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MICROBIOLOGICAL COMPOSITION OF THE AIR IN DAIRY FARMS USING DIFFERENT TYPES OF BEDDING

ÕHU MIKROBIOLOOGILINE KOOSTIS ERINEVAT LIIKI ALLAPANU KASUTAVATES PIIMAFARMIDES

Final Thesis
Curriculum in Veterinary Medicine

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Bedding material is one of the fundamental elements of animal housing environment, as animals like to spend a significant amount of time on it. The main interest in previously published studies concerning bedding materials has been in laying comfort, milking performance, and stall hygiene. Surprisingly, the present understanding of the bedding material's effect on the microbiological quality of the air is scarce. This study aimed to compare microbiological air quality in two free-stall dairy cattle farms using different kinds of bedding materials – peat and sawdust. Both of the study farms (Farm A = peat; Farm B = sawdust) were visited three times from spring 2022 to winter 2023. The air samples were collected by using two different methods: sedimentation and filtration. In total, 30 sedimentation samples and 72 filtration samples were collected. To apply the sedimentation method, five agar plates were placed on a polystyrene box at a height of one metre from the floor surface, after which all plates were opened for a specified time. Filtration samples were collected by using a portable air sampler (Sartorius AirPort MD8). The air samples were taken to the microbiology laboratory and cultivated on four types of growth media (Columbia agar, Columbia blood agar, MacConkey agar, and Sabouraud dextrose agar) to identify mesophilic bacteria, pathogenic bacteria, Gram-negative and enteric bacteria, and also fungal species. According to the results of the study, the two investigated bedding materials caused different microbial composition of the air. The results indicate that the bedding material may substantially affect the microbial composition of the air and thus the quality of the animal housing environment, but the field needs relevant studies more.

Keywords: bedding, air quality, bioaerosols, air sampling, animal welfare

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Allapanumaterjal on üks tähtsamaid elemente loomapidamise keskkonnas, kuna loomad tahavad sellel palju aega veeta. Varem avaldatud allapanumaterjale käsitlevate uuringute põhihuvi on olnud loomade lamamismugavus, piimatoodang ja aseme hügieen. Praegune arusaam allapanumaterjali mõjust ümbritseva õhu mikrobioloogilisele kvaliteedile ei ole piisav. Käesoleva uuringu eesmärk oli võrrelda õhu mikrobioloogilist kvaliteeti kahes vabapidamisega piimakarjafarmis, kus kasutati erinevat tüüpi allapanu – turvast ja saepuru. Mõlemaid uuringufarme (farm A = turvas; farm B = saepuru) külastati ajavahemikus kevad 2022 kuni talv 2023 kolm korda. Õhuproovide kogumiseks kasutati kahte erinevat meetodit: sedimentatsiooni ja filtreerimist. Kokku koguti 30 sedimentatsiooniproovi ja 72 filtreerimisproovi. Sedimentatsioonimeetodi rakendamiseks asetati viis Petri söötmetassi polüstüreenkastile ühe meetri kõrgusele põrandapinnast, misjärel avati kõik plaadid kindlaksmääratud ajaks. Filtreerimisproovid koguti portatiivse õhuproovide kogujaga (Sartorius AirPort MD8). Õhuproove analüüsiti mikrobioloogia laboris, kus need kultiveeriti neljale erinevale kasvusöötmele (Columbia agar, Columbia vereagar, MacConkey agar ja Sabouraud dekstroosi agar), et tuvastada mesofiilsed bakterid, patogeensed bakterid, gramnegatiivsed ja enterobakterid, samuti seeneliigid. Uuringu tulemuste kohaselt põhjustasid kaks uuritud allapanu materjali õhu erineva mikroobse koostise. Tulemused näitavad, et allapanumaterjal võib oluliselt mõjutada õhu mikroobset koostist ja seega ka loomapidamiskeskkonna kvaliteeti, kuid antud valdkond vajab rohkem vastavaid uuringuid.

Märksõnad: allapanu, õhu kvaliteet, bioaerosoolid, õhuproovide kogumine, loomade heaolu

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BRD	bovine respiratory disease
CBP	compost-bedded pack
CFU	colony forming unit
HTP	concentrations known to be harmful
MAC	*Mycobacterium avium* complex
ODTS	organic dust toxic syndrome
RMS	recycled manure solids

INTRODUCTION

Bedding materials are an essential part of animal welfare and performance in commercial practice. Bedding is used in animal husbandry to maintain hygiene, add comfort, and enable animals to behave as naturally as possible in a given housing environment. Good quality bedding does not only add comfort and well-being for animals but also increases productivity – the more time cows spend lying down comfortably, the more they ruminate and more milk is produced (Calamari *et al.*, 2009; Ferraz *et al.*, 2020).

We may have an idea of what bedding characteristics are desirable. However, none of the bedding materials are perfect. There is evidence that dusty or contaminated bedding material can pose health risks to animals and workers as shown in many studies (Samadi *et al.*, 2012; Saastamoinen *et al.*, 2015; Mönki *et al.*, 2021). Nevertheless, there is limited information available on the influence of bedding materials on microbial air quality in animal facilities. Previous studies on bedding materials have mainly been performed in connection with the pathogenesis of mastitis and hoof diseases, and for a reason. Milking cows spend on average 12 to 14 hours a day lying down in cubicles, making bedding a considerable source of teat and udder contamination (Hogan and Smith, 2012). A significant portion of mastitis cases are caused by environmental pathogens that originate from contaminated bedding material, including coliform bacteria and streptococcal species (Godden *et al.*, 2008). The effects of bedding on equine respiratory health has been investigated in several studies (Fleming *et al.*, 2008; Kwiatkowska-Stenzel *et al.*, 2017; Mönki *et al.*, 2021). To the author's knowledge, no similar studies have been conducted on adult dairy cattle. Hence, there is a significant reason to test the air quality in dairy farm environments. Surprisingly there is almost a complete paucity of information concerning bovine respiratory disease (BRD) in adult dairy cattle, even though respiratory diseases remain to be a key reason for welfare and economic concern for dairy producers (Gorden and Plummer, 2010).

Consumers are becoming increasingly conscious about the sustainability of their consumption habits. Society has encouraged people to invest in products originating from housing environments where the welfare of livestock has been ensured. Animal welfare is an important aspect of sustainability and product quality. If quality has been compromised, it may foster foodborne outbreaks (OIE Animal Production Food Safety Working Group, 2006). In addition, people working in animal holdings must be protected from zoonotic animal diseases and occupational infections. As bedding materials play a key role in the production animal

welfare and people working in animal shelters (Samadi *et al.*, 2012), there is a need for further investigation into how bedding materials influence the microbial quality of air. The aim of the study is to compare the influence of two bedding materials, peat and sawdust, on the microbiological composition of the air in the loose housing environment for dairy cattle. We hypothesise, that having peat as bedding produces more fine dust particles and therefore more microbes are attached to those particles in the air. To the author's knowledge, this study was the first of its kind in Estonia.

