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Research Article

Digestive Tract of Beavers (*Castor fiber*) Associated with Staphylococcal Species Variability and Their Properties

Andrea Lauková[1,*], Jana Ščerbová[1], Anna Kandričáková[1], Renata Miltko[2], Grzegorz Belzecki[2]

[1]Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice, Slovakia

[2]The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Instytucka 3, Jablonna 051 10, Poland

[*]**Corresponding Author:** Dr. Andrea Lauková, Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice, Slovakia, Tel: +421 55 7922964; E-mail: laukova@saske.sk

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Abstract

Objective: Staphylococci from beavers' gut (*Castor fiber*) were analysed as a contribution in the basic microbiology as well as in the part of beavers' microbiome study.

Methodology: Free-living beavers (12), both male and female (aged 4-5 years) were caught with a net in north-east part of Poland Województwo (Provincie) Podlaskie Gmina-Wizajny, GPS:22º 52E:54º22N and placed in wire cages. Sampling was provided with the Polish colleagues. Samples of jejunum (12), colon (12) and caecum (6) were done according to all ethic rules for animal handling.

Results: Staphylococci detected in jejunum reached 2.73 ± 1.16 cfu/g (log 10) on average; their counts in caecum reached 1.87 ± 0.37 cfu/g on average and in colon 2.89 ± 1.70 cfu/g. After score evaluation using MALDI-TOF spectrometry, a high variability in staphylococcal strains/species distribution in beavers'

gut was found; in total, including 18 identified strains, nine species were detected belonging into five clusters; all strains were allotted in the coagulase-negative staphylococcal species. The most frequently detected species were *S. hominis* and *S. haemolyticus* (five strains for each). The other species included two strains-*S. epidermidis* and *S. lentus, S. pasteuri, S. cohnii, S. vitulinus, S. warneri, S. xylosus*, one strain for each one species. Fifteen strains were DNase and almost hemolysis negative. Ten strains (not depending on the species) showed low-grade biofilm ability. Most of strains were methicillin susceptible with high production of lactic acid.

Conclusion: The study is original contribution in staphylococcal microbiome of beavers and gives opportunity in more detail study of individual strain species.

Keywords: Beaver; Staphylococci; Species; Diversity; Properties

1. Introduction

Beavers (*Castor fiber* L., 1758) are among more numerous animal species inhabiting aquatic-terrestrial ecosystems in Europe [1]. This is the largest rodent in Eurasia. In Poland, beavers were almost completely eradicated; however, artificial reintroduction and natural migration from Lithuania and Belarus led to the increase in their population. In 2014 year, the estimated beaver population in Poland was about 50,000 individuals [2]. The places of localization of the beavers are mainly in north-eastern part of the country. An increase in the number of beavers and the damage to plough caused by them are the reasons why they are being captured. It was an opportunity for our Polish colleague to catch them and to have material for studying because there is many „open" tasks in beavers microbiota research. Based on the co-operation with the Polish colleagues, material was allowed to sample authorized by the regional Direction of Environment Protection. In our previous studies characterization of *Enterococcus thailandicus* or partially also *Streptococcus gallolyticus* was reported [3, 4]; however, no information exists regarding detail studies of the genus Staphylococcus. This genus belongs to the Phylum Firmicutes, the Class Bacilli, the Order Bacillales, the Family Staphylococcaceae. Their pathogenity correlates with the production of coagulase. In general, coagulase-positive species of staphylococci are supposed to be pathogenic and coagulase-negative staphylococci as nonpathogenic respectively facultative pathogenic [5]. Based on comparative 16S rRNA sequence analyses staphylococci are divided into 11 clusters with individual species [6]. The clusters are represented by *S. aureus* cluster/group, cluster *S. auricularis, S. carnosus* cluster, clusters *S. epidermidis, S. haemolyticus, S. hyicus-intermedius, S. lugdunensis, S. saprophyticus, S. sciuri* and clusters *S. simulans* and S. *warneri* [6]. In this study, variability in the staphylococcal species is reported and selected properties of identified staphylococci from beavers gut. This is a contribution in basic microbiology associated with microbial environment of beavers' gut.

