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1 **Over 2000-fold increased production of the leaderless bacteriocin garvicin KS by genetic**
2 **engineering and optimization of culture conditions**

3

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10 **Running Head: Optimization of bacteriocin production**

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20 **Abstract**

21 The leaderless bacteriocin Garvicin KS (GarKS) is a potent antimicrobial, being active against a
22 wide range of important pathogens. GarKS production by the native producer *Lactococcus*
23 *garvieae* KS1546 was however relatively low (80 BU/ml) under standard laboratory growth
24 conditions (batch culture in GM17 at 30°C). To improve the production of GarKS, we
25 systematically evaluated the impact of different media and media components on bacteriocin
26 production. Based on the outcomes a new medium formulation was made to greatly improve
27 bacteriocin production. The new medium composed of pasteurized milk and tryptone (PM-T),
28 increased GarKS production about 60-fold compared to that achieved in GM17. GarKS production
29 was increased further 4-fold (i.e., to 20,000 BU/ml) by increasing gene dose of the bacteriocin
30 gene cluster (*gak*) in the native producer. Finally, a combination of the newly composed medium
31 (PM-T), an increased gene dose and a cultivation at a constant pH 6 and a 50-60% dissolved
32 oxygen level in growth medium, gave rise to a GarKS production of 164,000 BU/ml. This high
33 production, which is about 2000-fold higher compared to that initially achieved in GM17,
34 corresponds to a GarKS production of 1.2 g/L. To our knowledge, this is one of the highest
35 bacteriocin production reported hitherto.

36 **Importance**: Low bacteriocin production is a well-known bottle-neck in developing bacteriocins
37 into large-scaled and useful applications. The present study shows different approaches that
38 significantly improve bacteriocin production. This is an important research field to better exploit
39 the antimicrobial potential of bacteriocins, especially with regard to the decreasing effect of
40 antibiotics in infection treatments due to the global emergence of antibiotic resistance.

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42 **Introduction**

43 The decreasing effectiveness of antibiotics has become a serious worldwide problem due to the
44 emergence of multidrug-resistant bacteria (1, 2). Despite that, the number of new commercially
45 available antibiotics is dwindling. This is partly due to the fact that developing new antibiotics is
46 a very costly process (3), and the biopharma companies are therefore often reluctant to invest large
47 money in new antibiotics that soon may be useless because of resistance development.
48 Consequently, there is an urgent need of cost-effective and efficient antimicrobial agents with
49 different killing mechanisms to overcome multidrug-resistant bacteria.

50 Bacteriocins are ribosomally synthesized antibacterial peptides produced by bacteria, probably as
51 a means to compete for nutrients and habitats (4). So far hundreds of bacteriocins have been
52 isolated and characterized. Most of them have narrow-spectrum activity but some are active
53 against a broad-spectrum of bacteria including food-spoiling bacteria as well as important
54 pathogens (5, 6). Bacteriocins produced by lactic acid bacteria (LAB) are particularly interesting
55 due to LAB's safe status as they are commonly found in our foods (7, 8) and the gastrointestinal
56 tract of man (9) and animals (10). Most bacteriocins are membrane-active peptides, killing
57 sensitive bacteria by membrane disruption after selective interaction with specific membrane
58 receptors (11-15). This mode of action is different from most antibiotics which often act as
59 enzyme-inhibitors (16, 17). For this reason, antibiotic-resistant pathogens are often sensitive to
60 bacteriocins, thus making the latter very attractive as alternative or complementary drugs for
61 therapeutic use, especially to fight antibiotic resistance. Nevertheless, poor production is often a
62 bottleneck in large-scaled production of bacteriocins. Previous studies have shown that bacteriocin
63 production can be increased by optimization of growth conditions such as cultivation temperature,

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64 pH, aeration and growth medium (18-25). In addition, various heterologous expression systems
65 have been reported for increased bacteriocin production (26-30).

66 Recently we have reported the identification and characterization of a novel three-peptide
67 bacteriocin called garvicin KS (GarKS), produced by *L. garvieae* KS1546, a strain isolated from
68 raw bovine milk in Kosovo (31). A gene cluster (*gak*) containing the three structural genes
69 (*gakABC*) and genes likely involved in immunity (*gakIR*) and transport (*gakT*) has been identified
70 in the genome (31). GarKS is active against a broad spectrum of bacteria such as *Listeria,*
71 *Staphylococcus*, *Bacillus*, *Streptococcus* and *Enterococcus* (31). Despite its great potential,
72 production of GarKS is relatively moderate in standard laboratory growth conditions. To overcome
73 this problem, we conducted a multi-factorial optimization study that resulted in over 2000-fold
74 increased bacteriocin production. This approach includes medium optimization, genetic
75 engineering and cultivation optimization,

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79 **Results**

80 **GarKS production in complex media**

81 *L. garvieae* KS1546 (hereafter referred to KS1546) was routinely grown in the complex medium
82 GM17 at 30°C without agitation, and GarKS production was typically of 80 BU/mL after 7-12 h
83 growth. The bacteriocin production by KS1546 was examined in different complex media (MRS,
84 BHI and TH). Highest production was found between 7-12 h of growth in all tested media except
85 for TH where bacteriocin production appeared constantly low for all time-points tested (Fig. 1A).
86 Relative to GM17, GarKS production increased 2 to 4-fold in MRS, while it was about 2 to 4-fold
87 less in BHI and TH (Fig. 1A). Cell growth was the best in GM17 (3×10^9 CFU/ml) but the poorest
88 in MRS (1×10^9 CFU/ml) after 24 h at 30°C (Table 1).