1. LITERATURE REVIEW

1.1 Organic bedding materials

Organic bedding material is a popular choice for the production animal husbandry. A few of the main reasons why organic materials remain popular are: (1) easy to keep animals clean, handling is fairly easy and they yield good quality manure compared to inorganic bedding materials (Ferraz *et al.*, 2020) (2) generally, they are well available (Munir *et al.*, 2019), and (3) they may be used diversely in many types of housing systems (Lensink *et al.*, 2013). On the other hand, there are a few drawbacks to using organic bedding materials. Organic bedding alternatives appear to have more available nutrients, higher moisture content, and pH ideal for bacterial growth compared to inorganic bedding materials (Hogan *et al.*, 1989; Zdanowicz *et al.*, 2004). In a study by Hogan *et al.* (1989) scholars observed significantly higher gram-negative, coliform, *Klebsiella* spp., and streptococcal counts from organic beddings compared to inorganic bedding materials. Bacterial counts in organic bedding, such as sawdust and straw, generally contain more than 10^6 colony forming unit (CFU) per gram of coliforms (Hogan and Smith, 1997). The incidence of mastitis cases has been shown to increase around this level (Zehner *et al.*, 1986). Table 1 shows examples of recorded microbial counts in used organic and inorganic bedding alternatives.

1.1.1 Peat

Peat may be defined as an accumulation of partially degraded vegetation and other organic matter, mostly composeding of *Sphagnum* moss (Mönki *et al.*, 2021). Peat is used as bedding material in many different production animal holdings for such as cattle, horses, swine, and poultry (Aro *et al.*, 2021). Peat is commonly used in Nordic and Baltic countries due to its good availability and qualities as a bedding material (Mönki *et al.*, 2021). In Finland, the production animal sector uses annually approximately 0.6–1.3 million cubic meters of peat (Aro *et al.*, 2021). Peat's popularity is largely due to its good hygroscopicity and ability to bind noxious gases, such as ammonia (Saastamoinen *et al.*, 2022). In addition, peat yields good quality manure as it composts faster compared to other bedding materials (Airaksinen *et al.*, 2001). Peat seems to have some beneficial effects on gastrointestinal health. It has been used in pig husbandry as an iron supplementation for suckling piglets and for supporting newly weaned piglets' intestinal function (Johansen *et al.*, 2014).

Table 1. Some examples of reported microbial counts in used bedding materials

Material	Unit (log_{10} CFU)	Coliforms	Gram-negative	*Klebsiella* spp.	*Streptococcus* spp.	Mesophilic fungi	References
Peat	per g	-	-	-	-	7,4	Mäittälä *et al*., 2001
Sawdust	per g	6,2 - 6,5	7,0 - -	4,8 - 6	7,1 - 6,2	- 4 -	Hogan *et al.*, 1989 Airaksinen *et al.*, 2001 Zdanowicz *et al*., 2004
Straw	per g	6,3 -	7,1 -	3,7 -	7,8 -	- 5,6	Hogan *et al.*, 1989 Airaksinen *et al*., 2001
Compost	per g	6,3	-	-	7,2	-	Black *et al*., 2014
RMS*	per ml	6	8,2	6,5	8	-	Hogan *et al*., 1999
Sand	per g	5,7 6	6,3 -	3,2 4,1	7 6,5	- -	Hogan *et al.*, 1989 Zdanowicz *et al*., 2004
Crushed limestone	per g	5,8	6,5	3,8	6,6	-	Hogan *et al.*, 1989

*RMS, recycled manure solids

Peat's ability to bind ammonia is superior compared to other commonly used bedding materials like sawdust and straw (Airaksinen *et al.*, 2001). Great ammonia binding capacity is due to peat's low pH (Sommer, 2013). Saastamoinen *et al.* (2015) found out in their study that the ammonia level in the horse stable boxes was non-existent or very low when peat was used as bedding. Several studies have come to the same conclusion (Airaksinen *et al.*, 2001; Misselbrook and Powell, 2005). Ammonia levels in facilities using peat have shown to be as low as one-tenth of those measured in facilities using straw (Sommer, 2013). Dairy cattle farms are one of the most significant sources of ammonia emissions (Misselbrook and Powell, 2005). Therefore, peat could be used as means of decreasing fugitive ammonia emissions.

Peat has bacteriostatic properties due to naturally occurring humic acids and antibiotic microflora (Mellegård *et al.*, 2009). This has made it possible to reduce the amount of antibiotics used in the production animals (Aro *et al.*, 2021). Bacteriostatic properties found in peat may also be beneficial for the dairy industry. One area where it has shown to be beneficial is in inhibiting some of the spore-forming bacteria, such as *Bacillus cereus.* Spores of *B. cereus*

are very persistent in the farm environment and can contaminate raw milk and consequently alter the final product (Magnusson *et al.*, 2007a). Magnusson *et al.* (2007b) reported that the growth of *B. cereus* was inhibited completely in peat. Good results were also obtained when peat was mixed with sawdust. Mönki *et al.* (2021) state that peat bedding should be a good choice for horses suffering from respiratory disease. The researchers concluded in their study that peat bedding induces less airway inflammation in healthy horses compared to wood shavings.

There are, however, also some negative aspects to using peat. The drawbacks of peat are inconsistent hygienic quality, dark colour, and dustiness (Airaksinen *et al.*, 2001). In one study, conducted by Airaksinen *et al.* (2005), the researchers observed variations in the level of dustiness and hygienic quality of four different sorts of peat bedding. According to the study results, light and weakly decomposed *Sphagnum* peat contained significantly more endotoxin and might cause higher bacteria exposure compared to the other studied peat materials. In addition to high endotoxin load, fungi such as *Penicillium* spp. and *Aspergillus* spp. have been reported in air samples in high concentrations in piggeries using composted peat bedding (Mäittälä *et al.*, 2001; Airaksinen *et al.*, 2005). Mäittälä *et al.*, (2001) found that fungi concentrations were 20–80 times higher in air samples in piggeries while using peat compared to the previous time when sawdust was used. They suggested that the small particle size and dustiness might be one of the major reasons for higher airborne exposure. Peat may also contain high numbers of mycobacteria (Mönki *et al.*, 2021). These pathogens have remained a serious public health concern as there is evidence that subspecies belonging to *Mycobacterium avium* complex (MAC) may cause zoonotic and occupational infections (Eslami *et al.*, 2019). Johansen *et al.* (2014) investigated whether there was a link between the use of peat as bedding and *Mycobacterium avium* subsp. *hominissuis* outbreaks in the Norwegian swine population as there was an increase in condemnation of pig carcasses with tuberculous lesions at the meat inspection in 2009 and 2010. The researchers concluded that outbreaks of *M. avium* subsp. *hominissuis* seemed to be related to the use of contaminated peat bedding. Such outbreaks might cause greater economic losses for the farmer and may provoke public health concerns since the zoonotic aspect of *M. avium* infections has not been ruled out (Johansen *et al.*, 2014). The peat industry has an infamous reputation due to the negative environmental impact it causes. During peat harvesting, transportation, and storage, particulate matter emissions may rise resulting in dust dispersion over long distances (Tissari *et al.*, 2006).

As the peatland ecosystem is an efficient carbon sink and a slowly renewable energy source (Mönki *et al.*, 2021), there is cause to investigate the pros and cons of using peat as

bedding materials for animals. Peat harvesting in Finland is already on the scale down due to strict environmental regulations (Saastamoinen *et al.*, 2022). Alternative bedding materials are under development to replace peat with the aim of reducing the environmental impact, without compromising animal welfare (Aro *et al.*, 2021; Saastamoinen *et al.*, 2022).