2.Materials and Methods

2.1 Sampling and strains identification

Free-living beavers (12), both male and female (aged 4-5 years) were caught with a net in north-east part of Poland Województwo (Provincie) Podlaskie Gmina-Wizajny, GPS:22º 52E:54º22N and placed in wire

cages. Sampling was provided with the Polish colleagues. The sex of beavers was determined by secretion of anal glands (Cool Gray 1U-2U and 609U-610U-Pantone matching system, NJ, 1991). Weight of beavers varied between 16.0 and 23.5 kg (the average been 18.8 ± 2.2 kg); length of body was between 104-120 cm (the average been 111.2 ± 4.2 cm). There was no differences in weight and body mass between sex. The animals were euthanized by injection of 2.5 ml xylazine and 1 ml ketamine (Biowet, Poland). The administered doses were consistent with the procedure for farming farm-grown beavers adopted by the research station in Popielno (Polish Academy of Sciences, Poland). The diet of beavers was composed mainly of deciduous trees (bark and woody) and insignificant amounts of Graminoids and Forbs. Sampling of jejunum (12), colon (12) and caecum (6) was done according to all ethic rules for animal handling. Samples were transported in our laboratory and treated using a standard microbiological method (International Organization for Standardization-ISO 7899); one g of sample was mixed in Ringer solution (Merck, Darmstadt, Germany) by stirring with an use of the Stomacher–Masticator (Spain) and diluted. Mannitol Salt agar (ISO 6888, Difco, Detroit, Maryland, USA) or Baird-Parker agar with supplement (Becton and Dickinson, ISO 6888-2) were used for cultivation. Plates were incubated at 37º C for 24-48 h. Bacterial richness was calculated as an average count of colonies grown in the highest dilution per sample and expressed in colony-forming units per gram of sample (log10 cfu/g ± SD). Fifty appropriate colonies were picked up, they were spread on Brain Heart agar (Becton and Dickinson, Cockeysville, USA) enriched with 5% of defibrinated sheep blood prior identification.

Isolated pure strains were identified by the use of MALDI–TOF BioTyper™ identification system (Bruker Daltonics, USA) based on protein „fingerprints“ measured by matrix–assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Lysates of bacterial cells were prepared according to the instruction of producer, Bruker Daltonics [7] prior to identification. For other tests, identified strains were kept on MSA or Brain heart infusion/agar and/or stored using Microbank™ system (Pro-Lab Diagnostics, Canada).

After identification score evaluation, identical strains were excluded from analyzing and selected 18 identified strains by MALDI-TOF system were then phenotyped following the parameters involved and reported for the individual species in Bergeys' Manual of Systematic Bacteriology [8]. The conventional biochemical identification was provided by BBL Crystal tests for Gram-positive bacteria (Becton and Dickinson, Cockeysville, USA) involving 30 various tests (e.g. DNase, hemolysis, esculin, urease, lactic acid, fermentation of sucrose, trehalose, arabinose, arginine, etc.) and compared with the type strain involved in manual.

2.2 DNase, hemolysis test and biofilm formation

Individual identified strain was inoculated on the surface of DNase agar (7129, Oxoid, United Kingdom). Production or non production of thermo-stable deoxyribonuclease was confirmed after a 24 h incubation at 37º C. Streak of strains producing DNase hydrolysed the deoxyribonucleic acid contained in the medium. After medium flooding and acidifing with 1 N HCl (hydrochloric acid), the DNA precipitated, clear zones appeared around DNase positive streak of strain. No zone in negative strains

for DNase. Positive control was *Staphylococcus aureus* ATCC 25923.

Hemolysis was controlled by streaking the cultures on Brain heart agar supplemented with 5% of defibrinated sheep blood. Plates were incubated at 37º C for 24-48 h. The presence or absence of clearing zones around the colonies was interpreted as α, β and negative $_{\gamma}$ hemolysis [9].

