

Temporal dynamics of the fecal microbiota in veal calves in a 6-month field trial

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Abstract

Background

Maintaining a gut homeostasis during calf growth remains a challenge in the dairy industry, as enteric infections due to opportunistic pathogens are a main burden. Because of their high sensitivity to infections, calves are frequently treated with antibiotics, and the impact on the maturation of their microbiota is largely unknown. We conducted a field study on 45 calves distributed in three veal farms to follow the longitudinal dynamics of their fecal microbiota and of their commensal *E. coli* populations. We quantified the impact of collective antibiotic treatments received in a 15-days window before samplings on the microbiota diversity and the quantity of *E. coli*. In two farms, we also searched for associations between recommended daily doses of milk powder and bacterial abundances.

Results

The time had a significant effect on the microbiota composition. A high heterogeneity was observed between calves after their arrival in farms, followed by an increase in similarity over time, which was concomitant with a decrease in proportion of newly detected OTUs in the samples. A switch of dominant genera, such as that of *Lactobacillus* and *Bifidobacterium* at early stages to *Prevotella* and *Alloprevotella* were observed as the calves aged. The Shannon index sharply increased during the first month, and then its increase slowed-down, and even stagnated in one farm. Calves receiving antibiotics had a less diverse microbiota and a reduced number of *E. coli* during the treatment or in the fifteen days following it compared to calves not exposed, although these effects were limited. We found moderate to strong positive associations between the dose of milk powder and the relative abundance of several genera, and also with the absolute number of *E. coli*.

Conclusions

Massive and continuous bacterial successions happen during the maturation of the fecal microbiota of veal calves, resulting in major changes in the diversity and taxonomic composition. This observational study suggests that in such dynamical ecosystems, administration of collective antibiotic treatments results in a limited reduction of diversity and of the size of *E. coli* population, and highlights the need of additional work to fully understand the impact of antibiotic treatments.

Background

In ruminants, the postnatal microbial colonization of the hindgut shows conserved developmental trends, with a massive colonization of facultative anaerobes such as *Escherichia coli* and *Streptococcus* [1, 2]. These first colonizers are supplanted by obligate anaerobes communities soon afterward [3]. This microbial succession is associated with a massive increase of the bacteria load in the gut, as a 100 fold increase of the number of bacteria per gram of feces has been reported between the end of the first week and seven weeks of age [4]. This increase of bacterial load as calves age happens concomitantly with an

increase of the gut microbiota diversity during the pre-weaning period [5–9]. A major alteration of the microbiota composition in both foregut and hindgut occurs during the weaning period [10–12], when introduction of solid feed into the diet leads to structural and physiological changes in the rumen that enable an efficient digestion of plant fibers. While gut bacterial communities are changing, the gut immunity has to maintain a proper response to newly acquired bacteria and environment diversity. One study in calves from birth to 6 weeks of age found that copy numbers of microRNA regulating expression of genes involved in immune response were associated with the abundance of beneficial bacteria *Lactobacillus* and *Bifidobacterium* [13]. This study highlights the potential role of regulating factors involved in the interactions between developing calves and their gut microbiota.

Maintaining a gut homeostasis during calf development remains a challenge in the dairy industry, as enteric infections are one of the main causes of morbidity and mortality in pre-weaned calves [14–16]. *E. coli* is one of the most common agent of diarrhea in calves, but is also found as a commensal in the gut of healthy calves [12, 17]. Commensal *E. coli* populations are presumably composed with different strains [3] that can act as a barrier to infection, through competition for the same growth limiting nutrients [18, 19]. Although pathogenic strains have been extensively investigated, few studies have focused on commensal *E. coli* populations in pre-weaned calves, resulting in a bias towards pathogenic strains in the literature [20]. However, the variations of niche size and the selective pressures in the habitats of commensal strains may coincidentally promote the emergence and persistence of pathogenic bacteria, rendering commensal *E. coli* populations reservoirs of virulent strains [21]. Therefore, deciphering the ecological forces that shape the commensal *E. coli* population is necessary to wholly understand the etiology of enteric infections in pre-weaned calves.

Because of their high sensitivity to pathogens, calves are frequently exposed to antibiotics to treat infections, which has been shown to result in a loss of fecal microbiota diversity in cattle [22, 23]. Antibiotic treatments can increase the sensitivity of hosts to enteric infections through the collapse of commensal bacterial populations that act as a barrier and contribute to maintain an anaerobic environment that limit the expansion of facultative anaerobic pathogens [24–26]. A study reported a dysbiosis, defined as an alteration of the microbiota composition, in neonatal dairy calves treated with bacitracin methylene disalicylate towards an increase of *Escherichia* and *Enterococcus* abundances together with a decrease of beneficial bacteria compared with calves of the same age not exposed to antibiotics [27]. To our knowledge, a few attempts have been made to investigate the effect of antibiotics on the gut microbiota composition of pre-weaned dairy calves in commercial farms [28, 29].

The veal-calf industry is an intensive farming system that produces meat from milk-fed calves. Calves are purchased in dairy farms by “integrators”, companies that are involved at all stages of the production process [30]. They are collected at two weeks of age, batched, and placed without delay in fattening farms for approximately six months. Integrators provide farmers with feed, and technical and health back-up. Calves are mainly fed with milk replacers, and a small amount of solid feed is introduced during the first weeks for the welfare of the animals. Collective antibiotic treatment is frequent, particularly at the start of the fattening period. In France, which is a major European producer of veal, calves receive an

average of more than eight antibiotic treatments during the fattening process, mainly by the oral route [31, 32]. Collective treatment represents almost 96% of the administered treatments and 88% of the prescriptions are made in advance of an anticipated outbreak of disease, when some animals in the batch develop symptoms of infection [16, 31, 32].

Based on a field trial, we provide new data on the temporal dynamics of bacterial communities in veal calves highly exposed to antibiotics at early age, with an additional focus on commensal *E. coli* population. We hypothesized that the developmental trajectory of the microbiota would be influenced by the use of antibiotics during growth, resulting in the development of a community with lower diversity and persistent shifts in taxonomic composition. We addressed this question by performing 16S rRNA gene sequencing and quantitative PCR (qPCR) of *Escherichia* on 312 rectal swabs collected from 45 veal calves distributed in French veal farms during six months.

Results

Animal sampling and follow-up

We collected fecal samples from fifteen calves in three veal farms in Brittany, France, during the fattening. These fifteen calves per farm were sampled by rectal swabbing at day 7 and at day 21 in the farms, then monthly for five months until the departure of the batch to slaughterhouse. Calves were 14 day old when they arrived in the farms, and were mainly fed with milk replacer throughout the fattening period, which is reconstituted from milk powder with hot water. Calves stayed 161 days in farms A and C and 147 days in farm B. Collective antibiotic treatments were recorded by the three farms throughout the fattening period (Fig. 1). Antibiotics were always used at therapeutic doses and administered orally in feed. All calves received antibiotics more than once, and calves from farms A and C received several consecutive antibiotic treatments during the first month (Fig. 1).

All calves from farms A and B included in the study were followed until the end of the fattening period. One calf from farm C died during fattening and was excluded from the study. For the 44 remaining calves, there were no missing samples during the follow-up and thus downstream analyses were performed on 308 samples.

16S amplicon sequencing

After processing reads using the mothur pipeline, 34,153,188 quality amplicons were generated with an average of $111,248 \pm 63,002$ per sample (Additional file 1: Fig. S1a). One sample had mostly poor-quality reads and a very small number of amplicons, and thus was excluded from the downstream 16S sequencing analyses. The minimum number of operational taxonomic units (OTUs) detected in a sample was 119 and the maximum 1,302 (Additional file 1: Fig. S1b). The rarefaction threshold was set to 47,000 sequences (Additional file 1: Fig. S1c). Seven samples were below this threshold and excluded from the α - and β -diversity analyses.

Weighted and unweighted Unifrac distances

The similarity of microbiota composition among calves was tracked using β -diversity measures, which represents the dissimilarity between samples. The weighted Unifrac distances between calves were the highest on day 7, with an overall mean of 0.45 (\pm 0.10) (Fig. 2a) and started to decrease at the next sample, the overall mean reaching 0.33 (\pm 0.06) on day 21 (Fig. 2b). Weighted Unifrac distances remained low until the end of fattening, with a mean of 0.31 (\pm 0.08) on the last day (Fig. 2c). The sampling time had a significant effect on the calf microbiome composition ($p = 0.001$, PERMANOVA on weighted Unifrac distances). It explained 15.5% of the between-sample variation in calves, indicating extensive sharing of the microbial community among calves at a given time of the fattening period. Individuals belonging to the same farm had not more similar microbiota relative to individuals from different farms ($p = 0.4$, PERMANOVA on weighted Unifrac distances). Despite the use of different antibiotic molecules and different times for collective antibiotic treatments between farms, the farm affiliation explained only 4.0% of the between sample variation (Fig. 2). We obtained similar results for the unweighted Unifrac distances (Additional file 2: Fig. S2, $p = 0.001$ and $p = 0.5$ for the sampling time and the farm in the PERMANOVA test, respectively). The farm affiliation explained 2.9% of the between samples variation.

The weighted Unifrac distances between consecutive samplings showed a trend over time, with a downward trajectory for almost all calves, indicating that the magnitude of changes tended to decrease on a monthly scale (Additional file 3: Fig. S3).