1.1.2 Wood-based beddings

Wood derivative bedding materials' absorption capabilities depend on (1) moisture content, (2) particle size (the smaller the particle size the bigger the surface area and thus better absorption), (3) species and part of the wood used in making the product (Munir *et al.*, 2019). The light colour of the wood products increases light in the animal housing compared to the darker bedding materials such as peat (Airaksinen *et al.*, 2001; James, 2017). Wood has antimicrobial properties to some extent due to the extractives present in wood (Munir *et al.*, 2019). However, wood products are often heavily contaminated with coliforms; sawdust and wood shavings are infamous for yielding high *Klebsiella* spp. counts (Hogan and Smith, 1997).

1.1.2.1 Sawdust

Sawdust is commonly used in dairy cattle farms since its availability is generally good (Munir *et al.*, 2019), and it has several valuable features. Sawdust provides a clean, comfortable and soft stall, it has a fresh and pleasant smell and it is brightly coloured (James, 2017). Sawdust is typically produced as a by-product of wood sawing, is cut into small particles, resulting in a greater surface area and thus enabling more efficient moisture absorption (Munir *et al.*, 2019). Although sawdust has good absorbency, it must be changed regularly as sawdust will be saturated by excreta (*e.g.*, milk, faeces, and urine) relatively rapidly (Alasuutari and Palva, 2014; James, 2017). Likewise, cows are less willing to lay down on damp or wet bedding (Reich *et al.*, 2010). Gas binding capacity is also relatively good (Airaksinen *et al.*, 2001). Sawdust can be used in many kinds of housing systems, however, one should bear in mind that when sawdust is used together with liquid manure and slatted floors, build-up in the slurry pit should be avoided (Lensik *et al.*, 2013). The quality of sawdust may vary significantly. Ekesbo (2013) argues that certain types of sawdust qualities can cause small wounds, especially on the lateral part of the hoof whereas other types of bedding might not do so. Skin injuries can occur especially when the sawdust or wood shavings are used as thin layers on concrete surfaces.

Some sawdust varieties have been found to be dusty – fine particle size can be hazardous to both staff and animals (Alasuutari and Palva, 2014).

Sawdust is a valuable bedding material, yet one must bear in mind the microbiological risks involved in using sawdust as a bedding material. It has been proven that sawdust has a high bacterial count and therefore affects the higher incidence of environmental mastitis (Zdanowicz *et al.*, 2004). These contaminants are not only harmful to animals but could also possibly have zoonotic potential (Munir *et al.*, 2019). Several studies have shown that the incidence of *Klebsiella* spp. and *Escherichia coli* related mastitis are strongly correlated with the use of sawdust as bedding material (Hogan and Smith, 1997; Zdanowicz *et al.*, 2004; Ericsson Unnerstad *et al.*, 2009). Fresh sawdust has been shown to contain the greatest quantities of *Klebsiella* spp. (Hogan *et al.*, 1989). Also, wet sawdust could be a source of different moulds (James, 2017). On the other hand, Ericsson Unnerstad *et al.* (2009) also observed that using sawdust as bedding might reduce the risk of isolating *Streptococcus uberis* compared to using straw or peat.

1.1.2.2 Woodchips

Woodchips have a coarse texture and they are made of bark, sawdust and post-peelings (Munir *et al.*, 2019). Woodchips are more commonly used in open wintering pens where they are used to improve bedding drainage (Lensink *et al.*, 2013). Woodchip causes a low dust burden (James, 2017), animals tend to say cleaner due to abrasiveness and woodchip does not stick to the hair (Larney *et al.*, 2008). On the other hand, the large particle size can be an issue because animals may find it uncomfortable and chips may even harm the animal (Lensink *et al.*, 2013). Like other wood-derived bedding materials, woodchips also have inhibiting properties for bacterial growth (Munir *et al.*, 2019). If woodchips are derived from recycled wood products, it is important to thoroughly screen the bedding for foreign objects as they may contain physical hazards such as nails or staples (James, 2017). Woodchips have a good water-holding capacity and moderate ammonia absorption capacity, while woodchip's composting capability is lower compared with peat (Airaksinen *et al.*, 2001).

1.1.3 Straw

Barley or wheat straw is commonly used as bedding for livestock, depending on local availability (Lensink *et al.*, 2013). At best, bedding made of straw is soft, provides good

insulation (Tuyttens, 2005), and does not produce dust (Airaksinen *et al.*, 2001). The main known disadvantages of straw are its poor hygienic properties, costs, and limited availability in some regions (Tuyttens, 2005; Lensink *et al.*, 2013). Due to straw's physio-chemical properties *i.e.*, high lignin content and being low in hydrophilic groups, straw's capability to absorb moisture is limited (Diarra *et al.*, 2021). In addition, gas-binding properties are weak in straw (Airaksinen *et al.*, 2001).

The bacterial population typical for straw appears to vary between different studies. By way of illustration, Hogan *et al.* (1989) claimed streptococcal counts to be greater in straw compared to sawdust while Robles *et al.* (2020) reported that gram-negative bacteria and *Klebsiella* spp. were predominating in straw compared to other bedding materials used in the study. Kotimaa *et al.* (1991) investigated how farming methods, including the choice of bedding material, may have an impact on exposure to airborne microbes. In their study, it was observed that straw and baled hay emitted the greatest number of microbes, including mesophilic bacteria, xerophilic fungi, mesophilic fungi, thermotolerant fungi, and thermophilic actinomycetes. They also found *Aspergillus fumigatus*, one of the causative agents of farmer's lung disease, to be most abundant in straw harvested in spring (Kotimaa *et al.*, 1991).

1.1.4 Paper

Recycled paper can be used as bedding material by itself or in combination with another bedding material such as straw or sawdust (Airaksinen *et al.*, 2001; Lensink *et al.*, 2013). The ink from newspapers has been considered a possible health concern, as it could contaminate the milk (Tuyttens, 2005). Zehner *et al.* (1986) observed that chopped newspaper and softwood sawdust supported the least amount of microbial growth compared to recycled dried manure, chopped straw or fine hardwood chips. They suggested that paper contains only little available nutrients for bacteria and the toxic properties of the ink residues in the paper may hinder bacterial growth as well. Airaksinen *et al.* (2001) also observed that fungal and bacterial counts were lower in shredded newspaper and wood-based bedding materials compared to straw, peat, linen, and hemp. The water-holding capacity of paper is also good, and cattle may stay dryer with paper bedding (Misselbrook and Powell, 2005; Ward *et al.*, 2000). The drawback of paper as a bedding material is its tendency of caking when it gets too wet (Ward *et al.*, 2000). Airaksinen *et al.* (2001) found that the paper's ammonia holding capacity was moderate, whereas Ward *et al.* (2001) argue that ammonia levels in horse stable were the highest when the pelleted newspaper was used. Using newspaper as bedding material may result in higher

costs since the bedding has to be changed relatively often and cows seem to prefer sawdust to shredded newspaper (Misselbrook and Powell, 2005).

1.1.5 Manure-based bedding material

At the time of writing, there were two commonly used manure-derived bedding materials – compost and recycled manure solids (Lensink *et al.,* 2013). In compost-bedded pack (CBP), an organic bedding material, commonly fine wood shavings, is regularly added and the pack is stirred to remove urine and faecal matter from the bedding top layer, to leave a dryer surface for the animals to lay on and to aid the composting process (Lensink *et al.,* 2013). From the udder health perspective, this kind of bedding material use may decrease the level of hygiene and potentially put the milking cows at risk of mastitis (Leso *et al.*, 2020). The high bacteria count of the composting bedding material may predispose animals and people to pathogens. Mastitis-causing bacteria, including coliforms, *Staphylococcus,* spp., *Streptococcus* spp., *E. coli*, and *Bacillus* spp. can thrive in moist, nutrient-rich CBP environments (Black *et al.*, 2014). Samadi *et al.* (2012) stated in their study that the number of airborne bacteria was highest in farms where deep compost bedding was used and thus the usage of compost was significantly associated with greater occupational exposure levels. Compost bedding was also linked with higher personal endotoxin levels (Samadi *et al.,* 2012).

