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*Article*

# The Diversity of Gut Microbiota at Weaning Is Altered in Prolactin Receptor Null Mice

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**Abstract:** Maternal milk supports offspring development by providing microbiota, macronutrients, micronutrients, immune factors, and hormones. The hormone prolactin (PRL) is an important milk component with protective effects against metabolic diseases. Because maternal milk regulates microbiota composition and an adequate microbiota protects against the development of metabolic diseases, we aimed to investigate whether PRL/PRL receptor signaling regulates gut microbiota composition in newborn mice at weaning. *16SrRNA* sequencing of feces and bioinformatics analysis were performed to evaluate gut microbiota in PRL receptor-null mice (*Prlr*-KO) at weaning (postnatal day 21). The normalized colon and cecal weights were higher and lower, respectively, in *Prlr*-KO mice relative to wild-type mice (*Prlr*-WT). Relative abundance (Simpson Evenness Index), phylogenetic diversity, and bacterial concentration were lower in *Prlr*-KO. Eleven bacteria species, out of 470, differed between *Prlr*-KO and *Prlr*-WT mice with two genera (*Anaerotruncus* and *Lachnospiraceae)* related to metabolic diseases' development being the most common in *Prlr*-KO. A higher metabolism of terpenoids and polyketides was predicted in *Prlr*-KO mice compared to *Prlr*-WT mice; these metabolites have antimicrobial properties and are present in microbe-associated pathogenicity. Concluding, the absence of the PRL receptor alters gut microbiota, resulting in lower abundance and richness, which could contribute to metabolic diseases' development.

**Keywords:** bacterial diversity; gut microbiota; prolactin receptor; lactation; weaning

## 1. Introduction

Trillions of microbial cells, located in the intestinal compartment known as the "colonic microbiota", develop during childhood and adulthood and play important roles in promoting the host´s health [1]. Several factors, such as birth mode (vaginal or cesarean) and feeding method (breast milk or formula), influence the composition of the gut microbiota [2]. Cesarean-born neonates have higher bacterial diversity than those born vaginally but are more prone to developing asthma, allergies, and obesity. This has been attributed, at least in part, to the gradual replacement of the *Bacteroides* genus, which helps regulate the immune system, by the *Firmicutes* genus in the first year of life [3]. Moreover, formula-fed children have less bacterial diversity and richness in the first 12-24 months than those fed breast milk, with lower levels of *Lactobacillus, Staphylococcus, Megasphaera,* and Actinobacteria [3].

Within the main microbial reservoirs that sustain the early neonate's colonization, maternal milk is considered the second most abundant source after the mother's areolar skin, contributing $8\times10^5$ bacteria daily during lactation, particularly *Lactobacillus, Bifidobacterium, Staphylococcus spp.,* and *Streptococcus* [4]. Milk components, such as milk oligosaccharides, serve as an energy source for selected bacterial populations, which in turn produce short-chain fatty acids (SCFAs) and other metabolites that act as protectants against pathogens such as *Streptococcus pneumoniae* or *Campylobacter jejuni* [5].

However, not only are microbiota and macromolecules in milk delivered from the mother to infants, but so are immune components, including immune cells, regulatory cells, and antibodies [6];

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and hormones like prolactin (PRL), a protein that is known for its stimulatory effect on milk secretion and exerts a wide range of metabolic and immune actions [7]. Although PRL levels change depending on sex and physiopathological status, it has been recognized that the maintenance of a metabolically beneficial PRL level (HomeoFIT PRL: 7-100 μg/L) [8] could prevent metabolic diseases development. Whereas too low and too high PRL levels associate with increased prevalence of metabolic diseases [7,8]. During lactation, obesity is associated with reduced PRL action since the obese condition lowers the expression of PRL receptors (PRLRs) in mammary epithelial cells, hindering PRL signaling and causing a reduction in the production of milk components like β−casein and α−lactalbumin, leading to lactation insufficiency and precocious mammary gland involution [9]. Additionally, high-fat diet (HFD) feeding in rats reduces PRL levels in maternal milk, and their pups consuming obesogenic and hypoprolactinemic milk develop exacerbated adiposity, fatty liver, and insulin resistance at weaning, whereas PRL administration to the HFD-fed mothers or directly to the pups ameliorates those metabolic alterations [10]. PRL treatment in HFD-fed lactating rat dams normalized mammary gland function and restored milk yield and PRL levels [10]. Therefore, PRL in maternal milk favors metabolic homeostasis in the offspring, whereas lack of adequate PRL actions derived from an obesogenic environment contribute to the development of metabolic diseases.

Previous studies suggested that communication between the endocrine system and microorganisms regulates the host's hormonal homeostasis [11]. For instance, germ-free rats have 25% higher PRL levels than gnotobiotic (standard microbiota) animals [12]. Moreover, a 24 h SCFAs treatment can inhibit *Prl* expression in primary dairy cow anterior pituitary cells (DCAPCs) *in vitro* [13], supporting the idea that bacterial metabolites can influence endocrine factors. However, there are no reports about the relationship between the absence of PRLR signaling and gut microbiota composition in neonates, particularly during the weaning period, which is a critical time frame for producing microbial adaptations that shape the neonates' health and prevent the development of chronic conditions [14]. Altered microbiota has been implicated in several metabolic diseases such as cardiovascular diseases, obesity, and type 2 diabetes [15], and microbiota transplantation has been shown to reverse obesity, type 2 diabetes, or being used in the treatment of persistent and severe infections due to *C. difficile* [16].