Taxonomic composition of the microbiota

We investigated the taxonomic composition at different levels from phyla to OTUs. We detected 19 phyla in the samples. Among them, only *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* were present at a relative abundance above 1% in all samples. The phyla *Firmicutes* and *Bacteroidetes* were dominant in all farms and over time. Their overall mean relative abundances were 47.7% (\pm 10.2) and 40.3% (\pm 11.3), respectively, whereas the overall mean relative abundances of *Actinobacteria* and *Proteobacteria* were 5.2% (\pm 7.2) and 3.7% (\pm 4.3), respectively. In farm A, *Firmicutes* were predominant at the beginning and then decreased slightly towards relative abundances similar to those of *Bacteroidetes* (Fig. 3).

We detected 349 genera and 50 (14% of the genera) were part of the 5 most abundant genera found in at least one sample. On day 7, the mean of the cumulative relative abundances of the five dominant taxa was 73.9% (\pm 12.3). Some genera, such as *Lactobacillus*, *Megamonas*, and *Bifidobacterium*, were found at relative abundances above 50% in a few calves (Fig. 4a). On day 21 and the last day, the mean of the cumulative relative abundances of the five dominant taxa were 59.0% (\pm 7.4) and 60.2% (\pm 8.4), respectively (Fig. 4b and Fig. 4c). From the second month, some dominant taxa were detected in all calves and persisted until the end of fattening. These taxa included *Prevotella*, *Alloprevotella*, *Bacteroides*, *Faecalibacterium*, and unclassified taxa from *Ruminococcaceae* (Fig. 4c). The relative abundances of these taxa became gradually more evenly distributed among calves over time (Additional file 4: Fig. S4).

Within each calf, the proportion of newly detected OTUs was higher than the proportion of OTUs detected in the previous sample throughout the fattening period (Fig. 5). Moreover, the proportion of OTUs detected

in the previous sample varied between 7.2% and 48.6%, suggesting that the temporal dynamics of the calf microbiota is driven by massive and continuous replacement of autochthonous OTUs by newly detected ones. The proportion of newly detected OTUs tended to decrease over time, concomitantly with an increase in the proportion of OTUs detected in two consecutive samples (Fig. 5). Only 50 OTUs simultaneously persisted in more than 97% of calves between consecutive samples. Two OTUs from the genera *Faecalibacterium* and an unclassified OTU from the *Ruminococcaceae* family persisted from the first to last month in more than 97% of calves (Additional file 5: Table S1). No OTU was newly detected or lost simultaneously by more than 50% of calves (Additional file 5: Table S1).

No over-abundance or massive depletion of specific taxa was detected at the phylum (Fig. 3) or genus level (Fig. 4a and Fig. 4b) in samples of calves under collective antibiotic treatment or which had received antibiotics in the fifteen days before the sampling compared to calves not exposed in the same period.

Shannon index and number of observed OTUs

We evaluated the temporal dynamics of microbiota diversity in each sample by examining the α -diversity metrics, the Shannon index, and the number of observed OTUs. The Shannon index showed an increasing trend over time, with an overall mean of 3.05 (\pm 0.82) on day 7 and 4.23 (\pm 0.59) at the end of fattening. The temporal dynamics of microbiota diversity were best described by a two-slope model (Likelihood Ratio Test between the two candidate models, $p < 10^{-15}$, Fig. 6a, Additional file 6: Table S2). The coefficient of the first slope was higher than that of the second, suggesting a large increase in diversity during the first month, followed by a lowering of the rate of increase, and even stagnation on farm A. The estimates for the intercept and second slope for farms B and C were significantly higher than those for farm A, suggesting that the dynamics of microbiota diversity can be modulated by farm affiliation. We found a similar temporal trend and a significant farm effect on the number of observed OTUs (Additional file 7: Fig. S5a, Additional file 6: Table S3).

A variable representing antibiotic treatments in the previous 15 days of a sampling (still ongoing or not at the time of sampling) was added to the two-slope model and tested for significance for both indices. For Shannon index, a significant but limited effect of antibiotics was found, with an estimated decrease of -0.17 $CI_{95\%}[-0.27; -0.06]$ ($p = 0.003$, Additional file 6: Table S2, Fig. 6b). This indicates an antibiotic-induced reduction of bacterial diversity during exposure. The effect of antibiotic exposure on the number of observed OTUs was also significant and showed a similar effect of decreasing diversity, with an estimated decrease of 101.3 OTUs $CI_{95\%}[39.9; 170.4]$ ($p = 1 \times 10^{-6}$, Additional file 6: Table S3, Additional file 7: Fig. S5b)

Absolute number of *E. coli* per gram of feces

We quantified the absolute number of *Escherichia* / g by qPCR, which can be considered as a fair proxy of the absolute number of *E. coli* in calves (see Methods). *E. coli* is the main facultative anaerobic bacteria in the large intestine and a marker of dysbiosis [33]. We first compared these absolute numbers

to the relative abundances of the *Escherichia* genus estimated by 16S rRNA gene sequencing. The number of *E. coli* estimated by qPCR was strongly and positively associated with the relative abundance of the *Escherichia* genus (Spearman's correlation, $r_s = 0.80$, $p < 10^{-15}$, Fig. 7a), confirming the strong relationship between the two variables. The temporal dynamics of the number of *E. coli* / g was similar for the three farms, with an overall mean of $7.81 \log_{10}$ (*E. coli* / g) (± 0.67) at day 7, followed by a transient but massive increase (around $2 \log_{10}$) during the second month (Fig. 7b). The dynamics of the number of *E. coli* / g were best described by a quartic function of time (LRT between cubic and quartic function of time models, $p = 0.01$). The final model gave the same estimates for the parameters of farms A and C, which suggests the presence of an additional factor in shaping the number of *E. coli* / g in these farms (Fig. 7b and Additional file 6: Table S4).

As for α -diversity indices, a variable representing antibiotic treatments in the previous fifteen days of a sampling (still ongoing or not at the time of sampling) was added to the quartic model and tested for significance. We found a significant but limited reduction in the *E. coli* population in calves treated in the previous fifteen days relative to those that were not, with an estimated decrease of $-0.37 \log_{10}$ (*E. coli* / g) $CI_{95\%}[-0.66; -0.08]$ ($p = 0.01$, Additional file 6: Table S4, Fig. 7c).

Association between the estimated dose of milk powder given in two farms and bacterial abundances

In farms B and C where the info was available, we explored associations between the relative abundances of genera and the daily dose of milk powder recommended by the integrator that we were able to estimate in farms B and C. Calves were almost exclusively fed with milk replacer throughout the fattening period, which is reconstituted from milk powder with hot water. Their diet was also supplemented with a small amount of solid feed from the first weeks. We conducted 349 Spearman's rank correlation tests and we found 17 taxa at the genus level for which the Spearman's correlation coefficient was positive and significantly different from zero (Additional file 8: Table S5). The genus with the strongest correlation was *Megasphaera*, with a moderate to strong association ($r_s = 0.60$, Bonferroni adjusted $p < 10^{-15}$, Additional file 9: Fig. S6). The next genera with the highest positive correlation coefficients were *Enterococcus*, *Dialister* and *Mitsuokella* ($r_s = 0.44$, 0.42 and 0.41 , and Bonferroni adjusted $p = 2 \times 10^{-8}$, 3×10^{-7} and 6×10^{-7} , respectively, Additional file 9: Fig. S6) indicating a weak to moderate association with the dose of milk powder. Although at a lesser extent, the genus *Escherichia* was also positively associated with milk powder ($r_s = 0.28$, Bonferroni adjusted $p = 0.02$) (Additional file 8: Table S5).

As lactose is one of the main components of milk powder (approximately 45% of the dry weight), we first looked for the presence of a β -galactosidase enzyme, which hydrolyzes the lactose in monosaccharides glucose and galactose, in available annotated genomes from the NCBI genome database of the *Megasphaera*, *Enterococcus*, *Dialister* and *Mitsuokella* genera. The sequence of the *E. coli* LacZ β -galactosidase was also compared to the protein sequences found in members of these genera using the

blastp program [34, 35]. A β -galactosidase gene sequence was found in the genome of *Mitsuokella multacida*, which was isolated from human feces [36], and several species of the *Enterococcus* genus which have been isolated in cattle (*E. faecalis*, *E. faecium*, *E. hirae*, *E. thailandicus*, *E. malodoratus*, *E. devriesei*, *E. casseliflavus*, *E. italicus*) [37–40]. We didn't find a β -galactosidase annotated gene in the 24 *Megasphaera* annotated genomes nor in the 18 *Dialister* annotated genomes available on NCBI [35]. The bacteria of these two genera didn't carry a similar protein to the *E. coli* LacZ β -galactosidase.

As *E. coli* is a β -galactosidase positive species, we compared the estimate dynamic profile of the absolute number of *E. coli* / g in the three farms and the dose of milk powder recommended by integrators in farms B and C and found them very close, particularly during the second month, with the peak of the dose of milk powder superimposed over that for the number of *E. coli* / g (Fig. 7d). We searched for an association between the estimated daily doses of milk powder and the farm predicted numbers of *E. coli* / g in farms B and C, using Spearman's correlation test. We found a significant strong positive association between the farm predicted numbers of *E. coli* / g and the estimated dose of milk powder ($r_s = 0.77$, $p < 10^{-15}$).

