Dynamic establishment of the gut bacterial ecology of cows from birth to adulthood

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Abstract

Background: The gut microbiota plays multiple critical roles in maintaining the health of the host, especially in ruminants; however, our understanding of the process of gut microbiota establishment from birth to adulthood remains limited. Here, we investigated the bacterial ecology in the gastrointestinal segments (rumen, abomasum, duodenum and rectum) of Holstein cows from 1 week to 5 years of age by 16S rRNA gene sequencing to illustrate the dynamic establishment of gastrointestinal microbiota of cows. Results: We revealed a major change in the composition, diversity, and abundance of bacteria with age increase. Remarkably, the gut bacterial community was found to be age-discriminatory, and microbiota gradually matured in each segment of the gut which followed a natural logarithmic function by applying a maturity index algorithm, that meant gut microbiota was established quickly in young animals, then slowed with age. Using the Bayesian algorithm of Source-Tracker, we demonstrated that the proportion of bacterial transfer from the foregut to the hindgut was also higher in young animals, and then decreased sharply with age. Conclusions: Our findings demonstrated gastrointestinal microbiota was established quickly during young period of cows and highlighted the “window period” of 18 weeks after birth for cows to establish their gut bacterial community. This insight into gut microbiota might have important ecological applications in protecting gut health or manipulating the gut microbiota of cows.

Background

The gut microbiota has been demonstrated to be important for health of host, especially for ruminants such as cows that rely on gut fermentation to convert indigestible plant biomass into food products[1-3]. Considerable research has focused on the bovine intestinal microbial composition[4-6], compositional differences in each gut segment[7-11], as well as the function of the gut bacterial community[12]. These previous studies have expanded our understanding of the gut bacterial community.

The gastrointestinal microbiota is a dynamic system that changes with host development[13]. The gut microbiota of animals is believed to be established by capturing xenomicrobiota from surroundings[14-16], as animals are exposed to complex surroundings after birth[17-19], thus gut
microbiota is gradually established with age increasing from birth to adulthood, and eventually transits to an adult-like microbiota[13, 14, 20-22]. For ruminants, previous studies also observed the alpha diversity increase and predominant bacterial taxa change as animals aged[23-26]. Although this notion of gradual establishment of the gut bacterial community is well accepted, the details of this process remain to be elucidated from an ecological perspective.

The aim of our study was to explore the gradual establishment of the gut bacterial ecology of cows from birth to adulthood. By analyzing the 16S rRNA gene sequences of the microbiota from different gut segments, we were able to characterize dynamic changes in the bacterial community with increased age. Then, by adopting a maturity index[13], which depicts how fast the gut microbiota of calves approached that of adult cows, we illustrated the dynamic process of gut bacterial community establishment. Capturing bacteria from the surroundings (i.e. bacteria transfer) is believed to play an important role in gut microbiota establishment[14, 16]. We further introduced the Bayesian algorithms of Source-Tracker[27] to calculate the proportion of bacteria in the hindgut being transferred from the foregut at different ages. Our findings provide insight into the establishment of the gut bacterial community in cows from an ecological perspective.

Methods

Sample collection

Experiments were performed at the Zhong Di breeding stock dairy farm located in Beijing, China. Forty-two historically healthy Holstein female cattle were enrolled in the study with ages ranging from 1 week to adulthood. The cattle were raised under the following conditions and weaned at their 2 months old: 1-week-old calves (n=12) had free access to milk; 1-month-old calves (n=8) had free access to milk replacer and a solid starter diet (granules:flaking corn, 3:2); 2-month-old calves (n=8) had free access to milk replacer, solid starter diet and hay (50% alfalfa and 50% oats); 2-year-old (n=8) and 5-year-old (n=6) Holstein cows had free access to a total mixed diet consisting of 60% concentrated feed and 40% roughage. Each animal was reared in the same manner historically and being free access to fresh water and fasted for 12 h before harvest. The experimental design and procedures were approved by the Animal Care and Use Committee of the College of Animal Science
and Technology of China Agricultural University (Project number: 31772628), in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988)

