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IN VITRO TREATMENT OF DIFFERENT ISOLATES FROM CATTLE DUNG AND PIG SLURRY BY NISIN

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Abstract

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The antimicrobial effect of nisin against selected environmental isolates (from cattle dung and pig slurry) was tested using two methods: well diffusion test and agar spot test. Following isolates were included in the tests: enterococci (33), staphylococci (11), *Bacillus* spp. (2), *Providencia* spp. (3), *Citrobacter freundii* (1), *Morganella morganii* (2) and *Acinetobacter* -like sp. (1). Using well diffusion test all indicator organisms were sensitive to nisin at a concentration of 1 mg ml^{-1}, except of species *Morganella morganii* and *Citrobacter freundii.* Inhibition zones measured from 11 up to 26 mm (in diameter). The nisin activity towards indicators expressed in AU ml^{-1} ranged from 100 AU ml^{-1} up to 6400 AU ml^{-1}. The most sensitive to nisin was found *Enterococcus faecalis* V24 strain and *Bacillus pumilus* BP17 strain (3200 – 6400 AU ml^{-1}). Moreover, in our *in vitro* tests, the Gram-negative species of genera *Providencia* and *Acinetobacter* were sensitive to nisin (200, 400 AU ml^{-1}); in spite of the predominat effect of nisin against Gram-positive bacteria.

Animal excrements, antimicrobial substance, effect of treatment

In today's world, the contamination of the environment, in general is serious concern. Large volumes of pollutants are introduced into the ecosystem every day because of the production of agricultural waste and industrial chemicals. The excrements of farm animals, in the form of slurry or dung are accumulated in large quantities and forms organic manure which is a source of a diverse bacterial environment (Boopathy 1997). Thus, such excrements represent a potential health hazard mainly from the aspect of their using for land application (Paulsrud and Nedland 1997). Aerobic and/or anaerobic treatment in the thermophile and mesophile zones are used in dealing with a wide range of problems associated with slurry stabilization (Novák et al. 1994; Jepsen et al. 1997). However, these treatments e.g. in mesophile zone were found not sufficiently effective for complete decontamination (stabilization) of the excrements (Jepsen et al. 1997). Therefore, new ways are experimented and searched to reach higher effectiveness of stabilization processes. And, one of possible ways to solve this problem could be to utilize the ability of some bacteria to produce antimicrobial substances. Nisin is a bacteriocin (antimicrobial substance) of the lantibiotic type produced by certain strains of *Lactococcus lactis* subsp. *lactis.* It is composed of 34 amino acids. Nisin displays a bactericidal mode of activity and finds exclusive use as a biological food preservative to prevent Gram-positive but also Gram-negative spoilage bacteria (Blackburn et al.1990; De Vuyst and Vandamme 1994). Nisin showed inhibitory effect also against ruminal isolates (Lauková 1995). Because of its strongly bactericidal effect towards mastitis pathogens, it was also used for this type of treatment (Delves-Broughton et al. 1996). Moreover, the effect of other bacteriocins was reported for experimental treatment in slurry (Lauková et al. 1998a). Therefore, in this paper the antimicrobial effect of nisin against environmental isolates (from cattle dung and pig slurry) was tested. The aim of the

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study followed a confirming of nisin inhibitory effect; because of its further application e.g. in combination with the other bacteriocin in the animal excrement treatment.