Discussion

We characterized the temporal dynamics of the fecal microbiota of calves from two weeks of life to six months of age, in three commercial veal farms representative of management practices in the veal calf industry in France. The calves were mainly fed with milk replacers throughout the follow-up, and received several collective antibiotic treatments at therapeutic doses, most of them administered in the first weeks of fattening (Fig. 1). We did 16S rRNA gene sequencing to study the composition of the microbiota and quantitative PCR of the genus *Escherichia* as a proxy of *E. coli* to quantify the commensal populations of this bacteria of medical relevance. In two farms, we estimated the daily dose of milk powder recommended by the integrator to search for association with the relative abundances of genera. The most striking results of this study are (i) the homogenization of the fecal microbiota composition among calves, which began during the first month of life and was characterized by an increase of the α -diversity and of the proportion of the same dominant genera, (ii) the significant but limited effect of antibiotic treatments on the microbiota diversity and on the *E. coli* population size, and (iii) the significant associations of the estimated daily doses of milk powder with the relative abundances of some genera and with the farm level predicted number of *E. coli* / g from our model.

The development of the microbiota of these calves was characterized by the dominance of the *Firmicutes* and *Bacteroidetes* phyla (Fig. 3) and a switch of dominant genera, such as that of *Lactobacillus* and *Bifidobacterium* at early stages to *Prevotella* and *Alloprevotella* as the calves aged (Fig. 4), with a simultaneous increase in microbiota diversity (Fig. 6 and Additional file 7: Fig. S5a). These developmental features have already been described for calves with the same characteristics (age, sex, and breed) fed with milk replacers [12], as well as for females of the same breed in Canada [11, 12], USA [7, 41] and Japan [4] and for dairy calves of a different breed in Austria [5]. These shared findings suggest that the fecal microbiota of calves undergoes a predictable age-dependent trend that is common to

distinct calf populations. The high heterogeneity of the microbiota composition at day 7 in the farms (corresponding to three weeks of age) could be attributable to the distinct origin of the calves, as they came from different dairy farms. Prior studies have noted the importance of exposure to dam's bacterial communities and to environmental bacterial communities in influencing microbiota composition throughout the gut of the newly born calf [42, 43]. The transport from dairy farms could also be responsible of the high heterogeneity between calves at the beginning of the fattening, as its disrupting effect on gut microbiota has been reported in young beef cattle five days after their transport to feedlot [23]. From as soon as the end of the first month after arriving in the farms, massive and continuous bacterial successions (Fig. 5) gradually increased the similarity of the microbiota composition among calves on all farms, both in terms of presence and relative abundances of bacterial members, as it was shown by unweighted Unifrac distances and weighted Unifrac distances (Additional file 2: Fig. S2 and Fig. 2, respectively). This convergent pattern occurred in the absence of environmental or dietary change such as weaning, the calves being reared in dedicated closed buildings and drinking milk replacers throughout the fattening. This suggests that the influence of environmental factors and dietary factors on this trend of convergence is probably limited, and highlights the likely role of host physiology. Convergence related to age has also been observed in the ruminal microbiota of calves between one day and two years of life [10] and in both the ruminal and fecal microbiota of dairy cows receiving different diets before weaning [44]. The variability of the composition of fecal microbiota of pre-weaned dairy calves has been shown to be higher than fecal microbiota of lactating cows, suggesting the higher influence of the surrounding environment on calves compared to adults' more mature microbiota [29, 44]. These studies suggest that such changes in composition are not restricted to the lower part of the gut and are not strongly driven by diet. An interesting hypothesis could be that the convergent stabilization of the microbiota composition over time (Fig. 2, Additional file 2: Fig. S2) may be linked to age-dependent shifts of the gut mucosal immune system, as it has been shown that the expression of Toll Like Receptors in both the rumen and colon changes as calves aged [45]. Constraints imposed by the gut environment and autochthonous microbiota on allochthonous bacterial settlement might become less permissive, resulting in more specific requirements as the calves aged.

The microbiota of calves in farms where collective antibiotic treatments were given in the previous fifteen days or during sampling underwent a reduction of microbiota diversity and *E. coli* number relative to calves of the same age that haven't been exposed during the same period (Fig. 6b, Additional File 7: Fig. S5, Fig. 7c). We chose to take into account and pool in our analyses both long and short antibiotic treatments and molecules with different spectra to focus on common disruptive effects of antibiotics on microbial ecosystems. As all calves of the same farm received the same antibiotic treatment at the same time, the design of our study didn't allow the analysis of each molecule's specific effects. Indeed, we didn't have negative controls in the same farm, so the results could have been biased by potentially environmental or diet variables, which could have modulated the relative abundances of some genera. The antibiotic-induced loss of diversity has already been reported in young beef cattle [23] as well as pre-weaned calves [28], and was often found associated with the depletion of beneficial bacteria and/or the

increase of opportunistic pathogens [27, 28]. Antibiotic induced dysbiosis is also observed when pre-weaned calves are fed with low doses of antibiotic molecules [46].

The effect of the antibiotic treatments was limited relative to the longitudinal changes (Additional file 6: Tables S2, S3 and S4), which is consistent with findings in dairy pre-weaned calves which received enrofloxacin and tulathromycin metaphylactic treatments [47] and beef cattle which received oxytetracycline or tulathromycin injection [23]. These findings can be explained by the existence of a natural resistome independently of any antibiotic treatment, carried by some abundant families in the fecal microbiota of pre-weaned calves, as it has been recently shown [3]. Genera of these antibiotic-resistance genes carrying families were found as dominant in the feces of the veal calves, such as *Anaerostipes*, *Blautia* and *Roseburia* (*Lachnospiraceae* family), *Enterococcus* (*Enterococcaceae* family), *Faecalibacterium* and *Pseudoflavonifractor* (*Ruminococcaceae* family), *Bacteroides* (*Bacteroidaceae* family) and *Streptococcus* (*Streptococcaceae*). Members of the *Enterobacteriaceae* family, such as *E. coli*, were also found to be a major reservoir of antibiotic resistance gene within the microbiota resistome.

Two recent studies found that the resistome of fecal microbiota in pre-weaned dairy calves was composed of resistance conferring genes against tetracycline, sulfonamides, trimethoprim, β -lactams and macrolides [3, 29]. These results suggest the existence of a natural resilience of fecal bacterial communities to collective antibiotic treatments in veal farms, the antibiotics used to treat the calves in our study belonged to these classes. None of these studies reported the presence of *mcr-1* gene, which confers resistance to colistin, another molecule used to treat calves, in microbial communities, although another study detected the gene in calves' commensal *E. coli* [32]. Moreover, it has been shown that this natural resistome was shaped by the bacterial phylogeny of the fecal microbiome and decreases as calf aged, one of the main driver been the decrease in abundance of the *Enterobacteriaceae* family, in which 90% of the members were classified as *E. coli* [3]. It is well-known that commensal *E. coli* populations of veal calves harbor high levels of antibiotic resistance genes [32, 48], and that these genes are diverse. Antibiotic treatments may have promoted the increase in numbers of specific pre-existing *E. coli* strains in the beginning of the fattening period, as the extended treatments during the first month did not result in massive depletion of *E. coli* population.

Among others, we found a possible link between *E. coli* population dynamics and milk replacer, which is reconstituted from dry milk powder and is rich in lactose (Fig. 7d) [49]. Lactose allows the growth of the vast majority of *E. coli* strains [50]. It has been shown that the lag time and generation time of *E. coli* strains, which depend on metabolic efficiency and are crucial for gut colonization and persistence [51, 52], are influenced by the type and abundance of available nutrients in the habitat [53]. Our findings are consistent with another study in which the fecal microbiota of Simmental calves was followed during their first three months of life, although only six calves were included in the study and the sampling was sparse [5]. The relative abundance of the genus *Escherichia* was found to be maximal during the milk-feeding period and decreased before weaning. Associations between the estimated dose of milk powder and four other genera were also found. The concomitant fluctuations of this lactose rich source and the relative abundances of *Enterococcus* and *Mitsuokella* genera strongly suggest the direct role of host diet

on members of the fecal microbiota, although the β -galactosidase enzyme that enables lactose utilization was not found in two of them. The discrepancy between the associations found for the *Megasphaera* and *Dialister* genera, and the absence of the β -galactosidase enzyme sequence in the genome of known members of these genera could be explained by the utilization of another nutrient present in the milk powder by the members of these taxa, or a small redundancy of carbon sources among members of these genera.

Our study had some limitations. First, we followed the fecal microbiota of calves reared in commercial veal farms, so the calves were not randomly assigned to the different farms (nor antibiotic treatments) and neither the environmental nor diet variables could be controlled as in a randomized trial. Nevertheless, one of the aim of this study was to characterize the fecal microbiota of calves reared under common veal farm practices. These three farms, in which field studies had already been made [32], were representative of management practices in the veal calf industry in France. Second, calves were only sampled after seven days spent in farms, so no information regarding their microbiota composition before any antibiotic treatment was available. They were also sampled on a monthly basis, whereas high-frequency sampling has been recommended in early life microbiome studies in infants [54]. Furthermore, sampling was performed independently of antibiotic treatment. Hence, some short-term and mid-term age- and antibiotic-associated changes may have been missed. Third, although we tracked the dynamics of microbiota using 16S rRNA gene sequencing and *E. coli* qPCR, we only focused on specific features of this complex ecosystem and may have missed specific patterns at other levels. For example, we had no information concerning the dynamics of fecal bacterial loads, which has been shown to vary in newborn calves [1, 2, 17] and be linked with microbiota composition [55]. We also have no information concerning changes at the species level, although genus-level metabolic diversification has been shown within the microbiota [56, 57], nor intra-species level, as the similar absence of a link between nutritional preferences and phylogenetic distance has been shown within *E. coli* species [50].

