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Assessment of microbial contamination of sandboxes and toys left in sand

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Sand is one of children's favourite playing objects, thus sandboxes can be found in almost every playground next to apartment buildings. However, sand is also an excellent medium for the proliferation of microorganisms due to its favourable organic matter content, relative humidity, and pH. Therefore, children playing in sandboxes where toys have been left behind are at risk of becoming infected with microorganisms that cause infectious diseases. The main infectious pathogens *Esheriahia coli*, *Staphylococcus aureus*, *Salmonella* spp. and *Enterococcus* spp. were analysed in the sandboxes of children's playgrounds set up in the yards of apartment buildings. The test material came from the surfaces of the toys left in the sand, the edges of the sandboxes, and sand samples collected in autumn and winter. *Enterococcus* spp., *Salmonella* spp. and *E. coli* were detected in the samples from the edges of sandboxes and surfaces of sand-covered toys and surfaces collected in autumn. *S. aureus* (on the surface of the toys left in sand) and *E. coli* were detected in the samples collected from the surfaces of toys and sandboxes in winter. During the study, no infectious agents were detected in the sand samples.

Keywords: sandbox, sand, *Esheriahia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Enterococcus* spp.

INTRODUCTION

Due to its organic matter content, relative humidity, and pH, sand is a perfect environment for the development of microorganisms. As it is one of the main objects for children's play, sandboxes can be found in almost each yard and playground. However, sand is biologically active and, if contaminated, can serve as a reservoir for pathogenic microorganisms (Weiskerger et al., 2019).

Children, who are a high-risk group, can acquire infections from sand in sandboxes: by eating sand while playing in sandboxes, which they tend to do, or through dirty hands they can become

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infected with enteric infections (Romão et al., 2015). Sandboxes are often polluted with excrements of dogs, cats, and rodents, or bird droppings, and that is why enteric infections are common among children (Gotkowska-Płachta, Korzeniewska, 2015). Wright et al. (2009) found that from a few hundred to several millions of intestinal bacteria such as *Esheriahia coli* can be found in one gram of animal faeces. For example, a higher number of enterococci are found in the faeces of animals (dog, cats) than in bird droppings.

These microbes include bacteria such as *Staphylococcus aureus*, *E. coli*, *Enterococcus* spp. and *Salmonella* spp., which can be particularly dangerous to human health. At any time of the year, parasitic bacteria and human-associated pathogenic microbes can be detected in sand samples (Buczek, 2017). The survival rate of those microorganisms in the sand environment depends on temperature, pH, humidity, insolation, and nutrient availability (Gotkowska-Płachta, Korzeniewska, 2015; Romão et al., 2015).

The ability of microorganisms to cause disease is related to their virulence, quantity, and resistance of the organism. The number of bacteria in sand also depends on the season. Any contact with pathogenic microorganisms is a potential threat to human health. Symptoms of infection caused by microorganisms can be mild (mild gastrointestinal disease), severe (inflammation of the urinary tract, respiratory tract, and gastrointestinal tract, bone inflammation) or even fatal, especially in infected children (Mahon et al., 2010; Gotkowska-Płachta, Korzeniewska, 2015).

Epidemiological studies show that children who play in the sand are more likely to catch infection than those who do not play in it. Thus, sand can serve as a means of spreading diseases through direct contact, via sand containing microbes, or indirectly, via contact with toys in the sand which contain bacteria adhering to sand (Vennewald et al., 2010; Delgado, French, 2012; Whitman et al., 2014; Gotkowska-Płachta, Korzeniewska, 2015; Romão et al., 2015; Solo-Gabriele et al., 2016).

The aim of this study was to evaluate the microbial contamination of sandboxes and sand toys in the yards of apartment buildings.

MATERIALS AND METHODS

Qualitative microbiological tests were performed at the microbiology laboratory of the Department of Medical Technologies and Dietetics, Kaunas University of Applied Sciences, Department of Medical Technologies and Dietetics, in accordance with the HN 131: 2015 and LST EN ISO 14698-1 standard in compliance with occupational safety rules.

The study searched for the main infectious agents: *E. coli*, *S. aureus*, *Salmonella* spp. and *Enterococcus* spp.

