

1 Title: Lactolisterin BU, a novel Class II broad spectrum bacteriocin from *Lactococcus lactis*  
2 subsp. *lactis* bv. diacetylactis BGBU1-4

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4 Authors: Jelena Lozo<sup>1,2#</sup>, Nemanja Mirkovic<sup>2#</sup>, Paula M. O'Connor<sup>3,4</sup>, Milka Malesevic<sup>2</sup>,  
5 Marija Miljkovic<sup>2</sup>, Natalija Polovic<sup>5</sup>, Branko Jovcic<sup>1,2</sup>, Paul D. Cotter<sup>3,4</sup>, Milan Kojic<sup>2\*</sup>

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7 <sup>1</sup> Faculty of Biology, University of Belgrade, Belgrade, Serbia; <sup>2</sup> Institute of Molecular  
8 Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia; <sup>3</sup>Teagasc Food  
9 Research Centre Cork, Moorepark, Fermoy, Co. Cork, Ireland; <sup>4</sup>APC Microbiome Institute,  
10 Cork, Ireland; <sup>5</sup>Faculty of Chemistry, University of Belgrade, Belgrade, Serbia.

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12 <sup>#</sup>these authors contributed equally

13 **Running title:** Aureocin like bacteriocin in lactococci

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15 **\*Corresponding author:** Milan Kojic, [mkojic@imgge.bg.ac.rs](mailto:mkojic@imgge.bg.ac.rs)

16

17 **ABSTRACT**

18 *Lactococcus lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 produces a novel bacteriocin,  
19 lactolisterin BU, with strong antimicrobial activity against many species of Gram-positive  
20 bacteria, including important food spoilage and food-borne pathogens such as *Listeria*  
21 *monocytogenes*, *Staphylococcus aureus*, *Bacillus* sp. and streptococci. Lactolisterin BU was  
22 extracted from the cell surface of BGBU1-4 by propan-2-ol and purified to homogeneity by  
23 C18 solid phase extraction and reversed phase HPLC. The molecular mass of the purified  
24 lactolisterin BU was 5160.94 Da and an internal fragment, AVSWAWQH, as determined by  
25 N-terminal sequencing, showed low level similarity with existing antimicrobial peptides.  
26 Curing and transformation experiments revealed the presence of a corresponding bacteriocin  
27 operon on the smallest plasmid pBU6 (6.2 kb) of strain BGBU1-4. Analysis of the bacteriocin  
28 operon revealed a leaderless bacteriocin of 43 amino acids that exhibited similarity to  
29 bacteriocin BHT-B (63%), from *Streptococcus ratti*, a bacteriocin with analogy to aureocin A.

30

31 **IMPORTANCE**

32 Lactolisterin BU, broad spectrum leaderless bacteriocin produced by *L. lactis* subsp. *lactis* bv.  
33 diacetylactis BGBU1-4 strain, expresses strong antimicrobial activity against food spoilage  
34 and food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus*  
35 sp. and streptococci. Lactolisterin BU showed highest similarity with aureocin like  
36 bacteriocins produced by different bacteria. Operon for synthesis is plasmid located on the  
37 smallest plasmid pBU6 (6.2 kb) of strain BGBU1-4, indicating possible horizontal transfer  
38 among producers.

39

40 **Keywords:** bacteriocin, lactolisterin BU, antilisterial activity

41 **INTRODUCTION**

42 Bacteria have the ability to produce an extraordinary array of different antagonistic  
43 compounds. These include bacteriocins, described as ribosomally synthesized hydrophobic  
44 peptides (1, 2) usually active against bacteria closely related to the producer. In addition,  
45 some bacteriocins have broader inhibitory spectra against medically important pathogens and  
46 food-spoilage bacteria (3). Based on structure, mechanism of action, biochemical and genetic  
47 characteristics, bacteriocins from lactic acid bacteria (LAB) are generally classified into two  
48 different groups: Class I bacteriocins (lantibiotics) contain unusual amino acids such as  
49 lanthionine and dehydrated amino acids as a result of post-translational modifications and  
50 Class II bacteriocins consisting of unmodified or peptides with minor modifications.  
51 Furthermore, Class II bacteriocins are subdivided into four subclasses: pediocin-like  
52 bacteriocins (class IIa), two-peptide bacteriocins (class IIb), cyclic bacteriocins (IIc) and  
53 linear non-pediocin-like bacteriocins (class IId) (4). Bacteriocins produced by LAB have been  
54 intensively explored from a fundamental perspective, for their potential applications as food  
55 preservatives and, more recently, in veterinary and human medicine as possible alternative to  
56 antibiotics.

57 Positive properties of bacteriocins, which make them suitable for application in the food  
58 industry, they are inactive and non-toxic to eukaryotic cells and are sensitive to digestive  
59 proteases and so have little influence on gut microbiota. The application of bacteriocins (nisin  
60 and pediocin PA-1 are commercially available for food preservative uses) in the food  
61 industry provide many benefits such as the replacement of chemical preservatives or allowing  
62 the reduction of the intensity of heat treatment resulting in food that is more naturally  
63 preserved and with better sensorial and nutritional properties. Furthermore, bacteriocins are  
64 relatively thermostable and some of them can retain antimicrobial activity following  
65 pasteurization or sterilization. Also, some bacteriocins have a broad spectrum of antimicrobial

66 activity, so they can be used in foods as an effective method for extending shelf life and to  
67 control food-borne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* (5,  
68 6). *L. monocytogenes* is of particular concern since it is the causative agent of listeriosis (it  
69 can traverse the intestinal, placental and blood/brain barriers in humans) a relatively rare  
70 disease with high fatality rates (12%) in Europe and (25%) in the United States. Because of  
71 that, in the most European countries and in the United States, there are zero-tolerance  
72 standards for the *L. monocytogenes* in ready-to-eat (RTE) food (7–10).

73 Traditional fermented foods, such as cheeses produced from raw milk, are a rich ecological  
74 niche from which bacteriocin-producing LAB can be isolated (11). The indigenous LAB  
75 isolated from white brined cheeses from Serbia are good candidates for screening for  
76 antimicrobial substances as they are well adapted to the microbial environment's in cheese  
77 and could therefore be the source of novel properties (12).

78 Aureocins are new group of leaderless class II bacteriocins with broad spectrum of activity  
79 firstly isolated from *Staphylococcus aureus*. Aureocins act bactericidally on sensitive cells  
80 causing rapid lysis (13–15). According to their structure they could be classified into two  
81 main groups: multi-peptide (aureocin 70 like) and one peptide aureocins (aureocin A53 like).

82 In a previous study, it was demonstrated that the crude extract obtained from cell free  
83 supernatant of the natural isolate *Lactococcus lactis* subsp. *lactis* bv. diacetylactis BGBU1-4  
84 inhibited growth, biofilm formation and reduced 24 h old biofilms of coagulase negative  
85 staphylococci and *Listeria monocytogenes* clinical isolates (16). The objective of this work  
86 was to purify and biochemically and genetically characterise the broad spectrum bacteriocin  
87 lactolisterin BU, produced by *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4.

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## 91 RESULTS

### 92 Localization of the genes coding bacteriocin(s) production

93 The activity spectrum of *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 is broad, inhibiting  
94 different strains of *Lactococcus*, *Lactobacillus*, *Enterococcus* species and some pathogenic  
95 strains (16). Standard biochemical methods confirmed the proteinaceous nature of  
96 antimicrobial agent as it was found to be sensitive to proteinase K and pronase E. In addition,  
97 it was active against B464, a man-PTS deletion mutant derivative of IL1403 (17) suggesting  
98 that man-PTS is not a receptor for its antilisterial activity. To determine the bacteriocin coding  
99 genes location, a plasmid curing assay was performed. It was interesting to note that three  
100 types of plasmid-cured derivatives were obtained which differed in activity spectrum and size  
101 of the inhibition zone in agar well diffusion assays. It was noticed that derivative BGBU1-4/2,  
102 showed a reduced zone of inhibition against *L. lactis* subsp. *lactis* BGMN1-596 and *L.*  
103 *monocytogenes* ATCC 19111 compared to the parental strain and were sensitive to the  
104 parental strain. Derivatives BGBU1-4/29 and BGBU1-4/8 did not show antimicrobial activity  
105 against BGMN1-596 and ATCC 19111 and were sensitive to the parental strain BGBU1-4  
106 and derivative BGBU1-4/2. The plasmid profile analysis showed differences between parental  
107 strain BGBU1-4 and derivatives; derivative BGBU1-4/2 lost the plasmids pBU12 and pBU20,  
108 BGBU1-4/8 lost the smallest plasmid (pBU6) and pBU12, while in derivative BGBU1-4/29  
109 three plasmids (pBU6, pBU12 and pBU20) were absent (Fig. 1). These results indicate that  
110 strain BGBU1-4 synthesizes at least two bacteriocins active against *Lactococcus* sp. and *L.*  
111 *monocytogenes* strains that are encoded on plasmids'. It was possible to conclude that there is  
112 a direct correlation between the presence/absence of plasmid pBU6 and bacteriocin activity  
113 and most likely that the operon for the synthesis of the second bacteriocin is located on the  
114 plasmid pBU12.