Nevertheless, veal calves as studied in this work have relevant attributes to explore the microbiota maturation process. First, batches are composed of male calves of the same age and breed (usually Holsteins), so there is high genetic and physiological homogeneity among them. Second, they share the same living environment and diet, which are not subject to major changes, as they are reared in dedicated closed buildings in which the conditions are stable and controlled to optimize their growth. Moreover, they do not experience any drastic shift in their diet, as it remains predominantly composed of milk replacers during the six months of fattening. Third, the systematic administration of antibiotics at therapeutic doses to all members of the batch is common practice to prevent the spread of infectious diseases [31]. As healthy young subjects sharing similar controlled conditions over a long time period and who experience common antibiotic exposure, veal calves represent a unique chance to disentangle the driving factors of microbiota assembly in real conditions.

Conclusion

Our study demonstrated the (i) the early homogenization of the fecal microbiota composition among calves, (ii) the significant but limited effect of antibiotic treatments on the microbiota diversity and on the *E. coli* population size, and (iii) the significant associations of the estimated daily doses of milk powder with the relative abundances of some genera and the farm predicted numbers of *E. coli* / g. This observational study suggests that host developmental factors and diet can outweigh antibiotic treatment in shaping the taxonomic composition of the fecal microbiota of veal calves, and highlights the need of additional work to fully understand how antibiotic treatments impact such dynamical ecosystems. To our knowledge, this is the first field study that aims to follow the microbiota composition and the commensal *E. coli* population size of veal calves all over the fattening period.

Methods

Animal handling and sampling

We collected fecal samples from veal calves during a cohort study dedicated to monitor the excretion of extended spectrum β -lactamase (ESBL) producing *E. coli*, which has been shown to be frequent in veal calves [32, 58]. The fecal excretion of ESBL producing *E. coli* was followed in 45 veal calves distributed in three French veal farms (named A, B, and C) located in the region of Brittany [32], within a 100 km radius around Rennes. We streaked swabs on selective ChromID ESBL agar (bioMérieux, Marcy l'Etoile, France) and classified calves as "ESBL-producing *E. coli* high level excretor", "low level excretor" or "ESBL-producing *E. coli*-free" based on the number of colonies that grew after 24 hours at 37 °C (more than 100 colonies = high level excretion, less than 100 colonies = low excretion or no colonies = no excretion) [32]. We took this cohort as an opportunity to follow in real conditions the dynamics of veal calves' fecal microbiota by collecting additional fecal samples. Characterization of the ESBL-producing *E. coli* study will be published elsewhere.

Sampling began upon the arrival of batches of new calves in October and November 2015. A batch was defined in the study as a group of calves entering the farm at the same time and reared together until slaughter (Additional file 10: Fig. S7). In each farm, fifteen calves were randomly selected over 50 calves and included in the study: five calves with high levels of ESBL producing *E. coli* excretion, five calves with low level excretion and five calves with no excretion, at seven days in the farms. These fifteen calves per farm were sampled by rectal swabbing bimonthly until the departure of the batch to slaughterhouse for ESBL-producing *E. coli* excretion follow-up. To study the dynamics of the fecal microbiota, additional samples were collected at day 7 and at day 21 in the farms, then monthly for five months, which brings the number of samples per calf to seven (Fig. 1). Swabs were placed immediately in portable coolers with ice packs, shipped to the ANSES lab and stored at -80 °C.

Calves were 14 days old when they arrived in the farms, and were mainly fed with milk replacer throughout the fattening period, which is reconstituted from cow milk powder with hot water. Their diet was also supplemented with a small amount of solid feed from the first weeks. Information about the daily feeding quantities of milk powder were available for farms B and C. The quantity of milk powder to

feed the calves each day recommended by the integrators was obtained for farms B and C. This information was not available for farm A. The daily quantity of milk powder was divided by the mean weight of a male Holstein calf of the corresponding age at each day (assuming that the calves arrived to the farms at 14 days of age), which enabled us to estimate a proxy of the dose of milk powder consumed on these farms throughout the entire fattening period.

Thus, the integrator's recommended doses of milk powder, which is approximately constituted of 45% of lactose, were estimated for farms B and C. Collective antibiotic treatments were recorded by the three farms throughout the fattening period (Fig. 1). Antibiotics were always used at therapeutic doses and administered orally in feed. All treatments at entrance in the fattening farm were set up to prevent gastrointestinal disorders whereas treatments during the course of the fattening process were used to treat respiratory diseases. All calves received antibiotics more than once, and calves from farms A and C received several consecutive antibiotic treatments during the first month (Fig. 1).

DNA extraction, 16S rRNA gene sequencing, and *Escherichia*-specific quantitative PCR

Genomic DNA was extracted from rectal swabs using the DNEasy PowerSoil kit (QIAGEN, Venlo, Netherlands). The cotton tips of frozen swabs were broken off directly into bead tubes. The tubes were incubated at 70 °C for 10 min, as previously described [59]. The remaining steps were performed according to the manufacturer's instructions, with an additional overnight incubation step with elution buffer at 4 °C. Extracted DNA was stored at -20 °C. The V4 region of the 16S rRNA gene from each sample was amplified using the primers 515fB (GTGYCAGCMGCCGCGGTAA) and 806rB (GGACTACNVGGGTWTCTAAT) modified to contain a barcode sequence between the primer and the Illumina adaptor sequences as previously described [60, 61]. Dual-barcoded libraries were sequenced on an Illumina MiSeq machine (MiSeq Reagent Kit V3, 600 cycles) according to the manufacturer's specifications to generate paired-end reads of 300 bases in length.

Quantification of *E. coli* was done by qPCR targeting the 16S rRNA gene sequence specific to the *Escherichia* genus. The *Escherichia* genus is a good proxy of *E. coli* in cattle as *Escherichia* cryptic clades and other species represent less than four percent in non-human mammal feces [62]. We verified this assumption by plating on Drigalski plates three randomly selected swabs from distinct calves of our study. We determined the species of 50 colonies per plate using MALDI-TOF and the PCR Clermont typing PCR [63]. All the colonies were confirmed as *E. coli*. The sequences of forward primer (CATGCCGCGTGTATGAAGAA), reverse primer (CGGGTAACGTCAATGAGCAAA) and probe (FAM-TATTAACCTTTACTCCCTTCCTCCCGCTGAA-TAMRA) were obtained from [64]. DNA sample (approximately 20 ng) was added to 30 µl of PCR mixture containing 25 µl of TaqMan Universal PCR master mix II 2X (Applied Biosystems, Life Technologies, Carlsbad, California, USA), 300 nM each primer, 100 nM fluorescent probe, and bovine serum albumin at a final concentration of 0.1 µg/µl (New England BioLabs, Evry, France), as previously described [65]. In each experiment, a standard curve was performed

using known amounts of DNA of the archetypal ED1a *E. coli* strain. Products were detected with an Applied Biosystems Prism 7500 instrument.

Processing of 16S rRNA gene sequences

The quality of the reads was checked using FASTQC [66] and they were processed using mothur (version 1.35.1) [74, 75]. Contigs were generated by assembling forward and reverse reads. Low-quality contigs were discarded if the total length was outside 289 to 292 bases, if there were more than five ambiguous bases ("N"), or if homopolymer runs exceeded five bases. After a clean-up step, sequences were aligned to those of the SILVA reference database (February 2017 release) [76]. OTU assignment was made after clustering the sequences with a similarity cutoff of 97%. Singletons, duplicates, and triplicates were discarded. The taxonomy of each detected OTU was obtained using the RDP quality-controlled, aligned, and annotated Bacterial and Archaeal 16S rRNA gene sequences database [70]. Chimeric sequences were removed after *de novo* chimera detection using the VSEARCH tool, version 2.3.4 [71]. Sequences flagged as chloroplasts, mitochondria, or eukaryota were discarded from the dataset. We assessed the microbiota taxonomic composition, focusing on the phyla and genera, and focused on the five most abundant genera in each sample. For each calf, the OTUs detected in a sample were classified according to their detection in the previous sample to determine the monthly degree of change of the calf microbiota at the OTU level. We determined at each time point the proportion of OTUs that were not previously detected, and the proportion of OTUs that were not detected in the previous sample. This analysis was done to identify concurrent patterns of acquisition and persistence of OTUs among calves.

A rarefaction step was done before computation of the α - and β -diversity metrics. Several candidate rarefaction thresholds were assessed for several characteristics, such as the number of samples below the threshold, the number of samples for which the sampling effort would result in more than 25% and 50% of the total number of sequences, and the proportion of sequences sampled in the most abundant sequence sample. The threshold was chosen as the best compromise between these four characteristics. Then α -diversity and β -diversity metrics were computed from the rarefied samples. α -diversity is defined as the ecological diversity within samples, whereas β -diversity is defined as the dissimilarity between samples. The Shannon diversity index and the number of observed OTUs were computed as α -diversity metrics. Unweighted and raw weighted Unifrac distances were computed as β -diversity metrics [72]. For each calf, distances were computed between consecutive samples, and at the first, second, and last sampling, distances were computed between all calves.

In summary, the available data for each sample were (i) the relative abundance of taxa at the phylum level and the genus level, (ii) the α -diversity metrics Shannon index and the number of observed OTUs, (iii) the weighted and unweighted Unifrac distances to the previous and the next sample of the same calf, and (iv) the absolute number of *E. coli* per gram of feces. In addition, at the first, second, and last sampling, Unifrac distances between calves were computed.