Since maternal milk is a key regulator of gut microbiota composition, and microbiota protects against the development of metabolic diseases, in this work, we investigated whether PRL/PRL receptor signaling regulates gut microbiota composition in newborn mice at the end of lactation. Our hypothesis was that the absence of PRLR induces changes in gut microbiota diversity and composition, promoting a microbial profile potentially linked to an increased risk of developing metabolic diseases.

## 2. Materials and Methods

### *2.1. Animal husbandry, care, and macroscopic measurements*

All animals were housed under standard laboratory conditions (12/12 h light-dark cycle, 20 ℃, and 40-50% relative humidity). The animals were cared for following the guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The experiments conducted were approved by the Bioethics Committee of the Institute of Neurobiology of the National Autonomous University of Mexico (ID: 075). All mice were fed a standard diet with pellets from Rodent Lab Chow 5001 (Purina, St. Louis, MO, USA).

Male and female C57BL/6J *Prlr* +/- mice from The Jackson Laboratory were bred and maintained for several generations in the Vivarium of the Institute of Neurobiology of the National Autonomous University of Mexico (UNAM). After birth, litter sizes were adjusted to 6-8 animals, and male and female *Prlr* +/+ or *Prlr* -/- pups (n: 15 pups/group) were maintained for 21 days until the lactation period ended. The pups were then anesthetized by $CO_2$ inhalation and euthanized by decapitation. The pups' body weight was measured every two days starting from the fifth day of birth. The pups' colon was excised and weighed.

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*2.2. Fecal and cecal DNA extraction and quality control*

Before conducting the extractions, to avoid contamination, the 21-day old pups were placed in cages containing clean sawdust previously irradiated with UV for 15 min in a standard biosafety level 1 cabinet. Surfaces were cleaned with 70% ethanol. The fecal and cecal contents (200-300 mg) were extracted from the euthanized pups (n=3/tube) and stored in DNA/RNA shield collection tubes (R1101, Zymo Research Corp., Irvine, CA, USA) at -80 ºC. Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep kit (D4300, Zymo Research Corp.) following the manufacturer's instructions. Once extracted, the DNA samples were quantified in a NanoDrop 1000 Spectrophotometer (Thermo Fisher, Waltham, MA, USA), and electrophoretic running was conducted in a 1.5% agarose gel for 30 min for 1:10 diluted samples to verify DNA integrity.

*2.3. Library preparation, 16S rRNA sequencing, and diversity index analyses*

A total of five samples per group (each sample being a pool of DNA from three mice) were diluted in sterile water with a concentration of 20 μg/μL. Samples were then processed and analyzed by ZymoBIOMICS' Targeted Metagenomics Sequencing (Zymo Research) service. The DNA samples were prepared for sequencing with the Quick-16S™ NGS Library Pep Kit (Zymo Research) and the V3-V4 Primer Set. The sequencing library was prepared through real-time PCR reactions to quantify pooled qPCR readings based on equal molarity. The library was then cleaned up with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research) and quantified with TapeStation® (Agilent Technologies, Santa Clara, CA, USA) and Qubit® (Thermo Fisher). The ZymoBIOMICS® Microbial Community Standard (Zymo Research) was used as a positive control for each targeted library preparation. The final library was sequenced on Illumina® MiSeq™ with a V3 reagent kit (600 cycles) and a 10% PhiX spike-in. The unique amplicon sequences were identified from the raw reads, and the chimeric sequences were removed using the DADA2 pipeline [17]. The taxonomy assignment was performed using Uclust from Qiime v. 1.9.1, following the Zymo Research 16S database. Composition visualization, α-diversity, and β-diversity analyses were also performed in Qiime v. 1.9.1 [18]. A quantitative real-time PCR was set up to quantify the absolute abundance, with a standard curve made with plasmid DNA containing one copy of the *16S* gene in 10-fold serial dilutions. The same primers used in the Targeted Library Preparation were used. The number of genome copies per DNA sample was calculated by dividing the gene copy number by an assumed number of gene copies per genome. The amount of DNA per microliter of sample was calculated using an assumed genome size of $4.64\times10^6$ bp, the genome size of *Escherichia coli*. A two-dimensional Principal Coordinate Analysis (PCoA) was conducted for the visual hierarchical clustering and community ordination using the web-based tool MicrobiomeAnalyst [19]. For the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to predict metabolic pathways [20], the whole genomes of the annotated species were searched using the Kyoto Encyclopedia of Genes and Genomes (KEGG) genome database (https://www.genome.jp/kegg/ko.html) and MicrobiomeAnalyst.

*2.4. Statistical analysis*

Except for the microbiota composition, where data is presented as the media ± SD of five values (each representing three animals), all other data are presented as the media ± SD of 15 mice/group. After assessing the normality of data using normal distribution, normal quantile plots, and the Shapiro-Wilk's test, an Analysis of Variance (ANOVA) followed by a post-hoc Kruskal-Wallis multiple test were conducted to assess differences, with a cut-off p-value < 0.05 or 0.01. GraphPad Prism v. 8.2 (Dotmatics, Boston, MA, USA) and MicrobiomeAnalyst were used to plot data, and JMP v. 16.0 (SAS, Cary, NC, USA) was used to perform the statistical analysis.