The calves and cows were euthanized with an intravenous injection of Euthanyl (240 mg/mL; Sigma-Aldrich, Castle Hill, New South Wales, Australia). Each gastrointestinal compartment (rumen, abomasum, duodenum, and rectum) was isolated with sterile surgical thread to avoid the contents being mixed. Samples of digesta from rumen, abomasum, duodenum, and rectum were collected. In total, 152 samples were collected from 42 animals. All samples were immediately frozen in liquid nitrogen before being analyzed.

DNA extraction and PCR amplification

Genomic DNA was extracted with 0.5g digesta samples using the E.Z.N.A.® stool DNA Kit (Omega BioTek, Norcross, GA, U.S.) according to the manufacturer's instructions. The quality of the extracted DNA was detected by 1% agarose gel electrophoresis and spectrophotometry (optical density at 260/280 nm ratio). Extracted DNA was stored at -20°C until further analysis. The V3-V4 regions of the 16S rRNA genes were amplified with the universal primers (341F:5'-CCTAYGGGRBGCASCAG-3'; 806R :5'-GGACTACNNNGGGTATCTAAT-3') These primers additionally contained a set of 8-nucleotide barcode sequences that were unique to each sample. PCR reactions were performed in triplicate 25 µL mixture containing 2.5 µL of 10 × Pyrobest Buffer, 2 µL of 2.5 mM dNTPs, 1 µL of each primer (10 µM), 0.4 U of Pyrobest DNA Polymerase (TaKaRa, Kyoto, Japan), and 15 ng of template DNA. The PCR program was as follows: 95 °C for 5 min, 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s with a final extension of 72 °C for 10 min.

Illumina MiSeq sequencing and sequencing data processing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.), according to the manufacturer's instructions, and were quantified using QuantiFluor™-ST (Promega, U.S.). The amplicons of the V3–V4 hypervariable regions
of the 16S rRNA genes were then subjected to sequencing using the Illumina Miseq PE300 sequencing platform (Illumina, Inc., CA, USA) of Beijing Allwegene Tech, Ltd (Beijing, China).

Raw sequences were assigned to each sample by barcodes and then low-quality sequences were filtered: (1) raw reads that were shorter than 110 nucleotides; (2) the 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (3) exact barcode matching, 2 nucleotide mismatches in primer matching, and reads containing ambiguous characters were removed. Only clean sequences with an overlap longer than 10 bp were assembled using the FLASH-1.2.11 software[28]. Reads that could not be assembled were discarded. Chimera sequences were detected by usearch6.1[29]. Then high-quality sequences were first analyzed using the QIIME2 pipeline[30].

The representative sequence datasets were classified into operational taxonomic units (OTUs) using a threshold of 97% identity and the UCLUST algorithm[29]. The taxonomy of each 16S rRNA gene sequence was assigned by UCLUST against the GreenGenes database[31].

Core bacterial population, maturity index, and transfer proportion

A total of 562 bacteria were detected in all of the samples at the genus level, and among these, core bacteria were identified as being present in more than 80% of samples in each group. A meta-dataset of the core bacterial population in each gut segment was generated using the core bacteria identified.

The maturity index is defined as the similarity between the gut microbiota of the young to that of the adult[13]. Here we assumed that the microbiota in adult cows at 5 years old was fully developed, thus we could infer that a gut microbiota more similar to that of adult cows (at 5 years old) indicated that
the animal was more developed. The maturity index was calculated from the modified algorithm of Bray–Curtis similarity: Maturity index = ; where x is the bacteria abundance, n is the number of identified core genera in each gut segment, i is the list of core genera, j is the given sample for which the maturity index was measured, and k is the sample of each adult cow (of 5 years old).

