

Transfer of intestinal bacterial components to mammary secretions in the cow

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Results from large multicentre epidemiological studies suggest an association between the consumption of raw milk and a reduced incidence of allergy and asthma in children. Although the underlying mechanisms for this association are yet to be confirmed, researchers have investigated whether bacteria or bacterial components that naturally occur in cow's milk are responsible for modulating the immune system to reduce the risk of allergic diseases. Previous research in human and mice suggests that bacterial components derived from the maternal intestine are transported to breast milk through the bloodstream. The aim of our study was to assess whether a similar mechanism of bacterial trafficking could occur in the cow. Through the application of culture-independent methodology, we investigated the microbial composition and diversity of milk, blood and feces of healthy lactating cows. We found that a small number of bacterial OTUs belonging to the genera *Ruminococcus* and *Bifidobacterium*, and the *Peptostreptococcaceae* family were present in all three samples from the same individual animals. Although these results do not confirm the hypothesis that trafficking of intestinal bacteria into mammary secretions does occur in the cow, they support the existence of an endogenous entero-mammary pathway for some bacterial components during lactation in the cow. Further research is required to define the specific mechanisms by which gut bacteria are transported into the mammary gland of the cow, and the health implications of such bacteria being present in milk.

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21 Introduction

22 Epidemiological studies have shown that growing up on a farm is associated with a lower
 23 risk of developing allergy (Braun-Fahrlander et al., 1999; Ehrenstein et al., 2000; Waser et al.,
 24 2004; Alfvén et al., 2006; Mutius & Vercelli, 2010) and that the consumption of raw milk is
 25 strongly correlated with this effect (Perkin & Strachan, 2006; Waser et al., 2007). The
 26 commercialisation of fresh raw (unpasteurised) milk remains a controversial issue (Press
 27 release, 2014; Astley, 2014), and although its consumption has been discouraged due to
 28 potential health risks associated with pathogens (Allerberger et al., 2003), there is a growing
 29 body of evidence suggesting that consumption of unprocessed milk decreases the risk of
 30 asthma in children (Riedler et al., 2001; Loss et al., 2011). The mechanism for this effect are
 31 not yet fully understood, but it may be related to bacteria or bacterial components in the milk
 32 that modulate the immune system and which are modified during milk processing (Gehring et
 33 al., 2008; Hodgkinson et al., 2014).

34 Although rural families generally skim or heat raw milk before consumption, this milk differs
 35 in many aspects from commercially available milk. Whereas commercial milk is usually
 36 homogenised and pasteurised, milk obtained from the farm for local consumption is not
 37 processed in these ways, potentially resulting in a higher microbial load. Results from
 38 PASTURE, a large global study examining the effects of farm and urban living on asthma and
 39 allergy, showed no differences in endotoxin levels of raw farm and commercially processed
 40 milk (Lluis et al., 2014); however, samples were not analysed for other bacterial components
 41 or presence of viable organisms.

42 It has been reported that human breast milk is not completely sterile (Heikkilä & Saris,
 43 2003; Martín et al., 2003; Beasley & Saris, 2004; Jiménez et al., 2008), and that bacterial

44 components derived from the maternal intestine are transported to the lactating breast by
 45 phagocytic cells in the blood (Pérez et al., 2007). This fascinating observation suggests that
 46 milk acts as a source of natural inoculum provided by the mother for the breast-fed newborn
 47 that programs the neonatal immune system to respond appropriately when challenged with
 48 specific environmental and dietary bacterial antigens.

