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Interspecific and intraspecific variation in gastrointestinal microbiota composition of parrots and its association with incidence of selected disorders

Mezidruhová a vnitrodruhová variabilita ve složení mikrobioty trávicího traktu u papoušků a její souvislost s incidencí vybraných poruch

Diploma thesis

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Abstract

Captive parrots are susceptible to various digestive, metabolic, and behavioural disorders. Recent research in other vertebrates has suggested that these disorders can be linked to the gut microbiota, directly or through the microbiota-gut-brain axis. Although many commercial probiotic products intended for the use in parrots advertise beneficial effects on digestion and mental well-being, these statements are not sufficiently supported with publicly available scientific evidence, but probably rather based on the extrapolation of the knowledge of microbiota-gut-brain interactions in poultry, mice, and humans. However, there are substantial differences in the gastrointestinal tract morphology and gut microbiota composition between parrots and these model vertebrates. This thesis aimed to describe the interspecific and intraspecific variation in the gut microbiota composition of parrots and to link the variation in microbial communities to the incidence of eight selected behavioural and physiological disorders. The gut microbiota composition of 491 individuals from 85 parrot species was analysed using 16S rRNA metabarcoding. The host species, type of feed, and housing environment were identified as the main factors influencing the diversity and composition of the gut microbiota of parrots. A stronger phylogenetic signal was detected in oral microbiota compared to faecal bacterial communities. The core microbiota of parrots was described, but high interspecific and intraspecific variability in gut microbiota composition was observed. Decreased diversity of oral microbiota was detected in association with all the studied digestive, metabolic, and behavioural disorders. The potential association of *Lactobacillus*, *Suttonella*, *Globicatella*, *Streptococcus*, and other bacterial genera with the development of the studied disorders was revealed. Analysis of the content of commercial probiotics for parrots revealed in some cases considerable differences between the declared and actual content. The results also supported the assumption that these products are not designed specifically for parrots, as their composition does not take into consideration the natural composition of gut microbiota of healthy parrots.

Keywords: Parrots (Psittaciformes), gut microbiota, microbiota-gut-brain axis, digestive disorders, behavioural disorders, probiotics

Abstrakt

Papoušci chovaní v zajetí mohou trpět různými poruchami trávení, metabolismu a chování. Výzkum posledních let ukázal, že tyto poruchy by mohly souviset se střevní mikrobiotou, buďto přímo, nebo přes osu mikrobiota-střevo-mozek. Přestože mnoho komerčních probiotických produktů určených papouškům inzeruje prospěšné vlivy na zažívání a duševní pohodu, tyto vlivy nejsou dostatečně podpořeny veřejně dostupnými vědeckými pracemi. Jejich existence je předpokládána spíše na základě znalostí o ose mikrobiota-střevo-mozek získaných ve studiích prováděných na drůbeži, myších a lidech. Papoušci se však od těchto modelových obratlovců významně liší morfologií trávicího traktu, i složením střevní mikrobioty. Tato práce měla za cíl popsát mezidruhovou a vnitrodruhovou variabilitu ve složení střevní mikrobioty papoušků, a propojit tuto variabilitu s incidencí osmi vybraných poruch chování a fyziologie. Složení střevní mikrobioty 491 jedinců 85 druhů papoušků bylo analyzováno pomocí 16S rRNA metabarcodingu. Druh hostitele, typ potravy, a prostředí chovného zařízení byly identifikovány jako hlavní faktory ovlivňující diverzitu a složení střevní mikrobioty papoušků. Silnější fylogenetický signál byl zaznamenán u orální mikrobioty než u mikrobioty trusu. Byly popsány některé bakterie vyskytující se u většiny jedinců napříč druhy, ale mezidruhová i vnitrodruhová variabilita ve složení střevní mikrobioty byla vysoká. V souvislosti se všemi studovanými poruchami trávení, metabolismu a chování byla pozorována snížená alfa diverzita orální mikrobioty. Dále byly odhaleny potenciální vztahy mezi těmito poruchami a některými bakteriálními rody, jako jsou například *Lactobacillus*, *Suttonella*, *Globicatella* nebo *Streptococcus*. Analýzy složení komerčních probiotik pro papoušky odhalily v některých případech významné rozdíly mezi deklarovaným a skutečným složením. Výsledky také podpořily předpoklad, že tyto produkty nejsou designované speciálně pro papoušky, protože jejich složení nereflektuje přirozené složení střevní mikrobioty zdravých papoušků.

Klíčová slova: Papoušci (Psittaciformes), střevní mikrobiota, osa mikrobiota-střevo-mozek, poruchy trávení, poruchy chování, probiotika

Prohlášení

Přestože je můj podíl na předkládané práci hlavní, vzhledem k jejímu rozsahu nebylo možné provést vše zcela samostatně. Mikrobiální vzorky použité v této práci byly získány ve spolupráci s veterinárními lékaři (viz poděkování) a během sběru vzorků v zoologických zahradách nebo u soukromých chovatelů, kterého se účastnila řada spolupracovníků z Laboratoře evoluční a ekologické imunologie PřF UK. Extraktce DNA z přibližně poloviny vzorků byla provedena Mgr. Sylvií Dlugošovou. Přípravu na sekvenaci jsem provedla v laboratořích Detašovaného pracoviště Studenec Ústavu biologie obratlovců AV ČR pod vedením Mgr. Lucie Schmiedové, Ph.D. Následná sekvenace proběhla v institutu CEITEC v Brně. Statistické analýzy jsem provedla samostatně, pod vedením Mgr. Jakuba Kreisingera, Ph.D. a Mgr. Lucie Schmiedové, Ph.D.

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 10. srpna 2023

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1. Introduction

Parrots (Psittaciformes) are the most favourite pet birds bred throughout the world (Forshaw 2010). It is estimated that today, captive individuals account for half of the world parrot population (Mellor et al. 2021). Parrots usually live several tens of years, depending on species, and individuals living in captivity can live longer than individuals living in the wild (Veger 1988). However, captive parrots can be more susceptible to certain health issues, like digestive and behavioural disorders. Impaired digestion can develop as a consequence of infectious diseases or unsuitable feed (Bielfeld 1998; Girling 2004). Common behavioural disorders include feather damaging behaviour (Figure 1), aggression, apathy, stereotype behaviour, anorexia, chronic egg laying, or excessive screaming. Gaskins and Bergman (2011) published a survey, in which 71% of parrot owners reported that their bird performed at least one of these behaviours.

Today, there is a growing body of evidence that behavioural disorders might be connected to gut health linked to microbiota through microbiota-gut-brain axis (Morais, Schreiber, and Mazmanian 2021). Most research on this topic has been done in rodents and human, but some evidence exists also from poultry (Birkl et al. 2018; Hu et al. 2022). Based on these studies, it has been proposed that probiotics could be used to cure or prevent various behavioural problems. Regarding very high prevalence of behavioural disorders in parrots, it would be extremely interesting and potentially useful to study microbiota-gut-brain interactions also in this group, but to my knowledge, such research has not yet been carried out. Existence of relationship between gut microbiota and behaviour has been revealed in all animal models studied so far, therefore, it is highly probable in parrots as well. This is apparently also expected by producers of parrot probiotics, as some of them recommend using their products when birds are stressed. But applicability of our knowledge from mammals or poultry to parrots is very limited by the fact that parrots significantly differ from these animals in anatomy of their gastrointestinal tract (GIT), composition of gut microbiota, ecology, and other aspects.

Which bacteria form gut microbiota of healthy individuals of different parrot species? Are parrot behavioural disorders linked to changes in microbial composition or diversity? And if yes, do the currently available probiotic products for parrots contain bacterial species that could help to restore healthy gut microbiota?



Figure 1: Feather damaging behaviour is more common in big parrot species, but it can occur also in budgerigar. Author: Martin Těšíký

1.1. Parrots

Parrots (Psittaciformes) are an order of birds, a sister clade to passerines (Prum et al. 2015). According to the most up-to-date phylogenetic tree of parrots (Provost, Joseph, and Smith 2018), basal group of parrots is formed by Strigopoidea, comprising New Zealand kakapos and nestors. Both other superfamilies include parrots that are often kept in human households – Cacatuoidea (cockatoos and cockatiels) and Psittacoidea (“true parrots”). Parrots most commonly kept as pet birds are budgerigars (*Melopsittacus undulatus*) and cockatiels (*Nymphicus hollandicus*), followed by grey parrots (*Psittacus erithacus*), macaws (e.g. *Ara*, *Primolius*, *Diopsittaca*), lovebirds (*Agapornis*) and others.

Parrots are originally tropical birds, inhabiting Australia, Oceania, south-east Asia, Central and South America and sub-Saharan Africa (Figure 2). They rank among the most threatened bird orders, mainly due to the loss of their natural habitat and in some species also because of illegal exotic-bird trade (Olah et al. 2016). Species living in tropical rainforests (e.g. some macaws) feed on various types of fruits, seeds and green parts of plants. There are also nectarivorous parrots, like lorikeets or swift parrots (Gartrell et al. 2000). Macaws can even consume toxic fruits and neutralize the toxins by licking clay (Olah et al. 2017). Some other parrots (e.g. budgerigars) inhabit grasslands, where they feed on grass seeds (Veger 1988). Parrots can also feed on oil palm fruits and other crops, leading to conflicts with farmers (e.g. Congo Gray Parrots (*Psittacus erithacus*) (Dueker et al. 2020; Veger 1988)).

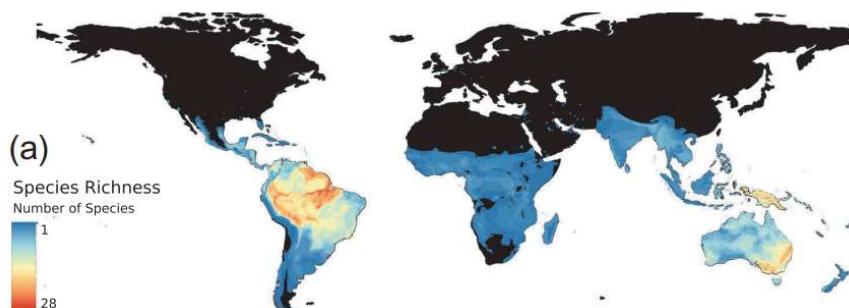


Figure 2: Species richness of parrots across the world

Parrot digestive tract is adapted to the type of feed, leading to differences in GIT morphology e.g. between granivorous and nectarivorous species (Gartrell et al. 2000). Most species have a well developed crop for storage of feed before digestion. However, all parrots lack caeca (Baumel 1979 according to Prinzinger and Schleucher 1998), part of the GIT that serves an important role for fermentation in other bird species. Other characteristic traits of parrots include zygodactyl feet, strong beak and psittacofulvin colouration. Psittacofulvins, pigments that have not been found anywhere else in nature (Berg and Bennett 2010), are responsible for yellow and red colours of parrots and unlike carotenoids, they are synthesized at the site of the growing feathers (McGraw and Nogare 2004).

Another typical trait of parrots is a large brain. Together with corvid birds, parrots feature the largest brains in avian fauna. While corvids evolutionarily increased their body and brain size simultaneously, parrots have rather increased brain-body ratio (Ksepka et al. 2020). Thanks to their large brains, parrots have developed advanced cognitive and learning abilities that help them in food foraging (Diamond and Bond 1991), predator avoidance (White, Brown, and Collazo 2006) and reproduction (Medina-Garcia and Wright 2021). Their high intelligence also makes them a good experimental model for behavioural research.

1.2. Behavioural and digestive disorders of parrots

Captive parrots can suffer from various health problems, like injuries, infectious diseases, helminthiasis, ectoparasites or behavioural disorders. Good state of health of pet birds is important also for humans, as some of the infections (e.g., psittacosis) can be transmittable to pet owners (Bielfeld 1998; Sterneberg-van der Maaten et al. 2016). Exhaustive description of all parrot health issues is beyond the scope of this thesis. Here, I would like to focus only on those that can be related to gut health and gut microbiota.

Structure, colour, and odour of faeces is one of the main diagnostic tools for assessment of parrot's health. However, impaired digestion (including diarrhoea or constipation) can be brought about by various infections, presence of helminths, unsuitable feed, or stress, and therefore, it can be very difficult to determine the right cause (Girling 2004; Vít 1970). Other symptoms of digestive disorders are vomiting (food is refluxed from proventriculus) and regurgitation (food is brought back from the crop). Vomiting occurs less frequently, but it is more dangerous, because vomits contain gastric acids (Girling 2004). The main bacterial pathogens causing digestive disorders in parrots are similar to those that infect poultry and other birds – mainly *Salmonella*, enteropathogenic *Escherichia coli*, or *Clostridium perfringens*, while the knowledge about the prevalence and impacts of other common poultry pathogens as *Campylobacter jejuni* in parrots is limited (Kim et al. 2021; Al Hakeem et al. 2022). Several subspecies of *Salmonella enterica* (Gram-negative bacilli from the family Enterobacteriaceae) cause diarrhoea and loss of appetite, and these bacteria can spread from the intestine to the liver, spleen, heart, lungs, or kidneys, and cause mortality (Vigo et al. 2009; Dunkley et al. 2009). However, *Salmonella* can also be detected in clinically healthy parrots (Allgayer et al. 2008). Despite the fact that some species of *Escherichia/Shigella* (Gram-negative, rod-shaped bacteria from the family Enterobacteriaceae) are dominant bacteria in the GIT of certain parrot species (West et al. 2023), there are also pathogenic strains of *E. coli* that cause diarrhoea and mortality, beside other birds also in parrots (Schremmer et al. 1999; Seeley et al. 2014). *Clostridium perfringens* (Gram-positive bacterium belonging to Clostridiaceae) is an opportunistic pathogen that has been shown to cause necrotic enteritis, for example in macaws (de Santi et al. 2020).

The idea of the relationship between behavioural disorders and the digestive tract has already existed for many decades – in a 35 years old handbook for parrot breeders (Veger 1988), it is mentioned that analysis of faeces can sometimes help to discover the cause of feather pecking and that feed change may cure this disorder. However, the mechanisms of this relationship are not fully understood until now. Almost nothing is known about which bacterial species interact with the host enteric nervous system and the components of the immune system, and how does it affect the neural regulation of behaviour. Captive parrots can exhibit several pathological behaviours – feather damaging, aggression, apathy, stereotype behaviour, anorexia, chronic egg laying, screaming and others (Gaskins and Bergman 2011). Several hypotheses have been proposed, to explain why these behaviours often develop in captive parrots. Parrots are highly social birds, living in stable flocks under natural conditions, and keeping them alone can lead to lack of social contact (van Zeeland et al. 2009). It has been shown that parrot species with larger brains are more likely to develop behavioural disorders (Mellor et al. 2021). Captive birds also spend much less time on searching, selecting, and manipulating food than individuals living in the wild. Parrot species that naturally rely on diets needing extensive handling, are more susceptible to behavioural disorders, when living in captivity (Mellor et al. 2021). Parrots even seem to prefer feed that requires foraging and manipulation from freely available feed (Coulton, Waran, and Young 1997). Another factor could be the short history of keeping parrots in captivity. Animals that have been domesticated thousands of years ago are already adapted to the new environment, including reduction of certain types of behaviours exhibited in the wild. But parrots are considered to be in the early stages of the domestication process, and it is likely that captive individuals share the behavioural capacities and the response thresholds of their wild counterparts (Meehan, Millam, and Mench 2003). Parrot species with relatively longer (around 150 years) domestication history, as budgerigars or cockatiels, are less susceptible to behavioural disorders (Ebisawa et al. 2021). However, it should be kept in mind that these species also rank among the smaller and less intelligent parrot species, and therefore, this could be the effect of already mentioned relationship between brain size and the probability of development of pathological behaviours. Lately, another hypothesis has been proposed, why parrots might be susceptible to behavioural disorders. Divin et al. (2022) has shown that parrots suffer from neuroinflammation more than passerines, probably because they are missing functional gene for CNR2 (cannabinoid receptor 2), which is a negative regulator of inflammatory responses. That could lead to stronger effects of peripheral inflammation (e.g., gut infections) on brain physiology and development.

One of the most common behavioural issues that bring parrot breeders to veterinary clinics is the feather damaging behaviour (FBD) (Gaskins and Bergman 2011). Its prevalence is estimated around 10 – 16% of captive parrot population (Grindlinger 1991 according to Meehan et. al. 2003; Kinkaid et

al. 2013), with some species suffering more often than others. FDB is most common in grey parrots, cockatoos and eclectus parrots (van Zeeland et al. 2009), in grey parrots it is present in ca 40% of all captive individuals (Mellor et al. 2021). FDB is usually regarded as a multifactorial disorder that may be influenced by a number of medical, genetic, neurobiological and socio-environmental factors (van Zeeland et al. 2009). FDB is more common in adult parrots than in young individuals (Kinkaid et al. 2013; Ebisawa et al. 2021), and it often develops at the beginning of sexual maturity (van Zeeland et al. 2009).

It has been proposed that the underlying motivation for FDB might be redirected foraging – when parrots are lacking the possibility to forage for food, their activity redirects into pecking own feathers (van Zeeland et al. 2009). Therefore, it is not surprising that hiding feed into objects requiring manipulation to get it, was shown to decrease manifestation of FDB (Meehan, Millam, and Mench 2003). Other possible ways of eliminating FDB include rehoming into new (bigger) cage or changing the cage position in house (Gaskins and Bergman 2011; van Zeeland et al. 2009), enriching the cage environment with novel objects (Meehan, Millam, and Mench 2003) or application of antidepressants, e.g., clomipramine (Seibert et al. 2004).

FDB is sometimes compared to feather pecking in hens or trichotillomania (TTM) in humans. FDB is usually self-inflicted, while feather pecking in hens consists of pecking and pulling out feathers of other individuals (van Zeeland et al. 2009). The underlying motivation of feather pecking is probably not redirected foraging as in FDB, but rather allopreening. But environmental enrichment might help in both cases. TTM is a human behavioural disorder, when people are compulsively pulling out and often chewing their own hair. FDB has been proposed as a potential animal model for the research of TTM (Bornick, Thyer, and Ritchie 1994).

Relationship with gut microbiota has been studied in feather pecking of hens, but not in FDB or TTM yet. It has been shown that hens prone to severe feather pecking have altered gut microbial composition (higher relative abundance of Clostridiales, lower relative abundances of *Lactobacillus* and *Staphylococcus*, and lower overall microbial diversity), compared to individuals less prone to this behavioural issue (Birkl et al. 2018; van der Eijk et al. 2019). On top of that, severe feather pecking can develop as a consequence of antibiotic treatment and this effect can be reversed by administration of *Lactobacillus rhamnosus* (C. Huang et al. 2023). This finding indicates that changes in the composition of gut microbiota might be the cause and not the consequence of feather pecking in hens, and therefore, presumably also of FDB and other behavioural disorders of parrots.

1.3. Microbiota, condition, and the gut-brain axis

In the previous chapter, I have shown that digestive and behavioural disorders can be caused by gut bacteria. These bacteria, together with archea, microscopic fungi, algae, and protists, are called microbiota. Including viruses, plasmids and mobile genetic elements into microbiota is controversial, as these are not perceived as living organisms (Berg et al. 2020). Microbiota inhabits GIT, respiratory tract, urogenital tract, skin, and other sites of animal bodies. In this thesis, I will focus on bacteria of GIT¹, because bacteria are the major and the best explored part of microbiota (Grond et al. 2018), and those living in GIT considerably influence host condition and behaviour (Belkaid and Hand 2014; Gonzalez-Santana and Heijtz 2020).

Gut bacteria are a source of several chemical compounds that can interact with host nervous system, immune system, and metabolism. Bacterial cell wall contains peptidoglycans and lipopolysaccharide (LPS) – these molecules are released during cell degradation, and they are absorbed through the intestinal epithelium from where they can pass into the bloodstream (Gonzalez-Santana and Heijtz 2020). Likewise, host body is transporting bacterial products of metabolism. Producers of short-chain fatty acids (SCFAs) (e.g., Lactobacillaceae, *Bacteroides* or *Prevotella*) ferment dietary fibre and produce mainly acetic, butyric, and propionic acid. These SCFAs can then serve as a source of energy for growth and development of enteric cells or enter the blood (Schwartz et al. 2010; Han et al. 2018). Another product of gut bacteria are neurotransmitters - noradrenalin, dopamine, serotonin, or gamma-aminobutyric acid (GABA). Serotonin is produced directly by gut bacteria (Clarke et al. 2014), or its production by host enterochromaffin cells is induced via SCFAs (Reigstad et al. 2015). Neurotransmitters can enter the bloodstream, or directly interact with afferent neurons of enteric nervous system (ENS) (Amzar et al. 2022).

There are several mechanisms by which gut microbiota-derived compounds can influence brain and behaviour – through the interaction with immune system, ENS, vagus nerve or by crossing the blood-brain barrier. In the other direction, brain influences gut and its microbiota through efferent pathways of the vagus nerve, changes in immune regulation, and by production of hormones (through hypothalamic-pituitary-adrenal axis), which can change the gut environment, affect intestinal barrier integrity, and alter the microbiota composition (Morais, Schreiber, and Mazmanian 2021). This bi-directional communication is called the microbiota-gut-brain axis. ENS is a branch of the autonomic nervous system, located throughout the length of the GIT. It forms a sensorimotor circuit including primary afferent neurons, interneurons, and motor neurons within the gut wall – this circuit is responsible for the coordination of gut peristalsis and secretion of gastrointestinal enzymes, and it also

¹ In this thesis, I am using the term “gut microbiota” for bacteria and other microorganisms inhabiting whole gastrointestinal tract, not only intestine. The term is widely used in this way also in scientific literature.

interacts with intestinal immunity (Geng 2022). The vagus nerve is the tenth cranial nerve running from medulla oblongata to several organs including the heart, lungs, and GIT. It forms synapses with enteroendocrine cells (neuropods), enabling direct transduction of sensory information from the gut to the brain (Kaelberer et al. 2018). Lately, it has been shown that not only information about nutrient availability, but also signals from gut bacteria (e.g., presence of tryptophan catabolites), can be transduced via this connection (Ye et al. 2021). Certain gut bacteria-derived compounds (peptidoglycans, SCFAs) are sensed by the pattern-recognition receptors (PRRs) like Toll-like receptors (TLRs), Nod-like receptors (NLRs), or peptidoglycan receptors (PGLYRPs). These receptors are expressed on the surface of enteric immune cells, which play a major role in microbiota-gut-brain axis by production of cytokines (Morais, Schreiber, and Mazmanian 2021). Interestingly, it has been shown that some of these PRRs are expressed also by neurons in mouse cerebellum, prefrontal cortex, and striatum (Arentsen et al. 2017).

Our knowledge about the previously described mechanisms of microbiota-gut-brain signalling comes from experiments with mice or zebrafish, but the functional relationship between microbiota and behaviour has been observed also in birds. In poultry, high relative abundance of *Lactobacillus* sp. in gut microbiota is often connected with increased feed intake (Stanley et al. 2016). Kraimi et al. (2019) performed an experiment in Japanese quail, in which they transplanted gut microbiota to germ-free chicks from a line selected for high emotional reactivity from quails of the same line or a line with low emotional reactivity. The second group was less active in behavioural tests compared to the first group, but this effect of microbiota transplantation lasted only two weeks. In other experiment with Japanese quail, administration of probiotics (*Pediococcus acidilactici*) improved memory (Parois et al. 2017). Similarly, *Bacillus amyloliquefaciens* can decrease aggressive behaviour and increase feeding frequency and duration of turkey poult (Abdel Azeem 2013). Another example of microbiota-gut-brain signalling in birds is the sickness behaviour – birds infected with gastrointestinal pathogens are less active, spend more time by sleeping and they can develop anorexia (Calefi et al. 2016; Marais, Maloney, and Gray 2013). Sickness behaviour as a reaction to gastrointestinal infection (or its simulation by injection of bacterial LPS) has been observed also in wild bird species (Coon, Warne, and Martin 2011; Moyers et al. 2015; Owen-Ashley et al. 2006).

