The composition of the microbiota in the full-term fetal gut and amniotic fluid: a bovine caesarean section study

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Abstract

The fetal development of the intestinal immune system is stimulated by the maternal microbiota, but it is still unclear whether viable bacteria exist in the healthy fetus. Analysis of such low microbial biomass environments are challenging due to contamination issues. The aims of the current study were to assess the bacterial load and characterize the bacterial composition of the amniotic fluid and meconium of full-term calves, leading to a better knowledge of prenatal bacterial seeding of the fetal intestine. Amniotic fluid and rectal meconium samples were collected during and immediately after elective caesarean section, performed in 25 Belgian Blue cow-calf couples. The samples were analyzed by qPCR, bacterial culture using GAM agar and 16S rRNA gene amplicon sequencing. To minimize the effects of contaminants, we included multiple technical controls and stringently filtered the 16S rRNA gene sequencing data to exclude putative contaminant sequences. The meconium samples contained a significantly higher amount of bacterial DNA than the negative controls and 5 of 24 samples contained culturable bacteria. In the amniotic fluid, the amount of bacterial DNA was not significantly different from the negative controls and all samples were culture negative.

Bacterial sequences were identified in both sample types and were primarily of phyla Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, with some individual variation. We conclude that most calves encounter *in utero* maternal-fetal transmission of bacterial DNA, but the amount of bacterial DNA is low and viable bacteria are rare.

Introduction

Characterizing the very first intestinal bacteria is essential for a better understanding of the co-development of the newborn and its intestinal microbiome. Host-microbiome interactions enable early-life education and maturation of the immune system during a *window of opportunity*. The use of prenatal and intrapartum antibiotics may disturb this process (Dierikx et al., 2020). However, the timing of the microbial colonization of the mammalian gut is still unclear (Perez-Munoz et al., 2017; Korpela and de Vos, 2018; Liu et al., 2019; Guzman et al., 2020). Vertical as well as environmental transmission of bacteria occur during and after birth and seed the neonatal gastrointestinal (GI) tract (Funkhouser and Bordenstein, 2013; Korpela and de Vos, 2018). Transmission of an orally inoculated *Enterococcus faecium* strain in pregnant mice to the meconium of their fetuses has been described (Jimenez et al., 2008).

Recent studies in humans, mice and cattle have reported the identification of microbial DNA or culturable bacteria in the mammalian fetal environment (Aagaard et al., 2014; Severovic et al., 2019; Al Alam et al., 2020; Rackaityte et al., 2020; Guzman et al., 2020). Nevertheless, others interpret such observations as intrauterine infections or contamination (Lauder et al., 2016; Eisenhofer et al., 2018; de Goffau et al., 2019; Hockney et al., 2020; Olomu et al., 2020; Theis et al., 2020a, 2020b). The newborn's GI tract is a low microbial biomass environment, implying multiple challenges for performing reliable microbiome analyses (Glassing et al., 2016; Eisenhofer et al., 2018; Stinson et al., 2018). Besides the technical limitations inherent to DNA sequencing, there are practical and ethical concerns while collecting samples in

49 humans. First-pass meconium has been used as a proxy to assess the fetal intestinal
50 microbiome, although samples are often collected hours after birth and even breastfeeding,
51 suggesting potential postnatal effects on the microbial composition of the meconium (Collado
52 et al., 2016; Liu et al., 2019; Stinson et al., 2019). Mammalian animal models can overcome
53 several of these ethical and practical difficulties, providing insights in the fetal gastrointestinal
54 microbiome.

55 In the current study, the microbial composition of the full-term fetal gut and the corresponding
56 amniotic fluid was assessed in Belgian Blue cow-calf couples. In this double-muscled beef
57 breed, caesarean sections (C-sections) are performed on a routine basis during the very early
58 stages of parturition, while fetal membranes are still intact, rendering this breed a highly
59 suitable model for full-term gestation microbiome studies.

60 Besides the potential role of the cow model for research, there is also a significant interest in
61 the composition and the development of the calf's intestinal microbiome. Gut health and
62 growth performance during the first weeks of life are main drivers for cost effective livestock
63 rearing (Thornton Philip, 2010; Lorenz et al., 2011). Few studies have been conducted on the
64 intestinal microbiome in vaginally born neonatal calves. These have reported low numbers of
65 bacteria, mostly belonging to the phyla Proteobacteria, Firmicutes, Actinobacteria and
66 Bacteroidetes (Alipour et al., 2018; Klein-Jöbstl et al., 2019; Guzman et al., 2020). Shared
67 microbiota were found between calf meconium and the maternal vaginal vestibulum (Alipour
68 et al., 2018; Yeoman et al., 2018; Klein-Jöbstl et al., 2019). To the best of our knowledge, no
69 microbiome studies have yet been performed on calves born by C-section, and no studies are
70 available to assess the association between the microbiome of the calf's meconium and the
71 microbiome of the corresponding amniotic fluid.

72 Our aims were to assess the bacterial load and characterize the bacterial composition of the
73 amniotic fluid and meconium of full-term neonatal calves. To this end, samples were collected
74 during elective C-section, and analyzed by qPCR, bacterial culture under carefully controlled
75 and validated conditions, and 16S rRNA gene amplicon sequencing. To minimize reagent and
76 environmental bacterial DNA contaminants, we included multiple technical controls and
77 stringently filtered the 16S rRNA gene sequencing data to exclude putative contaminant
78 sequences.

79 Materials and methods

80 All experimental procedures were approved by the institutional ethics and animal welfare
81 committee of the Faculty of Veterinary Medicine (EC2018/002 - Ghent University, Belgium).
82 The cows' owners were informed about the study and gave their written consent.

83 Study design

84 The sampling of this study was performed at the teaching hospital of the Department of
85 Reproduction, Obstetrics, and Herd Health of the Faculty of Veterinary Medicine in Ghent
86 (Belgium), where pregnant Belgian Blue beef cows of different herds (parity between 1 and 5)
87 were housed for an elective C-section.

88 In total 25 Belgian Blue cows and their calves (7 males and 18 females) were sampled from
89 November 2017 until March 2019. The cows were housed in tie-stalls at the facility for 9.5 d
90 ± 5.8 (mean ± standard deviation) prior to C-section and had ad libitum access to hay and water.
91 During this period, rectal temperature was measured twice daily, and calving indicators such
92 as udder distension, teat filling, pelvic ligament relaxation, vaginal discharge, vulvar edema,
93 and behavioral changes were monitored every two hours by graduate veterinary students.

94 Prior to elective C-section, in cows that exhibited a drop in temperature, cervical dilation was
95 assessed by manual palpation. The vulvar region was cleaned with iodine soap and water. A
96 gloved hand was inserted vaginally and the opening of the portio vaginalis cervicis was
97 estimated. For the present study, elective C-section was performed when the cow had a minimal
98 cervical dilation of eight centimeters, with no rupture of the fetal membranes prior to surgery.
99 All cows were healthy according to their vital parameters (heart rate, temperature, respiratory
100 rate) and there was no clinical evidence of intrauterine infection or contamination.