49 We hypothesise that a similar mechanism of bacterial trafficking from the intestinal tract to
 50 the mammary gland, previously reported in lactating mothers, also occurs in the cow,
 51 contributing to the bacterial components found in raw cow's milk and facilitating bacterial
 52 imprinting of the neonatal immune system. The identification of bacteria or bacterial
 53 components in unprocessed cow's milk thought to originate from the intestinal tract would
 54 support this hypothesis. Therefore we investigated the microbial composition of feces, milk
 55 leukocytes and blood leukocytes in lactating cows by pyrosequencing barcode-tagged 16S
 56 rRNA amplicons to identify bacterial groups common to all three microbial pools. Bacterial
 57 imprinting of the neonate via trafficking of gut bacteria into mammary secretions in the cow
 58 may also contribute to the protective effects that consuming raw cow's milk has been shown
 59 to have on development of allergy in children. An understanding of the mechanisms by which
 60 gut bacteria in milk imprint the neonatal immune system may provide the foundation for new
 61 strategies on how to shape the intestinal microbiota of the infant to aid in the prevention of
 62 immune disorders.

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65 **Materials and methods**

66 **Animals.** All experimental procedures were approved by the Ruakura Animal Ethics
67 Committee, AgResearch, New Zealand (application #12345). A total of 35 lactating cows
68 (Friesian, Jersey or Friesian × Jersey cross) located at the Tokanui Dairy Research farm
69 (Waikato, NZ) were screened for the presence of subclinical/clinical mastitis infection. Only
70 cows identified as having a low pooled quarter somatic cell count (SCC), defined as <100,000
71 cells/mL, at previous routine herd testing (conducted by Livestock Improvement Corporation,
72 Hamilton, NZ) were screened. As part of the screening procedure, individual quarter milk
73 samples were collected from all cows and subjected to a rapid mastitis test (Shoof
74 International Ltd., Cambridge, NZ) and somatic cell count. Those animals showing any signs
75 of subclinical or clinical mastitis (rapid mastitis test [RMT] score >1 [gel formation detected] or
76 SCC >200,000 cells/mL) in any quarter were not eligible for the study. A subset of 12 cows,
77 not showing signs of subclinical or clinical mastitis as defined above, were subsequently
78 randomly selected to participate in the study. These lactating cows (age: 2-4 years old; parity:
79 1-3) were 180 days in milk on average at the time samples were collected. Cows were on
80 twice-daily milking. Five (5) aged-matched non-lactating, non-pregnant cows of the same
81 breed and showing no signs of disease were selected as control animals for blood analysis.
82 All cows were grazing and fed on the feed pad.

83 **Sample collection.** Udders were thoroughly cleaned and disinfected with 70% ethanol and
84 methylated spirit-impregnated swabs (Meths Clear, Vetpak), paying particular attention to the
85 teat end by polishing the teat orifice with the swabs. Afterwards, a 14-gauge Teflon cannula
86 (Terumo Surflo) was inserted into the teat canal and connected to a sterile sample container
87 by a drip extension similar to that described by Vangroenweghe and colleagues

88 (Vangroenweghe et al., 2001). The cannula had a bevelled edge which curled inwards to
 89 minimise any potential damage to the teat canal. A total of 800 mL of milk was collected from
 90 each cow and placed in the cold (4 °C) until transport to the analytical laboratory in Hamilton,
 91 NZ. Immediately following the removal of the cannula, each quarter was sprayed with
 92 chlorhexidine teat spray (Teat X, Deosan). Somatic cell counts were performed frequently
 93 after the study to check for intramammary infections caused by the sampling procedure.
 94 Blood samples (approximately 450 mL) were collected via jugular venepuncture under local
 95 anaesthetic (lignocaine hydrochloride monohydrate 2%, PhoenixPharm Ltd.) and sedation
 96 (detomidine, Zoetis NZ Ltd.) into sterile blood collection bags (Fenwal Inc.) containing citrate
 97 phosphate dextrose as anticoagulant and Adsol as a red cell nutrient solution. The bags were
 98 placed in the cold (4 °C) and transported chilled to the analytical lab in Fielding, NZ. Skin was
 99 disinfected with 70% ethanol before venepuncture. Fecal samples were collected directly
 100 from the rectum of each animal with a gloved hand, placed into a sterile container, and stored
 101 at -80 °C until further analysis in Palmerston North, NZ.