Brain and behaviour are not the only aspects of host physiology that are influenced by gut bacteria. Microbiota also impacts host health and metabolism, playing a major role in determining host condition. Some authors distinguish commensal, mutualistic, and parasitic microbiota within a body space or other environment (e.g., Lederberg and McCray 2001). However, this distinction is simplified, as some bacterial species cannot be strictly assigned to any of these groups. *Enterococcus faecalis* is an example of opportunistic pathogen – it is a part of commensal microbiota in healthy individuals,

but it can turn into pathogen during immunosuppression (Moreno et al. 2003). Some bacteria can also change their strategy during evolution. Segmented filamentous bacteria (SFBs) are commensals that are linked to improved performance in mice (Schnupf et al. 2015), as well as in poultry (Danzeisen et al. 2013; Fu et al. 2022). However, SFBs are attached to enterocytes by holdfast structures destructing intestinal microvilli, which very much resembles the adherence mechanism of pathogenic *Salmonella enterica* (Hedblom et al. 2018). Therefore, it is probable that segmented filamentous bacteria developed from enteric pathogens into highly beneficial commensals. And finally, the term “beneficial” is also problematic, because it depends on the context of the study. For example, bacteria linked to increased body weight will be perceived as beneficial in studies focused on wild animals, where higher weight means higher fitness, but detrimental in human or household pets, where obesity is one of the main risk factors of many diseases.

Commensal bacteria can affect host condition by various mechanisms. Some species of *Lactobacillus*, *Bacillus* or *Enterococcus* can induce histomorphologic changes leading to increased absorptive surface of intestine (Sefcova et al. 2021; Cao et al. 2013; Bahrampour, Afsharmanesh, and Bami 2020). These changes could be caused by bacteria-induced altered expression of genes encoding several cytoskeletal proteins in enterocytes (Luo et al. 2013). Enterococci can also increase expression of mRNA for claudin-1 and 9occludin (proteins fundamental for integrity of epithelium) or mucin-2 (major component of protective layer of intestinal epithelium) (L. Huang et al. 2019). Gut bacteria can also induce changes in hormone levels (e.g., *Bifidobacterium*, hormones of thyroid gland (Abdel-Moneim et al. 2020)). *Lactobacilli* can alter cholesterol levels by metabolising it (Yazhini et al. 2018) or by inhibiting expression of cholesterol-binding protein NPCIL1 on enterocyte surface (Y. Huang and Zheng 2010).

Commensal gut bacteria also suppress or outcompete pathogenic microorganisms. They compete for nutrients and binding sites (van der Wielen et al. 2000) – binding to enterocytes or compounds of mucus layer reduces the risk of being cleared away by peristaltic movements (El-Sharkawy et al. 2020). Some commensals produce bacteriocins – e.g., bacteriocin EF55-producing *Enterococcus faecium* can suppress growth of *Listeria* sp. (Strompfova and Laukova 2007). Production of SCFAs by commensals acidifies intestinal contents, which decreases counts of *Escherichia* (Hossain, Begum, and Kim 2015), *Staphylococcus* (Gheorghe et al. 2020) and *Salmonella* (Casarin et al. 2015). And SCFAs also regulate cytokine production of intestinal immune cells (Sun et al. 2018; Yang et al. 2020).

There is a huge body of evidence, that gut bacteria interact with host immune system (for a review see Belkaid and Hand 2014 or Hooper, Littman, and Macpherson 2012). But interpreting isolated information about gut-bacteria-induced changes e.g., in gene expression of certain cytokines

as being beneficial or detrimental for the host is almost impossible (though not rare in scientific literature) due to the high complexity of mucosal immune system (Guo et al. 2016). Indeed, pathogenic bacteria induce local inflammation during infection. And some commensals can reverse this response by having anti-inflammatory properties (Casarin et al. 2015; Deutsch et al. 2017). However, that does not mean that lower inflammation would always be advantageous. Some pathogens (e.g., *Salmonella enterica*) suppress immune responses in order to escape defensive mechanisms of the host (Halici et al. 2008). And on the other hand, even beneficial bacteria can induce inflammation under particular conditions, which may be interpreted as advantageous for the host, because stimulated immune system is then fighting more effectively against pathogens (Sefcova et al. 2021). Therefore, gut microbiota has the potential to induce both pro- and anti-inflammatory responses, and the desired state is a well-regulated balance rather than one or the other extreme (Round and Mazmanian 2009).

This balance can be disrupted when the composition of microbiota is deviated from its homeostasis (this deviation is called dysbiosis). That may happen as a consequence of intestinal inflammation, major diet changes or administration of antibiotics (Zeng, Inohara, and Nunez 2017; Froehlich et al. 2016). Dysbiosis often includes expansion of one or few bacterial taxa, typically opportunistic pathogens that are low-abundant under homeostatic conditions. These changes in microbial composition are associated with functional changes in the microbial transcriptome, proteome, or metabolome (Zeng, Inohara, and Nunez 2017). Gut microbiota dysbiosis is typical for many diseases, however, it can be difficult to distinguish whether it is the cause or the consequence of the disease.

To be able to detect dysbiosis, we first have to define the healthy microbial composition. Here, several complications can arise. Birds have a high intraspecific variation in microbiota composition (Kropáčková et al. 2017b). On top of that, effects of certain gut bacteria on host condition can vary between sexes (K.-C. Lee, Kil, and Sul 2017) or individuals from different geographical locations (Siegerstetter et al. 2017). One of the commonly used ways how to overcome these issues, is to define core microbiome – group of bacteria that are shared among all individuals (or at least vast majority) of the host species/population/study group (Turnbaugh et al. 2007). Core microbiome has been already described e.g., in mice (Pedron et al. 2012) or chicken (Viso et al. 2021), and also in parrots (H. Liu et al. 2019).

1.4. Composition of gut microbiota of parrots

Gut microbiota of birds contains similar bacterial phyla as in mammals, but there are differences in their abundances. Birds accommodate less Firmicutes and Bacteroidetes than mammals, but more Proteobacteria and Actinobacteria (Grond et al. 2018). Gut microbiota is also less host species-specific, compared to mammals – many bacterial species are shared across different bird species (Song et al. 2020). However, vast majority of our current knowledge about the composition and function of bird gut microbiota comes from poultry-focused studies (Waite and Taylor 2015).

Studies focused on the description of gut microbiota composition of wild parrots are very rare. In 2008, Pacheco et al. (2008) analysed the composition of crop microflora of 3 adult individuals of wild-caught dusky-billed parrotlets (*Forpus modestus*) in Venezuela. Crop samples were cultured under aerobic or anaerobic conditions and 10 species of bacteria were then isolated. Predominant strains included Gram-positive *Bacillus*, *Streptococcus*, *Enterococcus* and *Staphylococcus*, as well as Gram-negative species such as *Escherichia coli*, *Pseudomonas* or *Klebsiella*. McDonald et al. (2010) evaluated health and nutritional status of individuals of 3 species of Australian parrots. In one of the species – galahs (*Eolophus roseicapilla*, n=16), they also analysed microbial composition of their faeces, but only using Gram's staining and light microscopy. Gram-positive rods were more numerous than Gram-positive cocci, Gram-negative rods were present only in half of the samples.

Several studies have focused on the composition of gut microbiota of kakapo (*Strigops habroptilus*) – a critically endangered herbivorous parrot that is endemic to New Zealand. These studies already benefited from the modern sequencing methods using 16S rRNA gene to analyse bacterial diversity in samples. Faecal microbiota of adult and chick kakapos has low diversity (Waite, Deines, and Taylor 2012; Waite, Eason, and Taylor 2014) and it is dominated by genera *Escherichia/Shigella*, and *Tyzzerella* (West et al. 2023). However, there are several facts that make kakapo a unique parrot species – it is flightless, nocturnal, lek-breeding and it has a very small population, numbering around 140 individuals (West et al. 2023; Cockrem 2002). All these factors could influence its gut microbiota composition and diversity.

Microbiota of captive parrots is different than that of wild populations, because they have different composition of feed, they can be cured by antibiotics and they come into contact with humans and sometimes also other species of parrots, if housed together (Xenoulis et al. 2010). Young captive birds are sometimes developing without contact with their mother, which is the case of hand-reared parrots and also commercial poultry (Kubasova et al. 2019). The source of microbiota is then limited to the environment because bacterial colonization of the gut of birds happens mainly after hatching (Těšický et al. 2023). In kakapo, hand-reared chicks had significantly lower bacterial richness

and higher relative abundance of *Lactobacillus gasseri* and *Streptococcus gallolyticus* (West et al. 2022).

Gut microbiota composition of other parrot species is known only from the captive birds. (Garcia-Mazcorro et al. 2017) analysed microbial composition of flock-pooled faecal samples of budgerigars (*Melopsittacus undulatus*, 12 flocks) and cockatiels (*Nymphicus hollandicus*, 4 flocks). In budgerigars, Lactobacillaceae accounted for 40 – almost 100% of all sequences, followed by Lachnospiraceae, Pseudomonadaceae, Enterobacteriaceae and Clostridiaceae. Cockatiel flocks varied in the dominant family – Lactobacillaceae were most abundant only in the samples from one flock, while others were dominated by Lachnospiraceae, Clostridiaceae or Erysipelotrichaceae.

Other nine parrot species (3-5 individuals per species) were used for faecal microbiome description by (H. Liu et al. 2019). Only 3.6% of OTUs were shared among all nine species, forming the core microbiota. These included *Lactobacillus* and *Clostridium* species. Also, *Ralstonia*, *Escherichia/Shigella*, *Candidatus Arthromitus* and phylum Actinobacteria were present in most of the parrot species, together forming dominant part of gut microbiota in all of them. All sampled parrots were living in the same zoological garden and were fed the same diet. Interestingly, individuals of the same species had more similar gut microbiota to each other than to individuals from other species, indicating a potential role of host genetics on gut microbiomes. However, differences in gut microbial composition among species did not reflect host phylogenies.

Lately, we compared microbial composition of different parts of GIT of six species of parrots (2 individuals per species) (Schmiedová et al. 2023). Faecal microbiota was dominated by *Ureaplasma* and *Lactobacillus* and resembled bacterial composition of colon and ileum. Oral microbiota had higher alpha diversity than faecal, and its composition was more similar to microbiota of crop, proventriculus, gizzard and respiratory tract, with Pasteurellaceae, Neisseriaceae and *Corynebacterium* being the dominant taxa. These results suggest that faecal samples are suitable for the investigation of the microbiota of lower GIT, while oral swabs are applicable for upper GIT.

Nowadays, several probiotics for parrots are available on the market. They advertise beneficial effects on health, proper digestion, appearance, and sometimes also mental well-being (reduced stress). These products often contain bacterial species that are also present in probiotics for humans, mammals, and poultry (e.g., *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Enterococcus faecium*, or *Bifidobacterium* sp.), which raises the question, whether their composition was designed with regard to the healthy composition of gut microbiota of parrots. While *Lactobacillus* is one of the dominant bacterial species in GIT of many parrot species, *Enterococcus* is usually not present (or at least not in high abundance) in their gut microbiota (H. Liu et al. 2019; Schmiedová et al. 2023).

2. Aims and hypotheses

Aim 1) To describe interspecific and intraspecific variation in gut microbiota composition of parrots.

The host-species specificity of bacteria inhabiting GIT of birds is generally low (Song et al. 2020). However, previous research has shown that parrots of the same species have more similar microbiotas compared to individuals of different species. Yet, the differences among species do not appear to reflect host phylogeny (H. Liu et al. 2019).

Hypothesis: I expect only minor effect of host phylogeny on the composition of microbial samples in my dataset. The intraspecific variation is predicted to be high, and it could be partly explained by environmental factors such as diet and the type of housing, or by sex and age.

Aim 2) To link the variation in microbial communities to the incidence of selected disorders.

Microbiota influences many aspects of host physiology and health, and the microbiota-gut-brain axis plays an important role also in forming the host behaviour. The existence of a relationship between gut microbiota and host digestion and metabolism is now widely accepted in vertebrates, but recent findings from poultry focused research indicate that changes in the gut microbiota composition might also cause behavioural disorders in birds (C. Huang et al. 2023).

Hypothesis: The faecal microbiota composition of individuals suffering from digestive, metabolic, or behavioural disorders (e.g., FDB, apathy or anorexia) is different from that of healthy parrots. I assume that bacterial taxa associated with increased incidence of these disorders will be detected.

Aim 3) To compare the composition of commercial probiotics for parrots with the composition of gut microbiota of healthy parrots.

Probiotics manufactured for parrots often contain the same bacterial species as probiotics for poultry or humans. Therefore, it is probable that these products are not designed specifically for parrots to which they are delivered, and that their composition does not take into consideration the natural composition of GIT microbiota of healthy parrots.

Hypothesis: Some of the bacterial taxa that are used in probiotics for parrots are not represented in faecal or oral samples of healthy parrots.

3. Material and methods

3.1. Sample and data collection

Between 2017 and 2022, 491 individuals of parrots from 85 species were sampled. All parrots were captive birds, bred in zoological gardens or by private breeders in the Czech Republic. Part of the animals was sampled through collaboration with veterinary practitioners. These included individuals suffering from various disorders, as well as healthy individuals being screened preventively. During sampling, all known relevant information was recorded (see the Recording card in Supplement 1). Basic information included parrot species, variety, origin, age, sex, feed, and type of breeding facility (outdoor / indoor, cage / aviary, kept individually / in pair / in a flock). Diagnostic information included symptoms of infectious diseases (e.g., cough, dyspnoea, conjunctivitis), metabolic and digestive issues (e.g., vomiting, defective digestion, obesity, loss of weight), and behavioural disorders (e.g., stereotype behaviour, FDB, anorexia, apathy).

Microbiota composition was analysed from faecal and oral samples. Faeces were collected (using sterile tweezers) from a fresh filter paper, which was placed at the bottom of the cage. Faecal samples were stored in tubes with 99.8% ethanol in a freezer (-20°C). Beak was swabbed with a sterile swab, which was then placed into a tube with 99.8% ethanol, and it was cut using sterile scissors. These tubes were also stored at -20°C. Sex of most individuals was determined using sexually dimorph traits or information provided by the breeder. Where this was not applicable, sex was determined molecularly from the blood, using CHD gene (Chromo Helicase DNA-binding gene) – this gene has already been used for sex determination of many bird species (Vucicevic et al. 2013). Blood was taken from the jugular vein using a heparinized insulin syringe. Samples of blood were stored in tubes with 96% ethanol in freezer (-20°C).

Parrots from 225 breeders were sampled in total. Some parrot genera were bred only by a single breeder, others by multiple (up to 72) (Figure 3 – each colour represents one breeder). Names of the breeders were replaced by a three-letter code in order to comply requirements on personal data protection. Microbiome composition was analysed from samples of faeces and from oral swabs. Both faecal and oral samples were available from 64% of sampled individuals, only faecal samples from 6% and only oral samples from 30% (for detailed information on sample counts see Table 1).

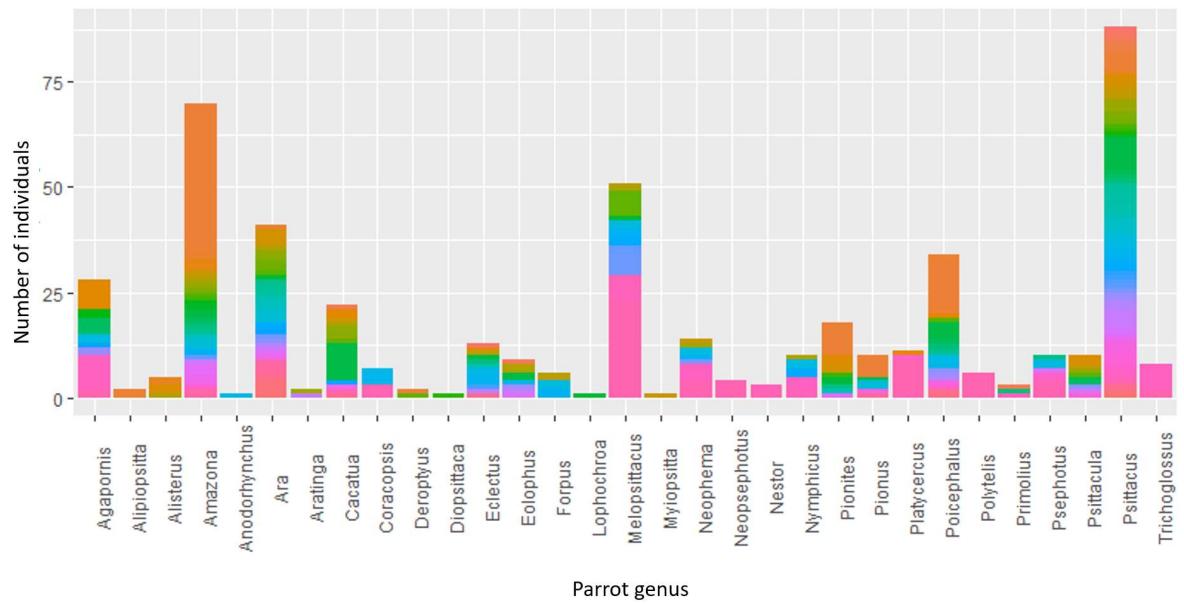


Figure 3: Proportions of parrot individuals from different genera bred by different breeders. Each colour represents one breeder.

Parrot genus	N° of faecal samples	N° or of oral samples	Parrot genus	N° of faecal samples	N° or of oral samples
<i>Agapornis</i>	25	27	<i>Myiopsitta</i>	1	1
<i>Alipiopsitta</i>	0	2	<i>Neophema</i>	8	14
<i>Alisterus</i>	3	5	<i>Neosephotus</i>	4	4
<i>Amazona</i>	32	68	<i>Nestor</i>	2	3
<i>Anodorhynchus</i>	1	1	<i>Nymphicus</i>	9	10
<i>Ara</i>	33	32	<i>Pionites</i>	6	18
<i>Aratinga</i>	1	2	<i>Pionus</i>	4	9
<i>Cacatua</i>	22	14	<i>Platycercus</i>	8	11
<i>Coracopsis</i>	7	7	<i>Poicephalus</i>	18	31
<i>Deroptyus</i>	2	2	<i>Polytelis</i>	4	6
<i>Diopsittaca</i>	0	1	<i>Primolius</i>	1	3
<i>Eclectus</i>	11	11	<i>Psephotus</i>	8	10
<i>Eolophus</i>	6	9	<i>Psittacula</i>	6	10
<i>Forpus</i>	4	5	<i>Psittacus</i>	70	85
<i>Lophochroa</i>	1	1	<i>Trichoglossus</i>	7	8
<i>Melopsittacus</i>	41	51			

Table 1: Numbers of individuals from each parrot genus, from which faecal and oral samples were collected and successfully sequenced.

3.2. Probiotics

Probiotic products for parrots were searched on the internet using key words “probiotic” and “parrot”. Eight selected products (Table 2) were purchased, and their content of bacterial DNA was analysed using metabarcoding.

Product name	Producer	Content	Web page
Ac-i-prim	Re-scha	<i>Lactobacillus acidophilus</i>	http://re-scha.de/products/dose-ac-i-prim-40g/
Aves	Aves Probiotics	<i>Enterococcus faecium</i>	https://www.aves-avian.com/aves-probiotics.html
Ema fauna Exotic	Bio Galaktik	Species of <i>Bacillus</i> , <i>Bifidobacterium</i> , <i>Lactobacillus</i> , <i>Lactococcus</i> , <i>Rhodopseudomonas</i> , <i>Sacharomyces</i> and <i>Streptococcus</i>	http://www.biogrunt.cz/exoticke-ptactvo/ema-fauna-exotic-1-l/
LactiFerm WS	Chr. Hansen	<i>Enterococcus faecium</i>	Sale terminated before the study finalisation
Nekton-Biotic-bird	Nekton	<i>Bacillus subtilis</i>	https://www.nekton.de/en/birds/product/nekton-biotic-bird/
Nutrimix	Versele-Laga	<i>Enterococcus faecium</i>	Sale terminated before the study finalisation
Probi-zyme	Versele-Laga	<i>Bacillus velezensis</i>	https://www.versele-laga.com/en/nl/oropharma/products/oropharma-probizyme
Proparrot	InProCo	<i>Lactobacillus fermentum</i>	http://www.inproco-bio.com/index.php?option=com_content&view=article&id=31&Itemid=59&lang=en

Table 2: List of analysed probiotic products for parrots

3.3. DNA extraction

Metagenomic DNA was extracted using PowerSoil DNA extraction kit (Qiagen), according to the manufacturer’s instructions. Extraction was done in a laminar flowbox, and all instruments and surfaces were sterilized with a flame or decontamination solution. DNA was stored in C6 solution of the extraction kit at -20 °C. Negative controls of extraction were added to every third or fourth extraction run. Samples of probiotics were processed in the same way as gut microbiota samples, but outside the laminar flowbox to avoid the risk of contamination of other samples. In the first step of the DNA extraction, 0.25 g of probiotic powder was added to the C1 solution of the extraction kit.

There were 54% of samples extracted using the version 07-2016 of PowerSoil DNA extraction kit that was not available later. Therefore, the rest of the samples was extraction using the version 05-2019. To check for any potential differences in sequencing results that could be caused by using a

different version of extraction kit, 24 randomly chosen faecal samples were divided into two duplicates (without homogenization) and each one of them was extracted using one of the versions of the extraction kit. Composition of these duplicates is shown in Figure 4. Duplicates were similar in alpha-diversity (Pearson's correlation coefficient = 0.62), as well as in composition (Figure 5). Distance matrix for PCoA was created based on Bray-Curtis distances, using square roots of abundances of OTUs. This transformation has been done also in all following analyses, where distance matrices based on Bray-Curtis distances were used.

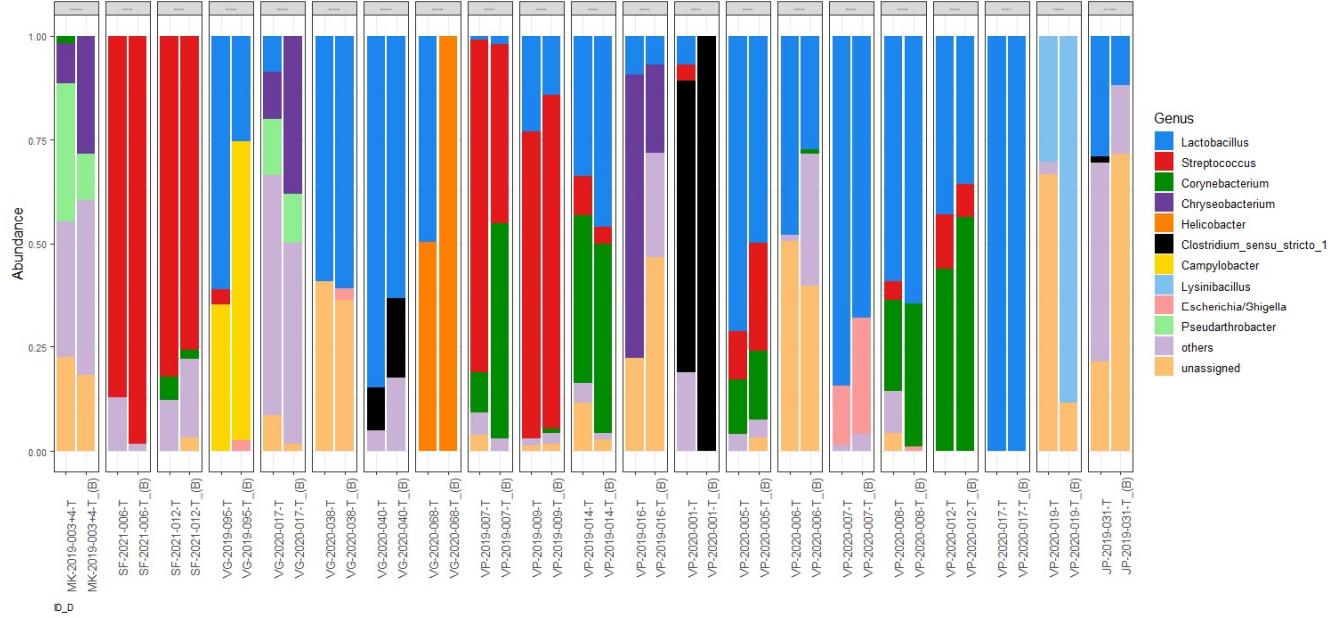


Figure 4: Taxaplot showing relative abundances of dominant bacterial genera in samples extracted by both versions of extraction kit. Each pair represents one sample, with extraction kit version 05-2019 on the left and 07-2016 on the right.