89

90 **GarKS production increased in milk-based media**

91 It is well known that bacteria are ecologically adapted to the environments where they normally
92 thrive. Since the producer KS1546 was isolated from raw milk (31), we examine the possibility to
93 use skim milk (SM) as growth medium. Bacteriocin production was increased 2-fold in SM (160
94 BU/ml) compared to GM17 (Fig. 1B). However, cell growth was remarkably poor in skim milk
95 (2×10^8 CFUs/ml) (Table 1), indicating that some growth factors were present in complex media
96 but absent in SM. Therefore, we tested the mixtures (50:50; v/v) of skim milk and complex media
97 (GM17, MRS, BHI and TH). As a result, the bacteriocin production was increased 16 times in
98 skim milk combined with TH (SM-TH) and 8 times in SM-GM17, compared to the production in
99 skim milk (Table 1 and Fig. 1B). The bacteriocin production in SM-TH and SM-GM17 was 2600
100 BU/ml and 1280 BU/ml after 9 h of incubation, respectively. On the other hand, no significant
101 increase of GarKS in SM-MRS (320 BU/ml) and SM-BHI (160 BU/ml) was found in all time

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102 points (Fig. 1B). All medium formulations gave approximately a similar cell density, i.e., between
103 2.8×10^9-3×10^9 CFUs/ml (Table 1).
104 The results above indicate that bacteriocin production was significantly influenced by some
105 specific factor(s)/nutrient(s), which are present in TH and GM17, but absent in MRS and BHI.
106 Tryptone, a tryptic digest of milk protein casein (32), is one of the nutrients found in GM17 and
107 TH, but not in MRS and BHI. The final concentration of tryptone in GM17 and TH broth is 0.5%
108 and 2%, respectively. To examine whether tryptone could improve cell growth and bacteriocin
109 production in combination with SM, we made formulations with different v/v ratios of SM and
110 10% tryptone (w/v). Highest bacteriocin production (about 2,600 BU/ml) was achieved when they
111 were mixed in equal volumes (50% ; v/v); this mixture had a final concentration of tryptone of 5%
112 (w/v) (Figure 2). Under these circumstances, final cell density was comparable to that in GM17,
113 i.e., about 3×10^9 CFU/ml (Table 1). The formulation composed of SM (50 %; v/v) and a final 5%
114 of tryptone (w/v) is hereafter called SM-T.
115 Yeast extract is a rich source of vitamins, minerals, and amino acids, which often improves
116 bacterial growth. We examined the effect of yeast extract (YE) in combination with SM-T. The
117 resulting formulation, SM-T-YE (SM-T containing 1% (w/v) yeast extract) yielded the same cell
118 density as in SM-T (3×10^9 CFU/ml), but bacteriocin production was reduced by 50% (Table 1).
119 Yeast extract was therefore excluded from the growth medium.
120 Although SM-T appeared as a good medium for the producer, we constantly encountered the
121 problem associated with caramelization of milk sugars in skim milk during autoclaving, which
122 might have detrimental effects on milk nutrition value. To avoid this problem, the autoclaved skim
123 milk in SM-T was replaced with equal amount of pasteurized milk, resulting in a new medium
124 termed pasteurized milk–tryptone (PM-T). The contents in milk (Q-milk) according to the

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125 manufacturer (Q-Meieriene AS, Bergen, Norway) are, g/l: fat, 5; carbohydrate, 45; protein, 35;
126 salt, 1; calcium, 1.3; vitamin B_2, 0.001; and vitamin B_{12}, 0.7×10^{-5}. Indeed, cell growth in PM-T
127 was increased to 3.5×10^9 CFU/ml and GarKS production increased two-fold in comparison to that
128 in SM-T (Table 2).
129

130 **GarKS production increased by genetic engineering**

131 The three structural genes (*gakABC*) encoding the three peptides that constitute GarKS are
132 clustered with genes probably involved in immunity (*gakIR*) and transport (*gakT*). First we
133 explored the possibility to increase bacteriocin production by increasing only the gene dose of
134 structural genes *gakABC* in the native producer. The recombinant plasmid pABC carrying
135 structural genes *gakABC* was constructed to deliver high gene dose in the native producer (Fig.
136 3A). However, we failed to get any transformants even after several attempts. Similar negative
137 result (i.e., no transformants) was obtained when we attempted to transfer pABC into the
138 heterologous host *L. lactis* IL1403 (data not shown). Probably, increased gene dose of the
139 structural genes might override the immunity or/and the transporter in the native producer, leading
140 to toxicity to cells. Consequently, the plasmid pA2T carrying the entire *gak* locus including the
141 genes involved in immunity and transport was constructed pA2T (Fig. 3B). The plasmid was first
142 transferred into *L. lactis* IL1403. As expected, transformation was successful and bacteriocin
143 production was detected in transformed cells (data not shown), confirming the functionality of the
144 *gak* locus. Next the plasmid was transferred into the native KS1546 and the clone (KS1546-PA2T)
145 was assessed for bacteriocin production. Using PM-T as growth medium, GarKS production by
146 the recombinant producer KS1546-pA2T was found to increase to 20,000 BU/mL, which is about

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147 4 times more than the production without increased gene dose (native KS1546 in PM-T), and about
148 250-fold more than that initially obtained in GM17 (native KS1546 in GM17) (Table 1).
149 To compare the growth patterns, the native and recombinant producers were grown under similar
150 growth conditions. The recombinant producer KS1546-pA2T showed a prolonged lag growth
151 phase compared to the native GarKS producer (with or without empty plasmid). Nevertheless,
152 KS1546-pA2T reached eventually about the same high cell density as the wild type control cells
153 when it entered stationary growth phase (see Fig.4).
154

155 **Optimization of culture conditions in a bioreactor increased GarKS production**