102 ***White blood cell separation from blood and milk samples.*** Milk somatic cells (MSCs)
 103 were isolated as previously described (Daley et al., 1991). Briefly, milk was allowed to warm
 104 to room temperature, and centrifuged in 200 ml aliquots, at 250 × *g* for 30 min. Milk cells were
 105 then washed with 80 mL phosphate buffered saline [PBS] (Dulbecco A, Oxoid Ltd.) and
 106 centrifuged again for 15 min. Cells were then resuspended in 80 mL of PBS containing 100
 107 µg/mL gentamycin sulphate (Boehringer Ingelheim Bioproducts) for 10 min to kill extracellular
 108 bacteria and in suspension. Following incubation, cell suspensions were spun for 10 min, and
 109 washed with 40 mL PBS. After another 10 min spin, cells were resuspended in 1 mL PBS,
 110 and stored at -80 °C until processed for DNA extraction as described below. All steps were

111 completed at room temperature in sterile conditions in a laminar flow cabinet appropriate for
112 cell culture work.

113 White blood cells (WBCs) were isolated as follows. Blood bags were centrifuged at 15 °C
114 at low speed ($2,000 \times g$ for 5 min with no brake applied). Plasma supernatant, buffy coat layer
115 and the upper layer of red cells were transferred to a platelet bag using a Fenwal plasma
116 extractor (Baxter Healthcare), and centrifuged again ($4,500 \times g$ for 7 min). Supernatant was
117 again removed with the manual extractor and discarded leaving approximately 80 mL of
118 pelleted cells in a minimal volume of plasma in the bag. The centrifuged cellular pellets
119 containing WBCs were resuspended in the remaining plasma, treated with gentamycin as
120 described above, washed and processed for bacterial DNA extraction as described below.

121 **Skin swabs.** After disinfection with ethanol but before milk collection, each teat and a defined
122 area immediately around the teat were swabbed using Amies charcoal swabs (Raylab NZ
123 Ltd.), and plated on Columbia sheep blood agar and McConkey agar plates (Fort Richard
124 Laboratories Ltd.) to check for bacterial contamination. Plates were incubated at 37 °C for 24
125 h in aerobic conditions. One (1) Columbia sheep blood agar plate from each sample was also
126 incubated at 37 °C for 48 h in anaerobic jars using an anaerobic GasPak generator (BBL
127 Becton Dickinson) for facultative anaerobes.

128 **Bacterial DNA extraction.** Total DNA was extracted from 200 mg of fecal samples using
129 NucleoSpin Soil kits (Macherey-Nagel GmbH) according to manufacturer's instructions, but
130 with the following modification. Fecal samples were diluted in 700 μ L of NucleoSpin lysis
131 buffer SL2 and 150 μ L SX buffer, and homogenised using a FastPrep FP120 Cell Disrupter
132 (Qbiogene Inc.) set to speed 6.5 for 45 sec prior to column purification of DNA. Milk and blood

133 cells were pelleted by centrifugation and DNA extracted from the cell pellets using the same
134 method described for fecal samples.

135 **High-throughput sequencing.** Isolated DNA was then used to amplify the V3-V5 regions of
136 16S ribosomal DNA, with universal bacterial primers (Claus et al., 2011) containing GS FLX
137 adapter sequences, a unique 8 nucleotide 'barcode', and template specific sequences;

138 forward primer

139 5'-CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNNAGGCCAGCAGCCGCGGTAA-3',

140 and reverse primer

141 5'-CTATGCGCCTTGCCAGCCCGCTCAGGCCRRACGAGCTGACGAC-3', with 'N'

142 indicating barcode nucleotides. Amplification reactions were completed on a MasterCycler

143 ProS thermocycler (Eppendorf AG). Fecal DNA was amplified using the following conditions;

144 95 °C for 4 min, 25 cycles of [95 °C for 30 sec; 49 °C for 30 sec; 72 °C for 60 sec] and 72 °C