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## 3. Results

### *3.1. Body weight and macroscopic measurements*

Macroscopic characteristics of wild-type (*Prlr* +/+, *Prlr*-WT) and knockout (*Prlr* -/-, Prlr-KO) mice showed no differences in body weight evolution (Figure 1A) during lactation. However, KO mice displayed a higher normalized colon weight ($p<0.01$) (Figure 1B) and a reduced cecal weight ($p<0.05$) (Figure 1C) compared to WT mice.

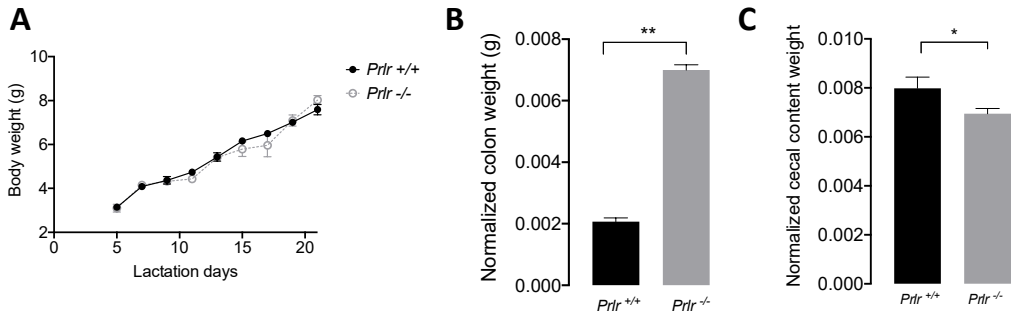


**Figure 1.** Macroscopic measurements in pups. (A) Body weight evolution during lactation. (B) Normalized colon weight to body weight, and (C) Normalized cecal content weight to body weight. Data were presented as the mean ± SD of 15 mice. The asterisks indicate significant differences (* $p<0.05$; ** $p<0.01$) according to the Kruskal-Wallis test.

### *3.2. General microbial diversity analysis from weaned Prlr-WT and Prlr-KO mice*

The α-diversity of the bacterial composition showed that both mouse genotypes shared 522 amplicon sequence variants (ASVs) (Figure 2), but *Prlr*-KO pups displayed a higher number of unshared ASV (+2.96%) than *Prlr*-WT animals (Figure 2A). Although no differences were found for the Shannon index (Figure 2B), *Prlr* +/+ pups exhibited a higher ($p<0.01$) Simpson Evenness (Figure 2C) and phylogenetic diversity (Figure 2D) than *Prlr* -/- pups. The Shannon index is a quantitative indicator of the number of different bacteria in the samples, indicating that higher Shannon indexes equal increased community diversity [21]. On the other hand, the Simpson Evenness index indicates the probability that individuals will belong to the same species, and a high Simpson Evenness value indicates a less diverse bacterial community [21,22].

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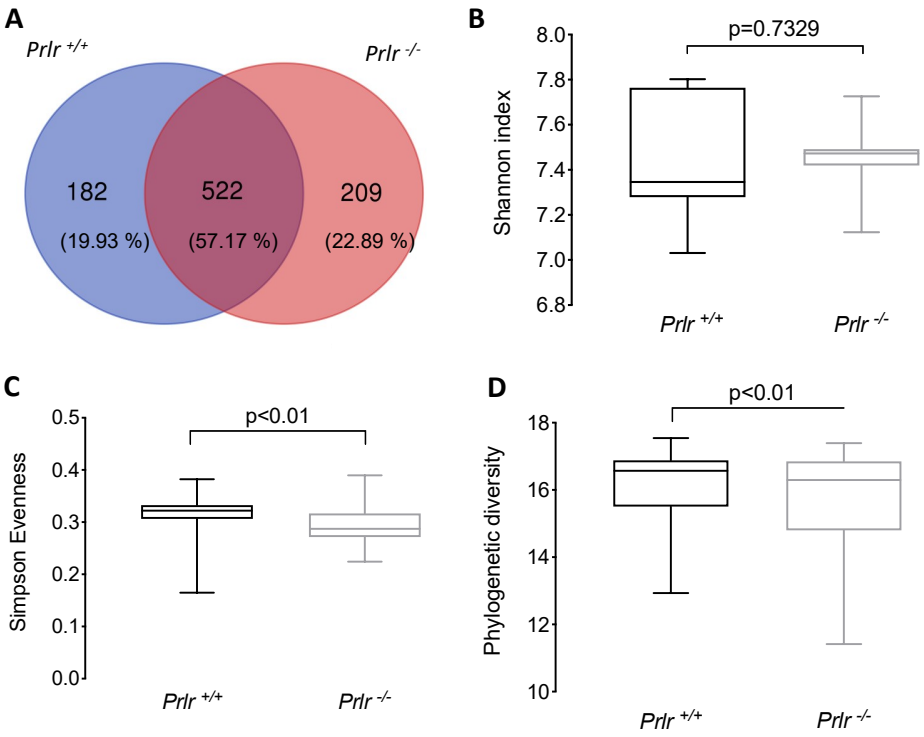


**Figure 2.** Alpha-diversity of the bacterial composition of weaned 21-day-old pups. (A) Venn diagram of the shared and unshared amplicon sequence variants (ASVs) between the groups; (B) Shannon index; (C) Simpson evenness; (D) Faith's phylogenetic diversity. Data (B-D) was presented as the mean ± SD of five samples (three mice each). Differences were assessed through a Kruskal-Wallis test.