Source-Tracker software (version 1.0.1) was developed to track the microbial source in the surrounding environment using a Bayesian approach[27]. We performed Source-Tracker analysis on OTU level with foregut (rumen) microbiota as source samples and hindgut (rectum) microbiota as sink samples and default parameters. This analysis provided a further understanding of the proportion of bacterial transfer from the foregut to the hindgut at different ages of the cows.

Data analysis

The diversity matrix was calculated using the QIIME Pipeline[30]. Venn plot and hypergeometric test were performed to present the distribution differences in the core bacterial community using the VennDiagram package (1.6.17) in the R software (version 3.3.0). The maturity index changes with increased age were fitted with the natural logarithmic function in the R software (version 3.3.0). Comparisons between groups were performed using the Mann–Whitney U test or the Kruskal–Wallis test, and a P-value <0.05 was considered significant.

Results

Datasets

To explore the dynamic establishment of the bacterial ecology in different gut segments of cows, we collected samples of digesta from rumen, abomasum, duodenum, and rectum from 42 Holstein cows with ages ranging from 1 week to 5 years old. In total, 152 samples were collected and after 16S rRNA gene sequencing on the Miseq platform and quality control, 7,627,293 clean sequences (50,179 ± 11663 sequences per sample) were obtained. When we assigned these sequences to operational
taxonomic units based on 97% nucleotide sequence similarity and different taxonomic levels based on
the Greengene database, we identified 157,619 operational taxonomic units (OTUs), 562 genera, 249
families, 131 orders, 64 classes, and 30 phyla in this meta-dataset. The majority of these sequences
belonged to Firmicutes (~49.2%), Bacteroidetes (~28.8%), Proteobacteria (~7.5% in relative), and
Actinobacteria (~3.0%) in relative abundance in all samples, and we also observed that the relative
abundance of these major bacterial taxa changed in different gut segments as well as with different
chronologic ages for each of the segments (Figure 1).

Dynamic changes in the bacterial composition of different gut segments with increased age

The Chao1 and Shannon indexes were used to assess the alpha diversity changes with increased age
in each of the gut segments. The Chao1 index reflects the number of expected species, while the
Shannon index accounts for both the abundance and evenness of the species presented in a given
community. We observed that the Chao 1 and Shannon indexes roughly increased with increased age
in each segment, and dichotomy between young calves (1 week, 1 month, and 2 months old) and
adult cows (2 years and 5 years old) was detected (P<0.05, Figure 2).

To further explore the bacterial changes in the different gut segments of cows, meta-datasets of the
core bacteria at the genus level for each gut segment of the rumen, abomasum, duodenum, and
rectum were established, and as a result, we identified 170 core genera in rumen, 176 core genera in
abomasum, 203 core genera in duodenum, and 119 core genera in rectum (see Material and
Methods). We first observed a low level of overlap in the core bacteria observed for the different ages
for each gut segment, with the number of core bacteria that persistently presented in the different
aged cows being 24 taxa (14.1% of the detected core bacteria) in the rumen (Figure 3a), 42 taxa
(23.9% of the detected core bacteria) in the abomasum (Figure 3b), 33 taxa (16.3% of the detected
core bacteria) in the duodenum (Figure 3c), and 24 taxa (20.2% of the detected core bacteria) in the
rectum (Figure 3d). Statistically, we also observed a difference in the distribution of core bacteria in different aged cows in each of the gut segments including the rumen, abomasum, duodenum, and rectum by a hypergeometric test (P<0.05). In a given gut segment, few bacterial genera were shared by different aged cows. Furthermore, we revealed that the abundance of core bacterial genera in each gut segment changed with increased age, and the core bacteria in each particular segment of the gut (rumen, abomasum, duodenum, and rectum) could be roughly clustered into three groups (Figure 3e–h): the abundance of bacterial genera in cluster 1 increased, that in cluster 2 decreased, and that in cluster 3 followed a quadratic curve with increased age (Figure 3i–k). These observations demonstrated that the composition and abundance of core bacteria in each gut segment differed with increased age, and these changes were therefore classified as age-associated changes.

