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CHEMICAL CHARACTERISTICS AND FUNCTIONAL CONTRIBUTIONS OF *ENTEROCOCCUS* SPP. ISOLATED FROM RAW GOAT CHEESE: METABOLITE PRODUCTION AND ENZYMATIC ACTIVITY

Mirjana Ž. Grujović[1], Katarina G. Marković[1], Teresa Semedo-Lemsaddek[2,3,4], Jelena M. Mašković[5]*

Abstract: This study investigated the enzymatic activity and metabolite production potential of autochthonous *Enterococcus* strains. The isolates demonstrated extracellular proteinase activity, curd formation, acidification ability, and diacetyl production, highlighting their potential role in cheese fermentation. Additionally, some strains produced bacteriocins, inhibiting the growth of indicator bacteria, suggesting their possible application in food preservation. These findings contribute to understanding the technological potential of *Enterococcus* in dairy fermentation and its implications for food safety and quality.

Keywords: *Enterococcus*, extracellular proteinases, biogenic amines, bacteriocins, dyacetil

Introduction

Lactic acid bacteria (LAB) play a crucial role in dairy fermentation, contributing to the texture, flavor, and safety of fermented products (Grujović *et al*., 2024a). Among LAB, *Enterococcus* species are frequently found in raw milk and traditional cheeses, where they influence both technological and microbiological properties (Grujović *et al*., 2024b). These bacteria exhibit diverse metabolic activities, including proteolysis, acidification, and the production of

[1]*University of Kragujevac, Institute for Information Technologies, Department of Science, Jovana Cvijića bb, 34 000, Kragujevac, Serbia*

[2]*University of Lisbon, Faculty of Veterinary Medicine, CIISA – Center for Interdisciplinary Research in Animal Health, 1300-477 Lisbon, Portugal*

[3]*Associate Laboratory for Animal and Veterinary Sciences (AL4AnimalS), Portugal*

[4]*University of Lisbon, Faculty of Sciences,BioISI - Biosystems & Integrative Sciences Institute, 1749-016 Lisbon, Portugal*

[5]*University of Kragujevac, Faculty of Agronomy, Department of Chemistry and Chemical Engineering, Cara Dušana 34, 32000 Čačak, Serbia (jelenav@kg.ac.rs)*

volatile compounds such as diacetyl, which enhances the sensory characteristics of dairy products (Terzić-Vidojević *et al*., 2013; 2021; Kondrotiene *et al*., 2024).

In addition to their role in fermentation, *Enterococcus* strains can produce bacteriocins, antimicrobial peptides capable of inhibiting spoilage and pathogenic bacteria. This property makes them potential candidates for natural food preservation. However, despite their technological advantages, concerns exist regarding their safety, particularly the potential for antibiotic resistance gene transfer, which may pose food safety risks (Grujović *et al.*, 2024b).

This study aims to evaluate the enzymatic activity, metabolite production, and antimicrobial potential of autochthonous *Enterococcus* isolates. By assessing their proteolytic capacity, acidification ability, diacetyl production, and bacteriocin synthesis, we aim to determine their suitability for application in cheese fermentation.

Materials and methods

Tested strains

The isolation and characterization of bacteria followed the methodology of Grujović *et al*. (2024a), while their safety assessment was detailed in Grujović *et al*. (2024b). The identified microorganisms included *E. faecalis* (49 isolates), *E. faecium* (13 isolates), and *E. hirae* (9 isolates). All tested isolates exhibited α-hemolysis, with varying resistance profiles across species (Grujović *et al.*, 2024b). The investigation of metabolite production and enzymatic activities was conducted using methods described in both studies, as are outlined below.

Enzymatic Activity

Growth in the presence of methylene blue

To assess the ability of isolates to reduce methylene blue, pasteurized milk containing 0.1% methylene blue was inoculated with 2% (v/v) bacterial inoculum and incubated at 37°C for 24 hours. A color change indicated bacterial reduction activity. Positive and negative controls were included.

Proteolytic Activity

Proteolytic activity was evaluated by incubating isolates on nutrient agar supplemented with milk (1.6% fat). Transparent zones around colonies indicated protease enzyme activity. *Bacillus subtilis* ATCC 6633 was used as the positive control, while *Escherichia coli* ATCC 25922 served as the negative control.

Production of decarboxylase

Histamine and tyramine production were assessed using a decarboxylase medium supplemented with histidine and tyrosine. A color change from yellow to purple during anaerobic incubation at 37°C indicated positive biogenic amine (BA) production.

Metabolite production of tested isolates

Milk acidification and curd formation

Isolates were tested for their ability to acidify and form curd in pure and enriched goat milk (supplemented with glucose and yeast extract). The pH was measured after 6 and 24 hours of incubation at 32°C, and curd or gas formation was monitored.

Production of Aromatic Compounds

Diacetyl production was evaluated by inoculating isolates in reconstituted skimmed milk for 16 hours. The presence of a red ring at the top of the tubes, after the addition of creatinine and NaOH, confirmed diacetyl generation.

