The bifidobacterial distribution in the microbiome of captive primates reflects parvorder and feed specialization of the host

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Bifidobacteria, which commonly inhabit the primate gut, are beneficial contributors to host wellbeing. Anatomical differences and natural habitat allow an arrangement of primates into two main parvorders; New World monkeys (NWM) and Old World monkeys (OWM). The number of newly described bifidobacterial species is clearly elevated in NWM. This corresponds to our finding that bifidobacteria were the dominant group of cultivated gut anaerobes in NWM, while their numbers halved in OWM and were often replaced by Clostridiaceae with sarcina morphology. We examined an extended MALDI-TOF MS database as a potential identification tool for rapid screening of bifidobacterial distribution in captive primates. Bifidobacterial isolates of NWM were assigned mainly to species of primate origin, while OWM possessed typically multi-host bifidobacteria. Moreover, bifidobacterial counts reflected the feed specialization of captive primates decreasing from frugivore-insectivores, gummivore-insectivores, frugivore-folivores to frugivore-omnivores. Amplicon sequencing analysis supported this trend with regards to the inverse ratio of Actinobacteria and Firmicutes. In addition, a significantly higher diversity of the bacterial population in OWM was found. The evolution specialization of primates seems to be responsible for Bifidobacterium abundance and species occurrence. Balanced microbiota of captive primates could be supported by optimized prebiotic and probiotic stimulation based on the primate host.

Primates are a remarkably species-rich order of mammals1. Their anatomical differences and natural habitat allow their arrangement into two main parvorders. Platyrrhines, referred as New World monkeys (NWM), naturally occurring in central and southern American tropical and subtropical regions and catarrhines (Cercopithecoida and Hominoida), referred as Old World monkeys (OWM), coming from tropical, subtropical, and temperate regions of Asia and Africa2. Many primate species are endangered3 and they must be protected. The conservation of threatened species is a complex and demanding process consisting of elaborated breeding programs and providing of habitat sanctuaries in captive or semi-captive centres, e.g. zoological institutions or forest corridors, which usually aim to reintroduce these species back into their natural habitat4,5. Unfortunately, health of captive animals is compromised by emerging recurring infectious diseases mediated through human contact and habitat modifications, and frequent therapeutic doses of antibiotics6,7. Furthermore, captive breeding modifies primate microbiome8,9 and these microbial shifts can substantially affect the host's health10,11. Captivity may be also associated with the occurrence of potential pathogens that further increase risk of gut dysbiosis and illnesses12,13.

Besides exposure to antibiotics, dietary changes and lifestyle seem to be significant modifiers of primate gut microbiome14. To provide nutritional needs, primates consume a wide range of plants and animal tissues and possess a variety of dietary specializations based on the proportion of individual dietary components (one type of feed component is dominating only), such as generalist feeders or omnivores, e.g. Cercopithecines15–18.
The generalist feeders are adapted to receive a wide variety of feed components, depending on their availability in the environment, and can be split by extension into groups classified by their majority feeds, with seasonal variation in their ratio. Among the generalist feeders, there are highly frugivorous representatives, namely chimpanzees9–23. If there is a lack of fruit, these primates consume various feed reaching from plants, nectar, seeds to insects or small vertebrates. Such a feeding type can be described as frugivore-omnivore. If the preferred fruit is less available during the season, primates start to consume more leaves or other parts of plants. Gibbons, for instance pursue this frugivore-folivore feeding strategy22–25. Similarly, if the second major component alongside fruit consists of insects, primates are classified as frugivores-insectivores (tamars)36–38. Exudates are another important nutritious feed apart from fruit and animal prey. Some primate species have specially adapted teeth for gum intake31,32. This type of feeding behaviour is called gummivory. It is typical for marmosets and can either be dominant or it can be supplemented with insect intake33–37. These primates are counted in the gummivore-insectivore feeding category.

Unfortunately, despite all efforts of breeders, composition of diet in captivity does not completely simulate that in the wild, in which primates consume a wider range of natural local plant and animal species9,38. In addition, Amato et al.39 points out the seasonality that is one of the natural phenomena of wild primate diet, which results in a seasonal variation of the gut microbiome.

Deviation from the natural lifestyle in captivity and associated modified diet led to a shift of native gut microbiota and a decrease in diversity and an increased relative abundance of Bacteroidetes9,39,40,41. Furthermore, the microbiome of captive primates displays a reduction in Actinobacteria compared to wild groups14,41. However, members of the Bifidobacteriaceae family (Actinobacteria phylum) are important natural commensals, which possess a large amount of adaptive genes involved in carbohydrate metabolisms12–44. Moreover, bifidobacteria can utilize a diverse range of dietary carbohydrates that escape degradation in the upper parts of the intestine45. Although, bifidobacterial abundance in the gut microbiota usually decreases with host aging46, bifidobacteria persist throughout the lifespan of primates42,47. Moreover, their abundance is confirmed by a recent boom of novel bifidobacterial species isolation and characterization connected to primate gut environment48–50.