Although there were no differences in the genotype clustering for the β-diversity (Figure 3) using a Permutational Analysis of Variance (PERMANOVA), two differentiated clusters were shown for each genotype, where *Prlr*-KO mice variation was contained within *Prlr*-WT.

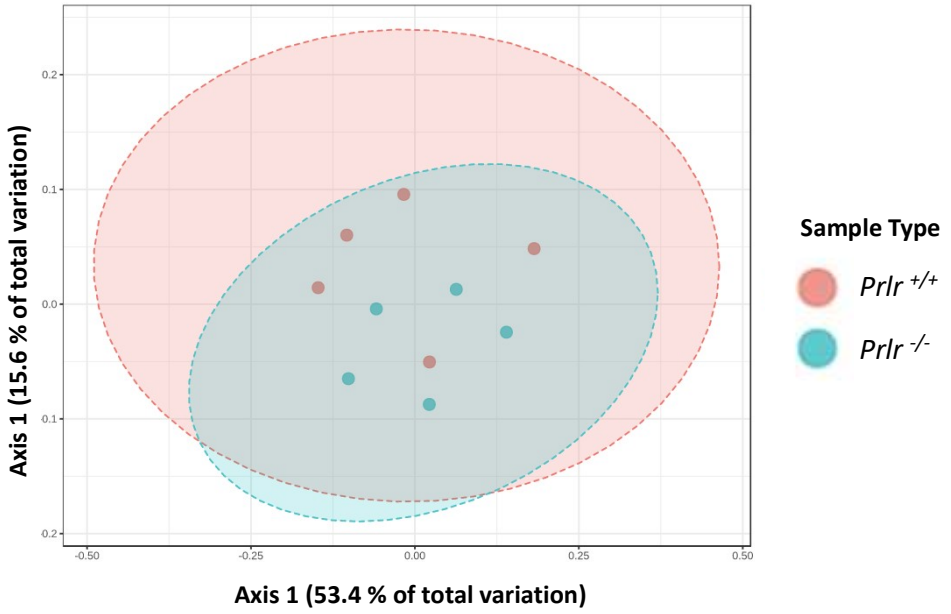


**Figure 3.** Bray–Curtis distance matrix PCoA plot. Each dot represents the pooled data from three mice (n=15/group). Analysis was conducted considering the species diversity, and data were not significant (p=0.075) based on a Permutational Analysis of Variance (PERMANOVA) analysis (p>0.05).

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*3.3. Taxonomical bacterial composition*

Next, we evaluated the relative abundance of phylum (Figure 4A), Class (Figure 4B), Order (Figure 4C), and Family (Figure 4D) distribution between the pups' genotypes. Only the Betaproteobacteria class (Figure 4E), Burkhoderiales order (Figure 4F), *Alcaligenaceae* family (Figure 4G), and *Anaerotruncus* genus (Figure 4H) were different between genotypes ($p<0.05$). Overall, 9 phyla, 14 classes, 14 orders, and 23 families were found.