Recycled manure solids (RMS) are made by separating liquid and solid matter from the slurry yielded at the farm (Bradley *et al.*, 2018). RMS is soft and non-abrasive, and it has been observed to create less dust compared to straw and sawdust (Leach *et al.*, 2015). The benefits of RMS include its availability, it is generally considered a cost-effective renewable bedding material, and it provides cleaner udders compared to peat (Frondelius *et al.*, 2020). A known disadvantage is that RMS has a heavy bacterial load compared to other bedding materials (Godden *et al.*, 2008; Bradley *et al.*, 2018), and this may pose a health risk for both animals and people from the food safety point of view (Leach *et al.*, 2015). There are indications that the use of RMS could compromise the udder health (Rowbotham and Ruegg, 2015). RMS has been reported to contain many different pathogens, including *Klebsiella* spp., *Pseudomonas* spp., *E. coli, B. cereus* (Leach *et al.*, 2015; Rowbotham and Ruegg, 2016), and oocysts of *Cryptsoporium* spp. and *Eimeria* spp. (Lasprilla-Mantilla *et al.*, 2019). The capability to emit pathogenic airborne microorganisms for animals and humans is yet to be shown in clinical studies (Leach *et al.*, 2015).

1.2 Inorganic bedding materials

Inorganic bedding materials have some advantages over organic ones in that their bacterial counts are significantly lower (Hogan *et al.*, 1989) and they do not foster a good environment for bacteria to grow and therefore decrease the incidence of mastitis (Hogan and Smith, 2012). Hogan *et al.* (1989) concluded that it could be useful to utilise inorganic bedding materials instead of organic alternatives during the summer and autumn months when the incidence of gram-negative bacteria is highest. Inorganic bedding materials however produce a lower quality manure compared to organic bedding alternatives (Ferraz *et al.*, 2020). Inorganic bedding materials, such as sand, are not suitable to be used at all farms, as it demands a comparable manure-handling system (Hogan and Smith, 2012).

1.2.2 Sand

Sand has several good properties as a bedding material, and it has a good reputation for promoting animal welfare. A sand bedding is clean, dust-free, relatively dry, has good drainage, and ideally should contain very low levels of organic matter (Ray *et al.*, 2022). As an inorganic bedding material, it does not support bacterial growth compared to organic alternatives (Hogan and Smith, 2012). Washed sand has up to 100-fold fewer bacteria compared to sawdust and straw (Hogan *et al.*, 1989). For this reason, washed sand is the gold standard alternative for farms encountering environmental mastitis (Hogan and Smith, 2012). Dairy cows have been observed to have fewer hock injuries when sand bedding was used compared to sawdust, straw, or geotextile mattresses (Norring *et al.*, 2008; Calamari *et al.*, 2009; Lombard *et al.*, 2010).

The downside of sand as a bedding material is the poor quality of manure it produces and manure handling is also more challenging as sand tends to collect on the bottom of the manure collection pit where it needs to be manually removed (Bewley *et al.*, 2001). Sand is also a more expensive bedding alternative. The cost can however be reduced by recycling it, but recycled sand may contain more bacteria if it contains more organic matter (Justice-Allen *et al.*, 2010; Ray *et al.*, 2022). Furthermore, sand tends to cause undesirable wear on surfaces and equipment (Lensink *et al.*, 2013).

1.3 Indoor determinants of microbial air quality

The dairy farm environment consists of different compartments, such as milking parlours, feeding lots, and resting areas for the animals. Due to the complexity of the farm, it has a vast

microbial diversity (Quintana *et al.*, 2020). Besides the bedding material, there are multiple factors affecting the microbial air quality. The air itself does not support microbial proliferation, but it does work as an efficient vehicle – microbes are dispersed within bioaerosols and dry matter particles (Quintana *et al.*, 2020). Bioaerosols are a mixture of solid or liquid particles suspended in gaseous media and are derived from animal, plant, and microbial origins (Mandal and Brandl, 2011). Bioaerosols may contain several harmful substances, including endotoxins produced by bacteria and fungi, volatile organic compounds, and gases like ammonia (Hartung and Schulz, 2011; Samadi *et al.*, 2012). Bioaerosols are present in dairy farms in high concentrations (Lange *et al.*, 1997). Thereby, people working in production animal facilities are exposed to bioaerosols and may be subject to many occupational respiratory diseases, such as asthma, bronchitis, organic dust toxic syndrome (ODTS), and hypersensitivity pneumonitis (Lange *et al.*, 1997; Hartung and Schulz, 2011).

The concentration of bioaerosols depends on farm design and peculiarities of housing and management such as animal density, ventilation system, type of bedding, moisture content of feed at storage, and overall level of hygiene (Lange *et al.*, 1997; Quintana *et al.*, 2020). Generally, the larger the operational unit (*i.e.*, the greater number of animals, feed, and manure) the higher the overall microbial load, and subsequently airborne materials are emitted to the immediate environment (Millner, 2009). Samadi *et al.* (2012) concluded that the level of airborne endotoxins, dust, bacteria, and fungi was linked to the type of bedding. They observed significantly higher exposure levels in farms where compost bedding was used, suggesting that the choice of bedding material could predict the level of bioaerosols' exposure. Zdanowicz *et al.* (2004) observed that streptococcal counts in air samples were higher when sand bedding was used while *Klebsiella* spp. and coliforms were predominating when sawdust was used. Feed, silage particularly, is a notable source of mould spores' contamination, which will eventually lead to higher spore counts in faecal matter and consequently further contamination of the environment (Calamari *et al.*, 2018). In addition, milking frequency is a notable contributing factor. A longer milking interval will result in a higher microbial load since microorganisms are given more time to multiply and accumulate on the milking equipment (Quintana *et al.*, 2020). Relative humidity is another important determinant in bioaerosol formation and distribution. Droplets remain airborne for longer periods in low relative humidity, and consequently facilitating the transmission of pathogens (Xiong *et al.*, 2017).

1.4 Microclimatic factors of microbial air quality

The ambient environment surrounding of the farm building can also affect the indoor air quality (Maasikmets *et al.*, 2019). Namely, season and weather conditions are one of the main factors pertaining to the microbiological composition of air on the farm (Quintana *et al.*, 2020). Farm doors are often kept closed during the colder season. Thus, lower ventilation rates are expected in winter, resulting in higher indoor ammonia and particulate matter concentrations (Maasikmets *et al.*, 2019). Microbial populations appear to vary among seasons. For instance, several studies have observed higher bacterial counts in warmer seasons (Quintana *et al.*, 2020). Hogan *et al.* (1989) observed in their study that gram-negative bacterial and coliform counts in bedding were higher during summer and autumn months, while they did not observe differences in streptococcal counts among seasons. We could similarly predict seasonal variation in the microbial composition of the air to that of bedding (Quintana *et al*., 2020).