156 The initial pH at 7 was declined to 4.8 when the recombinant producer KS1546-pA2T was grown
157 in PM-T for 6-7 h at 30°C (data not shown). To examine whether pH reduction could have a
158 negative impact on bacteriocin production, we grew the recombinant producer (KS1546-pA2T) in
159 PM-T in a bioreactor with constant pH at 5, 6 or 7. Indeed, pH had a great impact on cell growth
160 and bacteriocin production. Highest cell growth (0.7×10^{10} CFU/ml) and bacteriocin production
161 (82,000 BU/ml) were found at constant pH 6 (Table 1). Bacteriocin production measured at all
162 time-points was also highest at constant pH 6 (Fig. 5). Cell growth and bacteriocin production
163 were lowest at constant pH 5.
164 Aeration is defined as dissolved oxygen (DO) percentage in a culture medium. We observed that
165 the initial DO level at 50-60% was declined to 10% after 2 hours of cell growth in PM-T medium
166 and at constant pH 6. The effect of aeration on GarKS production was therefore examined by
167 purging the atmospheric sterile air into the growth medium (constant pH at 6). With aeration kept
168 at 50-60%, highest cell growth (1×10^{10} CFU/ml) and bacteriocin production (164,000 BU/ml)

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169 were obtained (Table 1 and Fig. 5). This level of bacteriocin production (164,000 BU/ml) was
170 about 2000-fold more than the initial production in GM17 which was 80 BU/ml.
171 We have previously shown that synthetic GarKS is functionally comparable to the biologically
172 produced counterpart (31). Synthetic GarKS (with > 90% purity) has a specific activity of 130-
173 140 BU/μg. Hence, the production of 164,000 BU/ml is equivalent to 1.2 g GarKS per liter which
174 is a level of commercial importance.
175

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176 **Discussion**

177 GarKS is potent against a set of important pathogens including *Staphylococcus*, *Bacillus*, *Listeria,*
178 *Streptococcus* and *Enterococcus*, making it very attractive in diverse antimicrobial applications
179 from food to medicine. Unfortunately, as also for many other bacteriocins, GarKS is produced at
180 relatively low levels during normal laboratory growth conditions (31). The low production by the
181 native producer can dramatically hamper potential applications of GarKS as industrial use of
182 bacteriocins requires high and cost-effective production. We have shown that optimization of
183 bacteriocin production by a bacterial strain is multi-factorial process, which involves the
184 systematic evaluation of nutritional ingredients and growth conditions e.g., temperature, pH and
185 aeration. The type of growth medium is probably one of the key factors in bacteriocins production
186 (33). The complex media e.g., GM17, MRS, BHI, and TH have been used in cultivation of LAB
187 because they give relatively good cell growth in laboratory conditions but not necessary for
188 bacteriocin production (34). This is illustrated in our study, GarKS production was the best in MRS
189 (320 BU/ml) but the lowest in BHI and TH (both 20 BU/ml) while the cell growth appeared about
190 in the same range in these media (1-2×10^9 CFU/ml).

191 To choose the optimal medium for bacteriocin production is often an empirical matter. The
192 components from complex media influencing bacteriocin production are often elusive and the
193 outcomes might vary significantly dependent on the type of producers. Nevertheless, some media
194 components have been shown to enhance bacteriocin production by inducing stress conditions due
195 to nutrient limitation (35) or stabilizing the bacteriocin molecules (36). The use of commercial
196 complex media (e.g., MRS) is not a cost-effective approach for large-scale bacteriocin production.
197 For instance, culture medium could account for up to 30% of the total production cost in
198 commercial biomolecule production (37). Accordingly, high costs of complex media will reduce

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199 attractiveness of bacteriocins for commercial application. Our bacteriocin producer is a strain of
200 *L. garvieae* isolated from raw milk and it has the capacity to ferment milk-associated sugars such
201 as lactose and galactose while another strain of *L. garvieae* isolated from intestine of Mallard duck
202 can not (31). Milk is a low-cost product relative to complex media and could be an ideal medium
203 for GarKS producer. However, the native producer appeared to grow poorly in sole skim milk that
204 might be the reason for reduced GarKS production. Skim milk is enriched in lactose and galactose
205 as carbon source but does not contain easily accessed nitrogen-containing components for bacteria.
206 Thus, the combination of tryptone and pasteurized skim milk which was found best for cell growth
207 was in line with the notion that tryptone serves as an enriched source of nitrogen. Further, this
208 formula also increased bacteriocin production over 30 fold compared to the growth in GM17.
209 Tryptone is composed of short peptides that are derived from enzymatic digest of milk protein
210 casein and serves as an enriched source of nitrogen in bacterial growth media.
211 Increase of gene dose is another means to enhance the production of biomolecules (38). In the
212 present study, we observed a 4-fold increase in bacteriocin production when a plasmid carrying
213 the entire *gak* locus was introduced into the native producer. Interestingly, when we attempted to
214 increase gene dose by introducing the structural genes only (using the plasmid pABC), no
215 transformed cells were obtained. One possible explanation for this negative outcome is that
216 expression of genetic determinants involved in bacteriocin iosynthesis is often highly fine-tuned
217 to secure immunity and efficient export. The extra gene dose of the structural genes alone might
218 override either immunity and/or transporter proteins, leading to toxicity in cell and cell death. It is
219 worth mentioning that most bacteriocins are expressed with a leader sequence which is necessary
220 not only for export but also to keep the bacteriocins in inactive form before export. For leaderless
221 bacteriocins like in the case of GarKS, they are produced in mature active forms before export,

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222 therefore an intracellular dedicated protection mechanism (immunity) available is crucial for cell
223 survival.