Sandboxes of children's playgrounds installed in the public yards of Dainava residential area of the city of Kaunas were investigated.

Samples were collected in autumn (in October 2022) and winter (in December 2022) in the same place. A total of 60 samples were collected: 30 samples in autumn and 30 samples in winter. Each group of the 30 samples consisted of ten samples from sandbox edges, ten samples from toys in sandboxes, and ten sand samples.

Different media and reagents were used for bacterial identification: Oxidase Reagent Dropper, TSI agar, Kovacs' reagent, Simons citrate agar medium , Urea medium, Mannitol Salt Agar (MSA), lyophilised rabbit plasma, medium containing DNA, group D serum (all manufactured by Liofilchem, Italy), 3% hydrogen peroxide, and 1% hydrochloric acid (HCl) (Labochema, Lithuania).

The microbiological test relied on the washing method (Mandell et al., 2010). Samples from sandboxes and toys in the sand were collected with a sterile swab into 1% peptone water (PW) and the sand samples themselves into sterile vessels. The sand samples were filtered through a filter with distilled water and seeded before sowing in nutrient media. The evaluation of the microbiological contamination test for washing consisted of six steps: (1) sampling, (2) preparation and quality control of the media, (3) primary inoculation, (4) inoculation

of the samples into secondary media, (5) identification of the microorganisms detected in the samples, and (6) evaluation of the results.

Primary inoculation of samples into the selective media. The media were prepared according to the manufacturer's recommendations on the media packaging (Liofilchem, Italy). Samples were seeded on Tryptone Bile X-Gluc (TBX) agar for detected *E. coli*, mannitol salt (MDA) agar for *S. aureus*, Bile esculin azide (TEAZ) agar for *Enterococcus* spp., and Xylose Lysine Deoxycholate (XLD) agar for *Salmonella* spp. Samples were incubated for 24 to 48 hours with a control (unseeded) Petri dish +37°C thermostat.

Primary evaluation of the results and inoculation of samples into secondary nourishing media. After 24 to 48 hours of incubation, Petri dishes showing growth of bacteria were removed from the thermostat and re-selected. The colour, size, and abundance of the grown colonies were assessed by visual inspection of the Petri dishes. The purity of bacterial cultures was then assessed: a portion of the colony was removed from the culture medium using a sterile microbiological loop, placed on a slide, and distributed by mixing with physiological saline (NaCl). The smear was fixed with a spirit light and stained using Gram stain. The stained smear was microscopically immersed and evaluated morphologically. The size, arrangement, and the colour of the bacteria were observed: the walls of gram-negative (–) bacteria turned red, the walls of gram-positive (+) bacteria turned blue. The growth of *E. coli* with blue-green colonies was observed on TBX agar, the growth of *S. aureus* was observed on MDA agar: bacteria broke down mannitol to acids, resulting in a change in the medium colour from pink to yellow; in TEAZ agar, *Enterococcus* spp. black colonies were visible and *Salmonella* spp. pink colonies with a black centre were evident in XLD.

After evaluating the morphological and cultural properties of the cultured bacteria, biochemical properties studies were performed to facilitate more accurate identification of the grown bacterial colonies (Mandell et al., 2010).

***E. coli* identification tests:**

Oxidase test. Oxidase activity was assessed by adding Oxidase Reagent Droppers to a single colony. *E. coli* bacteria did not degrade oxidases, so after adding the reagent, the colour of the colony does not change within 1–3 min.

Carbohydrate degradation. Part of the colony was taken from TBX agar in a sterile microbiological loop and inoculated into three-sugar iron (TSI) agar *E. coli* broke down all carbohydrates in TSI – lactose, sucrose, and glucose – to acids and gases.

Protein degradation. Kovacs reagent was added dropwise to a tube of tryptone water and the formation of a pink ring was observed, indicating the degradation of *E. coli* proteins to indole.

Citrate degradation. Decomposition of citrates was observed in Simans medium. *E. coli* did not degrade citrates, so the Simans medium did not change and remained dark green.

Urea degradation. Urea medium did not degrade urea in *E. coli*, so the medium did not change and remained yellowish in colour.

***S. aureus* identification tests:**

Mannitol degradation. *S. aureus* bacteria broke down mannitol to acids causing the medium to change from pink to yellow.

