *L. plantarum* ST31 produced limited plantaricin levels when grown in the presence of glycerol concentrations higher than 2 g/L (Todorov et al. 2000), while up to 5.0 g/L glycerol did not affect bacteriocin secretion by *Lactobacillus sakei* strains (Todorov et al. 2013). The fact that glycerol seems to inhibit bacteriocin production by several LAB strains may be ascribed to the lowering of water activity due to the increase of glycerol concentration in the medium (Todorov and Dicks 2007). Accordingly, the decrease in production of bacteriocins SD1, SD2, SD3 and SD4 may be due to changes in osmotic stress or to the binding of bacteriocins with cell membranes or other molecules, initiated by the presence of glycerol.

Optimal production of bacteriocins SD1, SD2, SD3 and SD4 was recorded in the presence of 1 g/L Tween 80 while higher concentrations had a negative effect on bacteriocin

production (Table 3). In contrast with the results of this study, the addition of Tween 80 to the growth medium increased production of bacteriocin ST194BZ by over 50 % (Todorov and Dicks 2005b). This is entirely consistent with results obtained for pediocin AcH (Biswas et al. 1991), lactocin 705 (Vignolo et al. 1995) and plantaricin 423 (Verellen et al. 1998).

Optimal production of bacteriocins SD1, SD2, SD3 and SD4 was recorded in the presence of 5 g/L sodium acetate while the other concentrations tested resulted in lower levels of bacteriocins. Production of bacteriocins SD1, SD2, SD3 and SD4 was reduced when growing the strains without magnesium or manganese sulphate (Table 3). This finding agrees with that of Todorov (2008), who reported that the production of bacteriocin AMA-K requires the presence of magnesium sulphate and manganese sulphate as medium components. Similar data were obtained for the effect of tri-ammonium citrate. Normally, this component is present in MRS medium at a level of 2 g/L. At this concentration, optimal production of bacteriocins SD1, SD2, SD3 and SD4 was recorded. Exclusion of tri-ammonium citrate from the medium formulation resulted in reduced bacteriocin activity (Table 3). However, increasing the concentration to 4 g/L had similar effects on production of bacteriocins SD1, SD2, SD3 and SD4. Similar results were described for production of bacteriocin AMA-K (Todorov 2008).

Conclusions

Optimal parameters for the production of enterocins SD1, SD2, SD3, and SD4 by *E. faecium* strains isolated from Sardinian goat's milk were investigated. This study confirmed that bacteriocinogenic capacity should be considered a strain-related trait. Each of the four strains and their related bacteriocins were affected strongly by physical and chemical factors such as growth medium, pH, temperature and time of incubation. Moreover, the effect of MRS medium components and glycerol on growth and bacteriocin production by *E. faecium* SD1, *E. faecium* SD2, *E. faecium* SD3 and *E. faecium* SD4 was examined. This study advances our knowledge towards achieving cost-effective production of high bacteriocins levels for use in the food processing industry. To our knowledge, this study is the first report indicating that cheese whey—a major world-wide disposal and pollution problem for the dairy industry—could be an interesting low-cost substrate for the production of bacteriocins by *E. faecium* strains. However, optimisation of MRS components seems to be crucial to the cost-effective production of high bacteriocins levels. As an alternative, supplementing cheese whey with some components of MRS will be an interesting strategy to be investigated for the optimisation of bacteriocin production from *E. faecium* strains. Techno-economical evaluations will be useful to determine the actual feasibility of both perspectives in order to develop a cheap and efficient process for the large-scale production of bacteriocins for use in the food industry.

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