Statistical analyses

Comparing community structures between and within calves over time

We performed permutational multivariate analysis of variance (PERMANOVA) tests [73] using weighted and unweighted Unifrac distance matrices to evaluate the effects of the time and the farm on the calf's microbiota. Tests were done using 1,000 permutations. To account for repeated measures, we constrained the permutations within each calf. The weighted and unweighted Unifrac distances between calves at the first, second, and last samplings were represented in heatmaps. Moreover, the Unifrac distances between consecutive samples for each calf were represented by spaghetti plots to assess the temporal variability of its microbiota composition.

Temporal modelling of α -diversity indices

We built linear mixed-effects models to study the temporal dynamics of the microbiota diversity. One-slope and two-slope models were tested. For the two-slope model, the break was set at the day 21 for the farms after visual inspection of the raw data. The equation of the two-slope model is shown below:

$$y_{i,j,k} = \theta_{0i,k} + \theta_{1i,k} \times t_{1i,j,k} + \theta_{2i,k} \times t_{2i,j,k} + \varepsilon_{i,j,k}$$

where $y_{i,j,k}$ is the observed Shannon index or the number of observed OTUs at the j^{th} day of calf i from farm k . $\theta_{0i,k}$, $\theta_{1i,k}$, $\theta_{2i,k}$ are the intercept, first slope, and second slope, respectively. $t_{1i,j,k}$ and $t_{2i,j,k}$ represent the time before or on the 21st day and after, respectively, and $\varepsilon_{i,j,k}$ the residual error.

The farm effect was introduced for each parameter and the calves set as random effects. The likelihood ratio test (LRT) was used to compare the fit between the candidate models. For each parameter of the final model, farms were grouped when the effects were not significantly different. We assumed that the random effects and residual errors were independent and had a normal distribution with a mean of 0. Evaluation of the final model was conducted using basic goodness-of-fit plots.

Temporal modelling of absolute number of *E. coli* per gram of feces

First, we performed Spearman's correlation between the number of bacteria from the *Escherichia* genus, estimated by quantitative PCR, and the relative abundance of the *Escherichia* genus to assess the consistency of the two techniques. Second, we built linear mixed-effects models to study the temporal dynamics of the absolute number of *E. coli* and tested polynomial functions of time. The farm effect was introduced for each parameter and the calves set as random effects. The equation of the quartic model is shown below:

$$y_{i,j,k} = \theta_{0i,j,k} + \theta_{1i,j,k} \times t_{i,j} + \theta_{2i,j,k} \times t_{i,j}^2 + \theta_{3i,j,k} \times t_{i,j}^3 + \theta_{4i,j,k} \times t_{i,j}^4 + \varepsilon_{i,j}$$

where $y_{i,j,k}$ is the observed number of *E. coli* / g and $\theta_{0i,j,k}$, $\theta_{1i,j,k}$, $\theta_{2i,j,k}$, $\theta_{3i,j,k}$, $\theta_{4i,j,k}$ the coefficients of each term of the polynomial function of time; $t_{i,j}$ the j^{th} day of calf i , and $\varepsilon_{i,j,k}$ the residual error.

The number of random effects was reduced by a backward approach using the Akaike Information Criterion (AIC), starting with the random effect of the coefficient with the highest degree. Selection of the final model and grouping by the farm effect were performed as for the α -diversity indices. We also made the same assumptions of normality and independence and evaluated the final model using basic goodness-of-fit plots.

Determination of the influence of antibiotic treatment on temporal predictions of α -diversity indices and the absolute number of *E. coli*

Alterations of fecal microbiota, such as selective depletion of bacterial populations and reduction of ecological diversity, are generally regarded as gut microbiota dysbiosis markers following antibiotic treatment [74–76]. We investigated such effects on the temporal dynamics of the calves' fecal microbiota by considering that dysbiosis linked to antibiotic could occur for all samples for which a collective antibiotic treatment was given within 15 days before sampling, antibiotic treatment being or not still ongoing at sampling time (Fig. 1).

The influence of antibiotic treatment on the intercept of α -diversity indices and the absolute number of *E. coli* models was tested by introducing a covariable representing the existence of treatment within the 15 days before sampling. Samples were collected in such a time window on two dates at farm A (days 7 and 21), one date at farm B (day 106), and four dates at farm C (days 7, 21, 35, and 91, Fig. 1).

Exploring associations between abundance of genera and the dose of milk powder

A link between the relative abundances of genera and the dose of milk powder was explored in farms B and C. The Spearman correlation test was used to look for positive associations between the dose of milk powder and the relative abundances of genera. As multiple tests were performed, the P values were adjusted using the Bonferroni correction. For genera that were found to have significant moderate to strong positive association with the dose of milk powder ($r_s > 0.4$), we searched for the presence of a β -galactosidase enzyme, which hydrolyzes the lactose in the monosaccharides glucose and galactose, in the NCBI genome and protein databases. We searched for the presence of a β -galactosidase enzyme in available annotated genomes of the corresponding genera in the NCBI genome database [35]. We also

blasted the protein sequence of the LacZ β -galactosidase enzyme in the NCBI protein database using the program blastp [34, 35].

As *E. coli* is a lactose fermenting species, we searched for an association between the estimated daily doses of milk powder and the farm predicted numbers of *E. coli* /g in farms B and C, using Spearman's correlation test. The farm predicted numbers of *E. coli* /g were obtained by adding to the intercept the estimates of the farm parameters in our final model.

All statistical analyses were performed using R software (R version 3.1.0) [77]. Mixed effects models were built using the nlme package [78] and PERMANOVA tests were done using the "adonis" function in the vegan package [79].

Abbreviations

AIC
Akaike Information Criterion
ESBL
extended spectrum β -lactamase
LRT
Likelihood ratio test
OTU
operational taxonomic unit
PERMANOVA
permutational multivariate analysis of variance
qPCR
quantitative PCR
rRNA
Ribosomal Ribonucleic Acid

Declarations

Ethics approval and consent to participate

This study was declared to the CNIL, the French office responsible for protecting personal data, supporting innovation and preserving individual liberties. No further ethical approval was needed since this study did not involve any experimentation on animals (only rectal swabs were sampled) and since we did not collect and register any personnel opinion of the participants.

Consent for publication

Not applicable

Availability of data and material

Sequencing reads were deposited as entire raw data in the European Nucleotide Archive repository (ENA) under the BioProject ID PRJEB33072, and separately for each sample. The code used for processing the 16S rRNA gene sequences was originally developed by Kozich *et al.* [68] and is available at the website https://www.mothur.org/wiki/MiSeq_SOP (access on January 2017). The unrarefied OTU table and the corresponding taxonomic classification analyzed during the current study are available from the corresponding author on reasonable request. *Escherichia*-specific qPCR dataset has been included as Additional file 11: Table S6.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MH, JYM, FM and ED conceived and designed the study. MH and JYM collected samples. MM performed the laboratory assays and carried out the bioinformatics analyses. MM, TTN and FM carried out the statistical analyses of the data. MM generated the figures. MM and ED wrote the manuscript. MH, TTN, JYM and FM revised and edited the draft. All authors read and approved the final manuscript.

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Additional Files

Additional file 1 (format PPTX): **Fig. S1**. Sequence and OTU distributions after bioinformatics processing. **(a)** Distribution of the number of 16S rRNA gene V4 region sequences in samples after quality filtering. **(b)** Distribution of the number of OTUs in samples after clustering sequences with a similarity cutoff of 97%. The inner lines in the boxplots represent the median, the edges show the first and third quartiles, and the whiskers extend to the 5th and 95th percentiles in **(a)** and **(b)**. **(c)** Rarefaction curves for 16S rRNA gene V4 region sequences. Each curve corresponds to a sample. The red vertical line represents the chosen rarefaction threshold.

Additional file 2 (format PPTX): **Fig. S2**. Heatmaps of the β -diversity unweighted Unifrac distances matrix at **(a)** first sampling (day 7), **(b)** second sampling (day 21), and **(c)** last sampling (day 161 for farms A and B and day 147 for farm C). Yellow squares indicate low Unifrac distances, whereas dark red squares indicate high Unifrac distances. Calf IDs are provided at the left of panels. Calves are ordered according to farms in both lines and columns. The means \pm standard deviations for each sampling on each farm are shown in the lower triangles.

Additional file 3 (format PPTX): **Fig. S3**. Observed intra-calf β -diversity weighted Unifrac distances between consecutive samplings in **(a)** farm A, **(b)** farm B, and **(c)** farm C. The dots indicate the Unifrac distances between consecutive samples of the same calf.

Additional file 4 (format PPTX): **Fig. S4**. Relative abundances of the 5 most abundant taxa at the genus level for all calves throughout the fattening period. For each panel, the first and second days represent the sampling date for farm C and farms A and B, respectively. Relative abundances of the 5 most abundant taxa are given for **(a)** day 35, **(b)** days 63 and 77, **(c)** days 91 and 106, and **(d)** days 119 and 133. Other detected taxa are depicted by the white bars. Calf IDs are provided at the top of the panels and are ordered according to farm. The color scale of the dots beneath the bar graphs represents the distribution of the Shannon index values. The color key refers to the phylum of each taxa and each palette was built to maximize distinctiveness between shades.

Additional file 5 (format XLSX): **Table S1**. OTUs detected in or absent from previous samples and shared by calves over time. The sampling, ranges of proportions of calves, and OTUs taxonomy are presented in each layer. The lists of OTUs consist of **(a)** OTUs simultaneously detected in two consecutive samples in more than 25% of calves, **(b)** OTUs that have not been previously detected and that simultaneously appear in more than 25% of calves, and **(c)** OTUs that have been simultaneously lost by more than 25% of calves.