Catalase test. Staphylococci were confirmed by a positive catalase test: 3% hydrogen peroxide was applied to a single colony. The test was positive if foaming was observed, when hydrogen peroxide decomposed into water and oxygen.

Plasma coagulase test. Isolated pure bacterial culture was added to a tube containing lyophilised rabbit plasma. The sample was incubated at +37°C in a thermostat for 24 hours. After incubation, plasma coagulation was analysed: if plasma coagulated, then the plasma coagulase test was positive, and if did not, the micro-organism did not coagulate the plasma.

DNA testing. The bacterium *S. aureus* broke down DNA. Staphylococci were inoculated on medium containing DNA with a microbiological loop and the Petri dish was incubated at +37°C for 24 hours. After incubation, 1% hydrochloric acid (HCl) was added to the control and to the pure colony culture. The test was positive

if a clear area formed around the spotted colony and a grey area formed on the control dish.

***Enterococcus* spp. identification test:**

Latex agglutination reaction test. *Enterococcus* spp. bacterium is group D Streptococcus, so group D serum was used for the agglutination reaction. One drop of group D serum was applied to a special latex plate and a pure enterococcal culture was applied with a microbiological loop. A positive test was indicated by the precipitation of white precipitate during the reaction.

***Salmonella* spp. identification tests:**

Lysine degradation. *Salmonella* spp. bacteria broke down lysine, so the colour of lysine agar changed from yellow to brown-purple.

Mannitol degradation. *Salmonella* spp. broke down mannitol into acids and gases causing the medium to change colour from pink to yellow.

Carbohydrate decomposition. In TSI agar (contains three sugars: glucose, lactose, and sucrose) *Salmonella* spp. decomposed only glucose to acids and gases (CO_2 and O_2), but did not decompose lactose and sucrose.

Citrate degradation. Decomposition of citrates was determined in Simans medium; salmonella decomposed citrates, so the medium changed from green to blue.

Urea degradation. *Salmonella* spp. did not contain a urease enzyme, so it did not break down urea and the medium remained yellow.

The data were analysed using IBM SPSS Statistics v26 software. Descriptive statistics (frequency), group comparison (Chi-square), and relationship calculation (non-randomness coefficient) were used.

RESULTS AND DISCUSSION

A total of 60 samples from same sites were tested, 30 of which were taken in autumn and 30 in winter. In the washing method, indicators of *E. coli*, *S. aureus*, *Salmonella* spp. and *Enterococcus* spp. microbial contamination were searched for.

In the 60 analysed samples, the highest detected number was of *Enterococcus* spp. (26.7%, 16/60), *E. coli* (11.7%, 7/60), *Salmonella* spp. (6.7%, 4/60), and the lowest number was of *S. aureus* (1.7%, 1/60) (Fig. 1).

Microorganisms were detected on toys from sandboxes (23.3%; 14/60) and the edges of sandboxes (23.3%; 14/60) (Fig. 2). However, no microorganisms were detected in sand itself.

In this study, *E. coli* bacteria were found on the toys in the sand (4) and on sandbox edges (3) (Fig. 2). No statistically significant