### 115 Purification and identification of bacteriocin(s)

116 The bacteriocin(s) produced by strain BGBU1-4 were purified by RP-HPLC and the  
117 molecular mass of the active peptides determined by MALDI-TOF MS. The RP-HPLC  
118 chromatogram showed dominant peaks (Fig. 2) (fractions 30 and 37) that were active against  
119 *L. lactis* subsp. *lactis* BGMN1-596 and *L. monocytogenes* ATCC 19111. Mass spectrometry  
120 analysis determined the molecular mass of fraction 30 at 3642.62 Da and fraction 37 at  
121 5160.94 Da. As fraction 37 was most active it was selected for further characterization.

### 122 **N-terminal sequencing of protein fraction 37**

123 N-terminal sequencing of the native peptide in fractions 30 and 37 failed most probably due to  
124 a blocked N terminus. N-terminal sequencing was challenged two times without success. To  
125 circumvent this, native peptides were digested with trypsin and N-terminal sequencing of an  
126 internal fragments were done. Internal 1112.61 Da fragment of protein from fraction 37  
127 revealed the amino acid sequence AVSWAWQH, which corresponds to lactolisterin BU  
128 residues 16-23 (Fig. 4), while N-terminal sequencing of peptides from fraction 30 failed two  
129 times and work was continued only on fraction 37 (lactolisterin BU). Lactolisterin BU is a  
130 leaderless peptide and consequently the N terminal amino acid is formylmethionine rather  
131 than methioine as there is no cleavage of the leader peptide. The formyl group of  
132 formylmethionine blocks the alpha carbon of the amino acid making it inaccessible to  
133 phenylisothiocyanate (PITC), the reagent used in N terminal sequencing (18). MALDI TOF  
134 MS was also used to confirm the presence of formylmethionine. Addition of a formyl group  
135 results in a 28 Da increase in mass which is in good agreement with the 29 Da mass  
136 difference oserved when the mass of the native peptide (5160.94 Da) was compared with the  
137 theoretical mass (5131.67 Da).

### 138 **Heterologous expression of the bacteriocin in *Lactococcus lactis* subsp. *cremoris* MG7284**

139 Plasmid curing indicates that genes for the synthesis and immunity of bacteriocins in strain *L.*  
140 *lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 are located on plasmids. The smallest plasmid

141 named pBU6 (6.2 kb) from strain BGBU1-4 was isolated and used for transformation of the  
142 bacteriocin non-producer *Lactococcus lactis* subsp. *cremoris* MG7284 strain. GM17 agar  
143 plates containing lactolisterin BU, with concentration of 1,34  $\mu$ M, were used to select for  
144 bacteriocin-resistant transformants. Obtained transformants were designated MG7284/pBU6  
145 and were used for further purification of lactolisterin BU. The plasmid profile analysis of  
146 transformants MG7284/pBU6 revealed that all transformants possess the smallest 6.2 kb  
147 plasmid and were found to be active against indicator strains BGMN1-596, ATCC 19111 and  
148 B464, confirming that man-PTS is not a receptor for its antilisterial activity (Fig. S1).

#### 149 **Analysis of plasmid pBU6**

150 Plasmid pBU6 was sequenced in its entirety and submitted to the European Nucleotide  
151 Archive under accession No: LT629305. Sequence analysis of plasmid pBU6 revealed that it  
152 is a small rolling circle replicating (RCR) plasmid, which contains nine ORFs: *repB*, *lliBU*,  
153 *abcT*, *hyp1*, *hyp2*, *hyp3*, *mobC*, *relM*, *RnaseY* (Fig. 3, Table 2).  
154 *In silico* analysis revealed that RepB protein shows high similarity with lactobacilli and  
155 lactococcal RepB proteins (*Lactobacillus fermentum* and *Lactococcus lactis*;  
156 WP\_011117039.1, WP\_032951507.1, respectively). The N-terminal sequencing results of the  
157 peptide digest of fraction 37 identified *lliBU* as the structural gene, encoding a 43-amino acid  
158 peptide, which is responsible for production of the bacteriocin, lactolisterin BU. Lactolisterin  
159 BU is leaderless bacteriocin rich in amino acids glycine and tryptophan (each 11.6%) with pI  
160 10.16. Interestingly, this protein shows highest similarity with bacteriocin BHT-B from  
161 *Streptococcus ratti*, (63%, AAZ76605.1; (19)) and other aureocin A like bacteriocins (Fig. 4).  
162 Downstream of the *lliBU* gene ORF designated as *abcT* (212 aa) encodes a protein similar to  
163 the sugar ABC transporter ATP binding protein from *Streptococcus ratti* (62%,  
164 WP\_003089811.1). One or more of the next three genes, *hyp1* (212aa), *hyp2* (169 aa), *hyp3*  
165 (92 aa), may encode a protein(s) that plays a role in producer immunity. The three genes

166 *mobC* (127 aa), *relM* (333 aa) and *rnaseY* (169 aa) encode proteins similar to the Mobilization  
167 protein C, WP\_010729194.1, Relaxase/mobilization nuclease, WP\_010730379.1, and  
168 Ribonuclease Y, WP\_017865030.1, respectively.

#### 169 **Biochemical characterisation of lactolisterin BU**

170 Antimicrobial activity of purified lactolisterin BU was unchanged after heat treatment at  
171 60°C, 80°C and 100°C for 15 and 30 min when compared with the control with MIC values  
172 of 0.67 µM against *L. lactis* subsp. *lactis* BGMN1-596 and 1.34 µM against *L.*  
173 *monocytogenes* ATCC19111.

174 The growth kinetics and antimicrobial activity of transformant MG7284/pBU6 is shown in  
175 Fig. 5. Detectable levels of bacteriocin activity were recorded after 2 h of growth at 30°C.  
176 Maximum bacteriocin activity of the MG7284/pBU6 was achieved at the end of exponential  
177 phase while activity was lower against indicator strain *Listeria monocytogenes* ATCC 19111  
178 after 24 h growth (Fig. 5).

179 Purified lactolisterin BU was active against various Gram-positive bacteria in micromolar  
180 concentrations of MICs. Lactolisterin BU showed relatively strong activity against *Listeria*  
181 *monocytogenes* ATCC 19111 as the MIC value of the bacteriocin was 1.34 µM. In addition  
182 lactolisterin BU showed very strong antimicrobial activity against *Staphylococcus aureus*  
183 ATCC 25923, *Streptococcus pyogenes* A2941 and *Streptococcus pneumoniae* P156 (Table 3).

184 It is interesting that purified lactolisterin BU exhibited antimicrobial activity against  
185 lactolisterin BU producers (parental strain BGBU1-4, cured derivative BGBU1-4/2 and  
186 transformants MG7284/pBU6) with MIC value of 1.34 µM (Table 3).

#### 187 **Mode of action of lactolisterin BU**

188 Results of *L. monocytogenes* ATCC 19111 culture growth (started with different number of  
189 cells) in the presence of different concentrations of lactolisterin BU strongly indicated that the  
190 effect of lactolisterin BU on the growth is not growth phase dependent. The strongest lysis of



191 bacteria was obtained when the cells were in the early logarithmic phase. With the increase in  
192 the number of cells, the effect of lactolisterin BU on stopping growth of sensitive bacteria is  
193 gradually decreased (when it was used in concentration of 1.34  $\mu\text{M}$ , minimum inhibitory  
194 concentration), but at higher concentrations (4.02 and 13.4  $\mu\text{M}$ ) it exhibited strong inhibition  
195 of the growth of bacteria in all stages of growth (Fig. S2), indicating that it can be  
196 successfully used against pathogens and food contaminants that are in the stationary phase.

