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Preliminary *in vitro* assessment of enterococcal postbiotic substances activity against *Enterococcus* spp. isolated from cows with subclinical mastitis

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Summary

The aim of the study was to evaluate the antimicrobial activity of four postbiotic substances produced by *Enterococcus faecium* against enterococci isolated from milk of cows with subclinical mastitis. The study was conducted on 10 isolates of *Enterococcus* spp. isolated from milk collected from Holstein-Friesian (HF) cows. *In vitro*, the highest efficacy against *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) isolated from milk of cows with subclinical mastitis was observed for three postbiotic substances: PS-ENT-9296, PS-ENT-M and PS-ENT-A(P). They inhibited the growth of all tested isolates (100.0%). PS-ENT-9296 produced by silage strain of *E. faecium* and PS-ENT-A(P) produced by environmental strain of *E. faecium* inhibited isolates of *E. faecalis* and *E. faecium* isolated from mastitis in cows with inhibitory activity in the range from 100 to 12 800 AU/mL. The antibacterial activity of the PS-ENT-M produced by the second environmental strain of *E. faecium* ranged from 100 to 25 600 AU/mL. A slightly weaker effect was observed with PS-ENT-4231 produced by ruminal strain of *E. faecium* (100-6400 AU/mL), which inhibited the growth of 8 out of 10 isolates of *Enterococcus* (80.0%). The results of our research confirmed the high activity of enterococcal postbiotic substances against *E. faecalis* and *E. faecium* isolates obtained from the milk of cows with subclinical mastitis.

Keywords: cows, subclinical mastitis, postbiotic substances, *Enterococcus faecalis*, *Enterococcus faecium*

Mastitis remains the most common and, at the same time, the most costly disease in dairy herds worldwide (40, 43, 48). The incidence of mastitis is high and shows marked geographical variation, with the subclinical form consistently predominating, which is without visible clinical signs and is detected solely on the basis of an elevated somatic cell count (SCC) and the presence of microorganisms in the milk. For this reason, cows with subclinical mastitis represent the most difficult reservoir of infection to control within a herd (43, 49). The consequences of mastitis for the herd are multifaceted. The disease reduces milk yield and impairs the quality of the raw milk, necessitates the disposal of milk during the post-treatment withdrawal period, increases treatment costs and accelerates the culling of cows from production (14, 49). In addition to direct losses, there are implications for animal welfare, food safety and the rise in antimicrobial resistance resulting from the widespread use of antibiotics in treatment and dry-off prophylaxis (39). In the vast majority of cases, it is of bacterial aetiology, and the infectious agents are traditionally divided into contagious pathogens, transmitted primarily during milking, and environmental pathogens, for which the cow's surroundings act as a reservoir (24, 49).

The introduction of a five-point quality management plan for milk production led to a reduction in infections caused by pathogenic bacteria, but at the same time to

an increase in the proportion of infections caused by environmental microorganisms such as: *Streptococcus uberis* (*S. uberis*) and *Escherichia coli* (*E. coli*) (24, 25). *Enterococcus* spp. are also considered to be of increasing epidemiological importance, as they can cause subclinical mastitis in cows, while, on the other hand, as environmental bacteria, they can behave as a reservoir for resistance determinants, transferring them to opportunistic and pathogenic species, which limits the effectiveness of treatment and contributes to the spread of multidrug-resistant strains in animal husbandry (46, 47).