Additional file 6 (format DOCX): **Tables S2, S3 and S4**. Tables S2, S3 and S4 contain the estimated parameters for the final models of the Shannon index, the number of observed OTUs and the absolute

number of *E. coli*, respectively.

Additional file 7 (format PPTX): **Fig. S5.** Dynamics of the mean observed and predicted number of observed OTUs for each farm. Predicted dynamics of the number of observed OTUs with and without the antibiotic treatment effect in the final model are represented in panels (a) and (b), respectively. The mean values \pm standard deviations of the observed data for each farm are represented by the dashed bars. Model predicted profiles and their 95% confidence bands are represented by the solid lines and bands, respectively. Antibiotic treatments during sampling or within 15 days before sampling are colored-coded by farm and indicated above the x-axis in panel (b).

Additional file 8 (format XLSX): **Table S5.** List of the Spearman's rank correlation coefficients between genera found in calves' feces and the dose of milk powder estimated in farms B and C. The genera with a significant positive correlation and a Bonferroni corrected p-value inferior to 0.05 were highlighted in green.

Additional file 9 (format PPTX): **Fig. S6.** Relative abundance of the genera *Megasphaera*, *Enterococcus*, *Dialister* and *Mitsuokella* as a function of the dose of milk powder. Each point represents a sample. These four genera had the highest significant positive correlation with the estimated dose of milk powder in farms B and C. Values on the x-axis correspond to samples in which the corresponding genus was not detected by 16S rRNA gene sequencing.

Additional file 10 (format PPTX): **Fig. S7.** Veal calves on fattening farms (a) on the first day, corresponding to 14 days of age, and (b) at 115 days of age, during the third month of fattening.

Additional file 11 (format TXT): **Table S6.** Absolute number of *Escherichia coli* per gram of feces in all the samples estimated by *Escherichia*-specific quantitative PCR.

Figures

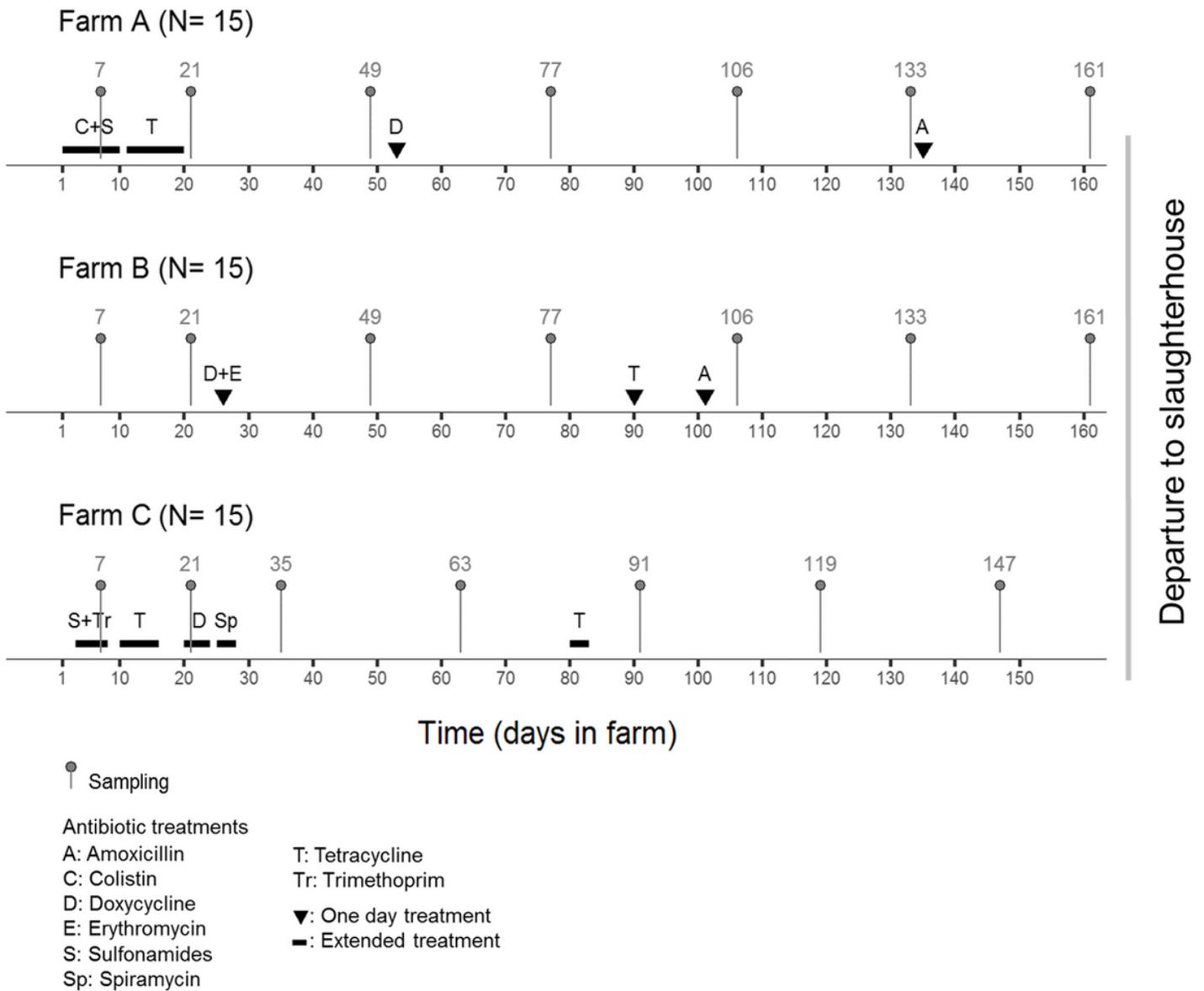


Figure 1

Scheme of sampling dates in each farm. Sampling points in farm A, farm B, and farm C are represented in the upper panel, middle panel, and lower panel, respectively. “N” indicates the number of calves studied in each farm. The days of sampling are indicated by grey dots. Of note, one calf on farm C died during the fattening period and was excluded from the study. Antibiotics treatment are indicated by bold dark lines or back triangles, and names of antibiotics are given in legend.

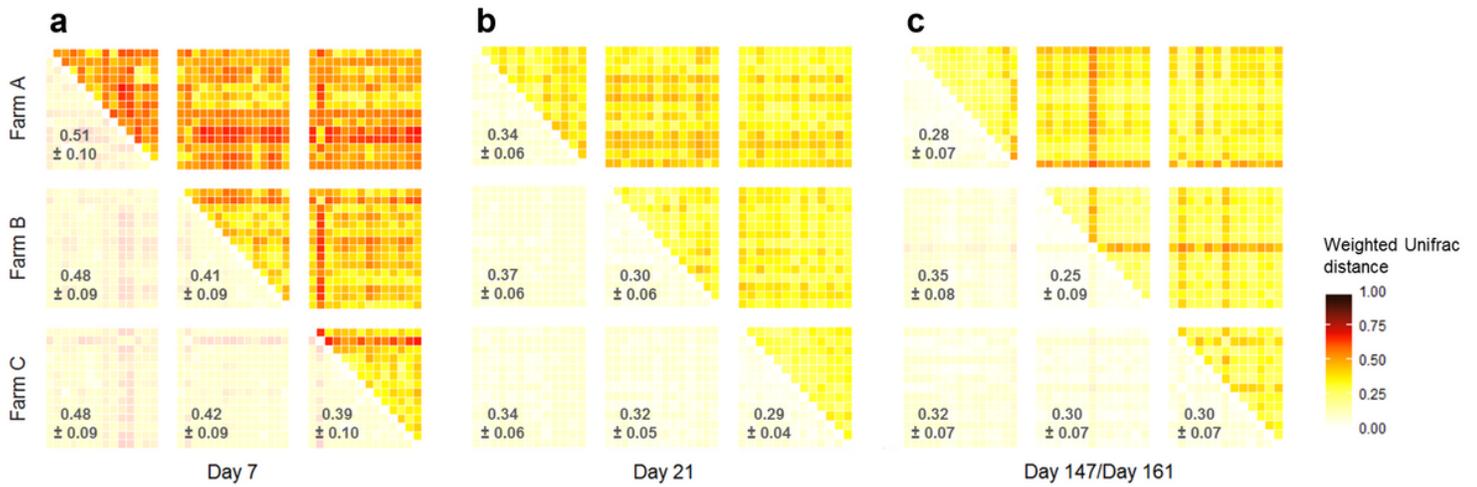


Figure 2

Heatmaps of the weighted Unifrac distances at the first month and last month of fattening. Heatmaps of the β -diversity weighted Unifrac distances matrix are represented at (a) first sampling (day 7), (b) second sampling (day 21), and (c) last sampling (day 161 for farms A and B and day 147 for farm C). Each square represents a pairwise distance between two calves. Pale yellow squares indicate low Unifrac distances, whereas dark red squares indicate high Unifrac distances. Calves are ordered according to the farms in both the lines and columns. Calf's distance from itself is represented by the white square on the main diagonal. The means \pm standard deviations for each sampling and each farm are shown in the lower triangles.