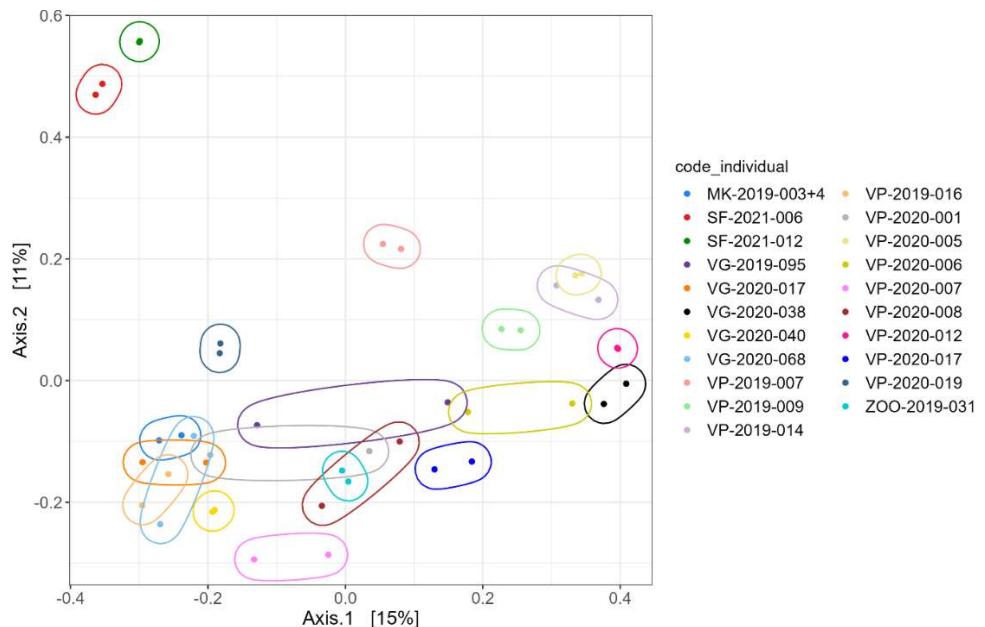


Figure 5: Principal coordinates analysis of samples extracted by both versions of extraction kit.

Parrot individual identity explained 93.6% of variation in the composition of these samples (adonis2 model; 999 permutations; distance matrix was created based on Bray-Curtis distances; p=0.001). There was also no significant difference in composition of samples extracted by old and new version of extraction kit in the main dataset (Figure 6). Therefore, we concluded that the usage of different versions of the DNA extraction kit did not affect our results.

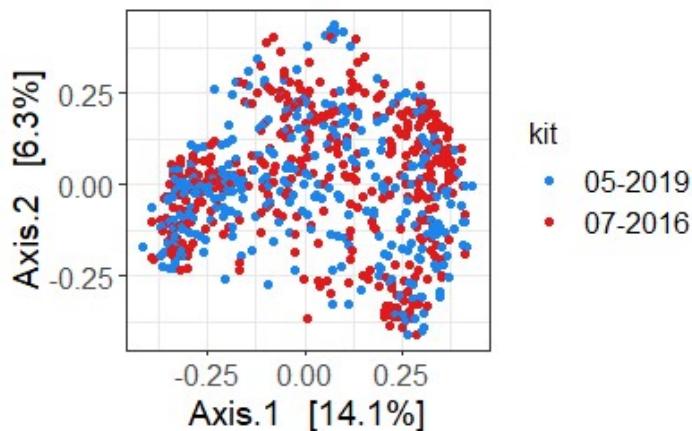


Figure 6: PCoA of all faecal and oral samples available in our dataset, coloured according to the kit used for DNA extraction. Distance matrix was created based on Bray-Curtis distances.

3.4. Metabarcoding

Sequencing libraries were prepared using two-step PCR, in technical duplicates. In the first step, V3 and V4 variable regions of 16S rRNA gene were amplified using universal primers S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC). Forward and reverse primers were bordered by oligonucleotides compatible with the Nextera adaptors (Illumina). In the second step, dual-indexed Nextera sequencing adaptors were attached to the products of the first PCR reaction. Positive and negative controls were added to each PCR plate. Reagents for the first and second PCR reaction are listed in Table 3.

First PCR reaction: Initial denaturation (3 min 95°C), 30 cycles (30 s 95°C, 30 s 55°C, 30 s 72°C), final extension (5 min 72°C)	
Reagent	Amount (per 1 reaction)
KAPA HIFI Hot Start Ready Mix	5 µl
Forward and reverse primers	0.2 µM + 0.2 µM
DNA template	4.6 µl
Second PCR reaction: Initial denaturation (3 min 95°C), 12 cycles (30 s 95°C, 30 s 55°C, 30 s 72°C), final extension (5 min 72°C)	
Reagent	Amount (per 1 reaction)
KAPA HIFI Hot Start Ready Mix	10 µl
Forward and reverse primers	0.2 µM + 0.2 µM
PCR product	6 µl

Table 3: Protocol of two-step PCR

Products of two-step PCR were used for gel electrophoresis and product concentration was estimated by GenoSoft software based on band intensities. Samples were divided into four groups according to their concentration and then pooled accordingly at equimolar concentration. The final library was cleaned using SPRIselect beads, extracted by PipinPrep (sequences 520 – 750 bp long) and sequenced by Illumina MiSeq (v3 kit, 300bp pair-end reads) at CEITEC institute in Brno.

3.5. Bioinformatical processing of sequences

Bioinformatical processing was done in R studio (version 4.1.1.) and Linux command line. Sequences were demultiplexed – assigned to samples according to barcodes and primers. Then, primers were trimmed by skewer software (Jiang et al. 2014). Forward and reverse reads were linked together. Using dada2, low-quality sequences (expected number of errors per read <1) were filtered out, the quality-filtered and fastq files were denoised. Then, an abundance matrix was constructed, presenting read counts for all amplicon sequence variants (ASVs) in each sample. Chimeric sequences were identified using uchime (Edgar et al. 2011) and the gold.fna database (available at <https://drive5.com/uchime/gold.fa>). ASVs were assigned to operational taxonomic units (OTUs) by the RDP classifier (80% confidence threshold) and taxonomy was added using Silva reference database (version 138). To eliminate other PCR or sequencing artefacts, all OTUs that were not consistently present in both technical duplicates of each sample were removed. Read counts for remaining OTUs from first and second duplicate were then merged, after consistence check. Sequences assigned as “Chloroplast”, “Mitochondria”, “Eukaryota” or those that were not assigned to any bacterial phylum were removed. Using the Decontam package, presumably contaminating OTUs were identified and eliminated. These included OTUs whose prevalence was increased in negative controls compared to microbial samples and ASVs that were more abundant in samples with a low concentration of DNA (based on concentration of PCR products). Samples with less than 500 sequences (mostly negative controls) were discarded. Finally, OTUs with abundance <0.01% were removed from each sample.

3.6. Statistics

Statistical analyses were performed in RStudio, using packages phyloseq, lme4, lmerTest, vegan, ape, performance, ggplot2, hmsc and default basic ones. Summary of most abundant OTUs, numbers of sequences/OTUs per sample and differences between sample types (oral swabs vs. faeces) were done on the full dataset, excluding only samples of probiotic products. Alpha diversity was always estimated based on the Shannon index, because given the filtering steps in sequence data processing (described above) other methods (Chao index, number of observed species) would provide biased results.

Analyses of the factors that influence microbial composition of faecal and oral samples were performed using permutational multivariate analysis of variance (PERMANOVA) independent of the order of predictors (function adonis2 from the R package vegan). Distance matrices were created based on Jaccard distances (presence/absence data) or Bray-Curtis distances (abundance data). Only healthy individuals were included, and all available and potentially relevant predictors were tested - parrot genus, sex, age, housing environment, feed and sampling day. To describe interspecific variation, parrot genus was used for the analyses instead of the parrot species, to minimise the effect of small sample size in most species sampled and to avoid overfitting of the statistical models (85 species were collapsed into 31 genera). Samples from parrot genera from which only one individual was sampled, were excluded from these analyses. Age was described by a categorial predictor with two levels – young individuals (less than one year old) and adult individuals (more than one year old). Feed was classified as “only grain”, “only granules” or “diverse” (containing at least two different types of feed from grain, granules, fruit and vegetables or green feed). The description of how the housing environment and type of nursing were scored is provided in detail below. The date of sample collection was included as a quantitative variable, with values representing the number of days from the 1st of January of each year. The year of sample collection (2017-2022) was not expected to influence our results. However, before further analysis, I checked for any differences between the sampling years. Samples collected in different years were not different either in their composition or alpha diversity (Figures 7 and 8). Only samples from the year 2022 were more similar to each other in their composition and had slightly higher alpha diversity than samples from other years. This was explained by the fact that only 23 samples were collected in 2022 and all of them were from budgerigars. Therefore, information about sampling year was not included in any of the subsequent analyses.

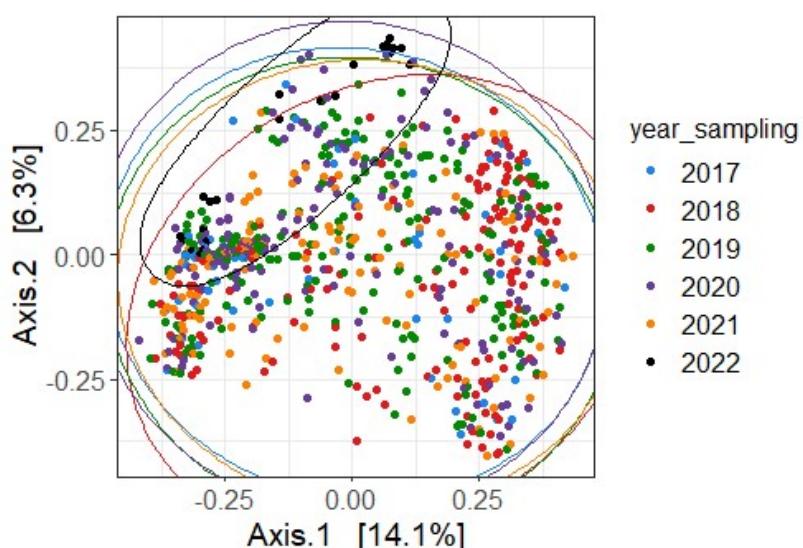


Figure 7: Principal coordinates analysis of all samples, coloured according to the year of sampling. Distance matrix was created based on Bray-Curtis distances.

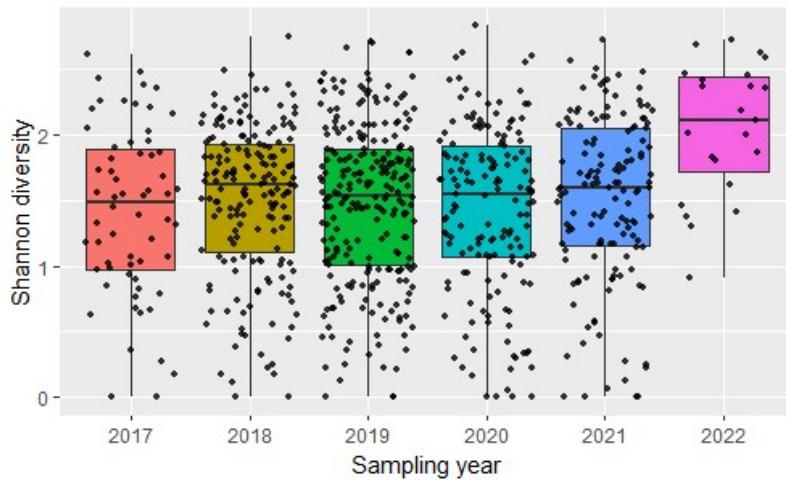


Figure 8: Comparison of alpha diversity (measured as Shannon index) of samples collected in different years. Only the year 2022 was significantly different from the other years, based on TukeyHSD post hoc test (95% family-wise confidence level).

Most of our tested predictors of microbial composition were not independent one to each other (Table 4). For example, birds housed in pairs were always adults. Birds housed in aviaries were usually kept outdoor in larger flocks, while those in cages were more often indoor and housed alone or in pairs. Parrots nursed artificially (without parents) were more often indoor than outdoor and were fed more diverse feed. Young birds (1. Year of life) were usually housed indoor. This unbalance in our data decreased our ability to distinguish between the individual effects of these predictors on the gut microbiota composition.

	Sex	Age	Feed	Nursing	Facility	Aviary	Housed
Age	1						
Feed	0.49160	0.04365					
Nursing	0.6969	0.4322	0.00288				
Facility	0.679	0.00187	0.0194	4.1e-6			
Aviary	1	0.02307	0.01049	0.00029	<2.2e-16		
Housed	0.02656	1.6e-8	0.01295	4.7e-8	<2.2e-16	<2.2e-16	
Health	0.6208	0.01211	0.01426	1.198e-10	1.114e-6	1.384e-8	2.446e-13

Table 4: Mutual dependency of predictors of gut microbiota composition was assessed based on contingency tables. Values represent p-values of Pearson's Chi-square test with Yates continuity correction.

Colour legend: p-value > 0.05 0.05 – 0.01 0.01 – 0.001 < 0.001

To avoid overfitting of our models, I decided to merge all the living-environment factors into one predictor called Housing. Levels of factors Nursing (reared by parents / artificially “on hand”), Facility (indoor / outdoor), Aviary (living in a cage / aviary) and Housed (alone / in a pair / in a flock) were grouped into two categories according to Redundancy analysis (Figure 9). All factors were arbitrary assigned the same weight – individuals received +1 point for being housed outdoor, in an aviary, in a flock, and for being nursed by parents; and -1 point for being housed indoor, in a cage,

alone or in pairs, and for being nursed artificially². Therefore, the new quantitative predictor Housing acquired values from -4 (hand-reared parrot housed alone or in pair in an indoor cage) to 4 (parent-reared parrot living in a flock in an outdoor aviary).

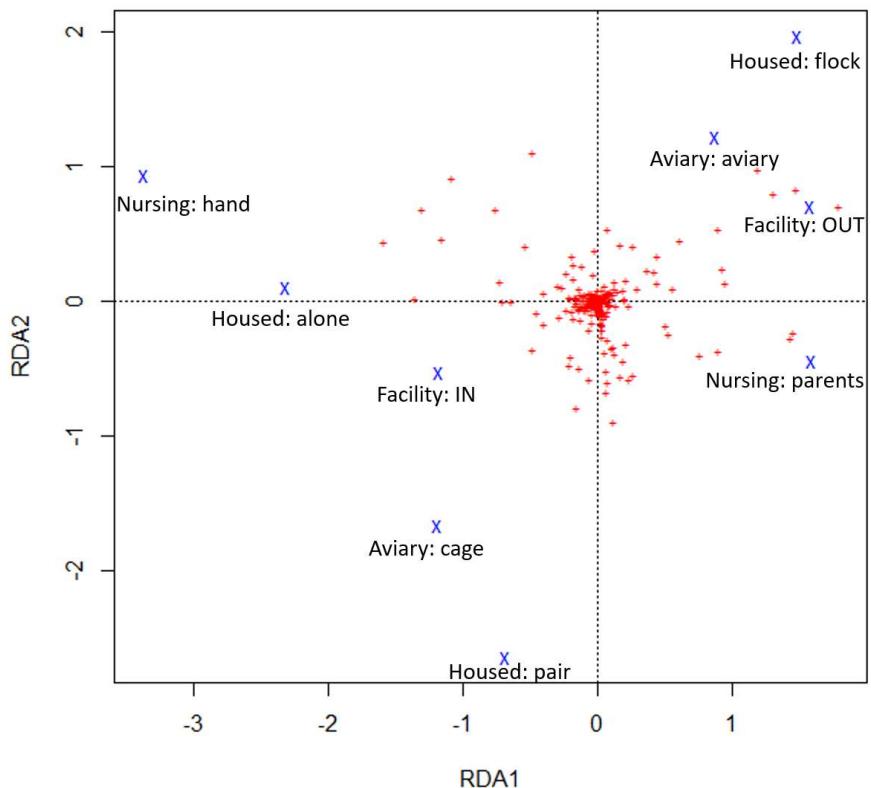


Figure 9: Redundancy analysis (RDA) of relationships between four predictors – blue “X” (reflecting housing environment and the degree of natural-like lifestyle) and response variables – red “+” (abundances of OTUs). Samples are not visualized for clear arrangement. RDA1 axis explains 44.1 % of variation, RDA2 axis 20.6 %. Linear statistics was used, because all values of axis lengths in decorana test were < 3.

Bacterial OTUs has been mapped using quimme program (Caporaso et al. 2010) and the reference database Greengenes (version 13.8, DeSantis et al. 2006) with 97% sequence similarity. The outputs of this mapping were then used as inputs for BugBase software (Ward et al. 2017) that predicts organism-level microbiome phenotypes in two steps. In the first step, it takes into account potential overestimation or underestimation of abundance of certain bacterial taxa caused by differences in 16S rRNA gene copy numbers between bacterial taxonomic groups. In the second step, it makes predictions for the proportions of bacteria with specific features (Gram-positive/negative, aerobic/anaerobic, potentially pathogenic etc.) in each sample, using normalized data, whole-genome reference data and phylogenetic reconstruction algorithms. The obtained proportions were logit transformed.

² Evaluating both groups of levels was necessary to avoid mistakes that would otherwise emerge due to missing values.

For the analysis of the factors related to faecal and oral microbiota alphadiversity, abundance of Gram-positive bacteria, abundance of potentially pathogenic bacteria and abundance of aerobic bacteria, linear mixed-effect models were used (`lmer` function from the package `lme4`). The same predictors as for microbial composition were tested – parrot sex, age, housing environment, feed and the day of sampling. Quantitative predictors (housing and sampling day) were centred. Parrot genus was used as a random factor, because if it was used as a fixed factor, the `lmer` models would test the effect of each level (genus) separately. Instead, the differences in gut microbiota alphadiversity, and abundances of Gram-positive, potentially pathogenic, and aerobic bacteria between parrot genera were visualized in boxplots.

For the purposes of the analyses of interspecific variation, only samples from individuals belonging to 21 parrot species were used. These species were chosen according to two criteria: 1) at least two faecal and two oral samples were available, and 2) the species were included in the phylogenetic tree of parrots by Provost, Joseph, and Smith (2018) that was downloaded in the newick format from the Supplementary material available on the journal's webpage. Phylogenetic tree of the 21 chosen species was extracted from this phylogenetic tree of parrots, by excluding all other tree tips, and it was converted into ultra parametric format (all branches have the same length). Then, this phylogenetic tree was compared with dendograms created according to the similarity of faecal or oral microbiota of these parrot species in a tanglegram (function `tanglegram` from the package `dendextend`). The dendograms based on microbiota similarities were created from a distance matrix (Bray-Curtis distances) of the parrot species, where bacterial abundances in faecal or oral samples from individuals of each species were averaged. Dendrogram was created using the function `pvclust` (package `pvclust`) with 1000 bootstrap replications. The entanglement function (package `dendextend`) was used to quantify the similarity of the compared trees.

For the examination of the intraspecific variation, subsets of oral and faecal microbiota samples were made. Only healthy individuals of those parrot species, from which at least five samples of the given type were available, were included (10 parrot species for faecal microbiota and 16 for oral). Distance matrix of these samples was created (separately for each sample type) based on Bray-Curtis distances. Distances between samples of the same parrot species were extracted and visualised in a boxplot. Mean values and variation in these distances were counted for each species and then correlated (Pearson's correlation coefficient) with the number of individuals and the number of breeders by which they were kept.

Smaller subsets of faecal and oral microbiota samples of healthy parrot individuals were selected for analyses of principal coordinates (PCoA). First, samples were filtered according to the breeder identity – we included only samples from breeders that kept more than one parrot species.

Then, the samples were filtered according to the parrot species, excluding all species that had been bred only by a single breeder. This resulted in subsets of 37 faecal and 51 oral samples of six parrot species bred by eight breeders. From these datasets, the PCoA was done using a distance matrix based on Bray-Curtis distances.

The core bacteria of parrots were defined separately for faecal and oral microbiota as bacterial genera detected in at least 50% of healthy individuals. Then, the microbiota of healthy parrots has been compared with the microbiota of parrots suffering from behavioural disorders (apathy, stereotype behaviour, anorexia and feather plucking) and digestive or metabolic disorders (indigestion, nausea, obesity, and weight loss) – sample counts are summarized in Table 5. Some individuals are included in more groups because they suffered from multiple disorders. The effect of each disorder on microbiota alpha diversity, and the relative abundances of Gram-positive, aerobic, and potentially pathogenic bacteria has been tested separately in Lmer models. The group of healthy parrot individuals was used as a control group (intercept) and the parrot genus and breeder were set as the random factors, in order to filter out the effects of factors that were previously shown to significantly influence microbiota composition. Finally, hierarchical modelling of species communities (HMSC) was used to identify bacterial genera predicting increased or decreased incidences of the selected disorders. Disorders with low numbers of individuals were excluded from the analysis (marked by an asterisk in Table 5). Only bacterial genera present in at least 5% of samples were included. Parrot genus and breeder were used as random factors. The posterior distributions were sampled with Markov Chain Monte Carlo chains and the parameter values that were estimated to be positive or negative with > 95% posterior probability were considered as significant.

Disorder	Nº of faecal samples	Nº of oral samples
Apathy	18	19
Stereotype behaviour*	4	3
Anorexia	36	41
Feather plucking	47	52
Indigestion*	10	11
Nausea	18	18
Obesity*	9	7
Weight loss	80	94

Table 5: Sample counts for the studied behavioural, metabolic, and digestive disorders. Asterisk represents disorders that were not included in HMSC models because of low sample counts.

4. Results

In total, 7 218 741 sequences clustered into 617 OTUs were retained after filtering steps described in Methods, chapter 3.5. Mean number of sequences per sample was 9 077, minimum 1 033, and maximum 31 135. Sequences were clustered into 617 OTUs. Mean number of OTUs per sample was 8.8, minimum 1, and maximum 25 (Figure 10). Most OTUs were detected only in low abundances. Only 20 most abundant OTUs (listed in table 6) accounted for 52% of all sequences.