Effectively combating antibiotic resistance requires the search for new naturally occurring antibacterial compounds (6). Bacteriocins represent a promising group of ribosomally synthesised peptides or proteins with bactericidal or bacteriostatic activity, produced by different species of Gram-positive and Gram-negative bacteria and usually released into the extracellular environment (6, 8, 34, 42). Their potential applications include the treatment of infections and the biopreservation of food (8, 42). Research into these compounds dates back to 1925, when colicin (the first known compound of this class) was described (5). However, the best-characterised representative remains nisin, produced by *Lactococcus lactis* subsp. *lactis* (*L. lactis* subsp. *lactis*): described in 1928, purified and named in 1947 it became the first lantibiotic and the first bacteriocin authorised for use in food – recognised as safe by the FAO/WHO in 1969 (9, 35). In contrast to bacteriocins, postbiotic substances have not been purified to a homogeneous state (1, 10, 11, 15, 16, 30, 34). According to the definition of the ISAPP (*International Scientific Association for Probiotics and Prebiotics*), postbiotic substances are generally defined as preparations of inactive microorganisms and/or their components that provide health benefits to the host (41).

The aim of the study was to evaluate the antimicrobial activity of four postbiotic substances produced by *Enterococcus faecium* against strains of enterococci isolated from the milk of cows with subclinical mastitis.

Material and methods

The sensitivity assessment of four selected postbiotic substances was conducted on 10 isolates of *Enterococcus* spp. obtained from the milk of Holstein-Friesian (HF) cows with subclinical mastitis on one farm located in the Lublin Province (Poland).

All procedures for collecting material for animal testing, carried out within the project, were recognized by the Local Ethical Committee for Animal Experiments in Lublin as routine veterinary services for dairy cows. Therefore, the study was conducted in accordance with Polish law (Act of November 17, 2021 amending the Act on the protection of animals used for scientific or educational purposes (Dz. U. 2021, poz. 2338)), as well as with European Union regulations contained of the Directive 2010/63/EU on the protection of animals used for scientific purposes.

The herd comprised 60 dairy cows housed in a tie-stall system on straw bedding and fed a Total Mixed Ration (TMR). The cows were milked twice daily using a high-line milking system, with an average annual milk yield of 7,946 kg. Before the morning milking, the teat skin was thoroughly washed; the first streams of milk were then collected onto a Shalm's plate, and a macroscopic examination of the milk was carried out, along with the California Mastitis Test (CMT). From each udder quarter where the test result was positive, a milk sample was taken for bacteriological examination after the teat skin had been disinfected with 70% alcohol. Milk for bacteriological testing was collected from the middle part into sterile, properly labelled tubes and delivered at 4 °C to the Department of Microbiology at the Faculty of Veterinary Medicine, University of Life Sciences in Lublin.

Classification of the subclinical form of mastitis. Mastitis was classified on the basis of a macroscopic assessment of the milk, a clinical examination of the cows, an elevated somatic cell count (SCC) and a bacteriological examination of the milk. The SCC will be measured in fresh milk using SomaCount FC Automatic, Bentley Instruments Inc., Chaska, MN. None of the cows in which subclinical mastitis caused by *Enterococcus* was diagnosed showed any visible changes in the milk or the udder, and no systemic symptoms were observed. However, an elevated SCC exceeding 200,000 cells/mL of quarter milk was recorded, as well as the presence of microorganisms in the milk, detected in microbiological tests.

Bacteriological examination of milk samples. A 10 µl aliquot of each milk sample was inoculated onto agar medium containing 5% defibrinated sheep's blood (Columbia agar, Oxoid, Basingstoke, Hampshire, England) (13). Following incubation at 37 °C for 24 hours under aerobic conditions, the morphology of the bacterial colonies was observed, a catalase test was performed, and Gram-stained microscopic preparations were prepared. In the next stage of the study, individual bacterial colonies were transferred to differential media. Slanetz-Bartley medium (Oxoid, Basingstoke, Hampshire, England) was used to isolate bacteria of the genus *Enterococcus*. Pure cultures of the isolated strains were identified using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF MS) (2).