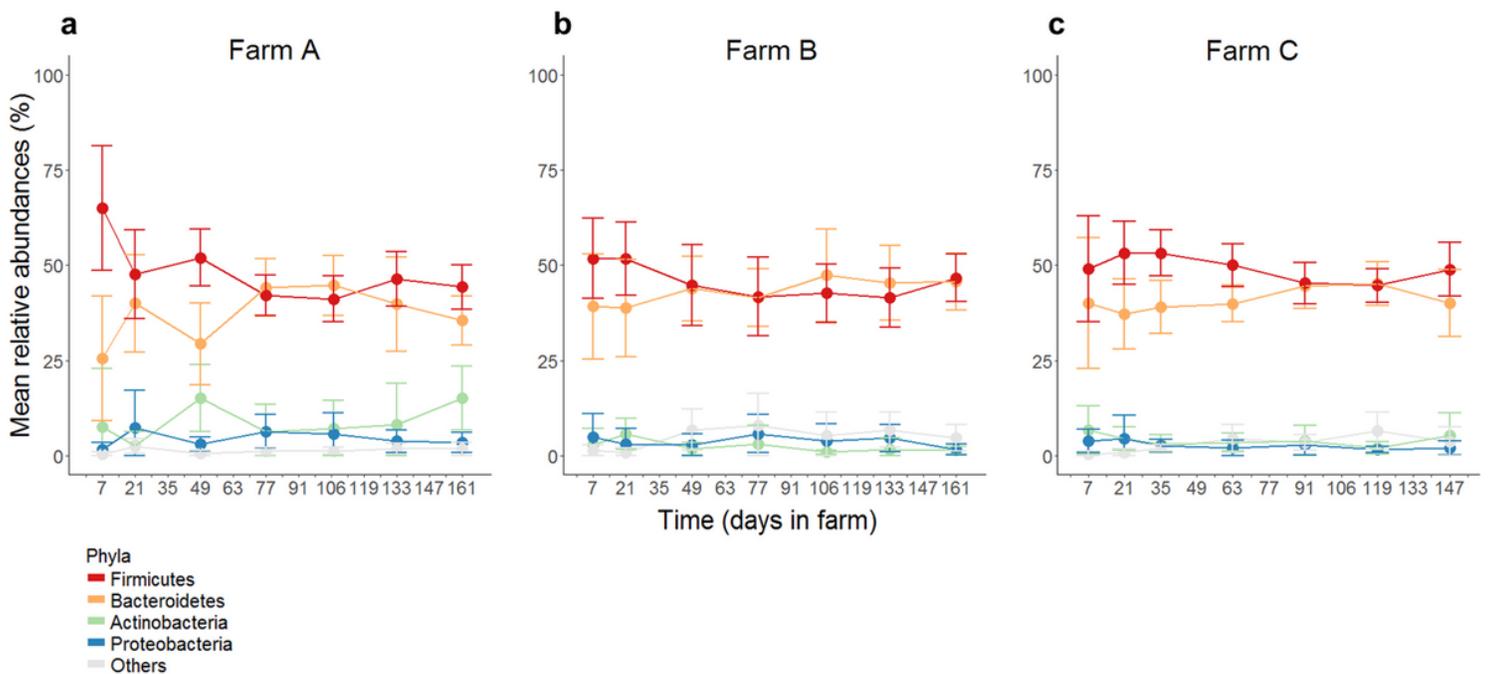


Figure 3

Mean relative abundances of the four main phyla over time in each farm. Mean relative abundance in farm A, farm B, and farm C are represented in panels (a), (b), and (c), respectively. Mean relative abundances \pm standard deviations of the data are represented by the bars.

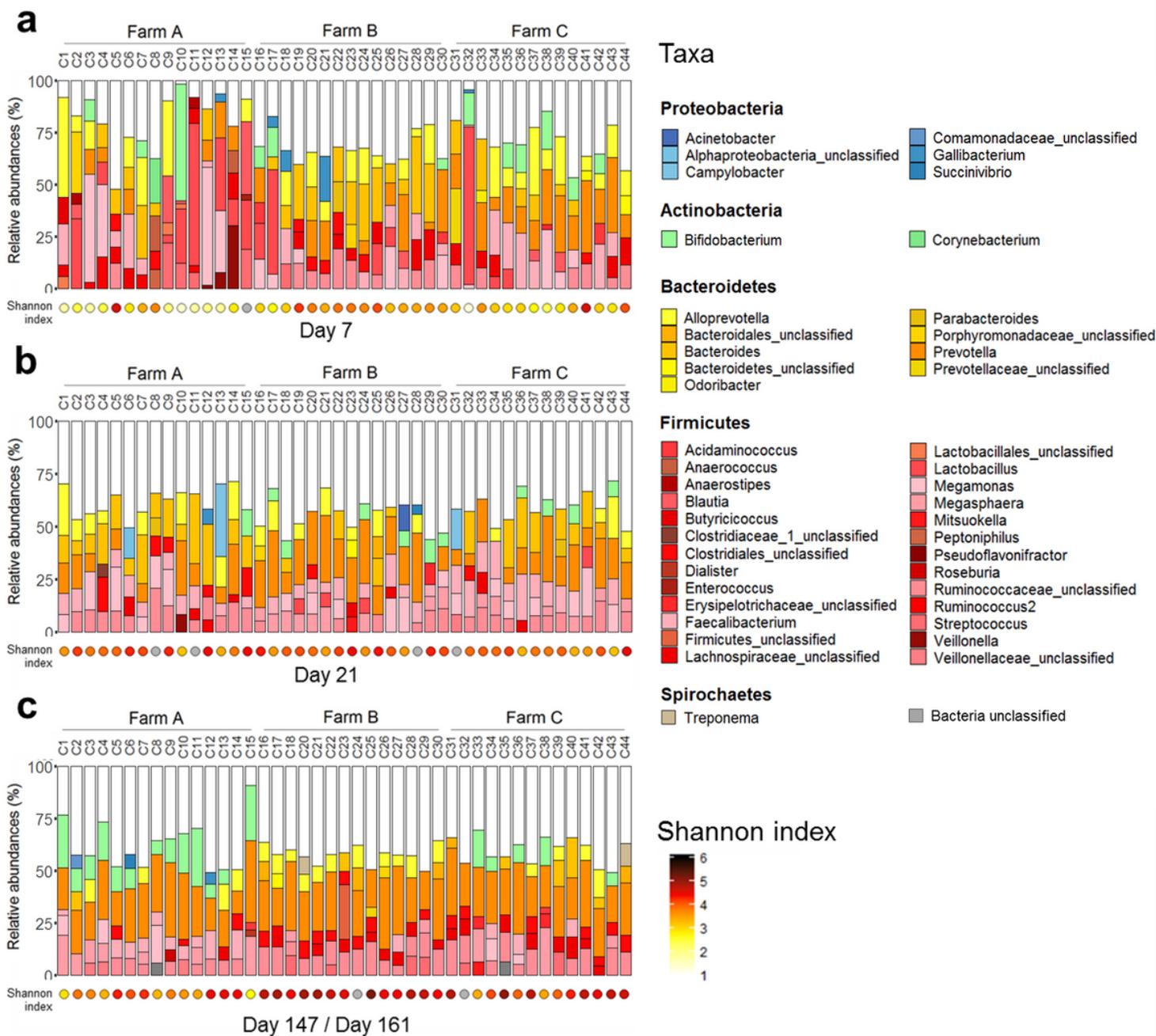


Figure 4

Individual microbiota composition at genus level at the first month and last month of fattening. Relative abundances of the 5 most abundant taxa at the genus level for all calves at (a) first sampling (day 7), (b) second sampling (day 21), and (c) last sampling (day 161 for farms A and B and day 147 for farm C). Other detected taxa are depicted in white. Calf IDs are provided at the top of the panels and are ordered according to farm. The color scale of the dots beneath the bar graphs represents the distribution of the Shannon index values. Grey dots indicate samples for which no index was computed because the

number of sequences was lower than the rarefaction threshold. The color key refers to the phylum of each taxa and each palette was built to maximize distinctiveness between shades.

