Casein and red meat proteins differentially affect the composition of the gut microbiota in weaning rats

Fan Zhao [a], Chong Wang [a,b], Shangxin Song [c], Chao Fang [d], Guanghong Zhou [b], Chunbao Li [b,*], Karsten Kristiansen [a,d,e,*]

[a] *Laboratory of Genomics and Molecular Biomedicine, Department of Biology, University of Copenhagen, Copenhagen 2100, Denmark*
[b] *Key Laboratory of Meat Processing and Quality Control, MOE, Key Laboratory of Meat Processing, MARA, Jiangsu Collaborative Innovation Centre of Meat Production and Processing, Quality and Safety Control, College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, PR China*
[c] *School of Food Science, Nanjing Xiaozhuang University, 3601 Hongjing Road, Nanjing 211171, PR China*
[d] *BGI-Shenzhen, Shenzhen 518083, PR China*
[e] *Institute of Metagenomics, Qingdao-Europe Advanced Institute for Life Sciences, BGI-Qingdao, Qingdao 166555, PR China*

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ABSTRACT

Casein and meat are food sources providing high-quality animal proteins for human consumption. However, little is known concerning potentially different effects of these animal protein sources during early stages of life. In the present study, casein and red meat proteins (beef and pork) were fed to young postweaning rats for 14 days based on the AIN-93G diet formula. Casein and red meat protein-based diets did not differentially affect the overall growth performance. However, they discriminately modulated the abundances of different potentially beneficial bacteria belonging to genus *Lactobacillus*. Intake of the casein-based diet increased the intestinal abundance of *Lactococcus lactis* with a pronounced potential for galactose utilization via the Tag6P pathway, and it also resulted in lower amounts of toxic ammonia in the rat cecum compared to red meat protein-based diets. We observed no adverse effects on colonic tissue in response to any of the protein-based diets based on histological observations.

1. Introduction

The diets consumed after weaning may have long-term effects on the development and health of infants by affecting the early establishment of the gut microbiota (Agostoni et al., 2019; Zhong et al., 2019). Following weaning and up to about 3–5 years of age, the microbiota of infants becomes more diverse following different trajectories with different individual dynamics sensitive to disturbances, and therefore this early period is crucial for the development of the gut microbiota with potential impact on long-term health (Roswall et al., 2021).

Consumption of high-quality animal protein has been reported to be associated with improved human nutrition, growth, development, and health (Wu, 2022). Bovine milk protein (with casein as a main ingredient) is the source of protein in most formula used before and after weaning (Zeng et al., 2020). The casein is rich in essential amino acids and gives rise to a variety of bioactive peptides upon hydrolysis by appropriate proteases (Guo et al., 2021). Some of these peptides have

been reported to modulate the composition of the gut microbiota (Miclo et al., 2012). However, about 2%–7.5% young individuals suffer from cow's milk protein allergy (Solinas, Corpino, Maccioni, & Pelosi, 2010), warranting the identification of proper replacements.

Beef and pork, as types of red meat, contain important micronutrients and are considered as sources of high-quality proteins (Williams, 2007). Although epidemiological studies have shown that high intake of red meat increases the risk of inflammatory bowel disease (IBD) (Li, Cui, Tan, Liu, & Yao, 2021) and colorectal cancer (Loke, Chew, Ngeow, Lim, & Peh, 2020), it still remains to be demonstrated whether more moderate intake of such proteins impacts negatively on health. Proteins from red meat and dairy are commonly included in infants' diets. Main differences between casein and red meat proteins include the content of branch chain amino acids (valine, leucine and isoleucine) with higher content being present casein and the slow release of casein from the stomach. (Liisberg et al., 2016). A recent study reported that red meat-based formula-fed infants showed higher length-for-age Z

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scores (LAZ) than those of diary-based formula-fed infants (Tang, Hendricks, & Krebs, 2018). However, the long-term effects of red meat-based formula and dairy-based formula on health in well-nourished infants remain to be elucidated.

Study on rodent model has compared the differential effects of animal-based (meat and dairy) and plant-based (soy) diets on growth, physiology, and the gut microbiota (Zhu et al., 2015), but less attention has been paid to the potentially different effects of diary and red meat, especially during the early important period after weaning.

As a first step in the present study, weaning Sprague Dawley (SD) rats were fed AIN-93G diets prepared with either casein or beef/pork proteins for 14 days. The objective was to compare the effects of casein and red meat proteins on the gut microbial composition and functional potential as well as the corresponding potential effects on host physiology in early development.

2. Material and methods

2.1. Diets and animals

Pork *longissimus dorsi* muscle and beef *longissimus dorsi* muscle were obtained from a commercial meat packing company (Sushi Group, Jiangsu, China). Meat was individually cooked in a 72 ℃ water bath till a central temperature of 70 ℃. During cooking, the central temperature was monitored by a digital thermometer fitted with a thin temperature probe (RM-113, Ruiming, Changzhou, China). Cooked meat was ground, freeze-dried and then ground again to obtain meat powder. Meat powder was passed through a 25-mesh sieve. Fat was removed by extraction with a mixture of methylene chloride/methanol (V/V = 2:1). Feed with casein, beef protein, or pork protein was obtained from Jiangsu-Xietong, Inc. (Jiangning, Nanjing, China) based on the AIN-93G formula (Reeves, Nielsen, & Fahey, 1993). The formulations were as follows: isolated proteins from pork or beef providing 17% protein content, 39.75% cornstarch, 13.20% sucrose, 7.00% soybean oil, 5.00% fiber, 3.50% mineral mix, 1.00% vitamin mix (AIN-93-VX), 0.3% L-cystine, 0.25% choline bitartrate and 0.0014% *tert*-butylhydroquinone. The diet composition is listed in Supplementary Table S1.

Animal experimental protocols were approved by the Animal Care Committee of Nanjing Agricultural University in accordance with the National Guidelines for Experimental Animal Welfare (MOST of People's Republic of China, 2006). Three-weeks old Male SD rats were obtained from Shanghai Laboratory Animal Research Center (Shanghai, China) and two rats were housed per cage with free access to diet and water in a specific pathogen free (SPF) animal facility (Permission ID: SYXK(Su)2011–0037) with a 12-h light–dark cycle. After 7-days acclimatization with the AIN-93G standard diet, the rats were assigned randomly to the three formulated diets: the AIN-93 standard diet, the pork protein-based AIN-93G diet, and the beef protein-based AIN-93G diet for 14 days. On days 2, 7 and 14, 10 rats in each diet group were decapitated after 4-h fasting (n = 10 rats per group per time point). Cecal content was carefully collected in sterilized tubes and stored at -80 ℃ for shotgun metagenomic sequencing analyses.

2.2. Metagenomic sequencing

DNA in the cecal samples was extracted using the NucleoSpin™®Soil kit (MN, Düren, Germany) as previously reported (Xiao et al., 2017). The DNA concentration was estimated by Qubit measurements (Invitrogen, USA).

2.2.1. Library preparation and sequencing

The extracted DNA was subjected to ultrasonic random fragmentation, end-repair, and adaptor ligation for DNA nanoball-based library construction. Then combined primer anchor synthesis-based shotgun metagenomic sequencing was applied using paired-end 150 bp mode on the DIPSEQ platform (Fang et al., 2018).