Microbiota maturity in the different gut segments increased with the increased age of cows

Given that the presence and abundance of core bacteria in each gut segment appeared to be associated with the age of cows, we hypothesized that the core bacterial genera in each gut segment may also be age-discriminatory. Thus, a maturity index that reflected the age-dependent changes in the microbiota was introduced in the current analysis (see Material and Methods), and a gradual increase in the maturity index was expected with increased age in each of the gut segments of the cows (Figure 4a). We found that the maturity index gradually increased with increased age following a natural logarithmic function (P<0.05) in each gut segment of the rumen (r=0.94), abomasum (r=0.82), duodenum (r=0.85), and rectum (r=0.88). These findings indicated that the maturity index would increase quickly during young, but this increase would slow in older animals.

To quantitatively illustrate the growth rate change in the maturity index, derivation of the achieved logarithmic function was established, which reflected how fast the maturity changed with increased age (i.e. the maturity growth rate). We observed that the maturity growth rate decreased with age
and this followed a power-law function in each gut segment. Two typical periods (the ‘quick-maturing period’ and the ‘moderate-maturing period’) were observed with a maturity growth rate cut-off at 0.005, and the shift between these two periods was predicted at around 18 weeks of age in the cows (Figure 4b). In addition, the duration of the quick-maturing period in the different gut segments also differed, with the microbiota quickly maturing until 21 weeks of age in the rumen, 19 weeks of age in the abomasum, 17 weeks of age in the duodenum, and 16 weeks of age in the rectum (Figure 4b), which suggested that the microbiota in the lower gut matured faster than that in the upper gut.

Influence of bacterial communities in the foregut (rumen) on those in the hindgut (rectum)

The bacterial community in the hindgut may be affected by that in the foregut due to bacterial transfer and this may be reflected by the similarity between the bacterial compositions in the foregut and hindgut because active bacterial transfer between these two habitats would result in similar bacterial communities. Here, by analyzing the similarity indices of unweighted (depicting the bacterial composition in the absence or presence of bacteria) and weighted UniFrac (depicting both the composition and abundance of bacteria) indexes, we observed that the similarity between bacterial communities in the foregut and hindgut was smaller than 30% with the unweighted UniFrac similarity index, and smaller than 50% with the weighted UniFrac similarity index (Figure 5). Meanwhile, the similarity indices were significantly affected by the age of the cows (P<0.05), with the unweighted UniFrac similarity being significantly decreased with age and the weighted UniFrac similarity also being generally decreased with age with the exception of cows at week 1 and year 5 (Figure 5). These observations of bacterial community similarity in the foregut and hindgut indicated that the foregut and hindgut harbor distinct bacterial communities; however, the bacterial community in the hindgut may be affected by that in the foregut, particularly in younger animals compared with older animals.

To further investigate the extent to which the microbiota in the foregut contributed to the
establishment of the microbiota in the hindgut, we used Source-Tracker[27] to estimate the proportion of bacteria in the hindgut that originated from the foregut at OTU level (Figure 6a). We observed that a large proportion (67.811.3%) of bacteria in the hindgut originated from the foregut in the 1 week old cows, but this transfer proportion sharply decreased with age (P<0.05; Figure 6a). Two typical periods of transfer activity (the ‘high-transferring period’ and the ‘moderate-transferring period’) were also observed with dichotomy at around 18 weeks of age (Figure 6a), which was in accordance with previous observations of microbiota maturity. This finding demonstrated that the transfer activity of the microbiota from the foregut to the hindgut was largely dependent on the age of the cows, with microbiota transfer being more active in calves under 18 weeks of age than in older cows.

The transferred bacterial OTUs were mainly belonged to Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria at the phylum level in cows of different ages; however, the proportion to which each phylum contributed to the transferred bacteria in the different aged cows varied (Figure 6b). Firmicutes was the dominant transferring bacteria in 1-week-old (constituting 54.1% on all transferred bacteria), 2-year-old (61.7%), and 5-year-old (95.4%) cows, whereas Bacteroidetes was the dominant transferring bacteria in 1-month-old (55.4%) and 2-month-old (60.0%) cows (Figure 6b). This finding demonstrated that the transfer activity of a given bacterial taxa also varied with the age of the animals.