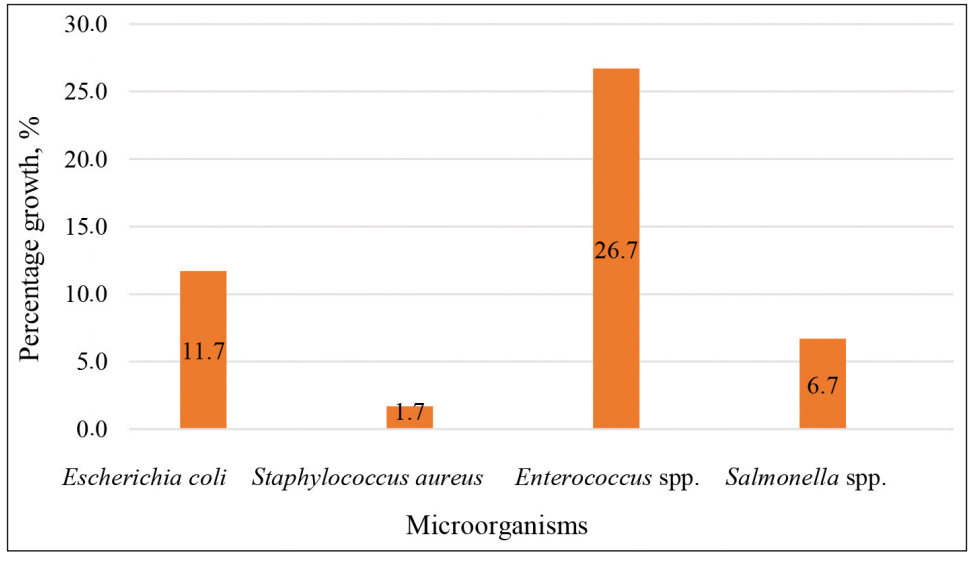

Fig. 1. Percentage of microorganism detection in samples ($N = 60$)

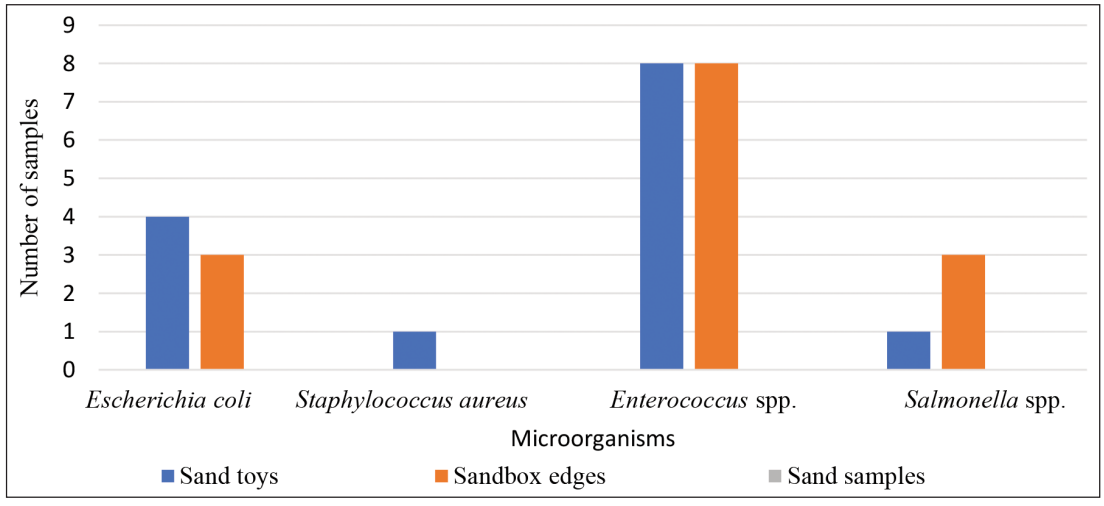

Fig. 2. Growth of microorganisms in different sites

association was found between the growth of *E. coli* and different locations of the samples ($p = 0.122$, $p > 0.05$). The higher concentration of bacteria on the toys in the sand was due to the fact that children were constantly touching the toys with their hands, and the toys could remain in the sandboxes for several years. The bacteria were not found in the sand samples and this may have been influenced by the fact that sand samples was taken from the surface.

Comparison of *S. aureus* bacteria found at different sites showed that only one sample containing *S. aureus* bacteria was found and it was taken from the toys in the sand (Fig. 2). No statistically significant association was determined between growth of *S. aureus* and different sample locations ($p = 0.362$, $p > 0.05$).

According to Shah et al. (2011), different pathogenic microbes could be found in sand: bacteria such as *S. aureus*, including methicillin-resistant strains, protozoa such as *Cryptosporidium* spp., enterovirus, and helminths and yeast such as *Candida parapsilosis*. All pathogenic microorganisms pose the risk of infectious diseases.

Vennewald et al. (2010) reported bacteria isolated in the cases of otitis externa in children who played with sand, such as *S. aureus*, *Streptococcus pyogenes*, gram-negative rods, fungal agents such as *Aspergillus* sp., and *Candida* sp.The highest number of infected samples in different sites contained *Enterococcus* spp. bacteria (Fig. 2): they grew in eight samples from both the toys in the sand and the edges of sandboxes. A statistically significant association was found between *Enterococcus* spp. bacterial growth in samples taken from different sites ($p = 0.004$, $p < 0.05$). *Enterococcus* spp. bacteria is commonly transmitted via animal (birds, domestic and wild dogs, and cats) faeces in the sand (De Graef et al., 2005). The highest risk of sand contamination is when sandboxes are near trees with bird nests (Delgado, French, 2012).

