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A 15-day pilot biodiversity intervention with horses in a farm system leads to gut microbiome rewilding in 10 urban Italian children

Daniel Scicchitano [a,b], Lucia Foresto [a,b], Cédric C. Laczny [c], Nicoló Cinti [a,b], Rosalba Vitagliano [e], Rashi Halder [c], Gaja Morri [d], Silvia Turroni [a], Federica D'Amico [a], Giorgia Palladino [a,b], Jessica Fiori [e,f], Paul Wilmes [c], Simone Rampelli [a,b], Marco Candela [a,b,*]

[a] Unit of Microbiome Science and Biotechnology, Department of Pharmacy and Biotechnology (FaBiT), Alma Mater Studiorum - University of Bologna, 40126 Bologna, Italy
[b] Fano Marine Center, The Inter-Institute Center for Research on Marine Biodiversity, Resources and Biotechnologies, 61032 Fano, Italy
[c] Systems Ecology Group, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg
[d] Psy D., Psychotherapist, Psychoanalyst, via M. Serenari, 7, 40033 Casalecchio di Reno, Bologna, Italy
[e] Department of Chemistry "Giacomo Ciamician", Alma Mater Studiorum - University of Bologna, 40126 Bologna, Italy
[f] Institute of Neurological Sciences of Bologna (IRCCS), Laboratory of Proteomics Metabolomics and Bioanalitical Chemistry, 40124 Bologna, Italy

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ABSTRACT

To provide some glimpses on the possibility of shaping the human gut microbiome (GM) through probiotic exchange with natural ecosystems, here we explored the impact of 15 days of daily interaction with horses on the GM of 10 urban-living Italian children. Specifically, the children were in close contact with the horses in an "educational farm", where they spent almost 10 h/day interacting with the animals. The children's GM was assessed before and after the horse interaction using metabarcoding sequencing and shotgun metagenomics, along with the horses' skin, oral and fecal microbiomes. Targeted metabolomic analysis for GM-produced beneficial metabolites (i.e., short-chain fatty acids) in the children's feces was also performed. Interaction with horses facilitated the acquisition of health-related traits in the children's GM, such as increased diversity, enhanced butyrate production and an increase in several health-promoting species considered to be next-generation probiotics. Among these, the butyrate producers *Faecalibacterium prausnitzii* and *F. duncaniae* and a species belonging to the order Christensenellales. Interaction with horses was also associated with increased proportions of *Eggerthella lenta*, *Gordonibacter pamelae* and *G. urolithinfaciens*, GM components known to play a role in the bioconversion of dietary plant polyphenols into beneficial metabolites. Notably, no increase in potentially harmful traits, including toxin genes, was observed. Overall, our pilot study provides some insights on the existence of possible health-promoting exchanges between children and horses microbiomes. It lays the groundwork for an implemented and more systematic enrollment effort to explore the full complexity of human GM rewilding through exchange with natural ecosystems, aligning with the One Health approach.

1. Introduction

The gut microbiome (GM) is deeply integrated into human physiology, providing the human body with essential functionalities that it did not evolve on its own [1]. Indeed, the human GM plays a strategic role in the development, functioning and regulation of the immune and metabolic systems, as well as protection against allochthonous pathogens [2]. However, it is now a fact that various modernization factors that characterize urban societies – such as processed food, antibiotic usage, sanitation, and reduction of physical interactions with animals and nature – have been poorly tolerated by human GM, resulting in a maladaptive process recently defined as "microbiome modernization" [3]. As documented by several GM studies and meta-analyses [4–7], this process has involved the loss of GM features characteristic of rural and traditional populations, leading to a decrease in ecosystem diversity and in the prevalence and abundance of the so-called "VANISH" (volatile and/or associated negatively with industrialized societies of urbanization/modernization) taxa [3,8]. These microorganisms are considered

* Corresponding author at: Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro, 6, 40126 Bologna, BO, Italy.
E-mail address: marco.candela@unibo.it (M. Candela).

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probiotic members of the human GM [8], capable of providing the host with health-promoting metabolites such as short-chain fatty acids (SCFAs, mainly acetate, propionate and butyrate), crucial multifunctional GM effectors for the maintenance of immune and metabolic homeostasis [9].

According to Sonnenburg and Sonnenburg [3], the GM modernization process may have led to unbalances in microbiome-dependent ecosystem services that affect human health and may be responsible – at least in part – for the increased incidence of non-communicable chronic diseases (NCCDs) in the Western world, including allergy, asthma, obesity, metabolic syndrome and type 2 diabetes, among others [10–14]. Particularly, in children living in an urban context, this modernization process could be responsible for the observed increase in allergic diseases [8], by compromising GM-dependent immune education and regulation processes in early life [15–17]. In this scenario, there is now a consensus on the urgent need to find sustainable solutions to facilitate the recovery of GM traits lost during the modernization process, supporting what has been defined as the "rewilding" of human GM [18].