101 Sampling

102 Prior to surgery, the cows were restrained in a standing position in a surgery crush specifically
103 designed for cattle. C-section procedure was done as described by Kolkman et al. (2007).
104 Briefly, the surgical area (left flank) was washed and disinfected, an abdominal incision was
105 made, and part of the uterus was exteriorized for uterotomy. The allantoic sac was opened up
106 to expose the intact amniotic sac. Amniotic fluid was aspirated through the amniotic membrane,
107 using a sterile 16 G needle (Agani, Terumo Europe, Hamburg, Germany) and sterile 20 ml
108 syringe (B. Braun, Melsungen, Germany). Within one hour after sampling, the retrieved
109 volume was aliquoted, under a laminar-flow hood, into 2 sterile 15 ml tubes (188271, Cellstar,
110 Greiner bio-one, Frickenhausen, Germany). In the first tube, 6 ml of amniotic fluid was
111 dissolved in 3 ml of glycerol (≥99 %, G2025, Sigma-Aldrich (Merck), Overijse, Belgium) and
112 subsequently stored at -80 °C to be used in culture experiments. In the second tube, 12 ml of
113 amniotic fluid was stored at -80 °C to be used for bacterial DNA extraction.

114 Meconium samples were acquired directly from the calves' rectum, immediately after birth (no
115 more than 30 minutes). Until the moment of sampling, calves laid on a clean concrete floor,
116 with access to neither the dam, nor colostrum. The perineum of the calf was dried with a clean
117 paper towel and disinfected with 70 % ethanol. A sterile double-guarded equine uterine culture

118 swab (Har-vet, 17705, Spring Valley, USA; or Minitube, 17214/2950, Tiefenbach, Germany)
119 was gently introduced in the rectum and the swab was exposed. Samples were taken in
120 duplicate, one stored immediately at -80 °C with no additives, and the other stored at -80 °C in
121 a sterile 2 ml cryovial containing 1 ml of a 30 % glycerol solution, prepared by diluting glycerol
122 (≥99 %, G2025, Sigma-Aldrich (Merck), Overijse, Belgium), in ultra-pure, nuclease-free water
123 (W4502, Sigma-Aldrich (Merck), Overijse, Belgium) to a final 30 % concentration.

124 Negative field controls were processed in the surgery room, using the same sampling
125 procedures and disposables. In total, 16 empty, sterile double-guarded equine uterine culture
126 swabs (10 Har-vet swabs and 6 Minitube swabs) were included for the meconium sampling, 5
127 of the Har-vet swabs stored in 1 ml of a 30 % glycerol stock solution, the others with no
128 additives. Additionally, 12 negative controls were included for the amniotic fluid sampling,
129 aspirating ultra-pure, nuclease-free water (W4502, Sigma-Aldrich (Merck), Overijse, Belgium)
130 instead of amniotic fluid. For 8 of them, the ultra-pure, nuclease-free water was stored with no
131 additives, and for 4 of them, 6 ml ultra-pure, nuclease-free water was dissolved in 3 ml of
132 glycerol.

133 All samples were shipped on dry ice to the laboratory of the Department of Veterinary
134 Biosciences of the Faculty of Veterinary Medicine in Helsinki (Finland) for further processing.

135 Culture

136 Validation of culture media

137 One of our aims was to define a single bacterial culture medium, capable of sustaining a
138 majority of the calf intestinal core microbiota. Consequently, this single medium could be used
139 for culturing the meconium and amniotic fluid samples, avoiding further splitting or dilution
140 of the low biomass samples. We first tested GAM "Nissui" (Gifu Anaerobic Medium Agar,

141 Code 05420, HyServe, Germany), YCFA (Yeast extract, Casitone and Fatty Acid), LB
142 (Lysogeny Broth, BD) and BB (Trypticase Soy Agar supplemented with Bovine Blood 211043,
143 Tammer BioLab Oy, Tampere, Finland) media for the ability to sustain calf intestinal core
144 microbiota, by plating each type of medium anaerobically with feces of a healthy 7 day old calf
145 (Lopez-Siles et al., 2012; Alipour et al., 2018). After 7 days of growth, mixed cultures were
146 extracted from the plates, 16S rRNA gene amplicon sequenced, and compared to the core
147 microbiota previously observed in fecal samples of young calves (Alipour et al., 2018).

148 ## Sample culturing

149 Bacterial culture on GAM-agar plates was performed for 24 meconium samples (5
150 corresponding negative controls) and 24 amnion samples (4 negative controls). The frozen
151 samples were first transferred to an anaerobic workstation (Ruskinn Concept Plus), mixed
152 thoroughly and plated, using an aseptic technique, at +37 °C. After this, the samples were
153 transferred to a laminar flow cabinet and plated in aerobic conditions at +37 °C. The culture
154 plates were checked for growth daily for 14 days. All visible bacterial colonies were
155 subcultured until pure isolates were obtained. Fresh cultures were then identified using
156 MALDI-TOF (Bruker Microflex LT) at the Central laboratory of the Faculty of Veterinary
157 Medicine (Helsinki, Finland).

158 Samples were prepared using MALDI Biotyper MSP Identification Standard Method v 1.1.
159 Mass spectra were analyzed in a mass/charge range from 2000 to 20 000 Da with MBT
160 Compass v4.1 on flexControl v3.4 (Bruker Daltonik GmbH) using BDAL-7311 as the
161 reference library. The Bruker Bacterial Test Standard (RUO) (Bruker Daltonik GmbH) was
162 used for instrument calibration. If the identification confidence score was <2.00, further
163 identification was done with 16S rRNA gene amplicon Sanger sequencing at the Institute of
164 Biotechnology (University of Helsinki, Finland).

165 ## DNA extraction

166 DNA from the meconium samples (N = 25) and corresponding negative controls (N = 11) were
167 extracted using ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA)
168 according to the manufacturer's instructions, with minor modifications to the protocol as
169 described previously (Alipour et al., 2018; Husso et al., 2020). ZymoBIOMICS™ Microbial
170 Community Standard and an in-house fecal standard were processed with the meconium
171 samples and no-template-controls were included in every batch.

172 For the amniotic fluid samples (N = 23) and the corresponding negative controls (N = 8), 2 ml
173 of each sample was first centrifuged in a microcentrifuge at 16100 × g 10 min in +4 °C. Most
174 of the supernatant was removed and 750 µl of ZymoBIOMICS Lysis Solution and 19 µl of
175 proteinase K (D3001-2-20/D3001-2-5, Zymo Research) were added to the remaining 200 µl of
176 the samples and incubated for 30 min at +55 °C. After these steps, the same protocol as applied
177 for the meconium samples was followed.

