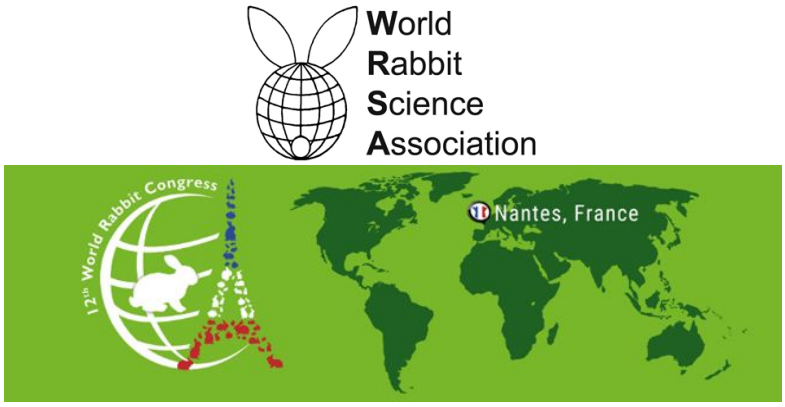

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STIMULATION OF EARLY SOLID FEED INGESTION IN THE NEST ACCELERATES THE MATURATION OF THE RABBITS CAECAL MICROBIOTA

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ABSTRACT

This study evaluated the relevancy of early life nutritional intervention for shaping the rabbit caecal microbiota. Thirty-two litters had free access to a pelleted feed from 15 days in addition with doe milk. In the early feeding group (EF), a starter feed in a gel form was added in the nest from 3 to 17 days, while rabbits from the control group (C) only had access to milk until 15 days. Caecal bacterial communities of 10 rabbits per group were assessed at 18, 25, 30, 38 and 58 days of age, by 16S rDNA sequencing. The intake of starter feed in the nest (1.1±0.4 g of DM/rabbit) had subsequent effects on alpha- and beta-diversities (+17 points for InvSimpson index at 30 days of age, P=0.018). From 18 days onwards, the structure of EF rabbit caecum bacterial communities was closer to the microbiota structure observed at 58 days than in C group, suggesting a higher maturity state at each age. With starter feeding, the microbiota acquired greater proportions of Ruminococcaceae at d18 (P=0.043, +5%) and it sped up the expected decrease of Bacteroidaceae at 25 and 30 days (-9% and -8% resp. P<0.05). Concomitantly, an increased production of volatile fatty acids was observed before weaning in EF group (+25% at 30 days). Our results suggest that early feeding can promote the maturity of the rabbit caecal ecosystem with long-term effects. A faster establishment of a stable microbiota would favor the maturation of rabbit's immune system and thus would reduce the risk of dysbiosis. Those effects will be investigated in the future to determine if early feeding strategy represents an effective tool to reduce the occurrence of enteritis around weaning.

Key words: early feeding, microbial diversity, young rabbit, colonization process, volatile fatty acids

INTRODUCTION

Gut microbiota engineering could represent an effective strategy to reduce antimicrobials use in rabbit breeding systems, since gut commensal bacteria positively promote rabbit's health through a barrier effect or the development of mucosal lymphoid tissue and antibody repertoire diversification (Fortun-Lamothe and Boullier, 2007). The intake of solid feed, and thus nutrient flow in the caecum, is the main driver of microbiota colonization. Since the suckling rabbit's microbiota is not established (Combes *et al.*, 2011), the onset of solid feed intake before weaning therefore represents a critical period to support the development of a desired microbiota. Previous attempts to stimulate early dry feed intake through the supply of maternal faeces in the nest (Combes *et al.*, 2014) or a pre-weaning diet in feeders (Read *et al.*, 2019) highlighted an acceleration of caecal microbiota maturation, with positive effects on health status reported in one trial.

In this study, we took advantages of the rabbit's ability to consume solid substrate shortly after birth on-farm (Paës *et al.*, 2019, 2020) to evaluate the effects of early solid feeding on rabbit's development and caecal microbiota composition. A starter diet in form of a gel processed with mash was provided to suckling rabbits from 3 to 17 days of age in the nest with a classical management afterwards. Microbiota development was investigated until 58 days of age.