As repeatedly shown in the literature, human GM is primarily shaped by the environment rather than by diet or genetics [19,20]. In particular, the rural environment and the contact with rural animals have been robustly associated with healthy GM profiles [19]. Consistently, the idea of shaping human GM through environmental determinants such as life sharing, interaction with the natural environment and contact with animals is consolidating, offering a new and still untapped perspective for human GM modulation towards eubiotic configurations [21,22]. As discussed by Robinson and colleagues [18], humans need to be reviewed as an ecological unit that openly interacts with the natural environment in a complex microbial process, including health-promoting microbial interactions. In particular, exposure to natural microbiomes would favor the acquisition of an eubiotic GM layout, leading to enhanced immunoregulatory activity. In this context, the idea of GM rewilding of Western urban citizens through prescribed health-promoting interactions with biodiverse natural microbiomes has been advanced [23–26]. A relevant and concrete step in this direction was taken by Roslund and colleagues [27]. In a 28-day biodiversity intervention in daycares, where urban daycares were enriched by covering the yards with forest floor and sod, the authors succeeded in increasing the GM diversity of the enrolled children, which also resulted in a higher diversity of butyrate producers and improved immune functions. Despite the promising results of this pioneering research, several questions remain unanswered, such as the optimal exposure to natural microbiomes, as well as the dynamics and mechanisms involved in health-promoting microbial interactions, requiring more systematic studies and trials [23].

In an attempt to shed some light in this direction, here we investigated the impact of a 15-day biodiversity intervention with horses in an educational farm on the GM composition and function of healthy urban Italian children. In particular, the GM and SCFA profile of 10 children aged 9–14 years were assessed before and after the intervention. The horses' gut, oral and skin microbiomes were also profiled, allowing the assessment of interspecies exchange of microbiome taxa. The findings of our pilot study suggest a probiotic exchange between horses and children, and a partial GM rewilding of the latter with the acquisition of health-promoting, butyrate-producing VANISH taxa.

2. Materials and methods

2.1. Biodiversity intervention with horses in an educational farm environment

The present study was approved by the Bioethics Committee of the University of Bologna on 27/02/2020, Prot. n. 0041442. Specifically, over a period of 15 days, the recruited children were in close contact with horses for at least 10 h/day in an educational farm (Prati di Amar, Bologna), petting and playing with them, supervised by qualified operators, without disturbing the animals' well-being. Written informed consent was obtained from the parent/legal guardian of each child enrolled. The children were also asked to complete an anonymous survey about their eating habits during the study period, which was used to assess the Mediterranean Diet Quality Index for children and adolescents (KIDMED).

2.2. Sample collection

On the day before and the day after the end of the biodiversity intervention, all children were asked to collect their first stool using an autosampler kit with specific instructions to avoid fecal contamination [28], for a total of 20 samples. During the 15 days of the intervention, a fecal sample, a skin swab and a mouth swab were collected from each of the 9 horses on the farm, for a total of 27 samples. All samples were immediately transported to the laboratory of the Unit of Microbiome Science and Biotechnology, Department of Pharmacy and Biotechnology, University of Bologna (Bologna, Italy) and stored at −80 °C until further processing.

2.3. Microbial DNA extraction

Microbial DNA was extracted from the 47 child and horse samples collected using different commercial kits depending on the starting matrix, namely the DNeasy PowerBiofilm Kit (QIAGEN, Hilden, Germany) for skin and oral swabs following the manufacturer's instructions, and the DNeasy Blood & Tissue Kit (QIAGEN) with a modified protocol [29] for fecal samples. Briefly, fecal material was supplemented with four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) and the homogenization step was performed three times using a FastPrep instrument (MP Biomedicals, Irvine, CA, USA) at 5.5 movements/s for 1 min, followed by a heating step at 95 °C for 15 min. DNA quality and quantity were assessed using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.4. Metabarcoding sequencing

PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene was performed in a total volume of 50 μL with 25 ng of total DNA, 2× KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland) and 200 nmol/L of 341F and 785R primers carrying Illumina overhang adapter sequences. The thermal cycle consisted of 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min [30]. PCR products were cleaned up using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA), and indexed libraries were prepared using Nextera technology and purified as above. The final libraries were quantified using a Qubit 3.0 fluorimeter (Invitrogen, Waltham, MA, USA), normalized to a final concentration of 4 nM and pooled. The pool was denatured with 0.2 N NaOH and diluted to a final concentration of 4.5 pM with a 20 % PhiX control prior to sequencing on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

2.5. Shotgun metagenomic sequencing

The QIAseq FX DNA library kit (QIAGEN) was used for library preparation, according to the manufacturer's instructions. Briefly, 450-bp size, end-repaired and A-tailed fragments were generated through fragmentation of 100 ng DNA of each sample using the FX enzyme mix with the following thermal cycle: 4 °C for 1 min, 32 °C for 10 min and 65 °C for 30 min. The obtained fragments were then incubated at 20 °C for 15 min to perform adapter ligation in the presence of DNA ligase and Illumina adapter barcodes. The subsequent purification step was

performed using Agencourt AMPure XP magnetic beads (Beckman Coulter). Libraries were pooled at an equimolar concentration (4 nM) to obtain the final pool, which was sequenced on an Illumina NextSeq2000 platform using a 2 × 150 bp paired-end protocol, following the manufacturer's instructions (Illumina). LCSB Genomics platform RRID: SCR_021931.

2.6. Bioinformatic analysis

For metabarcoding sequencing, raw sequences were analyzed using a pipeline combining PANDAsEq. [31] and QIIME 2 [32]. After filtering for length (min/max = 350/550 bp) and quality ("fastq filter" function of the Usearch11 algorithm) [33], reads were binned into amplicon sequence variants (ASVs) using DADA2 [34]. The VSEARCH algorithm [35] and the SILVA database release 138.1 [36] were used for taxonomic assignment. All unassigned and eukaryotic sequences were discarded. Alpha diversity was assessed using different metrics, namely Faith's Phylogenetic Diversity (PD whole tree), number of observed ASVs, and Shannon index, while beta diversity was computed using unweighted and weighted UniFrac distances.

