

Applicability of helveticin 34.9, a class III bacteriocin produced by *Lactobacillus helveticus* 34.9

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Introduction

Lactobacillus helveticus 34.9 was isolated from a Romanian home-made fermented milk. This strain has been shown to inhibit the growth of other bacteria, including other lactic acid bacteria, (potential) pathogenic bacteria, and a strain of *Halobacillus hunanensis* isolated from the degraded wall of Humor monastery (Romania). The present study aimed to identify the nature of inhibition, to isolate the inhibitory compound, and to characterize it, in terms of production under different environmental conditions, stability, mode of action, and potential applications.

Antibacterial activity assessment

Antibacterial activity of *Lb. helveticus* 34.9 grown in different conditions against *Lb. delbrueckii* subsp. *bulgaricus* LMG6901^T

Growth conditions	37°C		42°C	
	OD _{600nm}	Inhibitory activity (AU/ml)	OD _{600nm}	Inhibitory activity (AU/ml)
Control	3.8	200	2.3	100
Initial pH 4.5	1.5	200	0.7	<100
2% NaCl	3.4	800	2.8	100
0.1% BS	0.9	200	0.4	200
0.2% BS	0.3	800	0.2	<100
2% BS	0.2	100	0.2	<100
pH 4.5 + 2% NaCl	0.9	3,200	0.3	1,600
pH 4.5 + 0.2% BS	0.1	800	0.1	400
2% NaCl + 0.2% BS	0.4	3,200	0.2	3,200

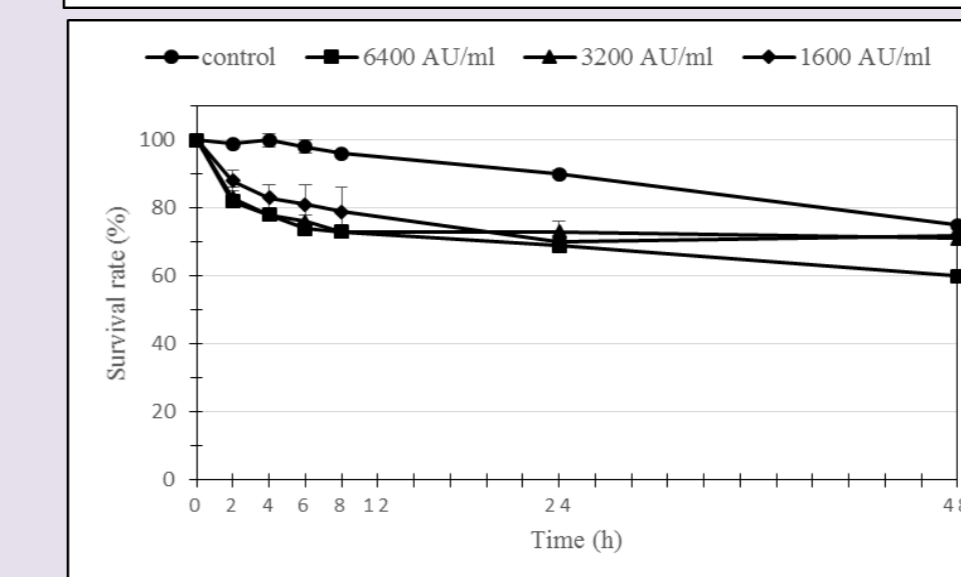
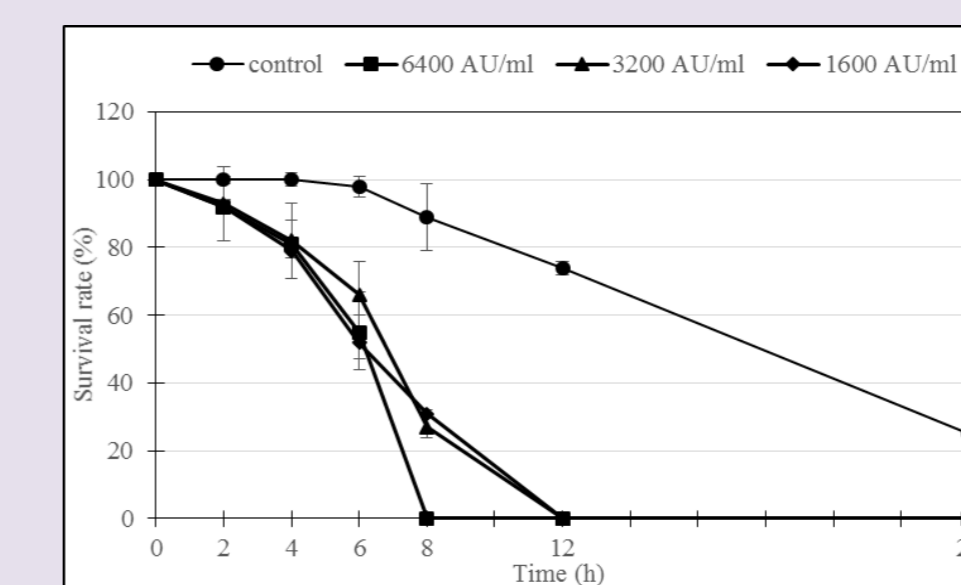
Bacteriocin isolation and characterization

