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Original article

Sensitivity to antimicrobials of faecal *Buttiauxella* spp. from roe and red deer (*Capreolus capreolus, Cervus elaphus*) detected with MALDI-TOF mass spectrometry

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Abstract

Wild ruminants are an interesting topic for research because only limited information exists regarding their microbiota. They could also be an environmental reservoir of undesirable bacteria for other animals or humans. In this study faeces of the 21 free-living animals was sampled (9 *Cervus elaphus*-red deer, adult females, 12 *Capreolus capreolus*-roe deer, young females). They were culled by selective-reductive shooting during the winter season of 2014/2015 in the Strzałowo Forest District-Piska Primeval Forest (53° 36 min 43.56 sec N, 21° 30 min 58.68 sec E) in Poland. *Buttiauxella* sp. is a psychrotolerant, facultatively anaerobic, Gram-negative rod anaerobic bacterial species belonging to the Phylum Proteobacteria, Class Gammaproteobacteria, Order Enterobacteriales, Family Enterobacteriacae and to Genus *Buttiauxella*. *Buttiauxella* sp. has never previously been reported in wild ruminants. In this study, identification, antimicrobial profile and sensitivity to enterocins of *Buttiauxella* strains were studied as a contribution to the microbiota of wild animals, but also to extend knowledge regarding the antimicrobial spectrum of enterocins. Five strains were identified using the MALDI-TOF identification system (evaluation score value was up to 2.224) and allotted to the genus *Buttiauxella* including the species *Buttiauxella gaviniae, B. ferragutiae*, *B. agrestis.* Strains were DNase negative, and they hydrolysed esculin; fermentation of L-arabinose, D-mannitol and D-mannose was positive. Dulcitol, inositol reaction, urea and indol were negative. *Buttiauxella* strains did not form biofilm. They were resistant to at least one of the 13 antibiotics tested. *B. agrestis* 2/109/1 was resistant to amdinocillin, clindamycin and penicillin. However, *Buttiauxella* strains were sensitive to the enterocins used (inhibition activity ranged from 100 to 25 600 AU/ml).

Key words: Buttiauxella, roe deer, red deer, antibiotics, sensitivity, enterocins

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Introduction

Wild ruminants are an interesting research topic. They live in small populations, in hard-to-reach areas, so it is very difficult to obtain material samples. On the one hand, they could be an environmental reservoir of undesirable bacteria for other animal species and humans alike; on the other, the meat from wild ruminants has delicious taste and health properties making it attractive for consumers. However, looking at the alimentary route, it could be stated among other things that meat from wild ruminants may be a potential source of some diseases for humans if it is not properly prepared (Bottone 1997).

Buttiauxella spp. are Gram-negative rod bacteria, which are psychrotolerant, and facultatively anaerobic. They belong in the Phylum Proteobacteria, Class Gammaproteobacteria, Order Enterobacteriales, Family Enterobacteriacae, and Genus *Buttiauxella* (Mueller et al. 1996). Previously, our studies of small ruminant microbiota were focused mostly on Gram-positive species, especially on lactic acid producing Firmicutes (Lauková 1993b, 1995). The taxonomical allotment of the species and properties was more detaily studied. In this study, however, identification, antimicrobial profile and sensitivity to enterocins of *Buttiauxella* strains were studied in order to contribute to the basic research related to wild ruminant microbiota as well as to study the antimicrobial spectrum of enterocins studied in our laboratory.

Materials and Methods

Sampling, strains isolation and identification

The material (faeces) was sampled in the Strzałowo Forest District (Piska Primeval Forest 53° 36 min 43.56 sec N, 21° 30 min 58.68 sec E) in Poland. Twenty-one free-living animals (9 *Cervus elaphus*-red deer, adult females and 12 *Capreolus capreolus*-roe deer, young females) were culled by selective-reductive shooting during the winter season of 2014/2015 (December/ /January) approved by the Polish Veterinary Administration. Each sample was labelled immediatelly after shooting of the animals (in the field) by marking the date and location of animal shooting/sampling, as well as the age and sex of the animal from which each sample was taken. The samples were stored at 4°C and then transported to our laboratory by Courier Company. The samples were provided by our colleagues in Poland (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jablonna, Poland, namely Renata Miltko, Barbara Kowalik, and Grzegorz Bełżecki, and additionally Marek Bogdaszewski, Institute of Parasitology of the Polish Academy of Sciences, Warsaw, Poland).