145 for 7 min. The PCR product size was 604 base pairs. Milk and blood cell DNA was amplified

146 using the following PCR conditions; 95 °C for 4 min, 40 cycles of [95°C for 30 sec; 49 °C for

147 30 sec; 72 °C for 60 sec] and 72 °C for 7 min. Several dilutions of template DNA were made if

148 the presence of PCR inhibitors was suspected. Samples were pooled and sent to the

149 commercial sequencing facility (Macrogen Inc., Seoul, South Korea). To control for

150 environmental contamination resulting from PCR with universal bacterial primers and high

151 cycle numbers (40), negative controls without template DNA were also sequenced.

152 **Sequence analysis.** Sequences were processed using QIIME 1.7. Reads were quality

153 filtered (quality score window > 50) and assigned to corresponding samples according to

154 barcode sequences using default values for minimum/maximum allowable length of read

155 (200/1000), allowed number of ambiguous reads (6), and allowable homopolymer length (6)

156 (split_libraries.py -w 50 -b 8 -g -r -f). The resulting demultiplexed sequences were denoised

157 and chimera checked using the Greengenes alignment as a database (release GG_13_5).
 158 Sequences identified as chimeric were removed from subsequent analyses. Sequences
 159 showing 97% or greater similarity were clustered into operational taxonomic units (OTUs)
 160 using UCLUST method. Representative sequences were assigned taxonomies using the rdp
 161 method against the Greengenes GG_13_5 database (default 0.8 confidence threshold).
 162 Alpha diversity and OTU networks were generated using QIIME 1.7. Hierarchical clustering
 163 analysis of bacterial profiles was performed in R 3.0.2 (R Core Team, 2013) using Euclidean
 164 distances and complete linkage clustering.

165 **Quantitative PCR.** Bacterial DNA was amplified by quantitative PCR (qPCR) with the
 166 bacterial 16S rRNA gene primers F_Bact 1369 (5'-CGG TGA ATA CGT TCC CGG-3') and
 167 R_Prok1492 (5'-TAC GGC TAC CTT GTT ACG ACT T-3') (Suzuki, Taylor & DeLong, 2000),
 168 using a Rotor-gene 6000 thermocycler (Qiagen). Samples were measured in duplicate using
 169 10 μ L reactions consisting of 1 μ L DNA template, 0.25 μ L forward primer (10 pmol/ μ L), 0.25
 170 μ L reverse primer (10 pmol/ μ L), 3.5 μ L nuclease-free water, and 5 μ L of KAPA SYBR® FAST
 171 Universal 2X qPCR Master Mix. Cycling conditions consisted of 95 °C for 3 min, followed by
 172 40 cycles of [95 °C for 20 sec, 60 °C for 30 sec, and 72 °C for 30 sec]. Calculated
 173 concentrations (ng/ μ L) were normalized to extracted DNA concentrations. The amount of
 174 DNA detected was expressed as equivalent number of *Escherichia coli* genomes per ng of
 175 total DNA to provide an estimate of the numbers of bacteria present.

176 **Statistical analyses.** Ninety five percent confidence intervals for bacterial DNA quantities
 177 were obtained using R version 3.0.2 (R Core Team, 2013). Significance of differences
 178 between mean DNA concentrations was determined using the non-parametric Kruskal-Wallis
 179 analysis of variance in R, with *P* values < 0.05 deemed to be significant. Power analysis

180 indicated that results from at least 10 cows with each cow categorised on whether it shows
 181 trafficking (whether at least one OTU is present in all three compartments [feces, blood and
 182 milk]) or not, will give 95% confidence intervals on the proportion of trafficking.

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185 Results

186 *Amplification and sequencing of bacterial DNA from the three biological pools.*

187 Bacterial DNA originating from MSCs and WBCs proved difficult to amplify using traditional
188 PCR conditions involving 25-30 cycles, probably due to its low abundance; thus, we
189 increased the number of PCR cycles to 40. This practice, however, might lead to false
190 positive results. Therefore, we sequenced the negative controls (no DNA template), and
191 filtered the sequenced samples to exclude the OTUs found in these control samples.