178 All manipulations of the tubes during the process were performed in a laminar flow cabinet,
179 and the workplace, instruments and pipettes were cleaned routinely with 10 % bleach. Certified
180 DNA, RNase, DNase and PCR inhibitor free tubes (STARLAB International, Germany) and
181 Nuclease-free Water (Ambion, Thermo Fisher Scientific, USA) were used for DNA extraction
182 and downstream analyses. All extracted DNA was stored at -80 °C.

183 ## Quantitative PCR

184 The bacterial 16S rRNA gene copy numbers in the meconium, amnion and negative control
185 samples were determined using quantitative PCR. The analyses were performed as described
186 previously (Alipour et al., 2018; Husso et al., 2020), with the exception that the PCR master

187 mix was first treated using a dsDNAse based decontamination kit (Enzo Life Sciences,
188 Farmingdale, New York). The amplification was performed using the Bio-Rad CFX96
189 instrument (Bio-Rad, Hercules, California) and universal eubacteria probe and primers
190 (Nadkarni et al., 2002). A standard series and negative controls were included in every run.
191 The data were analyzed using the Bio-Rad CFX Maestro software. The results were normalized
192 to the averages of absolute 16S rRNA gene copy numbers in the negative controls included in
193 each run.

194 Library preparation and 16S rRNA gene amplicon sequencing

195 The V3-V4 region of the 16S rRNA gene amplicons was sequenced using the Illumina MiSeq
196 platform in the DNA core facility of the University of Helsinki, as described previously
197 (Alipour et al., 2018; Husso et al., 2020). In total, 23 amniotic fluid samples and 23 meconium
198 samples of the same cow-calf couple were sequenced.

199 The ZymoBIOMICS Microbial Community Standard (Zymo Research, USA) and the in-house
200 adult cow fecal standard were pre-amplified with 12 cycles and all other sample types,
201 including negative controls, with 21 cycles.

202 The observed composition and abundances for the commercial standard matched the expected
203 composition provided by the manufacturer (data not shown).

204 Bioinformatics

205 The detailed bioinformatics pipeline is described in the Supplementary materials. Briefly, the
206 read quality was first inspected with FastQC and MultiQC (Andrews, 2011; Ewels et al., 2016).
207 Leftover primes and spacers were then trimmed with Cutadapt v1.10 (Martin, 2011).
208 A mapping file was created for QIIME2 and validated with Keemei (Rideout et al., 2016). The

209 FASTQ-files were imported to QIIME2 v2019.4, where the DADA2 plugin was used to
210 denoise and quality filter the reads, call ASVs and generate a feature table (Callahan et al.,
211 2016; Bolyen et al., 2019). A naïve Bayes classifier was trained in QIIME2 against SILVA
212 v132 99 % database, extracted to only include the V3-V4 region and used to assign taxonomy
213 to ASVs (Quast et al., 2013; Bokulich et al., 2018). Sequences derived from chloroplasts or
214 mitochondria were removed and singletons were filtered out, leaving only bacteria with at least
215 phylum-level identification.

216 The processed data was *in silico* filtered to remove ASVs which represented probable
217 contaminants, as described previously (Husso et al., 2020). Briefly, an ASV was removed if its
218 prevalence in actual samples was ≤2× its prevalence in instrument controls (DNA extracted
219 from empty sampling instruments only handled aseptically in a laminar flood cabinet) or its
220 prevalence in field controls (empty sampling instruments exposed to the surgery room
221 environment), and if its mean relative abundance in actual samples was ≤10× its mean
222 abundance in instrument controls or its mean abundance in field controls. The filtering was
223 performed separately for meconium and amnion samples. If less than 500 reads remained after
224 the decontamination, as was the case for six meconium samples, the sample was removed from
225 further analyses.

226 Statistics

227 The 16S rRNA gene qPCR results from samples and negative controls were compared using
228 two-tailed Mann-Whitney U test in IBM SPSS Statistics 25.

229 Shannon diversity indices were calculated for genus-level data with the R package phyloseq
230 (McMurdie and Holmes, 2013). The Kruskal-Wallis rank sum test was used to compare the
231 bacterial community structure of both sample types in RStudio (R Studio Team, 2020).

232 The PCoA figures were plotted using ASV and genus-level data and Bray-Curtis distances with
233 the R package phyloseq (McMurdie and Holmes, 2013). Permutational analysis of variance
234 (PERMANOVA) and multivariate homogeneity of group dispersions (betadisper) were
235 calculated using the R package vegan, using 9999 permutations (Oksanen et al., 2019).

236 DESeq2 was used to explore differentially abundant ASVS by calculating differential
237 expression between sample groups (Love et al., 2014).

238 The LEfSe (Linear discriminant analysis Effect Size) web application was used to identify the
239 taxons most likely explaining the differences between the sample types (Segata et al., 2011).

240 An ecologically organized heatmap of the top 40 most abundant ASVs was created with the R
241 package phyloseq (Rajaram and Oono, 2010; McMurdie and Holmes, 2013).

242 Spearman correlations and their Bonferroni corrected p-values were calculated using R-
243 package Hmisc 4.3.0 (Harrel, 2019).