2. AIM OF THE STUDY

The aim of the study is to compare the influence of two bedding materials, peat and sawdust, on the microbiological composition of the air in the loose housing environment for dairy cattle. The following hypothesis was raised: having peat as a bedding produces more fine dust particles to the air, to which more microbes can be attached.

3. MATERIALS AND METHODS

3.1 Description of the study farms

Air samples were collected from two loose-housing dairy farms located in Tartu County, Estonia. The farms bear some fundamental resemblance to each other, including natural ventilation and loose housing management. The similarities make these two farms fairly comparable to each other in terms of air quality and possible correlation with bedding material. The farms have different bedding materials of choice – the bedding material used in cow cubicles in Farm A consists of peat with straw added to it while in Farm B a mixture of sawdust and wood shavings is used. The first study Farm A is an experimental farm with approximately 125 dairy cows. Farm B, the second farm, has five times more dairy cows compared to Farm A (approximately 600 milking cows). The farm building in Farm A is semi-insulated while Farm B is uninsulated. Manure is removed in Farm A with autonomous manure scrapers that are continuously operating. Farm B relies on mobile manure removal, which is done three times a day. Manure tends to build up in Farm B (Figure 1).

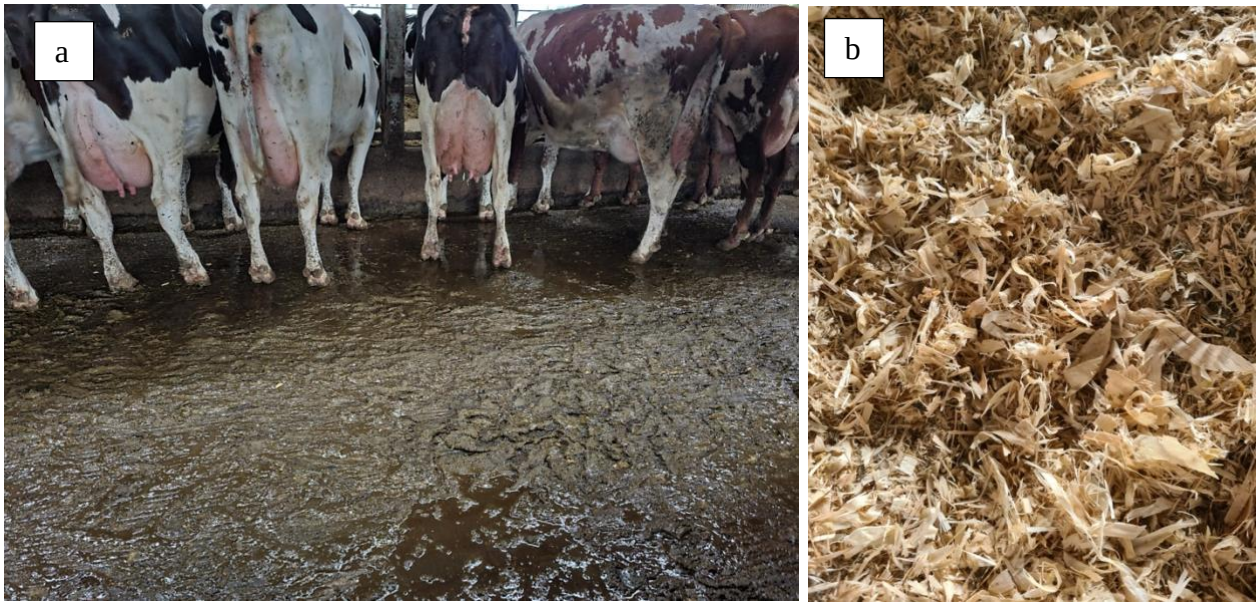

Figure 1. Manure builds up in Farm B (a). Bedding material (sawdust) (b) was added in abundant amounts to the mattresses.

3.2 Collecting air samples

Air samples were taken from two dairy farms located in Estonia (Farm A and Farm B). Each farm was visited three times during the study period from spring 2022 to winter 2023. Sampling

sites were kept the same throughout the study for each farm. Samples were collected in cubicles by using two different methods – sedimentation and filtration.

For the sedimentation method, five Columbia agar plates were placed on a polystyrene box approximately one metre above the floor level, representing the animal's breathing zone. After the study equipment was placed, airflow was waited to be normal again for approximately three minutes. Each of the agar plates was then opened in a calm manner and sequence for a defined time (30 sec, 1 min, 2 min, 6 min, and 10 min, accordingly).

The filtration samples were collected by using microbiological air sampler (Sartorius AirPort MD8). Air samples were collected onto 80 mm diameter soluble gelatine filters. Sterile and single-packaged filters were cut open on the side of the bag where the seam is located. The filters should be and were carefully removed from the package touching only the outer plastic edge of the unit and were then locked onto the air sampler's inlet with a special adapter. Air was drawn through the soluble gelatine filter with four different volumes of air (25 l, 50 l, 100 l, and 250 l) at a 30 l/min flow rate. The device was held stationary approximately 1 meter above the floor level. After air samples were collected, the filters were carefully removed from the inlet, and they were put back into a polyethylene bag. It was written on the bag what air volume was used for each sample. The soluble filters were afterward transplanted on three different types of agar plates (Columbia blood agar, MacConkey agar, and Sabouraud dextrose agar), and were incubated in a laboratory. Therefore, three gelatine filters were needed for each selected air volume (*i.e.*, 12 filters were required for each farm visit).

Temperature, air velocity, and relative humidity were concurrently measured from the same sites as the air samples were taken. It is shown in Figure 2 how the study equipment was placed at the cubicle.