2.2.2. Data processing

The adaptor and low-quality reads were eliminated from the raw reads and the quality was assessed by Phred quality score. Briefly, the following steps were performed. Reads containing > 3 "N" bases were removed. Contiguous bases were counted from the 3'-end of a read and those with a quality value < 20 were trimmed. The reads with a minimum length of 30 bp PE reads were kept. The remaining reads were then filtered to eliminate host DNA based on the rat reference genome (rn6) using SOAP2.22 (SOAPaligner/soap2, RRID:SCR 005503) (identity ≥ 0.9) as described previously (Fang et al., 2018; Li et al., 2009). The retained clean reads were aligned to the published gene catalog of the rat gut metagenome using SOAP2.22 (Pan et al., 2018).

2.2.3. Species composition and abundance analysis

Reads count assigned to genes were normalized. This gene profile was then classified at different taxonomic levels (including phylum, genus, and species) according to the annotations provided in the catalog of the rat gut metagenome (Pan et al., 2018). Wilcoxon rank-sum test was applied for differential abundance analysis using the R software. The Benjamini-Hochberg method was used for multiple testing correction (Benjamini & Hochberg, 1995), with a cutoff for adjusted P value at 0.05.

2.2.4. Gene function analysis

The gene profile was further classified at the KEGG Orthology (KO) level according to the catalog annotation. Wilcoxon rank-sum test was applied for differential analysis of KOs based on comparison of gene abundance profiles between any of the two dietary groups. Generally applicable gene-set enrichment (GAGE) was applied to evaluate changes in annotated gene related to metabolic processes (Luo, Friedman, Shedden, Hankenson, & Woolf, 2009). Gene sets were retrieved from the expert curated KEGG pathway database (https://www.genome.jp/kegg/) using the R software (gage package, version 4.1).

The α-diversity (within-sample diversity) was calculated using the Shannon index depending on the gene abundances at the phylum, genus and species levels. Gut microbial dissimilarities between groups were visualized by principal coordinate analysis (PCoA) using Bray-Curtis dissimilarities based on phylum, genus, species and KO abundance profiles, respectively (vegan package, version 2.5-7, R software).

2.3. Determination of short chain fatty acids and ammonia nitrogen

Acetic, propionic, butyric, isobutyric, valeric and isovaleric acids were detected by gas chromatography as previously described (Zhao, Nyman, & Åke Jönsson, 2006). The samples were analyzed using a Thermo Ultra trace gas chromatography system (Thermo Fisher Scientific, USA) equipped with a flame ionization detector (FID). A fused-silica capillary column with a free fatty acid phase (Inno-Wax, Agilent Technologies Inc., USA) of 30 m × 0.25 mm i.d. coated with 0.25 μm film thickness was applied. Ammonia nitrogen (NH_3-N) was determined by the phenol-hypochlorite method of Broderick and Kang (Broderick & Kang, 1980).

2.4. Histological observations

Colonic segments (1 cm long in the middle of colon) were cut and fixed in 10% formalin buffer for 24 h. The tissues were washed in distilled water (dH_2O), dehydrated in graded alcohol, embedded in paraffin, and transversely sliced into 4 μm thick sections. Sections were stained with hematoxylin and eosin (H&E). The sections from day 7 were stained with Alcian blue/periodic acid-Schiff (AB-PAS). The images of tissues were captured by a light microscope (ZEISS Axio Imager 2, Oberkochen, Germany). The mucosa depth and goblet cells were recorded, and 10 visual fields of each tissue sample were randomly selected for statistics analysis.

2.5. Statistical analysis

The effects of diet and feeding time on body weight, feed intake, feed efficiency, body length and mucosa depth were evaluated by two-way ANOVA analysis of variance in which protein diet and time point were set as independent, and the measured variables were set as the dependent. The effects of diets on all the other measured variables were evaluated by Wilcoxon rank-sum test. A p-value < 0.05 was considered significant.

3. Results

3.1. Effect of dietary proteins on the growth performance of young rats

The body weight of rats fed all three different protein diets consistently increased during the 14 days feeding period. Compared with the casein-based diet, rats fed a beef protein-based diet showed significantly higher accumulated body weight gain from day 6 to day 14 (P < 0.05), while rats fed a pork protein-based diet showed a significant difference from day 10 (P < 0.05, Supplementary Fig. S1A). The overall accumulated feed intake was found to be highest in the beef protein-based diet group, and lowest in the casein-based diet group. Significant differences were observed between the beef protein-based diet and casein-based diet groups (P < 0.05), and between the beef protein-based diet and pork protein-based diet groups (P < 0.05) from day 4 to day 14 (Supplementary Fig. S1B). The accumulated feed intake did not differ significantly between the pork protein-based diet group and the casein-based diet group until day 10 (P < 0.05). However, significant differences in feed efficiency were only observed in the first two days, where rats fed the pork protein-based diet showed a higher feed efficiency compared to rat fed the other two protein-based diets (P < 0.05, Supplementary Fig. S1C). Body length during the feeding period was also measured. Although rats fed beef protein-based diet and pork protein-based diet tended to show a higher body length than rats fed the casein-based diet on day 14, the differences did not reach a significant level (P > 0.05).
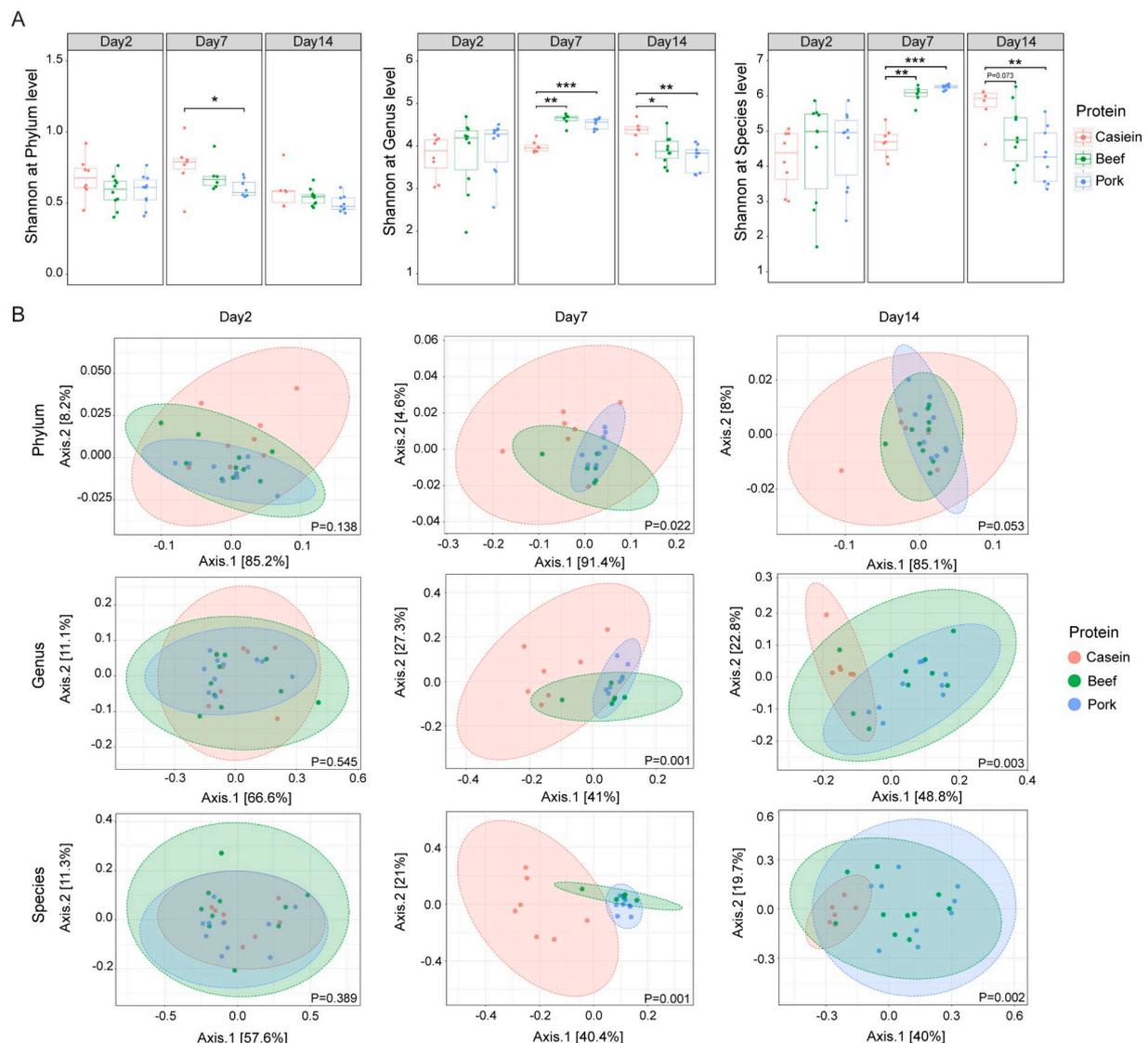