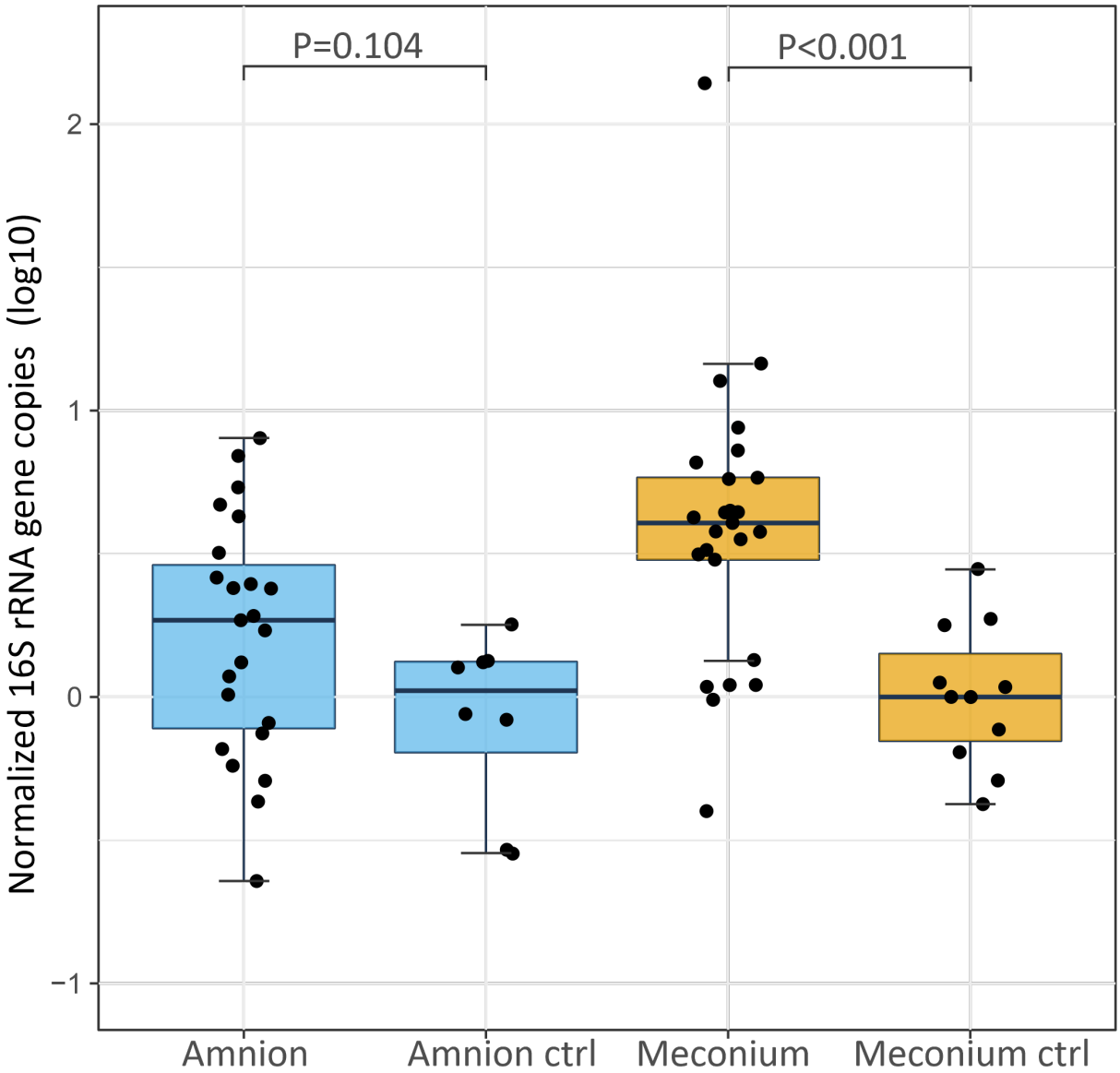
244 Differences were considered significant at $P < 0.05$.

245 Results

246 Quantification of bacterial 16S rRNA in amniotic fluid and meconium 247 samples by qPCR

248 The 16S rRNA gene copy numbers in amniotic fluid and meconium samples were assessed by
249 qPCR (Fig. 1). To minimize variation between qPCR runs, the copy numbers were normalized
250 to the averages of the negative controls in the same run as explained in Materials and Methods.

251 In the amniotic fluid samples, there was no significant difference (P = 0.104) between the
252 normalized 16S rRNA gene copy numbers of the samples (N = 23; mean = 2.4; SD = 2.1) and
253 the negative controls (N = 8; mean = 1; SD = 0.53).
254 In the meconium samples, the 16S rRNA gene copy number (N = 25; mean = 9.9; SD = 27)
255 was significantly higher (P < 0.001) than in the negative controls (N = 11; mean = 1; SD =
256 0.86).
257



258
259 *Figure 1. 16S rRNA gene copy numbers (fold change vs. negative controls) for amniotic fluid (n = 23,*
260 *negative controls n = 8) and meconium samples (n = 25, negative controls n = 11). Samples were*
261 *collected during elective C-section in Belgian Blue cow-calf pairs. The copy number of each sample*
262 *was normalized to the average copy number of the corresponding negative controls and log10*

263 *transformed. The midline of the box is the median, with the upper and lower limits of the box being the*
264 *third and first quartile. The whiskers extend up to 1.5 times the interquartile range from the box to the*
265 *furthest data point within that distance.*

266 Decontamination of the 16S rRNA gene amplicon sequencing data

267 We performed a stringent filtering of the 16S rRNA gene sequencing data to remove the
268 amplicon sequence variants (ASVs) potentially stemming from reagent contaminants and
269 environmental bacterial DNA (Table 1).

270 The decontamination was based on the prevalence and relative abundance of each ASV in the
271 samples and negative controls (see Materials and Methods).

272 *Table 1. Mean read counts (standard deviation) detected in meconium and amniotic fluid samples and*
273 *their negative controls. Only sequences with at least phylum-level identification were retained for*
274 *further analyses. If less than 500 reads remained after in silico decontamination, as in six meconium*
275 *samples, the sample was removed from further analyses.*

Sample type	Raw		Processed		Decontaminated	
	N	Reads	N	Reads	N	Reads
Meconium	23	92266 (36235)	23	57082 (25543)	17	4189 (8931)
Meconium control	11	126800 (21164)	11	84023 (14585)	-	-
Amnion	23	127797 (22376)	23	83279 (15120)	23	3338 (2922)
Amnion control	6	136488 (12663)	6	90802 (8998)	-	-

276

277 Microbial composition of amniotic fluid and meconium samples

278 The microbiota of both sample types consisted primarily of Proteobacteria, Firmicutes,
279 Bacteroidetes and Actinobacteria phyla, with individual variation (Fig. 2). In both sample
280 types, the inter-individual variation increased at lower taxonomic levels (Supplementary Table

1 and Fig. 3). The most abundant bacterial genera in meconium were *Delftia*, *Staphylococcus* and *Clostridium sensu stricto 1*, while the most prevalent genera were *Delftia*, *Acinetobacter*, unclassified *Burkholderiaceae*, *Staphylococcus* and *Corynebacterium 1* (Supplementary Table 1). In the amniotic fluid samples, *Staphylococcus*, *Streptococcus, Delftia, Sphingomonas* and *Enterococcus* were the most abundant genera, and *Delftia*, *Streptococcus*, *Staphylococcus, Sphingomonas* and *Acinetobacter* were the most prevalent genera (Supplementary Table 1). The observed composition and abundances for the commercial standard matched the expected composition provided by the manufacturer.