MATERIALS AND METHODS

Animals and experimental design

Thirty-two crossbred litters (commercial lines Hyplus PS19 x Hyplus PS59) were raised with their doe (INRAE, Castanet-Tolosan, France) using a mother-litter separate feeding system (Paës et al., 2019 adapted from Fortun-Lamothe *et al.*, 2000). All the litters had access to a specific pelleted feed from 15 days in addition with doe milk (see companion paper Paës *et al.* WRC2020). Two days after parturition (d2), litter size was standardized to 10 kits. At d3, litters were allocated to two experimental groups according to their body weight (kit weight: 99±9 g) and the doe's parity. In the early feeding group (EF), the litters were fed from d3 to d17 a starter feed in a gel form, while no starter feed was supplied to the control group (C). Starter feed gels were produced daily and processed with the kits' pellets transformed into mash, supplemented with vanilla flavor (0.06%, Phodé, Terssac, France) before being thoroughly mixed with three times its weight of hot water (80°C) and 0.6% of agar as described in Paës *et al.* (2020). Starter feed gels were provided to suckling rabbits directly in the nest inside plastic cups. The same diet composition was provided to the rabbits until sale and pellets were therefore formulated to cover both suckling and fattening rabbits' nutritional requirements. Taking into account the pellet presentation preferences of suckling rabbits (Paës *et al.*, 2019), 2.5-mm-diameter pellets were offered *ad libitum* before weaning (d36) while after weaning, rabbits received 4-mm-diameter pellets with a restricted feeding program. The chemical composition of the experimental diet (similar for gels and pellets) was the following: CP: 16.1%, NDF: 35.6%, ADF: 21.5%, Starch: 11.9%, Fat content: 2.8%. The DM content of starter feed gels equaled 26% while it amounted to 89% in the pellets. Mortality was registered daily and growth was regularly assessed by weighing litters or individuals.

Evaluation of caecal microbiota composition and activity

At days 18, 25, 30, 38 and 58, ten kits per group were sacrificed by electronarcosis and exsanguination. Healthy rabbits that exhibited an interest for starter feed (visual observations) were selected. To determine microbiota composition, caecum digesta was collected in sterile conditions and stored at -80°C. Fresh caecal content was also collected and diluted in H_2SO_4 (at 2% w/v) to quantify volatile fatty acid (VFA) concentrations at days 25, 30, 38 and 58. VFA were assessed with gas chromatography equipment (CPG HP 7890A, Agilent, Santa Clara, USA). The V3-V4 regions of 16S rDNA were amplified and sequenced on Illumina MiSeq at the Genomic and Transcriptomic Platform of GenoToul (INRAE, Toulouse, France). The raw sequences obtained (30376 per sample on average) were cleaned, clustered into operational taxonomic unit and affiliated to taxa (SILVA132 database) using the FROGS pipeline (Escudié *et al.*, 2018).

Statistical Analysis

Data were analyzed with R software (R Core Team, 2018). Weight and intake data were analyzed with linear mixed model (age and experimental group as fixed effects and litter as random effect). A phyloseq R package (McMurdie and Holmes, 2013) object was generated and diversity indices were calculated on rarefied count matrix. Additionally, three taxonomical datasets were generated for phylum, family and genus levels, respectively. To examine differences in community structure, Weighted Unifrac distances (Wunifrac), which incorporate both abundances and phylogenetic information, were calculated on rarefied matrix. To evaluate community evolution dynamic to reach stability i.e. maturity, the Wunifrac distances to reach 58 days microbiota structure were evaluated at the different sampling points. An ADONIS pairwise test with the Wunifrac distances was also performed to analyze group effects. Relative abundances of the phylum, family and genus levels were fourth root transformed and analyzed with linear mixed model (age and experimental group as fixed effects and litter as random effect) with false discovery rate adjustments.