224 We and others have observed that bacteriocin production by a certain strain is unstable, and
225 dependent on the culture conditions applied (39, 40). Consequently, different growth parameters
226 were examined to optimize the production of GarKS. LABs are well known for reducing culture
227 pH due to lactic acid production (41) and this is also true for the GarKS producer. We found that
228 culture conditions with constant pH 6 favors the cell growth and a high level of GarKS production.
229 Similarly, optimal nisin production has been reported at constant pH 6.5 (42). The availability of
230 oxygen also has a great influence on microbial cell growth and metabolic activities (43).
231 Microorganisms vary with respect to their requirements and tolerance toward molecular oxygen.
232 *L. garvieae* is a facultative anaerobic microorganism and its metabolic activities have been
233 reported to differ between aerobic and anaerobic conditions (44). We observed that the controlled
234 aeration had a positive effect on the cell growth and bacteriocin production. Similar results have
235 also been observed for other bacteriocins. For example, nisin A production by *L. lactis* UL719 was
236 enhanced with aeration (45). On the other hand, aeration has also been reported to be antagonistic to the
237 production of lactosin S (46) and LIQ-4 bacteriocin (47), suggesting that the effect of aeration on
238 bacteriocin production is strain-dependent.

239 In terms of cost-effectiveness, the medium PM-T contained tryptone which is a relatively costly
240 component; therefore we are searching for alternatives to replace tryptone. In preliminary studies,
241 we tested the chicken hydrolysate (processed from a waste product from meat industry) as an
242 alternative low-cost protein source to produce GarKS. We found that the recombinant producer
243 grew well in a medium based on Pasteurized milk and chicken hydrolysate (PM-CH), yielding a
244 cell density of 3×10^9 CFU/ml. However, although GarKS production in PM-CH was 8 times better

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245 than in the complex media GM17, the production was 8 times less than in PM-T. Thus, further
246 studies are necessary to optimize a PM-CH-based medium in order to achieve high level and cost-
247 effective bacteriocin production.

248 Low bacteriocin production is often a bottle-neck in large-scaled production of bacteriocins for
249 commercial use. Optimization of bacteriocin production is therefore an important research field to
250 better exploit the antimicrobial potential of bacteriocins, especially with regard to the decreasing
251 effects of antibiotics in infection treatments due to the global emergence of antibiotic resistance.
252 In the present study we have shown that we managed to achieve a very high level of GarKS
253 production, amounting to 164,000 BU/ml, by combining medium optimization, genetic
254 engineering and culture condition optimization. This amount is about 2,000 times higher compared
255 to the initial production in GM17 (80 BU/ml). A production of 164,000 BU/ml is equivalent to 1.2
256 g GarKS per liter. To our knowledge, this is one of the highest bacteriocin production achieved so
257 far. In comparison, nisin production has been reported to 0.40-0.80 g/L by *L. lactis* grown in a
258 medium composed of equal volume of skim milk and complex media GM17 (5). Finally, our study
259 and others' have shown that optimization of bacteriocin production is an empirical and multi-
260 factorial process and that it is highly strain-dependent. Only by systematic evaluation of different
261 aspects influencing growth and gene regulation one can find conditions suitable for high levels of
262 production.

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266 **Materials and Methods**

267 **Bacterial strains and growth conditions**

268 All bacterial strains and plasmids used in this study are listed in Table 2. Unless otherwise stated,
269 the native bacteriocin producer *L. garvieae* KS1546 was grown in M17 broth supplemented with
270 0.5% glucose (GM17) under static condition at 30°C. NEB® 10-beta *E. coli* (New England
271 Biolabs, Beverly, MA, USA) was grown in Luria-Bertani (LB) broth with shaking (200 rpm) at
272 37°C. Bacterial culture media and supplements were obtained from Oxoid Ltd (Hampshire, UK).
273 When necessary, erythromycin (Sigma-Aldrich Inc., St. Louis, MO, USA) was added at
274 200 µg/ml for *E. coli* and at 5 µg/ml for lactococcal species.

275

276 **Growth media for GarKS production**

277 The influence of different growth media on GarKS production was assessed in batch cultures under
278 static condition at 30°C. Following commercial complex media were used: GM17, deMan, Rogosa
279 and Sharpe (MRS), Todd-Hewitt (TH) and Brain Heart Infusion (BHI). To make new milk-based
280 medium formulations, skim milk (5%, w/v) or pasteurized skim milk was combined with an equal
281 volume of GM17, MRS, TH, and BHI, or with tryptone (10% w/v). Skim milk (SM) was prepared
282 by using milk powder (Oxoid, UK) while pasteurized milk (PM) was obtained from a dairy
283 company in Norway, Q-milk.

284

285 **DNA manipulation**

286 The *gak* cluster responsible for production of GarKS was amplified from genomic DNA of *L.*
287 *garvieae* KS1546 using Phusion High-fidelity DNA polymerase (New England Biolabs, UK) and
288 the primers gakF and gakR1 (Table 2). The genes *gakABC* encoding the three peptides constituting

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289 GarKS were amplified using the primers gakF and gakR (Table 2). Restriction sites SacI and
290 HindIII were introduced at the 5'end of forward and reverse primers. NEBuilder HiFi DNA
291 assembly cloning kit (New England Biolabs) was used to assemble the PCR fragments into the
292 plasmid pMG36e (48). Plasmid DNA was amplified in *E. coli* NEB® 10-beta before being
293 transferred into *L. garvieae* KS1546 or *L. lactis* IL1403 cells using a Gene Pulser™ (Bio-Rad
294 Laboratories, Hercules, CA, USA). Primers used in this study were obtained from Life
295 Technologies AS (Thermofisher Scientific, Oslo, Norway). The integrity of all recombinant
296 plasmids was confirmed by Sanger DNA sequencing (GATC Biotech AG; Constance, Germany),
297 which were sequenced using primers gakseqF, gakseqF1, gakseqF2, gakseqF3, gakseqR, pMGF
298 and pMGR (Table 2).