However, data are still scarce about the bifidobacterial microbiota of captive primates and the impact of different diets. We hypothesize that the quantity and species richness of bifidobacteria in captive primates are affected by the host and feed classification. The aim of this study was to compare the quantity and diversity of bifidobacteria in faecal microbiota of captive NWM and OWM by a combination of culture-dependent and culture-independent approaches.

**Results**

**Cultivation analysis.** *Quantification of cultivable bifidobacteria in primate faecal samples.* Non-selective and selective media were used for the quantification of anaerobic bacteria and bifidobacteria in primate faecal samples (FS) (Table 1). Cultivation counts significantly varied between the NWM and the OWM in each monitored group of bacteria (Fig. 1A, Suppl. Tab. 1). NWM harboured significantly more anaerobic bacteria (9.52 ± 0.62 log CFU g⁻¹) compared to OWM (8.62 ± 0.71 log CFU g⁻¹) (t(50) = 4.84, p = 1.30e-05). A similar statistically significant trend was found in colony forming units cultivated on WPS-MUP medium intended for bifidobacteria and a decrease in diversity and an increased relative abundance of Bacteroidetes8,9,40,41. Furthermore, the bifidobacterial species isolation and characterization connected to primate gut environment48–50.

Medium was not sufficiently selective also allowing the growth of clostridia51,52. Consequently, a notably greater number of bacteria (Fig. 1A, Suppl. Tab. 1). NWM harboured significantly more anaerobic bacteria (9.52 ± 0.62 log CFU g⁻¹) compared to OWM (8.62 ± 0.71 log CFU g⁻¹) (t(50) = 4.84, p = 1.30e-05). A similar statistically significant trend was found on WPS-MUP in gummivore-insectivores (9.63 ± 0.71 log CFU g⁻¹) and frugivore-insectivores (9.46 ± 0.57 log CFU g⁻¹) exhibited significantly higher numbers of anaerobic bacteria including bifidobacteria than frugivore-omnivores (8.72 ± 0.49 log CFU g⁻¹) and frugivore-omnivores (8.60 ± 0.78 log CFU g⁻¹). The same statistically significant trend was found on WPS-MUP in gummivore-insectivores (8.99 ± 1.19 log CFU g⁻¹) and frugivore-insectivores (9.19 ± 0.96 log CFU g⁻¹) in comparison with frugivore-omnivores (6.58 ± 1.05 log CFU g⁻¹) and frugivore-omnivores (7.07 ± 0.93 log CFU g⁻¹) in the NWM (t(50) = 5.17, p = 2.38e-07). Cultivation differences between parvorders were also reflected within the primate sub-division based on feed specialization (Fig. 1B). Specifically, gummivore-insectivores (9.63 ± 0.71 log CFU g⁻¹) and frugivore-insectivores (9.46 ± 0.57 log CFU g⁻¹) from bifidobacterial selective media were isolated for further identifications (Suppl. Tab. 1). From a total of 326 bifidobacterial species detected by MALDI‑TOF MS.

**Bifidobacterial species detected by MALDI-TOF MS.** Bacterial colonies with variable cultivation characteristics from bifidobacterial selective media were isolated for further identifications (Suppl. Tab. 1). From a total of 326 isolates, 210 were F6PPK-positive bifidobacteria and the remaining 116 isolates (isolated mainly from WSP-MUP) were F6PPK-negative gas producing clostridial rods or cells with sarcina morphology. All F6PPK-positive strains were also identified with MALDI-TOF MS using an expanded custom database for bifidobacterial identification. 54% of the strains (n = 112) were assigned to 18 different bifidobacterial species, 36% (n = 76) were assigned only to the Bifidobacterium genus, and 11% (n = 22) were not identified reliably (Fig. 2A, C).