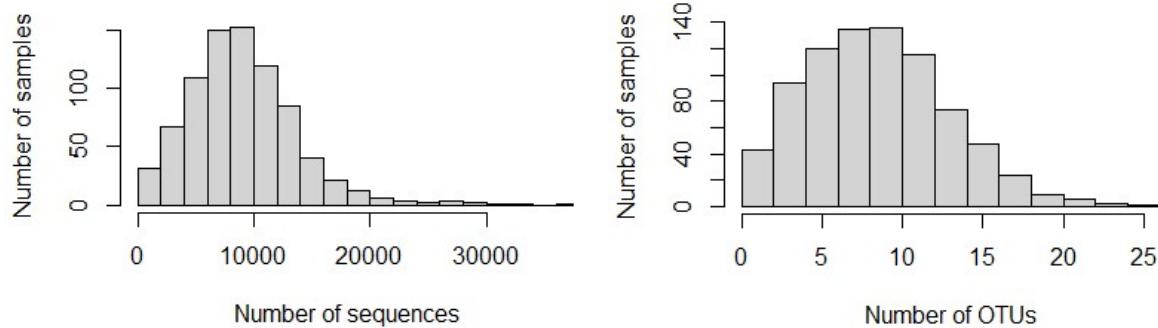


Figure 10: Histograms of numbers of sequences (left) and OTUs (right) per sample.

OTU	Phylum	Class	Order	Family	Genus	% of total sequences in dataset
OTU_1342	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	<i>Corynebacterium</i>	10.0
OTU_2396	Firmicutes	Bacilli	Lactobactillales	Lactobacillaceae	<i>Lactobacillus</i>	4.9
OTU_2061	Firmicutes	Bacilli	Lactobactillales	Streptococcaceae	<i>Streptococcus</i>	4.0
OTU_254	Proteobacteria	Gamma-proteobacteria	Pasteurellales	Pasteurellaceae	<i>Volucribacter</i>	3.4
OTU_993	Bacteroidota	Bacteroidia	Bacteroidales	NA	NA	2.5
OTU_146	Proteobacteria	Gamma-proteobacteria	Pasteurellales	Pasteurellaceae	NA	2.4
OTU_1008	Bacteroidota	Bacteroidia	Bacteroidales	NA	NA	2.4
OTU_2393	Firmicutes	Bacilli	Lactobactillales	Lactobacillaceae	<i>Lactobacillus</i>	2.2
OTU_2445	Firmicutes	Bacilli	Lactobactillales	Lactobacillaceae	<i>Lactobacillus</i>	2.2
OTU_180	Proteobacteria	Gamma-proteobacteria	Pasteurellales	Pasteurellaceae	NA	2.1
OTU_255	Proteobacteria	Gamma-proteobacteria	Pasteurellales	Pasteurellaceae	NA	2.1
OTU_2065	Firmicutes	Bacilli	Lactobactillales	Streptococcaceae	<i>Streptococcus</i>	2.0
OTU_1789	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto_1</i>	1.9
OTU_1319	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	<i>Corynebacterium</i>	1.7
OTU_1931	Firmicutes	Bacilli	Mycoplasmatales	Mycoplasmataceae	<i>Ureaplasma</i>	1.6
OTU_1809	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Candidatus_Arthromitus</i>	1.5
OTU_1311	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	<i>Corynebacterium</i>	1.4
OTU_2303	Firmicutes	Bacilli	Lactobactillales	NA	NA	1.3
OTU_1268	Fusobacteriota	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	NA	1.3
OTU_2608	Firmicutes	Bacilli	Lactobactillales	Lactobacillaceae	<i>Lactobacillus</i>	1.1

Table 6: Phylogenetic characterisation of 20 most abundant OTUs in our dataset (ordered from the most abundant ones).

29% of OTUs were present in faecal as well as in oral samples, 46% were unique to faeces and 26% were unique to oral samples (Figure 11 – left). Oral samples had significantly higher alpha-diversity compared to faecal samples (Figure 11 – right), and they were also different in composition (Figure 12). For these reasons, all following analyses were done separately for each sample type.

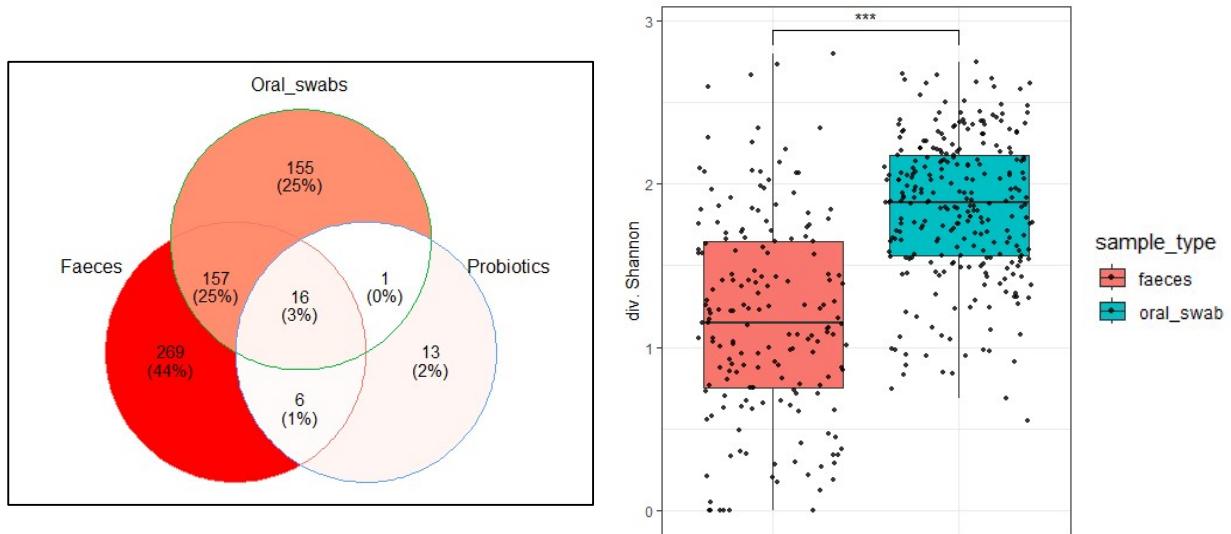


Figure 11: Left: Venn diagramm showing counts of OTUs unique for each sample type or shared among more types of samples. Right: comparison of alpha diversity (Shannon index) of faecal (n=345) and oral (461) samples.

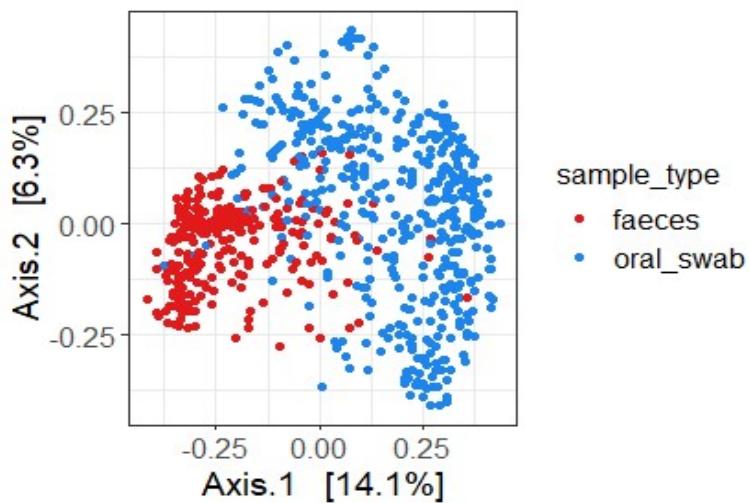


Figure 12: Principal coordinates analysis of faecal (n=345) and oral (461) samples. Distance matrix was created based on Bray-Curtis distances.

4.1. Predictors of the gut microbiota diversity and composition in healthy parrots

The composition of faecal and oral microbiota of healthy parrots was significantly influenced by parrot genus, housing environment, type of feed and sampling date (Table 7). Surprisingly, oral microbiota was also different between sexes, while the faecal one was not. All these predictors had similar effects on abundance of bacteria (models based on Bray-Curtis distance matrix – BC), as on their presence or absence (models based on Jaccard distance matrix – JA). Parrot genus explained around 30% of variation in microbial composition of faeces and 37% in oral samples. The type of feed had a more pronounced effect on faecal than on oral microbiota. Oral microbiota was more influenced by sampling day than faecal microbiota, suggesting lower stability of its composition over different year seasons. In faecal microbiota, the effect of sampling day was significant only in the model, where the presence or absence of bacterial taxa was assessed (JA), but not when the information about abundances was included (BC). This suggests that the presence of some minor bacterial taxa can vary throughout the year, but the abundances of major taxa do not change significantly. There was no significant effect of parrot age in any of the performed tests.

Predictor	Faecal microbiota (n=173)					Oral microbiota (n=264)				
	Df	BC – R ²	BC – p	JA – R ²	JA – p	Df	BC – R ²	BC – p	JA – R ²	JA – p
Parrot genus	19	0.301	0.001***	0.314	0.001***	22	0.365	0.001***	0.382	0.001***
Sex	2	0.010	0.104	0.010	0.159	2	0.008	0.006**	0.007	0.030*
Age	2	0.010	0.152	0.008	0.309	2	0.006	0.106	0.004	0.550
Housing	1	0.008	0.009**	0.007	0.010*	1	0.006	0.002**	0.004	0.011*
Feed	3	0.023	0.001***	0.025	0.001***	3	0.011	0.003**	0.011	0.005**
Sampling day	1	0.005	0.121	0.007	0.033*	1	0.006	0.001***	0.007	0.003**
Residual	144	0.582	---	0.561	---	232	0.493	---	0.473	---

Table 7: Results of PERMANOVA (adonis2 models, 999 permutations), where the effects of six potential predictors of microbial composition were tested. Faecal and oral samples were analysed separately. Distance matrix of samples was constructed using Bray-Curtis distances (BC; abundance data), or Jaccard distances (JA; presence/absence data). Df = degrees of freedom, R² = explained variation, p = p-value. The sum of all R² can be different from 1, when multiple factors are tested using marginal models (Bakker 2023).

Tables 8 and 9 are summarizing effects of tested predictors on faecal and oral microbiota alpha diversity, and the abundances of Gram-positive, aerobic, and potentially pathogenic bacteria. Alpha diversity of faecal microbiota was significantly influenced only by the feed – it was slightly increased in parrots fed only by grain, compared to parrots fed diverse feed (combination of grain, granules, fruits, or vegetables). Alpha diversity of oral microbiota was affected only by the type of housing. Birds housed in flocks in outdoor aviaries, or birds that were nursed by parents, had higher oral microbiota diversity compared to birds living alone or in pairs in indoor cages, or birds nursed artificially.

Faecal microbiota (n=173)								
Predictor	Alpha diversity (Shannon index)		Abundance of Gram-positive bacteria		Abundance of aerobic bacteria		Abundance of potentially pathogenic bacteria	
	Estim.	p	Estim.	p	Estim.	p	Estim.	p
Intercept	0.6766	<0.001 ***	4.8691	<0.001 ***	-2.8189	<0.001 ***	-2.0675	0.046 *
Sex_male	0.1874	0.197	-1.1573	0.006 **	0.3357	0.461	-0.0442	0.936
Age_adult	0.2353	0.151	-0.5738	0.235	0.1800	0.735	-0.3929	0.542
Housing ¹	-0.0582	0.068	0.0081	0.935	-0.1284	0.261	0.1093	0.445
Feed_grain	0.4722	0.008 **	-0.9133	0.098	0.7318	0.239	0.6283	0.414
Feed_granules	-0.3226	0.586	4.2373	0.012 *	-3.5404	0.055	-3.2354	0.144
Sampling day	0.0002	0.821	-0.0034	0.198	0.0018	0.549	0.0003	0.943

Table 8: Results of linear mixed-effect models showing the influence of six fixed factors on the alpha diversity (measured as Shannon index), and the abundances of Gram-positive, aerobic, and potentially pathogenic bacteria in faecal samples (all abundances were logit transformed). Quantitative predictors (*Housing* and *Sampling day*) were centred. Higher values of *Housing* represent more outdoor and natural-like housing environment. Reference level for *Sex* was female, for *Age* young (less than 1 year), and for *Feed* diverse. Parrot genus was used as a random factor. The models met the assumptions of linearity. Estim = estimate, p = p-value. Note 1: higher values of *Housing* represent more outdoor and natural-like lifestyle.

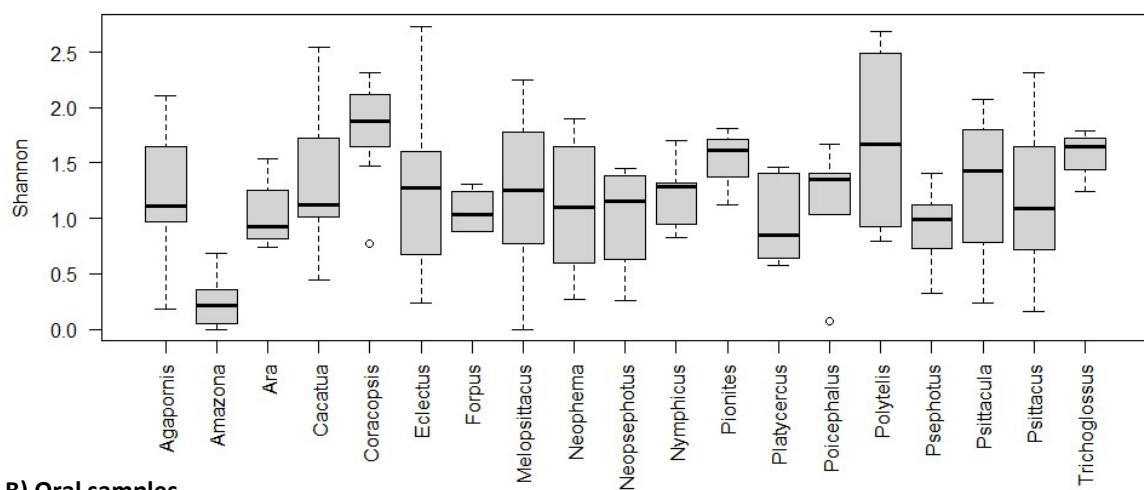
Oral microbiota (n=264)								
Predictor	Alpha diversity (Shannon index)		Abundance of Gram-positive bacteria		Abundance of aerobic bacteria		Abundance of potentially pathogenic bacteria	
	Estim.	p	Estim.	p	Estim.	p	Estim.	p
Intercept	1.945	<0.001 ***	-0.506	0.274	-1.802	0.001 **	-0.073	0.853
Sex_male	-0.105	0.127	0.436	0.099	0.245	0.410	-0.394	0.098
Age_adult	-0.100	0.285	0.095	0.793	-0.106	0.795	0.064	0.844
Housing ¹	0.055	0.002 **	-0.118	0.087	-0.071	0.365	0.121	0.049 *
Feed_grain	0.105	0.311	0.112	0.786	-0.495	0.294	0.192	0.601
Feed_granules	0.439	0.268	-0.932	0.543	2.841	0.103	1.129	0.413
Sampling day	<0.001	0.762	0.002	0.276	-0.002	0.302	-0.003	0.091

Table 9: Results of linear mixed-effect models showing the influence of six fixed factors on the alpha diversity (measured as Shannon index), and the abundances of Gram-positive, aerobic, and potentially pathogenic bacteria in oral samples (all abundances were logit transformed). Quantitative predictors (*Housing* and *Sampling day*) were centred. Higher values of *Housing* represent more outdoor and natural-like housing environment. Reference level for *Sex* was female, for *Age* young (less than 1 year), and for *Feed* diverse. Parrot genus was used as a random factor. The models met the assumptions of linearity. Estim = estimate, p = p-value. Note 1: higher values of *Housing* represent more outdoor and natural-like lifestyle.

The abundance of Gram-positive bacteria in faeces was lower in males, compared to females and increased in individuals that were fed only by granules. In oral microbiota, this abundance was not significantly affected by any of the studied factors. This was the case also for the abundance of aerobic bacteria in oral, as well as faecal microbiota. The abundance of potentially pathogenic bacteria in faeces was not influenced by any factors, while in oral samples it was higher in individuals that were housed in flocks in outdoor aviaries, or birds that were nursed by parents.

Differences in faecal and oral microbiota alpha diversity between different parrot genera are shown in the Figure 13. The highest alpha diversity of faecal samples was detected in *Coracopsis*, the lowest in *Amazona*. The diversity of oral samples was generally higher than that of faecal samples, but this was not true for *Trichoglossus* and *Pionites*.

A) Faecal samples



B) Oral samples

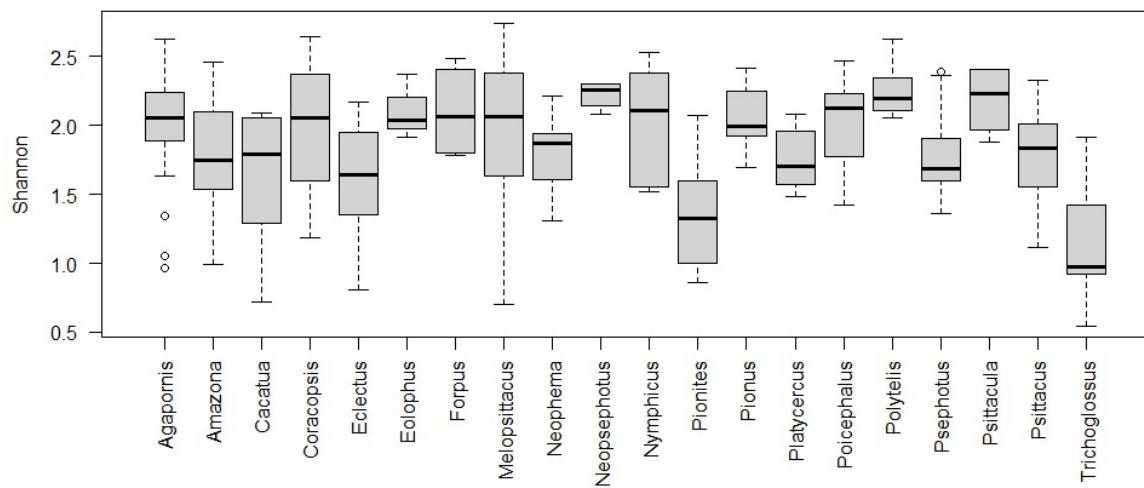
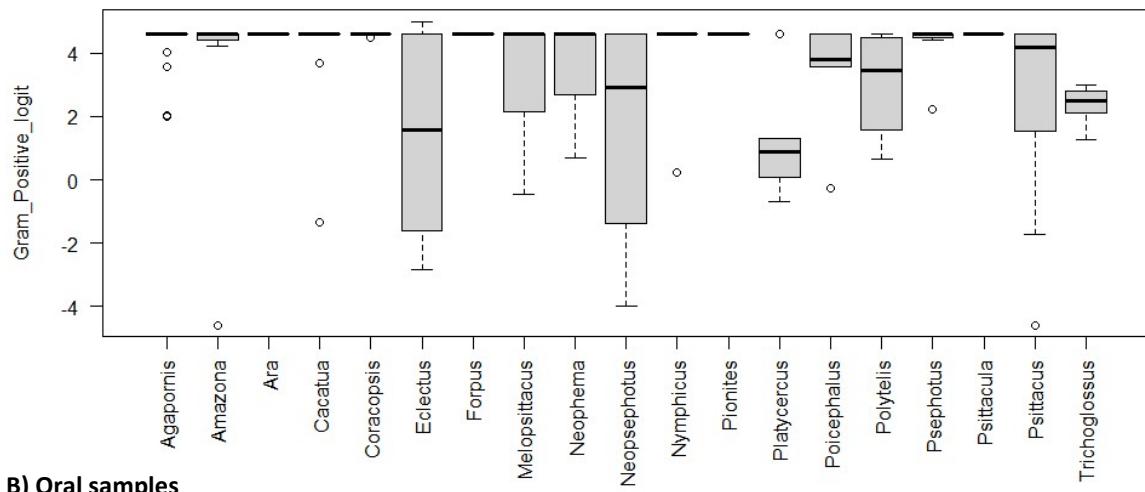


Figure 13: Comparison of alpha diversity of faecal (A, n=167) and oral (B, n=258) microbiota between parrot genera. Genera with < 3 samples were excluded from each dataset.

Figure 14 shows the abundances of Gram-positive bacteria in faecal and oral microbiota of different parrot genera. In approximately half of the studied genera, all or nearly all bacterial taxa detected in faeces were Gram-positive. Genera *Eclectus*, *Neopsephotus* and *Psittacus* had a high intraspecific variation in the relative abundance of Gram-positive bacteria. Faecal samples of *Platycercus* and *Trichoglossus* parrots contained significant abundance of Gram-negative bacteria. Surprisingly, *Trichoglossus* had the highest abundances of Gram-positive taxa in oral samples. There was no relation between these abundances in faecal and oral microbiota.

A) Faecal samples



B) Oral samples

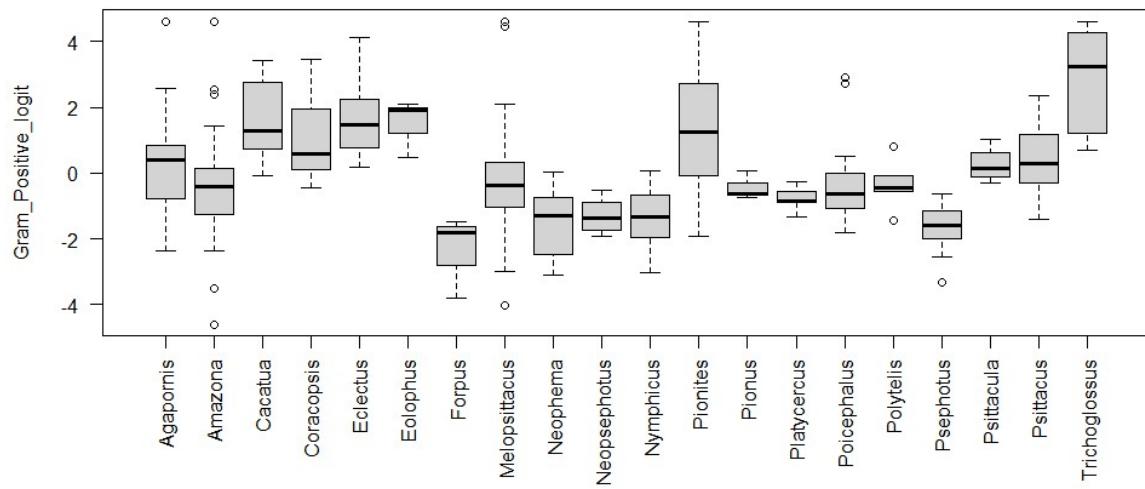
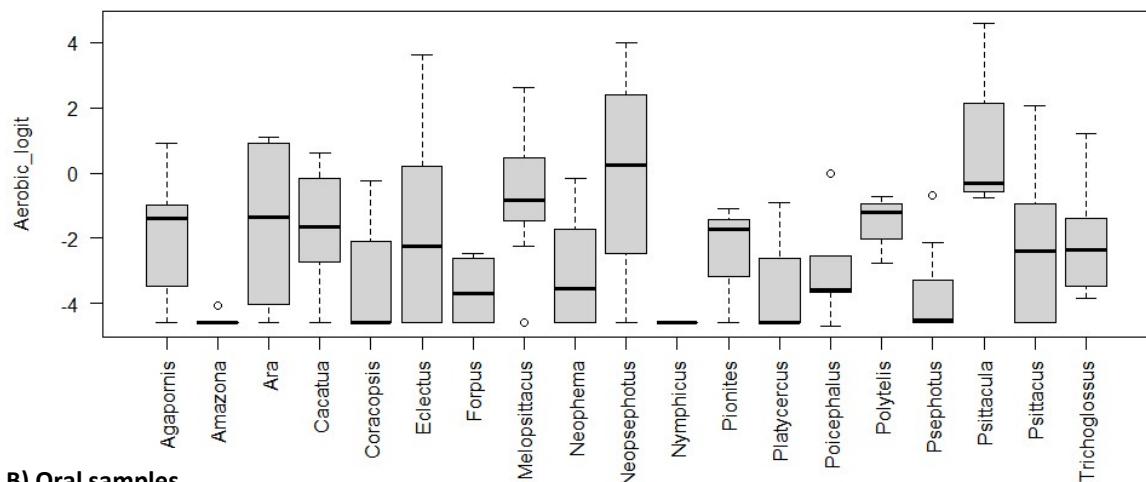


Figure 14: Comparison of the abundances of Gram-positive bacteria in faecal (A, n=167) and oral (B, n=258) samples between parrot genera. Genera with < 3 samples were excluded from each dataset. Abundances were logit transformed.