299

300 **Optimization of bacteriocin production in bioreactor conditions**

301 The effects of pH and aeration on GarKS production were tested at various constant pH (5, 6 and
302 7), and at controlled aeration in a fully automated 2.5 L miniforce bioreactor (Infors AG,
303 Switzerland). The pH was controlled by automatic addition of 5 M HCl or 5 M NaOH. The aeration
304 was maintained by purging sterile air into culture medium. Temperature (30°C) and agitation speed
305 of 150 rpm were maintained constant for all experiments. Samples of 2 ml were withdrawn
306 aseptically every 2 h for determination of bacteriocin production and cell growth (see below).

307

308 **Determination of bacteriocin production and cell growth**

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309 Bacteriocin activity was measured from heat-inactivated (100°C for 10 min) cell-free culture
310 supernatants. Bacteriocin activity was quantified using a microtiter plate assay as previously
311 described (27, 31). One bacteriocin unit (BU) was defined as the minimum amount of the
312 bacteriocin that inhibited at least 50% of growth of the indicator (*L. lactis* IL103) in a 200 µl
313 culture volume. Growth curve was determined by measuring turbidity of culture at OD_{600} every
314 30 min for 24 h or by counting colony forming units (CFU) from serially diluted bacterial cultures
315 on agar plates. A standard curve based on the activity of 98% pure synthetic GarKS peptides
316 (Pepmic Co., LTD, China) was used to define the specific bacteriocin activity (BU/mg) from cell
317 free supernatant.

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322 **ACKNOWLEDGEMENTS**

323 We thank Linda Godager and Line Degn Hansen for technical assistance. This study was financed
324 by the Research Council of Norway, Project nr 254784.

325

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469 # Figure legends

470

471 **Figure. 1**. GarKS production by the native producer in different complex growth media (A), and
472 in skim milk (SM) combined with complex growth media (B). Each culture was started by adding
473 1% (v/v) culture inoculum to a 5 ml growth medium and then incubated at 30°C without shaking.
474 Bacteriocin activity was measured at different time points. Standard deviations were based on
475 triplicate assays.

476 **Figure. 2**. Bacteriocin production in a medium composed of skim milk and tryptone. Different
477 ratios of skim milk and tryptone were made in the formulation by mixing an increasing portion
478 of skim milk (10%; w/v; from 1 volume to 9 volumes) with a corresponding decreasing portion
479 of tryptone (10%, w/v; 9 volumes to 1 volume). For growth conditions, see legend in figure 1.
480 The bacteriocin activity was measured after 9 h of culture incubation. Standard deviations were
481 based on triplicate assays.

482 **Figure 3**. The plasmid map of pABC (A) and pA2T (B), which were used to increase the gene
483 dose of the structural genes (*gakABC*) and the *gak* cluster in the native producer, respectively.

484 **Figure 4**. Temporal growth profile of the recombinant producer (KS1546-pA2T), and the native
485 producer with empty plasmid (KS1546-pMG) or without plasmid (*L. garvieae* KS1546). Data
486 were acquired from triplicate assays. Standard deviations are within a range ±0.01 to ±0.05.

487 **Figure 5**. GarKS production of the recombinant producer (KS1546-pA2T) in cultivation at
488 constant pH (pH at 5, 6, or 7) or at constant pH 6 and aeration (50-60 % dissolved oxygen). Each
489 culture was started by adding 2% (v/v) culture inoculum in 1.5 l of PM-T medium containing

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490 erythromycin at final concentration of 5 µg/ml. Standard deviations were based on triplicate
491 assays.
492

493 Table legends

494 **Table 1**. Influence of growth media, increased gene dose and culture conditions on bacteriocin
495 production.
496
497 **Table 2**. Bacterial strains, plasmids and primers used in this study
498

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499 # Tables:

500 **Table 1**. Influence of growth media, increased gene dose and culture conditions on bacteriocin
501 production.

Strain	Growth medium	Bacteriocin activity (BU/ml)	Cell growth (CFUs/ml)
Native producer *L. garvieae* KS1546	GM17[a]	80 ±20	3×10^9
	MRS[a]	320 ±20	1×10^9
	BHI[a]	20	1.5×10^9
	TH[a]	20	2×10^9
	SM[b] (10%, w/v)	160 ±20	2×10^8
	Tryptone[a] (10%, w/v)	80 ±20	3×10^8
	SM-TH[b]	2600 ±80	2.9×10^9
	SM-GM17[b]	1280	3×10^9
	SM-MRS[b]	320	2.8×10^9
	SM-BHI[b]	160	2.9×10^9
	SM-T[b]	2600 ±80	3×10^9
	SM-T-YE[b]	1300 ±40	3×10^9
	PM-T[b]	5100 ±80	3.5×10^9
The recombinant producer *L. garvieae* KS1546-pA2T	PM-T[b] (uncontrolled pH)	20,000 ±400	3.5×10^9
	PM-T[b] (constant pH 5)	2600 ±80	3.2×10^9
	PM-T[b] (constant pH 6)	82,000 ±400	0.7×10^{10}
	PM-T[b] (constant pH 7)	41,000 ±400	0.65×10^{10}
	PM-T[b] (constant pH 6 and aeration)	164,000 ±400	1×10^{10}

502 The bacteriocin activity and cell growth from complex growth media (a) and milk based media (b)
503 were measured after 7 and 9 h of incubation, respectively. SM- Skim milk, T- Tryptone, YE-Yeast
504 extract, and PM-Pasteurized milk. Standard deviations (±) were based on triplicate assays.