In this study, *Salmonella* spp. bacteria were found in samples taken from toys in the sand (1) and from the edges of the sandbox (3). More bacteria were found on the edges of the sandboxes than on the toys in the sand (Fig. 2), but no statistically significant association was found between *Salmonella* spp. bacterial growth and different sampling sites ($p = 0.153$, $p > 0.05$).

The presence of microorganisms in sandboxes was analysed in Poland. The sand collected from sandboxes in an unfenced housing estate was heavier infected compared with the sand collected from sandboxes near fenced houses and those farther from trees. The study reported that *Salmonella* spp., *E. coli*, and *Enetrococcus* spp. were detected (Gotkowska-Płachta, Korzeniewska, 2015).

Hauschild et al. (2002) performed an analysis of five sandboxes in Poland. The results revealed that six species of *Salmonella* and 16 species of *Staphylococcus* were detected in sandboxes. The highest number of microorganisms was found in summer (June and July). The increased risk of infectious diseases during summer may be due to the high activity of humans and domestic and wild animals at this time.

Further analysis shows that it is important to focus on the growth of bacteria with relation to the time of the year. The growth of *E. coli* bacteria in autumn and winter is shown in Fig. 3. The results vary, but only slightly: the number of bacteria found in winter (4) is slightly higher than in autumn (3). However, no significant difference could be detected ($p = 0.688$, $p > 0.05$). According to Schostag et al. (2015), seasonal variations in soil moisture, temperature, and nutrients can alter the number of bacteria and their structure. The increased number of bacteria in winter may have been due to melted snow, which is also rich in microorganisms, and considerable temperature changes (before sampling, the outdoor temperature was –15°C, the samples themselves were collected when the air became warmer and was about –2°C).

The growth of *S. aureus* bacteria is compared with regard to the season of the year (Fig. 3). No *S. aureus* bacteria were found in any of the samples in autumn; however, the bacteria grew in one sample collected in winter. Nevertheless, no statistically significant difference was found ($p = 0.313$, $p > 0.05$).

Enterococcus spp. bacteria grew from more samples collected in autumn (9) in comparison to those collected in winter (7) (Fig. 3). No statistically significant difference was detected ($p = 0.559$, $p > 0.05$).

Bacterial growth of *Salmonella* spp. was also evaluated with regard to the time of the year (Fig. 3). The bacteria grew in four samples collected in autumn and in none of the samples collected during in winter. This difference may have been due to higher temperatures or average humidity levels. Observing *Salmonella* spp., a statistically significant association for bacterial growth was found ($p = 0.038$, $p < 0.05$).

Regarding the results of the present study, bacteria were found both in autumn and in winter; however, the bacteria were different. The highest recorded number was of *Enterococcus* spp. and *E. coli* bacteria, which shows long-standing contamination with pathogenic microorganisms of faecal origin (Gotkowska-Płachta, Korzeniewska, 2015). In this study, *Enterococcus* spp., *Salmonella* spp., and *E. coli* were detected in the samples collected from the edges of sandboxes and surfaces of toys