*B. parmae, B. imperatoris/saguini, and B. ramosum were the most frequently identified species in the NWM, whereas B. dentium and B. catenulatum/pseudocatenulatum were most common in the OWM. Interestingly, B. adolescentis was equally represented in both primate parvorders. A more diverse species representation of bifidobacteria was found in the NWM (14 spp.) compared to the OWM (5 spp.). Genus-level assignment and the presence of not reliable identifications (NRI) was mainly detected in the NWM. Related presumed species compliance and the closest match of Bifidobacterium spp. strains was found predominantly with B. parmae and B. stellarboschense in the NWM, and B. angulatum/merycicum in the OWM (Fig. 2B).
| ID   | Primate host species                      | Family            | Parvorder | Zoo     | Feed category          |
|------|------------------------------------------|-------------------|-----------|---------|------------------------|
| PR1  | Common Marmoset (Callithrix jacchus)     | Cebidae           | New World Monkeys | Bojnice, SK | Frugivore-omnivore     |
| PR2  | Common Marmoset (Callithrix jacchus)     | Cebidae           | New World Monkeys | Pilsen, CZ | Gummivore-insectivore |
| PR3  | White-faced Saki (Pithecia pithecia)     | Hominidae         | New World Monkeys | Pilsen, CZ | Frugivore-omnivore     |
| PR4  | Emperor Tamarin (Saguinus imperator)     | Cebidae           | New World Monkeys | Pilsen, CZ | Gummivore-insectivore |
| PR5  | Moustached Tamarin (Saguinus mystax)     | Cebidae           | New World Monkeys | Pilsen, CZ | Gummivore-insectivore |
| PR6  | Brown-maned Tamarin (Saguinus fuscicollis) | Cebidae       | New World Monkeys | Pilsen, CZ | Frugivore-insectivore |
| PR7  | Red-handed Tamarin (Saguinus midas)      | Cebidae           | New World Monkeys | Pilsen, CZ | Frugivore-insectivore |
| PR8  | Red-handed Tamarin (Saguinus midas)      | Cebidae           | New World Monkeys | Pilsen, CZ | Frugivore-insectivore |
| PR9  | Emperor Tamarin (Saguinus imperator)     | Cebidae           | New World Monkeys | Pilsen, CZ | Frugivore-insectivore |
| PR10 | Silvery Marmoset (Mico argentatus)       | Cebidae           | New World Monkeys | Pilsen, CZ | Gummivore-insectivore |
| PR11 | Silvery Marmoset (Mico argentatus)       | Cebidae           | New World Monkeys | Pilsen, CZ | Gummivore-insectivore |
| PR15 | Silvery Marmoset (Mico argentatus)       | Cebidae           | New World Monkeys | Pilsen, CZ | Gummivore-insectivore |
| PR16 | Emperor Tamarin (Saguinus imperator)     | Cebidae           | New World Monkeys | Pilsen, CZ | Frugivore-insectivore |
| PR17 | Emperor Tamarin (Saguinus imperator)     | Cebidae           | New World Monkeys | Pilsen, CZ | Frugivore-insectivore |
| PR18 | Chimpanzee (Pan troglodytes)             | Hominoidea        | Old World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR19 | Northern White-cheeked Gibbon (Nomascus leucogenys) | Hylidae           | Old World Monkeys | Liberec, CZ | Frugivore-folivore     |
| PR20 | Golden-bellied Mangabey (Cercocebus chrysogaster) | Cercopithecidae   | New World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR21 | Diana Monkey (Cercopithecus diurnus)     | Cercopithecidae   | New World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR22 | Lion-tailed Macaque (Macaca silenus)     | Cercopithecidae   | New World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR23 | Hamadryas Baboon (Papio hamadryas)       | Cercopithecidae   | New World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR24 | Pygmy Marmoset (Cebuella pygmaea)        | Cebidae           | New World Monkeys | Liberec, CZ | Gummivore-insectivore |
| PR26 | Cotton-top Tamarin (Saguinus oedipus)    | Cebidae           | New World Monkeys | Liberec, CZ | Frugivore-insectivore |
| PR27 | Golden Lion Tamarin (Leontopithecus rufus) | Cebidae       | New World Monkeys | Liberec, CZ | Frugivore-insectivore |
| PR28 | Common Marmoset (Callithrix jacchus)     | Cebidae           | New World Monkeys | Olomouc, CZ | Gummivore-insectivore |
| PR29 | Patas Monkey (Erythrocebus patas)        | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR30 | Goeldi’s Marmoset (Callimico goeldii)    | Cebidae           | New World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR31 | White-headed Marmoset (Callithrix geoffroyi) | Cebidae       | New World Monkeys | Olomouc, CZ | Gummivore-insectivore |
| PR32 | White-headed Marmoset (Callithrix geoffroyi) | Cebidae       | New World Monkeys | Olomouc, CZ | Gummivore-insectivore |
| PR33 | Moustached Tamarin (Saguinus mystax)     | Cebidae           | New World Monkeys | Olomouc, CZ | Frugivore-insectivore |
| PR34 | Patas Monkey (Erythrocebus patas)        | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR35 | Silvery Marmoset (Mico argentatus)       | Cebidae           | New World Monkeys | Olomouc, CZ | Gummivore-insectivore |
| PR36 | Campbell’s Mona Monkey (Cercopithecus campbelli) | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR37 | Putty-nosed Monkey (Cercopithecus nictitans) | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR38 | Northern Talapoin Monkey (Miopithecus ogouensis) | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR39 | De Brazza’s Monkey (Cercopithecus neglectus) | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR40 | Northern White-cheeked Gibbon (Nomascus leucogenys) | Hylidae           | Old World Monkeys | Liberec, CZ | Frugivore-folivore     |
| PR41 | Chimpanzee (Pan troglodytes)             | Hominoidea        | Old World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR42 | Chimpanzee (Pan troglodytes)             | Hominoidea        | Old World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR43 | Chimpanzee (Pan troglodytes)             | Hominoidea        | Old World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR44 | Chimpanzee (Pan troglodytes)             | Hominoidea        | Old World Monkeys | Liberec, CZ | Frugivore-omnivore     |
| PR45 | Patas Monkey (Erythrocebus patas)        | Cercopithecidae   | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR46 | Southern Yellow-cheeked Gibbon (Nomascus gabriellae) | Hylidae           | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR47 | Southern Yellow-cheeked Gibbon (Nomascus gabriellae) | Hylidae           | Old World Monkeys | Olomouc, CZ | Frugivore-omnivore     |
| PR51 | Southern Yellow-cheeked Gibbon (Nomascus gabriellae) | Hylidae           | Old World Monkeys | Bratislava, SK | Frugivore-folivore     |
| PR52 | Green Monkey (Chlorocebus sabaeus)       | Cercopithecidae   | Old World Monkeys | Hodonin, CZ | Frugivore-omnivore     |
| PR55 | Hamlyn’s Monkey (Cercopithecus hamlyni)  | Cercopithecidae   | Old World Monkeys | Bojnice, SK | Frugivore-omnivore     |
| PR56 | Roloway Monkey (Cercopithecus roloway)   | Cercopithecidae   | Old World Monkeys | Bojnice, SK | Frugivore-omnivore     |
| PR57 | Lesser Spot-nosed Monkey (Cercopithecus petaurista) | Cercopithecidae   | Old World Monkeys | Bojnice, SK | Frugivore-omnivore     |
| PR58 | Southern Yellow-cheeked Gibbon (Nomascus gabriellae) | Hylidae           | Old World Monkeys | Bojnice, SK | Frugivore-folivore     |