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509 **Table 2**. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Description	Source/ reference
Strains		
L. garvieae KS1546	Wild type strain, native GarKS bacteriocin producer	[31]
L. garvieae KS1546-pA2T	*L. garvieae* KS1546 containing the recombinant plasmid pA2T	This study
L. lactis IL 1403-pA2T	*L. lactis* 1403 containing the recombinant plasmid pA2T	This study
L. garvieae KS1546-pMG	*L. garvieae* KS1546 containing the empty plasmid pMG36e	This study
L. lactis IL 1403-pMG	*L. lactis* IL 1403 containing the empty plasmid pMG36e	This study
Escherichia coli NEB® 10-beta	Subcloning host strain	New England Biolab
Plasmids		
pMG36e	Em^R, *E. coli-Lactococcus* shuttle vector	[48]
pABC	pMG36e containing the structural genes *gakABC*, Em^R	This study
pA2T	pMG36e containing the entire *gak* cluster; Em^R	This study
Primers		
gakF	5'-CGTAATTCGAGCTCCACCTC TGCTGTTTTTC-3'	This study
gakR	5'-AGACTTTGCAAGCTTGCAAT ATTACGTTTGTGGG-3'	This study
gakR1	5'-AGACTTTGCAAGCTTTTAATCC TGACTCATCAGATATTC-3'	This study
gakSeqF	5'-GTACATAGTACCTCAAAATTAT TTGAGC-3'	This study
gakseqF1	5'-GCAGAGCTTTAGTGTGGGAT-3'	This study
gakseqF2	5'-CGCTATTGCTTCTGAATATATA GTGGAC-3'	This study
gakseqF3	5'-GGCACTTTTACAAGAAATAGG ACT-3'	This study
gakseqR	5'-AGTAATTGCTTTATCAACTGCT GC-3'	This study
pMGF	5'-CATCCTCTTCGTCTTGGTAGC-3'	This study
pMGR	5'-GGCAGCTGATCTCAACAATG-3'	This study

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513 **Figures**

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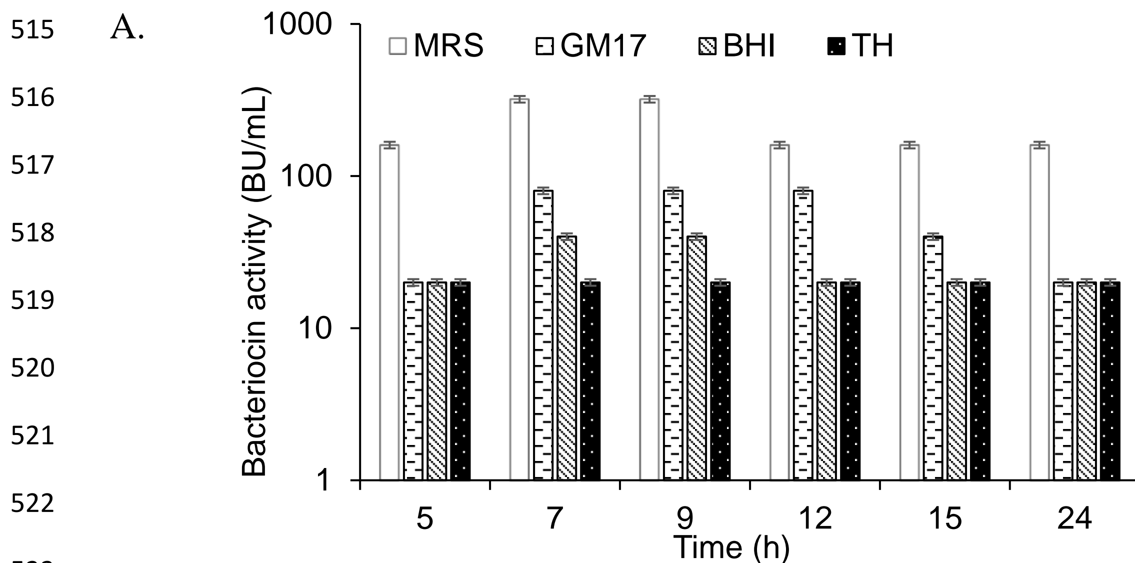
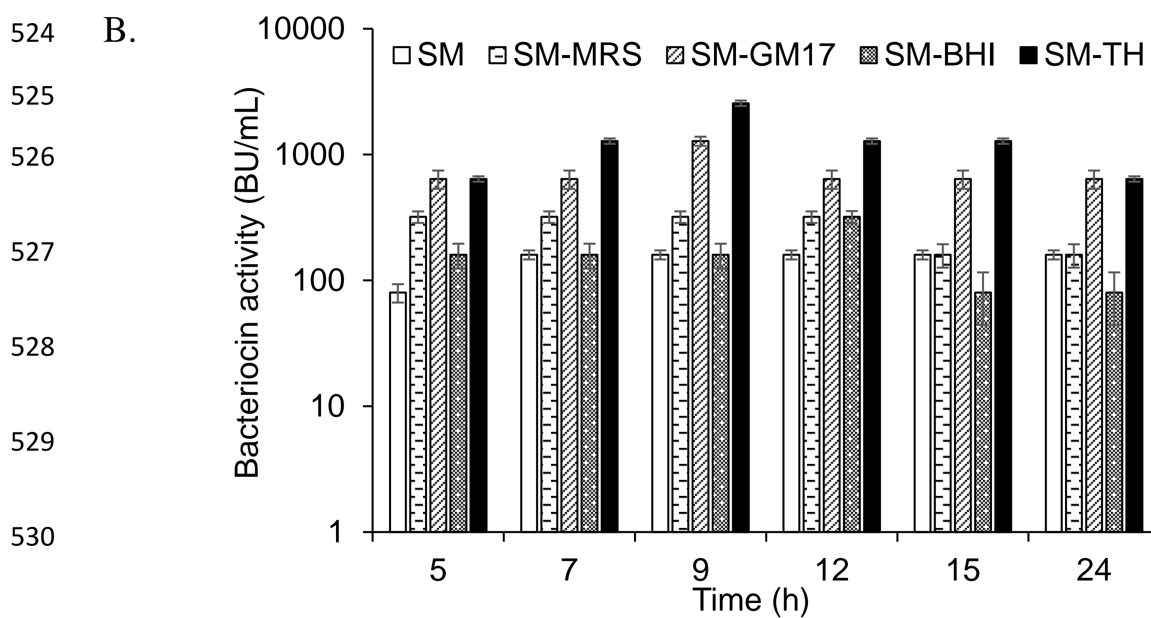
515 A.