To test biofilm formation ability in staphylococci, the quantitative biofilm plate assay was used as follows: one colony of the tested strain grown on Brain heart agar overnight at 37ºC (Difco, Maryland, USA) was transferred into 5 ml of Ringer solution (pH 7.0, 0.75% w/v) to reach McFarland Standard no 1. corresponding to 1.0 x 10^8 cfu/ml. A volume of 100 µl from that culture was then transferred into 10 ml of Brain- heart infusion (BHI). A volume 200 µl of standardized culture was inoculated into polystyrene microtiter plate wells (Greiner ELISA 12 Well Strips, 350 µl, flat bottom, Frickenhausen GmbH, Germany) and incubated for 24 h at 37ºC. The biofilm formed in the microtiter plate wells was washed twice with 200 µl of deionized water and dried at room temperature for 40 min. The remaining attached bacteria were stained for 30 min at room temperature with 200 µl of 0.1 % (m/v) crystal violet in deionized water. The dye solution was aspirated away and the wells were washed twice with 200 µl of deionized water and dried at room temperature for 30 min. After water removal and drying, the dye bound to the adherent biofilm was extracted with 200 µl 95% ethanol and stirred. A 150 µl aliquot was transferred from each well and placed in a new microplate well for absorbance (A) measurement at 570 nm using an Apollo 11 Absorbance Microplate reader LB 913 (Apollo, Berthold Technologies, USA). Each strain and condition was tested in two independent tests with 12 replicates. A sterile culture medium **(**BHI) was included in each analysis as negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as positive control (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy in Košice, Slovakia). Biofilm formation was classified as highly positive ($A_{570} \geq 1$), low-grade positive ($0.1 \leq A_{570} < 1$) or negative ($A_{570} < 0.1$) according to Chaieb et al. [10] and Slížová et al. [11].

2.3 Antibiotic phenotype profile and lactic acid testing

Disk diffusion method was used to test antibiotic phenotypic profile of identified staphylococci. Briefly, the strains were cultivated in Brain heart infusion (Oxoid) overnight at 37º C. The volume 100 µl was plated onto Brain heart agar (BHA) with 5% of defibrinated sheep blood and antibiotic disks were applied. The evaluation was done according to the manufacturers‘ instructions involved in the Clinical and Laboratory Standard Institute guidelines-CLSI [12]; the inhibition zones were expressed in mm. Antimicrobial free agar plates were included as a control to check growth of strains. Fifteen antibiotics were tested according to recommendation of disks‘ suppliers: oxacillin, (1µg, Ox, Oxoid), clindamycin, lincomycine (2 µg, Cli, L), novobiocin, neomycin (5 µg, Nov, N, Becton and Dickinson), penicillin (10 IU, P, Lach-Ner, Czech Republic), ampicillin, methicillin (10 µg, Met, Fluka, Oxoid), tobramycine (10 µg, Tob, Becton and Dickinson), erythromycin (Ery, 15 µg Becton and Dickinson), chloramphenicol, tetracycline, vancomycin, cefoxitine (30 µg, Lach-Ner and Oxoid) and gentamicin (Gn, Becton and Dickinson, 120 µg). Comparison with the

reference strains included in manufacturers' instruction (Becton and Dickinson) was used as positive control. Inhibition zones were evaluated and expressed in mm followed manufacturers' instruction.

Lactic acid (LA) production was evaluated by the spectrophotometric method (expressed in mmol/l) based on a conversion of LA to acetaldehyde by heat from sulfuric acid. Acetaldehyde reacts with *p*-hydroxybiphenyl forming colour complex.

3.Results

Staphylococci detected in jejunum reached 2.73 ± 1.16 cfu/g (log 10) on average; counts of staphylococci in caecum reached 1.87 ± 0.37 cfu/g on average and in colon was their amount 2.89 ± 1.70 cfu/g (log 10). After score evaluation using MALDI-TOF, eight identified strains were from jejunum, four strains from caecum and six strains from colon. High variability in staphylococcal distribution in beavers' gut was found (Table 1); in total, nine species were detected belonging into five clusters; all strains were allotted in the coagulase-negative staphylococcal species. The most frequently detected species were *S. hominis* (five strains) and *S. haemolyticus*, five strains as well (Table 1). The other species included two strains-*S. epidermidis* and the others species, one strain for each species such as *S. lentus, S. pasteuri, S. cohnii, S. vitulinus, S. warneri* and *S. xylosus*. In jejunum detected strains belong in six staphylococcal species (*S. hominis, S. haemolyticus, S. epidermidis, S. lentus, S. warneri* and *S. xylosus* and five clusters; four strains from caecum belong in three species (*S. pasteuri, S. hominis, S. haemolyticus*) and two clusters; finally, in colon four species (S. *haemolyticus, S. epidermidis, S. cohnii, S. vitulinus*) included six strains from four clusters (Table 1). SHo33 was evaluated having score corresponding with highly probable species identification (Table 1); eight out of 18 strains were evaluated in the range score 2.000-2.299 corresponding with secure genus identification, probable species identification and nine strains were evaluated with probable genus identification. Phenotyping test for each one species strain confirmed their taxonomical allottment. Esculin test is negative for *S. hominis, S. haemolyticus, S. epidermidis, S. xylosus* and *S. warneri* while in the case of *S. pasteuri, S. lentus* this reaction is positive and in *S. cohnii* can be this reaction variable. Oppositely, urea test in *S. hominis, S. epidermidis, S. pasteuri, S. warneri* and *S. xylosus* is positive and urea is negative in the strains *S. haemolyticus, S. lentus, S. cohnii*; variable reaction in urea is typical for *S. vitulinus*. Arabinose is fermented (positive reaction) in *S. lentus, S. vitulinus, S. cohnii*, and strains of *S. hominis, S. haemolyticus* and *S. xylosus* have negative reaction (arabinose negative);variable reaction in arabinose fermentation was found in the strain *S. pasteuri* Sp 42. Fermentation of trehalose, sucrose glucose, lactose and arginine is positive or variable.