The abundances of aerobic bacterial taxa in faecal and oral samples are shown in Figure 15. In faeces, the highest mean abundance was detected in genera *Psittacula*, *Neopsephotus* and *Melopsittacus*, and the lowest in *Amazona* and *Nymphicus*, where almost no aerobic bacteria were found. In oral microbiota, the highest mean abundance of aerobic bacteria was detected in parrots of the genus *Forpus*, and the lowest in *Psittacus* and *Platycercus*.

A) Faecal samples



B) Oral samples

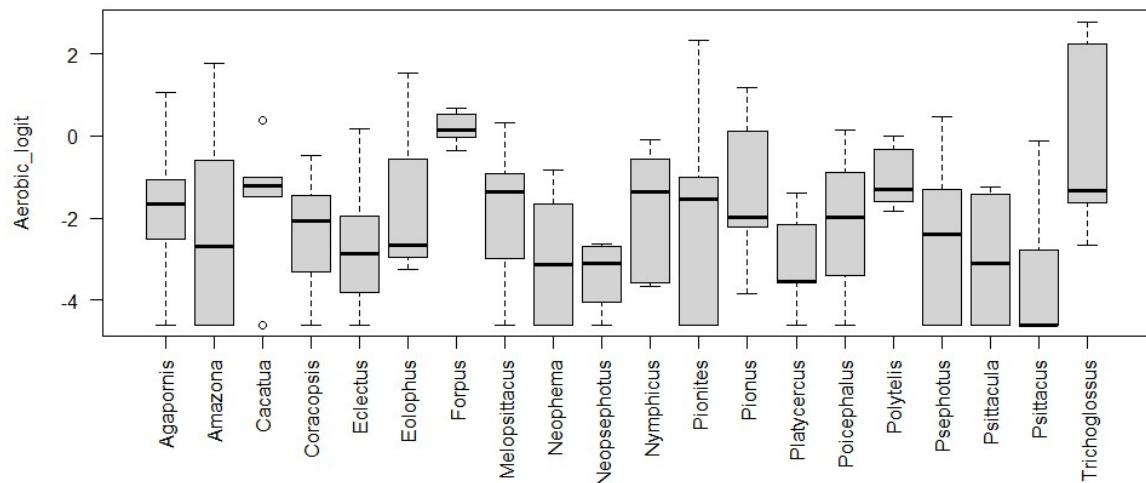
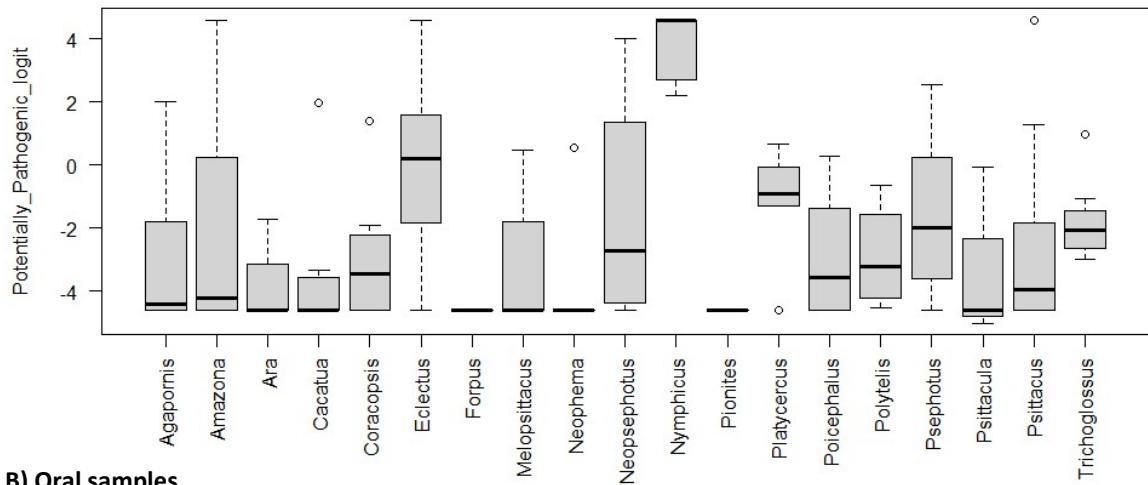


Figure 15: Comparison of the abundance of aerobic bacteria in faecal (A, n=167) and oral (B, n=258) samples between parrot genera. Genera with < 3 samples were excluded from each dataset.

Finally, the abundances of potentially pathogenic bacteria in faecal and oral samples are shown in the Figure 16. It is important to note, that only clinically healthy individuals were included in this analysis. Individuals of the genus *Nymphicus* had significantly more potentially pathogenic bacteria in faeces compared to all other genera. On the other hand, any of the individuals of *Forpus*, *Neophema* and *Pionites* did not have any potentially pathogenic bacteria in their faces. The mean abundance of potentially pathogenic bacterial taxa was higher in oral microbiota compared to faecal microbiota in most of the studied parrot genera.

A) Faecal samples



B) Oral samples

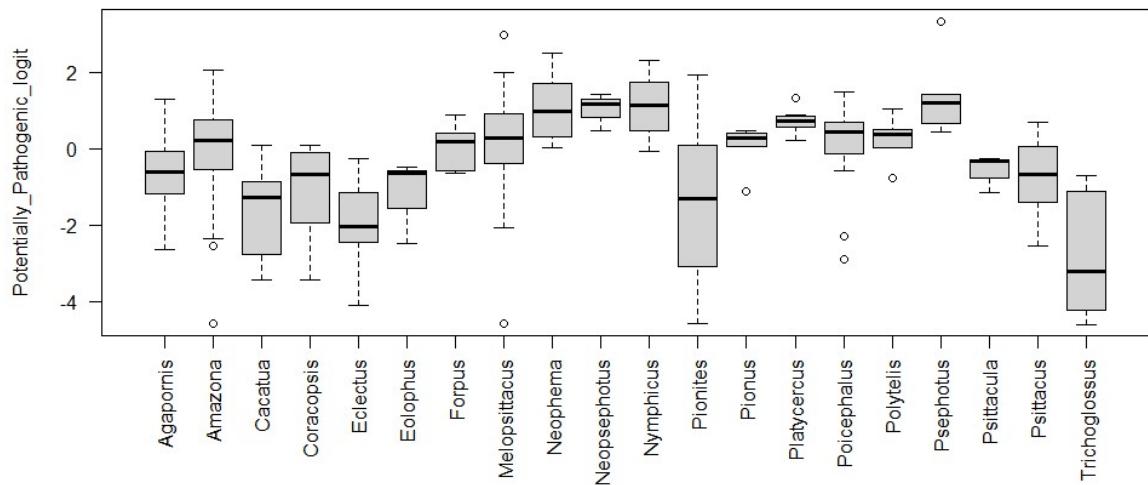


Figure 16: Comparison of the abundance of potentially pathogenic bacteria in faecal (A, n=167) and oral (B, n=258) samples between parrot genera. All individuals were clinically healthy. Genera with < 3 samples were excluded from each dataset.

To test for any relationship between alpha diversity and the three studied features, correlation coefficients were counted for all pairs of these variables (Table 10). The strongest negative association was found between abundances of Gram-positive and potentially pathogenic bacteria. This relation was detected in both sample types, but it was much stronger in oral microbiota. A weak positive correlation was found between the alpha diversity and the abundance of aerobic bacteria of both

sample types. In oral microbiota, alpha diversity was negatively correlated with the abundance of Gram-positive bacteria and positively correlated with the abundance of potentially pathogenic bacteria, but no such association has been found in faecal samples.

	Alpha diversity (Shannon index)	Gram-positive bacteria	Aerobic bacteria
Gram-positive bacteria	Faecal: -0.072 Oral: -0.406		
Aerobic bacteria	Faecal: 0.288 Oral: 0.226	Faecal: -0.130 Oral: -0.108	
Potentially pathogenic bacteria	Faecal: -0.040 Oral: 0.388	Faecal: -0.557 Oral: -0.900	Faecal: -0.187 Oral: 0.035

Table 10: Pearson's correlation coefficients of alpha diversity and the abundances of Gram-positive, aerobic, and potentially pathogenic bacteria in faecal and oral samples.

4.2. Interspecific variation in microbial composition

In the previous chapter, I have shown that the host genus is an important factor influencing the composition and diversity of faecal and oral microbiota in parrots. This result indicates that there might be a phylogenetic signal detectable in gut microbiota. In our dataset, some parrot genera were represented by only one species, while others included several species, and each species comprised different number of individuals. Therefore, for the assessment of interspecific variation in microbial composition, a subset of 21 parrot species has been made, from which at least two faecal and two oral samples were available. The relative abundances of bacterial genera in faecal and oral microbiota of these species are shown in taxaplots (Figures 17 and 18). Faecal microbiota of most species was dominated by *Lactobacillus*, while *Corynebacterium* was the most abundant in oral microbiota.

The similarity of faecal and oral microbiota composition between the selected parrot species was compared to their phylogenetic relationships in tanglegrams (Figures 19 and 20). The topology of the phylogenetic tree was considerably more similar to the topology of the microbiota-similarity dendrogram based on oral microbiota composition ($E = 0.285$) than to the one based on faecal microbiota ($E = 0.754$), as revealed by the entanglement number E , ranging from 0 (identical topology) to 1 (completely unrelated topology). The three species of *Agapornis* were clustered together in the faecal microbiota dendrogram and two of them also in the oral microbiota dendrogram. The sister species *Psittacus erithacus* and *Poicephalus senegalus* were clustered together according to the oral microbiota composition, and they were also closely related in the faecal microbiota dendrogram. In other branches, the phylogenetic signal was rather low. However, it should be taken into account that the topology of the deeper parrot phylogenetic lineages had only a low bootstrap support (represented by the dashed lines).

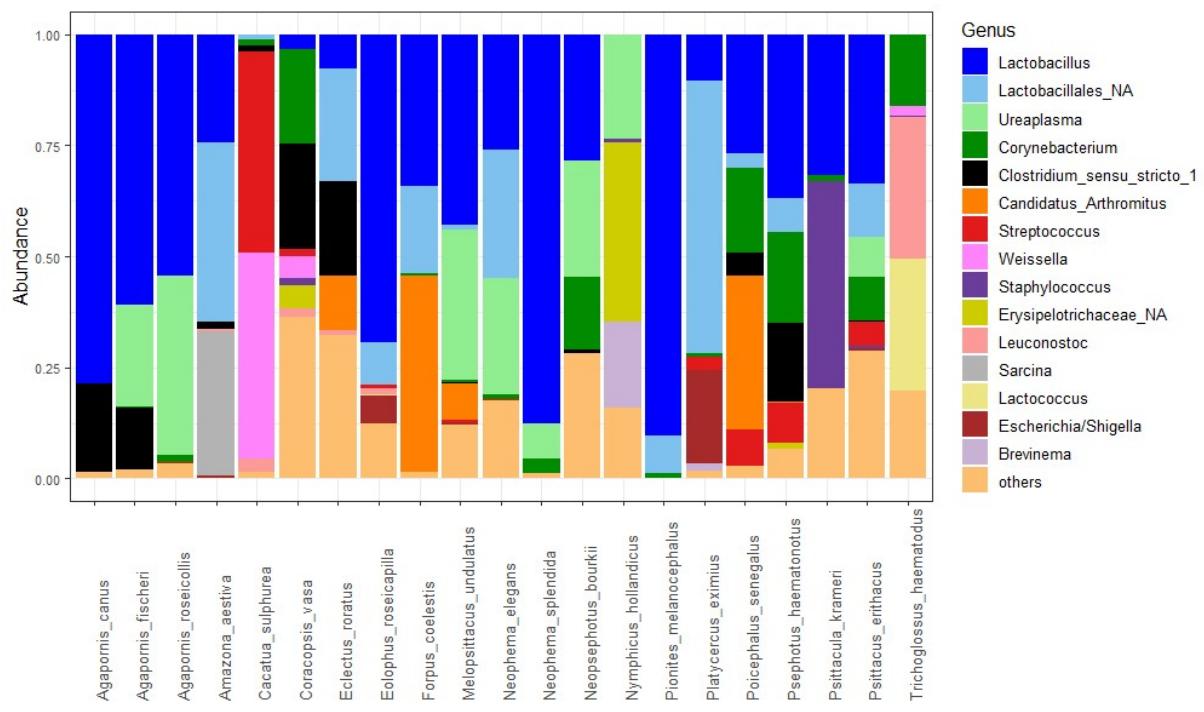


Figure 17: Taxaplot showing relative abundances of dominant bacterial genera in faecal samples from different parrot species.

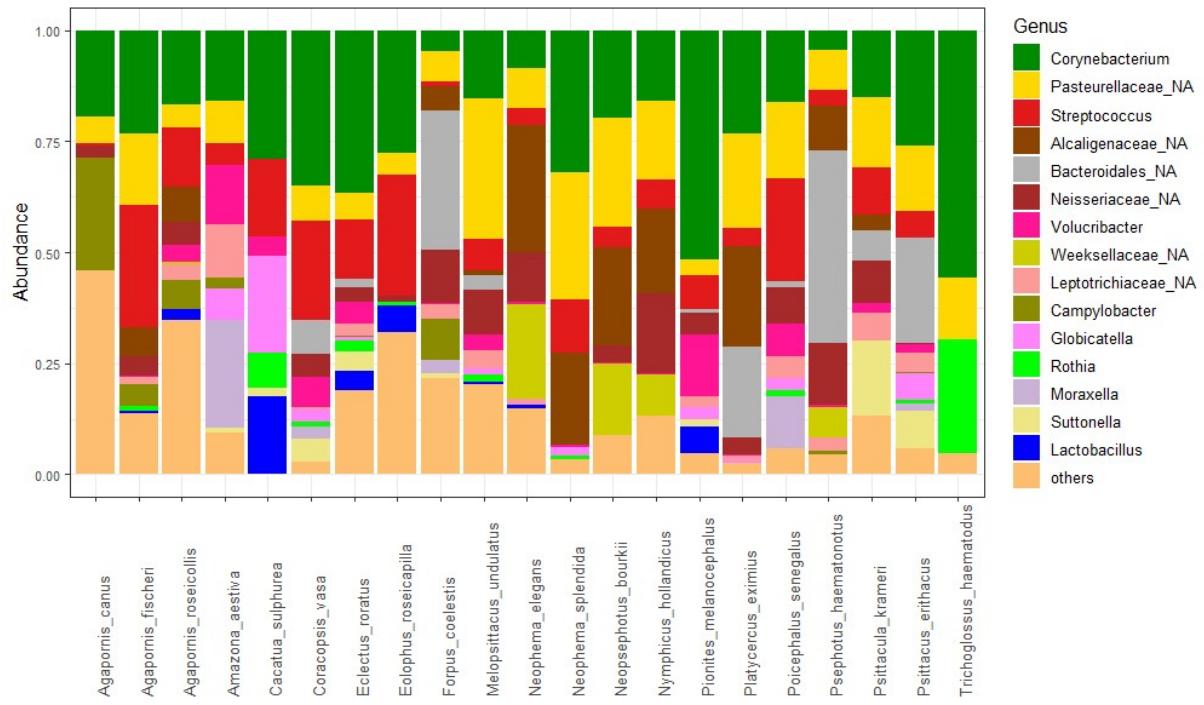


Figure 18: Taxaplot showing relative abundances of dominant bacterial genera in oral samples from different parrot species.

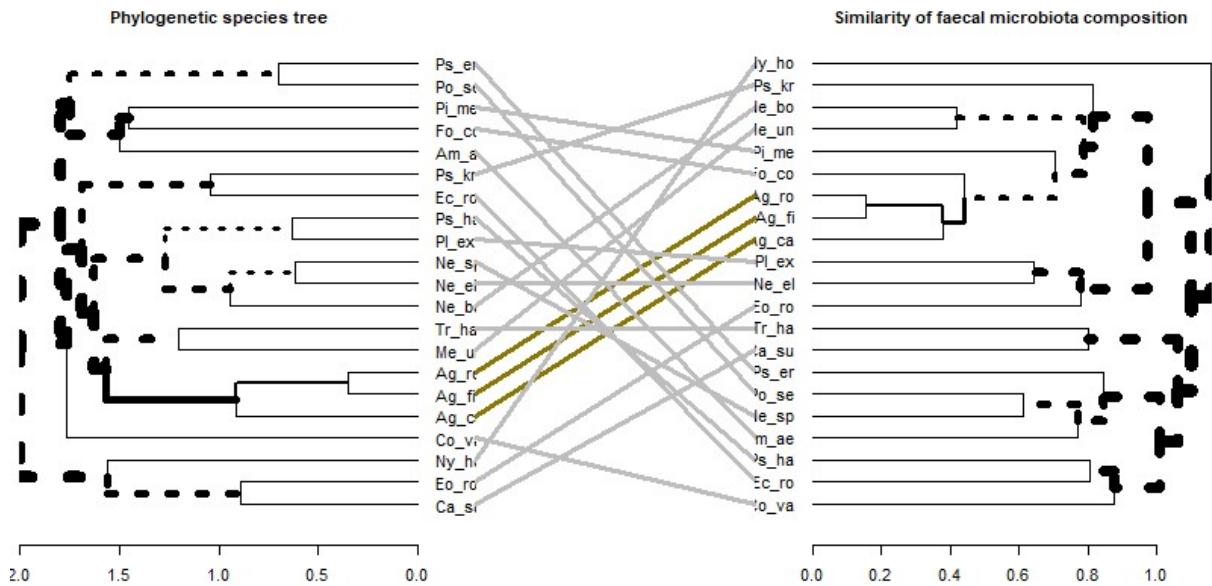


Figure 19: Tanglegram comparing the dendrogram of 21 parrot species based on their similarity in composition of faecal microbiota (right) and the phylogenetic tree according to Provost, Joseph, and Smith (2018) (left). Tip names are the abbreviations of the parrot genus and species name³.

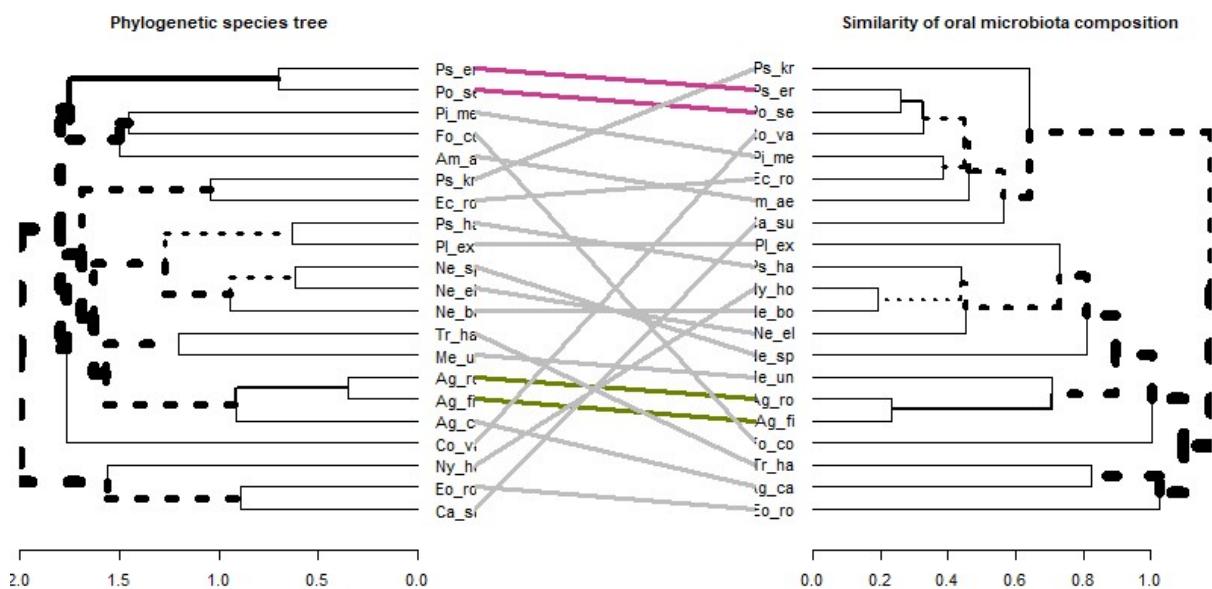
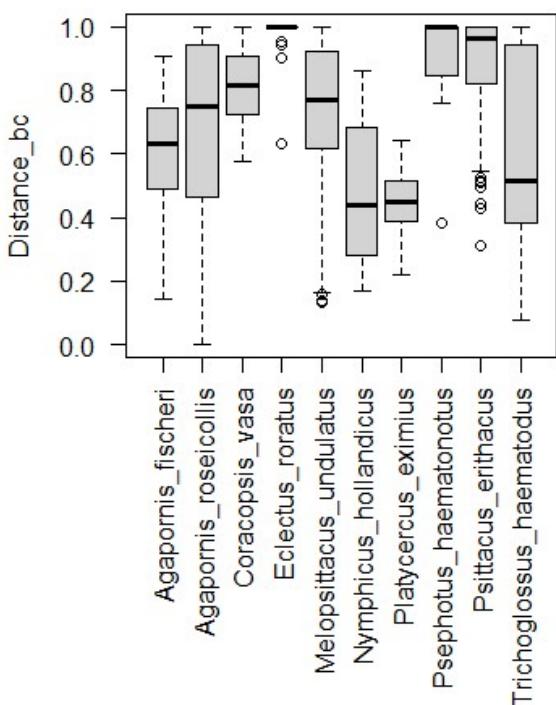


Figure 20: Tanglegram comparing the dendrogram of 21 parrot species based on their similarity in composition of oral microbiota (right) and the phylogenetic tree according to Provost, Joseph, and Smith (2018) (left). Tip names are the abbreviations of the parrot genus and species name³.

³ Ag_ca = *Agapornis canus*, Ag_fi = *Agapornis fischeri*, Ag_ro = *Agapornis roseicollis*, Am_ae = *Amazona aestiva*, Ca_su = *Cacatua sulphurea*, Co_va = *Coracopsis vasa*, Ec_ro = *Eclectus roratus*, Eo_ro = *Eolophus roseicapilla*, Fa_co = *Forpus coelestis*, Me_un = *Melopsittacus undulatus*, Ne_el = *Neophema elegans*, Ne_sp = *Neophema splendida*, Ne_bo = *Neopsephotus bourkii*, Ny_ho = *Nymphicus hollandicus*, Pi_me = *Pionites melanocephalus*, Pl_ex = *Platycercus eximius*, Po_se = *Poicephalus senegalus*, Ps_ha = *Psephotus haematonotus*, Ps_kr = *Psittacula krameri*, Ps_er = *Psittacus erithacus*, Tr_ha = *Trichoglossus haematodus*.