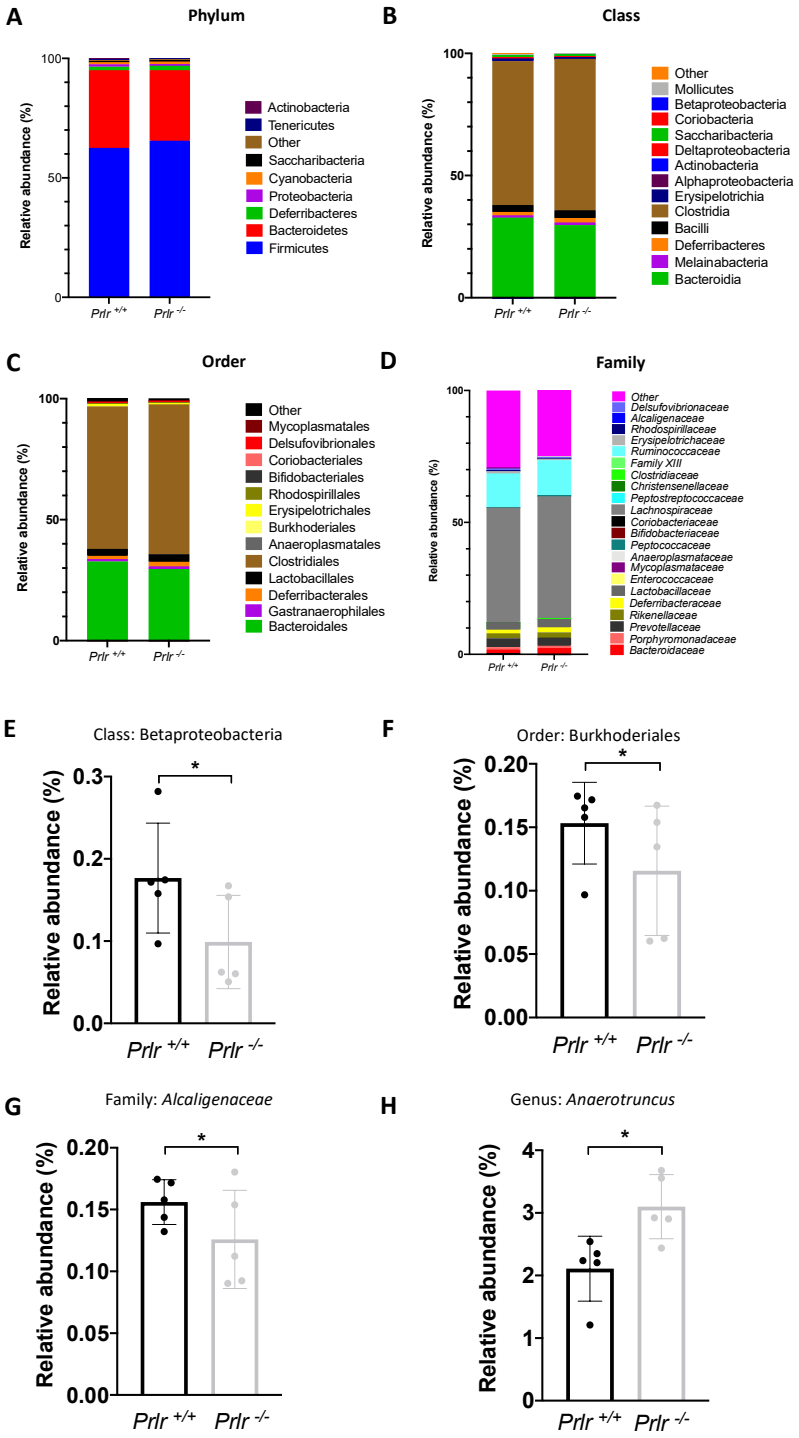


**Figure 4.** Taxonomic bacterial composition of the groups (21-day-old mice). Relative abundance of phylum (A), class (B), order (C), and family (D). Significantly different ($p<0.05$) class (E), order (F), family (G), and genus (H). Data was presented as the mean ± SD of five samples (three mice each). Differences were assessed through the Kruskal-Wallis test (* $p<0.05$).

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Out of 469 species, 11 species were different between genotypes (Figure 5). Although the identified species were unclassified according to the taxonomic database used, their known orders, families, or genus were placed accordingly. *Prlr* +/+ pups had greater abundance of species coming from the Bacteroidales and Clostridiales orders and the *Anaerotruncus* and *Ruminoclostridium* genera. On the other hand, *Prlr* -/- pups had the lowest abundance of species from the *Lachnospiraceae* family and the *Roseburia* genus.

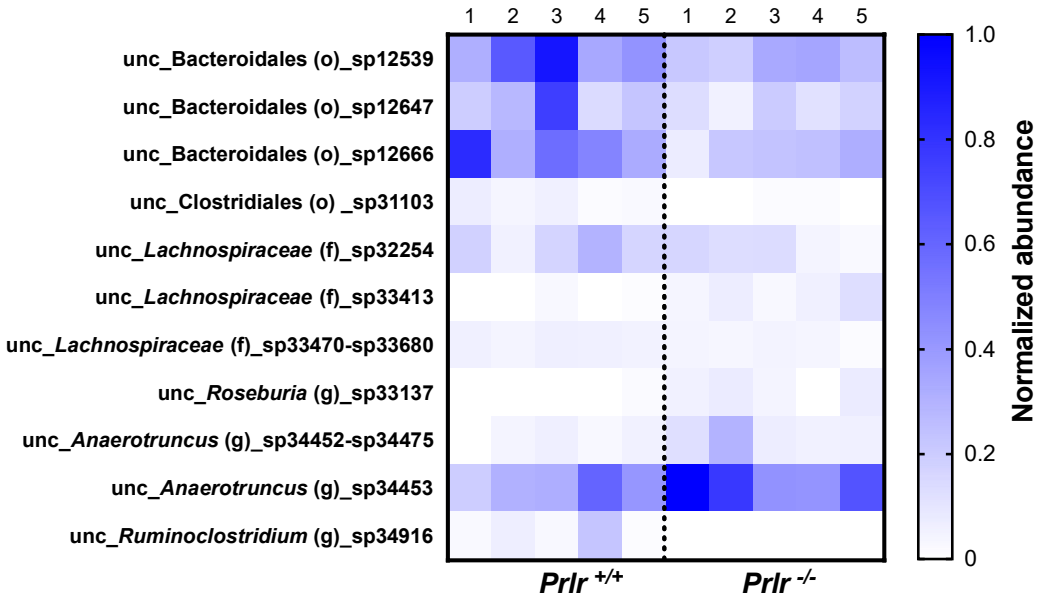


**Figure 5.** Significantly different bacterial species between 21-day-old pups of each group (five samples from each group were taken, each representing three mice). The values were presented in normalized abundance after a min-max normalization [(sample-min)/(max-min)]. F: family; G: genus; O: Order; UNC: unclassified.

### *3.4. PICRUSt metabolic prediction*

A PICRUSt metabolic prediction was performed based on the species classification obtained from the pups' genotypes (Figure 6). The prediction highlighted 11 probable metabolic pathways, but only the metabolism of terpenoids and polyketides was found to be different ($p<0.05$) (Figure 6A). A PCoA analysis showed differences in the overall metabolic performance of the genotypes ($p<0.05$), with *Prlr* +/+ samples being more like each other compared to *Prlr* -/- samples.