Strains	Score value	DNase	Hem	Biofilm	LA

Jejunum					
SHo 33	2.363	ng	γ	0.086 (0.03)	0.700 (0.49)
SHo 23	2.004	ng	β	0.121 (0.35)	0.540 (0.50)
SHo 2Tr2	2.016	ng	γ	nt	1.700 (0.50)
SHae 1Tr4	1.864	ng	α	0.127 (0.36)	0.850 (0.04)
SE 2Tr1	1.864	ng	γ	0.092 (0.02)	0.600 (0.21)
SL 72	2.000	ng	γ	0.074 (0.03)	1.630 (0.21)
SW 43	2.054	ng	γ	nt	2.100 (0.87)
SX 3Tr2	2.030	ng	γ	nt	1.130 (0.09)
Caecum					
Sp 42	1.922	ng	γ	nt	2.980 (0.18)
SHo 41	2.192	+	β	0.100 (0.31)	1.350 (0.13)
SHo 51	2.014	ng	γ	0.137(0.37)	0.960 (0.55)
SHae 32	1.900	ng	γ	0.095(0.03)	0.850 (0.04)
Colon					
SHae 64	1. 881	ng	α	0.098(0.03)	1.330 (0.19)
SHae 52	2.018	ng	γ	0.082(0.02)	1.400 (0.23)
SHae 62	1.999	ng	α	0.087(0.04)	1.600 (0.35)
SE 81	2.024	+	α	0.123(0.36)	1.600 (0.21)
Sco 131	1.821	+	α	0.160(0.40)	nt
Sv31	1.762	nt	nt	0.130(0.36)	1.300 (0.05)

Note: DNase activity, ng-negative; nt-not tested; (±SD), LA-lactic acid; SHo-*Staphylococcus hominis*, SHae-*S. haemolyticus*, SE-*S. epidermidis*, Sp-*S. pasteuri*, Sv-*S. vitulinus*, SW-*S. warneri*, SX-*S. xylosus*, SL-*S. lentus;* Sco-*S. cohnii;*

Table 1: Identification score, DNase activity, hemolysis, biofilm ability and lactic acid production of staphylococci from gut of beavers.

Fifteen strains were DNase negative, only *S. epidermidis* SE 81, *S. cohnii* Sco 131 from colon and SHo 41 from caecum were DNase positive. Majority of staphylococci were hemolysis negative (γ-hemolysis, ten strains, Table 1) or showed partial α-hemolysis (five strains). Only *S. hominis* SHo 41 and SHo 23 were evaluated with β-hemolysis (complete hemolysis).

Biofilm ability in staphylococci was evaluated as low-grade ($0.1 \leq A_{570} < 1$) positive in two strains from jejunum (SHae 1Tr4 and SHo 23, Table 1), in two strains from caecum, *S. hominis* SHo 41 and SHo 52

and in three strains from colon, *S. vitulinus* Sv 31, SE 81 and Sco 131 (Table 1). The rest of strains were negative (A_{570} < 0.1) and four strains were not tested for biofilm formation.