Screening for bacteriocin production

The agar-well diffusion method was used to screen for bacteriocin production against indicator bacteria, including *B. subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853. Soft nutrient agar (0.7% w/v) containing indicator strains was overlaid onto M17 plates, and wells were created in the solidified agar layer. Supernatants from isolated overnight *Enterococcus* spp. cultures were centrifuged (10,000 rpm, 30 minutes, 4°C), filtered, and adjusted to pH 6.2 before adding 100 µL aliquots into the wells. To confirm the proteinaceous nature of the antimicrobial substance, Pronase E (Sigma Chemie GmbH, Deisenhofen, Germany) was applied near the wells containing the supernatant. Plates were incubated overnight at 37°C, and clear inhibition zones, excluding areas near Pronase E, indicated the presence of proteinaceous antimicrobial substances.

Results and discussion

Enzymatic activity

The enzymatic activity of the tested isolates was evaluated based on their ability to reduce methylene blue, exhibit proteolytic activity, and produce decarboxylases.

The methylene blue reduction test operates on the principle that the color imparted to milk by adding a dye, such as methylene blue, gradually fades due

to oxygen removal and the subsequent formation of reducing substances during bacterial metabolism. Bacteria consume oxygen, and the higher the bacterial population, the faster oxygen is depleted, leading to a quicker disappearance of color. The bacteria responsible for this reduction produce various oxidoreductases and reductases, such as NADH dehydrogenase, flavoproteins, cytochrome reductases, and hydrogenases, which transfer electrons from metabolic substrates to methylene blue, reducing it from its oxidized blue form to a colorless state. This process occurs primarily under anaerobic conditions, where methylene blue acts as an alternative electron acceptor in the absence of oxygen. Therefore, the time taken for reduction serves as an indicator of the bacterial population and metabolic activity in the milk, with lactic acid bacteria being common examples of those capable of methylene blue reduction (Abele *et al*., 1945; Eltarahony *et al*., 2021). Consistent with this principle, all tested *Enterococcus* spp. demonstrated the ability to grow in the presence of methylene blue, suggesting their capacity to thrive in milk with significant bacterial counts.

Extracellular proteinases are essential in cheese production, as they hydrolyze milk proteins, particularly casein, facilitating curd formation and contributing to cheese texture (Kieliszek *et al*., 2021). Proteolysis further enhances flavor by releasing peptides and amino acids that act as precursors for flavor compounds. Additionally, it affects cheese firmness, elasticity, and smoothness (Konkit *et al*., 2016). Some peptides generated during this process also exhibit antimicrobial properties, contributing to an extended shelf life and maintaining cheese quality (Pessione and Cirrincione, 2016). Among the 71 *Enterococcus* spp. isolates analyzed, 12 (16.9%) demonstrated the ability to produce extracellular proteinases. Among them, *E. hirae* isolates exhibited the weakest transparent zones around colonies, indicating lower proteolytic activity. This aligns with previous findings that enterococci are less competitive in extracellular proteinase production compared to other LAB genera (Lim *et al*., 2019; Kieliszek *et al*., 2021; Kondrotiene *et al*., 2024).

Bacteria produce tyramine from tyrosine and histamine from histidine through decarboxylation reactions catalyzed by tyrosine decarboxylase (TDC) and histidine decarboxylase (HDC), respectively. These processes begin with the uptake of the corresponding amino acids into the bacterial cytoplasm via specialized transport systems. TDC removes the carboxyl (-COOH) group from tyrosine, converting it into tyramine, while HDC catalyzes the same reaction on histidine, producing histamine, with carbon dioxide (CO_2) released as a byproduct in both cases.

Several bacteria, including those from the *Enterococcus* genus, are known for their amino acid decarboxylase activity and production of BA such as tyramine and histamine, particularly in fermented foods (Barbieri *et al*., 2019). In our study, 12 isolates exhibiting proteolytic activity were further analysed for decarboxylase production. The results showed that *E. faecium* C28-2, isolated from goat cheese, produced TDC. Kalhotka *et al*. (2012) also reported significant TDC activity in *E. faecium* isolated from goat milk.

Metabolite production of tested isolates

The investigation of the metabolic production of the tested *Enterococcus* isolates included their ability to acidify milk and form curds, as well as their production of diacetyl and bacteriocins.

A key characteristic of LAB is their ability to ferment lactose, producing lactic acid. This process leads to several important effects: (a) a reduction in milk pH, resulting in casein coagulation; (b) an increase in acidity, which helps inhibit the growth of pathogenic and spoilage bacteria; (c) an improvement in the rheological properties of dairy products due to casein coagulation; and (d) the development of the final flavor profile in ripened cheeses as a consequence of acidification (Terzić-Vidojević *et al*., 2021).

The activity of isolates that exhibited proteolytic activity was evaluated in both pure and enriched goat milk. Initially, the activity of isolates from goat milk and cheese was limited after 6 hours of incubation but significantly improved after 24 hours. pH variation is presented in Figure 1.

Out of 71 isolates, 12 *Enterococcus* spp. isolates demonstrated curd formation after 24 hours of incubation in both pure and enriched milk. Acidification ability was also observed, with pH levels decreasing from approximately 6.2 at 6 hours to 5.6 at 24 hours in pure goat milk (initial pH 6.6). In enriched milk (initial pH 6.3), acidification was more pronounced, with pH levels dropping to about 5.9 at 6 hours and 5.2 at 24 hours.