192 A total of 190245 quality-checked bacterial 16S rRNA gene sequences were obtained by
193 pyrosequencing from all tested animals (min = 1276, max = 25175; median = 4369). The
194 mean number (\pm SEM) of sequences obtained from feces ($n = 11$), MSCs ($n = 12$), and
195 WBCs ($n = 11$) was 8062 ± 2097 , 4164 ± 510 and 3765 ± 653 , respectively. The mean read
196 length was 281 bp (min = 200; max = 564). The number of operational taxonomic units
197 (OTUs) at 97% similarity were 2163 (min = 16; max = 983; median = 262), excluding all OTUs
198 and sequences found in the negative control sample (15 OTUs).

199 Quantitative PCR analysis showed that mean amounts of bacterial DNA present per ng of
200 DNA extracted from MSCs (6.51×10^{-3} pg [95% CI, 1.02×10^{-2} to 2.87×10^{-3} pg]) were higher
201 than from WBCs (1.79×10^{-3} pg [95% CI, 2.66×10^{-3} to 9.07×10^{-4} pg]), which indicates
202 higher numbers of bacteria present in MSCs compared to WBCs.

203 No bacterial DNA was recovered from blood originating from the aged-matched, non-
204 lactating, non-pregnant control animals. Skin swabs collected from the teats after cleaning,
205 but before milk collection did not show signs of viable bacteria in the culture conditions
206 employed, ruling out any direct bacterial contamination from the skin during milk collection.

207 ***Microbial composition of the three biological pools.*** Analysis of DNA extracted from
 208 MSCs, WBCs and feces uncovered a small number of OTUs that were observed in all three
 209 biological samples from at least one cow (Table 1). Sequence assignment to the closest
 210 related taxa using the Greengenes GG_13_5 database indicated that sequences classified as
 211 *Ruminococcus* genus, *Peptostreptococcaceae* family, and *Bifidobacterium* genus were found
 212 concurrently in all three biological samples in a total of five, five and four cows, respectively.
 213 Members of 15 bacterial phyla were detected in the WBCs, whereas 22 bacterial phyla were
 214 represented in the MSCs. In comparison, fecal DNA contained representatives from only 13
 215 phyla despite having the highest overall diversity at the 0.97 OTU level.

216 The microbial diversity of each environment, as shown by the mean Chao1 index \pm SEM at
 217 a sampling depth of 1000 sequences was 596 ± 17 , 427 ± 59 and 107 ± 20 for feces, MSCs
 218 and WBCs respectively (Figure 1). As expected, fecal samples had greater microbial diversity
 219 compared to blood and milk samples ($P < 0.01$). MSCs also had significantly greater microbial
 220 diversity than WBCs ($P < 0.01$).

221 The profile of microbial sequences identified in the feces, MSCs and WBCs differed
 222 between the sampling sites as shown by the hierarchical cluster analysis of bacterial profiles
 223 (Figure 2). The most prevalent bacterial groups detected in the feces included, as expected,
 224 members of the *Firmicutes* and *Bacteroidetes*. In contrast, bacterial sequences in WBCs were
 225 predominantly from *Mycoplasma* (33.9%) and *Streptophyta* (24.1%). Bacterial profiles from
 226 milk more closely resembled that from feces (Figures 2-4), with the most abundant groups
 227 including *Staphylococcus* (27.6%), *Ruminococcus* (7.2%), *Peptostreptococcaceae* (6.5%),
 228 *Bifidobacterium* (5.6%), *Butyrivibrio* (2.3%) (Table 2).