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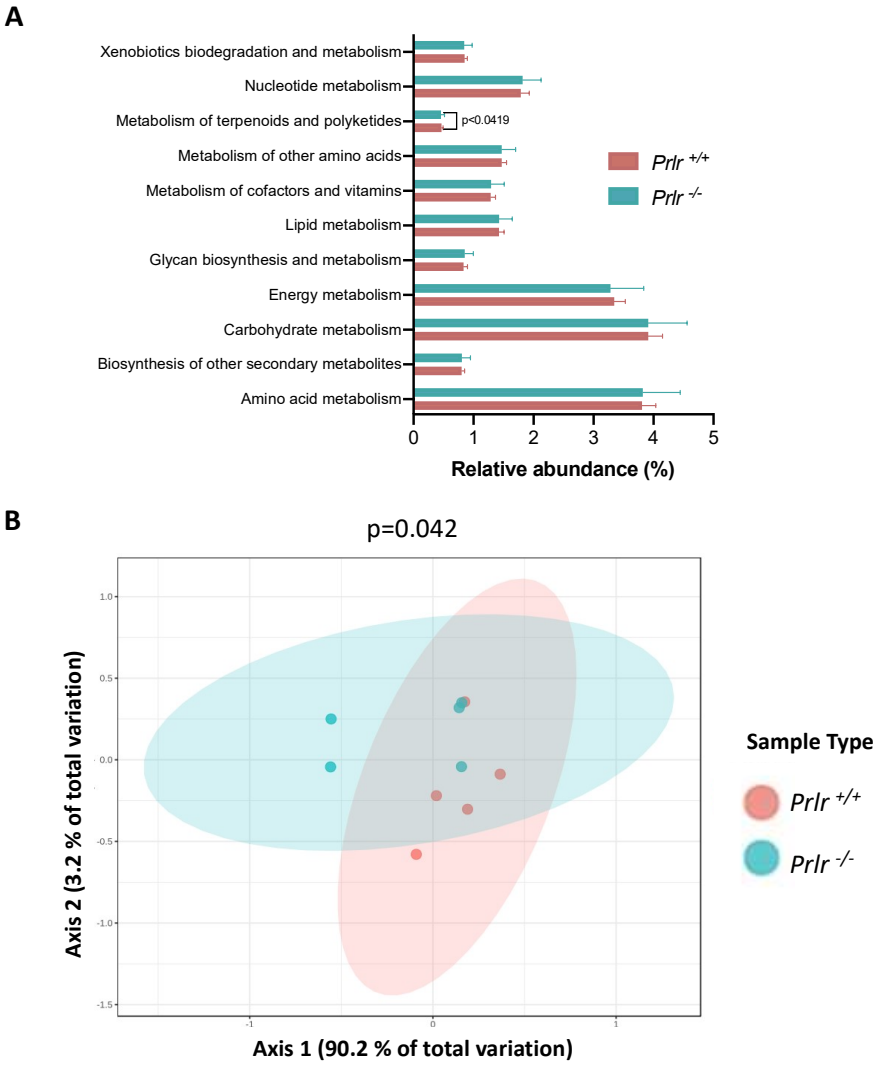


**Figure 6.** PICRUSt metabolic prediction based on species composition from 21-day-old, weaned mice. (A) Relative metabolic abundance for each group; (B) PCoA analysis of the metabolic performance. Data in (A) was presented as the mean ± SD of 469 identified species. Differences in (A) were evaluated through the Kruskal-Wallis test ($p<0.05$). Differences in (B) were assessed using PERMANOVA ($p<0.05$). Each dot in (B) represents three mice.

## 4. Discussion

This research was intended to assess the impact of the absence of PRL/PRL receptor signaling in gut microbiota development on mice at weaning. The evaluation was conducted in a well-established PRLR knockout mouse model, originally created through gene targeting in 129svj [23] and later on C57BL/6 mice, where a 1.5 kb fragment of the targeting vector containing exon 5 was replaced with the similarly sized thymidine-neomycin (Tk-NO) cassette, resulting in an in-frame stop codon mutation [21]. Immunological characterization of the *Prlr*-KO mice has indicated that these mice do not have a defective hematopoietic system [24] and mice are capable of normal humoral and cell-mediated immune responses after exposure to T-independent/dependent antigens [25,26]. Previous results from our research group have shown that *Prlr*-KO mice display slightly altered liver growth, with higher liver to body weight (LBW) ratio at 2 weeks of age but lower LBW ratio after 4 weeks of age, compared to WT mice [22,23]. Adult *Prlr*-KO mice (16-18 weeks) do not show any differences in the visceral and subcutaneous adipose tissue weight or adipocyte area, compared to their WT counterparts. However, when challenged by a high fat diet (HFD) feeding for 8 weeks, *Prlr*-KO adult mice showed increased adiposity, characterized by adipocyte hypertrophy, and exacerbated glucose

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intolerance and insulin resistance compared to HFD-fed WT mice [27]. Also, streptozotocin (STZ)-induced diabetes in *Prlr*-KO adult mice (5-7 weeks old) resulted in increased hyperglycemia and glucose intolerance ($p<0.05$), and lower insulin levels ($p>0.05$) than STZ-induced diabetic WT mice [24], whereas no abnormalities in glucose and insulin levels were observed in non-diabetic *Prlr*-KO mice compared to their WT counterparts. Thus, *Prlr*-KO mice show increased susceptibility to develop exacerbated metabolic diseases. Despite several metabolic and phenotypic parameters have been described in the *Prlr*-KO mice, there are no reports about gut microbiota characterization in this mouse model. The rationale for studying the impact of PRL/PRL receptor signaling on gut microbiota at weaning is that maternal milk is a key regulator of gut microbiota composition and gut microbiota is critical for metabolic homeostasis of the host. Prolactin is a component of maternal milk regulating metabolism in the offspring [10], and during lactation in rodents, maternal milk is the main source of prolactin, as pituitary prolactin secretion (the primary source of circulating prolactin) starts around weaning [28,29].