Continued
Species assignment verification by 16S rRNA gene sequencing. The MALDI-TOF MS identification was verified by 16S rRNA gene Sanger sequencing of 46 strains, whose selection was randomly executed based on determined species frequency and identification scores (Suppl. Tab. 2). Due to similar MALDI-TOF MS spectra, some bifidobacterial species could not be distinguished. However, the results consistently suggest an assignment to either of the two indistinguishable species. These indistinguishable groups were merged to produce consistent

| ID | Primate host species                                      | Family       | Parvorder | Zoo             | Feed category           |
|----|----------------------------------------------------------|--------------|-----------|-----------------|-------------------------|
| PR59 | Northern White-cheeked Gibbon (**Nomascus leucogenys**) | Hylobatidae  | OWM       | Liberec, CZ     | Frugivore-folivore      |
| PR60 | Northern White-cheeked Gibbon (**Nomascus leucogenys**) | Hylobatidae  | OWM       | Liberec, CZ     | Frugivore-folivore      |
| PR61 | Golden Lion Tamarin (**Leontopithecus rosalia**)       | Calitrichidae| NWM       | Olomouc, CZ     | Frugivore-insectivore    |

Table 1. List of monkey hosts kept in zoological gardens. General information about primate taxonomy, parvorder and feed classification. Primate general feeders (n = 52) were grouped to 4 individual feed categories based on proportion of dominating feed components – frugivore-omnivore, frugivore-folivore, frugivore-insectivore, and gummivore-insectivore. Zoo, zoological garden; CZ, Czechia; SK, Slovakia; NWM, New World monkey; OWM, Old World monkey.

Figure 1. Quantification of cultivable anaerobic bacteria (log CFU g⁻¹) in primate faecal samples. (A) Cultivation counts of bacteria per parvorder: New World monkeys (n = 24) and Old World monkeys (n = 28). (B) Cultivation counts of bacteria per feed category: frugivore-folivore (n = 8), frugivore-omnivore (n = 21), frugivore-insectivore (n = 13), gummivore-insectivore (n = 10). Asterisks (*) denote statistically significant differences as determined by t-test and ANOVA (p < 0.05).
MALDI-TOF MS assignment and are presented together in the following groups: B. angulatum/merycicum, B. breve/indicum, B. catenulatum/pseudocatenulatum, and B. imperatoris/saguini.