RESULTS AND DISCUSSION

From d3 to d17, each young rabbit (EF group) consumed 4.3±1.4 g of starter feed gel (1.1±0.4 g in DM). Intake of milk (on average 28±9 g/rabbit/day between d3 and d21) and pelleted feed (23±21 g of DM/rabbit/day between d18 and d35) did not differ between the two groups. Similarly, growth performance did not differ between groups (weaning weight: 976±123 g/rabbit). Those results were expected since milk intake remained the main source of nutrients until 3 weeks of age. For instance at d17,

starter feed gel only accounted for 2% of total dry matter intake. The good sanitary conditions did not allow us to study potential health benefits of early feeding (three deaths reported between d3 and weaning and no mortality observed during fattening).

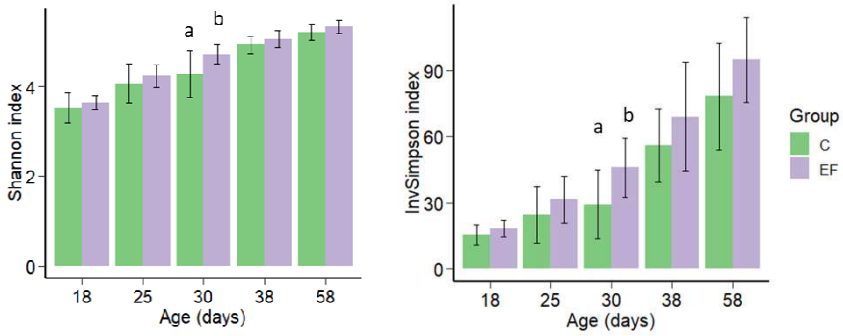

Figure 1: Effects of early feeding on caecum bacterial alpha-diversity (means ± sd). C: control, EF: early feeding

Not surprisingly, alpha-diversity measures increased with age (P<0.001). Microbiota richness, expressed as the number of observed OTUs, did not differ between groups (227±63 OTUs). Shannon and InvSimpson indices, complementary measures which integrate both evenness and richness of the caecal bacterial communities, were significantly higher in the EF group (P=0.002 and P=0.018 resp.). This suggest that feed consumption early in life promoted the growth of a greater number of species with a balanced distribution, while in the control group the microbiota was dominated by a moderate number of taxa. The investigation of the interaction age * group effect highlighted significant differences of Shannon and Simpson diversity indices at d30 (Figure 1). Similarly, higher production of VFA, the main end-products of microbiota fermentation, was observed in EF group at 30 days of age (101 *vs* 81 mM, P=0.029) due to greater acetate release (+19 mM, P=0.025), while at d38, higher amount of VFA were quantified in the caecum of C group (+18 mM, P=0.044).

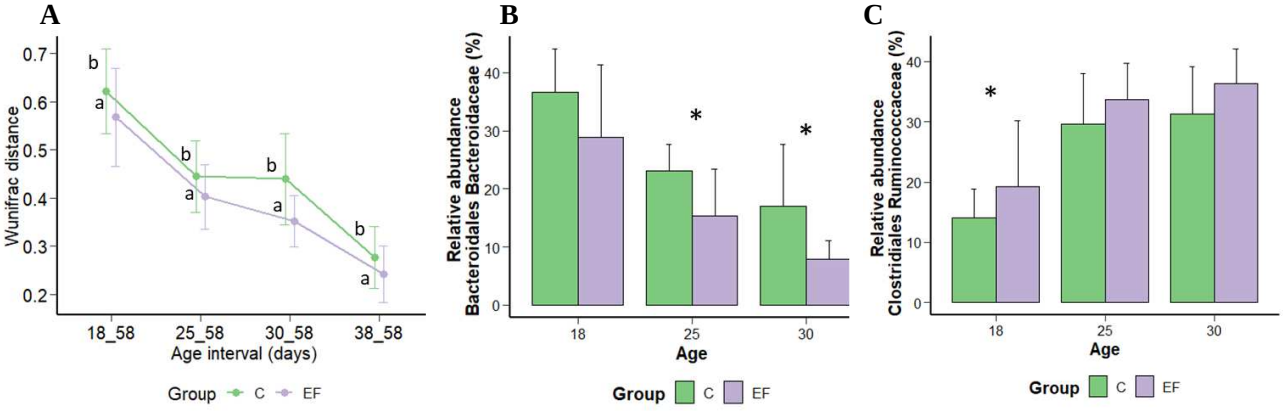

Figure 2: Maturation of caecal bacterial community (**A**): High values stand for high dissimilarity of the bacterial community from 58 days; means at the same age with different letters differ at P < 0.01. Distribution (means ± sd) of the families Bacteroidaceae and Ruminococcaceae in the caecum of suckling rabbits (**B and C**). C: control, EF: early feeding