4.3. Intraspecific variation in microbial composition

Besides the interspecific variation in microbial composition, I was also interested in the intraspecific variation. To examine this, I made subsets of oral and faecal microbiota samples of those parrot species, from which at least five samples of the given type were available (10 species for faecal microbiota and 16 for oral). The numbers of individuals and the numbers of different breeders for each parrot species are summarized in Figure 21 for faecal samples and in Figure 22 for oral samples. The boxplot shows distances in microbiota composition between all pairs of samples from individuals of the same parrot species. Mean values and variation in these distances were counted for each species and then correlated with the number of individuals and the number of breeders. Parrot species that were bred by a higher number of breeders had higher means of distances between individual faecal microbiota composition ($r = 0.544$), but not between oral microbiota composition ($r = -0.101$). Intraspecific differences were more pronounced in faecal microbiota composition than in oral. The highest variation was detected in faecal microbiota composition in *Eclectus roratus*, *Psephotus haematonotus* and *Psittacus erithacus*. Low intraspecific variation in faecal and oral microbiota composition was found in *Platycercus eximus*.



Parrot species	Nº of individuals	Nº of breeders
<i>Agapornis fischeri</i>	11	2
<i>Agapornis roseicollis</i>	9	4
<i>Coracopsis vasa</i>	6	2
<i>Eclectus roratus</i>	7	7
<i>Melopsittacus undulatus</i>	37	8
<i>Nymphicus hollandicus</i>	7	4
<i>Platycercus eximus</i>	6	1
<i>Psephotus haematonotus</i>	5	4
<i>Psittacus erithacus</i>	18	17
<i>Trichoglossus haematodus</i>	6	1

Pearson's correlation (r)	Mean distance	Variation in distance
Nº of individuals	0.156	-0.065
Nº of breeders	0.544	-0.228

Figure 21: Left: boxplot of pairwise distances of faecal microbiota composition between individuals of the same parrot species. Distance matrix was created based on Bray-Curtis distances. Right top: Table summarizing numbers of individuals of all species that were included in the analysis of intraspecific variation, and the numbers of breeders by which these individuals were bred. Right bottom: Pearson's correlation coefficients showing relations between the numbers of individuals or breeders of the parrot species, and the mean distances in faecal microbiota composition of the samples (or variation in this distance).

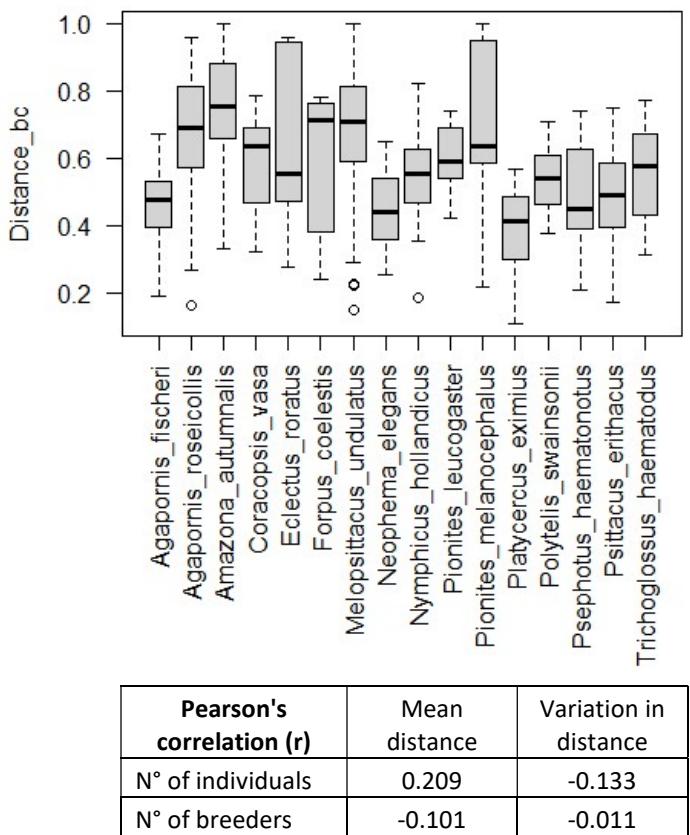


Figure 22: Left: boxplot of pairwise Bray-Curtis distances of oral microbiota composition between individuals of the same parrot species. Right top: Table summarizing numbers of individuals of all species that were included in the analysis of intraspecific variation, and the numbers of breeders by which these individuals were bred. Right bottom: Pearson's correlation coefficients showing relations between the numbers of individuals or breeders of the parrot species, and the mean distances in oral microbiota composition of the samples (or variation in this distance).

Given the fact that parrot breeders usually hold several individuals (often genetically related) of one or a few parrot species, house them in similar conditions, and give them the same feed, I expected that gut microbiota of parrots from one breeder would have more similar composition compared to individuals from other breeders. However, it was not possible to include breeder identity as a predictor of microbial composition into adonis2 models, due to the reasons described in *Material and methods*. Therefore, I visualized the effect of breeder identity in PCoA plots (Figures 23 and 24). Here, I included only the samples from parrot species that had been bred by multiple breeders, and only those breeders from whom multiple parrot species had been sampled. As shown earlier, gut microbiota composition is more similar in individuals of the same species compared to other species. From these PCoA plots, it can be concluded that an important part of the intraspecific variation is explained by the breeder, which includes the effects of feed and housing environment, as well as potential genetic relatedness and transmission of bacteria among co-housed individuals.

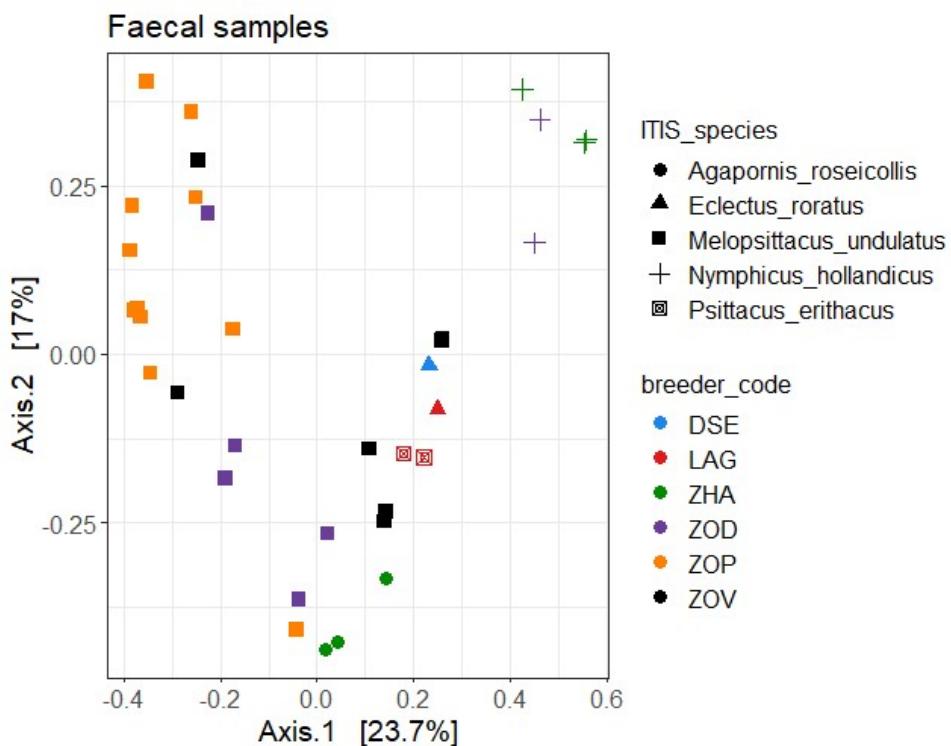


Figure 23: Principal coordinates analysis of faecal samples of selected healthy individuals (n=35). The symbol represents parrot species, and the colour represents the breeder. Distance matrix was created based on Bray-Curtis distances.

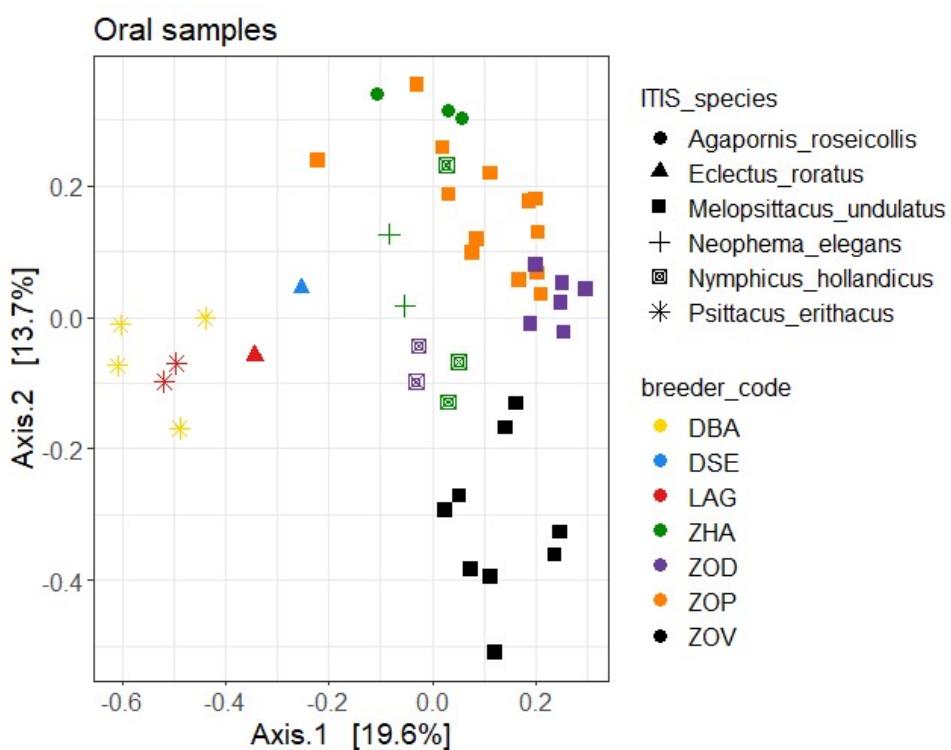


Figure 24: Principal coordinates analysis of faecal samples of selected healthy individuals (n=46). The symbol represents parrot species, and the colour represents the breeder. Distance matrix was created based on Bray-Curtis distances.

4.4. Core microbiota of parrots

The core microbiota of parrots was studied on the full dataset of healthy parrot individuals. Bacterial OTUs were collapsed into genera. Vast majority of genera has been detected in less than 10% of individuals (Figure 25). In faecal microbiota, only the genus *Lactobacillus* was present in majority (71.6%) of all samples (Table 11). In oral microbiota, 5 genera were detected in majority of samples (Table 12). *Corynebacterium* was present in 94.8%, *Streptococcus* and an unassigned genus of Pasteurellaceae family in 87% and two other genera – *Volucribacter* and an unassigned genus of Neisseriaceae has been detected in slightly over half of the samples. These genera were considered as core bacteria of parrot faecal and oral microbiota.

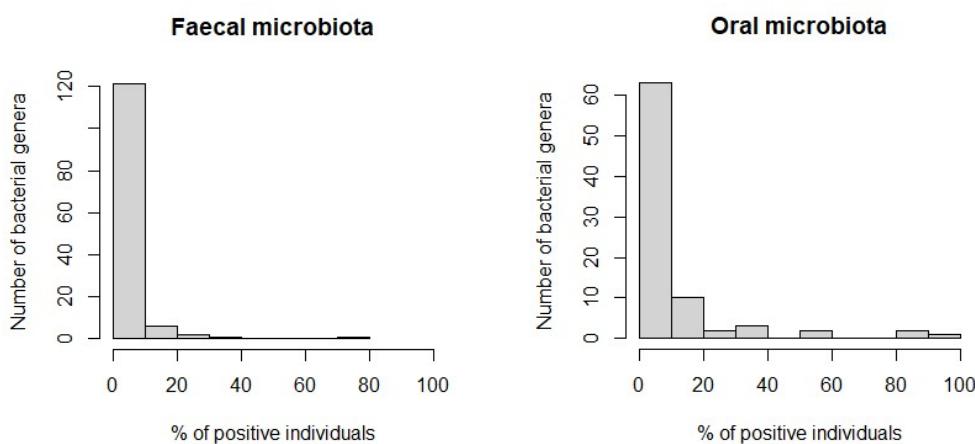


Figure 25: Histograms of the numbers of bacterial genera present in faecal (left) and oral (right) microbiota of healthy parrot individuals.

Phylum	Genus	Positive individuals (%)
Firmicutes	<i>Lactobacillus</i>	71.6

Table 11: Bacterial genera present in faecal microbiota of more than 50% of healthy parrot individuals.

Phylum	Genus	Positive individuals (%)
Actinobacteriota	<i>Corynebacterium</i>	94.8
Proteobacteria	Pasteurellaceae_NA	87.6
Firmicutes	<i>Streptococcus</i>	87.3
Proteobacteria	Neisseriaceae_NA	55.2
Proteobacteria	<i>Volucribacter</i>	53.1

Table 12: Bacterial genera present in oral microbiota of more than 50% of healthy parrot individuals.

4.5. Comparison of healthy individuals and individuals suffering from selected disorders

The composition and diversity of faecal and oral microbiota of healthy parrots has been compared with the microbiota of parrots experiencing selected behavioural (apathy, stereotype behaviour, anorexia and feather plucking) and digestive or metabolic issues (indigestion, nausea, obesity, and weight loss). The incidence of these disorders was not balanced across parrot genera

(Figure 26). For example, almost all *Melopsittacus* and *Agapornis* parrots in our dataset were healthy. On the other hand, most individuals of Ara suffered with one of the disorders.

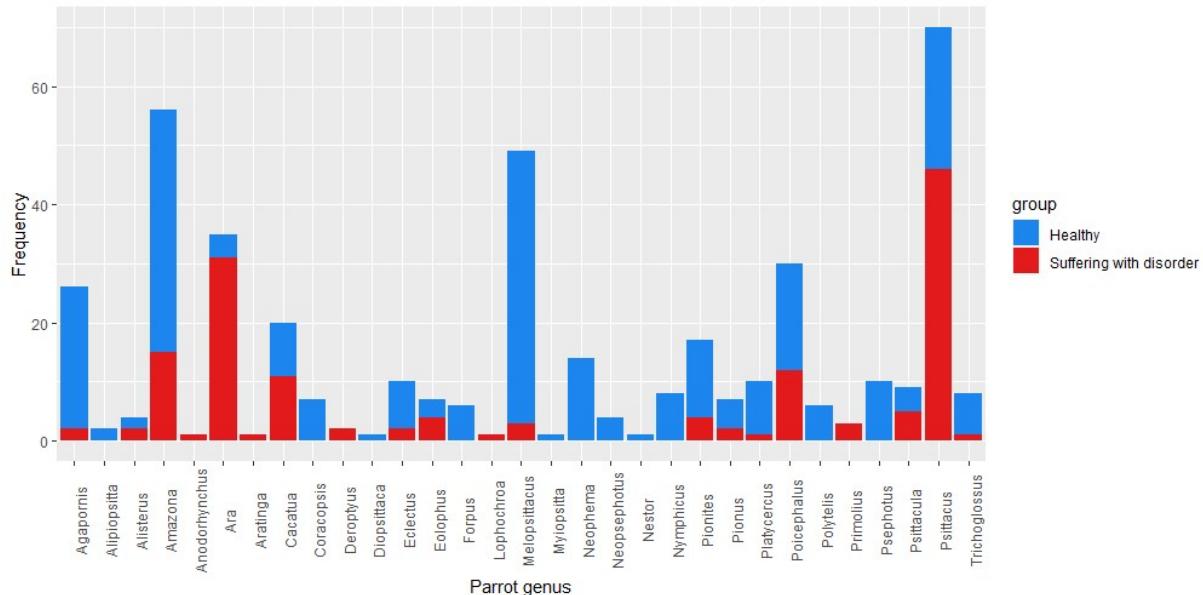


Figure 26: Incidence of the selected disorders in different parrot genera in our dataset.

Table 13 shows the differences in alpha diversity of faecal and oral microbiota of individuals suffering from these disorders, compared to the healthy group. The effect of each disorder was tested separately, and the host genus and breeder were used as random factors. There were no significant differences in the diversity of faecal bacteria. In oral microbiota, all disorders were linked to reduced alpha diversity, but this reduction was significant only for anorexia, feather plucking, obesity and weight loss.

Alpha diversity (Shannon index)						
Disorder	Faecal microbiota			Oral microbiota		
	n	Estim.	p	n	Estim.	p
Apathy	18	-0.056	0.736	19	-0.031	0.773
Stereotype behaviour	4	0.557	0.082	3	-0.112	0.644
Anorexia	36	0.080	0.546	41	-0.251	0.004**
Feather plucking	47	-0.099	0.389	52	-0.233	0.003**
Indigestion	10	-0.066	0.766	11	-0.282	0.053
Nausea	18	-0.093	0.588	18	-0.220	0.071
Obesity	9	0.136	0.517	7	-0.572	<0.001***
Weight loss	80	-0.041	0.679	94	-0.173	0.014*

Table 13: Results of linear mixed-effect models testing the effect of selected disorders on the alpha diversity of faecal and oral microbiota of parrots. Each disorder was tested separately against healthy individuals (n=176 for faeces and n=267 for oral samples). Parrot genus and breeder were used as random factors.

The differences between abundances of Gram-positive, aerobic, and potentially pathogenic bacteria in faecal and oral microbiota of individuals suffering from the selected disorders and healthy individuals are shown in Tables 14 and 15. No significant differences have been detected, with two exceptions – indigestion was connected to increased levels of potentially pathogenic bacteria in faeces, and anorexia with increased abundance of Gram-positive bacteria in oral microbiota. Surprisingly, all disorders were linked to decreased abundances of potentially pathogenic bacteria in oral microbiota. However, this effect was not significant for any of them.

Faecal microbiota							
Disorder	n	Abundance of Gram-positive bacteria		Abundance of aerobic bacteria		Abundance of potentially pathogenic bacteria	
		Estim.	p	Estim.	p	Estim.	p
Apathy	18	-0.109	0.854	-0.294	0.607	0.591	0.359
Stereotype behaviour	4	-1.294	0.271	0.311	0.777	1.722	0.158
Anorexia	36	-0.386	0.43	-0.224	0.644	0.750	0.167
Feather plucking	47	0.1489	0.695	0.312	0.449	-0.715	0.101
Indigestion	10	-0.146	0.845	-0.858	0.264	2.102	0.017*
Nausea	18	-0.115	0.842	0.801	0.207	0.251	0.716
Obesity	9	0.337	0.605	0.343	0.627	-0.710	0.382
Weight loss	80	-0.462	0.205	-0.009	0.981	0.712	0.094

Table 14: Results of linear mixed-effect models testing the effect of selected disorders on the abundances of Gram-positive, aerobic, and potentially pathogenic bacteria in faecal microbiota of parrots. Each disorder was tested separately against healthy individuals (n=176). Parrot genus and breeder were used as random factors.

Oral microbiota							
Disorder	n	Abundance of Gram-positive bacteria		Abundance of aerobic bacteria		Abundance of potentially pathogenic bacteria	
		Estim.	p	Estim.	p	Estim.	p
Apathy	19	0.497	0.223	-0.307	0.480	-0.532	0.128
Stereotype behaviour	3	0.943	0.324	0.359	0.729	-0.541	0.489
Anorexia	41	0.723	0.029*	-0.164	0.630	-0.450	0.122
Feather plucking	52	0.487	0.097	-0.303	0.330	-0.473	0.068
Indigestion	11	0.414	0.466	-0.100	0.865	-0.368	0.442
Nausea	18	0.612	0.188	0.464	0.333	-0.527	0.183
Obesity	7	0.846	0.157	0.018	0.978	-0.741	0.137
Weight loss	94	0.310	0.244	0.094	0.707	-0.121	0.615

Table 15: Results of linear mixed-effect models testing the effect of selected disorders on the abundances of Gram-positive, aerobic, and potentially pathogenic bacteria in oral microbiota of parrots. Each disorder was tested separately against healthy individuals (n=267). Parrot genus and breeder were used as random factors.

The HMSC analysis revealed several bacterial genera in faecal (Figure 27) or oral (Figure 28) microbiota significantly associated with the selected disorders. The last row of the heatmap (intercept) represents the healthy group. There, the blue colour represents genera with higher abundances in healthy individuals compared to individuals suffering from any of the disorders, and the red colour represents genera with lower abundances. Each group of individuals suffering from one disorder was tested against the intercept with no influence of other disorders. The HMSC models predicted the incidence of each disorder (rows) in the conditions of increased or decreased abundance of each bacterial genus (columns). In faecal samples, feather plucking was significantly associated with increased abundances of *Globicatella*, *Lactobacillus* and *Streptococcus*. Weight loss was connected to increased abundances of *Clostridium* and *Escherichia/Shigella*, and decreased abundances of *Leuconostoc* and *Weissella*. And anorexia was connected to higher abundances of *Kocuria* and *Streptococcus*. No significant association was found for apathy or nausea.

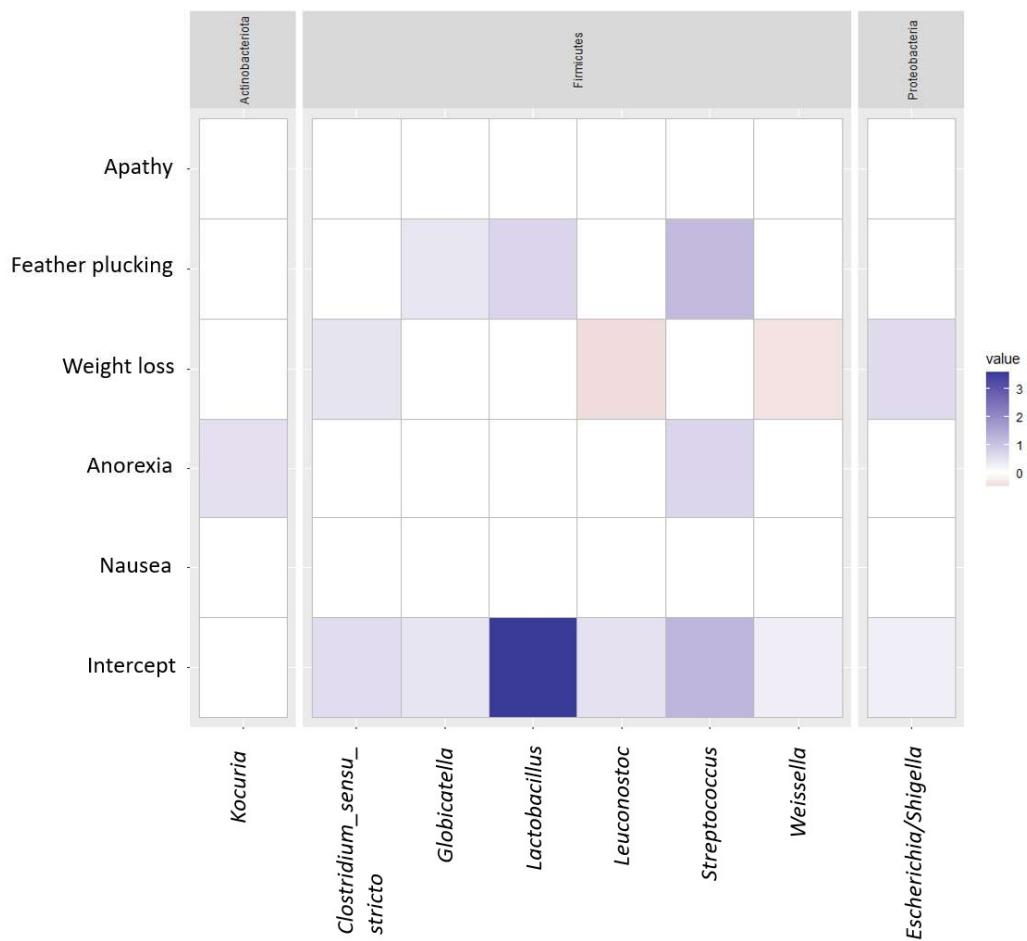


Figure 27: Heatmap summarizing results of HMSC analysis of faecal samples (n=297). Blue colour represents disorders that are predicted to have increased incidence in samples with higher abundance of the specific bacterial species than intercept. Red colour represents disorders that are predicted to have increased incidence in samples with lower abundance of the specific bacterial species than intercept. White colour represents parameter values that were estimated to be positive or negative with < 95% posterior probability. Intercept represents healthy individuals. Parrot genus and breeder were used as random factors. Only bacterial species present in at least 5% of samples were included.

