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EXPLORING THE BACTERIOCINOGENIC PROPERTIES OF *Lactococcus lactis* R152 ISOLATED FROM A TRADITIONALLY MADE CHEESE

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Abstract

Lactococcus lactis R152 was isolated from a traditionally made cheese and identified through 16S rRNA gene sequencing. This strain exhibited antibacterial activity against five bacterial strains, including four potential pathogenic strains. In this study, we investigated the nature of the inhibition and characterized the active compound. The pH neutralization of the culture supernatant did not affect the inhibitory activity against Lactobacillus delbrueckii subsp. bulgaricus LMG6901^T, but this was lost after proteinase K treatment, indicating that the antibacterial compound is proteinaceous, likely a bacteriocin. Ammonium-sulfate precipitation of the culture supernatant increased the activity from 1,600 AU/ml to 12,800 AU/ml. Tricine-SDS-PAGE analysis indicated an estimated molecular mass of the presumptive bacteriocin of less than 6,500 Da. This compound was resistant to heat (preserving activity after autoclaving) and to pH variation in the pH 1 and 9 interval. These findings, combined with the identification of nisin A gene in the bacterial genome, suggest that Lactococus lactis R152 produces nisin A, a bacteriocin with potential applications in the food industry.

Key words*: antimicrobial activity, bacteriocin, food-borne pathogens, Lactococcus lactis.*

INTRODUCTION

The increasing prevalence of antibiotic-resistant bacteria poses a significant threat to global health, driving the search for novel antimicrobial agents (WHO, 2023). One promising avenue of research lies in the exploration of naturally occurring antimicrobials produced by microorganisms, particularly by lactic acid bacteria (LAB). Because food fermentation relies heavily on LAB, these bacteria have a well-established history of safe use in food production. Moreover, they can inhibit bacteria responsible for food spoilage or pathogenic bacteria derived from food. Several metabolites produced by LAB, such as organic acids, bacteriocins, hydrogen peroxide, reuterin, diacetyl, are involved in this activity (Ibrahim et al., 2021). Among the inhibitory compounds, bacteriocins stand out as a particularly promising group by enhancing the ability of LAB to limit the growth of undesirable bacteria, especially in food products (Dal Bello et al., 2012; Kondrotiene et al., 2018). Many LAB have been shown to produce bacteriocins, which usually exhibit antimicrobial activity against closely related bacterial species (Simons et al., 2020). Among LAB, *Lactococcus lactis* gained considerable reputation due to its widespread use in dairy fermentations and its ability to produce nisin, a well-characterized bacteriocin with broad-spectrum antimicrobial activity (Liu et al., 2021). Discovered in 1947 by Mattick and Hirsch (Mattick & Hirsch, 1947) from a strain of *Lactococus lactis*, nisin is a class I lantibiotic (Wayah & Philip, 2018) and it was approved by the EU, WHO, and FDA to be used in over 48 countries for the preservation of food, especially in cheese production (Favaro et al., 2015; Singh, 2018). Nisin has a bactericidal mode of action against many Gram-positive bacteria, such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus*, and *Listeria monocytogenes*, among others. It works by disrupting the bacterial cell membrane and interfering with cell wall

synthesis in the sensitive strains (Tavares et al., 2024). In contrast, Gram-negative bacteria demonstrate significantly greater tolerance to nisin, primarily due to their less permeable cell walls (Gong et al., 2018). Along with its applications in food preservation, nisin is also being explored for its potential therapeutic uses, including its role in combating antibiotic-resistant bacteria (Field et al., 2016). Overall, nisin is a valuable tool in food safety and preservation, contributing to the reduction of foodborne illnesses and enhancing the quality of various food products (Dal Bello et al., 2012; Kondrotiene et al., 2018).

Traditional fermented foods (such as fermented vegetables, fermented dairy products or fermented cereals - *bors*), particularly those produced using artisanal methods, represent a rich reservoir of diverse microbial communities, potentially harboring novel LAB strains with unique functional properties (Grosu-Tudor et al., 2014; Grosu-Tudor S. & Zamfir M., 2014).

Lact. lactis R152 was isolated from a traditionally made cheese from Romania and was selected for further investigation due to its observed antimicrobial activity. This paper presents the results of a study investigating the antibacterial activity of *Lact. lactis* R152 and the characterization of the antibacterial compound, aiming to assess its potential for applications in food preservation and as a possible alternative to conventional antimicrobials.