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231 Discussion and Conclusion

232 The general convention dictates that mammalian milk, including that of human and bovine
 233 origin, is at its origin free from microorganisms. According to the Food and Agriculture
 234 Organization of the United Nations, milk secreted into a cow's udder is sterile (FAO, 1990).
 235 This theory, however, has been recently challenged. A few scientific studies using nucleic
 236 acid-based methodologies have started to reveal that colostrum and human breast milk
 237 contain microorganisms (Martín et al., 2003; Beasley & Saris, 2004; Pérez et al., 2007),
 238 becoming potential sources of bacterial exposure for the breast-fed newborn. Nevertheless,
 239 the origin of such microorganisms, as well as their health implications, are still a controversial
 240 issue. Although it is generally accepted that the presence of bacteria in milk can result from
 241 contamination with bacteria from the mother's skin or the infant's mouth, a newly proposed
 242 endogenous pathway to explain the origin of some milk bacteria is under debate. An entero-
 243 mammary pathway has been suggested by which selected bacteria from the maternal
 244 gastrointestinal microbiota reach the mammary secretions via the blood leukocytes (Martín et
 245 al., 2004; Pérez et al., 2007; Fernández et al., 2013; Rodríguez, 2014). We hypothesised that
 246 a similar mechanism of bacterial trafficking from the intestinal tract to the mammary gland,
 247 previously reported in lactating mothers and mice, also occurs in the cow, contributing to the
 248 bacterial components found in raw cow's milk. Bearing in mind that the lactational physiology
 249 of humans and rodents is different to that of ruminants, the occurrence of this endogenous
 250 bacterial circulation would lead to new scientific insights of bovine lactation physiology.

251 We employed an established culture-independent methodology to investigate the bacterial
 252 composition in the three biological compartments. Our results suggest that there are bacterial
 253 components belonging to the *Ruminococcus* genus, the *Peptostreptococcaceae* family, and

the *Bifidobacterium* genus that can be found in common in feces, WBCs and MSCs from the same lactating cow. It is therefore reasonable to speculate that members of these bacterial groups may have been transferred from the gut to the mammary gland via circulating white blood cells. Pérez and colleagues observed DNA from *Bifidobacterium longum* in milk samples from lactating mothers which was also present in their blood and feces (Pérez et al., 2007). These researchers also found sequences from *Bacteroides*, *Clostridium*, and *Eubacterium* in human milk. Martín et al. have also isolated bifidobacterial species in human milk (Martín et al., 2003).

Two aspects in our approach require attention: namely, milk collection and PCR amplification. Common practise for milk sampling from cows is by hand stripping. When hand-stripping, it is very difficult to collect milk aseptically due to skin flakes, dust, and hair in the environment, which can all introduce bacterial contamination to the sample. Rather than hand-stripping, we used a catheter inserted into the teat canal which was connected to the sample container by a drip extension set that allowed milk collection by gravity. This procedure not only prevented the teat canal from being stretched or damaged (historical post-sampling SCC records suggest no intramammary infections were caused by the procedure, data not shown), but also avoided external microbial contamination of the milk. Although we cannot exclude that bacteria of skin origin or from the keratin lining could have colonised the milk duct of the cow's udders and then transferred to the milk, we have designed our experiment to exclude any bacteria in suspension by collecting only white cells (the majority of the somatic cells in blood) by centrifugation. Additionally, these cells were treated with the bactericidal gentamycin, and consequently only internalised bacterial signatures or those coming from membrane bound bacteria were measured by high throughput sequencing. Finally, all three biological samples were processed in three laboratories located in different

278 sites which significantly reduces the possibility of introducing the same type of contamination
279 to each of the samples.

280 Although environmental contaminants can become disproportionately represented when
281 amplifying sequences from samples containing low copy numbers of bacterial DNA compared
282 to non-target DNA, our results show that high-throughput sequencing is a useful method for
283 assessing microbial composition in milk and blood. Despite the presence of contaminant
284 sequence in the negative control samples subjected to 40 rounds of PCR amplification, the
285 number of OTUs observed was substantially lower than that in blood and milk samples. Taxa
286 found in common in all three environments were identified in a maximum of 5 cows out of the
287 total tested animals. Because very small quantities of bacterial DNA were amplified in blood
288 and milk, sampling error could come into play. Quantitative PCR analysis showed higher
289 numbers of bacteria present in MSCs compared to WBCs, suggesting that cells with bacteria
290 accumulated in the mammary gland as there was more bacteria per unit of mammalian DNA
291 in the milk than there was in the blood.