In oral samples, apathy was linked to increased counts of *Lactobacillus* and other Lactobacillales of unassigned genus, and decreased counts of *Suttonella*. Feather plucking was significantly associated with decreased abundance of unassigned genus of Gracilibacteria, while weight loss with the decrease in *Moraxella*. Anorexia was linked to lower abundances of *Suttonella*. And nausea was connected to increased abundances of *Lactobacillus* and other Lactobacillaceae, and decreased abundances of *Globicatella* and *Suttonella*.

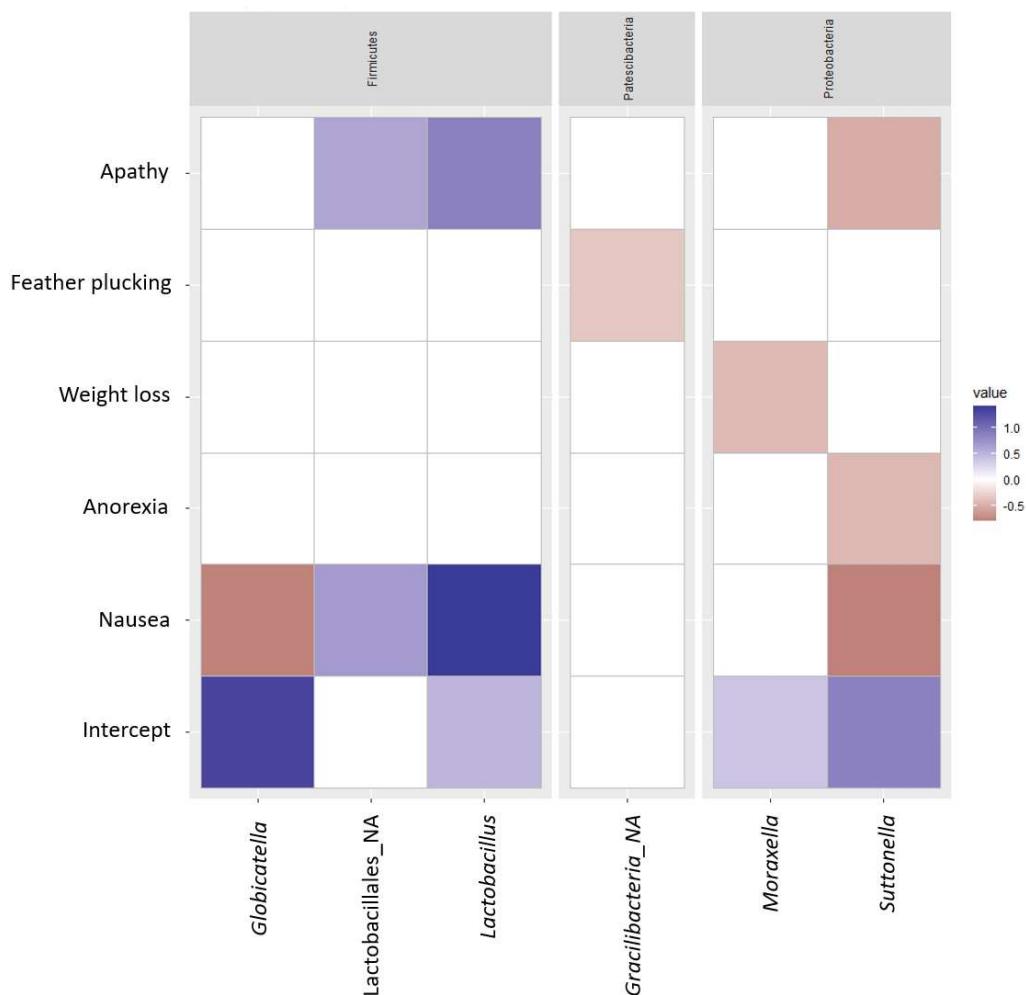


Figure 28: Heatmap summarizing results of HMSC analysis of oral samples (n=397). Blue colour represents disorders that are predicted to have increased incidence in samples with higher abundance of the specific bacterial species than intercept. Red colour represents disorders that are predicted to have increased incidence in samples with lower abundance of the specific bacterial species than intercept. White colour represents parameter values that were estimated to be positive or negative with < 95% posterior probability. Intercept represents healthy individuals. Parrot genus and breeder were used as random factors. Only bacterial species present in at least 5% of samples were included.

4.6. Probiotics for parrots

Content of eight commercial probiotics for parrots was analysed using metabarcoding. Only negligible differences between extraction duplicates were found. Thirteen OTUs were detected only in probiotics, while six were detected also in parrot faeces, one in probiotics and oral samples and other sixteen were shared among all three sample types (Figure 11). One of the main aims of this thesis was to compare the composition of probiotics for parrots with their declared content and also with the gut microbiota composition of healthy parrot individuals. 13 out of 36 OTUs detected in analysed probiotic products were not detected in any of our parrot samples. That brought me to the question, if these are dominant OTUs of these products, or if they are present only in very low abundances that would indicate potential contaminations. Following tables are summarizing information about individual probiotic products, providing a deeper insight into abundances and phylogenetic classification of OTUs detected in them.

Ac-i-prim

The declared content of the probiotic product Ac-i-prim was *Lactobacillus acidophilus* CECT 4529 (4b1715) 5×10^{11} CFU/kg. Our results show that 100% of sequences were assigned to a single OTU from the genus *Lactobacillus* (Table 16), which was in accordance with the declared content. This OTU was also present in the faecal and oral microbiota of healthy parrots in our dataset.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2378	100.0	Firmicutes	<i>Lactobacillus</i> _NA	Yes	Yes

Table 16: Results of metabarcoding of the probiotic product Ac-i-prim. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Aves

The declared content of the product Aves was *Enterococcus faecium* NCIMB 11181 (4b1708) 200.000×10^6 CFU. No sequences obtained by metabarcoding were assigned to *Enterococcus* (Table 17). Despite the declared content, three OTUs belonging to Lactobacillales and two OTUs of *Bifidobacterium* were found. Only two of these OTUs were present in faecal microbiota and one also in oral microbiota of the parrots in our dataset.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2445	35.0	Firmicutes	<i>Lactobacillus</i> _NA	Yes	Yes
1412	33.1	Actinobacteriota	<i>Bifidobacterium</i> _NA	No	No
2303	29.1	Firmicutes	<i>Lactobacillales</i> _NA	Yes	No
1410	1.4	Actinobacteriota	<i>Bifidobacterium pseudolongum</i>	No	No
2455	1.4	Firmicutes	<i>Lactobacillus</i> _NA	No	No

Table 17: Results of metabarcoding of the probiotic product Aves. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Ema fauna Exotic

The declared content of the probiotic product Ema fauna Exotic was: species of *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Rhodopseudomonas*, *Saccharomyces* and *Streptococcus*. Of these genera, only *Lactobacillus* was detected (Table 18). It was represented by six different OTUs, from which only one was detected in the faecal microbiota of healthy parrots.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2453	41.0	Firmicutes	<i>Lactobacillus</i> _NA	No	No
2536	18.0	Firmicutes	<i>Lactobacillus</i> _NA	No	No
2605	13.6	Firmicutes	<i>Lactobacillus</i> _NA	Yes	No
2531	11.7	Firmicutes	<i>Lactobacillus</i> _NA	No	No
2537	9.6	Firmicutes	<i>Lactobacillus</i> _NA	No	No
459	3.9	Proteobacteria	<i>Sphingomonas mucosissima</i>	Yes	Yes
487	1.6	Proteobacteria	<i>Endobacter</i> _NA	No	No
2535	0.6	Firmicutes	<i>Lactobacillus</i> _NA	No	No

Table 18: Results of metabarcoding of the probiotic product Ema fauna Exotic. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Lactiferm WS

The declared content of the product Lactiferm WS was *Enterococcus faecium* (NCIMB 11161) 200x10⁹ CFU. All sequences detected by metabarcoding were assigned to a single OTU from the genus *Lactobacillus* (Table 19), which was in contradiction with the declared content. This OTU was present in the faecal microbiota of healthy parrots in our dataset, while not in oral microbiota.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2303	100.0	Firmicutes	<i>Lactobacillales</i> _NA	Yes	No

Table 19: Results of metabarcoding of the probiotic product Lactiferm. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Nekton-Biotic-bird

The declared content of the probiotic product Necton was *Bacillus subtilis* C-3102, min. 1×10^{11} CFU/kg. Sequences assigned to the genus *Bacillus* (two OTUs) together accounted for 89.1% of all detected sequences (Table 20). These two OTUs were not present in faecal nor oral microbiota of healthy parrots in our dataset. The remaining 10.9% of sequences belonged to the genera *Lactobacillus*, *Pseudomonas*, *Prevotella*, *Leuconostoc* and *Terribacillus*.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2185	85.6	Firmicutes	<i>Bacillus</i> _NA	No	No
2378	4.7	Firmicutes	<i>Lactobacillus</i> _NA	No	No
2225	3.5	Firmicutes	<i>Bacillus</i> _NA	No	No
111	1.9	Proteobacteria	<i>Pseudomonas</i> _NA	Yes	Yes
958	1.4	Bacteroidota	<i>Prevotella</i> _NA	No	No
1993	1.2	Firmicutes	<i>Leuconostoc</i> _NA	Yes	Yes
2237	1.2	Firmicutes	<i>Terribacillus</i> _NA	Yes	Yes
109	0.6	Proteobacteria	<i>Pseudomonas</i> _NA	Yes	Yes

Table 20: Results of metabarcoding of the probiotic product Nekton-Biotic-bird. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Nutrimix

The declared content of the probiotic product Ema fauna Exotic was 100% *Enterococcus faecium*, but any of the sequences obtained by metabarcoding were not assigned to the genus *Enterococcus* (Table 21). The two most abundant OTUs belonged to the order Lactobacillales and were not assigned to any known family. They were also detected in the faecal samples of healthy parrots, but not in oral samples.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2303	97.9	Firmicutes	Lactobacillales_NA	Yes	No
2264	1.6	Firmicutes	Lactobacillales_NA	Yes	No
1873	0.6	Firmicutes	<i>Selenomonas lacticifex</i>	No	No

Table 21: Results of metabarcoding of the probiotic product Nutrimix. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Probi-zyme

The declared content of the probiotic product Probi-zyme was *Bacillus velezensis* DSM 15544 2×10^{11} CFU. In accordance with the declared content, 80.1% of sequences were assigned to the genus *Bacillus* (Table 22). However, seven more OTUs belonging mainly to the phylum Proteobacteria were

also detected. Some of them were also present in the faecal and/or oral microbiota of healthy parrots, but the dominant *Bacillus* OTU was not.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2185	80.1	Firmicutes	<i>Bacillus</i> _NA	No	No
303	8.8	Proteobacteria	<i>Pantoea</i> _NA	Yes	No
301	3.3	Proteobacteria	Enterobacteriales_NA	Yes	Yes
117	2.6	Proteobacteria	Pseudomonadaceae_NA	No	No
2237	1.6	Firmicutes	<i>Teribacillus</i> _NA	No	No
302	1.5	Proteobacteria	<i>Pantoea</i> _NA	No	Yes
459	1.4	Proteobacteria	<i>Sphingomonas mucosissima</i>	Yes	Yes
116	1.1	Proteobacteria	<i>Pseudomonas monteili</i>	Yes	No

Table 22: Results of metabarcoding of the probiotic product Probi-zyme. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

Proparrot

The declared content of the probiotic product Proparrot was *Lactobacillus fermentum*. Only half of the sequences were assigned to two OTUs from the genus *Lactobacillus* (Table 23). The more abundant one was also present in the oral microbiota of healthy parrots in our dataset, but not in their faecal microbiota. Second half of the sequences obtained by metabarcoding belonged to bacteria from different taxonomical groups, but most of them were detected also in faecal and oral microbiota of healthy parrots.

OTU	Relative abundance (%)	Phylum	Species	In parrot faecal microbiota	In parrot oral microbiota
2545	47.3	Firmicutes	<i>Lactobacillus</i> _NA	No	Yes
2097	29.8	Firmicutes	<i>Streptococcus</i> _NA	Yes	Yes
290	8.1	Proteobacteria	Enterobacteriaceae_NA	Yes	Yes
1135	4.6	Bacteroidota	<i>Chryseobacterium</i> _NA	Yes	Yes
2011	3.0	Firmicutes	<i>Lactococcus</i> _NA	Yes	Yes
2098	2.7	Firmicutes	<i>Streptococcus</i> _NA	No	Yes
1136	2.4	Bacteroidota	<i>Chryseobacterium</i> _NA	No	No
2543	2.0	Firmicutes	<i>Lactobacillus</i> _NA	No	No

Table 23: Results of metabarcoding of the probiotic product Proparrot. Last two columns specify, if the OTU has been detected in faecal or oral microbiota of any of the healthy parrot individuals in our dataset.

5. Discussion

In this study, the gut microbiota of healthy parrots and parrots suffering from digestive, metabolic, and behavioural disorders has been studied by means of faecal and oral samples. Based on our results, the oral microbiota of parrots has significantly higher alpha diversity compared to faecal samples, and these two bacterial communities differ in their compositions. This corresponds with our previously published results obtained in a smaller subset of parrot individuals, but a larger spectrum of different tissues (Schmiedová et al. 2023). The same result was found also in the great tit (*Parus major*) (Kropáčková et al. 2017a). On the other hand, the alpha diversity of faecal microbiota can be higher compared to the oral one in some reptile species (Du et al. 2022). In humans, there are also significant differences in the composition of oral and faecal microbiota. The alpha diversity of bacteria in faeces is comparable to the diversity in saliva, but buccal mucosa hosts less diverse bacterial communities (Huttenhower et al. 2012). Due to these differences between oral and faecal microbiota composition and diversity in parrots and other hosts, all our analyses were done separately for faecal and oral microbiota samples.

5.1. Factors influencing gut microbiota composition and diversity

The first aim of this thesis was to describe the interspecific and intraspecific variation in the microbiota composition of parrots. I hypothesized that the effect of host phylogeny on the composition of microbiota samples would probably be low, and the high intraspecific variation could be partly explained by environmental factors such as diet and type of housing, or by sex and age. As predicted, we found that the composition of faecal and oral microbiota of healthy parrots was significantly influenced by the host genus, housing environment, type of feed, and sampling date. Oral microbiota was also different between sexes, while faecal was not (there was only a difference in the abundance of Gram-positive bacteria). This contrasted with my expectations which were based on the fact that faeces are eliminated from the body through cloaca, where they could be contaminated by bacteria originating from the urogenital tract that are more likely to be sex-specific. In the study by West et al. (2023), sex was the most influential predictor of faecal microbiota composition and diversity in kakapos. The explanation for this difference from our data could be that kakapo is the only parrot with the lek type of mating system and that can be associated with high levels of testosterone in males (Moore et al. 2019; Wingfield 1984). As testosterone levels were shown to influence cloacal and gut microbiota composition (Escallón et al. 2017; d’Afflitto et al. 2022), this hormonal regulation could cause significant differences also between the faecal microbiota composition of male and female kakapos. Our dataset was comprised of faecal samples of parrot species with different types of mating systems, but it was dominated by monogamous species (*Psittacus erithacus*, *Melopsittacus undulatus*,

Ara ararauna, *Amazona aestiva*, etc.), where the differences in testosterone levels between sexes could be lower, diminishing also the sex-specific differences in microbiota composition.

The significant effect of sampling day (continuous predictor acquiring values from 1 to 365) can be partly explained by the interrelation with other predictors, as the samples taken on the same day were usually taken from individuals bred by the same breeder. However, given the high number of breeders included in this study (225), at least part of this variation is most likely biologically relevant. Interestingly, the effect of sampling day on faecal microbiota composition was significant only in the model, where the presence or absence of bacterial taxa was assessed, but not when the information about abundances was included. This suggests that the presence of some minor bacterial taxa can vary throughout the year, while the abundances of major taxa do not change significantly. The effect of sampling day was more pronounced in oral microbiota compared to the faecal one. West et al. (2022) found a marginally significant effect of season on the variation among bacterial communities in the faecal samples of kakapos, but they attributed these signals to the heterogeneity in group dispersions. To my knowledge, no other research has been done on the long-term stability and seasonal variation of microbiota composition of parrots. However, the seasonal variation in gut microbiota composition in captive parrots will probably be lower than in wild birds, where the changes in gut microbiota can be caused by seasonal changes in diet (Gongora, Elliott, and Whyte 2021).

The effect of parrot age was not significant in any of the performed tests. This agrees with the studies conducted on kakapo (Waite, Deines, and Taylor 2012; Waite, Eason, and Taylor 2014; West et al. 2023). On the other hand, changes in gut microbiota composition during development were described e.g., in chicken, great tits (*Parus major*), little penguins (*Eudyptula minor*), and short-tailed shearwaters (*Ardenna tenuirostris*) (Richards-Rios et al. 2020; Y. Liu et al. 2021; Dewar et al. 2017; Teyssier et al. 2018). However, these studies were focused on gut microbiota composition changes in the first few days or weeks of life, while our dataset did not include parrots of such a young age.

As I expected, variables connected to the breeder, the type of feed, and the housing environment, had a significant effect on the composition and diversity of the faecal and oral microbiota of parrots. The composition of both sample types was significantly influenced by both of these variables. In terms of alpha diversity, housing affected only oral microbiota. Specifically, birds housed in flocks in outdoor aviaries had higher oral microbiota diversity compared to birds living alone or in pairs in indoor cages, and they had higher abundance of potentially pathogenic bacteria. This result suggests that oral microbiota can be enriched by bacteria from other individuals in the flock, probably including trans-species transmission in aviaries, where multiple parrot species are co-housed. Potentially, bacteria could be transmitted to outdoor housed parrots also by contact with wild bird species. Transmission of bacteria has been proved between wild birds and poultry (Xiang et al. 2023;

Ayala, Yabsley, and Hernandez 2020). But poultry is flightless and, therefore, often housed in open runs, where direct contact with wild birds (e.g., on the feeding dish) is possible. In the case of closed aviaries, the transmission of bacteria from wild birds is less probable. However, if this type of microbiota transmission was proved, it would be of great epidemiological interest. Notably, alpha diversity and the abundance of potentially pathogenic bacteria negatively correlated with the abundance of Gram-positive bacteria, suggesting that these minor bacterial taxa contributing to the increased microbiota diversity of outdoor flocks are often Gram-negative species with the pathogenic potential.

One of the limitations of this thesis is the impossibility of differentiating between the effects of indoor/outdoor housing, number of co-housed individuals, and artificial nursing, given by the understandable co-distribution of these variables. Unfortunately, these predictors were highly interrelated and had to be merged. Therefore, the differences in microbiota composition and diversity between individuals nursed by parents and individuals nursed artificially by humans could not be properly assessed. However, one of the potential explanations for the higher abundance of Gram-negative and potentially pathogenic bacteria in the oral microbiota of birds that were housed outdoors, in flocks, or nursed by parents, is that this effect is driven by the type of nursing. It has been discovered that in chicken, Gram-negative bacteria are less abundant in individuals raised without contact with hens than in naturally raised chicks (Kubasova et al. 2019).

The type of feed had a more pronounced effect on faecal microbiota composition than on the oral microbiota. In terms of diversity, it influenced only faecal microbiota, but not the oral one. This could be explained by the fact that faecal microbiota composition well represents the composition of the microbiota of the lower intestinal tract (Schmiedová et al. 2023), where the feed is already broken down into molecules that substantially influence the gut environment and that can serve as nutrients for the bacteria. Surprisingly, parrots fed with diverse feed had lower alpha diversity of faecal microbiota compared to individuals that were fed only grain.

Importantly, all the factors discussed so far explained only a small part of the variation in gut microbiota composition (less than 2.5%). Markedly more variation has been explained by the parrot genus – around 30% of the variation in the microbial composition of faeces and 37% in oral samples. This can be partly caused by the high number of parrot genera – although parrot genera with only one individual were excluded, 27 remained for faecal and 29 for oral samples (all other tested predictors had a maximum of three levels), and most genera were represented by low numbers of individuals. However, the residual numbers of degrees of freedom were high enough (144 for 173 faecal samples and 232 for 264 oral samples) to conclude that the high proportion of variation explained by the parrot genus is not caused by the overfitting of the models.

For further analysis of the phylogenetic effect, I compared the phylogenetic and microbiota composition clustering using tanglegrams. I detected a significantly stronger phylogenetic signal in oral microbiota compared to faecal microbiota. However, in both dendograms based on the similarities in microbial composition, only the clustering on species level showed good bootstrap support, while the support for the topology of deeper phylogenetic lineages was low. These results confirm that the similarity in microbial composition is relatively high among closely related (recent) lineages but diminishes fast in the phylogenetic history. This result does not correspond with the results of Song et al. (2020) that found constantly low similarity in gut microbiota composition of birds across divergence time. They compared the level of phylosymbiosis⁴ and the proportion of host species-specific bacterial taxa between mammals and birds (represented by 491 species across phylogenetic tree). The effect of host phylogeny was significant in both groups but explained only 2% of the variation in gut microbiota composition of birds, compared to 17% in mammals.

Although gut microbiota in birds is less specific than in mammals, this does not disprove the existence of phylosymbiosis in birds. The effect of host species on faecal or intestinal microbiota has been detected in parrots (H. Liu et al. 2019), passerines (Kropáčková et al. 2017b), neotropical birds of several orders (Hird et al. 2015), waterbirds (Laviad-Shitrit et al. 2019) and New World vultures (Roggenbuck et al. 2014). Contrary to Song et al. (2020), Hird et al. (2015) even found a significant effect of all tested phylogenetic categories (host species, genus, family, and order). Even the order explained more than 20% of the variation in microbial composition (Weighted UniFrac, min. 2 individuals per species), while ecological variables such as feed or habitat, which were also significant, explained only 7 – 16% of the variation. However, the clustering of microbial samples in dendograms that would correspond to the phylogenetic tree of host genera or higher taxonomical categories was reported only in waterbirds and vultures (Laviad-Shitrit et al. 2019; Roggenbuck et al. 2014).