After delivery, the faeces were immediatelly treated with the standard dilution microbiological method according to ISO (International Organization for Standardization). Faecal samples (21) were stirred using a Stomacher (Masticator, Spain) and diluted in Ringer solution (pH 7, Merck, Darmstadt, Germany) at the ratio 1:9. The appropriate dilutions were spread on Mac Conkey agar (Oxoid, Ltd., the United Kingdom) and incubated at 37°C for 24 h. Bacteria numbers were estimated as the average count of colonies grown at the highest dilution per sample and expressed in colony-forming units per gram of sample (CFU/g ± SD). Grown colonies were then picked up, and controlled for purity by plating on Trypticase soya agar (TSA, Difco, USA) supplemented with 5% defibrinated sheep blood. Species identification was done by means of the MALDI Biotyper TM identification system (Bruker Daltonics USA) based on analysis of bacterial proteins using MALDI-TOF mass spectrometry. Lysates of tested strains/bacterial cells were prepared according to the producers instruction (Bruker Daltonic 2011) prior to identification. This method is used especially for research microbiology. The results were evaluated using the identification database as follows: value score 2.300-3.000 means highly probable species identification, value score 2.000-2.299 corresponded with secure probable species identification/probable species identification, and value score 1.700-1.999 is associated with probable genus identification. Positive controls were those included in the identification system. In addition, phenotypic characterization was performed using the commercial BBL Enteric/Nonfermenter ID Crystal kit (Becton and Dickinson, Cockeysville, USA) to confirm the species according to the properties reported by Mueller et al. (1996) and Brenner and Farmer (2001). For subsequent analyses the strains were stored using the Microbank system (Pro-Lab Diagnostic, Richmond, Canada).

Determination of nuclease activity and biofilm formation

Because representatives of the genus *Buttiauxella* are DNase-negative, each strain was inoculated on the surface of DNase agar (Oxoid, USA). There the production of deoxyribonuclease was expected to be confirmed or excluded (after 24 h incubation at 37°C). Colonies producing DNase hydrolysed the deoxyribonucleic acid (DNA) contained in the medium. After medium flooding and acidifying with 1 N HCl (hydrochloric acid), the DNA precipitated, the medium became turbid and cleared zones formed around DNase-positive colonies.

Table 1. Identification score, antibiotic phenotype profile and sensitivity to enterocins of *Buttiauxella* strains.

Strain	Score	AMD	AK	DA	P	Ent4231	EntM	EntA (P)	Ent 55	Ent2019	Ent 9296	Ent412
B. gaviniae												
8/143/2	2.224	+15	+15	R	R	800	800	3 200	800	3 200	3 200	25 600
5/107/1	2.145	+25	+30	+22	R	100	100	200	100	400	200	100
B. agrestis												
4/112/2	1.784	+15	R	R	+11	800	1 600	3 200	3 200	3 200	3 200	3 200
2/109/1	1.798	R	+10	R	R	100	100	400	400	400	800	200
B. ferr.												
1/1/2	1.952	R	+10	R	R	100	100	100	100	100	200	3 200

Buttiauxella gaviniae, Buttiauxella agrestis, Buttiauxella ferragutiae; AMD-amdinocillin/mecillinam (10 μg), amikacin-AK (30 μg), clindamycin-DA (2 μg), penicillin-P (10IU). To other antibiotics were strains sensitive (listed in material and Methods)-inhibition zones in the range from 11 to 33 mm. Ent 4231 (by ruminal *Enterococcus faecium* CCM4231, Lauková et al. 1993), Ent M, Ent A (P) (by environmental strains *E. faecium* CCM8558, CCM7419; Mareková et al. 2003, 2007), Ent55 (by chicken strain *E. faecium* EF55; Strompfová and Lauková 2007), Ent 2019 (by rabbit *E. faecium* strain CCM7420; Pogány Simonová et al. 2009), Ent 9296 (by *E. faecium* EF9296 from silage; Marciňáková et al. 2004), Ent 412 produced by *E. faecium* EF412 from horese (Lauková et al. 2008). The inhibition activity in Arbitrary Units per ml. R-resistant, + sensitive, size of inhibition zone in mm.

To test biofilm formation, the qualitative method according to Freeman et al. (1989) was used. Briefly, Brain heart infusion/broth with sucrose and agar is supplemented with 0.8 g/l of Congo red. Agar plates are inoculated with tested strains. Plates are checked at 24 h after incubation at 37°C, then they are kept in laboratory conditions for 48 and 72 h and checked again. Strains producing black colonies with dry crystalline consistency are evaluated as forming biofilm (slime producers). Pink-red colonies are those not forming biofilm, what means they are negative. *Streptococcus equi* subsp. *zooepidemicus* CCM7316 (biofilm forming) was used as a positive control, kindly supplied by Dr. Styková (University of Veterinary Medicine and Pharmacy, Košice, Slovakia).