Raw shotgun reads were pre-processed for quality, adapter content and deduplication using fastp v. 0.23.4 with the following parameters "–detect_adapter_for_pe –cut_tail -c -D". Reads were then filtered for eukaryotic host DNA using bmtagger software with *Equus caballus* (NCBI GenBank accession: GCA_002863925.1) and *Homo sapiens* (GCA_000001405.29). The resulting reads were taxonomically assigned using kraken2 v. 2.1.3 with default parameters, and species-level abundances within each sample were estimated using Bracken v. 2.9. The output tables were used to calculate alpha diversity indices (Shannon, Simpson, and observed features) and beta diversity based on Bray-Curtis distances at species level. Resulting reads were also used to obtain a general functional annotation for each sample using HUMAnN v. 3.0.1 [37]. HUMAnN output tables were normalized to Copies per million (CoPM) using humann_renorm_table. Normalized tables were merged and processed to remove UNMAPPED IDs, UniRef90 classification was converted into KEGG Orthology (KO) classification and collapsed to the corresponding KEGG pathway. The final table was used to compute alpha diversity indices (Shannon, Simpson, and observed features) and beta diversity based on Bray-Curtis distances, and to assess specific SCFA-related KOs [38] and KEGG pathways assigned to "Infectious disease: bacterial". In addition, high-quality reads were assembled using metaspades.py v. 3.15.3 with default parameters, with horses' metagenomes co-assembled into a single assembly file. Each resulting assembly was annotated using prokka v. 1.14.6 with default parameters and "—addgenes" to retrieve all families of Carbohydrate-Active enZYmes (CAZymes), according to the latest version of the online CAZy database, namely glycoside hydrolases (GHs, EC 3.2.1-), glycosyl transferases (GTs, EC 2.4-), polysaccharide lyases (PLs, EC 4.2.2.-), carbohydrate esterases (CEs) and auxiliary activities (AAs). Using prokka output files, nucleotide sequences referring to the open reading frames (ORFs) of each CAZyme were retrieved and used to build a reference database, dereplicated at 90 % similarity and then used to assess the abundance of each CAZyme in our samples. Alignment was performed using Bowtie2 v. 2.3.4.3 [39] with the parameters "–end-to-end" and "–very-sensitive"; the number of aligned reads for each sample was retrieved using Samtools v. 1.16. Reads per kilobase of gene per million reads mapped (RPKMs) in each sample and for each gene were calculated by summing the number of reads of all mapped ORFs as follows:

$$\frac{Total\ reads\ mapped\ to\ gene}{Total\ reads * Mean\ gene\ length} * 10^9$$

The abundance table, in terms of RPKMs, of each CAZyme family identified in our dataset was used to assess the differential abundance of these enzymes after the intervention in terms of log2[Fold Change] (log2FC).

2.7. Binning of metagenome-assembled genomes (MAGs)

Assemblies from each sample were used to construct Metagenome-Assembled Genomes (MAGs) using the metawrap binning module (metawrap v. 1.3.2). Only MAGs with >50 % completeness and < 5 % contamination, as assessed using the checkm lineage_wf workflow [40], were retained. High-quality MAGs were dereplicated into species-level genome bins (SGBs) using dRep v. 3.2.2 and the following parameters: "–ignoreGenomeQuality -pa 0 -sa 0.95 -nc 0.30 -cm larger -centW 0". SGBs were taxonomically classified using the gtdbtk classify_wf workflow with default parameters [41] and the latest available Genome Taxonomy Database (GTDB) release (release 214), while the abundance of each SGB in each sample was obtained using the metawrap quanti_bins module. The resulting table of Genome copies per million reads of each SGB was used to assess the log2FC of SGBs after the intervention. MinHash sketches implemented in the mash tool v. 2.3 were used to compare the resulting SGBs overrepresented in the subjects after the intervention with the VANISH reference genomes from [8], where genomes with a mash distance ≤0.05 were defined as closely related species.

2.8. Gas chromatography-mass spectrometry determination of short-chain fatty acids in children's feces

An aliquot of each human fecal sample was weighted (approximately 250 mg) and processed as described in Fiori et al. [42] for the characterization of SCFAs. Briefly, samples were homogenized in 10 % perchloric acid and centrifuged at 15,000 rpm for 5 min at 4 °C. Supernatants were diluted 1:10 in water and added with D8-butyric acid (internal standard) to 20 μg/mL. Headspace solid-phase microextraction (HS-SPME) was carried out using a 75-μm CarboxenTM/polydimethylsiloxane fiber (Supelco, Sigma-Aldrich, Milan, Italy) at 70 °C, with 10-min equilibration and 30-min extraction time. Gas chromatography–mass spectrometry analysis was carried out on a TRACE GC 2000 Series (Thermo Fisher Scientific, Waltham, MA, USA) gas chromatograph, interfaced with GCQ Plus (Thermo Fisher Scientific) mass detector with ion-trap analyzer. A phenomenex ZB-WAX (30 m × 0.25 mm ID, 0.15-μm film thickness) capillary column was used.

2.9. Statistical analysis

Statistical analyses and graphical representations were performed using R software v. 4.3.1 (https://www.r-project.org/), implemented with the "Made4" [43], "vegan" (https://cran.r project.org/web/packages/vegan/index.html), "pairwiseAdonis" [44] and "gplots" (https://cran.r-project.org/web/packages/gplots/index.html) packages. Data separation in the Principal Coordinates Analysis (PCoA) plots was assessed using a permutation test with pseudo-F ratio (functions "adonis" in the vegan package and function "pairwiseAdonis" in the homonymous package). The Kruskal-Wallis test was used to assess significant differences in alpha diversity among groups. The Wilcoxon signed-rank paired test was used to assess feature variations in the children's GM before and after the intervention. *P*-values were corrected for multiple testing, when necessary, using the Benjamini-Hochberg method, with a false discovery rate (FDR) ≤0.05 considered statistically significant and a value lower than 0.1 considered as a trend.