Discussion
The gut microbiota has been demonstrated to be of importance to the host[32, 33], especially in ruminants such as cows[1, 3]. Understanding the dynamic establishment of the bacterial ecology in different gut segments from birth to adulthood has potentially important ecological and medical applications. From an ecological perspective, our study sheds light on the dynamic establishment of the bacterial ecology in the different gut segments of cows.
Previous studies demonstrated that although rumen of preweaning calves were not well developed, bacteria colonized in rumen at least at first day after birth[15, 24, 25], these early colonizers also known as pioneer species played important roles in shaping the rumen environment and rumen microbial succession[23], thus preweaning calves should be enrolled to explore the gut microbiota establishment.

We used different gut segments (rumen, abomasum, duodenum, and rectum) of cows to explore the establishment of the microbiota, because spatial heterogeneity in the microbial community along the gut has been widely demonstrated in mice[11, 34], chickens[35], pigs[36], and humans[37], as well as cows[23, 25, 38]. The function of the bacterial community was also reported to differ between gut segments[12]. Although the majority of bacteria in different gut segments belonged to a few taxa such as the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, these previous studies suggested that the establishment of bacterial ecology in the different gut segments may differ[25, 26].

With increased age, we observed comprehensive bacterial community changes in each of the gut segments in terms of alpha diversity, composition, and the abundance of dominant and core bacteria. These findings were also supported by previous studies in cows[24, 39] and humans[13, 16, 40]. Despite the complexity of function and bacterial communities in the gut of cows[1], age-discriminatory bacteria were detected. These observations indicated that the establishment of the gut bacterial community could be described effectively from an ecological perspective[13].

The maturity index was defined as a measure of how similar the gut microbiota of calves was to that of adult cows, and therefore reflected the establishment of a gut bacterial community[13]. We found that the maturity index in each gut segment gradually increased with age following the natural
logarithmic function. This indicated that the gut microbiota established quickly in young animals, then slowed with age. A previous study detected two pivotal times in establishing the microbiota in the mammal gut, the first was at birth when the mammal gut captured bacteria from the vagina, colostrum, and surrounding environment to establish its own bacterial community[15, 16], the second was at weaning when animals transited from liquid food to solid food and the gut microbiota shifted quickly to that of adulthood[41]. With derivation of the achieved logarithmic function, we also observed two typical periods of microbiota maturation, the ‘quick-maturing period’ and the ‘moderate-maturing period’, with the shift occurring at around 18 weeks of age in the cows. Similar trends but with different shift times were observed for humans, with the gut microbiota quickly established before 6 months of age[20], the gut microbiota of 12-month-old infants being similar to those of their mothers[14], and the gut microbiota of a 3-year-old resembling that of an adult[22, 40].

Capturing bacteria from the surroundings (i.e. bacteria transfer) has been proposed to play an important role in gut microbiota establishment[14-16]. To quantitatively assess the proportion of microbiota transferred between the foregut and hindgut, we adopted the Bayesian algorithms of Source-Tracker[27]. This software has previously been successfully employed to calculate the proportion of gut bacteria transferred from the surrounding environment in adults[42], infants[43, 44], and in a mouse model[45]. Here, we demonstrated that the proportion of bacterial transfer from the foregut to the hindgut was high in young animals, then decreased sharply with age increase. The activity of bacterial transfer could also be roughly dichotomous at around 18 weeks of age in cows. These findings implied that the hindgut was able to capture bacteria more easily from the foregut in young animals than in older animals. Combined with our previous findings regarding microbiota establishment, our results inferred that cows had a “window period” of 18 weeks after birth in which to establish their gut bacterial community in an efficient way; this may also be the “window period” for us to manipulate the gut microbiota of cows.
The limitations of current experiment are follow-up design with the same individual along the trial was not used and the big gap in time of each age groups. Further studies with following-up design and with larger sample sizes might give more precise and detailed information, however, the major conclusions are unlikely to be changed as the microbiota change along age increasing is notable and the observation of curves of maturity is also typical by comparing with previous studies on rumen of cows[23, 24] or human[13, 14, 40].