524 B.



533 Figure. 1

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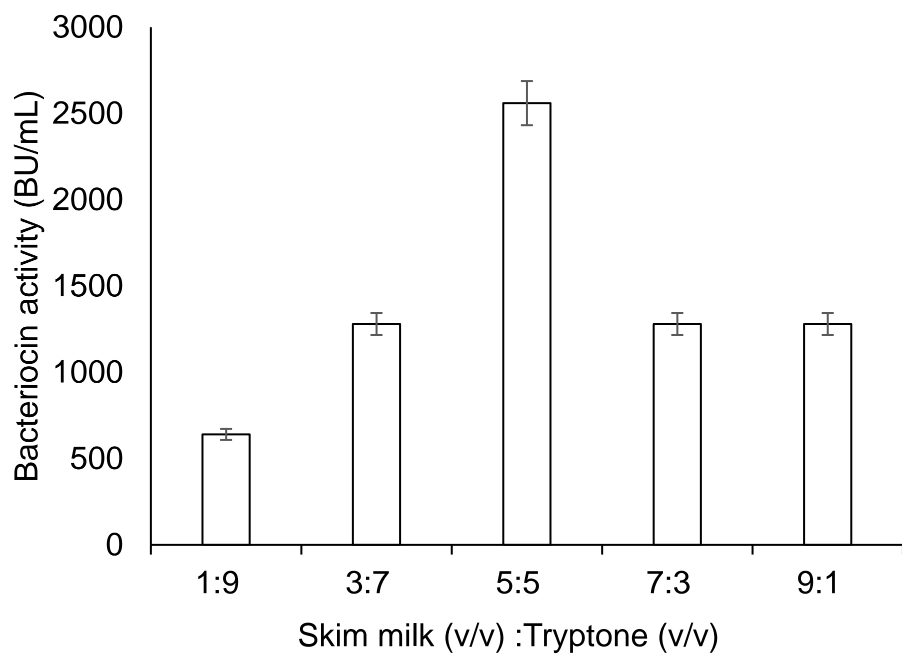

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536 Figure. 2.

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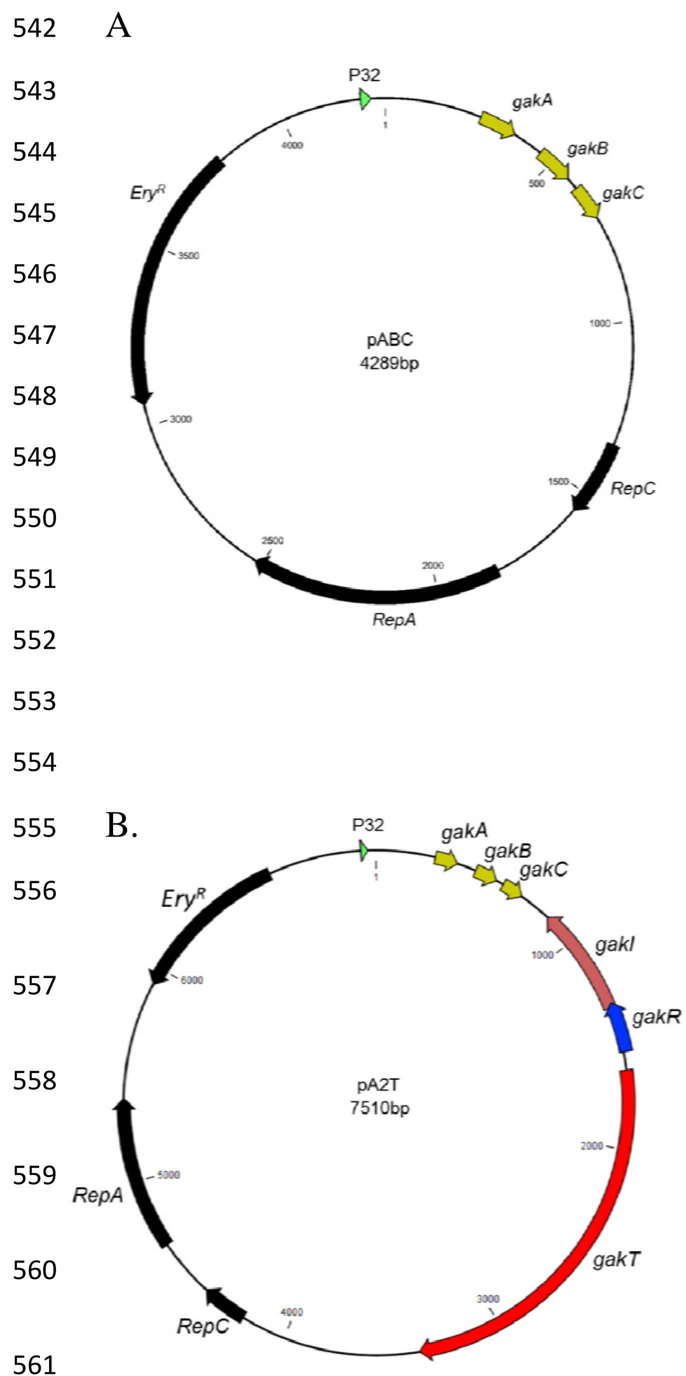
542 A