3. Results

3.1. Biodiversity intervention with horses in farm environment resulted in increased GM diversity in urban children

The metabarcoding sequencing of the 16S rDNA was performed on the GM of 10 children before (timepoint 0) and after the 15-days of biodiversity intervention with horses in a rural farm environment (timepoint 1), for a total of 20 samples. During the intervention, the

enrolled children were showing a moderate adherence to the Mediterranean diet, according to Mediterranean Diet Quality Index for children and adolescents (KIDMED) [45] (Supplementary Table S1). Twenty-seven samples, representing the skin, oral and fecal microbiomes of the 9 horses involved in the biodiversity intervention were also sequenced. For the 47 samples (human and animals) a total of 521′926 high-quality reads were obtained (mean per sample ± SD: 10′467.9 ± 3444.8), corresponding to a total of 2862 ASVs.

According to our findings, the biodiversity intervention resulted in a significant increase in the GM diversity of the enrolled children, as estimated by the Shannon index (Wilcoxon signed-rank test, $p = 0.049$) (Fig. 1A). The PCoA plot of both weighted and unweighted UniFrac distances showed segregation between the pre- and post-intervention samples of children, although not statistically significant (permutation test with pseudo-F ratio "Adonis", $p > 0.05$) (Fig. 1B). As expected, the horse skin, oral and fecal microbiomes showed a different compositional structure, well segregating from each other and from the children's GM in the PCoA plot (Supplementary Fig. 1). The horse-associated microbiomes also differed from the children's GM in alpha diversity, with the fecal and skin microbiomes being the most diverse (Supplementary Fig. 1).

3.2. Compositional and functional changes in the children's GM following intervention with horses

To provide a high-resolution compositional and functional dimension to our study, the entire sample set was further processed by shotgun metagenomics, resulting in a total of 1.3 billion high-quality reads (mean per sample ± SD: 27.1 ± 7.7 million). The species-level compositional structure of the children's GM and horses' microbiomes – oral, skin and gut – is shown in Fig. 2. Several components of the children's GM showed significant variation following intervention (Wilcoxon signed-rank paired test, $p \leq 0.05$) (Fig. 3). In particular, *Faecalibacterium* spp., including *F. prausnitzii* and *F. duncaniae*, *Gordonibacter pamelaeae*, *Gordonibacter urolithinfaciens* and *Eggerthella lenta* increased, while *Akkermansia muciniphila* and *Bifidobacterium adolescentis* decreased. When focusing on the GM species assigned to VANISH taxa according to Carter and colleagues [8], we found that the intervention resulted in a significant increase in the total diversity of *Faecalibacterium* species in the children's GM, while no changes were observed in the diversity of *Prevotella*, *Phascolarctobacterium* and *Blautia* species (Supplementary Fig. 2).

With the aim of providing functional insights into the impact of the intervention on the children's GM, we focused on KO genes and pathways involved in: (i) degradation of complex polysaccharides (eg. CAZymes); (ii) production of SCFAs and (iii) infectious diseases and toxin biosynthesis. According to our findings, the intervention resulted in a decrease of 23 CAZymes families (log2FC < 1.5) and an increase of 4 CAZymes families (log2FC > 1.5). Specifically, gene families belonging to the classes Glycosyl Transferases (GTs), Carbohydrate Esterases (CEs) and Polysaccharide Lyases (PLs) increased, while functions belonging to Glycoside Hydrolases (GHs) and Auxiliary Activities (AAs) decreased (Supplementary Table 2). With respect to SCFA-related KOs [38], 23 genes were more abundant (log2FC > 1.5), while 15 were less abundant (log2FC < 1.5) (Supplementary Table 3), overall retaining the full genomic potential for the supply of pyruvate, succinate and acetate and apparently acquiring more lactate-producing genes, such as D-lactate dehydrogenase (quinone) [EC:1.1.5.12] and lactaldehyde dehydrogenase [EC:1.2.1.22]. Only 3 (out of 11) KEGG pathways for infectious disease and toxin biosynthesis were detected in the children's GM, none of which underwent variation following the intervention (Supplementary Table 4). Finally, the intervention resulted in a trend towards increased fecal levels of butyrate (Wilcoxon rank-sum paired test, $p = 0.093$), while no changes were observed for acetate, propionate, valerate, isobutyrate and isovalerate (Fig. 4).