Staphylococci were susceptible to chloramphenicol, clindamycin, gentamicin, lincomycine, cefoxitine and vancomycin. Strains Sv31, Sp42, SW 43, SHae 1Tr4, SHo 23 were susceptible to all antibiotics except penicillin, to which these strains were resistant; it means they were resistant to one out of 15 antibiotics meaning they were mostly susceptible (Table 2). The most resistant were strains SHo 33 and Sco 131, resistant to 6 out of 15 antibiotics; SL 72 and SHae 52 were resistant to five out of 15 antibiotics. SHo 51, SHae 32 and SE 81 were resistant to four antibiotics. Three strains (SHo 2Tr2, SHo 41, SHae 64) were resistant to three antibiotics and SHae 62 and SX 3Tr2 were resistant to two antibiotics. Resistance to Ox was detected in the strains SHo 41, SHae 32 and Sco 131; moreover, Sco 31 and SHo 41 were also resistant to methicillin. The most frequently was detected resistance to Amp, in 9 strains (Table 2); in five strains was found resistance to neomycin, erythromycin and novobiocin. Four strains showed resistance to Tc. To Tob and Ox were resistant three strains and Met resistance was found only in two strains. Majority of strains ecxept SHae 41 and Sco 131 were Met susceptible.

Strains	Ox	N	Amp	Met	E	Nov	Tc	Tob
Jejunum								
SHo 33	24	R	R	10	R	R	R	21
SHo 23	22	16	27	26	23	15	26	21
SHo 2Tr2	19	17	R	25	R	20	28	22
SHae 1Tr4	28	15	30	28	17	17	27	22
SE 2Tr1	25	13	R	26	22	20	15	20
SL 72	36	R	R	30	22	R	30	R
SW 43	19	17	26	22	24	16	31	21
SX 3Tr2	20	17	R	24	20	R	19	27
Caecum								
Sp 42	20	16	30	23	21	20	20	20
SHo 41	R	17	30	R	19	20	26	27
SHo 51	21	16	R	20	R	19	R	24
SHae 32	R	20	R	24	15	R	25	27
Colon								
SHae 64	18	21	R	25	R	21	27	25
SHae 52	21	R	R	27	R	17	R	24
SHae 62	20	13	R	23	30	25	30	23
SE 81	20	R	19	30	19	16	R	R
Sco 131	R	R	13	R	22	R	26	R
Sv 31	17	14	17	26	22	18	27	22

Note: R-resistant, (no)-inhibition zone size in mm; Ox-oxacillin (1 µg), N-neomycin, Amp-ampicillin, Met-methicillin , Tob-tobramycine (10 µg), E-erythromycin (15 µg), Nov-novobiocin (10 µg), Tc-tetracycline (30 µg), vancomycin (30 µg); Strains were resistant to penicillin and susceptible to gentamicin, clindamycin, cefoxitine, lincomycin, chloramphenicol and vancomycin. SHo-*Staphylococcus hominis*, SHae-*S. haemolyticus*, SE-*S. epidermidis*, Sp-*S. pasteuri*, Sv-*S. vitulinus*, SW-*S. warneri*, SX-*S. xylosus*, SL-*S. lentus*

Table 2: Antibiotic profile of identified staphylococci.

Lactic acid (LA production) values were high, 1.330 mmol/l (0.15) on average. *S. pasteruri* Sp 42 reached the highest LA production (2.980 (0.18) mmol/l, Table 1) followed with *S. warneri* SW 43 (2. 100 (0.87 mmol/l). Eleven out of 18 strains produced high value of LA not depending on the species.

4. Discussion

Staphylococci are one of the major groups of bacterial commensals and as mentioned in our previous study they includes 52 known species with 28 subspecies [13]. The total counts of staphylococci from beavers' gut are comparable with the staphylococcal counts detected in the other animals such as deers or horses [14, 13]. What it can differ, it is their species variability; in gut of beavers high species variability in staphylococci was noted. Based on 16S rRNA sequence analysis [6], staphylococci from jejunum were allotted as follows*: S. hominis* and S. *haemolyticus* in S. haemolyticus group/cluster, *S. epidermidis* SE 2Tr1 in S. epidermidis group, *S. lentus* in S. sciuri group, *S. warneri* SW 43in S. warneri group and S, xylosus SX 3Tr2 in S. saprophyticus group. *S. pasteuri* Sp42 from caecum is from S. warneri cluster and SHo 41, SHo 52 and SHae 32 are from S. hemolyticus cluster. Similarly as SHae 64, SHae 52 and SHae 62 from the colon. *S. vitulinus* belong in S. sciuri cluster and *S. cohnii* Sco131 in S. saprophyticus cluster, *S. epidermidis* SE81 in S. epidermidis cluster. Among 11 clusters following classification according to Takashi et al. [6], nine species from five clusters were present in beavers' gut samples. Variability in staphylococci was also reported in faeces of deers or horse [14, 13].