Fig. 1. Effects of intake of a casein-based diet, a beef protein-based diet, and a pork protein-based diet on α diversity (A), and β diversity (B) of the cecal microbiota at the phylum, genus and species levels, respectively. (A), n = 10 per group (outliers were removed in some groups). *: indicates significant differences between the corresponding groups at the indicated time point according to Wilcoxon rank-sum test with P < 0.05, **: P < 0.01, ***: P < 0.001. (B), principal coordinate analysis was performed based on Bray-Curtis dissimilarity at the phylum, genus, and species levels. Permutational multivariate analysis of variance was applied to assess the significance of effects of different dietary proteins on the microbial composition at each time points. P value is shown in the right bottom of each figure.

3.2. Effect of dietary casein and red meat proteins on the cecal microbiota of young rats during a short-term feeding period

Metagenomic sequencing yielded 2073.22 Gb of high-quality non-host data for the 74 samples with an average of 28.02 Gb per sample (Supplementary file 2). 99.13% of the raw reads remained as high-quality reads, which attained an average length of 134.8 bp. Gene identity and taxonomic classification were based on mapping onto the established rat gut metagenome catalog (Pan et al., 2018).

Based on the established rat catalog, we were able to identify a total of 34 phyla, 671 genera and 1404 species. α-diversity and β-diversity analyses were performed based on all three different taxonomic levels. Shannon index was selected to represent α-diversity. At the phylum level, the Shannon index remained relatively stable during the feeding

period in all three groups (Fig. 1A), except for a significant difference between the pork fed group and the casein fed group on day 7 (P < 0.05). At the genus and species level we observed that the Shannon index of the cecal metagenome of rats fed beef protein-based diet and pork protein-based diet interestingly exhibited a significantly higher Shannon index on day 7 (P < 0.05), contrasting a lower Shannon index on day 14 compared to the casein-based diet group (Fig. 1A). PCoA analysis using Bray-Curtis dissimilarity based on abundance profiles at the phylum, genus and species levels revealed no significant difference on day 2 (PERMANOVA, P > 0.05) (Fig. 1B). However, on day 7, we observed a significant separation of the beef protein-based and pork protein-based diet groups from the casein-based diet group at the phylum, genus, and species levels (PERMANOVA, P < 0.05). On day 14, the two red meat protein-based diet groups could still be distinguished from the
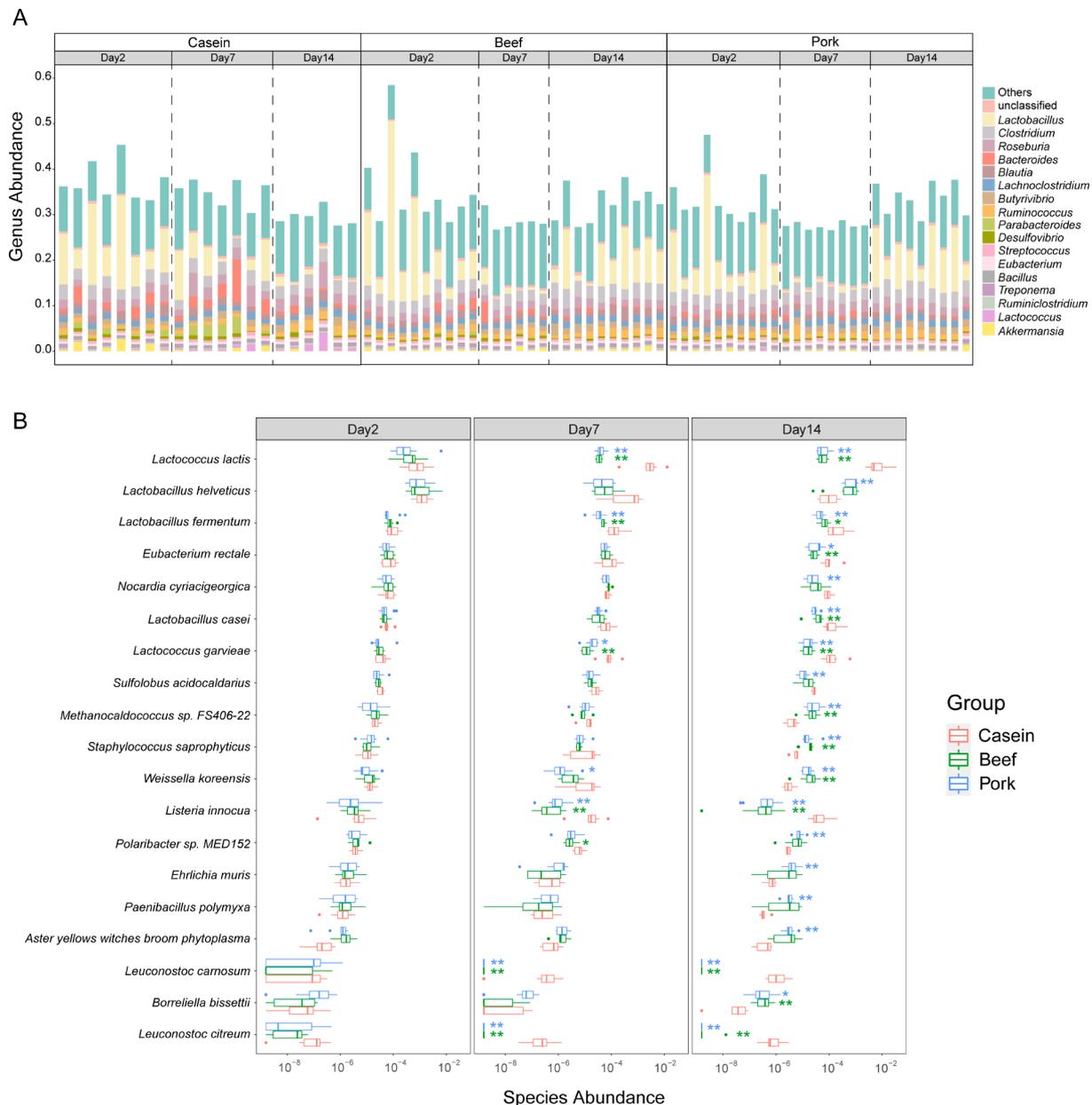

Fig. 2. Effects of intake of a casein-based diet, a beef protein-based diet, and a pork protein-based diet on the microbiota at the genus level (A), and the species level (B). (B), only species exhibiting different abundances on day 14 between rats fed the casein-based diet and the beef protein-based diet or between rats fed the casein-based diet and pork protein-based diet are shown in the figure. * in blue color represents significant differences between the casein-based diet and the beef protein-based diet. * in green color represents significant differences between the casein-based diet and the pork protein-based diet. Statistical analyses were performed by using Wilcoxon rank-sum test. * $P_{adj} < 0.1$, ** $P_{adj} < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

casein-based diet group at genus and species level (PERMANOVA, P < 0.05), but the effect of the different proteins seemed to diminish. We observed a high similarity of the cecal metagenome in rats fed the two red meat-based diets at all three taxonomic levels during the entire feeding period.