Beside the overwhelming age effect (P<0.001, R^2=68%), bacterial community structure was affected by the early feeding stimulation (ADONIS test on Wunifrac distances, P=0.012, R^2=2%), with significant differences between the two groups observed at d30. Previous studies (Combes *et al*., 2011, Read *et al.*, 2019) observed the establishment of a stable and homogeneous community between 49 and 70 days of age. Our final time point (d58) can therefore be considered as a steady state, desirable to reach faster since subadults rabbits are less sensitive to digestive troubles. From d18 until d38, the distance to reach 58-day-old community structure was shorter (P<0.01) when early feed intake was stimulated (Figure 2A), suggesting that early nest feeding with gel accelerated the maturation of the bacterial community in the

caecum, with persistent effects after weaning. Similarly, Read *et al.* (2019) observed differences of ecosystem maturity according to the pre-weaning diets tested.

Six bacterial phyla were found across all the samples, with a trend for higher proportions of Firmicutes and lower relative abundances of Bacteroidetes in the caecums from EF group (P=0.074 and P=0.077 resp.). Among the 10 most abundant families detected, we observed lower proportions of Bacteroidaceae in EF group at d25 (P=0.041, -8%) and d30 (P=0.016; -9%) as well as higher abundances of Ruminococcaceae at d18 (P=0.043, +5%) (Figures 2B, 2C). The latter represents a prevalent family of the caecal ecosystem of rabbits fed with solid feeds, that is able to degrade complex plants material from complete feeds (Biddle *et al.*, 2013). On the contrary, Bacteroidaceae is the dominant family of 18-days-old rabbits, presumably in relation with the capacity of those bacteria to break down milk carbohydrates (Read *et al.*, 2019). Consequently, those results indicate that early feed intake may drive caecal microbiota colonization towards ecosystems functionally adapted to plant-based substrates. The distribution of the 69 genus identified (64% with taxonomic affiliation) was similar between the two groups.

CONCLUSIONS

Early feed intake supplied fermentable plant substrates for caecal bacteria even though the corresponding intake accounts for limited nutrients intake compared to milk. Altogether, nest feeding seemed efficient to reshape bacterial community towards a more mature state.

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Stimulation of early solid feed ingestion in the nest accelerates the maturation of the rabbits caecal microbiota

Paës C., Gidenne T., Barilly C., Bébin K., Duperray J., Gohier C., Guené-Grand E., Rebours G., Aymard P., Beaumont M. Combes S.

Experiment Group on Rabbit ''GEC''

Context

Gut microbiota engineering → an effective strategy to preserve health, since commensal bacteria promote

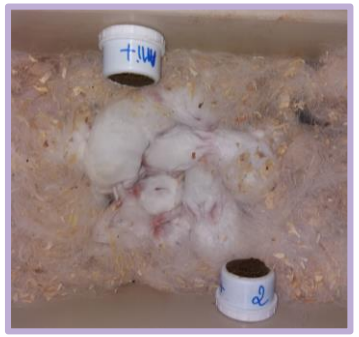
- barrier effect (host and microbiota)
- the development of mucosal lymphoid tissue & antibody repertoire diversification