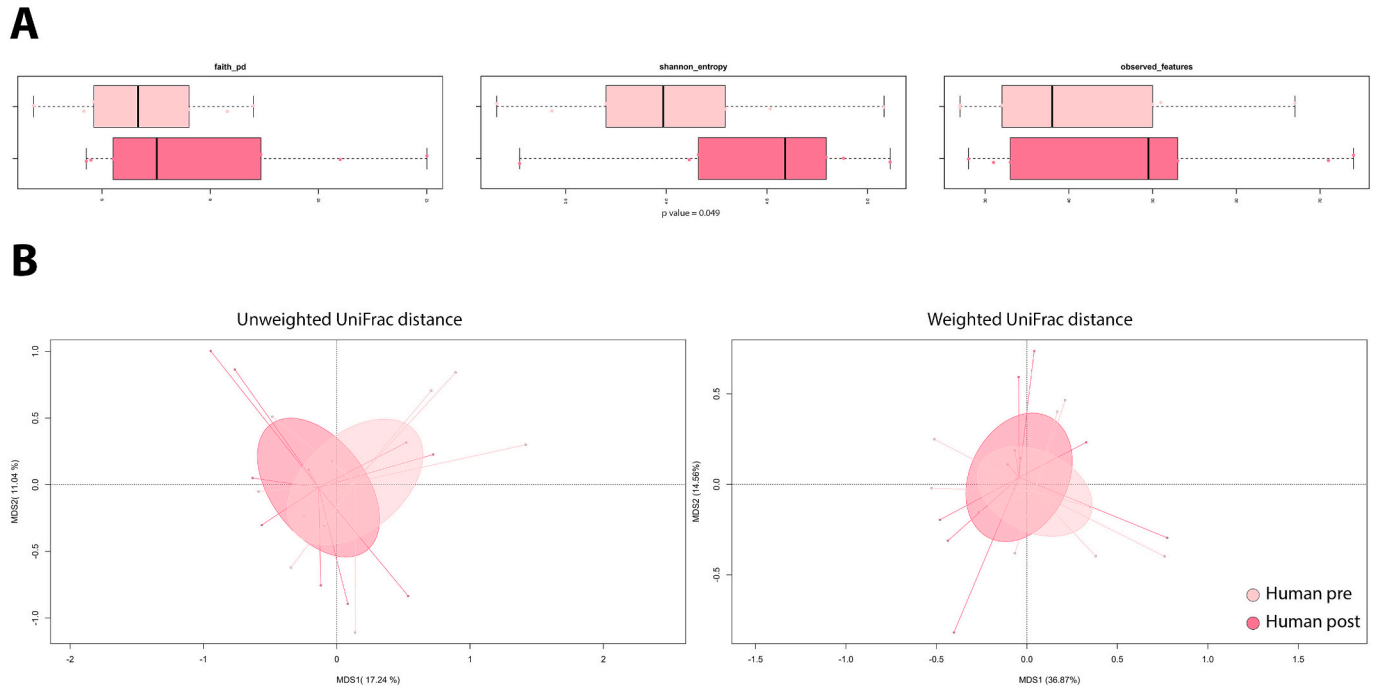

Fig. 1. Diversity of the children's gut microbiome before and after interaction with horses. A, Boxplots showing the distribution of alpha diversity, as assessed by Faith's phylogenetic diversity, Shannon index and number of observed features, in the children's GM before (Human pre) and after (Human post) interaction with horses (color legend bottom right). A significant increase in diversity was observed for the Shannon index (Wilcoxon signed-rank paired test, $p = 0.049$). B, Principal coordinates analysis (PCoA) plots based on unweighted and weighted UniFrac distances between study groups. No significant differences highlighted, permutation test with pseudo-F ratio "Adonis", $p > 0.05$.