Among all the identified genera, zero, 240, and 13 genera differed significantly in abundances between the rats fed the beef protein-based diet and the casein-based diet on day 2, day 7 and day 14 respectively (P < 0.05, P_{adj} < 0.1). Five genera showed significant differences in abundances on both day 7 and day 14. Zero, 75, and 11 genera differed significantly in abundances between rats fed the pork protein-based diet and the casein-based diet (P < 0.05, P_{adj} < 0.1, Supplementary file 3), with two genera exhibiting significant differences in abundances on both day 7 and day 14. No significant differences in the abundances at the genus level were found comparing the beef protein-based diet and the pork protein-based diet groups. Of note, as shown in Fig. 2A, the abundance of *Lactobacillus* tended to decrease in rats fed the casein-based diet during the feeding period. By contrast, a sharp decrease in the abundance of *Lactobacillus* was observed in rats fed either the beef protein-based diet or the pork protein-based diet from day 2 to day 7,

surprisingly, followed by a recovery from day 7 to day 14. The abundance of *Parabacteroides* in the casein-based diet group was significantly higher than in the beef protein-based diet and the pork protein-based diet groups only on day 7 (P < 0.05, P_{adj} < 0.1). In addition, an increase in abundance of *Lactococcus* was observed in rats fed the casein-based diet, but not in rats fed the two red meat protein-base diets, which resulted in a significantly higher abundance in the casein-based diet group compared to the other two protein-based diet groups on day 7 and day 14.

Among all the identified species, zero, 365, and 43 species differed significantly in abundances between the beef protein-based diet and the casein-based diet groups on day 2, day 7 and day 14 (P < 0.05, P_{adj} < 0.1, Supplementary file 4), with 22 species found to differ significantly in abundance on both day 7 and day 14. Zero, 191, and 71 species differed significantly in abundances between the pork protein-based diet and the casein-based diet groups (P < 0.05, P_{adj} < 0.1, Supplementary file 4), with 19 species exhibiting significant differences in abundances on both day 7 and day 14. Again, no species were found to differ significantly in abundance between the beef protein-based diet and the pork protein-based diet groups. The species that differed in abundance
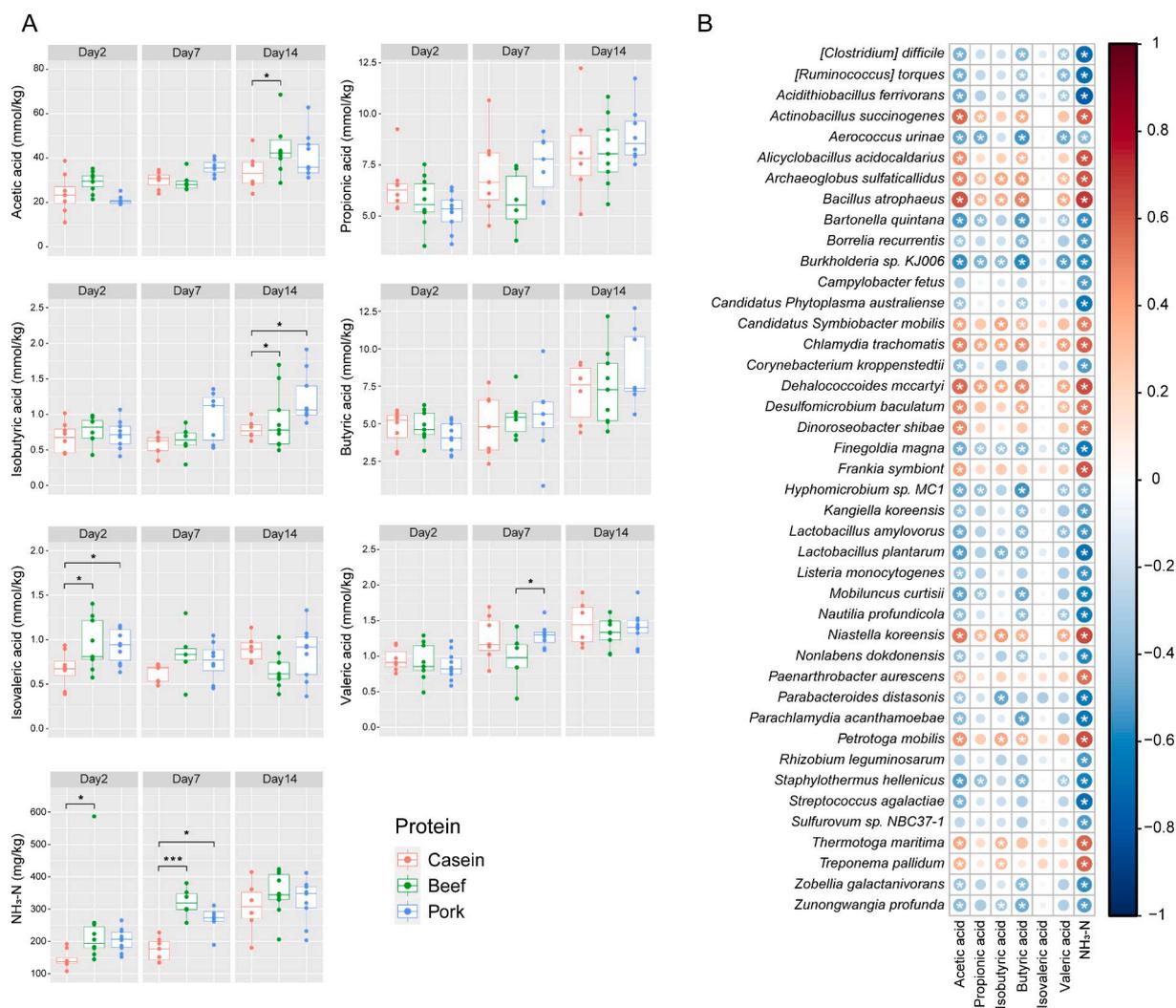

Fig. 3. Effects of intake of the casein-based diet, the beef protein-based diet and the pork protein-based diet on SCFAs and ammonia nitrogen (A), and correlations with the gut microbiota (B). (A), differences were analyzed by two-way ANOVA in which protein diet and time point were set as independent, and the measured variables were set as dependent. *: indicates significant differences between the corresponding groups at each time point with P < 0.05, ***: P < 0.001. (B), the correlation was analyzed by using Spearman's correlation analysis. The species differing in abundance according to protein sources are shown. Red color in the heatmap represents positive correlation while blue color represents negative correlation. *: indicates significant correlation (P < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

between the beef protein-based diet and casein-based diet groups or between the pork protein-based diet and the casein-based diet groups with P adj values < 0.05 on both day 7 and day 14 are shown in Fig. 2B. *Lactococcus lactis* and *Lactococcus garvieae* belonging to the genus *Lactococcus* exhibited significantly higher abundances in the gut microbiota of rats fed the casein-based diet than rats fed the red meat protein-based diets on day 7 and day 14. Several species belonging to *Lactobacillus* varied in abundance between the casein-based diet and the red meat protein-based diet groups during the experimental period. The abundance of *Lactobacillus helveticus* was found to be significantly higher in the casein-based diet group than in the other two groups on day 7, but significantly lower on day 14. However, the abundances of *Lactobacillus fermentum* and *Lactobacillus casei* were found to be significantly higher in the casein-based diet group on both day 7 and day 14. We noticed that *Leuconostoc arnosum* and *Leuconostoc citreum* might serve as a marker for the casein-based diet group as these two species were almost non-detectable in the two red meat protein-based diet groups.