Materials and Methods

Bacterial cultures, media and growth conditions

The collection of 53 strains including enterococcal isolates (33), staphylococci (11), *Bacillus pumilus* (1), *Bacillus* sp. (1), *Providencia alcalifaciens* (2), *P. rettgeri* (1), *Citrobacter freundii* (1), *Morganella morganii* (2) and *Acinetobacter* - like sp. (1) from the cattle dung as well as from pig slurry were tested. Cow dung waters were collected from the basins of 25 local farms in 15 north-eastern Slovakia districts. Pig slurry samples were collected from a pig farm at Figa in the district Rimavská Sobota (Slovakia). Regarding to our previous study (Lauková et al. 1998b), different representatives of enterococci isolated from the cattle dung water were used (Table 1). Samples of pig slurry were kept in sterile plastic bottles and transferred to the laboratory. Then tenfold dilution series were made in saline solution for plating. Gram -positive enterococci were selected using M-Enterococcus agar. Staphylococci were isolated on Mannitol salt agar. *Bacillus* sp. and *Acinetobacter* - like were cultivated on Trypticase soy agar enriched with 0.6% of yeast extract (TSYA) and 5% of defibrinated sheep blood (TSAB). Plates were cultivated at 32-37 °C for 24-48 h. *Providencia* sp., Morg*anella* sp. and *Citrobacter* sp. were selected on TSAB after their pre-cultivation in Rappaport-Vassiliadis enrichment broth (BioMerioux, France) at 30-37 °C for 24 h. Media mentioned were supplied from Becton & Dickinson (Cockeysville, USA). Phenotypical determination of selected isolates was performed (without the principal tests) by BBL Crystal Gram-positive ID kit as well as by Enteric/Nonfermenter ID kit (Becton & Dickinson). Enterococci for testing were grown in MRS broth and staphylococci in Brian heart infusion at 37 °C for 18 h (OD_{600} – 1.0). The other bacteria were grown in Trypticase soy broth with 0.6 % of yeast extract (Becton & Dickinson) at 30-37 °C for 18 h.

Bacteriocin assay

Nisin used for treatment was the commercial preparation Nisaplin (Aplin & Barrett, Ltd, Dorset, England) containing 25 mg nisin g^{-1}. Solutions of nisin were prepared at a concentration of 1 mg·ml^{-1} suspended in 0.02 mol·l^{-1} HCL (pH2) and stored at -20 °C. Antimicrobial effects were tested using two methods: the agar well diffusion method (Tagg 1976) and agar spot test (De Vuyst et al. 1996). In well diffusion test 100 µl of nisin solution was added into wells cut into the Brain heart agar and/or TSYA plates containing 500 µl of indicator (tested) strain. After incubation at 30-37 °C for 18 h, nisin activity was observed as clear zones surrounding wells with nisin. In agar spot test, serial twofold dilutions of nisin were spotted (10 µl) onto indicator lawns. The lawns were prepared by adding fresh cultures of indicator strains with an optical density at 600 nm of 1.0 to 3.5 ml of BHI or TSYB overlay agar. Overlaid agar plates were incubated at 30-37 °C. And, activity was expressed in arbitrary units (AU), corresponding to 10 µl of the highest dilution causing a definite zone of inhibition on the lawn of the indicator organism.

Results and Discussion

Enterococci - isolates from the cattle dung water were mentioned previously by Lauková et al. (1998b). Among enterococci isolated from pig slurry *Enterococcus faecium* and *Ent. faecalis* were detected and used as indicators in our test. Phenotypical determination of staphylococci showed their belonging to following species: *Staphylococcus xylosus, Staph. capitis, Staph. warneri* and *Staph. aureus*. Enterococci represent obligatory component of slurry. Because they are considered to be good indicators of fecal contamination (Godfree et al. 1997, Frahm et al. 1998). *Bacillus* spp. and *Acinetobacter* spp. were chosen as the target group of bacteria due to their ubiquitous distribution in the environment (Bifulco et al. 1989, Guardabassi et al. 1998; Mansour et al. 1999). *Citrobacter* spp. were detected e.g. from well water (Páčová et al. 1999). And, the representatives of the other Gram-negative bacteria are frequently occurred in the excrements and/or waste (Kearney et al. 1994; Lauková et al. 2000). It means, that the bacterial selection of all isolates to serve such as indicator organisms for nisin treatment was developed from real status. Table 1 summarizes the results obtained after the isolates treatment by nisin. Using well diffusion test all indicator organisms were sensitive to nisin at a concentration used (Table 1) except of species such as *Morganella morganii* or *Citrobacter freundii.* Inhibition zones measured from 10 up to 26 mm (in diameter). The nisin activity towards indicators expressed in AU ml^{-1} ranged from 100 AU ml^{-1} up to 6400 AU ml^{-1}. The most sensitive to nisin was

Table 1
The numbers of species used for nisin effectiveness tests and activity expressions