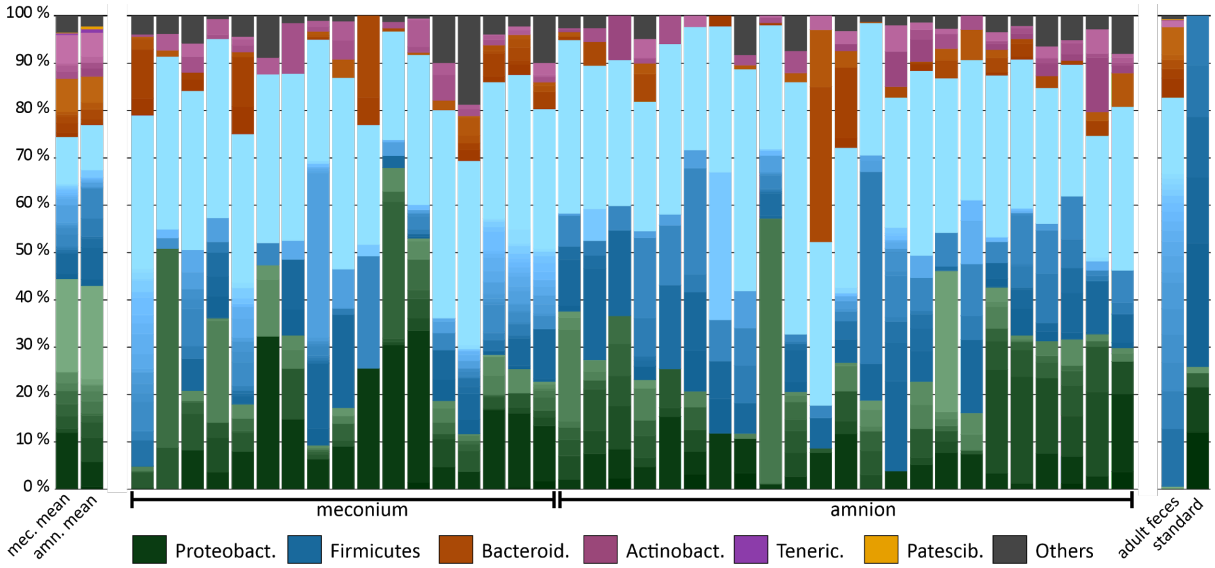


Figure 2. Microbiota composition in bovine meconium and amniotic fluid samples collected during elective C-section, adult feces, and a commercial community composition standard. The main colors indicate the bacterial phyla. Within phyla, the shades indicate bacterial genera. The lightest shade of each phylum shows the combined abundance of the least abundant genera (with a maximum of <0.5% of total).

A heatmap analysis of the 40 most abundant ASVs shows the difference between the sample types in more detail (Fig. 3).

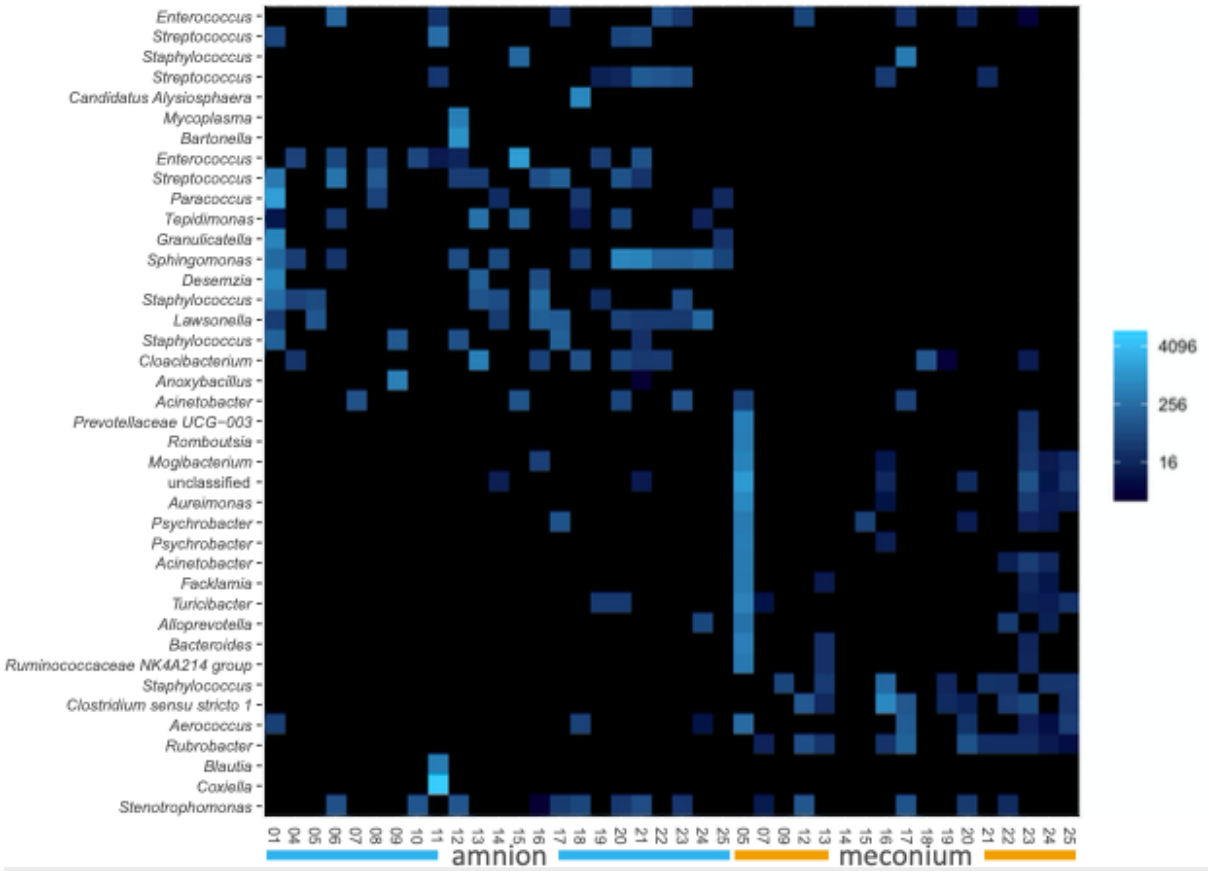
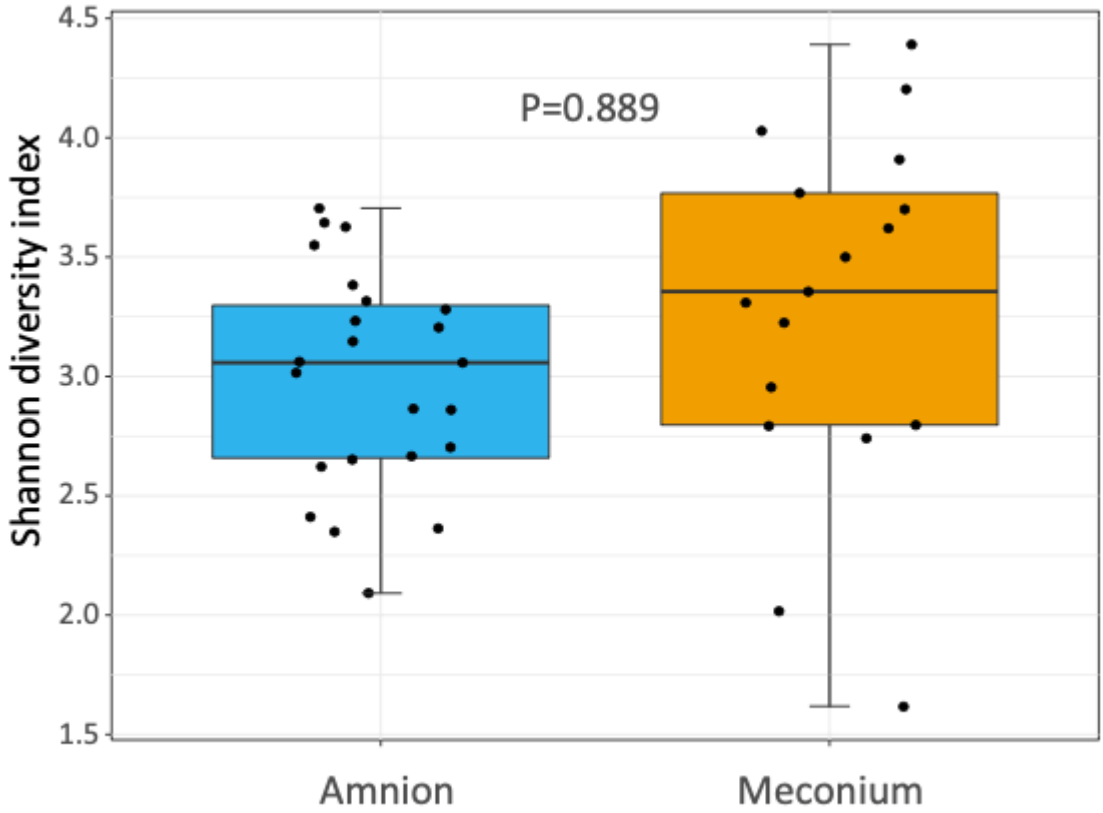