An agreement between the MALDI-TOF MS species assignment and the sequencing of 16S rRNA gene was confirmed for 38 strains. Only 3 strains were identified differently by the two methods. Namely, strain N127 identified as B. faecale by 16S rRNA gene sequencing was mistaken for B. adolescentis by the MALDI-TOF MS, B. imperatoris for NRI (N40), and PEBJ_s for B. imperatoris/saguini (N50). Interestingly, mentioned strain N50 together with N74, N94, N97, and N115, exhibiting MALDI-TOF MS NRI score (< 1.69), were considered potential novel species of bifidobacteria. In addition, this sample set also contained 5 problematic strains (N16, N70, N81, N119, and N125), whose 16S rRNA gene sequencing failed repeatedly and thus their MALDI-TOF MS identity was not confirmed.

Amplicon sequencing analysis. Amplicon sequencing profiles of the FS collected from captive primates were determined by sequencing the V4 region of the 16S rRNA gene. The bacterial α-diversity was expressed as an ASV count, Shannon diversity, and Pielou evenness. Each diversity parameter between the primate parvorders was significantly higher in the OWM (ASV count: F(1,50) = 30.47, p = 1.21 × 10–6, η2 = 0.379, Shannon: F(1,50) = 38.01, p = 1.21 × 10–6, η2 = 0.432, Pielou: F(1,50) = 38.41, p = 1.08e–07, η2 = 0.434) (Fig. 3A). Similarly, there was a significantly higher diversity, evenness, and richness of the bacterial population in the frugivore-folivores and frugivore-omnivores compared to the frugivore-insectivores and gummivore-insectivores (Fig. 3B, Supplementary S1).

Microbial community shifts were found between the NWM and OWM parvorders. The relative abundance of phylum Actinobacteriota (W = 13) and Campylobacterota (W = 12) was significantly higher in the NWM.
compared to the OWM as confirmed by the ANCOM statistics. Meanwhile, the phylum Firmicutes showed an opposite trend, which was however not statistically significant (Fig. 4A). The difference in the Actinobacteriota can be attributed specifically to the family Bifidobacteriaceae which was significantly higher in the NWM (16%) compared to the OWM (3%) (W = 139) (Fig. 4B, Supplementary S2). These findings corroborate the cultivation results.

The proportion of the phylum Actinobacteriota was statistically significantly different among the primate feed categories (W = 12) and it was the highest in the frugivore-insectivores followed by gummivore-insectivores, frugivore-omnivores, and frugivore-folivores. Furthermore, the phyla Proteobacteria and Campylobacterota were statistically significantly different among the categories (W = 8, W = 7 respectively) with a notable enrichment of both in the frugivore-insectivores followed by gummivore-insectivores compared to frugivore-omnivores and frugivore-folivores. Moreover, although not statistically significant, the opposite ratio of Firmicutes was also detected (Fig. 4C). The relative abundance of Bifidobacteriaceae was significantly different across the categories (W = 140); the most abundant in the frugivore-insectivores (19%), followed by the gummivore-insectivores (12%), the frugivore-omnivores (4%), and the frugivore-folivores (2%) (Fig. 4D).

By comparing 16S rRNA gene sequencing data of cultured bifidobacterial isolates with the results of 16S rRNA gene amplicon sequencing of the FS, we retrospectively confirmed the presence of 18 species within this sample set. B. callitrichos and B. parmae were significantly enriched in the NWM (W = 38, W = 38 respectively), followed by B. saquini (W = 34), B. biavatii (W = 34), B. vansnderenii (W = 34), B. aerophilum (W = 34), unclassified II ASV (W = 33) and sp. I ASV (W = 30). The distribution of bifidobacteria corresponds to the proportion of Bifidobacteriaceae among the total relative bacteria in samples normalized to 42 134 sequences/sample in the primate feed categories as determined by amplicon sequencing.
Discussion

Dynamic microbial communities aid the living and surviving of animals in changing environmental conditions, including habitat degradation, captive breeding, and diet. If microbial balance of the host is disturbed and dysbiosis occurs, there is a presumption of disease development\(^5\)\(^,\)\(^53\)\(^,\)\(^54\). Among others, commensal microorganisms, such as bifidobacteria, play a crucial role in maintaining the gut homeostasis\(^55\)\(^–\)\(^57\). Bifidobacterial diversity and adaptation are connected to their hosts and environments with possession of specific genomic traits\(^58\)\(^–\)\(^60\) which includes primates\(^42\).