MATERIALS AND METHODS

Bacterial strains and growth media

Lact. lactis R152 was isolated from an artisanal sheep cheese sourced from Romania and identified through 16S rRNA gene sequencing (results not published). The antibacterial activity of this strain was tested against another LAB strain, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901^T, and against six potential pathogenic bacteria: *Listeria monocytogenes* ATCC1911, *Escherichia coli* ATCC25922, *Salmonella enterica* ATCC14028, *Bacillus cereus* CBAB, *Bacillus subtilis* ATCC6633, and *Staphylococcus aureus* ATCC25923. All strains were preserved at -80°C in the presence of glycerol (25%). LAB strains were grown in MRS medium (De Man et al., 1960), while the others in BHI (Brain Heart Infusion) medium (Carl Roth GmbH, Germany). For solid media, 1.5% agar was added to the broth, while for the indicator top-layer medium, 0.7% agar was added.

Antibacterial activity of *Lact. lactis* R152

Evaluation of antimicrobial effect

The inhibitory activity against the seven bacterial strains mentioned above, was initially evaluated by using the spot-on-the-lawn method (Lewus & Montville, 1991). Briefly, 10 µl of an overnight culture of *Lact. lactis* R152 were spotted onto the surface of MRS agar and incubated at 28°C to allow bacterial growth in the spots. A thin top-layer containing 100 µl of overnight indicator culture inoculated in 5 ml of MRS/BHI supplemented with 0.7% agar was then added and further incubated for 24 h. The antibacterial activity was indicated by the presence of a clear halo around the spots of *Lact. lactis* R152.

Bacteriocinogenic activity of Lact. lactis R152

To investigate the nature of the inhibition, the supernatant obtained from an overnight culture of *Lact. lactis* R152 was further used. After removing the bacterial cells by centrifugation (13000 x g, 10 min, 4°C), the pH of the supernatant was adjusted to approximately 6.5-7.0, to eliminate any inhibition caused by organic acids. The antibacterial activity was then assessed using the agar spot method (De Vuyst et al., 1996). In this method, the indicator top-layer was poured onto the solid medium, allowed to solidify, and then 10 µl of the neutralized supernatant were spotted on top.

Additionally, the activity of the neutralized supernatant was determined after treatment with proteinase K (Carl Roth, Karlsruhe, Germany), for 1 h, at 65°C. This test would allow to establish if the inhibition was due to a proteinaceous compound.

Concentration and quantification of the putative bacteriocin

Ammonium sulphate was added to the neutralized cell-free supernatant to 40% saturation and allowed to precipitate overnight with a gentle agitation. The resulting precipitate, along with the floating pellicle, was then dissolved in

5 mM potassium phosphate buffer, pH 6.5, to yield the crude bacteriocin.
Antibacterial activity was quantitatively measured against *L. delbrueckii* subsp. *bulgaricus* using the agar spot method, as described elsewhere (De Vuyst et al., 1996). One unit of activity (AU/ml) was defined as the reciprocal of the highest dilution that shows inhibition.

Characterization of the putative bacteriocin

Inhibition of (potential) pathogenic bacteria

The inhibitory activity of the crude bacteriocin was evaluated against the indicator strains used in the initial screening. The well diffusion method (Valgas et al., 2007) was employed, as it allows the use of larger sample volumes. Briefly, the indicator strains were inoculated onto BHI agar medium using sterile cotton swabs. After inoculation, wells were cut in the medium and filled with 50 µl of the crude bacteriocin. Plates were left for about 30 min to allow the bacteriocin to diffuse into the medium, then incubated for 24 h at the optimal growth temperature of each indicator.

Influence of pH and temperature on the inhibitory activity of the crude bacteriocin

The pH stability of the inhibitory compound was assessed by adjusting the pH to various values ranging from 1 to 9, and testing after one hour of treatment.
To evaluate the heat stability, the crude bacteriocin sample was incubated at different temperatures, as shown in Table 3. The residual activity was quantitatively determined by the agar spot method.

Molecular mass

The molecular mass of the putative bacteriocin was estimated by Tricine-SDS-PAGE (Tricine sodium dodecylsulphate-polyacrylamide gel electrophoresis), according to Schägger and von Jagow (1987). Electrophoresis was performed in a Biometra Minigel Twin (Biometra, Germany). The gel was then thoroughly washed for 5 h with sterile ultrapure water and covered with a top-layer, containing *L. delbrueckii* subsp. *bulgaricus* LMG6901^T. Molecular mass was estimated in comparison with polypeptide SDS-PAGE molecular weight standards (BioRad Laboratories, Hercules CA, USA).