Bacteriocin activity determined by agar spot method, after various treatments

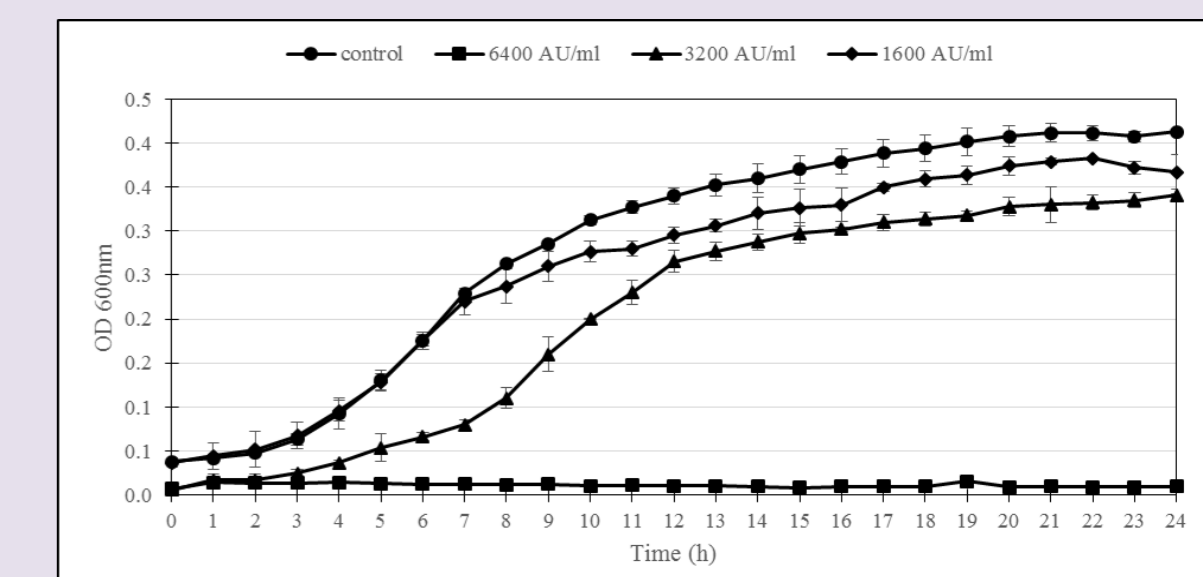
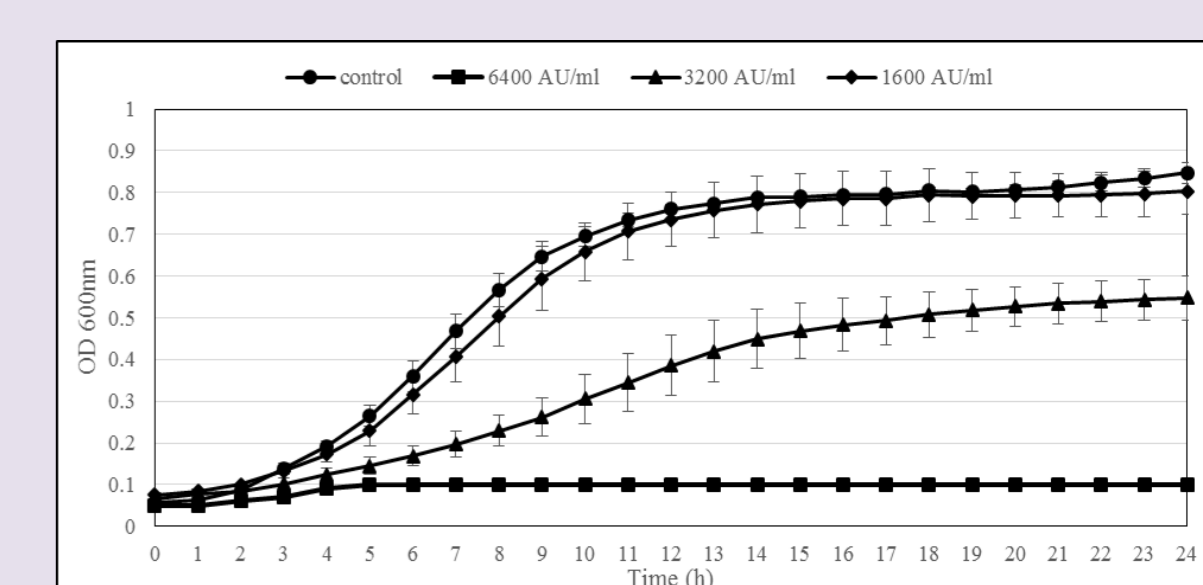
Treatment	Bacteriocin activity (AU/ml)
CFCS*	3,200
Ammonium sulphate precipitation (Crude bacteriocin - Control)	25,600
pH 2.0	6,400
pH 4.0	25,600
pH 6.0	25,600
pH 8.0	6,400
pH 10.0	3,200
60°C 15 min	400
30 min	200
60 min	100
100°C 15 min	0
30 min	0
60 min	0
Chloroform/methanol extraction	6,400