Two independent approaches, cultivation with subsequent MALDI-TOF MS identification and amplicon sequencing of the V4 region of the 16S rRNA gene, were used to analyse the microbiome composition and the prevalence of bifidobacterial species in primate gut microbiota. NWM are a significant source of cultivable bifidobacteria with average counts of 10^8 CFU g^-1 of faeces compared to the OWM with four orders of magnitude lower counts. Interestingly, although no health complications were evident, FS of primate individuals with reduced or undetectable cultivation counts of bifidobacteria contained Clostridiaceae, mainly displaying sarcina morphology. This was mainly observed in individuals belonging to the OWM parvorder (Suppl. Tab. 1). Spore-forming bacteria identified as Sarcina ventriculi (syn. Clostridium ventriculi) were previously isolated also from primates without apparent health problems\(^61\)\(^–\)\(^63\). Although they are considered pathogens\(^64\), this may indicate sarcina as common bacteria of the primate gut microbiota. In the gut of NWM, the abundance of sarcina is probably decreased by the presence of bifidobacteria, which exhibit potential to hamper growth of clostridia\(^65\)\(^–\)\(^67\). The inverse ratio and balancing of the bifidobacteria and clostridia are typically described in the gut microbiome of infants\(^68\)\(^–\)\(^70\).

Figure 4. Relative abundance of primate gut microbiota. (A) Relative abundance of bacteria within parvorders on phylum level. (B) Relative abundance of bacteria within parvorders on family level. (C) Relative abundance of bacteria within feed categories on phylum level. (D) Relative abundance of bacteria within feed categories on family level. ANCOM statistically significant differences are denoted with grey links.
Timperio et al. showed that the screening of bacterial isolates from environmental samples can be performed efficiently, quickly, and inexpensively using MALDI-TOF MS and should be refined by implementation of environmental strains into the database. Within our study, the use of an extended custom database for MALDI-TOF MS allowed reliable species differentiation and identification of wild bifidobacterial isolates. Higher species diversity was observed in NWM. Interestingly, the multi-host species B. adolescentis was present among most screened captive primates. In OWM B. dentium and B. catenulum/pseudocatenulatum, that are common species of the human gut microbiota, as well as B. adolescentis, were found. Lugli et al. detected B. adolescentis and B. dentium in OWM as well, and indicated possible joint development and evolutionary relatedness. In contrast, NWM exhibited the presence of cultivable bifidobacteria mainly with primate origin. Interestingly, Brown et al. pointed out that marmoset bifidobacteria are closely related to those in tamarins. Furthermore, we found that bifidobacterial species variability in NWM significantly exceeds that in OWM. Furthermore, we hereby confirmed that we can re-isolate recently described primate Bifidobacterium spp. also from primate species with various captive locations other than those from which bifidobacteria were originally isolated.

Moreover, MALDI-TOF MS screening allowed us to identify 5 potential novel species of bifidobacteria isolated from tamarins that were confirmed by 16S rRNA gene sequencing. That indicates primate gut as a promising environment for the discovery of novel species of bifidobacteria. To achieve an accurate identification of potential novel species, a combination with other methods, such as sequencing of phylogenetic markers, multi- locus sequence typing, and genome sequencing, should be included.

The significantly lower species richness and high relative abundance of bifidobacteria in NWM compared to OWM was confirmed by sequencing of the V4 region of 16S rRNA gene. The relative abundance of Bifidobacteriaceae reached 16% in the NWM and only 3% in the OWM. The same trend was also detected for Prevotellaceae and Veillonellaceae. In particular, marmosets and tamarins exhibited 32% bifidobacterial abundance compared to wild marmosets in the OWM. This high relative bifidobacterial proportion in adult marmosets could be a consequence of their housing as family groups and their constant subjection to the gut microbiota of other individuals. Conversely, Lachnospiraceae, Oscillatoriaceae, Ruminococcaceae, and Spirochaetaceae showed an opposite trend with high abundances in OWM. Interestingly, we showed that the captive NWM have high relative levels of bifidobacteria, which is similar to what they display in the wild. It indicates that NWM gut is a rich bifidobacterial environment that is also supported by other studies. In contrast to our results in captive individuals, some microbiome studies point to a slightly increased bifidobacterial relative proportions in wild OWM as well. Although the captivity was previously described as a factor influencing the presence of Actinobacteria in the primate gut microbiome, our results suggest that it is probably not as strong as the affiliation to the primate parvorder, which seems to be considerably more significant.

Primate gut microbiome seems to be significantly modified by dietary changes of the host species and geography. Frugivore-insectivores and gummivore-insectivores possessed significantly more abundant Bifidobacteriaceae compared to frugivore-omnivores and frugivore-folivores. Interestingly, if insects constitute an important component of the diet, bifidobacteria are highly abundant. Ecologically beneficial symbionts leading to host evolutionary dependence have been previously described in other animal taxa, such as sap-feeding insects, which generate essential amino acids exclusively for their microbial symbionts. Bifidobacteria are known as a commensal bacterial group of insects with social life, whereas the importance of insects in the diet of primates in relation to bifidobacterial occurrence remains unclear.