Figure 3. Ecologically organized heatmap (NMDS, Bray) of the 40 most abundant ASVs in bovine meconium and amniotic fluid samples collected during elective C-section, sorted by sample type. The taxon names are presented as genus level identifications. Color scale indicates relative abundance and is a log transformation with base 4.

The alpha diversity (Shannon index) at the genus level was not significantly different between the meconium and amniotic fluid samples (Fig. 4, P = 0.889).



308

309 *Figure 4. Shannon diversity index showing the bacterial community structure (at genus level) of*
310 *amniotic fluid and meconium samples, collected during elective C-section in Belgian Blue cow-calf*
311 *pairs. Boxplot as in Fig 1.*

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313
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315

Comparison of amniotic fluid and meconium microbiota composition

The microbiota composition was significantly different for amniotic fluid and meconium samples at the ASV (PERMANOVA, $P < 0.001$, $R2 = 0.049$, Fig. 5 A), and genus level (PERMANOVA, $P < 0.001$, $R2 = 0.062$, Fig. 5 B).

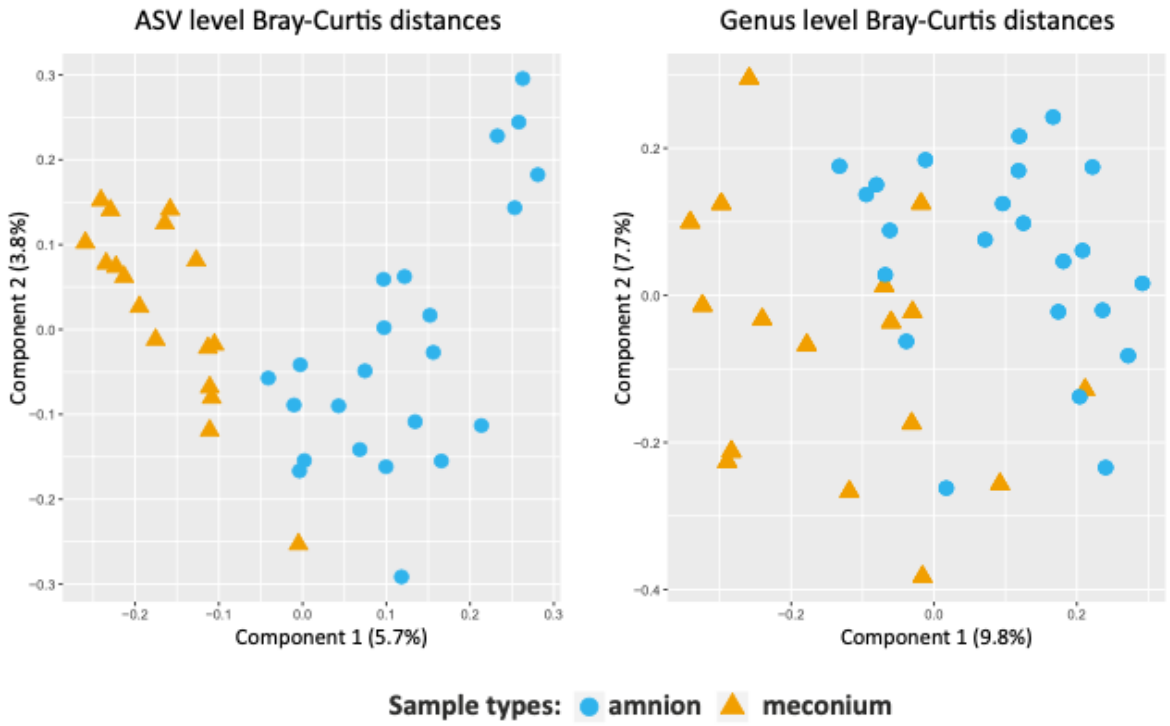

Figure 5. Comparison of the bacterial community structure of amniotic fluid and meconium. 5 A: PCoA on Bray-Curtis distances based on ASV level data. Figure 5 B: PCoA on Bray- Curtis distances based on genus level data. Colors and shapes indicate the sample types.

According to the DESeq2 analysis, three ASVs were more abundant in meconium than in amniotic fluid: one *Staphylococcus* ASV (log2 Fold Change = 25.084, $P_{adj} < 0.001$), one *Rubrobacter* ASV (log2 Fold Change = 8.337, $P_{adj} < 0.001$), and one *Clostridium* ASV (log2 Fold Change = 8.055, $P_{adj} = 0.004$).

329 In amniotic fluid, one *Sphingomonas* ASV (log2 Fold Change = -8.420, $P_{adj} < 0.001$) and one
330 *Staphylococcus* ASV (log2 Fold Change = -23.438, $P_{adj} < 0.001$) were more abundant than in
331 meconium.

332 By LefSe (Linear discriminant analysis effect size) analysis including all taxonomic levels,
333 meconium samples had a greater relative abundance of *Clostridiales* and *Rubrobacter* (LDA
334 score (log10) > 4.0) than the amniotic fluid, but a lower relative abundance of Bacillales and
335 *Corynebacteriales* (LDA score (log10) < -4.0).

336 Correlations between meconium and amniotic fluid from the same 337 animal

338 We calculated Spearman rank correlations for the microbiota between meconium and amniotic
339 fluid samples, taking into account ASVs that were present >100 times in all of the samples.
340 Average correlations (ρ) at ASV (N = 17, $\rho_{avg} = -0.0201$, $P_{avg} < 0.05$) and genus level (N = 17,
341 $\rho_{avg} = 0.1685$, $P_{avg} < 0.05$) were very weak and biologically not significant. All p-values were
342 Bonferroni corrected for multiple testing.