Mode of action

Decrease of the survival rate of *L. delbrueckii* subsp. *bulgaricus* LMG6901^T (a) and *H. hunanensis* 5Hum (b) in the presence of helveticin 34.9



Growth of *L. delbrueckii* subsp. *bulgaricus* LMG6901^T (a) and *H. hunanensis* 5Hum (b) in the presence of helveticin 34.9



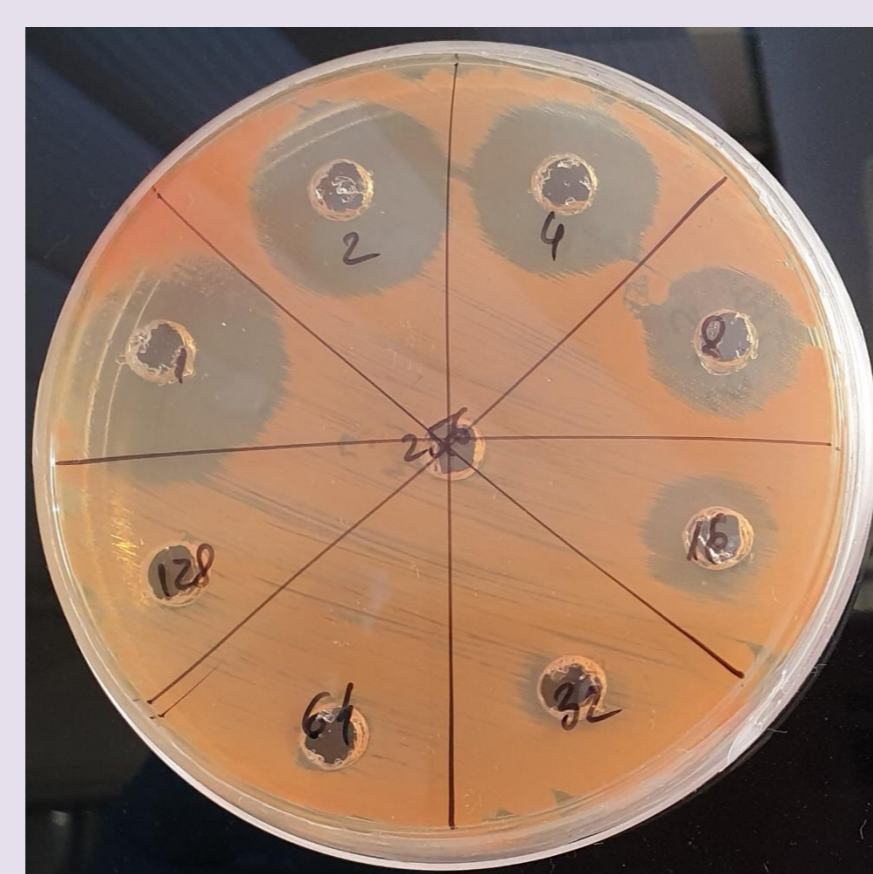
Results

- The highest production and inhibitory activity were detected in the presence of combined stress conditions;
- The antibacterial activity is due to a high molecular mass (about 35 KDa) protein, as shown by Tricine-SDS-PAGE;
- The bacteriocin was extracted after bacterial cultivation in MRS broth (pH 4.5) supplemented with 2% NaCl;
- Helveticin 34.9 is stable at pH between 2.0 and 10.0, with a slight activity decrease at pH above 8.0; it is highly sensitive to heat and proteinase K treatment;
- Helveticin 34.9 inhibits the growth of strains belonging to *L. delbrueckii*, *L. helveticus*, *L. sakei*, *E. faecium*, *B. subtilis*, *B. cereus*, *S. aureus*, and *Halobacillus hunanensis* species;
- The mode of action and degree of inhibition depended on the indicator strain used. Helveticin 34.9 killed *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T cells from a suspension within 8 h, but it only reduced to 60% the viability of *H. hunanensis* 5Hum cells.
- When the bacteriocin was added to the growth medium prior inoculation with the indicator strain, it prevented the bacterial growth of both strains.
- Helveticin 34.9 induced morphological changes in the sensitive strains, more severe in the case of *L. delbrueckii* subsp. *bulgaricus*, as observed by scanning electron microscopy.