563 Figure 3.

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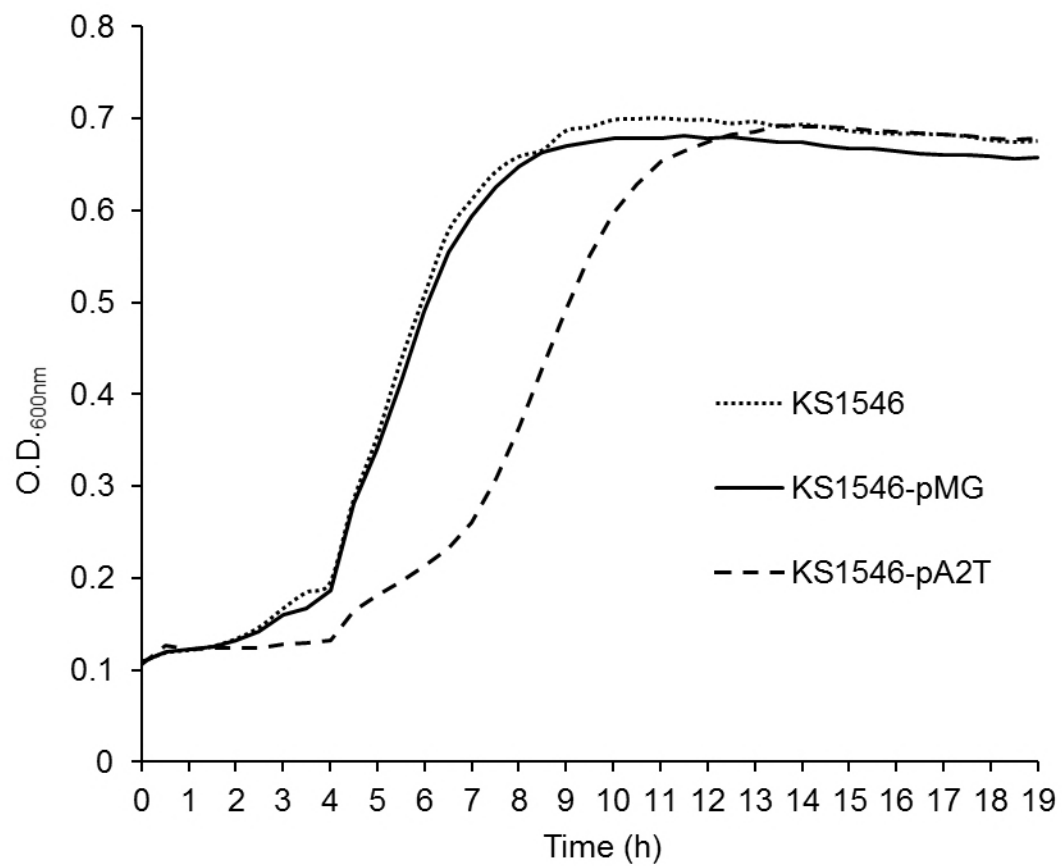

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567 Figure 4.

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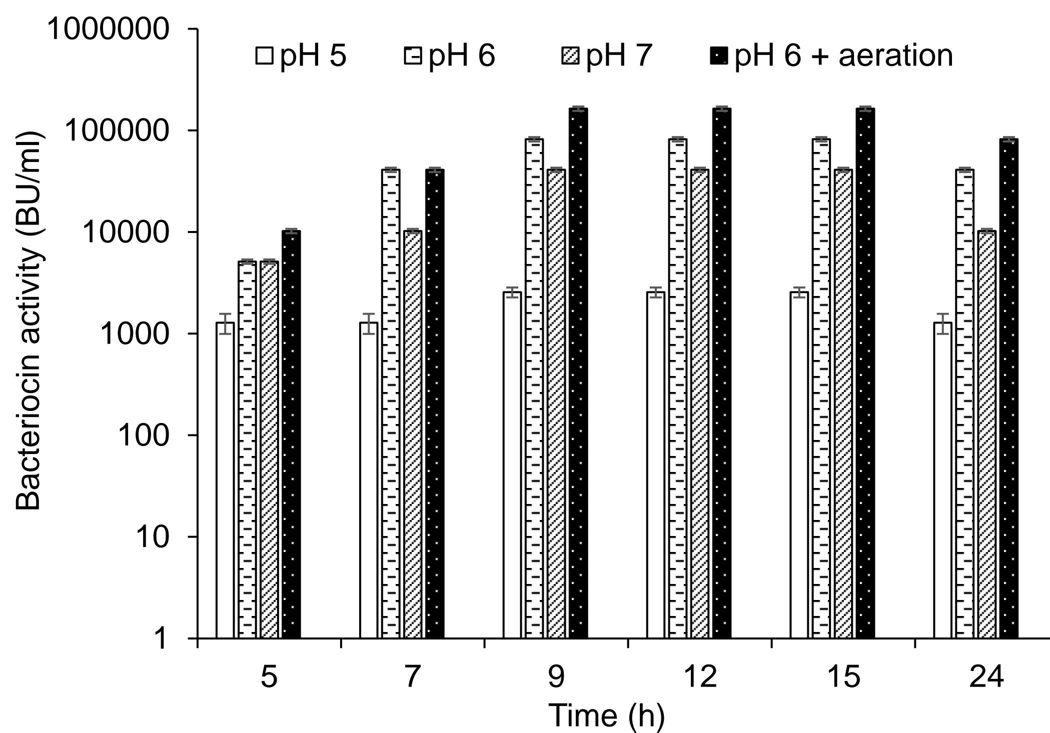

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572 Figure 5.

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