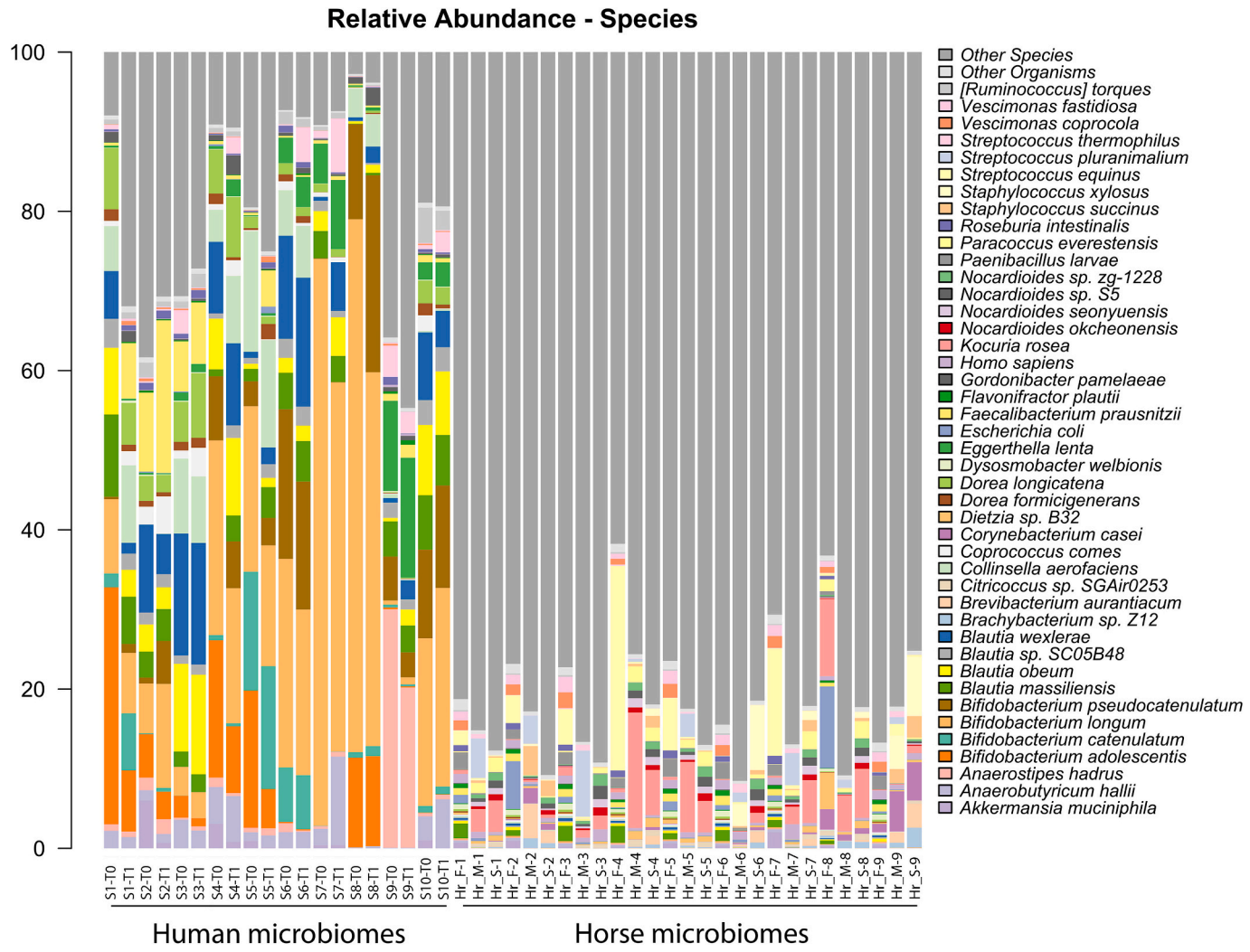

Fig. 2. Species-level composition of horse (oral, skin and gut) microbiomes and child gut microbiomes before and after intervention. Relative abundance (%) of microbiome species in both human and horse samples. Only taxa with relative abundance >0.5 % in at least 13 % of samples are represented. Relative abundance of unassigned reads was grouped under the name "Other Organisms", while taxa below the relative abundance filter were grouped within the "Other Species" name. S = Subject, T0 = Pre-Intervention, T1 = Post-Intervention, Hr_S = Horse Skin, Hr_M = Horse Mouth, Hr_F = Horse Feces.

3.3. Species-level genome bins reconstruction and inter-host microbiome exchange

To account for possible inter-host exchanges of microbiome components, we assembled Metagenome Assembled Genomes (MAGs) from the entire set of assembled metagenomes. Four hundred high-quality Metagenome-Assembled Genomes (MAGs) were obtained and dereplicated into 190 Single-Genome Bins (SGBs), using 95 % similarity as the minimum threshold for MAG clustering. Taxonomic identification of the obtained SGBs is provided in Supplementary Table 5. Twenty-three SGBs increased in the children's GM after the intervention (log2FC > 1.5), 9 of which were below the detection limit at baseline (Genomes Copies per Million reads (GCMs) < 1), thus potentially acquired through contact with the animals (Supplementary Table 6). These SGBs included *Linivicinus* spp., *Rhodococcus* spp., *Ocillospiraceae* spp., Christensenellales spp., *Angelakisella* spp., *Vescimonas* spp., Clostridia spp., *Scatavimonas* spp., and *Prevotella rara.* Thirteen of the 23 SGBs, assigned to *Linivicinus* spp., *Rhodococcus* spp., *Ocillospiraceae* spp., Christensenellales spp., *Angelakisella* spp., *Vescimonas* spp., *Faecalibacterium hattorii*, *Bifidobacterium pullorum*, *Bifidobacterium animalis*, *F. prausnitzii* and *F. duncaniae*, were detected in the horse microbiomes (skin, mouth or gut) with at least 1 GCM. Interestingly, the SGB assigned to *F. prausnitzii* was closely related to a *Faecalibacterium* VANISH genome detected in the Hadza Tanzanian hunter-gatherer GM by Carter et al. (2023) (mash distance = 0.05). The remaining 10 of the 23 SGBs increased in the children's GM after the intervention (assigned to Clostridia spp., *Scatavimonas* spp., Christensenellales spp., *P. rara*, *Latilactobacillus sakei*, *Acetatifactor intestinalis*, *Ruminococcus bicirculans*, *Hominisplanchenecus faecis*, *Oliverpabstia faecicola*, *Dialister invisus*) were not detected in the horse microbiomes with at least 1 GCM. Among the SGBs that decreased in the children's GM after the intervention (Supplementary Table 6), we detected SGBs belonging to the *Streptococcus* genus, which was highly abundant in the horse microbiomes, as well as to *Vescimonas*, *Collinsella intestinalis* and *Acutalibacteraceae* species, all of which were detected at high abundance in horses. Furthermore, several SGBs exclusively present in the children's GM were depleted after the intervention, namely *A. muciniphila*, *Gemmiger avicola*, *Ruminococcus* spp., *Aphodocola* spp., *Lactococcus cremoris*, *Lentihominibacter excrementipullorum*, *Enterococcus lactis*, *Coprococcus eutactus*, *Prevotella copri*, *Wujia chipingensis*, *Blautia* spp. and *Lactiplantibacillus plantarum*.

4. Discussion

In the present work, we explored the impact of a 15-day pilot biodiversity intervention with horses on the GM composition and function of 10 Italian children living in an urban context, using