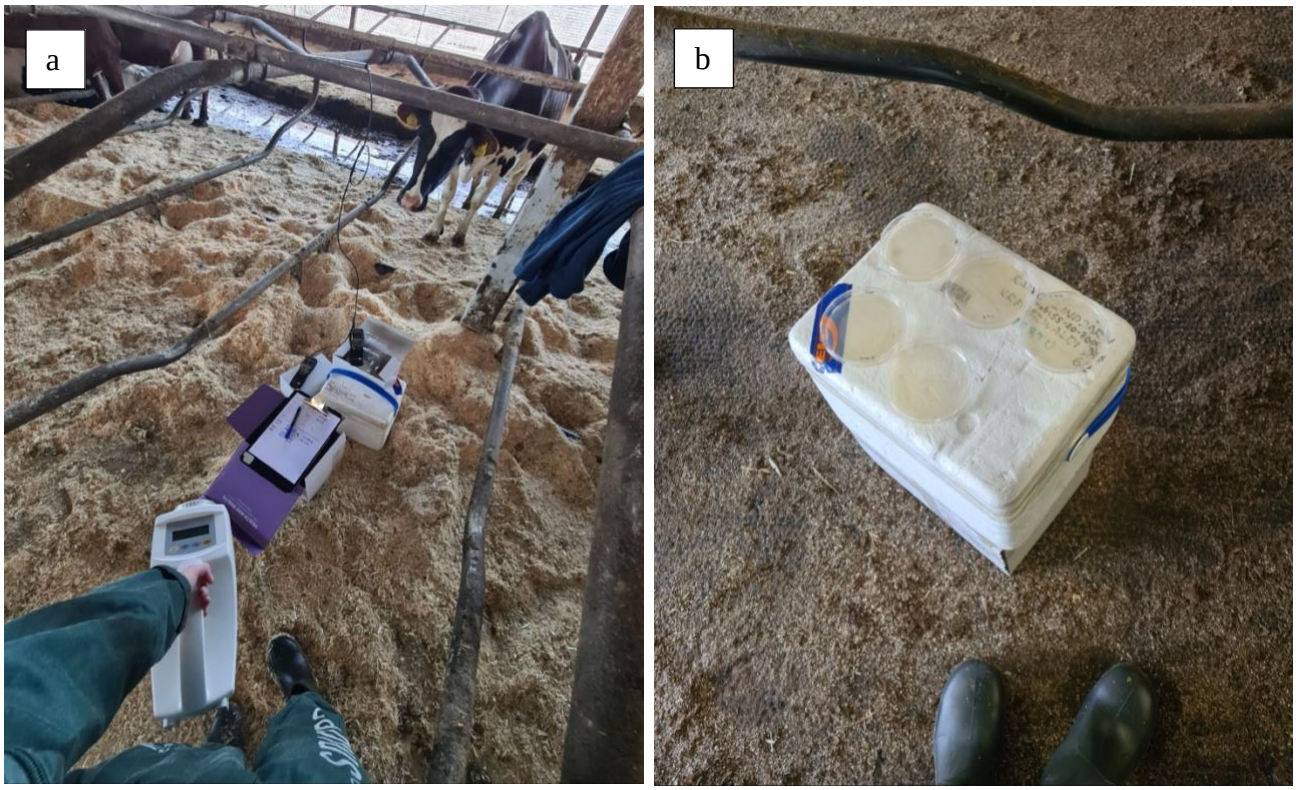

Figure 2. Study equipment setups. Air sampler (a) and Columbia agar plates (b) were kept approximately at the breathing zone level of the cow.

3.3 Handling of samples and microbiological assays

After each farm visit, samples were taken to the microbiology laboratory at the Estonian University of Life Sciences and stored in a refrigerator. Samples were then promptly further processed. Four different types of culture media were used in the study to cultivate and identify airborne bacteria and fungi from the air samples (Table 2). Columbia agar, a non-selective growth medium that was used in the sedimentation method, was for cultivating mesophilic bacteria to give an idea of the total bacterial count. Columbia agar plates were incubated at 37°C for 24 hours. Selective growth media (Columbia blood agar, MacConkey agar, and Sabouraud dextrose agar) were used to identify pathogenic bacteria, Gram-negative and enteric bacteria, and fungal species, respectively. Columbia blood agar and MacConkey agar plates were incubated for 24-48 hours at 37°C, while Sabouraud dextrose agar was kept at 35°C for up to seven days. Gelatine filters obtained from the filtration method were adhered to each type of selective media and then placed in the incubator. Specifications on the type of growth media, incubation times, and temperatures used in the study are listed in Table 2.

Table 2. Growth media used in the study

Microbe groups	Growth media	Temperature (°C)	Incubation time
Mesophilic bacteria	Columbia agar base (VWR Chemicals)	37	24 h
Haemolytic bacteria (e.g., *Staphylococcus*)	Blood agar: Columbia agar base + 5% defibrinated bovine blood (VWR Chemicals)	37	24-48 h
Gram-negative bacteria and enteric bacteria	MacConkey LAB AGAR (Biomaxima)	37	24-48 h
Fungi	Sabouraud Dextrose with Chloramphenicol (Biomaxima)	35	up to 7 days

After incubation was completed, colonies were counted manually using a pen and a click counter. In case Petri plates had several colonies, the lid of the plate was divided into four quadrants by using a marker pen and colonies were counted from a single quadrant. The number of colonies was then multiplied by four. In addition, cultivated samples were sent for species recognition to Estonian National Centre for Laboratory Research and Risk Assessment. The species recognition was done by a mass spectrometry assay (MALDI-TOF).

3.4. Permits and approvals required for the thesis

Farm data were used anonymously according to the oral permission of the owner. There is no conflict of interest.

4. RESULTS

4.1 Sedimentation method

A total of 30 samples were altogether acquired by the sedimentation method. Generally, the number of CFUs was highest on Columbia agar plates that had been opened for 10 minutes and lowest on plates that had been opened only for 30 seconds. The greatest peak in the bacterial concentration was obtained in early summer from Farm B (640 CFU), where sawdust is utilised as a bedding material. According to the results, the bacterial concentrations were higher in samples that were collected from Farm A in spring and winter. The greatest difference between total bacterial counts was observed in winter. Mean total bacterial counts were nearly two times higher in Farm A compared to Farm B (270 CFU and 139 CFU, respectively). As shown in Figure 3, it is noteworthy to mention that the ambient temperature at the sample collecting site was lower in Farm B (4,9°C) compared to Farm A (10,6°C). Air velocity appeared to be very similar in the two study farms throughout the study (Figure 3).

4.2 Filtration method

4.2.1 Columbia blood agar

Altogether, 72 samples were taken by air sampler, from which 24 were cultivated on Columbia blood agar plates. With the filtration method, it is possible to utilise higher sampling volumes compared to the sedimentation method. Microbial counts were higher in Columbia blood agar plates than in Columbia agar plates. The highest number of CFUs in the samples were obtained in spring (840 CFU in Farm A and 1400 CFU in Farm B). Microbial counts were generally slightly higher in Farm A farm where peat was used. The biggest difference was seen in the winter (Figure 4).