343 Bacterial cultures

344 Based on our testing of multiple media, the GAM (Gifu Anaerobic Medium) agar was selected
345 to assess if there were live bacteria in the samples stored in glycerol (see Materials and
346 Methods), since it was able to sustain the largest number of bacterial core genera found in calf
347 feces, including some additional unique genera not found on any other plate type (Alipour et
348 al., 2018).

349 All amniotic samples and their negative controls were culture negative, both in anaerobic and
350 aerobic conditions. Five out of 24 meconium samples and 1 out of 5 negative controls were

351 culture positive on GAM agar. The results for each sample are presented in detail in Table 2.
352 Both gram negative and gram-positive strains were isolated from the samples.

353 *Table 2. Culture positive meconium samples and identified bacteria. If the identification confidence*
354 *score was <2.00 for MALDI-TOF, further identification was done with 16S rRNA gene amplicon Sanger*
355 *sequencing to achieve genus level identification for all isolates.*

Sample	Aerobic strains	Anaerobic strains
Negative control 1	*Staphylococcus* sp. (Gram+), *Kocuria* sp. (Gram+)	*Rothia* sp. (Gram+)
Meconium 5	*Roseomonas* sp. (Gram-)	-
Meconium 10	*Acinetobacter* sp. (Gram-)	*Streptococcus* sp. (Gram+), *Fusobacterium* sp. (Gram-)
Meconium 12	-	*Cutibacterium* sp. (Gram+)
Meconium 16	*Kocuria* sp. (Gram+)	-
Meconium 21	*Achromobacter* sp. (Gram-)	-

356

357 The Sanger sequence from the *Roseomonas* sp. isolate acquired from a meconium sample was
358 a 100 % identical match to the 16S rRNA gene amplicon sequencing data from the same
359 sample. In addition, the Sanger sequences of *Staphylococcus* sp., *Kocuria* sp., *Roseomonas* sp.,
360 and *Acinetobacter* sp. isolates matched the 16S rRNA gene amplicon sequencing data when
361 compared to all meconium samples.

362 Three bacterial genera could be identified in the negative controls: *Rothia* sp., *Staphylococcus*
363 sp. and *Kocuria* sp.. Of these, only *Kocuria* sp. was also identified from the actual meconium
364 samples.

365 Discussion

366 Vertical transmission of microbes during, and perhaps already before birth is a universal
367 phenomenon across the whole animal kingdom and plays an important role in shaping host
368 immunity and fitness (Funkhouser and Bordenstein, 2013; Vandenplas et al., 2020). Despite
369 the advances in our understanding of the assembly and lifelong effects of the perinatal
370 microbiota, the *in utero* exposure of the fetus to maternal bacteria and bacterial components
371 remains poorly understood (Perez-Munoz et al., 2017; Korpela and de Vos, 2018; Liu et al.,
372 2019).

373 In the present study, we sampled amniotic fluid during, and the corresponding calves
374 immediately after they were born by elective C-section. The elective C-section is a routine
375 procedure in Belgian Blue beef cattle, since the pelvis of the dam does not allow vaginal birth
376 of the relatively large double-muscled calf (Kolkman et al., 2007). We performed the surgery
377 during the very early stages of parturition, when both fetal membranes (amniotic and
378 allantochorionic membrane) were still intact, allowing us to sample the full-term fetus and the
379 intrauterine environment without exposure to the vaginal microbes and minimal exposure to
380 the environmental microbiota. Meconium samples were collected rectally. The relatively large
381 size of the calves permits the insertion of a double-guarded sampling device into the rectum,
382 minimizing the risk of contamination.

383 In contrast, in most human studies first-pass meconium was collected from diapers (Hansen et
384 al., 2015; Collado et al., 2016; Liu et al., 2019; Stinson et al., 2019). Because the first-pass

385 meconium is often collected several hours after birth, microbes from the environment and the
386 mother (especially during breastfeeding) have time to colonize the intestine. Hence, this
387 sampling procedure should be seriously questioned for researching the eventual intrauterine
388 transmission of bacteria to the neonatal gastrointestinal tract (Dornelles et al., 2020). In the
389 current study, amniotic fluid samples were collected *in vivo*, while the amniotic membrane was
390 still intact, as has been done in humans during pre-labor C-section (Collado et al., 2016).

391 Bacterial culture is traditionally used to determine the viable population in a bacterial
392 community. However, standard culture-dependent techniques are known to be inadequate to
393 identify all bacteria. Capturing a broad bacterial diversity would require the use of many
394 different selective media and multiple specific growing conditions (Lau et al., 2016; Lagier et
395 al., 2018). In the current study, one of our aims was to define a single bacterial culture medium,
396 capable of sustaining the majority of the calf intestinal core microbiota. We first tested four
397 different culture media: GAM "Nissui" (Gifu Anaerobic Medium Agar), mostly used for
398 anaerobic bacteria, YCFA (Yeast extract, Casitone and Fatty Acid), containing volatile fatty
399 acids typically supporting the growth of several gut bacteria, LB (Lysogeny Broth) as a general
400 media, and BB (Trypticase Soy Agar supplemented with Bovine Blood) supporting fastidious
401 bacteria by blood enrichment. The GAM medium was found optimal for both aerobic and
402 anaerobic culturing.

403 Bacteria were successfully cultured from 5 out of 24 meconium samples, representing both
404 gram-negative and gram-positive bacterial genera, known to live in mammalian hosts.
405 *Roseomonas* and *Achromobacter* species are known to act as opportunistic pathogens, but have
406 also been isolated from a wide variety of environmental sources (Reverdy et al., 1984; Rihs et
407 al., 1993). Various *Acinetobacter* and *Streptococcus* species have physiological functions in
408 their mammalian hosts, but the genera also include pathogenic and/or environmental species

409 (Krzyściak et al., 2013; Touchon et al., 2014). *Fusobacteria* species are typically closely related
410 to mucous membranes, especially the oral cavities, while *Cutibacterium* and *Kocuria* species
411 have most often been isolated from the skin (Hofstad, 1992; Savini et al., 2010; Scholz and
412 Kilian, 2016). One of our negative controls yielded *Kocuria* colonies. This was the only taxon
413 shared between the actual meconium samples and the negative controls, suggesting low-level
414 contamination from the sampling environment.