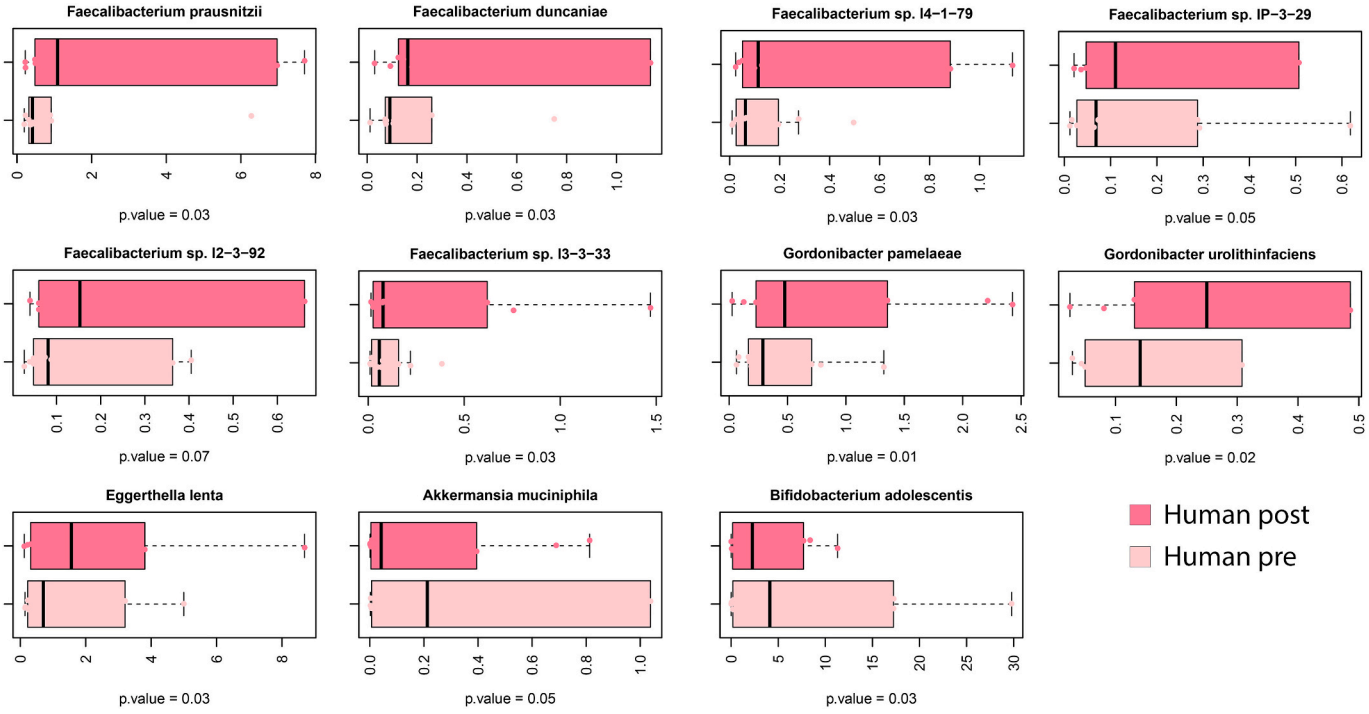

Fig. 3. Species-level Genome Bins (SGBs) differentially represented in the children's gut microbiome after interaction with horses. Boxplots showing the relative abundance (%) distribution of SGBs differentially represented in the children's GM before (Human pre) and after (Human post) interaction with horses. Only SGBs with a mean relative abundance of at least >0.1 % in one group were considered. *P*-values obtained by Wilcoxon signed-rank paired test are reported below each graph.

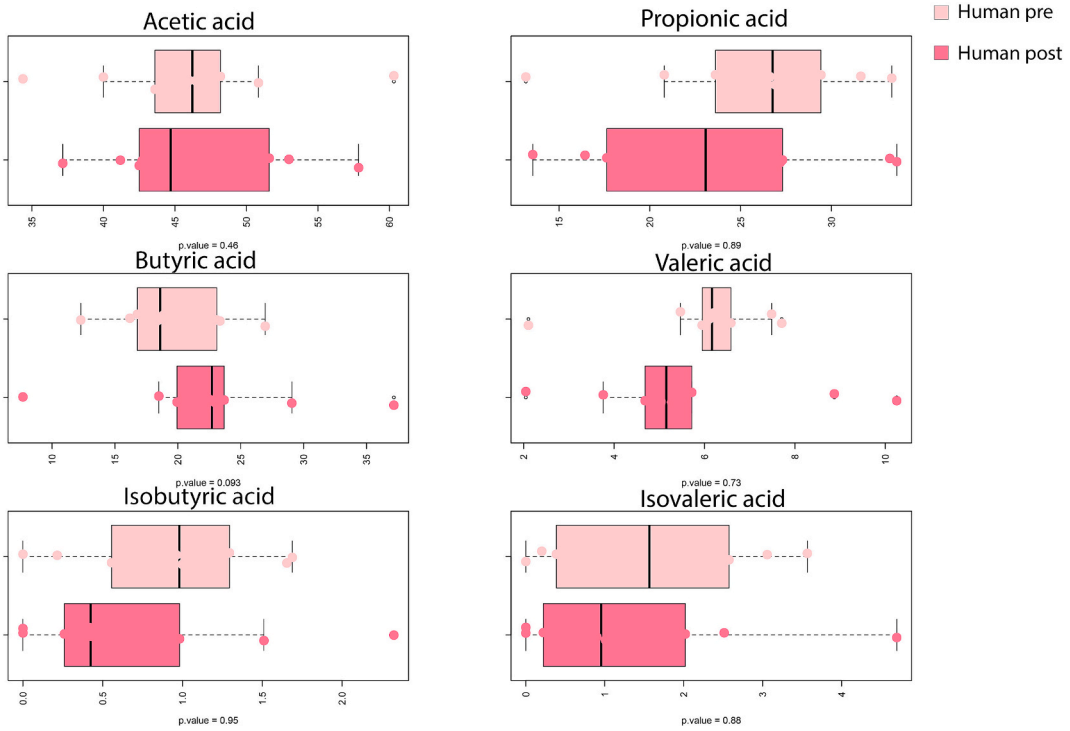

Fig. 4. Variation in levels of short-chain fatty acids in the children's feces before and after interaction with horses. Boxplots showing the relative abundance (%) distribution of levels of short-chain fatty acids in the children's feces before (Human pre) and after (Human post) interaction with horses. P-values obtained by Wilcoxon signed-rank paired test are reported below each graph.