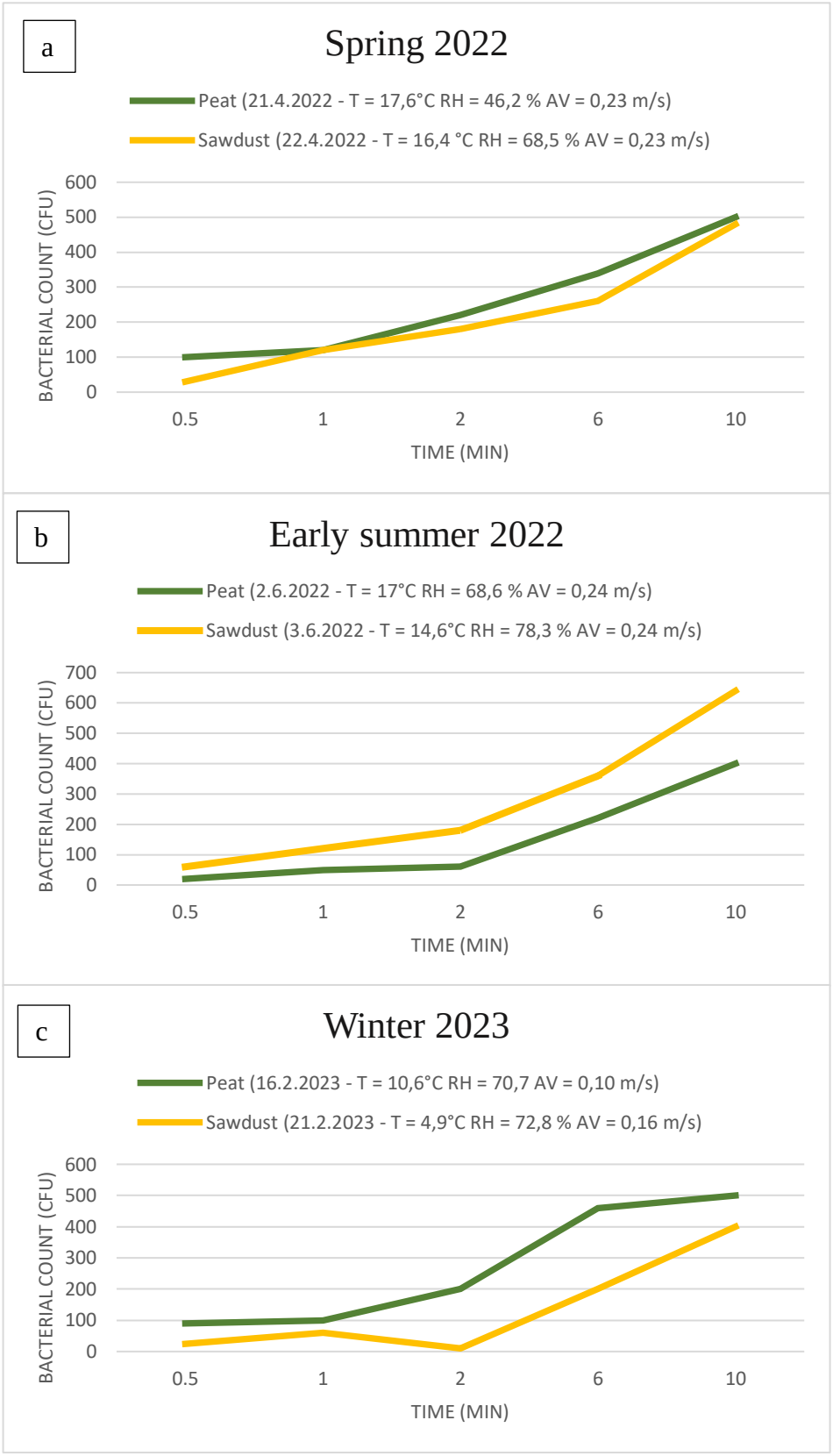

Figure 3. Bacterial counts recorded from air of two study farms (Farm A with peat; Farm B with sawdust). Samples (n = 30) were collected during spring (a), early summer (b) and winter (c). Mean values of microclimate parameters are included in the figures (T = temperature, RH = relative humidity, AV = air velocity).

Figure 4. Microbial counts recorded from air of two study farms (Farm A with peat; Farm B with sawdust) in Columbia blood agar plates. Samples (n = 72) were collected during spring (a), early summer (b), and winter (c).

4.2.2 MacConkey agar

The purpose of using MacConkey agar was to screen for Gram-negative and enteric bacteria. For most of the samples, there was no growth on MacConkey agar plates. The only samples with growth were collected from Farm A in the early summer (2.6.2022). One of the samples that was collected with a volume of 25 l of air had only mould in it. Other plates (50 l, 100 l, and 250 l) had mixed growth, two of them having colonies of *A. fumigatus*. The number of CFUs was 100 CFU with a 250 l sampling volume).

4.2.3 Sabouraud dextrose agar

Most of the Sabouraud agar plates had mixed growth. The highest fungal concentrations were obtained from samples that were collected in the early summer (100 CFU mixed growth collected with 250 l sampling volume in both farms). The number of fungi colonies dropped drastically in the winter samples. There was no growth in the samples that were collected from Farm B in February, and only one of the samples originating from Farm A had *Penicillium* spp. growth.

4.3 MALDI-TOF assay

A total of 12 different microbial species were recognised from air samples. Nine microbial species were collected from the samples originating from Farm A, whereas seven species were found from Farm B. *Bacillus* spp. was the predominating bacterial species found from the samples (Figure 5). Staphylococcal species were more common in Farm B compared to Farm A.

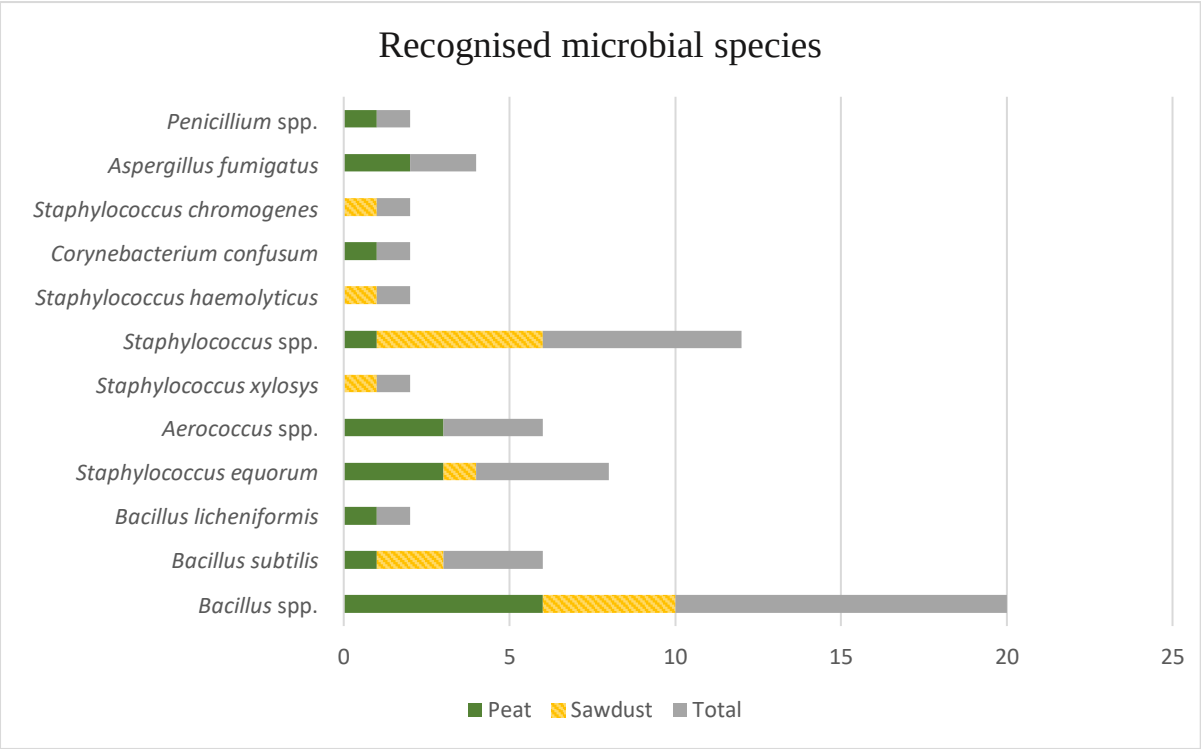

Figure 5. Bacterial and fungal species (n = 12) from air samples (n = 24) recognised from selective growth media by using a MALDI-TOF mass spectrometry. The x-axis shows from how many growth media (Columbia blood agar, MacConkey agar, Sabouraud dextrose agar) each microbial species was found.