#### 197 **An attempt to isolate a lactolisterin BU resistant mutant**

198 In our previous experiences in obtaining mutants resistant to bacteriocins we used two  
199 approaches: selection of spontaneous mutants and mutagenesis with N-methyl-N'-nitro-N-  
200 nitrosoguanidine (20). We have successfully isolated mutants from both approaches but using  
201 mutagenesis we isolated greater number of mutants that showed greater diversity. First, we  
202 tried to isolate spontaneous mutants by spreading 500  $\mu\text{l}$  of 10 times concentrated overnight  
203 cultures of sensitive strains on GM17 Petri dishes containing lactolisterin BU in  
204 concentrations of 1.34  $\mu\text{M}$  and 2.68  $\mu\text{M}$  (1 and 2 times MIC values). No mutant-resistant to  
205 lactolisterin BU was obtained indicating a possible different mechanism of action or that a  
206 mutation leading to resistance is lethal (target protein is essential). In order to confirm the  
207 impossibility of obtaining mutants resistant to lactolisterin BU, mutant banks of three  
208 sensitive strains (*L. lactis* subsp. *lactis* BGMN1-596, *L. lactis* subsp. *cremoris* MG7284 and  
209 *Enterococcus faecalis* BGZLS10-27) were constructed using N-methyl-N'-nitro-N-  
210 nitrosoguanidine (which increases the chance of getting more mutations in each of the genes)  
211 and used to isolate resistant mutants by spreading of aliquots on selective GM17 Petri dishes  
212 containing lactolisterin BU. We did not manage to select the mutant resistant to lactolisterin  
213 BU from the mutant banks after three attempts per each strain, confirming the treatment that  
214 we applied did not yield mutant resistant to lactolisterin BU, or it is very difficult to isolate  
215 such a mutant.

216 **DISCUSSION**

217 Lactic acid bacteria are found in different ecological niches and, as a result of their efforts to  
218 adapt and survive, they produce various secondary metabolites among which are bacteriocins.  
219 Although, bacteriocins have been studied for almost seven decades, researchers still find them  
220 interesting due to their potential applications. In the last decade this field was broadened by  
221 their possible use instead of and/or in synergy with antibiotics to overcome the immense  
222 problem of increase in prevalence of antibiotic resistant bacteria (4). Natural isolates from  
223 traditionally prepared food products are a tremendous source of highly diverse, unique  
224 metabolites. These isolates come from harsh environments and contain genes that are usually  
225 lost in industrial strains. Lactococci commonly produce more than one bacteriocin and  
226 *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* BGBU1-4 strain produces at least two  
227 bacteriocins (21–23). Crude extract from cell free supernatant of the strain BGBU1-4  
228 demonstrated growth inhibition, and reduction of 24 h old biofilms formed by clinical isolates  
229 of *Listeria monocytogenes* and coagulase negative *Staphylococcus* sp., while prevention of  
230 biofilm formation was demonstrated for *L. monocytogenes* clinical isolates (16). Genes for  
231 bacteriocin lactolisterin BU production were plasmid pBU6 located which is not unusual (22–  
232 25), but plasmid cured derivatives suggest that broad spectrum antimicrobial activity of strain  
233 BGBU1-4 is a consequence of production of at least two bacteriocins. Characterization of the  
234 anti-listerial bacteriocin purified by HPLC revealed that ORF *lliBU* on pBU6 encodes the  
235 lactolisterin BU structural gene. In addition, sequence analysis of lactolisterin BU, a 43-amino  
236 acid peptide, shows the highest similarity with BHT-B, a Class II bacteriocin from  
237 *Streptococcus ratti*. Although lactolisterin BU shows some characteristics of Class IIa (anti-  
238 listerial activity and absence of unusual amino acids) bacteriocins it does not possess the  
239 highly conserved motif T-G-N-G-V/L generally found in “pediocin-like” bacteriocins with  
240 antilisterial activity and so cannot be classified as a Class IIa bacteriocin (26–28). Therefore it

241 is classified as a Class IId leaderless bacteriocin and can be compared to LsbB, Lacticin Q,  
242 Lacticin Z, aureocins A70 and A53, bacteriocins known to be synthesized and exported  
243 without a leader sequence (14, 15, 23, 29, 30). A comparative analysis of the amino acid  
244 sequences has shown that lactolisterin BU shares a conserved region AKYGxKAV with the  
245 majority of known aureocin A53 like bacteriocins (Fig. 4). It can be assumed, considering the  
246 variation in length and primary structure, that the region AKYGxKAV is responsible for the  
247 activity of aureocin A53 like bacteriocins. It is interesting that lactolisterin BU shows higher  
248 identity with BHT-B bacteriocin from *Streptococcus ratti*, (63%) than with lacticin Q and  
249 lacticin Z (33.9%) isolated from lactococci. Antilisterial activity of lactolisterin BU is not  
250 mediated by interaction with man-PTS, like in other Class II bacteriocins, as it showed  
251 antimicrobial activity on mutant B464 (17).

252 Biochemical characterization showed that lactolisterin BU, like other bacteriocins, is sensitive  
253 to proteolytic enzymes, relatively thermostable, and has maximum production in early  
254 stationary phase (31–33). Lactolisterin BU has potential as a food preservative due to its  
255 strong antimicrobial activity (active in micromolar concentrations) against many species of  
256 Gram-positive bacteria, including important food spoilage and food-borne pathogens such as  
257 *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus* sp. and *Streptococcus* sp..  
258 Additionally, since production of many food products involves exposure to high temperature,  
259 the relative thermostability of lactolisterin BU is another desirable feature for its application  
260 in the food industry. The most desirable characteristic of lactolisterin BU for its use in  
261 controlling contaminants of food or pathogens is the inability to induce resistance that is most  
262 likely the result of a specific mechanism of action or an essential target molecule. Genes for  
263 bacteriocin production are often plasmid located enabling horizontal gene transfer between  
264 genera and it is expected that rearrangements during transfer results in novel peptides. It is  
265 assumed that a similar scenario happened with lactolisterin BU. The presence of a highly

266 conserved region between lactococcal bacteriocin lactolisterin BU, bacteriocin BHT-B from  
267 *Streptococcus ratti*, aureocin A53 from *Staphylococcus aureus* and aureocin A53 like  
268 bacteriocin from *Corynebacterium jeikeium* indicates a common origin of the bacteriocin  
269 operon. It is interesting that the homologous bacteriocin operon is present in such a wide  
270 variety of genera, which indicates that bacteriocin production confers an advantage to the  
271 carrier. The greater similarity between lactolisterin BU and aureocins from other bacteria than  
272 with lactococcal (lacticin Q and Z) indicates a different evolutionary pathway of these  
273 lactococcal bacteriocins.

274 This work provides insight into a new and unusual Class II lactococcal bacteriocin,  
275 lactolisterin BU. Genetic and biochemical characteristics, activity spectrum, protease  
276 sensitivity, thermostability and inability or very rare occurrence of resistance recommend  
277 lactolisterin BU as good candidate for a safe, cheap and natural food preservative. Further  
278 experiments on lactolisterin BU in preventing *L. monocytogenes* development in products  
279 obtained from raw milk are ongoing.

## 280 **MATERIALS AND METHODS**

### 281 **Bacterial strains and culture conditions**

282 The bacterial strains used in this study are listed in Table 1. The bacteriocin producer *L. lactis*  
283 subsp. *lactis* bv. diacetylactis BGBU1-4 was isolated from a three day old traditional semi-  
284 hard cheese made from mixed cow (20%) and sheep (80%) milk (34). The cheese was  
285 produced without the use of starter cultures in a household in the village of Buzina, located on  
286 the mountain Beljanica in eastern Serbia. Lactococcal strains were grown in M17 medium  
287 (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% w/v) (GM17) at  
288 30°C. *Lactobacillus* strains were grown in MRS medium (Merck GmbH, Darmstadt,  
289 Germany). Non-lactococcal indicator strains were grown aerobically in Luria-Bertani (LB)  
290 broth at 37°C. *Streptococcus* strains were grown in Brain Heart Infusion (BHI) medium

291 (Oxoid, Basingstone, Hampshire, England) at 37°C and an atmosphere of 5% CO<sub>2</sub>. Solid  
292 medium and soft-agar were made by adding 1.5% or 0.7% (w/v) agar (Torlak, Belgrade,  
293 Serbia), to the liquid media, respectively.