Sensitivity of isolates of *Enterococcus* spp. to postbiotic substances. The antimicrobial activity was tested using an agar spot test as described by De Vuyst et al. (45). A 24-hour culture of the tested isolates (200 µL, with an absorbance at A600 ranging from 0.6 to 1.0) was added to 4.0 mL of semi-solid Brain Heart agar (0.7%, w/v, Difco, USA). A layer of semi-solid agar containing the isolates was spread onto solid medium (BHA, 1.5% w/v, Difco). Ten µl of each serial dilution of the postbiotic substance was applied onto the surface of the soft agar containing the bacterial strain, and the plate was incubated at 37 °C for 18 hours. The postbiotic substances were diluted in phosphate buffer (pH 6.5, in a 1 : 1 ratio). Following incubation, the bacterial growth inhibition zones were assessed. Inhibitory activity was expressed in arbitrary units per millilitre (AU/mL) as the reciprocal of the highest two-fold dilution of the postbiotic substances that resulted in complete inhibition of the test strain.

The following postbiotic substances (PS) were used (Tab. 1): PS-ENT-4231 produced by the ruminal strain *Enterococcus faecium* (*E. faecium*) CCM4231 (29). PS-ENT-9296 produced by silage strain *E. faecium* EF9296 (31) and two PS produced by strains of *E. faecium* of environmental origin (PS-ENT-M produced by *E. faecium* strain AL41=CCM8558 (accession

number in GenBank: MW256492) and PS-ENTA(P) produced by *E. faecium* strain EK13 = CCM7419) (32, 33). The postbiotic substances used in the study were produced by the strains isolated, characterized, and studied at the Laboratory of Animal Microbiology, Centre of Biosciences of the Slovak Academy of Sciences, Institute of Animal Physiology in Košice, Slovakia (Tab. 1).

Tab. 1. The postbiotic substances used in the study

Substance	Producer strain	Source	Reference
PS-ENT-4231	*E. faecium* CCM4231	Ruminal strain	Lauková et al. (1993) (29)
PS-ENT-9296	*E. faecium* EF9296	Silage strain	Marciòáková et al. (2008) (31)
PS-ENT-M	*E. faecium* AL41 = CCM8558	Environmental origin	Mareková et al. (2007) (32)
PS-ENT-A(P)	*E. faecium* EK13 = CCM7419	Environmental origin	Mareková et al. (2003) (33)

Results and discussion

The bacteriological examination of milk sampled from cows with subclinical mastitis identified 10 isolates belonging to the genus *Enterococcus*: *Enterococcus faecalis* (*E. faecalis*) (8 isolates) and *E. faecium* (2 isolates).

Sensitivity of *Enterococcus faecalis* and *Enterococcus faecium* isolated from milk of cows with subclinical mastitis to postbiotic substances. The highest efficacy against *E. faecalis and E. faecium* isolated from milk of cows with subclinical mastitis showed three postbiotic substances: PS-ENT-9296, PS-ENT-M and PS-ENT-A(P) (Tab. 2). They inhibited the growth of all tested isolates (100.0%). PS-ENT-9296 produced by the silage strain of *E. faecium* and PS-ENT-A(P) produced by the environmental strain of *E. faecium* inhibited isolates of *E. faecalis* and *E. faecium* isolated from mastitis in cows with inhibitory activity in the range from 100 to 12 800 AU/mL. The antibacterial activity of the PS-ENT-M produced by the second environmental strain of *E. faecium* ranged from 100 to 25 600 AU/mL. A slightly weaker effect was observed with PS-ENT-4231 produced by the ruminal strain of *E. faecium* (100-6400 AU/mL), which inhibited the growth of 8 out of 10 isolates of *Enterococcus* (80.0%). One *E. faecalis* isolate and one *E. faecium* isolate were not inhibited by PS-ENT-4231 within the range of concentrations tested. The *E. faecalis* strain No. 8 was the most susceptible to all tested PS (6400-25600 AU/mL), whilst the *E. faecalis* strain No. 5 showed the lowest susceptibility (0-100 AU/mL). (Tab. 2).