Genetic screening

Pure Link Genomic DNA kit (Invitrogen, MA, USA) was used for DNA extraction from *Lact. lactis* R152 cells. PCR reactions were conducted with the extracted DNA, using specific primers (Table 1) for the genes responsible for encoding nisin A (Li & O'Sullivan, 2002), lactococcin 972 (Alegría et al., 2010), and plantaricin A (Diep et al., 1996). The reaction mixture and PCR protocol were previously detailed (Zamfir et. al, 2016). Amplification was carried out in a Mastercycler pro S (Eppendorf, Germany). The amplified products were visualized by agarose gel electrophoresis.

Table 1. List of primers and annealing temperatures

Primer	Sequence (5'-3')	Annealing temperature (°C)
NisA-F	GGATAGTATCCATGTCTG	54
NisA-R	CAATGATTTCGTTCGAAG	
plnA-F	GTACAGTACTAATGGGAG	54
plnA-R	CTTACGCCAATCTATACG	
Lcn972-F	TTGTAGCTCCTGCAGAAG GAACATGG	50
Lcn972-R	GCCTTAGCTTTGAATTCTT ACCAAAAG	

RESULTS AND DISCUSSION

Antibacterial activity of *Lact. lactis* R152

When testing the bacterial culture, *Lact. lactis* R152 showed a clear inhibition against *L. delbrueckii* LMG6901^T and against four of the (potential) pathogenic strains used as indicators (Table 2).
The largest inhibition zone was observed against the *Listeria monocytogenes* strain (approximately 50 mm in diameter) and *L. delbrueckii* subsp. *bulgaricus* LMG6901^T (around 40 mm). Inhibition zones greater than 30 mm in diameter were also observed against indicator strains belonging to *E. coli*, *S. enterica,* and *B. cereus* species. However, when the neutralized cell-free supernatant was tested, the inhibitory activity was only detected against *L. delbrueckii* LMG6901^T (results not shown).

Table 2. Antibacterial activity of *Lact. lactis* R152 against the bacterial strains used as indicators

Indicator strain	Diameter of the inhibition zone (mm)*
L. delbrueckii subsp. *bulgaricus* LMG6901^T	40
Listeria monocytogenes ATCC1911	50
Escherichia coli ATCC 25922	35
Salmonella enterica ATCC 14028	32
Bacillus cereus CBAB	33
Bacillus subtilis ATCC 6633	ni
Staphylococcus aureus ATCC 25923	ni

*ni = no inhibition

Furthermore, no inhibition of the supernatant was shown after proteinase K treatment (results not shown). These findings suggest that the antibacterial activity of *Lact. lactis* R152 against (potential) pathogenic strains is primarily due to the production of organic acids, while the activity against *L. delbrueckii* LMG6901^T is due to other compounds, most probably a bacteriocin.

In general, bacteriocins have a narrow inhibitory spectrum, typically affecting bacterial species closely related to the producing bacteria (Putri et al., 2024), while organic acids exert a more broad-spectrum, and non-specific antimicrobial effects. Various bacteria, but also yeasts and molds that contaminate food, are sensitive to the organic acids produced by LAB (Ibrahim et al., 2021). For instance, the organic acids produced by *Lactococcus lactis* may prevent contamination of food with *E. coli* and many spoilage bacteria (Alizadeh Behbahani & Noshad, 2024; Maaty et al., 2025; Sanca et al., 2023).

Characterization of the putative bacteriocin

The inhibitory activity of the culture supernatant was determined to be 1,600 AU/ml. Ammonium sulphate precipitation resulted in an increase of activity to 12,800 AU/ml (Table 3). A mild inhibitory activity of the crude bacteriocin was detected against *B. cereus* CBAB, through the agar well method (Figure 1), suggesting a combined mechanism of action against this particular indicator strain.