5. DISCUSSION

To the author's knowledge, this study was the first of its kind in Estonia. There are only a few studies that have been made with the aim of comparing bedding materials and microbiological air quality in a livestock building (Mäittälä *et al.,* 2001; Samadi *et al*., 2012). To date, there is no established common consensus regarding the methodology for air sampling and how to interpret the results (Viani *et al*., 2020). Detailed comparison of the present study results is hence not possible owing to the absence of similar data and lack of standard methodology. There are no strict limits for microbial air contamination in livestock houses. In Finland, the Ministry of Social Affairs and Health has published a list of the lowest concentrations of pollutants known to be harmful (in Finnish *haitalliseksi tunnettu pitoisuus*, HTP) for the health of employees under the Decree on Concentrations Known to be Harmful (654/2020). This publication has not included any HTP values concerning the permitted number of bacteria and fungi in indoor air. One of the reasons for the lack of international-level hygienic standards is the great variation in total bacterial counts recorded in the air of the farm buildings (Popescu *et al.,* 2011) and the diversity of number of uncontrollable factors contributing to the microbial count (Lange *et al.,* 1997; Quintana *et al.,* 2020). Moderately high bacterial concentrations are expected in cattle housing and the highest in poultry houses, hence microbial counts vary depending on the type of animal species housed (Hartung and Schulz, 2011). According to the study results made by a broad European Union wide study, the total bacterial count in cattle production units has been around 4.4 log_{10} CFU/m³ and fungi 3.8 log_{10} CFU/m³ (Hartung and Schulz, 2007). Permitted concentrations for farming facilities have been only proposed. Literature has suggested maximum concentrations of airborne bacteria of 5 log_{10} CFU/m³ and fungi concentration of 3.7 log_{10} CFU/m³ in cattle houses (Karwowska, 2005; Popescu *et al.,* 2011). Our results cannot be directly compared to the previous studies since the results are displayed as CFUs. Regardless of the air sampling method, it is worth to consider that the absolute total microbial count cannot be achieved (Jörg Hartung, *personal communication* 2022). The actual total number of airborne bacteria can be up to five times higher than cultivable bacteria yielded on the growth medium (Karwowska, 2005).

We hypothesised, that peat emits more fine dust particles into the air, and microbes are attached to those particles. Hence, we could expect higher microbial counts from air samples when peat bedding is used. In our study setting, recorded total bacterial counts were marginally higher in Farm A where the peat bedding is used, compared to Farm B where the sawdust is used. This finding supports our initial hypothesis. Also, microbial diversity was 1.3 times

greater in the obtained samples when peat was the bedding material of choice. These findings are consistent with a previous study reporting significantly higher microbial concentrations in air samples associated with the usage of peat and the microbial species spectrum was more variable compared to sawdust (Mäittälä *et al.,* 2001). Dustiness of the peat bedding was claimed to result in much higher dust levels and subsequently higher total bacterial and fungal counts compared to sawdust bedding. The type of bedding material has been suggested to be a predominant contributor to exposure to dust, endotoxin, bacteria, and fungi also by other studies (Airaksinen *et al*., 2005; Samadi *et al*., 2012). The same phenomenon could possibly explain our test results. However, the sample size should be significantly higher, a longer study period is required, and other contributing factors excluded to make any reliable conclusions from the present study results. If peat and sawdust would be compared in a controlled laboratory environment or in a smaller animal unit, this could help to exclude other contributing factors including animal activity and microclimatic factors (Maasikmets *et al.,* 2019). The greatest difference in results was seen in winter samples. Mean total bacterial counts were nearly two times higher in Farm A compared to Farm B (270 CFU and 139 CFU, respectively). Samples were taken almost one week away from each farm visit and the temperature at the sample collecting site was lower in Farm B (4,9°C) compared to Farm A (10,6°C). Thus, fluctuations in temperatures between two sampling intervals in addition to a small sample size could have biased the results. Previous studies have demonstrated greater microbial counts during warm weather and *vice versa* (Hogan *et al*., 1989; Quintana *et al.,* 2020). Sampling time could also have affected the study results. As discussed in the study of Popescu *et al.* (2011), the scholars concluded that daily variation exists, which should be considered when planning the sampling schedule. It is generally recommended to schedule sampling early in the morning before the beginning of the farm activities to avoid strong dust dispersion in the air at the time of sampling, yet the scholars claimed that for practical reasons it would be better to schedule the air quality determination to the evening when researchers can work without disturbing the farmers. In the case of our study, the timing of the sampling was variable, yet most of our samplings were scheduled for the mornings. For future studies, it could be beneficial to agree to schedule the sample collection always at the same time to minimise the effect of daily variation on the air quality determination.

Along with the bedding material, the concentration of bioaerosols is affected by the number of animals, feed, and manure (Lange *et al*., 1997). Even though the number of animals was greater in Farm B compared to Farm A, total microbial counts were slightly higher in Farm A. Due to the farm design, milking cows are required to walk long distances to the milking

parlour in Farm B, which means that animal activity and subsequent resuspension of previously settled dust into the air could be thus greater compared to Farm A (Maasikmets *et al.*, 2019). Manure removal was done less frequently in Farm B, which could have predicted a higher microbial load. The results could possibly indicate that the effect of bedding material on bioaerosol exposure is somewhat underestimated.

The most abundant microbial species found from the samples was *Bacillus* spp., and it was equally found from both study farms. According to the study conducted by Magnusson *et al.* (2007b), the growth of *B. cereus* was managed to inhibit completely in peat in a laboratory setting. According to our study results, no difference was found between the two bedding materials and the occurrence of *Bacillus* spp. in the samples. Spores of the members of the genus *Bacillus* are known to be very persistent in the environment (Magnusson *et al.*, 2007a). Two fungi species were recognised from the air samples: *Penicillium* spp. and *A. fumigatus*. These species were recognised from samples that were obtained from Farm A. This finding is consistent with the earlier study results where fungal concentrations and occurrence of these species were correlated with the use of peat bedding (Mäittälä *et al.*, 2001). Both *A. fumigatus* and *Penicillium* spp. have a pathogenic potential and are known components of farmer's lung disease and ODTS (Kotimaa *et al*., 1991; Lange *et al*., 1997). Perhaps using peat qualities that produce less dust could decrease the dust burden and subsequent bioaerosol exposure of workers and animals (Airaksinen *et al.*, 2005). High concentrations of airborne microorganisms might reduce the general welfare of farm animals (Ekesbo, 2013), but the field needs more relevant studies.

For some reason, most of the time there was no growth on MacConkey agar cultures. Due to this unexpected outcome, observing the occurrence of Gram-negative and enteric bacteria species, and detailed species recognition was incomplete. Despite the negative test results, coliforms and *Klebsiella* species could be present in the study environment (Hogan and Smith, 1997), especially in the case of Farm B where sawdust is used. It is long known from the literature that the use of sawdust as a bedding material has a strong correlation to increased risk of coliform and *Klebsiella*-related mastitis incidence in the dairy herd (Zdanowicz *et al.* (2004). As the use of sawdust is posing the risk of environmental mastitis, literature has suggested replacing the sawdust with alternative bedding materials as a control measure (Hogan and Smith, 2012).

CONCLUSIONS

In conclusion, the results of the conducted study show that the microbiological composition present in the air samples differs between the facilities using different bedding materials. The results of this study support the initial hypothesis of peat producing more airborne particles and subsequently more microbes are bound to particles. The results of the study could indicate that the effect of bedding material on bioaerosol exposure is somewhat underestimated. Nevertheless, more research on the effect of bedding material on air quality is required to draw consistent conclusions. The information gained from this study may assist future air quality studies regarding sampling methodology, which still lacks a common consensus. Microbial species recorded from the samples included the same species as those found in the bedding materials. This finding is consistent with previous study results. However, the results yielded with the MacConkey agar plates were inconclusive, and we cannot exclude the chance of having mastitis pathogens typical for sawdust, *Klebsiella* spp., or coliforms, present in the air. Thus, it should be acknowledged that sawdust might still pose the milking cows at increased risk of *Klebsiella* or coliform-related mastitis. Based on the obtained results, we consider using bedding material in a quality that produces less dust to lessen airborne particles and subsequent bioaerosol exposure to the animals and people working in the animal premises.

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APPENDIX

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