3.3. Effect of dietary casein and red meat proteins on cecal short chain fatty acids (SCFAs) and ammonia nitrogen (NH$_3$-N) content of young rats

Fig. 3A shows how the content of SCFAs and NH$_3$-N varies during the treatment period in the three diet groups. On day 2, the amount of isovaleric acid in rat cecal content was significantly higher in the two red meat protein-based diet groups than in the casein-based diet group (P < 0.05). The same trend was observed for NH$_3$-N, but the difference only reached significance comparing rats fed the beef protein-based diet with rats fed the casein-based diet. On day 7, the two red meat protein-based diet groups showed significantly higher amount of NH$_3$-N than the casein-based diet group. On day 14, the levels of acetic acid differed significantly between rats fed the beef protein-based diet and rats fed the casein-based diet (P < 0.05), and the levels of isobutyric acid differed significantly between rats fed the two red meat protein-based diet and rats fed the casein-based diet (P < 0.05). Spearman's correlation analysis revealed a strong positive correlation between production of NH$_3$-N and the abundances of *Bacillus atrophaeus*, *Dehalococcoides mccartyi*, and *Niastella koreensis*. By contrast, the production of NH$_3$-N was negatively correlated with the abundances of *Clostridium difficile*, *Ruminococcus torques*, *Acidithiobacillus ferrivorans*, *Lactobacillus plantarum*, and *Streptococcus agalactiae* (P < 0.01, |r| > 0.65) (Fig. 3B).

3.4. Effect of dietary casein and red meat proteins on the functional potential of cecal bacteria

To further understand the functional potential of the cecal microbiota in young rats fed the casein or red meat protein-based diets, mapped genes were annotated to KEGG orthologies (KOs). A comparison of KOs profiles based on gene abundances between the beef/pork protein-based diets and the casein-based diet groups are presented in supplementary file 5. Among these differentially abundant KOs, zero, 1139, and 256 differed in abundance between the beef protein-based diet and the casein-based diet groups on day 2, day 7 and day 14, with 65 differing both on day 7 and day 14 (P < 0.05, P$_{adj}$ < 0.1). Zero, 1656, and 960 KOs differed in abundance between the pork protein-based diet and the casein-based diet groups on day 2, day 7 and day 14, with 329 exhibiting significant differences in abundance on both day 7 and day 14 (P < 0.05, P$_{adj}$ < 0.1). No KO was found to differ significantly in abundance between the two red meat protein-based diet groups (P$_{adj}$ > 0.1). PCoA analysis using Bray-Curtis dissimilarities based on abundances of KOs exhibited profiles similar to those based on genus and species abundances (Supplementary Fig. S2).

GAGE analysis based on KEGG pathways was further conducted to explore potential metabolic processes in the microbiota affected by the different protein-based diets. A total of 43 metabolic pathways differed in abundance between the beef/pork protein-based diets and the casein-based diet groups (P < 0.05, FDR q < 0.1, Supplementary file 6) at any

one of the time points (Fig. 4A). For most of the KEGG pathways that differed significantly in abundance, the beef protein-based diet and pork protein-based diet groups exhibited the same direction of changes compared to the casein-based diet group. Among these pathways, the O-Antigen nucleotide sugar biosynthesis pathway was found to be enhanced in the two red meat protein-based diet groups compared to the casein-based diet group at all three time points, while pathways involved in sulfur metabolism and galactose metabolism were found to be decreased in abundance on day 7 and day 14 (P < 0.05, FDR q < 0.1). Comparison of profiles of KOs involved in galactose metabolism are shown in Fig. 4B (Supplementary Fig. S3). Most of these KOs exhibited significantly lower abundance in the two red meat protein-based diet groups when compared to the casein-based diet group, especially on day 7 and day 14. All the KOs involved in processes that metabolize lactose into D-glyceraldehyde-3P exhibited significantly higher abundances in the casein-based diet group than in the two red meat protein-based groups (Fig. 5A). The higher gene abundances of most of these KOs (including K02786, K02788, K01200, K01819 and K01635) in the casein-based diet group reflected a contribution from *Lactococcus lactis*, especially on day 7 and day 14 (Fig. 5B).

3.5. Histological examination of colonic mucosa and goblet cells

Hematoxylin and Eosin (H&E) staining was applied in order to examine possible microstructural differences of the colonic mucosa in response to the three dietary proteins in young rats (Fig. 6A). No significant difference in mucosa depth was found between any of the dietary groups at any time point (Fig. 6A). A tendency towards a slightly lower mucosa depth was found in rats fed the pork protein-based diet on day 14 compared to rats fed the casein-based diet (P = 0.059). Since the gut microbial composition varied the most at day 7, Alcian Blue-Periodic acid-Schiff (AB-PAS) staining was further performed to examine distribution and number of goblet cells in the rat colon (Fig. 6B). The number of goblet cells did not differ significantly between any of the groups (Fig. 6B), however, the color of the stained goblet cells in the casein-based diet group seemed to differ from that in the beef or pork protein-based diet groups, reflecting a different proportion of neutral goblet cells responding to PAS after staining with AB.

4. Discussion

While previous studies have demonstrated a differential effect of diets containing different plant-based and animal-based proteins (Zhu et al., 2015, Madsen, Myrmel, Fjære, Liaset, & Kristiansen, 2017; Holm et al., 2016; Liisberg et al., 2016; Fjære et al., 2019; Myrmel et al., 2019), the effects of different protein sources on growth and the gut microbiota just after weaning have received less attention. Here we show that young rats fed two red meat protein-based diets (beef and pork proteins) exhibited higher accumulated body weight gain and feed intake during a 14-days post-weaning period. This result is consistent with a previous study showing a higher body weight gain of rats fed red meat protein-based diet compared to a casein-based diet (Song et al., 2018). The current study further demonstrates dynamic changes during the immediate post-weaning period and that the higher body weight gain in rats fed the beef protein-based diet reflected a higher feed intake, as the overall feed efficiency did not differ significantly compared to the casein-based diet group and all three high-quality animal protein sources showed similar nutritional value in terms of the effect on growth performance. Still, we noted that a significantly higher feed efficiency was observed in rats fed the pork protein-based diet during the first 2 feeding days compared to rats fed the beef protein-based diet or the casein-based diet.