#### 294 **Spectrum and kinetics of bacteriocin activity**

295 The agar well diffusion assay was used to determine the antibacterial spectrum of the strain  
296 BGBU1-4 (33). Each indicator strain was inoculated into appropriate soft-agar, and wells  
297 (diameter 5 mm) were made in the plate. The wells were filled with 50 µL of sample and  
298 plates were incubated under appropriate conditions for the respective indicator strain (Table  
299 2). After 24 h of incubation, plates were examined for the presence of inhibition zones. A  
300 clear zone of inhibition around the wells was taken as evidence of bacteriocin production.  
301 To monitor kinetics of bacteriocin production/activity 100 mL of fresh preheated GM17 broth  
302 was inoculated with overnight culture (1% v/v) and incubated at 30°C. Samples were taken at  
303 0, 2, 4, 6, 8, 10, 12, and 24 h. Bacteriocin activity was determined by area zone of inhibition.  
304 *L. monocytogenes* ATCC 19111 and *L. lactis* subsp. *lactis* BGMN1-596 were used as  
305 indicator strains.

#### 306 **Genetic characterization**

#### 307 **Plasmid curing experiments**

308 Plasmid curing assays were done by growing the bacterial cells of BGBU1-4 strain in the  
309 presence of novobiocin at sub-lethal temperatures as described previously (35). Preheated  
310 GM17 broth (42°C) containing novobiocin (5 µg/mL) was inoculated with 10<sup>3</sup> cells per mL.  
311 After 2 h of incubation, the cells were collected by centrifugation and resuspended in the  
312 same volume of fresh preheated novobiocin containing GM17 broth to avoid a bacteriocin-  
313 killing effect on the cured cells. This step was repeated four times and end point aliquots (0.1  
314 mL) were plated onto GM17 agar plates, which were then incubated at 30°C for 48 h.

#### 315 **Molecular methods**

316 Derivatives obtained following plasmid curing experiments, and transformants were  
317 confirmed using pulsed field gel electrophoresis (PFGE) as described previously by Kojic et  
318 al., 2006 (23). For isolation of total DNA from lactococci, a modified version of the method  
319 described by Hopwood et al., 1985 (36) was used. Plasmid DNA from lactococci was isolated  
320 by the modified method previously described by O`Sullivan and Klaenhamer, 1993 (37).  
321 Plasmids were introduced into lactococci by electroporation using an Electroporator  
322 (Eppendorf, Hamburg, Germany) (38). Plasmid DNA was sequenced by the MacroGen  
323 Sequencing Service (MacroGen Europe, Amsterdam, The Netherlands). Nucleotide sequences  
324 were analysed using BLAST algorithm. The functions of the proteins encoded by the pBU6  
325 plasmid were attributed on the basis of homology with known proteins by using BLAST  
326 comparison with Entrez protein blast.

### 327 **Purification of the bacteriocin**

328 Lactolisterin BU was purified from the cells according to the method of Rea et al., 2007 (39)  
329 with the following modifications. Briefly, the cell pellet from a 2 litre culture grown in TY  
330 broth was resuspended in 250 mL of 70% (v/v) 2-propanol, 0.1% (v/v) TFA/L of broth and  
331 stirred at room temperature for 3-4 hours. Sample was centrifuged as at 8280 g for 20 minutes  
332 and cell supernatant retained for purification. The 2-propanol was evaporated using a rotary  
333 evaporator (Buchi Labortechnik AG, Flawil, Switzerland) and the sample applied to a 5 g (20  
334 mL) Strata C18-E SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol  
335 and water. The column was washed with 40 mL of 30% (v/v) ethanol, and the bacteriocin was  
336 eluted with 40 mL of 70% (v/v) 2-propanol, 0.1% (v/v) TFA. An aliquot of the cell C18 SPE  
337 70% 2-propanol, 0.1% TFA eluent was concentrated using rotary evaporation before  
338 separation of the peptides using RP-HPLC. Aliquots of approximately 4 mL were applied to a  
339 Phenomenex (Phenomenex, Macclesfield Cheshire, UK) Proteo Jupiter (RP)-HPLC column  
340 (250 × 10.0 mm, 4 $\mu$ , 90Å) previously equilibrated with 25% acetonitrile, 0.1% TFA. Peptides

341 were eluted in a gradient of 30% acetonitrile containing 0.1% TFA to 70% acetonitrile  
342 containing 0.1% TFA over 40 minutes where buffer A is Milli Q water containing 0.1% TFA  
343 and buffer B is 90% acetonitrile containing 0.1% TFA and the flow rate was 2.5 mL/min.  
344 Fractions were collected at 1 minute intervals and assayed on *Lactococcus lactis* BGMN1-596  
345 and *Listeria monocytogenes* ATCC 19111 indicator plates.

346 MALDI TOF Mass spectrometry was performed on fractions exhibiting positive inhibitory  
347 activity, using an Axima TOF<sup>2</sup> MALDI TOF mass spectrometer (Shimadzu Biotech,  
348 Manchester, UK), as described by Mills et al., 2011 (40).

#### 349 **N-terminal sequencing of protein from fractions 30 and 37**

350 Protein fractions 30 and 37 that showed antimicrobial activity were sent to Department of  
351 Molecular and Biomedical Sciences “Jozef Stefan” Institute (Ljubljana, Slovenia) for N-  
352 terminal sequencing by Edman degradation. N-terminal sequencing of the native peptides  
353 failed due to the presence of an N terminal formyl methionine so peptides were digested with  
354 trypsin and trypsin fragments were sequenced on the second attempt.

#### 355 **Determination of minimal inhibitory concentrations (MICs)**

356 The minimal inhibitory concentration of the lactolisterin BU was determined using the broth  
357 microdilution method proposed by Steinberg et al., 1997 (41). The microdilution testing assay  
358 used a mixture of the indicator strains (Table 1 and Table 3) and increasing concentrations of  
359 bacteriocin, lactolisterin BU. Microdilution testing, with in-house prepared panels was  
360 performed following the Clinical and Laboratory Standards Institute’s Performance Standards  
361 for Antimicrobial Susceptibility Testing (Twenty-Fourth Informational Supplement. CLSI  
362 document M100-S24). Indicator strains were diluted to 0.5 McFarland units from which 20  
363  $\mu$ L were distributed to wells of a clear 96-well flat bottom microtiter plate. Concentration of  
364 pure lactolisterin BU was determined by spectroscopic method using theoretical extinction  
365 coefficient calculated from the peptide sequence as described by Blanusa et al., 2007 (42).



366 Lactolisterin BU (47  $\mu\text{M}$ ) was two-fold serially diluted to give a dilution series from 43  $\mu\text{M}$   
367 to 0.67  $\mu\text{M}$ . The microtiter plates were incubated under appropriate conditions for 24 h, and  
368 the optical densities at 595 nm ( $\text{OD}_{595}$ ) were recorded at 30 min intervals (Infinite M200pro,  
369 Tecan, Switzerland). Values obtained were used to illustrate the antimicrobial activity of the  
370 bacteriocin lactolisterin BU. Control wells contained appropriate medium (blanks) and  
371 untreated culture. All experiments were done in triplicate.

### 372 **Effect of temperature treatment on lactolisterin BU activity**

373 Water dissolved purified lactolisterin BU (concentration of 47  $\mu\text{M}$ ), was incubated at 60°C,  
374 80°C and 100°C for 15 min and 30 min. After treatments, antimicrobial activity was  
375 determined using the broth microdilution method proposed by Steinberg et al., 1997 (41) as  
376 described above. The indicator strains were *L. monocytogenes* ATCC 19111 and *L. lactis*  
377 subsp. *lactis* BGMN1-596; untreated purified bacteriocin was used as a control. All  
378 experiments were done in triplicate.