The time and order in which microbiota colonize the gut, as well as the nutrients/substrates they encounter, are critical and highly contribute to the variation of microbiota between individuals [30]. Breast milk and the alveolar skin provide an abundant number of microorganisms to neonates, and the proportion of breast milk intake and its replacement with solid foods has a significant impact on microbiota diversity, an event that has been proposed as the major driver in the development of gut microbiota in human adults [2]. During weaning, *Lactobacillaceae* are gradually lost, followed by the expansion of *Clostridiaceae* [14], which are critical bacterial families that prevent colonization by bacterial pathogens [31]. Since mouse microbiota in early life cannot protect the host against pathogen colonization, *Lactobacillaceae* presence is important, as this bacterial family effectively and directly inhibits pathogens, contributes to barrier maintenance, and modulates the host's immune system [32].

Along with microbiota and macro or micronutrients, breast milk delivers immune and endocrine factors that contribute to the neonates' nutrition and development. This supports the concept of breast milk as a biological system [33,34]. Among the endocrine factors, PRL is delivered via maternal milk from the mother to the offspring in humans [35] and rodents [10], and studies suggest it regulates neonatal metabolic homeostasis [10], which could involve the modulation of gut microbiota composition and diversity. Although the relationship between microbiota changes and PRL activity has been scarcely explored, variations in PRL levels are linked to metabolic changes potentially implicating microbiota dysbiosis, as this is one of the reasons involved in the development of cardiovascular diseases, obesity and type 2 diabetes [15]. Moreover, microbiota transplantation has proven to reverse the severity of metabolic diseases [16]. Few reports have explored the association between endocrine factors and microbiota, and "microbial endocrinology" has been proposed as an emergent research area to study host-microbe interactions and how microbiota impact physiological processes, including endocrine and immune function, which are critical to preventing the development of chronic NCDs [36]. Here, we evaluated the impact of the absence of PRLR-PRL signaling on the gut microbiota composition of mice at weaning.

Macroscopically, both WT and KO mice displayed similar body weight development throughout lactation. However, at weaning, the *Prlr*-KO mice showed significantly higher colon weight, which is an early indicator of a pro-inflammatory state [37]. Changes in microbiota diversity have been associated with stress during weaning due to ecological mechanisms after the change in nutrient supply or maturation of the immune system (e.g., maternal IgA replacement with endogenous IgA) [38]. Operational taxonomic units (OTUs) were originally used to group bacterial reads into clusters, considering the microbial sequence identity [39]. However, the use of denoising methods migrated to identify exact sequence variants or ASVs, allowing to distinguish between the predicted "true" biological variation and those probably generated by sequencing error, and even a single nucleotide variation is defined as a separated ASV [40]. Hence, ASV variations in the tested animals indicate exact sequence variations interpreted as a differential bacterial composition for each mouse genotype, suggesting that *Prlr* -/- mice had a higher abundance of unique members of the bacterial community, but this does not reveal the size of the populations (absolute abundances), as

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observed by a lower Simpson Evenness and Phylogenetic diversity index in *Prlr* -/- mice compared to *Prlr* +/+ mice [41]. Lower microbial diversity and richness are linked to adverse conditions such as increased gut permeability, NCDs (e.g., insulin resistance and obesity), and pro-inflammatory phenotypes [42]. Since PRL activity is absent in *Prlr*-KO animals, it could be feasible that an excess of milk-derived PRL in the colon could influence bacterial populations as both *Prlr*-WT and *Prlr*-KO mice were subjected to the same potential stressful conditions to the gut microbiota, but differential bacterial profiles could be observed at several taxonomical levels. Another possibility is that PRL is converted into smaller fragments in the intestinal milieu, such as vasoinhibins (Vi) [43], and that the effects observed in *Prlr*-KO mice are the result of both a lack of PRLR signaling and an excess of Vi signaling. Excess Vi could be generated in the intestinal lumen derived from the cleavage of milk PRL or in the circulation of the pups resulting from elevated PRL levels known to be present in *Prlr*-KO animals. High PRL levels arose from absent PRLRs, which normally exert a negative feedback loop, stimulating dopamine release and inhibiting PRL production and secretion [44].