Table 3. Influence of pH and heat treatment on the inhibitory activity of the bacteriocin produced by *Lact. lactis* R152

Treatment		Inhibitory activity (AU/ml)
Cell free supernatant		1,600
Crude bacteriocin		12,800
60°C	10 min	12,800
	30 min	6,400
	60 min	6,400
100°C	10 min	6,400
	30 min	6,400
	60 min	6,400
121°C	15 min	3,200
pH 1		12,800
pH 3		12,800
pH 5		12,800
pH 7		12,800
pH 9		3,200

Figure 1. Antibacterial activity of the crude bacteriocin produced by *Lact. lactis* R152 against *Bacillus cereus* CBAB

It has been shown that *Lactococcus lactis* is able to produce several bacteriocins, including nisin, lacticin, and lactococcin. These antibacterial peptides have a broad spectrum of activity, including various spoilage or pathogenic bacteria that may develop in food (Negash & Tsehai, 2020; Putri et al., 2024). However, this broad spectrum of activity was not confirmed in our study.

The crude bacteriocin was further incubated at different temperatures and pH values, and the results are summarized in Table 3. As shown, the activity remained high (6,400 AU/ml) after heating for 1 h at 60°C and 100°C.

Additionally, 25% of the initial activity was retained even after autoclaving at 121°C for 15 min, indicating a high heat stability of the inhibitory compound. The compound also demonstrated remarkable stability to pH changes. The activity remained constant (12,800 AU/ml) between pH 1 and pH 7, with a decrease to 3,200 AU/ml only observed at an alkaline pH of 9.

The molecular mass of the crude bacteriocin was estimated using Tricine-SDS-PAGE. After electrophoresis, when the gel was overlaid with a top-layer inoculated with the indicator strain, a clear inhibition zone was detected, corresponding to a protein band of less than 6.5 KDa (Figure 2).

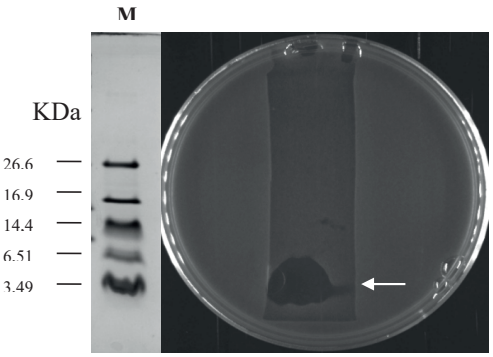

Figure 2. Estimation of the molecular mass of the bacteriocin produced by *Lact. lactis* R152. The protein band corresponding to the bacteriocin is indicated by the arrow

Given this very low molecular mass (< 6.5 KDa), and high stability at pH variations and at elevated temperatures, the bacteriocin produced by *Lact. lactis* R152 can be classified as a class I bacteriocin, which includes nisin, among others (Nes et al., 1996). This remarkable stability under different pH conditions and at high temperatures could offer a technological advantage for its potential use as a preservative in the food industry, (Grosu-Tudor et al., 2014).

Genetic screening

Using primers targeting genes encoding known bacteriocins, a specific amplification was observed for the nisin A gene, but no amplification occurred for lactococcin or plantaricin (Figure 3). The amplicon corresponded to the expected size of 298 bp. This confirms that *Lact. lactis* R152 possesses the genetic material required for nisin synthesis.

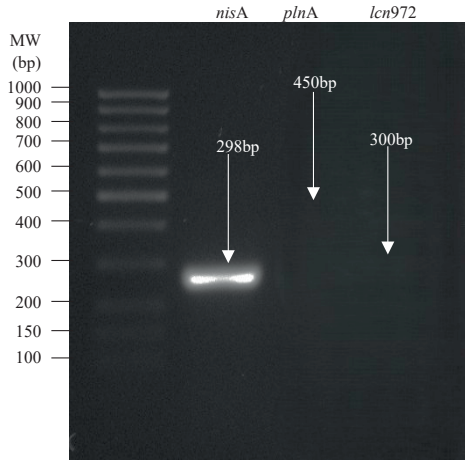

Figure 3. Amplification of genomic DNA extracted from *Lact. lactis* R152 using specific primers for *nis*A gene. First arrow indicates the amplified product of nisA, corresponding to about 298 bp; no specific amplicons were obtained for *pln*A and *lcn*972.

CONCLUSIONS

Based on our findings, we can conclude that *Lact. lactis* R152, isolated from artisanal sheep cheese, produces a small bacteriocin, most likely nisin A. This bacteriocin demonstrated excellent stability at high temperatures and at pH variations, making it promising for use in food preservation. Additionally, the antibacterial activity of *Lact. lactis* R152 against other bacteria may also be attributed to the production of organic acids.

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