Testing of antimicrobial profile

The identified *Buttiauxella* strains were tested for their antibiotic phenotype profile. The agar disk diffusion method was used, including antibiotics as recommended for the Family Enterobacteriacae in the Clinical Laboratory Standard Institute guidelines (CLSI 2017). TSY agar (Oxoid) supplemented with 5% sheep blood was used and the following antibiotic discs with concentrations recommended by the supplier: clindamycin-DA (2 μg), ampicillin-AMP, amdinocillin/mecillinam-AMD, gentamicin-GEN (10μg), penicillin-P (10IU), azithromycin-AZM (15μg), amikacin-AK, chloramphenicol-C, tetracycline-TE (30μg), ticarcillin-TIC, mezlocillin-MEZ (75μg), carbenicillin-CAR, piperacillin-PRL (100μg). These antibiotic disks were supplied by Oxoid and Becton and Dickinson (the United Kingdom and USA). The zone diameter for each individual antibiotic was assessed as susceptible or resistant according to the interpretation table of the antibiotic disks producer. The zones were evaluated and compared with the reference-type strain included in the supplier documentation.

Testing sensitivity to enterocins

The identified *Buttiauxella* strains were treated with partially-purified substances (PPS) of enterocins using the quantitative agar diffusion method (De Vuyst et al. 1996). Producer strains were isolated and characterized at our laboratory (Centre of Biosciences of the Slovak Academy of Sciences, Institute of Animal Physiology, v.v.i., Laboratory of Animal Microbiology, Košice, Slovakia). Enterocins (7) were prepared according to the protocols set up in the part of references for producing strains: ruminal *E. faecium* CCM4231 produced Ent 4231 (Lauková et al. 1993b), chicken strain *E. faecium* EF55 – Ent55 (Strompfová and Lauková 2007), environmental strains *E. faecium* CCM8558 and CCM7419 (Ent A, P) - Ent M and Ent A, P (Mareková et al. 2003, 2007), horse strain *E. faecium* EF412 – Ent 412 (Lauková et al. 2008), *E. faecium* from silage – Ent 9296 (Marciňáková et al. 2004) and Ent 2019 produced by rabbit *E. faecium* CCM7420 (Pogány Simonová et al. 2009). The inhibition activity was defined as the reciprocal of the highest dilution producing an inhibition zone against the indicator strain and expressed in Arbitrary Unit per ml (tested against the principal, the most sensitive indicator strain *Enterococcus avium* EA5 isolated from piglets) used as positive control. Activity of

PPS against the most sensitive indicator (EA5) was ranged from 3 200 to - 51 200 AU/ml. Testing was performed using Trypticase soya agar (1.5 %; 0.7 %) and broth (Becton and Dickinson, USA). Incubation was carried out at 37°C for 18-24 h.

Results

The total count of colonies grown on Mac Conkey agar reached on average 5.43 (2.33) log cfu/g. Among 21 colonies submitted for identification using MALDI-TOF mass spectrometry, five strains were taxonomically allotted to the genus *Buttiauxella* involving three species: *Buttiauxella gaviniae* (one from roe deer-BG8/143/2 and one from red deer-BG5/107/1), *B. ferragutiae* (BF1/1/2 from red deer), *B. agrestis* (one from roe deer-BA4/112/2 and one from red deer BA2/109/1). Among the indicated strains, three originated from red deer (*B. gaviniae* BG5/107/1, *B. agrestis*-BA2/109/1, *B. ferragutiae*-BF1/1/2) and two were isolated from roe deer (*B. gaviniae*-BG8/143/2, *B. agrestis*-BA 4/112/2, Table 1). Evaluation scores of *B. gaviniae* strains reached 2.145 and 2.224; this means secure probable species identification/probable species identification (Table 1). The rest of the strains ranged from 1.700-1.999 (probable genus identification). Following the phenotypization, strains were found DNase negative, all species hydrolysed esculin, and fermentation of L-arabinose, D-mannitol and D-mannose was positive. Basic biochemical reactions tested in taxonomical identification of bacteria such as dulcitol, inositol reaction, urea and indol reaction were negative. *Buttiauxella* strains did not form any biofilm.