### 379 **Mode of action of lactolisterin BU**

380 To analyze the effect of lactolisterin BU on the growth of the sensitive strain, purified  
381 lactolisterin BU (three different concentrations were used; 1.34  $\mu\text{M}$  – minimal inhibitory  
382 concentration, 4.02  $\mu\text{M}$  –three times higher than MIC, and 13.4  $\mu\text{M}$  – ten times higher  
383 concentration than MIC) was added to the cultures of *L. monocytogenes* ATCC 19111  
384 inoculated with different number of bacteria ( $3 \times 10^7$ ,  $3.3 \times 10^8$  and  $2 \times 10^9$  cells/mL) in  
385 microtiter plates. Before the addition of bacteriocin, diluted bacterial cultures were incubated  
386 for 1 hour at the optimal growth temperature to refresh the cells. The bacterial growth was  
387 monitored by measurement of optical densities at 595 nm ( $\text{OD}_{595}$ ) that were recorded at 30  
388 min intervals (Infinite M200pro, Tecan, Switzerland) and by determination of viable bacterial  
389 cells (CFU) at every hour of growth. Control wells contained appropriate medium (blanks)  
390 and untreated cultures. All experiments were done in triplicate.



391 **Mutagenesis of sensitive strains with N-methyl-N'-nitro-N-nitrosoguanidine.**

392 Cells from middle logarithmic growth phase ( $OD_{600} \sim 0.6-1$ ) of sensitive strains (*L. lactis*  
393 subsp. *lactis* BGMN1-596, *L. lactis* subsp. *cremoris* MG7284 and *Enterococcus faecalis*  
394 BGZLS10-27) were harvested by centrifugation at 10 000 g for 10 min at 4°C and washed  
395 two times in the same volume of 100 mM sodium phosphate buffer (pH 7). Ten times  
396 concentrated cells in phosphate buffer were exposed to different concentrations of N-methyl-  
397 N'-nitro-N-nitrosoguanidine (0, 25, 50, 100 and 200 µg/mL in phosphate buffer) for 1 h at  
398 30°C in dark. After treatment cells were washed two times with 10 times volume of phosphate  
399 buffer and finally resuspended in the same volume of GM17. Cultures were grown for 1 h at  
400 30°C in dark in order to recover cells and cell survive was determined by plating of 10 times  
401 dilutions on GM17 plates and incubation at 30°C for two days. Stabilised mutated cells were  
402 stored at -80°C by adding glycerol (final concentration 15%) until use.

403

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407

408

## 409 REFERENCES

- 410 1. **Montesinos E.** 2007. Antimicrobial peptides and plant disease control. *FEMS*  
411 *Microbiol Lett* **270**:1–11.
- 412 2. **Keymanesh K, Soltani S, Sardari S.** 2009. Application of antimicrobial peptides in  
413 agriculture and food industry. *World J Microbiol Biotechnol* **25**:933–944.
- 414 3. **Nes IF.** 2011. History, current knowledge, and future directions on bacteriocin research  
415 in lactic acid bacteria, p. 3–12. *In* Drider, D, Rebuffat, S (eds.), *Prokaryotic*  
416 *Antimicrobial Peptides - From Genes to Applications*. Springer Science & Business  
417 Media, New York.
- 418 4. **Cotter PD, Ross RP, Hill C.** 2013. Bacteriocins - a viable alternative to antibiotics?  
419 *Nat Rev Microbiol* **11**:95–105.
- 420 5. **Acuña L, Corbalan NS, Fernandez-No IC, Morero RD, Barros-Velazquez J,**  
421 **Bellomio A.** 2015. Inhibitory effect of the hybrid bacteriocin Ent35-MccV on the  
422 growth of *Escherichia coli* and *Listeria monocytogenes* in Model and Food Systems.  
423 *Food Bioprocess Technol* **8**:1063–1075.
- 424 6. **Thomas L V, Ingram RE, Bevis HE, Davies EA, Milne CF, Delves-Broughton J.**  
425 2002. Effective use of nisin to control *Bacillus* and *Clostridium* spoilage of a  
426 pasteurized mashed potato product. *J Food Prot* **65**:1580–5.
- 427 7. 2011. The European Union summary report on trends and sources of zoonoses,  
428 zoonotic agents and food-borne outbreaks in 2009. *EFSA J* **9**:2090.
- 429 8. 2014. WHO | Food and health in Europe: a new basis for action. WHO.
- 430 9. **Schlech WF, Acheson D.** 2000. Foodborne Listeriosis. *Clin Infect Dis* **31**:770–775.
- 431 10. **Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM,**  
432 **Tauxe R V.** 1999. Food-related illness and death in the United States. *Emerg Infect*  
433 *Dis* **5**:607–25.
- 434 11. **Cocolin L, Foschino R, Comi G, Graziafortina M.** 2007. Description of the  
435 bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian  
436 goat milk. *Food Microbiol* **24**:752–758.
- 437 12. **Topisirovic L, Kojic M, Fira D, Golic N, Strahinic I, Lozo J.** 2006. Potential of  
438 lactic acid bacteria isolated from specific natural niches in food production and  
439 preservation. *Int J Food Microbiol* **112**:230–235.
- 440 13. **Netz DJA, Bastos M do C de F, Sahl H-G.** 2002. Mode of action of the antimicrobial  
441 peptide aureocin A53 from *Staphylococcus aureus*. *Appl Environ Microbiol* **68**:5274–  
442 80.

- 443 14. **Netz DJA, Pohl R, Beck-Sickinger AG, Selmer T, Pierik AJ, Bastos M do C de F,**  
444 **Sahl H-G.** 2002. Biochemical characterisation and genetic analysis of aureocin A53, a  
445 new, atypical bacteriocin from *Staphylococcus aureus*. *J Mol Biol* **319**:745–756.
- 446 15. **Netz DJA, Sahl H-G, Marcolino R, dos Santos Nascimento J, de Oliveira SS,**  
447 **Soares MB, do Carmo de Freire Bastos M, Marcolino R.** 2001. Molecular  
448 characterisation of aureocin A70, a multi-peptide bacteriocin isolated from  
449 *Staphylococcus aureus* 11. Edited by M. Yaniv. *J Mol Biol* **311**:939–949.
- 450 16. **Cirkovic I, Bozic DD, Draganic V, Lozo J, Beric T, Kojic M, Arsic B, Garalejic E,**  
451 **Djukic S, Stankovic S.** 2016. Licheniocin 50.2 and bacteriocins from *Lactococcus*  
452 *lactis* subsp. *lactis* biovar. *diacetylactis* BGBU1-4 inhibit biofilms of coagulase  
453 negative staphylococci and *Listeria monocytogenes* clinical isolates. *PLoS One*  
454 **11**:e0167995.
- 455 17. **Diep DB, Skaugen M, Salehian Z, Holo H, Nes IF.** 2007. Common mechanisms of  
456 target cell recognition and immunity for class II bacteriocins. *Proc Natl Acad Sci U S*  
457 *A* **104**:2384–9.
- 458 18. **Miyatake N, Kamo M, Satake K, Uchiyama Y, Tsugita A.** 1993. Removal of N-  
459 terminal formyl groups and deblocking of pyrrolidone carboxylic acid of proteins with  
460 anhydrous hydrazine vapor. *Eur J Biochem* **212**:785–9.
- 461 19. **Hyink O, Balakrishnan M, Tagg JR.** 2005. *Streptococcus rattus* strain BHT produces  
462 both a class I two-component lantibiotic and a class II bacteriocin. *FEMS Microbiol*  
463 *Lett* **252**:235–241.
- 464 20. **Uzelac G, Kojic M, Lozo J, Aleksandrak-Piekarczyk T, Gabrielsen C, Kristensen**  
465 **T, Nes IF, Diep DB, Topisirovic L.** 2013. A Zn-dependent metallopeptidase is  
466 responsible for sensitivity to LsbB, a class II leaderless bacteriocin of *Lactococcus*  
467 *lactis* subsp. *lactis* BGMN1-5. *J Bacteriol* **195**:5614–21.
- 468 21. **Van Belkum MJ, Hayema BJ, Geis A, Kok J, Venema G.** 1989. Cloning of two  
469 bacteriocin genes from a lactococcal bacteriocin plasmid. *Appl Environ Microbiol*  
470 **55**:1187–91.
- 471 22. **Mirkovic N, Polovic N, Vukotic G, Jovcic B, Miljkovic M, Radulovic Z, Diep DB,**  
472 **Kojic M.** 2016. *Lactococcus lactis* LMG2081 produces two bacteriocins, a  
473 nonlantibiotic and a novel lantibiotic. *Appl Environ Microbiol* **82**:2555–2562.
- 474 23. **Kojic M, Strahinic I, Fira D, Jovcic B, Topisirovic L.** 2006. Plasmid content and  
475 bacteriocin production by five strains of *Lactococcus lactis* isolated from semi-hard  
476 homemade cheese. *Can J Microbiol* **52**:1110–20.
- 477 24. **Mirkovic N, Radulovic Z, Uzelac G, Lozo J, Obradovic D, Topisirovic L, Kojic M.**  
478 2015. Isolation and characterization of bacteriocin and aggregation-promoting factor  
479 production in *Lactococcus lactis* ssp. *lactis* BGBM50 strain. *Food Technol Biotechnol*  
480 **53**.