DNase is a virulence factor which catalyses the degradation of DNA. However, among 18 identified staphylococci, these virulence factor phenotype was present only in three strains of three species, SHo 41, SE 81 and Sco 131 from caecum and colon. Also hemolysis belong in virulence factors and it is also one of identification characteristic of these species. Alfa-hemolysis (partial hemolysis) was detected in the strains SHae 64, SHae 1Tr4, SHae 62, SE81 and Sco 131. SHo 41 showed β-hemolysis and other strains were negative (γ-hemolysis) [8]; Bergeys' Manual of Systematic Bacteriology 2009). Among our strains, *S. cohnii* is typical to have DNase activity while other species are reported DNase negative.

As previously noted, members of the genus Staphylococcus are commensals that can cause a variety of infections in animals [15]. Biofilm formation was e.g. described in *S. epidermidis* strains from subclinical mastitis [16] but also in staphylococci from faeces of horses [14]. In this study, one of two *S. epidermidis* strains was non biofilm forming (SE 2Tr1) and second one SE 81 strain was low-grade biofilm positive. It seems that biofilm formation is not-associated with the species but it depends on the character of strain itself. E.g. 14 faecal strains of *S. vitulinus* from horses were low-grade biofilm positive, similarly as *S. vitulinus* strain in this study. Following the faecal staphylococci from roe and red deers, *S. haemolyticus* strains were mostly negative or low-grade biofilm positive which is a similar status as in beavers' *S. haemolyticus* strains [13]. However, staphylococci from beavers were mostly biofilm non-forming. The emergence of methicillin resistant staphylococci in animals is a public health concern due to the high zoonotic potential of especially multidrug resistant strains [17]. Evaluation of oxacillin and cefoxitine disks are frequently used to predict methicillin resistance [18]. Therefore, in this study those antibiotics were tested. However, only three strains (SHo 41, SHae 32, Sco 131 showed Ox resistance and SHo 41 and Sco 131 showed Met resistance phenotype. SHo 41 and Sco 131 were also biofilm-forming strains, DNase positive with α and/or β-hemolysis indicating probably their

pathogenic character; Sco 131 was resistant also to other antibiotics; while SHae 32, Ox resistant strain was DNase negative, hemolysis negative and non biofilm forming. Those DNase and hemolysis negative strains being without biofilm formation ability predict their non pathogenic character. Moreover, majority of strains were Met susceptible.

In spite of the fact that staphylococci as representatives of the phylum Firmicutes belong in lactic acid bacteria group, they usually dont produce high amount of LA. However, staphylococci in this study showed high LA production. Similarly, high LA production was reported in faecal staphylococci isolated from roe and red deer [13]; there also more than 2.00 mmol/l value was measured in the species *S. pseudintermedius* and *S. hominis*. LA production in staphylococci can contribute in their antimicrobial and control ability together with another antimicrobial substances in their each microbiome itself.

5. Conclusion

High variability in staphylococcal strains/species distribution in beavers‘ gut was found; including 18 identified strains, nine species were detected belonging into five clusters; all strains were allotted in the coagulase-negative staphylococcal species. The most frequently detected species were *S. hominis* and *S. haemolyticus* (five strains for each). The other species included two strains-*S. epidermidis* and *S. lentus, S. pasteuri, S. cohnii, S. vitulinus, S. warneri, S. xylosus,* one strain for each one species. Fifteen strains were DNase and almost hemolysis negative. Ten strains (not depending on the species) showed low-grade biofilm ability. Most of strains were methicillin susceptible with high production of lactic acid. The study is original contribution in staphylococcal microbiome of beavers and gives opportunity in more detail study of individual strain species. Moreover, it seems that strains were not pathogenic, meaning not be a threaten for environment and beavers themselves.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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