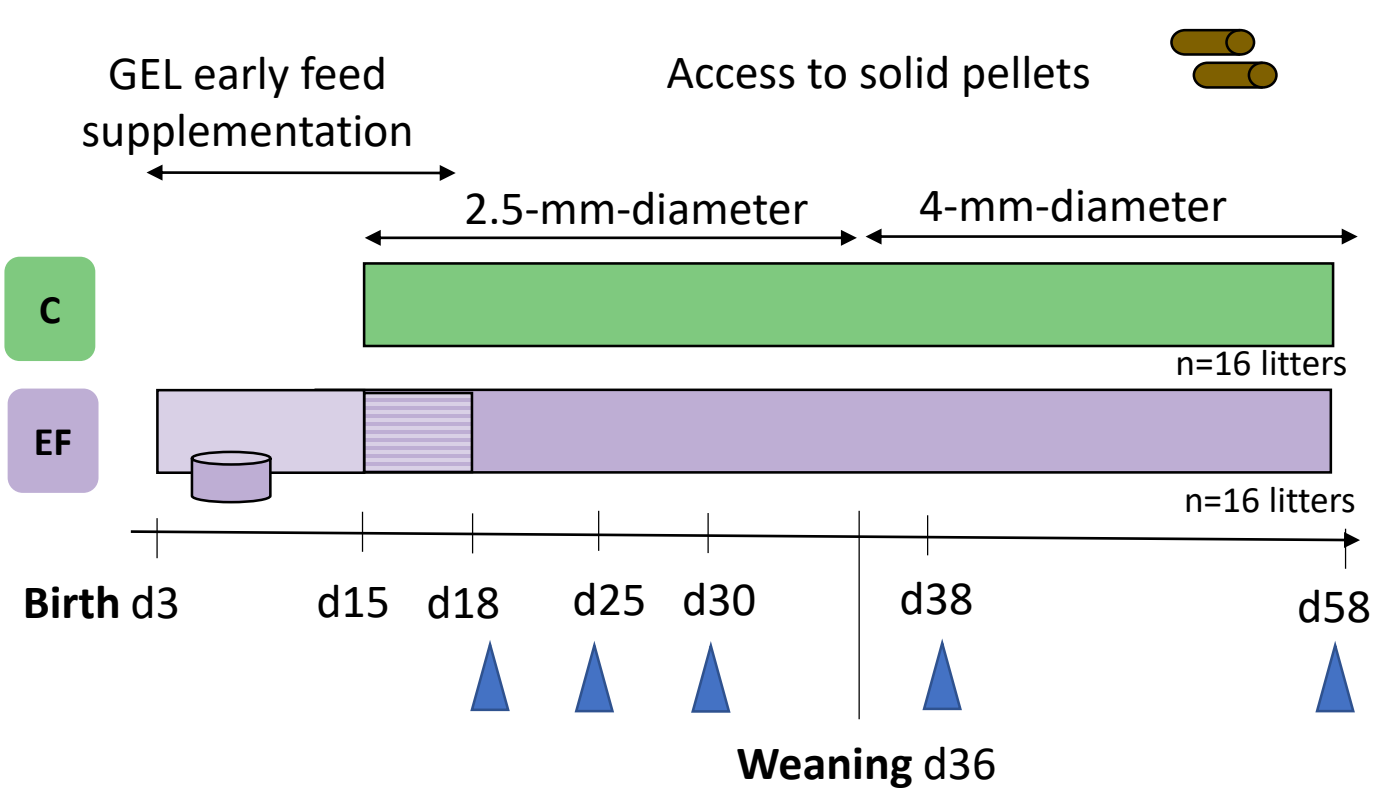
- Solid feed intake major driver of microbiota establishment *(Read et al., 2019)*
- Ingestion of solid feed starts at 8 days of age *(Paës et al 2019, 2020)*

→Development of early feeding strategies as **biomimetic strategy** to improve the health management of the young rabbit

We took advantages of the rabbit's ability to consume solid substrate shortly after birth to evaluate the effects of early solid feeding on rabbit's caecal microbiota establishment

Material and methods





From d21 to d36:

- Use of a **mother-litter separate feeding system** with controlled suckling
- **Solid and milk intake monitoring** every 2-3d

- feed pellet mash 30%
- Vanilla flavor 0,06%
- Agar 0,6%

Microbiota composition: 16S rRNA gene sequencing

Caecal content sampling (n=10/ age)