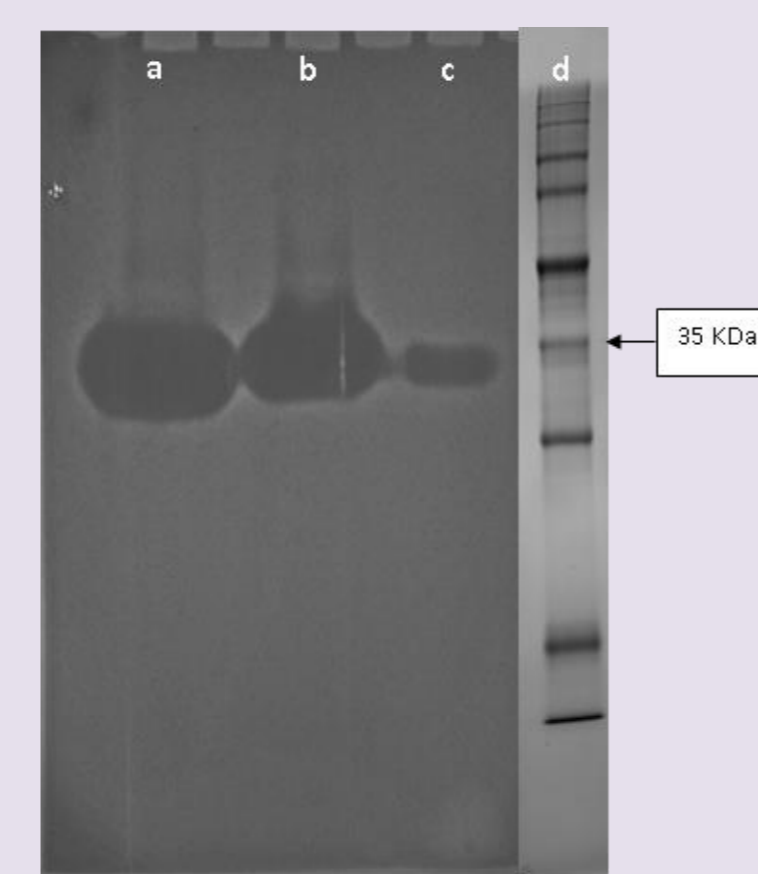
Inhibitory spectrum of the bacteriocin produced by *Lb. helveticus* 34.9

Indicator strain	Growth medium	Growth temperature	Inhibition zone* (mm)
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> LMG 6901 ^T	MRS broth	37 °C	11.8 ± 0.1
<i>Lactobacillus helveticus</i> 102	MRS broth	37 °C	10.4 ± 0.2
<i>Lactobacillus helveticus</i> 122	MRS broth	37 °C	n.i.
<i>Lactiplantibacillus plantarum</i> P26	MRS broth	37 °C	n.i.
<i>Enterococcus faecalis</i> LMG13566	MRS broth	37 °C	n.i.
<i>Enterococcus faecalis</i> LMG16216	MRS broth	37 °C	n.i.
<i>Enterococcus faecium</i> LMG14203	MRS broth	37 °C	8.5 ± 0.1
<i>Leuconostoc mesenteroides</i> LMG13562	MRS broth	28 °C	n.i.
<i>Latilactobacillus sakei</i> LMG13558	MRS broth	37 °C	10.8 ± 0.2
<i>Pediococcus pentosaceus</i> LMG13561	MRS broth	30 °C	n.i.
<i>Salmonella enterica</i> ATCC 14028	BHI	37 °C	n.i.
<i>Bacillus subtilis</i> B17	BHI	30 °C	8 ± 0.2
<i>Bacillus cereus</i> CBAB	BHI	30 °C	7 ± 0.2
<i>Escherichia coli</i> ATCC 25922	BHI	37 °C	n.i.
<i>Staphylococcus aureus</i> ATCC 25923	BHI	37 °C	8.4 ± 0.2
<i>Listeria monocytogenes</i> ATCC 1911	BHI	37 °C	n.i.
<i>Halobacillus hunanensis</i> 5Hum	MH (10% NaCl)	30 °C	12.6 ± 0.3