Species	Number	Source	Activity * A[1]	Activity * B[2]
Gram-positive				
Enterococcus				
faecium	8	cattle dung	10-20	100-800
Ent. faecium	3	pig slurry	10-18	100
Ent. faecalis	3	cattle dung	11-26	200-3200
Ent. faecalis	2	pig slurry	15	100
Enterococcus				
casseliflavus	9	cattle dung	10-25	100-800
Ent. durans	1	cattle dung	19	800
Ent. avium	2	cattle dung	17	100
Enterococcus sp.	5	cattle dung	10-18	400-1600
Staphylococcus				
xylosus	7	pig slurry	12-17	400-1600
Staph. warneri	1	pig slurry	10	100
Staph. capitis	1	pig slurry	13	200
Staph. aureus	2	pig slurry	14	100
Bacillus pumilus	1	pig slurry	16	6400
Bacillus sp.	1	pig slurry	16	400
Gram-negative				
Providencia rettgeri	1	pig slurry	ND	200
P. alcalifaciens	2	pig slurry	12	0
Acinetobacter-like sp.	1	pig slurry	17	400

The species *Morganella morganii* and *Citrobacter freundii* were not inhibited by nisin treatment. *Activity reached was expressed in zones of inhibition[1] in mm or in Arbitrary units per ml (AU ml^{-1})[2].

Ent. faecalis V24 strain as well as *B. pumilus* BP17 (3200 – 6400 AU ml^{-1}, Table 1). V24 strain also showed the highest inhibition zone in well diffusion test. Nisin is known to have bactericidal effect against a broad range of Gram-positive bacteria including also spore-forming bacteria e.g. like *Bacillus* spp. (Vandenbergh 1993; Jack et al. 1995). That is, this effect was confirmed here by both methods used. Interestingly, V24 strain itself is producer of an antimicrobial substance (Lauková et al. 1998b). However, in our *in vitro* tests also Gram-negative bacteria (*Providencia, Acinetobacter* – like sp.*)* were sensitive to nisin (Table 1). Although only a small reaction was detected (200, 400 AU ml^{-1}), it could be discussed that maybe by combining of nisin with other antimicrobial substance or the other agents (e.g. chelating agents) the restrictions for the practical application of nisin in different matrices (low stability, high pH) might be overcome (Vandenbergh 1993; Pol and Smid 1999). Combination effect of nisin and heat injury or essential oils was e.g. successfully used for *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Listeria monocytogenes* inactivation (Mendoza-Yepes et al. 1997; Boziaris et al. 1998). Further possible application of different bacteriocins for final re-treatment of e.g. animal waste using antimicrobial substances individually or in combination indicate also our previous results. That is, bacteriocin substance produced by *Ent. faecalis* V24 strain caused the reduction of 2.03 and 1.44 log cfu ml^{-1} of *L. monocytogenes* and *Yersinia enterocolitica* cells (Lauková et al. 2000). And, the exploitation of bacteriocins in agriculture will be focused in the near future on the retardation of spoilage e.g. by plant pathogens, for grain preservation, etc. (Paik et al. 1997).

Ošetrenie izolátov z hnojovice dobytka a ošípaných nisinom *in vitro*

Cieľom experimentu bolo otestovať antimikrobiálny efekt nisinu na vybrané environmentálne izoláty (z hnojovice hovädzieho dobytka a z hnojovice ošípaných) s použitím dvoch testovacích metód: tzv. „jamkového difúzneho testu" a tzv. „agar spot testu". Na testovanie boli vyselektované nasledovné baktérie: enterokoky (33), stafylokoky (11), *Bacillus* spp. (2), *Providencia* spp. (3), *Citrobacter freundii* (1), *Morganella morganii* (2) a *Acinetobacter*-like sp. (1). Pri použití „jamkového difúzneho testu" sa všetky testované indikátorové organizmy prejavili ako citlivé na nisin v koncentrácii 1 $mg \cdot ml^{-1}$, s výnimkou druhov *Morganella morganii* a *Citrobacter freundii.* Inhibičné zóny merali od 11 mm do 26 mm (v priemere). Pri použití druhej metódy, kde inhibičná aktivita nisinu bola vyjadrená v arbitrárnych jednotkách (AU ml^{-1}) sa hodnoty aktivity pohybovali v rozmedzí od 100 AU ml^{-1} do 6400 AU ml^{-1}. Najcitlivejšie na nisin boli kmene *Enterococcus faecalis* V24 a *Bacillus pumilus* BP17 (3200 - 6400 AU ml^{-1}). Napriek tomu, že nisin prejavuje inhibičný efekt predominantne na Gram-pozitívne baktérie, pri testovaní *in vitro* boli inhibované i Gram-negatívne druhy rodov *Providencia* a *Acinetobacter* (200, 400 AU ml^{-1}).

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