415 Although we detected cultivable bacteria in some of the meconium samples, most of the
416 samples were culture negative. This indicates that the presence of live bacteria in the fetal
417 intestine is rare, in contrast to the prevalent bacterial DNA. Individual differences in amounts
418 of viable bacteria retrieved from meconium were also described in a recent dog study.
419 Interestingly, puppies born without detectable meconium microbiota were shown to have a
420 slower growth rate than those in which meconium microbiota were detected (Pipan et al.,
421 2020). Both the mucosal surface of the intestine as well as the amniotic fluid contain
422 bacteriostatic substances, such as lactoferrin and salivary scavenger and agglutinin (SALSA),
423 which are able to suppress bacterial growth and viability (Reichhardt and Meri, 2016;
424 Lisowska-Myjak et al., 2019). In human meconium, SALSA amounts to 10 % of all proteins,
425 highlighting its potential role in antimicrobial defense (Reichhardt et al., 2014). Lactoferrin,
426 SALSA and other similar substances may explain the small number of viable bacteria observed
427 in our study, in contrast to the significant amount of detected bacterial DNA.

428 The culture-independent techniques were performed based on the 16S rRNA gene. Since
429 contamination is a major challenge in the analysis of low bacterial biomass samples, we
430 processed several types of negative controls alongside our biological samples, treated qPCR
431 mastermix with dsDNAse, and applied rigorous *in silico* filtering of potential contaminants
432 (Glassing et al., 2016; Eisenhofer et al., 2018). Many similar studies are published without or

433 with a very limited number of contamination controls, which makes our approach more
434 reliable, especially for assessing low bacterial biomass environments.

435 We observed a small but significant amount of bacterial DNA in the meconium samples by
436 16S qPCR, suggesting a prenatal transmission of bacterial DNA to the fetal intestine. This is
437 consistent with previous studies in cattle, in which calves born at term *per vias naturales*, were
438 sampled (Mayer et al., 2012; Alipour et al., 2018; Klein-Jöbstl et al., 2019). A profile
439 dominated by the phyla Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria was
440 identified in the meconium, in agreement with previous studies using 16S rRNA gene amplicon
441 sequencing (Alipour et al., 2018; Yeoman et al., 2018; Klein-Jöbstl et al., 2019; Husso et al.,
442 2020; Guzman et al., 2020). Additionally, some of the most prevalent genera observed in
443 meconium in our study, such as *Sphingomonas*, *Acinetobacter*, *Pseudomonas*, *Ruminococcus*
444 and *Stenotrophomonas,* have also been previously described in neonatal calves (Klein-Jöbstl
445 et al., 2019).

446 In contrast to the meconium, there was no significant difference between the 16S rRNA gene
447 copy numbers of amniotic fluid samples and the negative controls, and all were culture
448 negative. A recent caesarean section study in sheep stated amniotic fluid sterility during the
449 third trimester of pregnancy (Malmuthuge and Griebel, 2018). In other studies however, a low
450 biomass microbiome in the pregnant bovine uterus was described (Moore et al., 2017; Guzman
451 et al., 2020).

452 Using 16S rRNA gene amplicon sequencing, we found a bacterial signature in the amniotic
453 fluid, dominated by the phyla Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, in
454 accordance with previous studies (Karstrup et al., 2017; Moore et al., 2017; Guzman et al.,
455 2020). Deeper taxonomic identification, down to the family or genus level, revealed no further
456 agreements with these studies.

457 From a comparative point of view between mammalian species, the amniotic fluid sampled by
458 Collado et al. (2016) in women during pre-labor C-section was found to contain a distinct, low
459 diversity, low biomass microbiome, predominated by Proteobacteria (*Enterobacteriaceae*).
460 Despite this and other recent studies, the presence of microbial DNA and culturable bacteria in
461 the human placenta and fetal environment is still under debate (de Goffau et al., 2019).

462 The microbial DNA signatures in meconium and amniotic fluid were similar at the phylum
463 level. However, they presented a significantly different microbial composition at genus and
464 ASV level: genera *Staphylococcus*, *Rubrobacter* and *Clostridium* were relatively more
465 abundant in meconium samples than in amniotic fluid samples according to DESeq2 analysis.
466 Complementing this with the LefSe analysis using all taxonomic levels, also found
467 *Clostridiales* and *Rubrobacter* taxons relatively more abundant in the meconium. The
468 microbial compositions in the bovine amniotic fluid and meconium of the corresponding
469 neonate did not show a significant correlation, contrary to earlier studies in humans (Collado
470 et al., 2016; He et al., 2020).

471 Our analysis suggests that the bacterial DNA found in meconium does not necessarily originate
472 from the amniotic fluid. The maternal microbial components may be translocated via the
473 placental and umbilical blood vessels directly to the fetal internal organs. Microbial
474 macromolecules and even live bacteria may be transported by leukocytes via these blood
475 vessels (Perez et al., 2007).

476 A recent bovine study revealed differing microbial communities between the amniotic fluid
477 and the meconium (Guzman et al., 2020). As the fetus swallows the amniotic fluid, which is
478 concentrated and retained in the intestine as meconium, together with epithelial cells and
479 intestinal secretions, it is unexpected that the two would harbor completely different bacteria.
480 However, the amniotic fluid microbiota may fluctuate dynamically over time, while meconium

481 represents a more stable collection of substances accumulated over the gestation period.
482 Moreover, fetal excretion in the amniotic fluid is limited in cattle. Meconium is usually
483 expelled only after birth, and fetal urine largely accumulates in the allantoic cavity, between
484 chorion and amnion, rather than in the amniotic cavity (Bongso and Basrur, 1976).

485 The less permeable synepitheliochorial placenta evidenced by three distinct layers may restrict
486 the translocation of bacteria and their components from the dam to the fetus in cattle, in
487 comparison to humans. The very low levels of bacterial DNA in the bovine amniotic fluid limit
488 the sequencing depth and thus the precision of the compositional analyses. Our strict data
489 decontamination protocol furthermore minimized the artefactual similarity arising from shared
490 contaminants.

491

492 In conclusion, we found that the meconium of full-term calves delivered by elective caesarean
493 section in most cases contains a small amount of diverse bacterial DNA and eventually
494 although rather rare, culturable bacteria. In the amniotic fluid, bacteria were not observed by
495 16S qPCR or culturing, but a microbial DNA profile was distinguishable from controls by
496 amplicon sequencing. Based on these results, bacterial components are translocated to the fetus
497 *in utero*, but the prenatal acquisition of live bacteria is likely not physiologically significant.

498 Data availability

499 The raw 16S rRNA gene amplicon sequencing dataset is fully available in the NCBI Sequence
500 Read Archive (SRA) with the accession number PRJNA643145 after journal acceptance.

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Author contributions

MN, LL, AI and GO contributed to conception and design of the study. AH performed the bioinformatics, statistical analyses and bacterial culture. AH and TG performed MALDI-TOF analyses. LL and JG organized the sampling and collected the samples. AH and LL wrote the manuscript. All authors contributed to manuscript preparation, read, and approved the submitted version.

Ethics declaration

All experimental procedures were approved by the institutional ethics and animal welfare committee of the Faculty of Veterinary Medicine (EC2018/002 - Ghent University, Belgium). The cows' owners were informed about the study and gave their written consent for their cows' participation.

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