292 Milk sample collection techniques and cell differentiation methodologies can influence milk
293 cell differentiation results; however, it is generally accepted that macrophages are the
294 predominant cell type in bovine milk from healthy glands (Miller, Paape & Fulton, 1991;
295 Dosogne et al., 2003). Lymphocytes and neutrophils are also present along with a small
296 percentage of detached epithelial cells which together make up the total somatic cell
297 population. During inflammation, neutrophils are rapidly recruited to the mammary gland,
298 becoming the predominant cell type and increasing somatic cell counts in inflamed quarters
299 (Riollet, Rainard & Poutrel, 2000). Cows used in the study showed no signs of intramammary
300 infection; therefore, the phagocytic macrophages are expected to be the predominant cell
301 type present in milk from these animals. Macrophages in milk are thought to be derived from

302 blood monocytes which exit the bloodstream, migrate across the epithelium and enter the
 303 mammary gland (Goldman & Goldblum, 1997). Macrophages play a key role in immune
 304 surveillance, acting as scavenger cells with the ability to recognise pathogens and initiate
 305 innate responses through the secretion of pro-inflammatory mediators. Following
 306 phagocytosis of antigens, some tissue macrophages differentiate into dendritic cells and
 307 migrate to draining lymph nodes where they interact with T-cells to induce antigen-specific
 308 acquired responses (Randolph et al., 1999). Macrophages can also function as antigen
 309 presenting cells, a subset of which are able to induce oral tolerance through interaction with
 310 CD103⁺ dendritic cells (Mazzini et al., 2014). Breast milk macrophages express certain
 311 dendritic cell surface markers, spontaneously produce granulocyte-macrophage colony-
 312 stimulating factor and have the unique ability to differentiate into dendritic cells when
 313 stimulated with interleukin-4 (Ichikawa et al., 2003). Combined, these findings suggest that
 314 breast milk macrophages exhibit characteristics consistent with that of partially differentiated
 315 dendritic cells, and that such cells may play a role in mediating T-cell dependant immune
 316 responses in the mammary gland. However, as the enhanced functionality displayed by milk
 317 macrophages is thought to be induced by phagocytosis of milk components following entry to
 318 the mammary gland, such findings do not provide an explanation as to the likely mechanisms
 319 by which gut bacteria and bacterial components are transported from the intestinal lumen to
 320 the mammary gland. More recently it has been proposed that although the mononuclear
 321 phagocytes found in breast milk are largely derived from peripheral blood monocytes, a
 322 proportion of these mononuclear phagocytes are dendritic cell-like cells which arise in gut-
 323 associated lymphoid tissue, capture luminal microbiota and then transport these microbial
 324 components to the mammary gland. Such a mechanism is thought to exist to educate the

325 neonatal immune system to recognise commensal-associated molecular patterns of bacteria
326 and to respond to such bacteria appropriately (Donnet-Hughes et al., 2010).

327 The possibility of bacterial trafficking from the gut to the mammary gland in the cow opens
328 up interesting alternatives for probiotic use in the manipulation of the intestinal ecosystem for
329 animal health. Ingested probiotics with the ability to get access to the mammary gland
330 through the bloodstream could be employed to combat pathogenic microorganisms involved
331 in the development of mastitis. Finally, further research is required to unequivocally link the
332 biological activity of bacterial groups of interest in the development of allergy, in which case
333 technological strategies in milk processing could be directed towards maintaining the integrity
334 of such beneficial components.