Studies have demonstrated how consumption of different dietary proteins is reflected in the composition of gut bacterial communities, however, mainly between plant and animal protein-based diets (Zhu et al., 2015), whereas few studies have focused on the effects of different
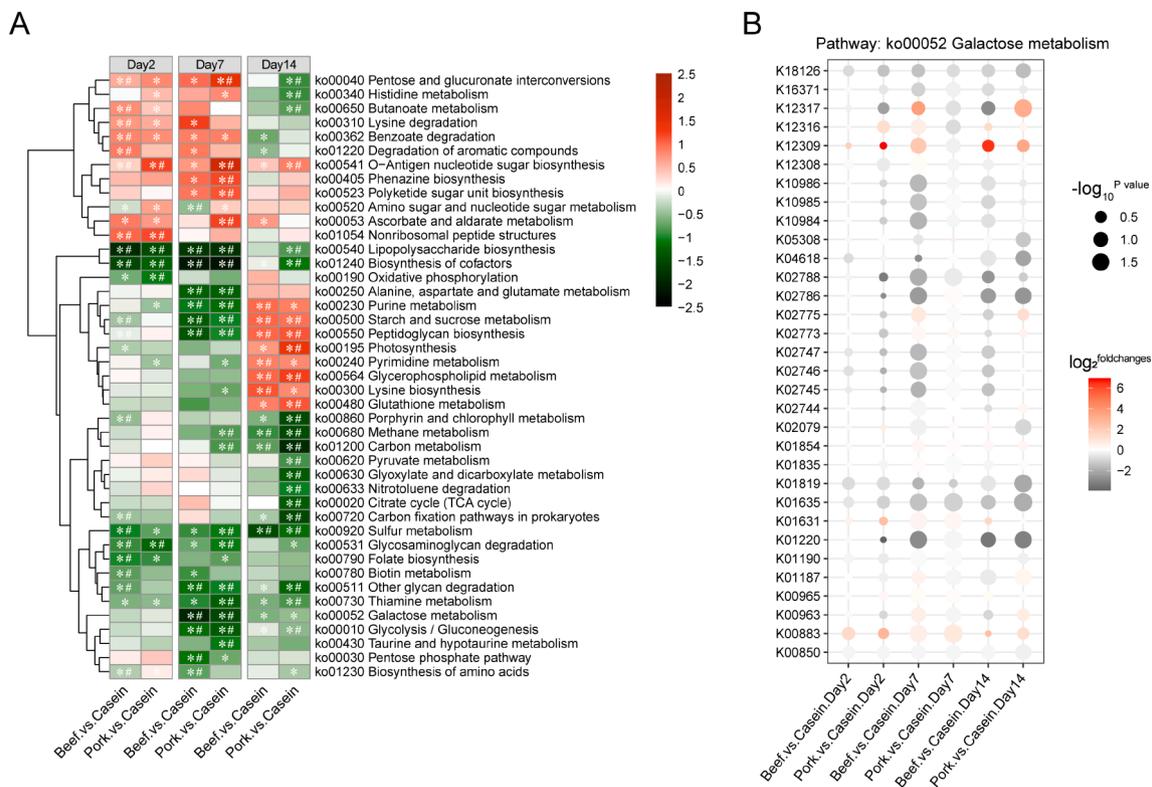
Fig. 4. Analysis of the functional potential of the cecal microbiota in the young rats fed casein, beef protein and pork protein-based diets based on KEGG pathways. (A), differentially enriched KEGG pathways were analyzed by using the generally applicable gene set enrichment (GAGE) method. Comparison between either casein-based and beef protein-based diets or casein-based and pork protein-based diets at each time points are shown in the heatmap. The red color represents enriched in the casein-based diet group, the green color represents enriched in the beef or pork protein-based diet groups. *: P < 0.05, #: FDR q value < 0.1. (B), comparisons of KEGG orthology (KO) involved in galactose metabolism between the casein-based diet group and the beef protein-based diet group/pork protein-based diet group. The red color represents higher abundance of KOs in the beef/pork protein-based diet group, while the black color represents higher abundance of KOs in the casein-based diet group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

animal protein sources on the gut microbiota (Zhao et al., 2017). Our previous study has shown that meat protein-based diets induced higher abundances of *Lactobacillus* in rats when compared to casein or soy protein-based diets (Zhu et al., 2015). However, due to the limitation of 16S rRNA sequencing methodology used in these studies, information at the species level was limited in these studies. Additional studies have reported that intake of dairy products could increase the abundance of species belonging to *Lactobacillus* and *Bifidobacterium* (Aslam et al., 2020). In the present study using shotgun metagenomics sequencing, which compared to 16S rRNA gene amplicon sequencing provides more information at the species level as well as better prediction of functional capacity, we observed a dramatic decrease in the abundance of *Lactobacillus* in the two red meat protein-based diet groups on day 7, with a recovery at day 14 where *Lactobacillus* became even more abundant than in the casein-based diet group. Our analysis showed that the higher abundance of the genus *Lactobacillus* in rats fed the beef and pork protein-based diets on day 14 mainly reflected the more predominant species *Lactobacillus helveticus*, whereas other species belonging to genus *Lactobacillus* were found to be more abundant in rats fed the casein-based diet, including *Lactobacillus fermentum* and *Lactobacillus casei*. Thus, the results indicated that different sources of animal proteins specifically promoted the growth of certain species belonging to the genus *Lactobacillus*.