Although captive feeding inevitably modifies primate gut microbiome to decreased diversity, the feed optimization could improve the animals health condition. In contrast to Amato et al., who state that the host phylogeny is stronger driver in shifts of microbial composition than the diet and geographic location, our results suggest that both diet and the host itself affect the microbiome composition, especially the relative abundance of Bifidobacteriaceae. Moreover, it is important to mention, that the diet of captive animals usually includes fruits, vegetables, and leaves that may not completely match the available components present in the wild. In addition, the natural microbiota reflects diet seasonality and location that may affect trophic interactions in the gastrointestinal tract of the host.

Clayton et al. confirmed that modified diet in captive primates is related to the alteration of microbiome composition and host health. Captive primate individuals susceptible to health disorders may show clinical signs including chronic diarrhoea, weight loss, lethargy, cardiac disease, and poor reproductive success. Therefore, it is necessary to further monitor the relationship between the microbiome, diet, and the health of captive primates. Microbiota modulation is an effective and affordable strategy for host health support of threatened animals. Therefore, applicable mitigation strategies such as optimized dietary and prebiotic interventions could be pursued towards supporting balanced microbiota in captive primates. Moreover, probiotic supplementation with focus on bifidobacteria, that naturally colonize primate guts, can be a further promising approach. Furthermore, this may provide a potential approach in human probiotic intervention. Due to the ever-decreasing diversity of the human microbiome through diet and antimicrobial intake, the microbiome of originally living evolutionarily close relatives has the potential to design a probiotic that is no longer part of the human microbiota and could have the potential to strengthen health. Probiotic intervention should be optimized according to the gut microbiota composition and should be supported by appropriately selected prebiotic stimulation in symbiotic mixtures for long-term maintenance of balanced microbiome and host health.

Materials and methods
Sampling and cultivation analysis. Faecal samples of primate hosts (n = 52) belonging to two parvorders, NWM (n = 24) and OWM (n = 28), were preliminary screened for quantitative content of cultivable bifidobacteria. The list of primate hosts and classification into parvorders and feed category is shown in Table 1. Sampling was performed in zoological gardens in Dvur Králové, Hodonin, Liberec, Olomouc, Pilsen
(all Czechia), Bojnice, and Bratislava (both Slovakia) between 2017–2019. FS were collected in tubes containing dilution buffer (5 g L⁻¹ tryptone, 5 g L⁻¹ nutrient broth No. 2, 2.5 g L⁻¹ yeast extract (all Oxoid, Basingstoke, UK), 0.5 g L⁻¹ L-cysteine, 1 mL L⁻¹ Tween 80 (both Sigma-Aldrich, St. Louis, Missouri, USA), 30% glycerol (VWR, Radnor, Pennsylvania, USA), and glass pearls for homogenization. Media were prepared in an oxygen-free carbon dioxide environment and then sterilized. After sampling, the tubes were stored at −20 °C and within the 14 days transported into the laboratory for analysis. Then, decimal serial dilutions of FS were spread on the following media.

Wilkins-Chalgren Anaerobe Agar was supplemented with 5 g L⁻¹ GMO-Free Soya Peptone (both Oxoid), 0.5 g L⁻¹ L-cysteine, and 1 mL L⁻¹ Tween 80 to determine total counts of anaerobic bacteria (WSP medium). Moreover, two selective media were used for bifidobacterial quantification and isolation: WSP-NORF (WSP agar supplemented with 100 mg L⁻¹ of mupirocin, 200 mg L⁻¹ of norfloxacin (both Oxoid), and 1 mL L⁻¹ of acetic acid (Sigma-Aldrich)) and WSP-MUP (WSP agar supplemented with 100 mg L⁻¹ of mupirocin and 1 mL L⁻¹ of acetic acid). All plates were incubated anaerobically using GENbag anaer (bioMérieux, Craponne, France) at 37 °C for 2 days.

Isolation and culture identifications. Based on variable cultivation characteristics, the isolation of colonies from selective media and consecutive sub-cultivation was performed in tubes containing WSP broth under anaerobic conditions at 37 °C for 1 day. Whether a culture belonged to Bifidobacterium spp. was verified by fructose-6-phosphate phosphoketolase (F6PPK) test with cetrimonium bromide for cell disruption according to Orban and Patterson (2000) enabling longer reads and thus more precise taxonomic identification. PCR products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, Georgia, USA) and sequenced by Eurofins Genomics (Ebersberg, Germany). The obtained sequences were processed in Chromas Lite 2.5.1 (Technelysium Pty Ltd., Tewantin, Australia), BioEdit with ClustalW algorithm, and compared with 16S rRNA gene sequences in BLAST rRNA/ITS (https://blast.ncbi.nlm.nih.gov/) and EZBioCloud databases (https://www.ezbiocloud.net/). The sequences of the 16S rRNA gene are available in the GenBank database under accession numbers MN736337–341, 342, 344–346, 348, 350–355, 357–360, 363–365, 367, 369, 372–378, 381, 387–388, 390–392, and MW678772–74.