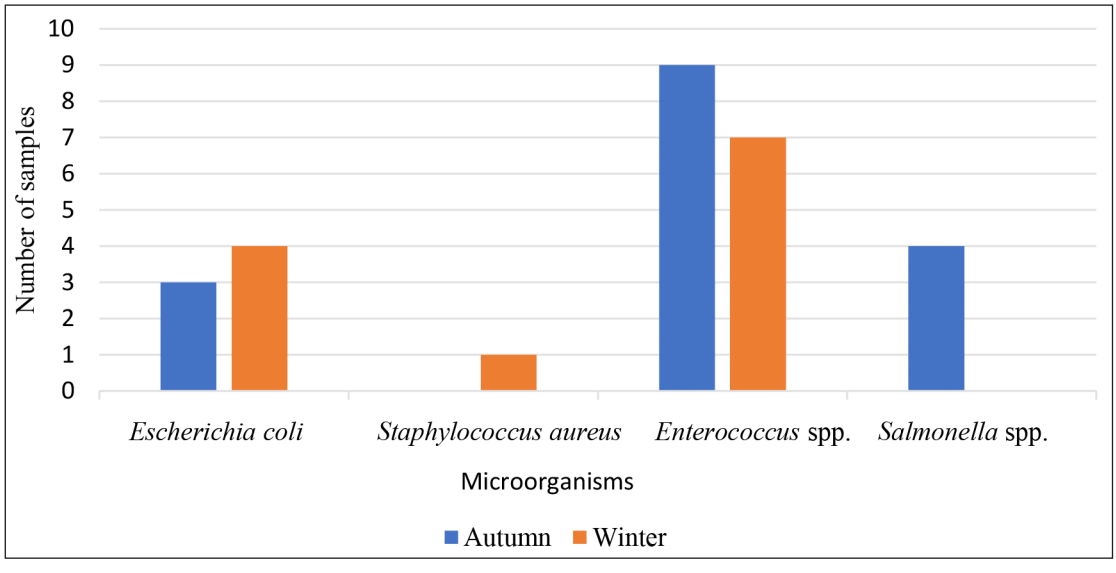

Fig. 3. Growth of microorganisms in different seasons of the year

during the autumn season. In winter, *S. aureus* were found on the surface of toys in the sand, *Enterococcus* spp. on the surfaces of the edges of sandboxes and the surfaces of the toys in the sand, and *E. coli* on the surfaces of the toys and edges of sandboxes. No infectious agents were detected in the sand samples during the study. Toys can stay in sand for a long time and more than one child plays with the same toys, therefore bacteria accumulate on them and there is a high risk of infection for the children playing with them. This may be because the sandboxes are not tightly closed and are frequented by wild and domestic dogs, cats, and birds.

Sand from sandboxes that are covered and protected from contamination by animal faeces meets the cleanliness category. Thus, protection of sand from faecal contamination by animals is an effective preventive measure. However, this may not be enough: it is necessary to de-worm pets, limit the number of unattended animals, and raise hygiene skills in children playing in the sandbox (Bozhko et al., 2018).

CONCLUSIONS

With regard to the diversity of microbes found in sand, further research is needed to identify the main microorganism and to link the microbes found in the sand with risks to human health. All classes of microbes should be assessed in an environmental assessment of sandboxes and playgrounds.

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SMĖLIO DĖŽIŲ IR SMĖLYJE ESANČIŲ ŽAISLŲ MIKROBINĖS TARŠOS VERTINIMAS

Santrauka

Smėlis yra vienas mėgstamiausių vaikų žaidimų objektų, todėl smėlio dėžių galima rasti beveik visose daugiabučių kiemuose įrengtose vaikų žaidimo aikštelėse. Tačiau smėlis dėl jame esančio organinių medžiagų kiekio, santykinės drėgmės ir pH taip pat yra puiki terpė mikroorganizmams vystytis. Vaikams žaidžiant smėlio dėžėse su jose paliktais žaislais kyla pavojus užsikrėsti mikroorganizmais, sukeliančiais infekcines ligas. Šio darbo tikslas buvo nustatyti skirtingus mikroorganizmus smėlio dėžėse, įrengtose daugiabučių kiemų vaikų žaidimo aikštelėse. Medžiaga buvo renkama rudens ir žiemos laikotarpiais nuo smėlyje paliktų žaislų, smėlio dėžių kraštų paviršių, taip pat paimti smėlio mėginiai. *Enterococcus* spp., *Salmonella* spp. ir *Esheriahia coli* bakterijos aptiktos mėginiuose, surinktuose nuo smėlyje esančių žaislų ir smėlio dėžių kraštų paviršių rudens sezono metu. Žiemą aptikta *Staphylococcus aureus* ant smėlyje esančių žaislų paviršių ir *E. coli* – ant žaislų ir smėlio dėžių paviršių. Smėlio mėginiuose tyrimo metu infekcinių ligų sukėlėjų nerasta.

Raktažodžiai: smėlio dėžės, smėlis, *Esheriachia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Enterococcus* spp.