Spearman's correlation analysis revealed a negative correlation between the abundance of *Lactobacillus plantarum* and the cecal content of NH_3-N. Rats fed the two red meat protein-based diets exhibited higher cecal NH_3-N content than rats fed the casein-based diet. The luminal ammonia concentration in the large intestine lumen is primarily the net result of microbial production through amino acid deamination and urea hydrolysis, microbial utilization of ammonia, and ammonia absorption through the epithelial cells (Tuśnio et al., 2021). Urea hydrolysis in the intestinal lumen is performed via bacterial urease activities (Wallace, 1996). Interestingly, the abundances of microbial gene mapped to urease KOs (K01428, K01429, K01430 and K14048) were found to be significantly higher in the casein-based diet group than the other two red meat protein-based diet groups at day 7 (Supplementary file 5), indicating an enhanced potential of NH_3-N production. However, significantly lower amount of cecal NH_3-N was observed in the casein-based diet group at this time point. Two processes could explain these apparent contradictory findings. Firstly, whereas large amounts of ammonia can be absorbed through the large intestine mucosa (Tuśnio et al., 2021), bacteria may additionally assimilate ammonia to form bacterial protein during carbohydrate fermentation (Diether & Willing, 2019). Thus, increased carbohydrate fermentation and bacterial growth can decrease ammonia concentrations in the gut due to higher incorporation of nitrogen into microbial cells. Moreover, studies have also shown that NH_3-N can be taken up and detoxified by probiotic *Lactobacilli* (O'keefe, 2016), which is consistent with the current negative correlation between *Lactobacillus plantarum* and the concentration of NH_3-N. Secondly, the amount of protein entering the colon depends to a large extent on the amount and digestibility of the protein (Silvester & Cummings, 1995). Thus, a decrease in protein digestibility increases the amount of protein reaching the colon and the rate of protein fermentation by colonic bacteria. Although animal and dairy proteins are both considered as relatively more digestible proteins compared to unpurified plant proteins (Gilbert, Bendsen, Tremblay, & Astrup, 2011), casein still
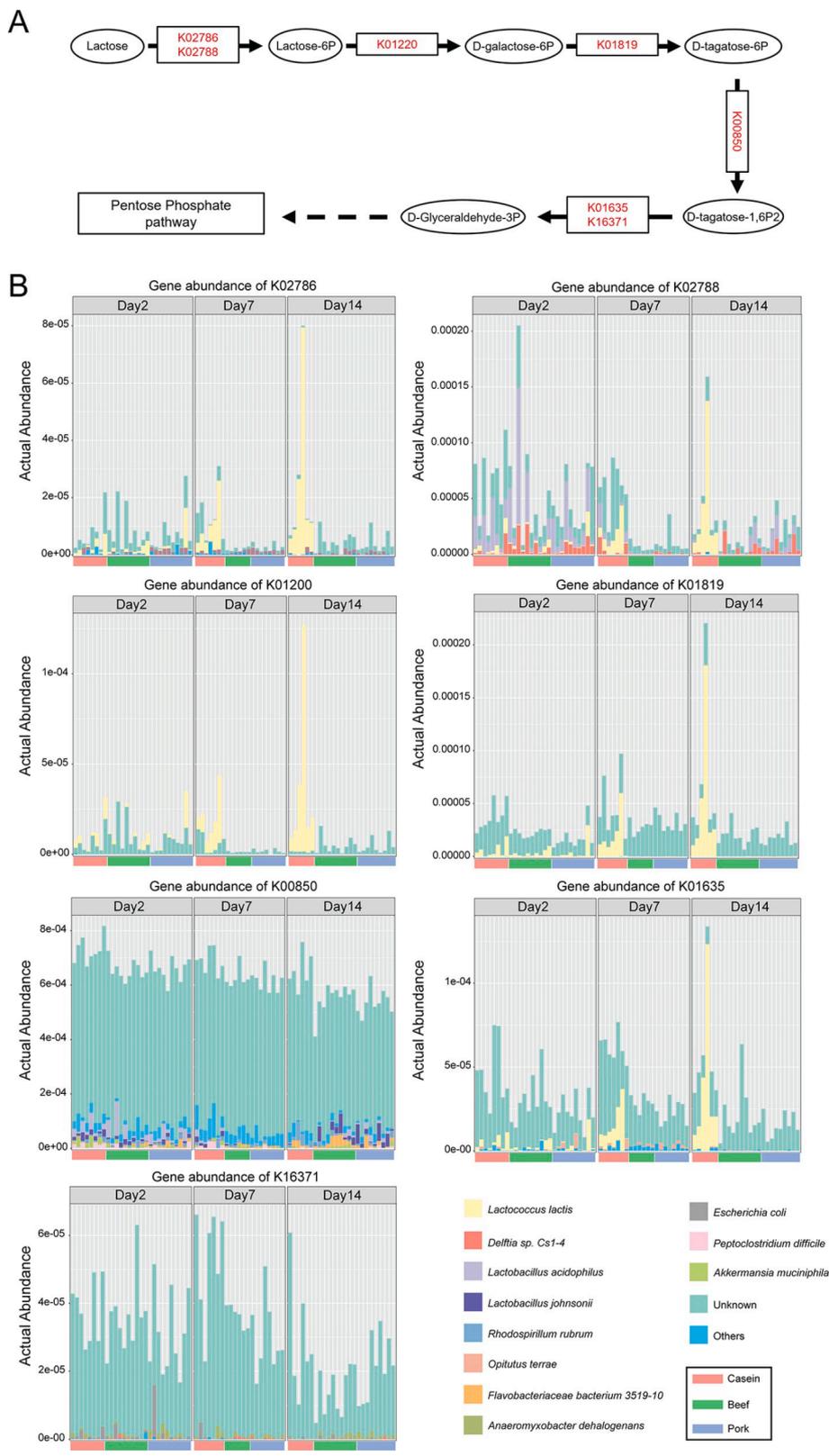
Fig. 5. The Tag6P pathway for utilization of lactose (A) and gene abundances of the KOs (B) involved in this pathway. (A), KOs labeled in red indicate significantly different abundances between the casein-based diet group and the beef/pork protein-based diet groups (P < 0.05, P adj < 0.1). (B), the abundances of genes mapped to KOs involved in the Tag6P pathway are shown with abundance information of the contribution by the corresponding species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

has been reported to have even higher digestibility than meat proteins (Gilbert et al., 2011). This could result in more substrates for bacteria to metabolize in the red meat protein-based diet groups. Excessive ammonia content in the colon lumen has been shown to be toxic and interfere with colonocyte metabolism and morphology (Andriamihaja et al., 2010). We assessed the colonic morphology of rats fed the three different animal protein sources, but no abnormal phenotypes were observed by H&E staining, indicating an overall acceptable net amount of NH_3-N induced by intake of the three different dietary protein-based diets. Of note, our analysis of functional potential of the gut microbiota revealed that genes involved in sulfur metabolism, especially genes included in the dissimilatory sulfate reduction module, were found to be
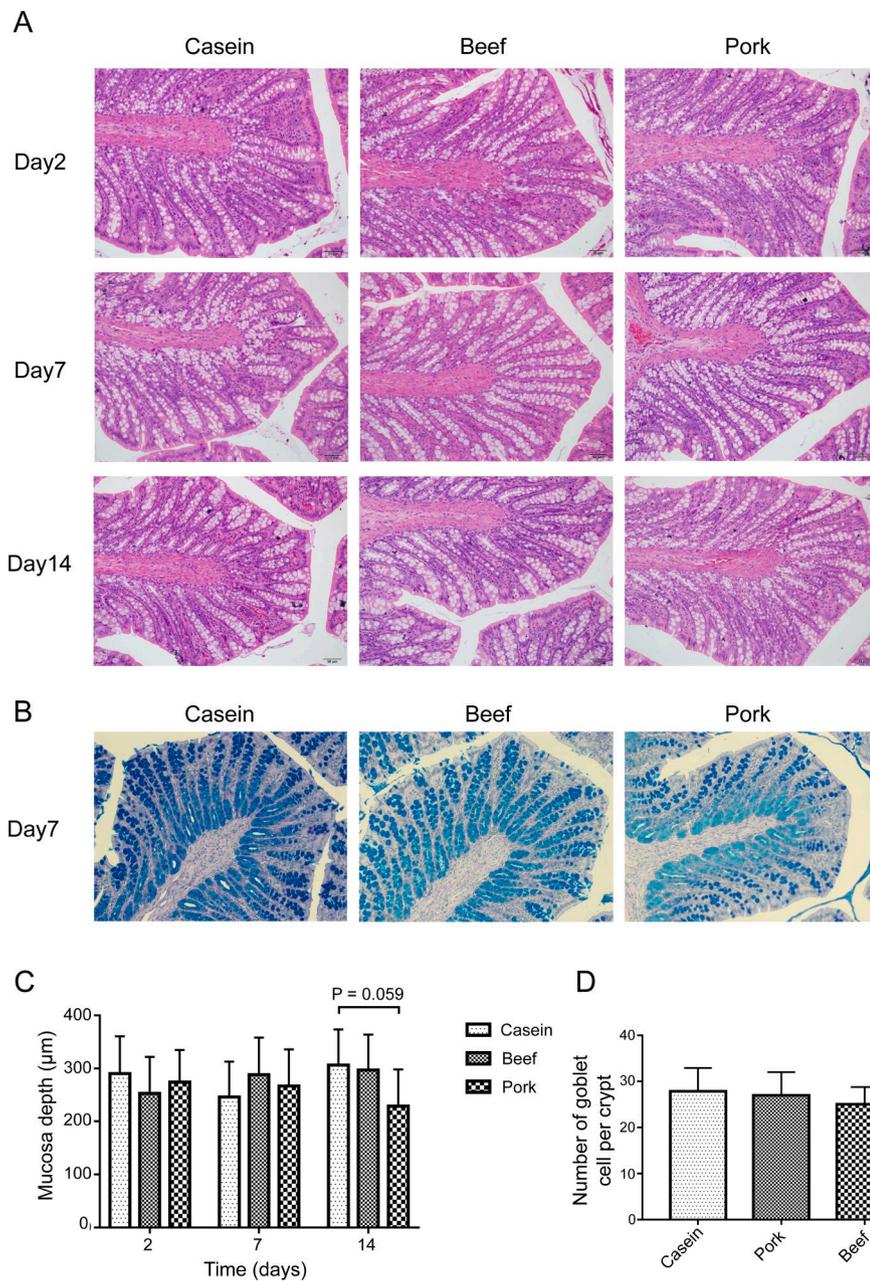
Fig. 6. Morphological examination of the colon in rats by H&E staining (A), and AB-PAS staining (B). The scale in the figure represents 50 μm. n = 10 per group (outliers were removed). The mucosa depth (C) and number of goblet cells (D) were calculated according to Fig. 6A and 6B. For mucosa depth, differences were analyzed by two-way ANOVA in which protein diet and time point were set as independent. For the number of goblet cells, Wilcoxon rank-sum test were applied for statistical analysis.