Amplicon sequencing analysis. Total genomic DNA was extracted from 200 mg of FS using the Fast DNA SPIN kit for soil (MP Biomedicals, Illkirch-Graffenstaden, France) according to the manufacturer’s instructions. The DNA concentration of each sample was determined using the Qubit 1X dsDNA HS Assay Kit (Invitrogen, Paisley, UK) and a Qubit fluorometer. Subsequent library preparation and sequencing were performed by Novogene (Cambridge, UK). As amplicon sequencing method supports only shorter fragments, the V4 region of the 16S rRNA gene (300 bp fragments) was amplified using primers 515F (5'-GAGGTTGATCTCCTGGCTCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') and a Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, Massachusetts, USA). The library was prepared using the NEB Next® UltraTM DNA Library Prep Kit for Illumina and paired-end 250 bp sequencing was performed using the NovaSeq 6000 DNA Sequencing machine (Illumina, San Diego, California, USA). The resulting sequences were submitted to the NCBI database with the accession number ERP128111. Amplicon sequence variants (ASV) were obtained using the DADA2 pipeline (biocorconductor-dada2 v1.16.0) and Silva non redundant database v138 (Supplementary S3) with custom manual species assignment. The depth of sequencing of the resulting data was normalized by rarefaction to the lowest sequencing depth (42 134 sequences/sample) and a relative abundance on several taxonomic levels in different variable groups were explored (Supplementary S4). Total bacterial diversity was expressed as Shannon entropy, the population richness was expressed as simple feature or ASV counts and the evenness was expressed as Pielou’s index.

Statistical analyses. Counts of bacterial colonies in log CFU g⁻¹ within the parvorders and feed categories are shown as boxplots. The normality of data was evaluated by Shapiro–Wilk test (α = 0.05). Differences in bacterial counts were assessed using a Mann–Whitney U Test (α = 0.05) within the parvorders, and a one-way ANOVA within the feed categories (α = 0.05) using STATISTICA software (StatSoft, Prague, Czechia) and Microsoft Office Professional Plus 2016.

To detect differentially abundant taxa between the sample categories, the ANCOM statistical test was used from the package skbio v0.5.2 (scikit-bio.org). The one-way F statistics from the scpy package v1.4.1 was used to determine that statistical significance with α = 0.05. Several categories of the data were explored on both the Phylum and Family level. Furthermore, the bifidobacterial sub-population was extracted for each sample and the differentially abundant species were calculated. Statistically significant results are presented in form of boxplots (Supplementary S2).
The statistical significance of difference in means in terms of the diversity metrics (Shannon, Pielou, and ASV counts) was assessed using the ordinary least squares method coupled with a pairwise T-test. The data was Box-Cox transformed and the resulting residuals were normally distributed (Jarque-Berra and Omnibus probability > 0.05), however, the groups were highly heteroskedastic. To mitigate this, we have used the ordinary least square method from the package statsmodels v0.11.10 with MacKinnon and White's heteroscedasticity robust standard errors\(^{310}\) (Supplementary S1).

**Ethical approval.** The sampling of primate faeces was performed during routine daily procedures. All procedures involving animals adhered to recommendations of the “Guide for the Care and Use of Animals” by the Czech University of Life Sciences Prague. The research conducted herein was approved by Ethic and Animal Care Committee of the Czech University of Life Sciences Prague (protocol number: CZU/17/19) and was performed in accordance with the relevant guidelines and regulations. All zoological institutions have rigorous standards for animal welfare and are accredited by the European Association of Zoos and Aquaria. The research adhered to the legal requirements of the Czech Republic for the ethical treatment of nonhuman primates as well as in accordance with European Directive 2010/63/EU.

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**Author contributions**
N.-B.V. designed experiments. M.N. and N.-B.V. carried out experiments. M.N. and S.A. participated at data analysis, interpretation, and visualization of results. M.N., N.-B.V., and S.A. drafted the manuscript. B.P. and K.J. were involved in the writing up of the manuscript. B.J. and D.K.J. participated at data evaluation and revised the manuscript critically. The study was supervised by N.-B.V. and D.K.J. All authors reviewed the manuscript and approved the version to be published.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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