- 481 25. **Ansari A, Siddiqui NN, Ghani M, Aman A, Qader SA.** 2015. Plasmid borne BAC-  
482 IB17: Localization of a potential antibacterial positive marker (Bac+) encoded broad  
483 inhibitory spectrum bacteriocin. *Pak J Pharm Sci* **28**:1331–5.
- 484 26. **Kalmokoff ML, Banerjee SK, Cyr T, Hefford MA, Gleeson T.** 2001. Identification  
485 of a new plasmid-encoded sec-dependent bacteriocin produced by *Listeria innocua*  
486 743. *Appl Environ Microbiol* **67**:4041–7.
- 487 27. **De Kwaadsteniet M, Fraser T, Van Reenen CA, Dicks LMT.** 2006. Bacteriocin T8,  
488 a novel class IIa sec-dependent bacteriocin produced by *Enterococcus faecium* T8,  
489 isolated from vaginal secretions of children infected with human immunodeficiency  
490 virus. *Appl Environ Microbiol* **72**:4761–6.
- 491 28. **Altena K, Guder A, Cramer C, Bierbaum G.** 2000. Biosynthesis of the lantibiotic  
492 mersacidin: organization of a type B lantibiotic gene cluster. *Appl Environ Microbiol*  
493 **66**:2565–71.
- 494 29. **Iwatani S, Zendo T, Yoneyama F, Nakayama J, Sonomoto K.** 2007.  
495 Characterization and structure analysis of a novel bacteriocin, lacticin Z, produced by  
496 *Lactococcus lactis* QU 14. *Biosci Biotechnol Biochem* **71**:1984–1992.
- 497 30. **Fujita K, Ichimasa S, Zendo T, Koga S, Yoneyama F, Nakayama J, Sonomoto K.**  
498 2007. Structural analysis and characterization of lacticin Q, a novel bacteriocin  
499 belonging to a new family of unmodified bacteriocins of Gram-positive bacteria. *Appl*  
500 *Environ Microbiol* **73**:2871–2877.
- 501 31. **Ribeiro SC, O'Connor PM, Ross RP, Stanton C, Silva CCG.** 2016. An anti-listerial  
502 *Lactococcus lactis* strain isolated from Azorean Pico cheese produces lacticin 481. *Int*  
503 *Dairy J* **63**:18–28.
- 504 32. **Lozo J, Jovcic B, Kojic M, Dalgalarrrondo M, Chobert J-M, Haertlé T, Topisirovic**  
505 **L.** 2007. Molecular characterization of a novel bacteriocin and an unusually large  
506 aggregation factor of *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8, a natural  
507 isolate from homemade cheese. *Curr Microbiol* **55**:266–271.
- 508 33. **Lozo J, Vukasinovic M, Strahinic I, Topisirovic L.** 2004. Characterization and  
509 antimicrobial activity of bacteriocin 217 produced by natural isolate *Lactobacillus*  
510 *paracasei* subsp. *paracasei* BGBUK2-16. *J Food Prot* **67**:2727–34.
- 511 34. **Golić N, Čadež N, Terzić-Vidojević A, Šuranská H, Beganović J, Lozo J, Kos B,**  
512 **Šušković J, Raspor P, Topisirović L.** 2013. Evaluation of lactic acid bacteria and  
513 yeast diversity in traditional white pickled and fresh soft cheeses from the mountain  
514 regions of Serbia and lowland regions of Croatia. *Int J Food Microbiol* **166**:294–300.
- 515 35. **Kojic M, Strahinic I, Topisirovic L.** 2005. Proteinase PI and lactococcin A genes are  
516 located on the largest plasmid in *Lactococcus lactis* subsp. *lactis* bv. diacetylactis S50.  
517 *Can J Microbiol* **51**:305–14.

- 518 36. **Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate**  
519 **CM, Smith CP, Ward JM, Schrempf H.** 1985. Genetic manipulation of *Streptomyces*  
520 — A laboratory manual: The John Innes Foundation, Norwich.
- 521 37. **O’sullivan DJ, Klaenhammer TR.** 1993. Rapid Mini-Prep Isolation of High-Quality  
522 Plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl Environ Microbiol*  
523 **59**:2730–3.
- 524 38. **Holo H, Nes IF.** 1989. High-frequency transformation, by electroporation, of  
525 *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media.  
526 *Appl Environ Microbiol* **55**:3119–23.
- 527 39. **Rea MC, Clayton E, O’Connor PM, Shanahan F, Kiely B, Ross RP, Hill C.** 2007.  
528 Antimicrobial activity of lacticin 3147 against clinical *Clostridium difficile* strains. *J*  
529 *Med Microbiol* **56**:940–946.
- 530 40. **Mills S, Serrano LM, Griffin C, O’Connor PM, Schaad G, Bruining C, Hill C,**  
531 **Ross RP, Meijer WC.** 2011. Inhibitory activity of *Lactobacillus plantarum* LMG P-  
532 26358 against *Listeria innocua* when used as an adjunct starter in the manufacture of  
533 cheese. *Microb Cell Fact* **10 Suppl 1**:S7.
- 534 41. **Steinberg DA, Hurst MA, Fujii CA, Kung AH, Ho JF, Cheng FC, Loury DJ,**  
535 **Fiddes JC.** 1997. Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in  
536 vivo activity. *Antimicrob Agents Chemother* **41**:1738–42.
- 537 42. **Blanusa M, Perovic I, Popovic M, Polovic N, Burazer L, Milovanovic M,**  
538 **Gavrovic-Jankulovic M, Jankov R, Cirkovic Velickovic T.** 2007. Quantification of  
539 Art v 1 and Act c 1 being major allergens of mugwort pollen and kiwi fruit extracts in  
540 mass-units by ion-exchange HPLC-UV method. *J Chromatogr B* **857**:188–194.
- 541 43. **Gasson MJ.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other  
542 lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**:1–9.
- 543 44. **Diep DB, Skaugen M, Salehian Z, Holo H, Nes IF.** 2007. Common mechanisms of  
544 target cell recognition and immunity for class II bacteriocins. *Proc Natl Acad Sci U S*  
545 *A* **104**:2384–9.
- 546 45. **Kojic M, Fira D, Banina A, Topisirovic L.** 1991. Characterization of the cell wall-  
547 bound proteinase of *Lactobacillus casei* HN14. *Appl Environ Microbiol* **57**:1753–  
548 1757.
- 549 46. **Valenzuela AS, Omar N ben, Abriouel H, Lopez RL, Veljovic K, Canamero MM,**  
550 **Topisirovic MKL, Galvez A.** 2009. Virulence factors, antibiotic resistance, and  
551 bacteriocins in enterococci from artisan foods of animal origin. *Food Control* **20**:381–  
552 385.
- 553
- 554

555 **FIGURE LEGENDS**

556

557 **Figure 1.** Plasmid profile analysis of parental strain *L. lactis* subsp. *lactis* bv. diacetylactis  
558 BGBU1-4 and its cured derivatives on 1% agarose gel. M - the upper part of GeneRuler 1kb  
559 DNA ladder Thermo Fisher Scientific (from top to bottom: 10 kb, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5,  
560 1, 0.75 kb), chrom – indicates position of chromosomal DNA. Only plasmids of the selected  
561 cured derivatives are shown in the figure, taken from the different positions of the gel.

562 **Figure 2.** Reverse-phase high performance liquid chromatography chromatogram (A) (RP-  
563 HPLC) and Matrix laser desorption ionization time of flight (MALDI-TOF) mass  
564 spectrometry data (B, C). Arrow indicates location of the antimicrobial peptide.