For an interspecific comparison of the gut microbiota composition of parrots, I constructed oral and faecal microbiota profiles of 21 parrot species. The faecal microbiota of three of them was also analysed by H. Liu et al. (2019), but the results they obtained were different from ours. In *Psittacus erithacus*, we similarly described *Lactobacillus* as the most abundant species (with a relative abundance of around 50%). However, the remaining 50% were formed by *Streptococcus*, *Ureaplasma*, and *Corynebacterium* in our dataset, while H. Liu et al. (2019) detected *Clostridium* and *Ralstonia*. In both studies, around 30% of faecal OTUs in *Amazona aestiva* were not assigned to any genus, and *Clostridium* was found to be a minor bacterial genus. However, in our samples, *Lactobacillus* formed

⁴ Phylosymbiosis is the non-random association of microbial communities with the evolutionary history of their host organisms (Kohl et al. 2016).

approximately 50% of the whole community, while in H. Liu et al. (2019) the dominant taxa were *Planococcus*, *Acinetobacter*, and *Escherichia/Shigella*. In *Eclectus roratus*, we found *Lactobacillus*, *Clostridium*, and *Candidatus Arthromitus* as the most abundant taxa, while H. Liu et al. (2019) detected besides *Candidatus Arthromitus* and *Clostridium* also high abundances of *Ralstonia*. I assume that these differences are caused by the high intraspecific variation in faecal microbiota composition that I described in all three of these parrot species, and certain differences in methodology. Compared to this thesis, H. Liu et al. (2019) used a different technique of DNA extraction and chose a different approach for data filtering. To avoid false positives, we sequenced our samples in technical duplicates and removed all OTUs that were not coincidentally detected in both of them, which resulted in the loss of 60 – 80% of OTUs per sample during the data filtering procedure. We also excluded OTUs assigned to chloroplasts and mitochondria and OTUs with relative abundance lower than 0.01% (usually less than 1000 sequences). We believe that these filtering steps helped us to remove sequencing artefacts with no biological relevance. H. Liu et al. (2019) did not perform any of these filtering steps, which led to a significant difference in the mean numbers of OTUs detected per sample (8.8 in this thesis, versus 353 in H. Liu et al. (2019)). However, consistent with our results, their samples were dominated by a few highly abundant OTUs, similar to those detected in faecal samples in this thesis. Most of our analyses of microbiota composition were based on Bray-Curtis distances, while H. Liu et. al (2019) calculated distances using weighted UniFrac. Both of these methods take into account species abundances, and thus the major bacterial taxa have a larger impact on the calculated distances between samples than minor taxa. Therefore, our results should be comparable despite the described differences in methodology and the mean counts of OTUs detected per sample.

To summarize the factors with main effects on the composition and diversity of microbiota of parrots, these were the host species, type of housing environment, and feed for faecal samples, while host species, sex, feed, type of housing, and the day of sampling had the major influence on oral microbiota.

5.2. Core microbiota

There are substantial differences in the definition of the core microbiota between studies. Waite, Eason, and Taylor (2014) defined core microbiota of kakapo as OTUs present in at least 90% of individuals, while (West et al. 2023) as bacterial species with a minimum prevalence of 85% and a minimum average relative abundance of 1% across all samples. H. Liu et al. (2019) described the core microbiota of parrots as OTUs present in at least one individual from every studied parrot species. This resulted in 105 core OTUs belonging to the genera *Lactobacillus*, *Ralstonia*, *Clostridium*, *Candidatus Arthromitus*, *Acinetobacter*, *Kocuria*, *Escherichia/Shigella*, *Planococcus*, *Rhodococcus*, and *Staphylococcus*. Most of these bacterial genera were present also in our results, but none of them was

detected in all the studied parrot species. I defined the core microbiota of each sample type as bacterial species detected in more than 50% of individuals. This limit was exceeded only by *Lactobacillus* in faecal samples, and five species in oral samples (*Corynebacterium*, *Streptococcus*, *Volucribacter*, and unassigned genera of Pasteurellaceae and Neisseriaceae family).

Lactobacilli are Gram-positive facultatively anaerobic rods that produce lactic acid, SCFAs, bacteriocins, and other compounds. They are present also in the gut microbiota of other birds like passerines (Kropáčková et al. 2017b), waterbirds (Laviad-Shitrit et al. 2019) or poultry (Wei, Morrison, and Yu 2013), but they usually do not represent the dominant bacterial genus in these groups of birds. We detected 67 OTUs that were assigned to *Lactobacillus* (with only three of them being assigned also to the species level), which forms 11% of all OTUs in our dataset. Concerning the dominant genera of oral microbiota of parrots, *Corynebacterium* (Gram-positive, order Actinomycetales) was found to be part of the commensal gut microbiota for example in ducks (Zhu et al. 2020), and it was represented by 26 OTUs in our dataset. *Streptococcus* (Gram-positive, order Lactobacillales) is also present in the gut microbiota of ducks and chickens, and some strains are even used in probiotic products for poultry because they showed positive effects on host health and production-related traits (Zhang et al. 2021; Naseem et al. 2012). However, some other *Streptococcus* species are pathogenic for birds and mammals (Roy et al. 2013; Aryasinghe et al. 2014). Our dataset contained 28 OTUs assigned to *Streptococcus*, but none of them was assigned on the species level, and therefore, we cannot say if these OTUs belonged to the commensal or pathogenic species. *Volucribacter* (Gram-negative), represented by 4 OTUs in our dataset, is a parrot pathogen, but it can be detected also in healthy individuals (Gregersen et al. 2010; Bisgaard et al. 2017). Other genera of Pasteurellaceae (from the class Gammaproteobacteria) can be either commensal or pathogenic for a variety of animal hosts, producing endotoxins and causing respiratory or reproductive tract infections (Frey 2011). The last bacterial taxon identified as a part of the core oral microbiota of parrots was an unassigned genus of Neisseriaceae, represented by 20 OTUs. However, we cannot say if all these OTUs belonged to a single genus or multiple genera. Neisseriaceae were detected in faecal samples from healthy individuals of several penguin species (Dewar et al. 2013), and they were shown to have higher abundances in the gut microbiota of chickens with high feeding efficiency, compared to those with low feeding efficiency (Lv et al. 2021). However, some species of *Neisseria* can also cause severe pathogenicity and even mortality in birds (C. Wang et al. 2016).

In conclusion, the core oral and faecal microbiota of parrots is formed by bacterial genera that can also be found in other bird and mammalian hosts. Unfortunately, the 16S rRNA metabarcoding does not provide a sufficient resolution to assign the obtained sequences on the species level, which greatly limits our understanding of the functional roles of these core bacteria.

5.3. Associations between the gut microbiota and digestive, metabolic, and behavioural disorders

The second aim of this thesis was to link the variation in microbial communities to the incidence of selected disorders. I hypothesized that the gut microbiota composition of individuals suffering from digestive, metabolic, or behavioural disorders would be different from that of healthy parrots, and I aimed to detect bacterial taxa associated with an increased incidence of these disorders. Our dataset included different numbers of individuals suffering from the selected types of disorders (apathy, stereotype behaviour, anorexia, feather plucking, indigestion, nausea, obesity, and weight loss). The incidence of these disorders can be influenced by several factors. Kinkaid et al. (2013) performed a survey among parrot owners (17 parrot species), where they found a significant effect of parrot species and age (more common in adult birds than in juveniles or adolescents) on the performance of feather plucking. They did not find any significant effect of hatch origin (wild-reared, parent-reared in captivity, or hand-reared). A similar study by Ebisawa et al. (2021; 13 parrot genera) also showed a higher prevalence of feather plucking in adult birds than in young birds, and indicated no effect of hand-rearing. This disorder was less common in birds that were kept by large families and families with children, which is in accordance with the generally accepted hypothesis that feather plucking often develops as a consequence of stimulation deficiency and loneliness (van Zeeland et al. 2009). However, including pellets or human food in feed was also found to be a risk factor for feather plucking, which supports the idea that the gut microbiota plays an important role in the development of this disorder.

The numbers of individuals suffering from different disorders that were sampled in this thesis cannot be compared to the results of these surveys because our sampling effort was not standardised. In accordance with the hypothesis presented by van Zeeland et al. (2009), the incidence of the selected disorders (when tested all together against the healthy group) was higher in individuals that were bred alone or in pairs in indoor cages or nursed artificially by humans. However, this association does not have to be direct, because we found complex connections between several of the studied factors and characteristics of gut microbiota composition (Table 24). It is important to note that in this thesis, I work with the relative abundances of bacteria, meaning that, for example the loss of certain Gram-negative bacterial taxa leads to relatively higher abundances of the Gram-positive ones, even if their absolute numbers do not change.

	Healthy	Suffering with disorders
Housing environment and type of nursing	More outdoor, in flocks, nursed by parents	More indoor, alone or in pairs, nursed by humans
Alpha diversity of oral microbiota	↑	↓
Abundance of potentially pathogenic bacteria	↑	↓
Abundance of Gram-positive bacteria	↓	↑
Most common parrot genera	<i>Melopsittacus, Agapornis, Psittacus, Cacatua, Psephotus</i>	<i>Psittacus, Ara, Amazona, Poicephalus, Cacatua</i>

Table 24: Summary of the differences between healthy individuals and individuals suffering from some of the selected disorders.

Surprisingly, the abundances of potentially pathogenic bacteria in oral microbiota were decreased in parrots suffering from the selected disorders. This result was not significant, but it was consistent across all of the studied disorders. The potential explanation of this observation lies in the complex connection described in Table 24. Some species are more susceptible to the development of these disorders, which can be caused by genetic factors. The majority of budgerigars (*Melopsittacus*) and lovebirds (*Agapornis*) in our dataset were healthy, which agrees with the observation that behavioural disorders are more common in larger parrot species (Van Zeeland et al. 2009). But at the same time, these little parrot species are more commonly bred in bigger flocks in outdoor aviaries, where contact with diverse environmental microbes is more common. That would explain our observation that healthy individuals host a more diverse oral microbiota. The low alpha diversity of oral microbiota of parrots suffering from the selected disorders is probably linked to the absence of minor bacterial taxa and thus increased relative abundance of the dominant ones, which could lead to the shift in abundances of potentially pathogenic bacteria and Gram-positive bacteria. It is also important to note, that bacteria assigned to the “potentially pathogenic” group in BugBase software might not cause any pathology in the context of bird oral microbiota. Because of these complex connections, it is impossible to conclude what are the causes and consequences of the observed differences between healthy individuals and individuals suffering from the selected disorders.

Surprisingly, associations between the composition and diversity of faecal microbiota and the selected disorders were weaker than in oral microbiota. We found no significant differences in faecal microbiota diversity or abundances of Gram-positive or aerobic bacteria. In terms of potentially pathogenic bacteria, their abundance was significantly increased only in parrots suffering from indigestion. But as I expected, although almost no whole-community characterizations of faecal microbiota were associated with the selected disorders, potential effects of some individual bacterial species were revealed by the HMSC models.

A higher incidence of weight loss was predicted by the HMSC predictions under the conditions of increased abundances of *Clostridium* and *Escherichia/Shigella*, and decreased abundances of *Leuconostoc* and *Weisella* in faeces. Interestingly, *Leuconostoc mesenteroides* and several species of *Weisella* are used in the food industry (for the production of sourdough or kimchi), and they exhibit properties beneficial for human health – for example, they produce bacteriocins that inhibit the growth of *Listeria* or enteropathogenic strains of *E. coli*. For this reason, they have been proposed as probiotics for humans (K. W. Lee et al. 2012; de Paula et al. 2015; Vogel et al. 2011). On the other hand, the two bacterial genera positively associated with the increased incidence of weight loss (*Escherichia/Shigella* and *Clostridium*) both include pathogenic species. As I have mentioned in the Introduction, while some species of *Escherichia/Shigella* are highly abundant in healthy parrot individuals, others can cause diarrhoea and even mortality (West et al. 2023; Schremmer et al. 1999; Seeley et al. 2014). Similarly, the genus *Clostridium* is part of the healthy gut microbiota of parrots, passerines, and poultry (H. Liu et al. 2019; Kropáčková et al. 2017a; Wei, Morrison, and Yu 2013), but one species, *Clostridium perfringens* is one of the main gastrointestinal pathogens of birds, including parrots (de Santi et al. 2020).

Anorexia was connected to increased abundances of *Kocuria* and *Streptococcus* in faecal microbiota. As stated above, the genus *Streptococcus* includes both pathogenic and commensal species (sometimes used in probiotics). Several species of *Kocuria* (Gram-positive, order Actinomycetales) have been identified as human pathogens, causing peritonitis mainly in immunocompromised individuals (Purty et al. 2013). *Kocuria rosea* is a keratinolytic bacterium causing the degradation of feathers in poultry (Bernal et al. 2003; Coello, Vidal, and Bretana 2000). Surprisingly, feather plucking was predicted to have a higher incidence under the conditions of increased abundances of the bacterial species that were dominant in the faecal microbiota of the healthy group – *Globicatella*, *Lactobacillus*, and *Streptococcus*, and this was not accompanied by any significant decrease in alpha diversity. As I have already mentioned in the context of the core microbiota, *Lactobacillus* was the most abundant bacterial genus detected in parrot faeces, present in the majority of individuals across various parrot species in our dataset, and it was represented by 67 OTUs. Its abundance was significantly higher in the healthy group compared to the group including individuals suffering from any kind of the studied digestive, metabolic, or behavioural disorders. Therefore, its association with feather plucking was surprising. This could be caused by the fact that the diversity of *Lactobacillus* species is very high and individual species can have different impacts on the host physiology, as has been shown in poultry-focused research (e.g., Torok et al. 2011; Yan et al. 2017). Unfortunately, the methods used in this thesis did not provide sufficient resolution to distinguish between individual species – most sequences were assigned only on the level of bacterial genera, but

not on the species level. Therefore, I can only hypothesize that there were probably differences in the species composition of lactobacilli between the healthy and feather plucking individuals. It is rather probable that lactobacilli play some role in this behavioural disorder, as oral administration of *Lactobacillus rhamnosus* was shown to reduce feather pecking in hens (C. Huang et al. 2023). For this reason, it would be very interesting to focus more closely on the species diversity of this bacterium in the context of feather plucking in parrots, by means of methods allowing higher resolution than 16S rRNA metabarcoding (e.g., shotgun metagenomic sequencing or qPCR). This is also applicable to *Globicatella*, which was the only bacterium with increased abundances in both sample types of the healthy group. However, its simultaneous association with feather plucking suggests that this genus may include species with diverse effects on host health as well. To my knowledge, the only study that revealed some information about the relationship between *Globicatella* and birds has been published by Song et al. (2022), who described an association between the abundance of this bacterium in the ileum of broilers and increased activity of the mucosal immune system. Some species of *Globicatella* are pathogenic in humans and livestock (Vela et al. 2000; Miller et al. 2017), however, its effects on the physiology and health of parrots might be different.

Regarding the associations between oral microbiota and the studied disorders, apathy was predicted to have a higher incidence under the conditions of increased abundances of *Lactobacillus* that was otherwise linked to the healthy group, similar to feather plucking in the context of faecal microbiota. Furthermore, apathy was associated with increased abundances of other unassigned genera of Lactobacillales and decreased abundances of *Suttonella*. Interestingly, the decreased abundance of *Suttonella* in oral microbiota was also connected to increased incidences of anorexia and nausea. On the other hand, one species of this genus, *Suttonella ornithocola* is a recently discovered pathogen, causing necrotic pneumonia in European tits (Paridae) (Lawson et al. 2011; Merbach et al. 2019). However, it is possible that this genus includes both commensal and pathogenic species, as is known for the genus *Streptococcus* and others.

Weight loss was significantly associated with the decreased abundance of *Moraxella* (Gram-negative, class Gammaproteobacteria, order Pseudomonadales) in oral microbiota. While nothing is known about the ecology of *Moraxella* in the gut microbiota of birds, some species were shown to cause conjunctivitis in sheep and cattle, or respiratory infections in humans (Vidakovics and Riesbeck 2009; LaCroce et al. 2019). And lastly, feather plucking was associated with the decreased abundance of an unassigned genus of the class Gracilibacteria, which is a group of bacteria discovered from the deep-sea sediments, known almost exclusively from the genomes obtained using metagenomics and other modern cultivation-independent methods (Li, Kato, and Horikoshi 1999; Sieber et al. 2019).

To summarize our findings concerning the associations between gut microbiota and digestive, metabolic, and behavioural disorders, the incidence varied between different parrot genera and could be potentially influenced by the type of housing or nursing, suggesting genetic and environmental effects. Importantly, these variables could influence the development of the studied disorders through gut microbiota, and the microbiota-gut-brain axis, as several associations were found between microbiota and the incidences of the selected disorders. Namely, we detected decreased alpha diversity of oral microbiota in individuals suffering from any of the disorders, and a potential role of *Lactobacillus*, *Suttonella*, *Globicatella*, *Streptococcus*, and other bacterial genera.

5.4. Probiotic bacteria

The third and last aim of this thesis was to compare the composition of commercial probiotics for parrots with the composition of gut microbiota of healthy parrots. I hypothesized that some of the bacterial taxa that are used in probiotics for parrots will probably not be present in faecal or oral samples of healthy parrots, because the products for parrots often contain the same bacterial species as probiotics for poultry or humans. Therefore, it is probable that these products are not designed specifically for parrots. To my knowledge, only one study evaluating the effects of probiotic use in parrots has been published so far – Wyss et al. (2009) described an increased ratio of Gram-positive rods to cocci in faecal samples of individuals of three Macaw species, after the administration of *Lactobacillus salivarius*.

In this thesis, the metabarcoding of the eight selected probiotics for parrots brought very unexpected results. According to our analysis of probiotic content, only a single product demonstrated a precise match between its actual and declared content. In two other cases, the declared bacterial taxa formed 80 – 90% of the detected sequences. The remaining five products had considerably different content than what had been declared by the producer. The presence of non-declared bacterial taxa in low abundances could be explained by environmental contamination during DNA extraction, as this was done outside of the laminar flowbox due to lab safety reasons. However, even if this was the case, declared bacterial taxa should still be detected in substantially higher abundances. Surprisingly, at least some of the declared bacteria were not detected at all in four out of eight probiotic products.

Sequences obtained by metabarcoding of the selected probiotics most frequently belonged to the genera *Lactobacillus*, *Bacillus*, *Streptococcus*, *Prevotella*, and *Pseudomonas*, all of these genera being part of the gut microbiota of healthy parrots. However, 13 out of 36 OTUs detected in probiotics were not detected in microbiota of any of the healthy parrots in our dataset. As I have already mentioned, the effects of *Lactobacillus* and *Streptococcus* on host physiology and health substantially

vary among different species of these bacterial genera, and the effects can also be host-specific. Therefore, the selection of the species and strain for use in probiotics should always be preceded by experimental verification of its effects on the specific recipient.

The second most commonly detected bacterial genus in probiotics for parrots was *Bacillus* (Gram-positive, order Bacilli). It has been shown that probiotics containing *B. subtilis* or *B. amyloliquefaciens* can reduce stress behaviour and aggression or increase the feeding frequency in poultry under normal or heat-stress conditions, which suggests that *Bacillus* can influence the host behaviour through the gut-brain-axis (W. C. Wang et al. 2018; Abdel Azeem 2013). However, bacteria of this genus were not associated with the incidence of any of the disorders tested in the HMSC analysis in this thesis.

In conclusion, the metabarcoding of the eight selected probiotics for parrots revealed substantial differences between the declared and actual content of most of the products. Approximately one-third of the OTUs detected in probiotics were not found in the oral or faecal microbiota of healthy parrots. The HMSC analysis revealed some positive associations between host health and several bacterial taxa that have not yet been used in commercial probiotics for parrots.

6. Summary

Captive parrots can suffer from a variety of digestive, metabolic, and behavioural disorders that can be associated with the gut microbiota. Although many available probiotic products for parrots proclaim beneficial effects on digestion and sometimes also mental well-being, the publicly available scientific evidence of microbiota-gut-brain interactions in parrots is scarce, and the current knowledge is primarily based on the extrapolation of the results of poultry or mammals-focused research. This thesis contributed to the present knowledge on the gut microbiota of parrots, the factors influencing its composition and diversity, and its association with digestive, metabolic, and behavioural disorders. We analysed the microbial content of oral and faecal samples from 491 individuals of 85 parrot species by means of 16S rRNA metabarcoding. We assessed the interspecific and intraspecific variation in composition and diversity of microbiota, and we linked this variation to the incidence of eight selected digestive, metabolic, and behavioural disorders (apathy, stereotype behaviour, anorexia, feather plucking, indigestion, nausea, obesity, and weight loss). We observed high interspecific and intraspecific variation in gut microbiota composition of parrots. The main factors influencing the composition and diversity of faecal microbiota were the host species, type of housing environment, and feed. Oral microbiota was mainly influenced by the host species, sex, feed, type of housing, and the day of sampling, suggesting higher seasonal variation compared to faecal bacteria. We also identified the core oral and faecal microbiotas of parrots, which were formed by bacterial genera commonly found also in other bird and mammalian hosts, such as *Lactobacillus*, *Corynebacterium*, *Streptococcus*, or genera belonging to Pasteurellaceae. All selected disorders were found to be characterized by decreased alpha diversity of oral microbiota. Hierarchical modelling of species communities revealed a potential role of *Lactobacillus*, *Suttonella*, *Globicatella*, *Streptococcus*, and other bacterial genera in the development of the studied disorders. Notably, substantial differences among the effects of specific species of these genera on the physiology and health of the host were proposed. Analysis of the content of probiotic products for parrots revealed considerable differences between the declared and the actual content of most of them. Approximately one-third of the OTUs detected in these products were not found in oral or faecal microbiota of healthy parrots. Therefore, our findings support the assumption that the composition of probiotic products for parrots does not take into consideration the natural composition of the gut microbiota of healthy parrots. Given the differences in GIT morphology and gut microbiota composition of parrots and other animals, and the host species-specific impacts of many bacterial species, treating health issues in parrots with probiotic bacteria that showed positive effects on health in poultry, mammals, and humans, does not appear fully appropriate. Unfortunately, the 16S rRNA metabarcoding did not provide a sufficient resolution to assign most of the obtained sequences on the species level. This greatly limits our understanding of

the species-specific effects of the studied bacterial genera on host health and the development of the studied disorders. Our results show that further research is needed, to disentangle the complex relationships between genetic and environmental factors, gut microbiota, and digestive, metabolic, and behavioural disorders. Application of methods allowing higher resolution than 16S rRNA metabarcoding, like shotgun metagenomic sequencing or qPCR, will be necessary to fill the described gaps in our current knowledge. Moreover, the results of correlational research such as this have to be verified experimentally. To my knowledge, almost no manipulative research focused on the gut microbiota of parrots has been conducted so far. However, such experiments are necessary to test for the suggested positive effects of the bacteria with the probiotic potential and to identify the ideal dosage and way of administration that would lead to successful colonization of parrot the gut by these species. It would also be interesting to compare the gut microbiota of wild and captive individuals of parrots to test whether the frequent development of behavioural disorders in captive parrots could be associated with the shifts in gut microbiota composition connected to domestication. In that case, probiotics mimicking wild parrot microbial composition could help to prevent and/or cure some of these disorders.

7. List of abbreviations

ASV	Amplicon sequence variant
CFU	Colony forming units
Df	Degree of freedom
DNA	Deoxyribonucleic acid
ENS	Enteric nervous system
FDB	Feather damaging behaviour
GIT	Gastrointestinal tract
HMSC	Hierarchical modelling of species communities
Lmer	Linear mixed-effects regression model
mRNA	Messenger ribonucleic acid
NLR	NOD-like receptor
OTU	Operational taxonomic unit
p	p-value
PGLYRP	Peptidoglycan receptor
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
qPCR	Quantitative polymerase chain reaction
R ²	Coefficient of determination
RDA	Redundancy analysis
RDP	Ribosomal database project
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acid
TLR	Toll-like receptor
TTM	Trichotillomania

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