Antagonistic effect of helveticin 34.9 against *H. hunanensis* 5Hum

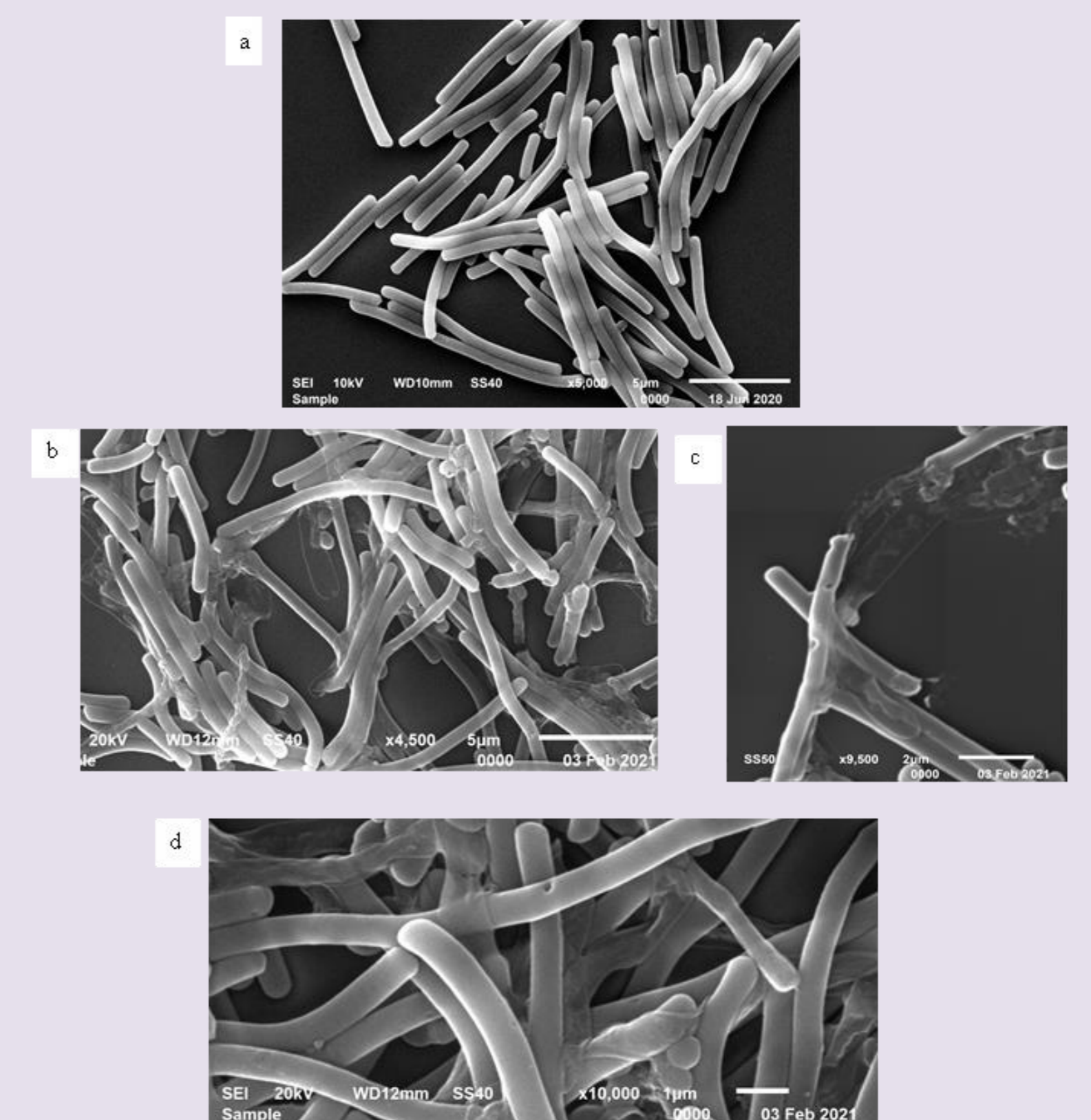


Molecular mass estimation by Tricine-SDS-PAGE

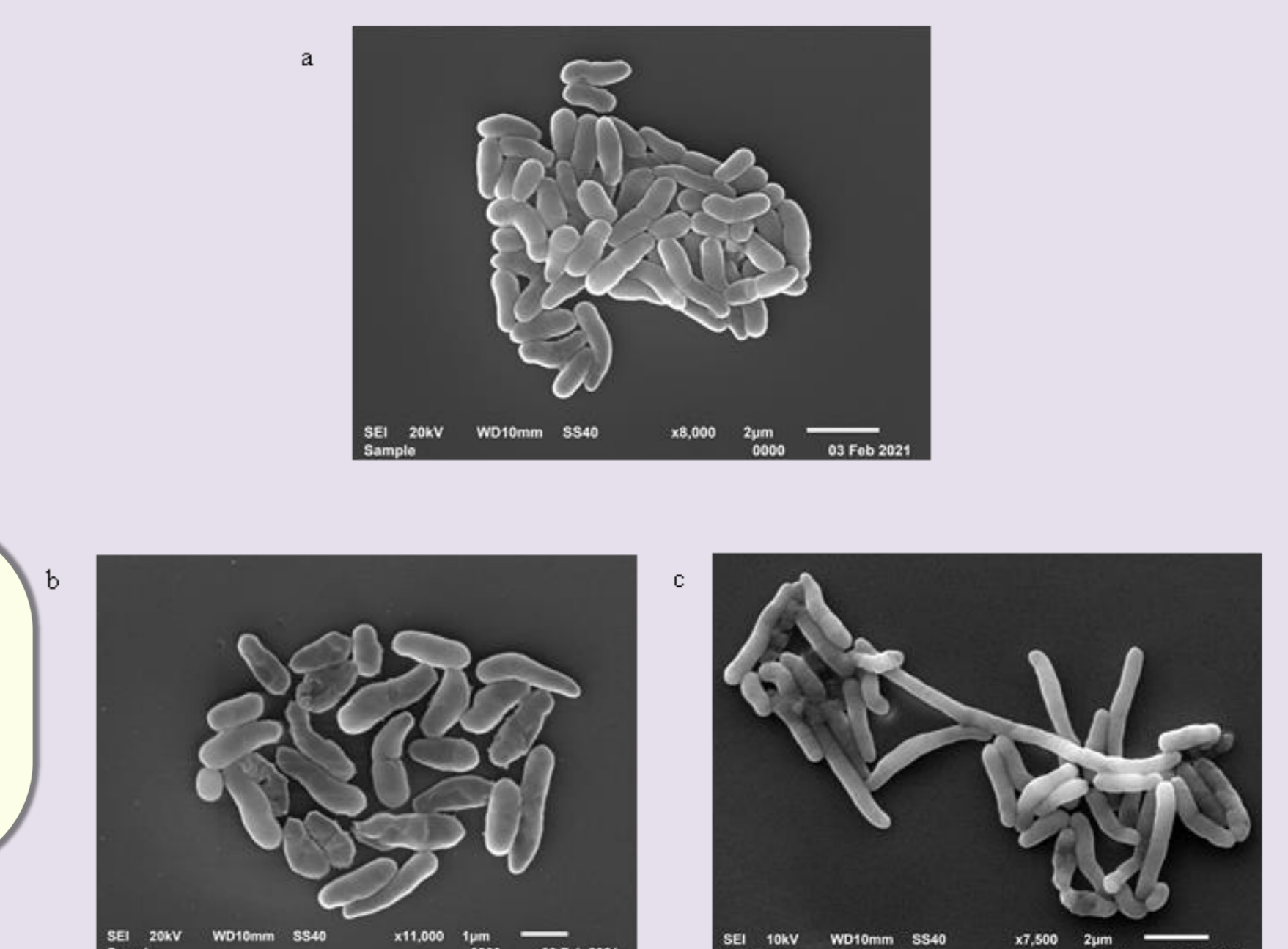


- 1 - cell free supernatant;
- 2 - ammonium sulphate precipitate;
- 3 - chloroform/methanol extract
- 4 - MW markers

Scanning electron microscopy of *L. delbrueckii* subsp. *bulgaricus* LMG6901^T: control cells (a); morphological changes of the sensitive cells induced by bacteriocin treatment (b-d)



Scanning electron microscopy of *Halobacillus hunanensis* 5Hum: control cells (a); morphological changes of the sensitive cells induced by bacteriocin treatment (b-c)



Conclusion

The present study opens new perspectives for the applicability of LAB bacteriocins, including in the non-food area. Results are of great importance given that class III bacteriocins is much less studied compared with the other classes, due to the narrow inhibitory spectrum and limited application.