Conclusion
In summary, we tracked the establishment of the gastrointestinal microbiota in cows from 1 week old to adulthood. Our findings shed light on the dynamic bacterial ecology of the gut of cows and highlighted the “window period” of 18 weeks after birth that is important for the establishment of the gut bacterial community. These data may be important in terms of ecological and medical applications that protect gut health or manipulate the gut microbiota of cows.

Declarations
Abbreviations

Not applicable.

Ethics approval and consent to participate

The experimental design and procedures were approved by the Animal Care and Use Committee of the College of Animal Science and Technology of China Agricultural University (Project number: 31772628), in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988)

Consent for publication

Not applicable.

Availability of data and material
Sequence data were deposited in the BigData Center (http://bigd.big.ac.cn) under accession number CRA 000782.

Competing interests

The authors declare no conflict of interest.

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Authors’ contributions

WJZ and SLL designed the study; CYG performed experiments with technical assistance from YJW, JJL, ZJC and HJY; HY performed sequencing and sequencing analysis; SKJ and HY performed statistical interpretation and analyses; SKJ and CYG took primary responsibility for writing the manuscript. All authors discussed the results and commented on the manuscript.

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Figures

Figure 1

Bacterial composition of predominant phylum in different gut segments with age change of cows. Only bacteria with mean abundance higher than 2.0% were shown.

Figure 2

Change of alpha diversity with age increase in different gut segments of cows. Alpha diversity was calculated with Chao1 index (a) and Shannon index (b), and boxes with different letters differed, P<0.05.
Dynamic change of core bacterial community at genus level in different gut segments with age increase of cows. (a-d) Compositional change of core genera in Rumen (a), Abomasum (b), Duodenum (c) and Rectum (d) with age increase, each circle represented one age group and overlaps represented the shared core genera. (e-h) Abundance change of core genera in rumen (e), abomasum (f), Duodenum (g) and rectum (h) with age increase, each row represented one age group and each column represented one detected core genus, taxonomic assignment at phylum level was indicated at the top of each column, and by adopting Pearson correlation, 3 clusters were detected based on the abundance change with age increase, which was marked as cluster 1, cluster 2 and cluster 3. (i-k) Representative patterns of bacterial abundance change with age increase, 3 clusters detected above were showed roughly increased (i), decreased (j) or followed a quadratic curve (k) in abundance with age increase.

Maturity of microbiota in different gut segments with age increase of cows. (a) Dynamic change of maturity index (see Material and Methods), the maturity indexes with different age of cows in each gut segments were fitted to a natural logarithmic function with P < 0.05 and r > 0.82. (b) Dynamic change of maturity growth rate, curves of maturity growth rate followed a power-law function for each gut segments were achieved, horizontal dash line represented the maturity growth rate cut-off at 0.005, vertical dash line and gray shade represented the cross points of cut-off line and maturity growth rate curves of each gut segments at around 18 weeks old, the insert panels showed the cross points of cut-off line and maturity growth rate curves for each gut segments.
Figure 5

UniFrac similarity of microbiota in foregut (rumen) and hindgut (rectum) of cows, boxes with different letters differed, P<0.05.

Figure 6

Bacterial transfer from foregut (rumen) to hindgut (rectum) of cows. (a) Changes of bacterial transfer proportions from foregut to hindgut with age increase, vertical dash line represented a cut-off at 18 weeks old of cows. (b) Contributions of the predominant phylum to bacterial transfer in each age group of cows, the contributions of each phylum reflected the rate of contribution in overall transferred bacteria.

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