more abundant in the casein-fed group than in the red meat protein-fed groups (Supplementary Fig. 4A&B). It has been reported that only a limited number of microbial groups are capable of dissimilatory sulfate reduction producing hydrogen sulfide (H_2S), which may exert a toxic action on colonocytes (Carbonero, Benefiel, Alizadeh-Ghamsari, & Gaskins, 2012; Attene-Ramos et al., 2010). However, the abundance of the gene encoding O-acetylhomoserine/O-acetylserine sulfhydrylase K17069 (MET17) was also significantly higher in the casein-fed group (Supplementary Fig. 4A&B), indicating a potential for consumption of H_2S to produce L-cysteine. As the amount of H_2S in the large intestine was not determined in the current study due to limited amount of sample, more work is needed to understand to what extent intake of the casein-based diet influences production and utilization of H_2S and how these activities may influence both the host and microbial communities of the colonic ecosystem.

AB-PAS staining was further used to examine the number and appearance of mucin producing goblet cells. Although the number of goblet cells did not differ among the three groups, a clear difference in

the color of stained goblet cells was found between the casein-based diet group and the two red meat protein-based diet groups. Mucins are classified into neutral and acidic subtypes (Święch, Tuśnio, Barszcz, Taciak, & Siwiak, 2019). By first staining all the acidic mucins with AB, remaining acidic mucins which are PAS positive will be masked and will not react further. The neutral mucins, which are solely PAS positive, will subsequently be stained as bright magenta. Where mixtures occur, the resultant color will appear dark blue or purple depending on the dominant mucin type (Vaiphei, 2022). Therefore, the darker blue color in the casein-based diet groups indicated the existence of a higher proportion of neutral to acidic mucins compared to the two red meat protein-based diet groups. Goblet cells in intestinal regions densely populated by microbes express predominantly acidic mucins (Deplancke et al., 2000), indicating that acidic mucins as a result of stimulation by the microbial environment could probably further protect against bacterial translocation (Deplancke & Gaskins, 2001). Therefore, we speculate that the higher proportion of acidic mucins in the colon of rats fed two red meat protein-based diets could reflect alterations of the gut

microbial environment induced by the change of dietary proteins from casein to red meat proteins. In fact, the physiologic relevance of distinct mucin subtypes is currently not well understood, and more work is required to understand the associated effects on host health.

Another interesting finding of the current study is the higher abundance of the genus *Lactococcus* in the casein-based diet group than in the two red meat protein-based diet groups. A casein-based diet has been reported to induce a higher abundance of *Lactococcus* when compared to a chicken protein-based diet in our previous study (Zhao et al., 2019). Combined with the present result, *Lactococcus*, especially *Lactococcus lactis* could be taken as a biomarker in the casein-based diet group compared to meat protein-based diet groups. *L. lactis* is the most widely studied species of lactic acid bacteria (Guillot, Gitton, Anglade, & Mistou, 2003). According to the result of the analysis of functional potential, we found that *L. lactis* might greatly contribute to galactose metabolism. This species was reported to be able to use the Leloir pathway, the Tag6P pathway, or both pathways for galactose utilization, but in a strain-dependent manner (Neves et al., 2010). Although in the present study we were unable to perform analysis on the strain level, we indeed found an enhancement of the Tag6P pathway in the casein-based diet group compared to the two red meat protein-based diet groups, as the abundances of genes encoding all enzymes involved in this pathway were significantly higher in the casein-based diet group. This indicated a regulatory effect of a casein-based diet on the *L. lactis* sub strains with a preference of galactose utilization through the Tag6P pathway. Although, lactose does not exist in casein, and we did not provide lactose in the diet formula, some glycoproteins (Gal and GalNAc) are present in kappa casein which might induce such type of metabolism (Głąb & Boratyński, 2017). Certain natural *L. lactis* isolates have been reported to be beneficial for host health (Mercier-Bonin & Chapot-Chartier, 2017). In addition, two other species, *Leuconostoc carnosum* and *Leuconostoc citreum* belonging to lactic acid bacteria were also found to be more abundant in the casein-based diet group, while they were almost non-detectable in the two red meat protein-based diet groups. Certain strains of *L. carnosum* are known to produce bactericides which could inhibit or kill the pathogenic bacterium *Listeria monocytogenes* (Budde, Hornbæk, Jacobsen, Barkholt, & Koch, 2003), and certain strains of *L. citreum* are known to secrete enzymes capable of converting sucrose to the well-established prebiotics inulin (Olivares-Illana, López-Munguía, & Olvera, 2003). All these previous reported studies point to the potential beneficial effects on host health by increasing abundances of beneficial gut bacteria.

5. Conclusion

The present study showed no significant difference between casein-based, beef protein-based, and pork protein-based diets in terms of growth performance after weaning. During the 14 days feeding period, the casein-based and two red meat protein-based diets promoted different potential beneficial bacterial species. The red meat protein-based diet groups showed significantly higher abundance of the genus *Lactobacillus* by specifically regulating the abundance of the predominant species *Lactobacillus helveticus*, while the casein-based diet induced higher abundances of the less dominant potential beneficial species *Lactobacillus fermentum* and *Lactobacillus casei*. In addition, intake of the casein-based diet specifically increased the abundance of *Lactococcus lactis* with a great potential for galactose utilization via the Tag6P pathway. Relatively lower amount of ammonia which could be toxic was observed in the casein-based diet group than in the two red meat protein-based diet groups. However, no aberrant phenotype and no negative effects on colonic tissue were observed in rats fed all three different protein-based diets according to the morphological observation. Our results provide new insight into the effect of dietary casein and red meat proteins on the growth development and gut microbiota during the early stage of life. The prolonged effects of these differentially shaped microbial patterns on host health remain to be further explored.

CRediT authorship contribution statement

Fan Zhao: Conceptualization, Methodology, Formal analysis, Writing – original draft, Data curation. **Chong Wang:** Resources, Methodology, Formal analysis, Data curation. **Shangxin Song:** Investigation, Methodology. **Chao Fang:** Formal analysis. **Guanghong Zhou:** Supervision, Funding acquisition. **Chunbao Li:** Supervision, Project administration, Funding acquisition. **Karsten Kristiansen:** Supervision, Writing – review & editing, Funding acquisition.

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

Data will be made available on request.

Data availability statements

The sequencing data are available on NCBI. The shotgun metagenomic sequencing accession ID is PRJNA793774.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133769.

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