Tab. 2. Sensitivity of *Enterococcus faecalis* and *Enterococcus faecium* isolated from milk of cows with subclinical mastitis to postbiotic substances expressed in arbitrary units per milliliter (AU/mL)

No.		PS-ENT-4231	PS-ENT-9296	PS-ENT-M	PS-ENT-A(P)
1	*E. faecalis*	100	400	3200	1600
2	*E. faecalis*	1600	200	1600	1600
3	*E. faecalis*	6400	12 800	12 800	3200
4	*E. faecalis*	3200	6400	1600	6400
5	*E. faecalis*	ng	100	100	100
6	*E. faecalis*	6400	800	12 800	12 800
7	*E. faecalis*	1600	3200	25 600	12 800
8	*E. faecalis*	6400	12 800	25 600	12 800
9	*E. faecium*	100	400	800	100
10	*E. faecium*	ng	100	1600	3200
Total		8/10 (80.0%)	10/10 (100.0%)	10/10 (100.0%)	10/10 (100.0%)

Explanations: PS – postbiotic substances

Enterococcus spp. belong to a group of environmental aetiological factors associated with mastitis in dairy cows, although until recently they were considered a commensal component of the gastrointestinal microbiota of humans and animals (20, 21, 46). Among the species isolated from the milk of cows suffering from mastitis, *E. faecalis* and *E. faecium* predominate; however, depending on the geographical region and the husbandry system, significant proportions of *E. hirae*, *E. durans* and *E. mundtii* are also recorded (27, 37, 46). In our studies, *E. faecalis* isolates were by far the most common (80.0% of all isolates). However, in a study carried out in China, 60 isolates of *Enterococcus* spp. belonging to four species were obtained from 280 milk samples, with *Enterococcus hirae* isolates accounting for the largest proportion (68.3%), followed by: *E. faecium* (25.0%), *E. mundtii* (3.3%) and *E. durans* (3.3%) (46). Enterococci most commonly cause subclinical mastitis, which is difficult to detect at an early stage and, at the same time, is resistant to treatment; this is due to their ability to survive in adverse environmental conditions, form biofilms and possess numerous virulence factors (17, 18, 44, 50). It should be emphasised, however, that the presence of *Enterococcus* in milk does not always indicate inflammation, these bacteria are also isolated from the milk of clinically healthy cows, which makes it difficult to interpret their pathogenic role clearly and requires a thorough diagnosis of mastitis (37).

A crucial epidemiological characteristic of *Enterococcus* spp. is their resistance to a wide range of antibiotic classes (β-lactam antibiotics, including cephalosporins, tetracyclines and lincosamides), which significantly limits the choice of effective antimicrobial agents (3, 23, 26). In a study conducted by Wu et al. (46), as many as 83.3% of isolates were resistant to penicillin, whilst 15.0% were resistant to ampicillin, 15.0% to vancomycin, 6.7% to tetracycline and 25.0% to ciprofloxacin (46). Resistance of enterococci to macrolides and chloramphenicol is also common, and high-level resistance to aminoglycosides (HLAR) is of particular clinical significance, as it eliminates the synergistic effect achieved in combination therapy (23, 27). However, the most serious problem from a public health perspective remains resistance to glycopeptides, with vancomycin-resistant enterococci (VRE) being isolated with increasing frequency. In the most

recent studies on subclinical mastitis, the proportion of multidrug-resistant strains, including those carrying the *vanA* gene, which encodes resistance to vancomycin, proved to be unexpectedly high (3, 26). In the study by Khasapane et al. (26), all *E. faecium* isolates from milk samples were resistant to vancomycin (100.0%), and the *vanA* gene was the most common antibiotic resistance gene (ARG), detected in 96.0% of *E. faecalis* isolates. The results of these studies also revealed multidrug resistance in 20.6% of *Enterococcus* spp. isolates from milk. The presence of enterococci in milk means that the mammary gland and dairy products may become a reservoir and a vector for the transmission of resistance determinants to the human population, in line with the "One Health" concept (7, 20).