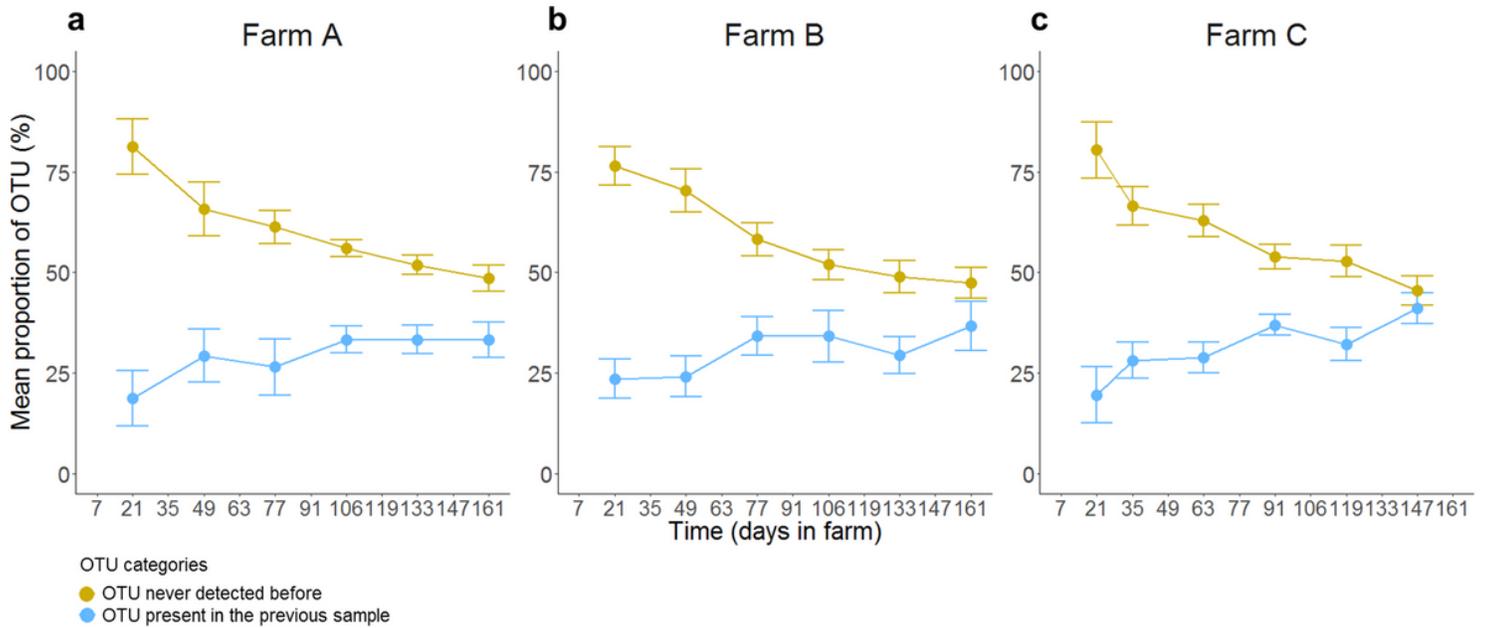


Figure 5

Mean proportions of OTUs relative to those detected in previous samples of the same calf. Mean proportions of OTUs in farm A, farm B, and farm C are represented in panels (a), (b), and (c), respectively. Mean proportions \pm standard deviations of the data are represented by the bars.

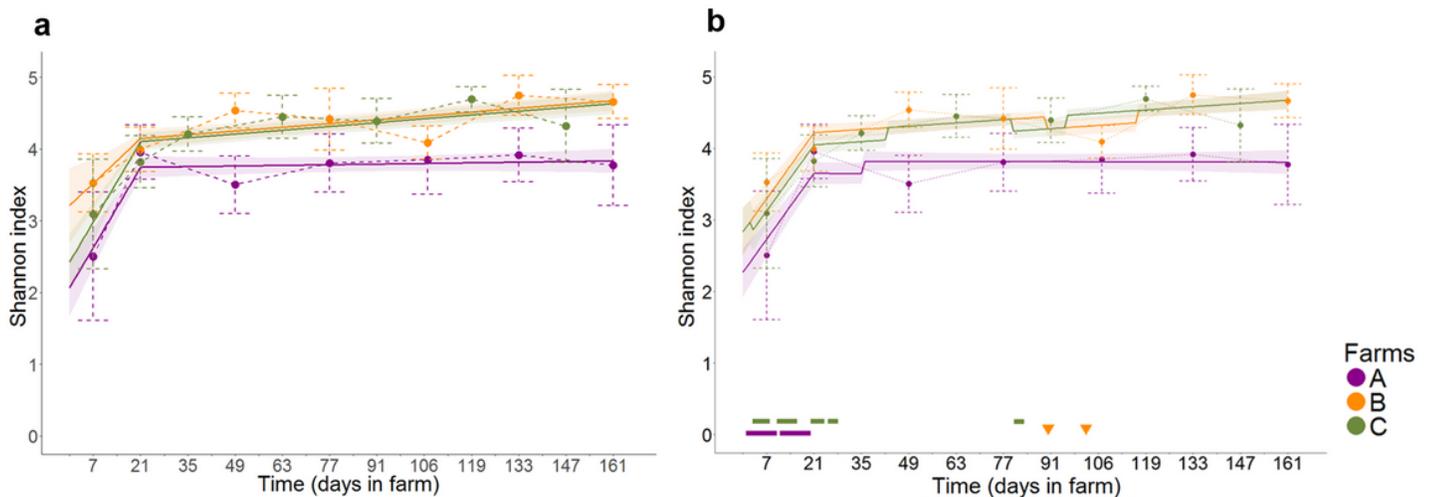


Figure 6

Dynamics of the mean observed and predicted Shannon index for each farm. Predicted dynamics of the Shannon index with and without the antibiotic treatment effect in the final model are represented in panels (a) and (b), respectively. The mean values \pm standard deviations of the observed data for each

farm are represented by the dashed bars. Model predicted profiles and their 95% confidence bands are represented by the solid lines and bands, respectively. Antibiotic treatments during sampling or within 15 days before sampling are colored-coded by farm and indicated above the x-axis in panel (b).

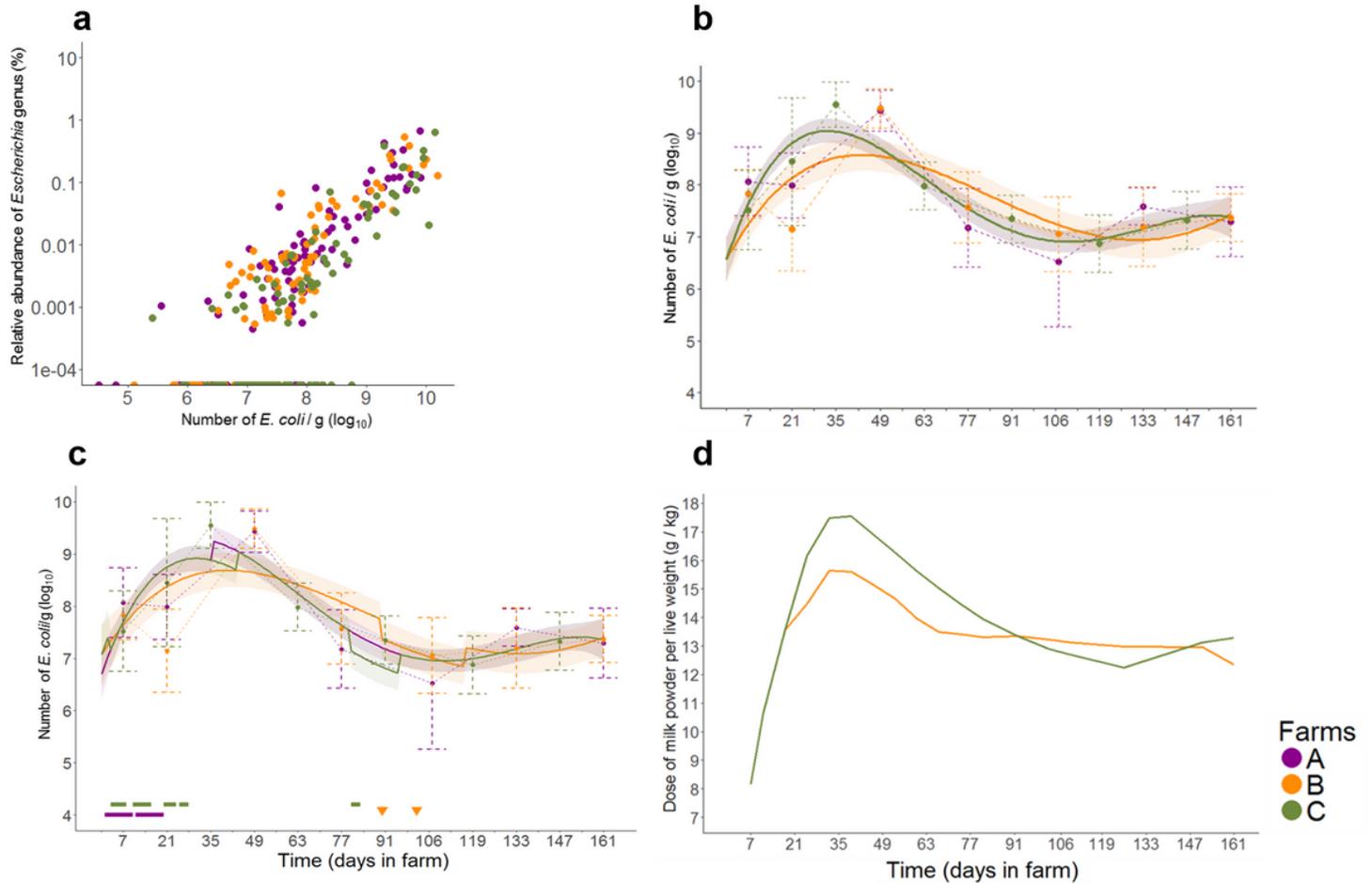


Figure 7

Absolute quantification of the *Escherichia coli* population by qPCR. (a) Relative abundance of the *Escherichia* genus as a function of the number of *E. coli* / g estimated by qPCR. Each point represents a sample. Values on the x-axis correspond to samples in which *Escherichia* genus was not detected by 16S rRNA gene sequencing. (b) Dynamics of the mean observed and predicted number of *E. coli* / g for each farm in the final model without the antibiotic-treatment effect. The mean values \pm standard deviations of the observed data for each farm at each sampling time are represented by the dashed bars. The model predicted profiles and their 95% confidence bands represented by the solid lines and bands, respectively. The predicted profiles of farms A and C are overlapping, which is why the predict profile of farm A does not appear. (c) Dynamics of the mean observed and predicted number of *E. coli* / g for each farm in the final model with the antibiotic-treatment effect. Antibiotic treatments during sampling or within 15 days before sampling are colored-coded by farm and indicated above the x-axis. (d) Temporal dynamics of the recommended dose of milk powder per kilo of live weight in farms B and C.

Supplementary Files

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