Terzić-Vidojević *et al*. (2013) reported that six out of 28 enterococci isolated from raw goat milk exhibited acidification activity and curd formation within 7.5–8.5 hours, whereas other isolates required significantly more time to curdle milk or failed to do so entirely. This suggests that the *Enterococcus* genus is less competitive compared to other LAB species investigated. As suggested Terzić-Vidojević *et al*. (2021), enterococci with low acidifying potential may not be suitable as primary starter cultures for cheese production. However, they could be valuable as adjunct cultures when used alongside high-acidifying strains, given their other beneficial technological properties.

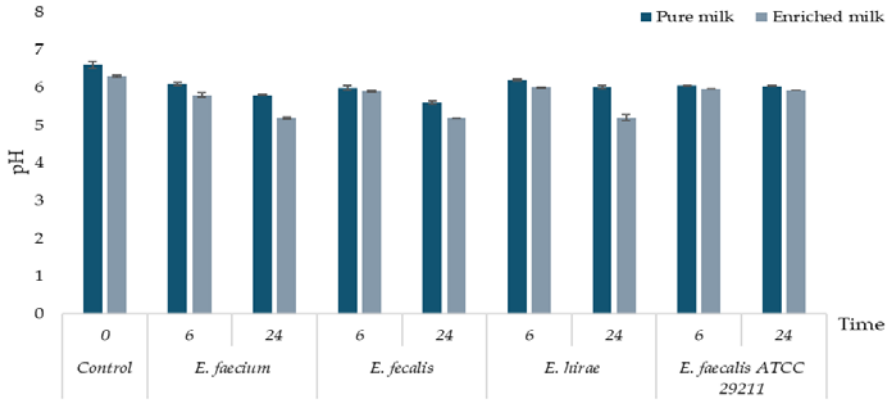

Figure 1. pH fluctuations throughout the experiment

Most aromatic compounds in cheese ripening are produced through citrate metabolism by LAB, especially enterococci. During raw-milk cheese production and ripening, citrate is degraded into several aromatic compounds, including acetate, acetaldehyde, acetoin, and diacetyl, contributing the distinct and robust flavor of raw-milk cheese. Diacetyl plays a key role in providing the buttery, "buttermilk" aroma and flavor (Terzić-Vidojević *et al*., 2021). The results from the investigation of diacetyl production ability showed that, out of 71 isolates, *E. faecalis* (13 isolates) and *E. faecium* (11 isolates) exhibited the ability to produce diacetyl, while E. *hirae* isolates did not show this ability. Our results are consistent with those found by Nieto-Arribas *et al*. (2011), who reported that the highest production of diacetyl was observed in *E. faecium* and *E. faecalis* strains.

In this study, we assessed the potential of isolated enterococci to produce bacteriocins and to inhibit the growth of indicator strains using the agar-well diffusion method. Among the six *E. faecalis* strains isolated from raw goat milk and cheese, three (C0-9, C28-3, and C28-7) produced bacteriocins that demonstrated antagonistic potential against the tested indicator strains (Table 1). Notably, only *E. faecium* C28-1 produced bacteriocins with antagonistic potential against the tested indicator strains (Table 1).

Enterococcus-derived antimicrobial compounds aid in food preservation by inhibiting bacteria and extending shelf life. However, concerns arise due to the potential for horizontal gene transfer, which may spread antibiotic resistance to pathogens, posing food safety risks (Grujović *et al.*, 2024b). The same authors also reported that *E. faecalis* C0-9 was active against various bacterial species, indicating its potential in bacteriocin production.

Table 1. The effect of partially purified *Enterococcus* bacteriocins on indicator strains

Specie	Isolate	Indicator strains					
		B. subtilis ATCC 6633		*E. faecalis* ATCC 29212		*P. aeruginosa* ATCC 27853	
		ZI	A	ZI	A	ZI	A
E. faecium	C14-2	8	T	6	C	/	/
E. faecium	C21-4	6	C	6	T	/	/
E. faecium	C28-1	8	T	8	C	6	T
E. faecium	C28-4	10	T	6	T	/	/
E. faecalis	M-4	8	T	6	C	/	/
E. faecalis	M-6	10	T	4	C	/	/
E. faecalis	C0-9	6	C	8	T	4	C
E. faecalis	C28-3	10	T	8	T	4	C
E. faecalis	C28-7	8	C	6	C	4	C
E. faecalis	C28-12	8	T	4	T	/	/

ZI*, zone of growth inhibition given in mm (millimeter); A*, zone appearance (C, clear zone of inhibition; T, turbid zone of inhibition; /, no zone of inhibition)

Conclusion

The findings of our investigation confirmed the enzymatic activity and metabolite production ability of autochthonous *Enterococcus* strains isolated from raw goat milk and cheese samples, indicating their potential application. However, it is essential to assess safety, regulatory requirements, and possible technological constraints when using *Enterococcus* strains in food production.

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