Results

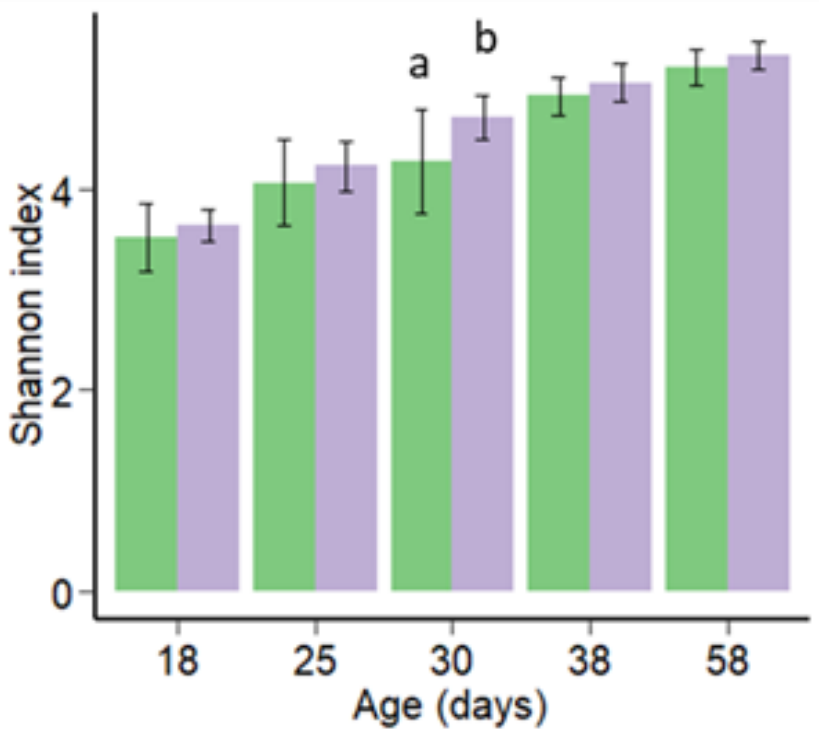
Intake:

- **Starter GEL** : 4.3 ± 1.4 g EF group d2-d17 (1.1±0.4 g in DM),).
- **Milk** : 28 ± 9 g/rabbit/day between d3 and d21
- **Pelleted feed** 23±21 g of DM/rabbit/day between d18 and d35

No difference between the two groups

Microbiota alpha- diversity:

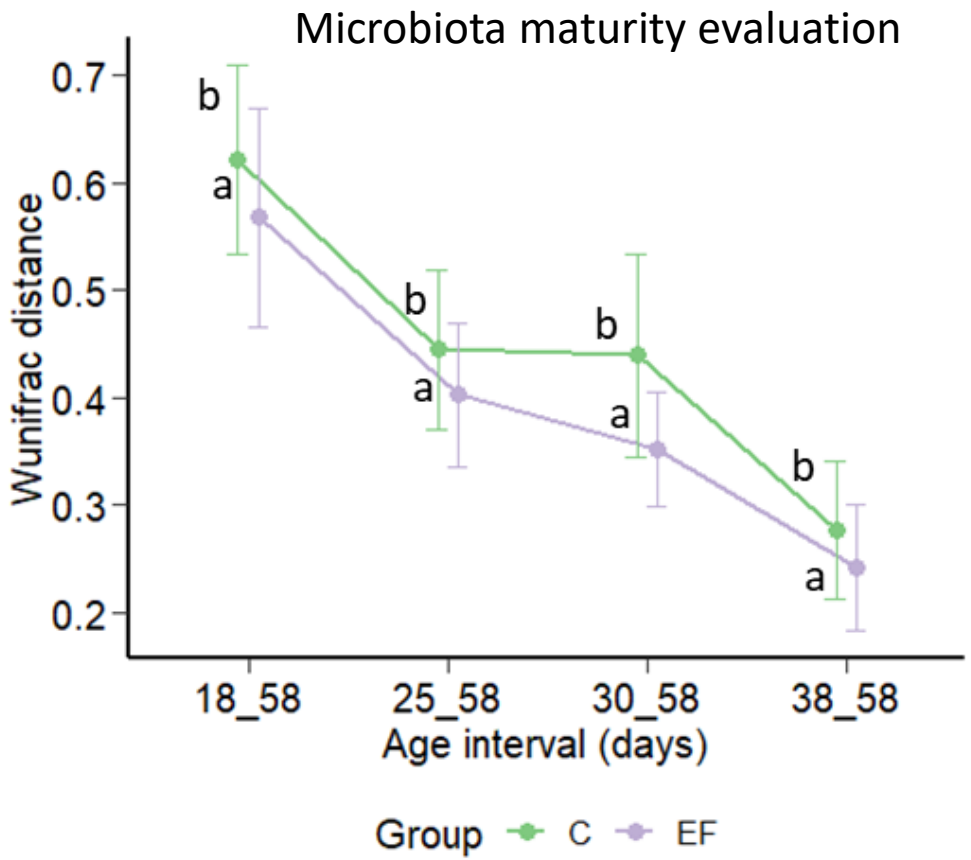
- **Increase with age** (P<0.001)
- **Highest in EF group** (P<0.002)