565 **Figure 3.** Circular restriction map of plasmid pBU6. Only relevant restriction sites and their  
566 positions are indicated; unique restriction sites are indicated by bold letters. The position and  
567 orientation of the genes are indicated by arrows.

568 **Figure 4.** Alignment of lactolisterin BU (SDR48784) amino acid sequence with homologous  
569 bacteriocins: lacticin Q from *Lactococcus lactis* QU5 (BAF57910.1), lacticin Z from  
570 *Lactococcus lactis* QU14 (BAF75975.1), bacteriocin BHT-B from *Streptococcus rattii*  
571 (DQ145753.1), aureocin A53 from *Staphylococcus aureus* (AAN71834) and aureocin A53  
572 like bacteriocin from *Corynebacterium jeikeium* (WP\_010976360). Highlighted residues  
573 indicate conservation in at least five of the peptide sequences while an asterisk indicates  
574 completely conserved residues. 'x' corresponds to unconserved residues, periods indicate  
575 amino acids belonging to similar groups, and colons indicate amino acids belonging to the  
576 same group.

577 **Figure 5.** Lactolisterin BU levels in culture relative to the cell density of *Lactococcus lactis*  
578 subsp. *cremoris* MG7284/pBU6 tested on *L. monocytogenes* ATCC 19111. Filled squares  
579 represent bacterial growth measured by colony forming units (CFU) circles indicate

580 corresponding bacteriocin activity determined by area of zone inhibition. Error bars represent  
581 standard deviations of three independent experiments.



**Table 1.** Strains used in this study

Strains or plasmids	Relevant characteristic(s)	Source or reference
<i>Lactococcus lactis</i>		
BGBU1-4	Bacteriocin producer (lactolisterin BU)	(34)
BGBU1-4/2	Derivate of BGBU1-4; Bac <sup>+</sup> ; Bac <sup>s</sup>	This work
BGBU1-4/8	Derivate of BGBU1-4; Bac <sup>+</sup> ; Bac <sup>s</sup>	This work
BGBU1-4/29	Derivate of BGBU1-4; Bac <sup>-</sup> ; Bac <sup>s</sup>	This work
BGMN1-596	Bac <sup>-</sup> , Bac <sup>s</sup>	(20)
MG7284	Bac <sup>-</sup> , Bac <sup>s</sup>	(43)
MG7284/pBU6	MG7284 transformed with pBU6 plasmid (producer of lactolisterin BU)	This work
B464	Man-PTS deletion mutant of strain IL1403	(44)
<i>Lactobacillus casei</i> BGHN14	Bac <sup>-</sup> , Bac <sup>s</sup>	(45)
<i>Listeria monocytogenes</i>		ATCC19111
<i>Staphylococcus aureus</i>		ATCC 25923
<i>Enterococcus faecalis</i>		ATCC 29212
<i>Enterococcus faecalis</i> BGZLS10-27		(46)
<i>Bacillus cereus</i>		ATCC 11778
<i>Bacillus subtilis</i> subsp. <i>subtilis</i>		ATCC23857
<i>Streptococcus pyogenes</i> A2941		Pasteur laboratory, Belgrade
<i>Streptococcus pneumoniae</i> P156		Pasteur laboratory, Belgrade
<i>Escherichia coli</i> H7:O157		ATCC 35150
<i>Pseudomonas aeruginosa</i>		ATCC 27853
<i>Salmonella</i> Typhimurium		ATCC 14028
<i>Salmonella</i> Enteritidis		ATCC 13076

Bac<sup>+</sup> = bacteriocin producer, Bac<sup>-</sup> = non-bacteriocin producer, Bac<sup>s</sup> = sensitivity to lactolisterin BU; ATCC= American Type Culture Collection, Manassas, VA, USA



**Table 2.** Results of BLAST comparison of proteins encoded by plasmid pBU6 with Entrez protein database. The number of amino acids and the position from the beginning of the sequence of plasmid pBU6 are given for each ORF.

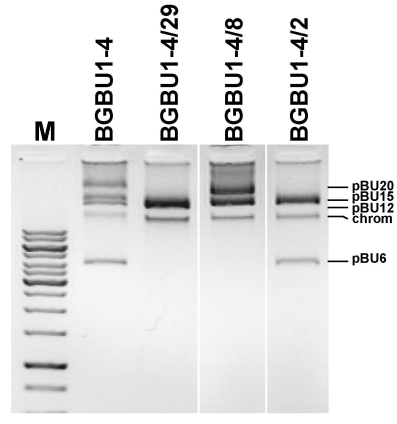
<i>orf</i> (No. of amino acids) position*	Proteins with the highest identity, accession number	Predicted domain(s) or superfamily in encoded ORF	Organism	Amino acid identity (%)
<b>RepB</b> (278 aa) 80-916	RepB, WP_011117039.1 RepB, WP_032951507.1	Rep_3 (pfam01051)	<i>Lactobacillus fermentum</i> <i>Lactococcus lactis</i>	90% 85%
<b>LiBU</b> (43 aa) 1034-1165	Aureocin-like bacteriocin, AAZ76605.1	Bacteriocin_iii (pfam11758)	<i>Streptococcus ratti</i>	63%
<b>AbcT</b> (212 aa) 1230-1868	Sugar ABC transporter ATP-binding protein, WP_003089811.1	ABC_DR_subfamily_A (CD03230)	<i>Streptococcus ratti</i>	62%
<b>Hyp1</b> (212 aa) 1861-2449	Hypothetical protein, WP_003089809.1	/	<i>Streptococcus ratti</i>	42%
<b>Hyp2</b> (169 aa) 2503-3012	Hypothetical protein, AAZ76608.1	/	<i>Streptococcus ratti</i>	43%
<b>Hyp3</b> (92 aa) 3531-3809	Hypothetical protein WP_027822861.1	/	<i>Lactobacillus plantarum</i>	51%
<b>MobC</b> (127 aa) 3829-4212	Mobilization protein C, WP_010729194.1 Hypothetical protein WP_011117545.1	MobC (pfam05713) /	<i>Enterococcus faecium</i> <i>Enterococcus faecalis</i>	41% 55%
<b>RelM</b> (333 aa) 4194-5195	Relaxase/mobilization nuclease, WP_010730379.1	Relaxase (pfam03432)	<i>Enterococcus faecium</i>	51%
<b>RNaseY</b> (169 aa) 5219-5728	Ribonuclease Y, WP_017865030.1	Phosphodiesterase (PRK12704)	<i>Lactococcus lactis</i>	29%