The growing problem of multidrug resistance is leading to a search for alternative strategies to control enterococcal mastitis, with particular promise being associated with bacteriocins and postbiotics (13, 24). Enterococci, and in particular *E. faecium* and *E. faecalis*, are among the most numerous producers of postbiotic compounds known as enterocins, which constitute an exceptionally diverse group of ribosomally synthesised antibacterial peptides and proteins (20, 35, 47). Among enterocins, the following are distinguished: class IIa pediocin-like peptides containing the conserved YGNGV motif, exemplified by enterocin A produced by *E. faecium* (4); bacteriocins secreted via the sec-type secretory pathway, such as enterocin P (12) and bacteriocin T8 (28); cyclic bacteriocins with a closed head-to-tail structure, represented by enterocin AS 48 (22), as well as large, thermolabile proteins with peptidoglycan hydrolase activity, such as enterolysin A (36). The genetic determinants of many of these substances are located on plasmids, which facilitates their spread within the population (20, 47). This structural diversity results in a wide range of mechanisms of action, ranging from the formation of pores in the cytoplasmic membrane to the enzymatic digestion of the cell wall (20, 47). A characteristic feature of bacteriocins produced by Gram-positive bacteria, including enterocins, is that they are most potent against microorganisms closely related to the producer (35). The activity of enterococcal postbiotics against multidrug-resistant enterococci (MDRE) and VRE strains, which have become a serious problem in hospital-acquired infections, is of particular interest (3). In a study by Fujii et al. (21), conducted on 80 strains of *E. faecalis* and 38 strains of *E. faecium* isolated from clinical cases, it was shown that enterolysin A inhibited *E. faecium* (including vancomycin-resistant strains), while bacteriocins T8 and BacAS9 were active against both *E. faecalis* and *E. faecium*, including VRE isolates (21). Phumisantiphong et al. (39) selected, from among the 10 000 isolates tested, an *E. faecalis* strain 478 that produces a bacteriocin exhibiting potent activity against numerous MDRE and VRE strains.

In our studies assessing the *in vitro* susceptibility of enterococci isolated from the milk of cows with subclinical mastitis, four different postbiotic substances produced by *E. faecium* were used. Undoubtedly, the greatest advantage of these compounds was their very high activity against the isolates tested, as well as the widespread availability of *E. faecium* producer strains in the cows' environment. The two postbiotic substances, PS-ENT-M and PS-ENT-A(P), are produced by the environmental strains of *E. faecium*, AL41 and EK13 respectively (32, 33). PS-ENT-9296 is produced by the *E. faecium* strain EF9296, isolated from silage, while PS-ENT-4231 is produced by the *E. faecium* strain CCM4231, obtained from a calf's rumen (29, 31). The three postbiotic substances PS-ENT-9296, PS-ENT-M and PS-ENT-A(P) inhibited the growth of all *E. faecalis* and *E. faecium* isolates obtained from the milk of cows suffering from mastitis. Research carried out by Lauková's team shows that all these postbiotic substances exhibit high thermal stability and resistance to changes in pH (29, 31-33).

In summary, a very interesting aspect is the dual role of enterococci, which, on the one hand, can cause mastitis, while, on the other hand, produce their own antimicrobial substances that offer numerous advantages compared to antibiotics: a lower risk of rapid resistance selection and no need for a long milk withdrawal period. The outcomes suggest that the examined postbiotic substances have great potential as antimicrobials for the treatment of enterococcal mastitis. More research should be carried out on the efficiency, safety, and applicability of their use in dairy cattle.

Study limitations. The use of a small number of isolates in the study results directly from the very difficult process of isolating *Enterococcus* bacteria from the milk of cows suffering from mastitis. This is due, among other things, to the fact that these microorganisms are commonly found in the barn environment including in bedding, feed and water which increases the risk of sample contamination and makes it difficult to unequivocally confirm that a given isolate originates from the udder rather than being a contaminant in the milk. Only *Enterococcus* isolates were used in the study for which there was no doubt that they were the aetiological agent of the subclinical form of mastitis in cows.

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