Buttiauxella strains were mostly sensitive to antibiotics; however, they were resistant to at least one of the 13 antibiotics used. All strains were sensitive to AZM, TIC, MEZ, PRL, GEN, C, TE, AMP and CAR. Three of the five strains tested (*B. agrestis* BA2/109/1 from red deer, *B. gaviniae* BG8/143/1 from roe deer and BG 5/107/1 from red deer) were resistant to P. Four strains (Table 1) were resistant to DA; on the other hand, only *B. gaviniae* BG5/107/1 was sensitive to DA (clindamycin). *B. agrestis* BA 2/109/1 was resistant to AMD (amdinocillin/mecillinam); BA 4/112/2 strain was resistant to AMK. *B. agrestis* 2/109/1 was resistant to three antibiotics (AMD, DA, P) and BA 4/112/2 strain was resistant to two antibiotics (AK, DA), *B. gaviniae* BG8/143/2 was also biresistant (DA, P). The strains *B. gaviniae* BG 5/107/1 and BF1/1/1 were monoresistant; one resistant to P and the other to DA.

Buttiauxella strains were sensitive to the enterocins used (inhibition activity ranged from 100 to 25 600 AU/ml, Table 1). The highest inhibition activity was noted against *B. gaviniae* BG 8/143/2; however, *B. agrestis* BA 4/112/2 was also inhibited with activity 800-3 200 AU/ml. *B. gaviniae* BG 5/107/1 (100-400 AU/ml), *B. agrestis* BA 2/109/1 (100-800 AU/ml) and *B. ferragutiae* BF 1/1/2 (100, 200, 3 200 AU/ml) were also inhibited due to enterocins, but with lower inhibition activity.

Discussion

As indicated above, the species *Buttiauxella* are Gram-negative, rod-shaped bacterial cells which may be commonly isolated from water and soil, but also from the appendix and the intestinal tract; however, their clinical significance is not completely known (Brenner and Farmer 2001). In our case, three different species related to five strains were detected; this indicates species variability within *Buttiauxella* occurrence in the animals tested. Moreover, no studies of *Buttiauxella* colonization in ruminants or wild ruminants have been published previously. MALDI-TOF score evaluation confirmed mostly secure probable species identification/probable species identification or probable genus identification. In addition, phenotypic properties were comparable as reported for reference-type strains of *Buttiauxella* by Brenner and Farmer (2001). The other *Buttiauxella* species identified up to now are *B. brennerae, B. izardii*, *B. noackiae*, *B. warmboldiae* (Mueller et al. 1996). Biofilm formation is an important defensive mechanism; in the case of the pathogenic strain it is important for combating the host response and remaining stable in the hostile environment. Our *Buttiauxella* strains did not form any biofilm. Biofilm formation can arise from different genetic determinants, so assessment of genetic level is important. The correlation between the phenotypic biofilm production and the existence of biofilm-associated genes (BAGs) has been reported in some studies as considerably good (Jiang-Zhong et al. 2014); our strains are probably BAG-negative. *Buttiauxella* is not the only new species novum of Gram-negative bacteria which can be found in the faeces of roe/red deer. For example, species novum pathogen *Rodococcus equi* has also been identified in roe/red deer occuring with the frequency of 0.7-0.9 % (Witkowski et al. 2015).

Identified strains were mostly sensitive to antibiotics; wild ruminants had no connection with antibiotic pressure. The resistance phenotype detected in the strains was surprising; this could probably be explained by acquired resistance from some environmental influence. We did not analyse resistant genes; however, such genes may move within a bacterium from its chromosome or plasmid, and to other bacteria via transposons and integrons. Bacteria can acquire resistance via transmissible genes, e.g. by conjugation. Resistant bacteria can be spread from animal to animal, from

animal to environment and from animals to humans e.g. via food (Pipová et al. 2012).

The enterocins used in this study are allotted to Class II enterocins which have a broad inhibiton (antimicrobial) spectrum involving Gram-negative bacteria (Franz et al. 2007, Lauková et al. 2012). In our previous studies, *in vivo* inhibition effect against coliforms was found involving the same enterocins which were used in this study (Lauková et al. 2012); and coliforms are representatives of the Family Enterobacteriacae. To date we have not found any information regarding the inhibition activity of enterocins against *Buttiauxella* strains. Our results are a contribution not only to knowledge about the microbiota of wild ruminants; they also extend the information regarding the antimicrobial spectrum of the enterocins tested. This contribution is mostly related to basic research into enterocins; but it could also be an indication for their potential use.

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