The effect of the hormonal milieu on the gut bacterial composition of newborn Wistar rats was recently evaluated, showing that daily oral administration of leptin and adiponectin decreased the Proteobacteria phylum and *Blautia* genus [45]. However, since a natural Proteobacteria decrease is presented along with intestinal maturation, the tested adipokines could enhance this process [46]. Moreover, leptin administration decreased *Sutterella* and increased *Clostridium* genera, while adiponectin decreased *Roseburia* and increased *Enterococcus* genera [45]. In another study, leptin concentrations in the maternal milk of women with obesity did not impact the neonates' microbial diversity or composition, but a higher insulin concentration from the mothers' milk was correlated with increased taxonomic diversity, particularly with Gammaproteobacteria, and was inversely correlated with *Streptococcaceae* [47]. It has also been reported that several bacterial genera could metabolize hormones such as progesterone or estradiol [48], and detectable plasmatic levels of progesterone (6-10 ng/mL) and 17β-estradiol (20-40 pg/mL) have been found in female rat pups at weaning [49], suggesting that colonic hormonal composition might influence bacterial growth based on their metabolism. There are no reports on the impact of PRL on gut microbiota, but germ-free rats were found to contain 25% more PRL plasma levels than gnotobiotic animals [50]. Moreover, bacterial families such as *Lactobacillaceae* (*Lactobacillus gasseri, L. crispatus,* and *L. jenesnii*), *Peptostreptococcus, Bifidobacteriaceae* (*Bifidobacterium longum*), and *Streptococcaceae* (*Streptococcus agalactiae, Streptococcus anginosus*) have been found to successfully grow in human follicular fluid [51], which contain several hormones, such as progesterone and PRL [52], advocating for a potential interaction between microbiota and PRL.

Regarding the mechanisms explaining the successful bacterial growth under hormone treatment, it has been found that the absence of estrogen receptor β signaling could differentially impact the overall abundance of bacterial phyla (e.g., Proteobacteria, Bacteroidetes, and Firmicutes) or orders (e.g., Lactobacillales) [53]. Moreover, the reported ability of *Clostridium scidens* to metabolize small traces of bile acids that easily escape from the small intestine into the cecum to produce adverse secondary bile acids, deoxycholic acid, lithocholic acid, and even glucocorticoids acting as signaling hormones in bacteria agrees with a proposed hormonal influence on bacteria [54]. Other reports indicate that epinephrine and norepinephrine from the host activate the transcription of virulence genes and flagella regulation in enterohemorrhagic *E. coli.,* involving the participation of a histidine kinase sensor located at the bacterial surface [55].

Betaproteobacteria or its related order (Burkhoderiales) has been linked to NCD development in human adults. For instance, Betaproteobacteria abundance is increased in humans with type 2 diabetes [56]. Five-year-old infants resulting from hypertensive pregnancies showed fewer *Alcaligenaceae* and *Coriobacteriaceae* families than those from normotensive pregnancies [57]. The *Anaerotruncus* genus, which we found increased in *Prlr*-KO animals, is a butyrate-producing group of bacteria associated with obesity and correlates negatively with high carbohydrate-based diets but positively with total fat and the consumption of saturated fatty acids [58]. In rabbits, the *Anaerotruncus* genus was found to be negatively correlated with weaning weight [59].

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Particularly for the differentially found species, the significant abundance of *Lachnospiraceae* species in the *Prlr*-WT mice could predict better intestinal health compared to *Prlr*-KO mice since *Lachnospiraceae* are largely believed to be health-promoting species critical to maintaining the colonic tissue due to their ability to produce SCFAs, the primary nutrition source for colonocytes [60], and known metabolites to decrease pro-inflammatory factors, protect the colonic mucosa, and inhibit NLRP3 inflammasome activation and reactive oxygen species (ROS) production [61]. Increased *Lachnospiraceae* abundance in 2-9–week-old children is considered one of the collective microbiota characteristics of appropriate growth after birth, together with augmented microbial diversity, a higher abundance of *Streptococcus* and strictly aerobic taxa, and a decrease in *Staphylococcus* and *Enterobacteriaceae* abundances [62].

The predicted functional analysis is consistent with maternal milk harboring bacteria linked to carbohydrates, amino acids, and energy metabolism [63]. Although reports are scarce, a recent article indicated that early exposure of neonates to elevated leptin and insulin concentrations from the maternal milk of women with obesity could impact the neonates' metagenomic profile, as high leptin concentrations are inversely correlated with bacterial amino acids, carbohydrates, vitamins, and amino acid metabolism [64]. Products such as polyketides, alkaloids, and terpenoids are only derived from plants and can be formed by microorganisms [65]. Most terpenoids are terpene derivatives not encoded by microbiome genomes but represent microbial metabolites from dietary products or bile acid derivatives, and some of them are the result of the activity of oxidizing enzymes, such as terpene cyclases or synthases, and the addition of carbohydrates, amino acids, and fatty acid chains into polycyclic terpene backbones [66,67]. Some terpenes are widely synthesized by Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes and display a wide range of biological properties, but little information regarding their colonic biosynthesis and effect has been reported [68]. On the other hand, polyketides are secondary metabolites produced mainly by Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes, exhibiting antimicrobial properties against selected populations [69].

## 5. Conclusions

The results obtained in this research suggest that the absence of PRLR signaling could promote a higher abundance of gut microbiota potentially linked to NCDs during weaning. Both *Prlr*-WT and *Prlr*-KO weaned mice shared similarities in bacterial diversity, taxonomic composition, and metabolic functionalities, but *Prlr*-KO mice displayed differential bacterial species that could predispose mice to adverse disease conditions. Although it is challenging to establish a mechanism of action based on the results presented in this research, the fact that lack of prolactin receptor signaling derives in altered gut microbiota at a key developmental time point, opens new research avenues that merit additional research. Moreover, these results support the hypothesis that an altered microbiota profile in *Prlr*-KO mice contribute to their observed susceptibility to develop aggravated metabolic diseases.

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