metabarcoding sequencing, shotgun metagenomics and targeted metabolomics. In particular, we explored potential health-promoting interactions with horses and their associated microbiomes. According to our findings, interaction with horses resulted in the acquisition of several health-promoting features in the children's GM, suggesting the occurrence of a rewilding process facilitated by exchange with natural

microbiomes. Such features included increased GM diversity, higher abundance of VANISH taxa belonging to the *Faecalibacterium* genus, and higher fecal levels of the beneficial GM metabolite butyrate. More specifically, several health-promoting taxa, such as *F. prausnitzii*, *F. duncaniae*, *G. pamelae*, *G. urolithinfaciens* and *E. lenta*, increased after interaction with horses, while *A. muciniphila* and *B. adolescentis* decreased. For *F. prausnitzii*, *F. duncaniae* and *A. muchiniphila*, the corresponding variations were confirmed at the highest possible level of metagenomic resolution, i.e., SGB. Interestingly, we also observed a correspondence of the SGB assigned to *F. prausnitzii* with an *F. prausnitzii* SGB assembled from the hunter-gatherer GM [8], further supporting a possible GM rewilding process.

When specifically focusing on the children's GM variation at the SGB level, we identified 23 SGBs that increased after interaction with horses. Thirteen of these SGBs, including *Limivicinus* spp., *Rhodococcus* spp., Christensenellales spp. and *Vescimonas* spp., were also detected in the horse microbiomes, suggesting inter-species microbiome exchange. The remaining 10 SGBs, including L. *sakei*, *R. bicirculans*, *H. faecis*, *O. faecicola* and *D. invisus*, were not detected in the horse microbiomes, suggesting an indirect mechanism of biostimulation. Finally, some highly abundant SGBs in the horse microbiomes (e.g., *Streptococcus* spp.) were depleted in the children's GM after the intervention.

Strikingly, different species found to be increased in the children's GM after interaction with horses, such as *F. prausnitzii*, *F. duncaniae* and Christensenellales spp., have been recognized as important candidates for next-generation probiotics. The first two are health-promoting species capable of degrading fiber and producing butyrate [46–48], which could help explain the intervention-related increase in fecal levels of this metabolite. Similarly, *Christensenellaceae* members are known to have anti-inflammatory and anti-obesogenic roles [49]. In addition, *E. lenta*, *G. pamelae* and *G. urolithifaciens*, which were also found to be increased in the children's GM after interaction with horses, are capable of bioconverting dietary plant polyphenols into health-promoting and protective bioactive metabolites with known anti-inflammatory and anticancer properties [50]. Specifically, *Gordonibacter* species have been reported to provide urolithin, whereas *E. lenta* is involved in enterolignan biosynthesis [51,52]. Similarly, *D. invisus*, which also increased after interaction with horses, has been associated with the production of the cytokine adiponectin [53], which protects against metabolic inflammation. On the other hand, exposure to horses resulted in an increased abundance of an SGB related to *Rhodococcus* spp. in the children's GM. Although this SGB was not assigned to the species *R. equi*, a possible zoonotic agent in immunocompromised subjects [54], this result deserves in-depth analysis, e.g. by culturomics, and suggests the need for caution in interactions between immunocompromised children and horses. However, supporting the safety of child-horse microbiome exchange, no metagenomic evidence of transmission of virulent and/or toxin-producing strains was observed in our pilot setting.

5. Conclusions

Despite the small sample size, our pilot study provides evidence for a non-neutral microbiome exchange between horses and urban children and a possible rewilding of the latter's GM, which acquired several health-promoting features, including increased diversity, higher abundance of beneficial components, such as VANISH taxa belonging to *F. prausnitzii*, and higher butyrate levels. Although the findings need to be confirmed on a larger scale and the mechanisms underlying the dynamics of inter-species microbiome exchange remain to be elucidated, our study supports the possibility of improving the human GM profile by recovering eubiotic traits through health-promoting interactions with natural microbiomes, including holobiont systems. This aligns with the One Health framework, which emphasizes the importance of balanced interactions between humans, animals, and the environment for supporting human and planet health. However, in consideration of these premises, we believe it is important to continue larger and more systematic studies aimed at exploring the beneficial role of natural microbiomes for human health. Focusing, moreover, on if and how such observed changes in the subjects' GM can be reversed and thus restored to an urbanized GM profile.

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Authors consent

All authors approved the new version of the manuscript and consented to publication.

CRediT authorship contribution statement

Daniel Scicchitano: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Lucia Foresto:** Writing – review & editing, Methodology. **Cédric C. Laczny:** Writing – review & editing, Methodology. **Nicoló Cinti:** Writing – review & editing, Methodology. **Rosalba Vitagliano:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Rashi Halder:** Writing – review & editing, Methodology. **Gaja Morri:** Writing – review & editing. **Silvia Turroni:** Writing – review & editing. **Federica D'Amico:** Writing – review & editing. **Giorgia Palladino:** Writing – review & editing. **Jessica Fiori:** Writing – review & editing, Methodology. **Paul Wilmes:** Writing – review & editing. **Simone Rampelli:** Writing – review & editing. **Marco Candela:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

High-quality reads from all samples are deposited in the European Nucleotide Archive under the project accession number PRJEB73511.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100902.

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