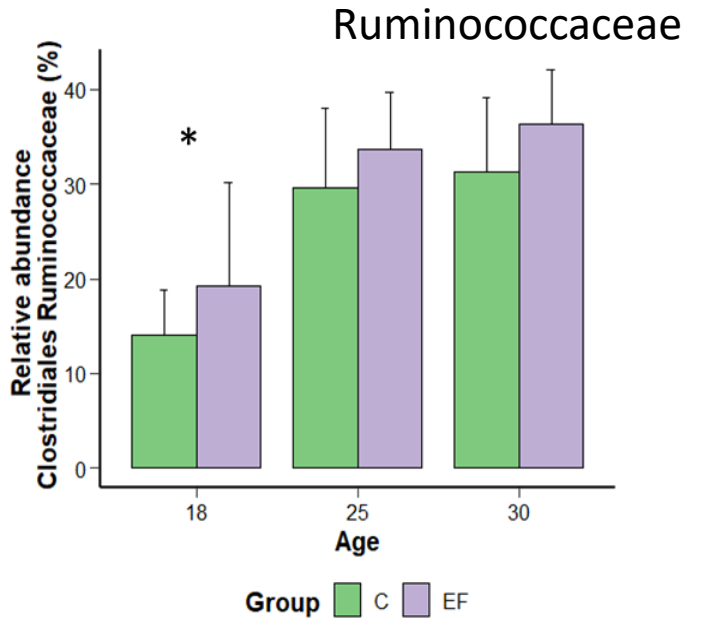


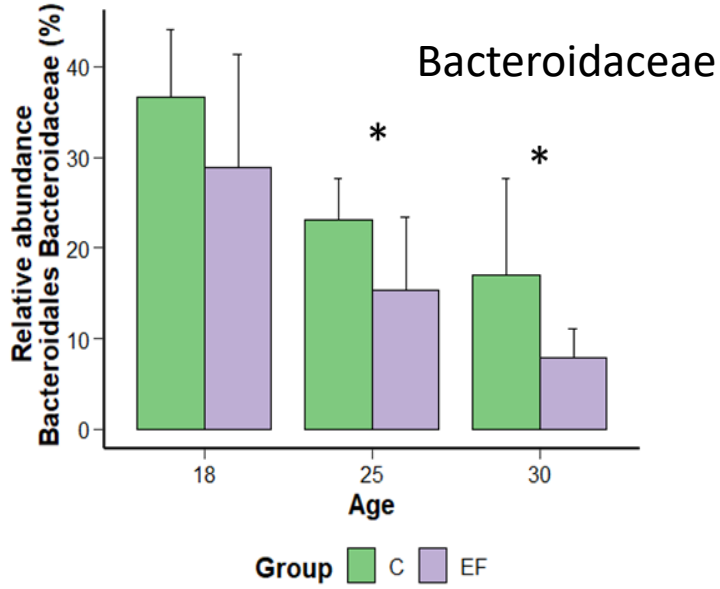
C Control

EF Gel Early feeding

Results







Early nest feeding with gel accelerated the maturation of the bacterial community in the caecum, with persistent effects after weaning

Conclusion

Early feeding in the nest

→ Increases microbiota diversity.

→ Accelerates microbiota maturity

- **Early feed intake supplied fermentable plant substrates for caecal bacteria, even though the corresponding intake accounts for limited nutrients intake compared to milk.**

- **Altogether, nest feeding seemed efficient to reshape bacterial community towards a more mature state.**

- **The health effect should be studied on a larger scale experiment**