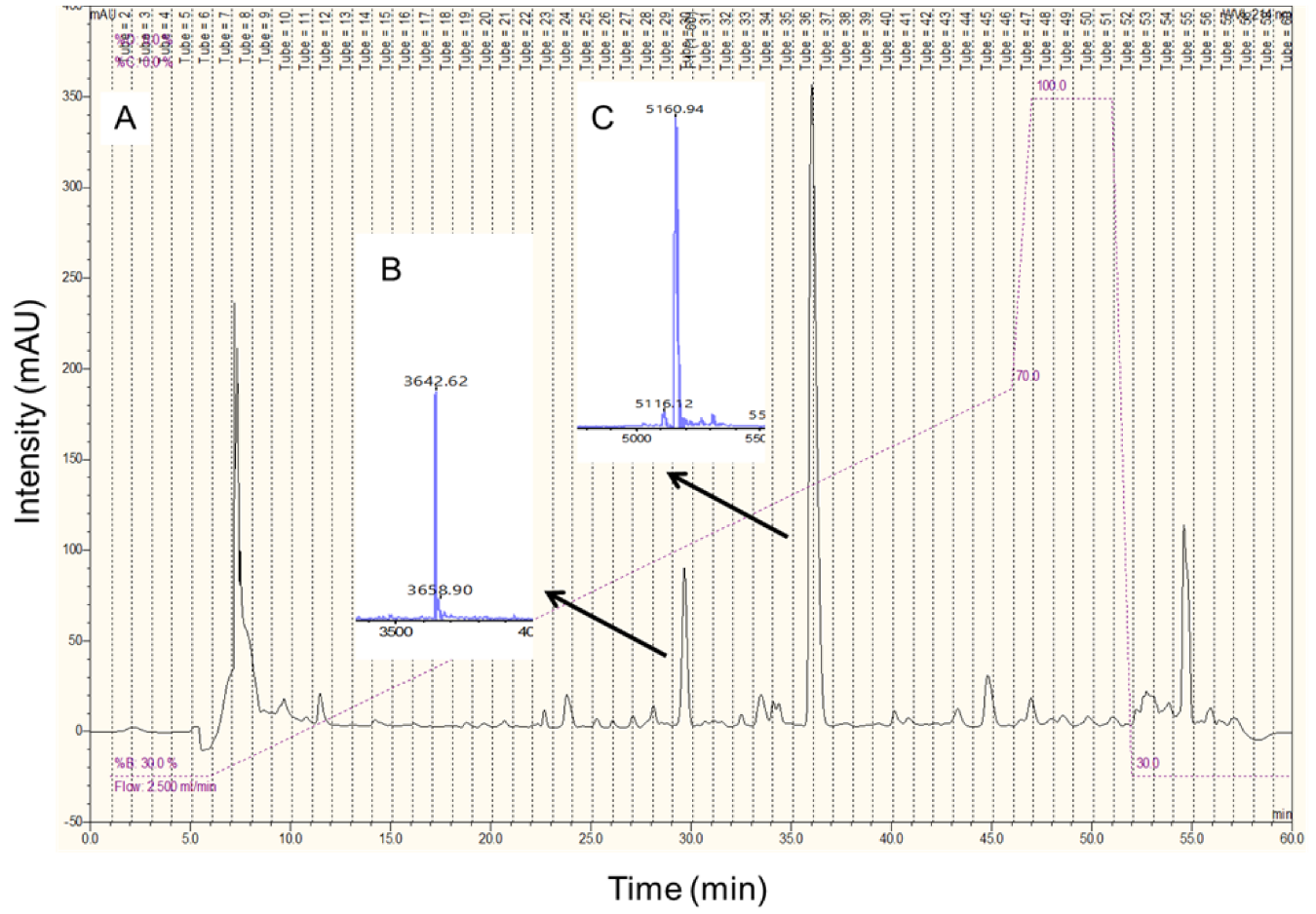
aa = amino acid, \* = position of gene on plasmid pBU6

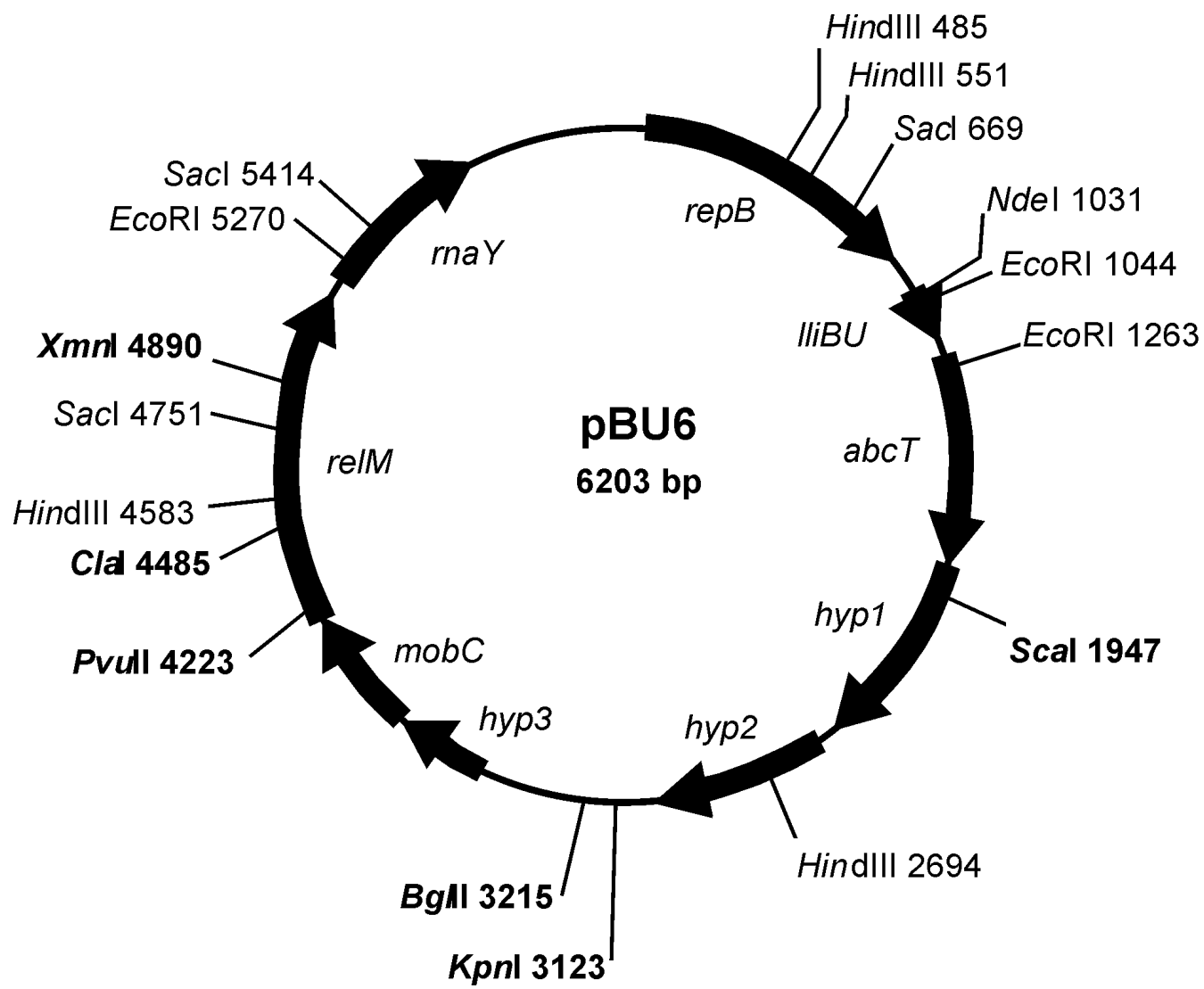
**Table 3.** Antimicrobial spectra of purified lactolisterin BU

Indicator strains	MIC ( $\mu$ M)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> BGMN1-596	0.67
<i>Lactococcus lactis</i> subsp. <i>lactis</i> BGBU1-4	1.34
<i>Lactococcus lactis</i> subsp. <i>lactis</i> BGBU1-4/2	1.34
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG7284/pBU6	1.34
<i>Lactobacillus casei</i> BGHN14	0.67
<i>Listeria monocytogenes</i> ATCC 19111	1.34
<i>Staphylococcus aureus</i> ATCC 25923	0.67
<i>Enterococcus faecalis</i> ATCC 29212	1.34
<i>Enterococcus faecalis</i> BGZLS10-27	1.34
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> ATCC23857	5.375
<i>Bacillus cereus</i> ATCC 11778	5.375
<i>Streptococcus pyogenes</i>	0.67
<i>Streptococcus pneumoniae</i>	0.67
<i>Escherichia coli</i> H7:O157 ATCC 35150	N.A.
<i>Pseudomonas aeruginosa</i> ATCC 27853	N.A.
<i>Salmonella</i> Typhimurium ATCC 14028	N.A.
<i>Salmonella</i> Enteritidis ATCC 13076	N.A.

N.A.- no activity







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Lactolisterin BU      --MWGRILGTVAKYGPKAVSWAQHKWE-LI--NMG---DLAFRYIQRIWG--
Lacticin Q            MAGFLKVVQLLAKYGSKAVQAWANKGKILDWLNAGQAIWVSKIKQILGIK
Lacticin Z            MAGFLKVVQILAKYGSKAVQAWANKGKILDWINAGQAIWVVEKIKQILGIK
Bacteriocin BHT-B    --MWGRILAFVAKYGTKAVQAWKNKWFL---SLG---EAVFDYIRSIWGG-
Aureocin A53         -MSWLNFLKYIAKYGKKAVSAWKYKGVLEWLVNVPTEWVWQKLKKIAGL-
Aureocin A53 like    MAGFLKVVKAVAKYGSKAVKWCWDNKGKILEWLVNIGMAVDWIVEQVRKIVGA-
                      : ..: :**** *. . * :* ::* : . :: * *
Identity consensus   xxxxxxxxxxxxAKYGxKAVxxxWxxKxxxLxxxxxGxxxxxxxxxxxxIxGxx

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