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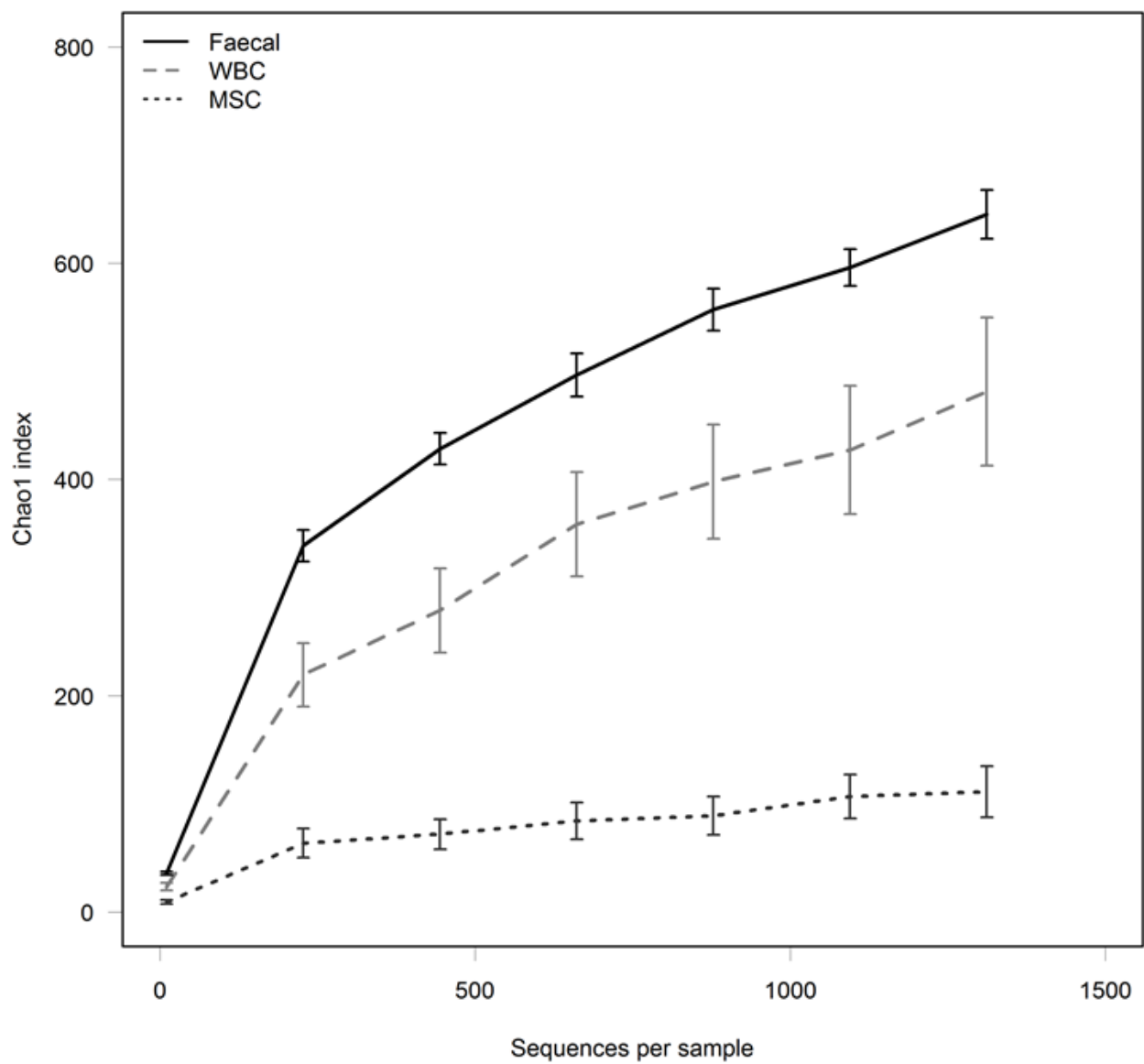
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Figure 1(on next page)

Analysis of diversity within communities.

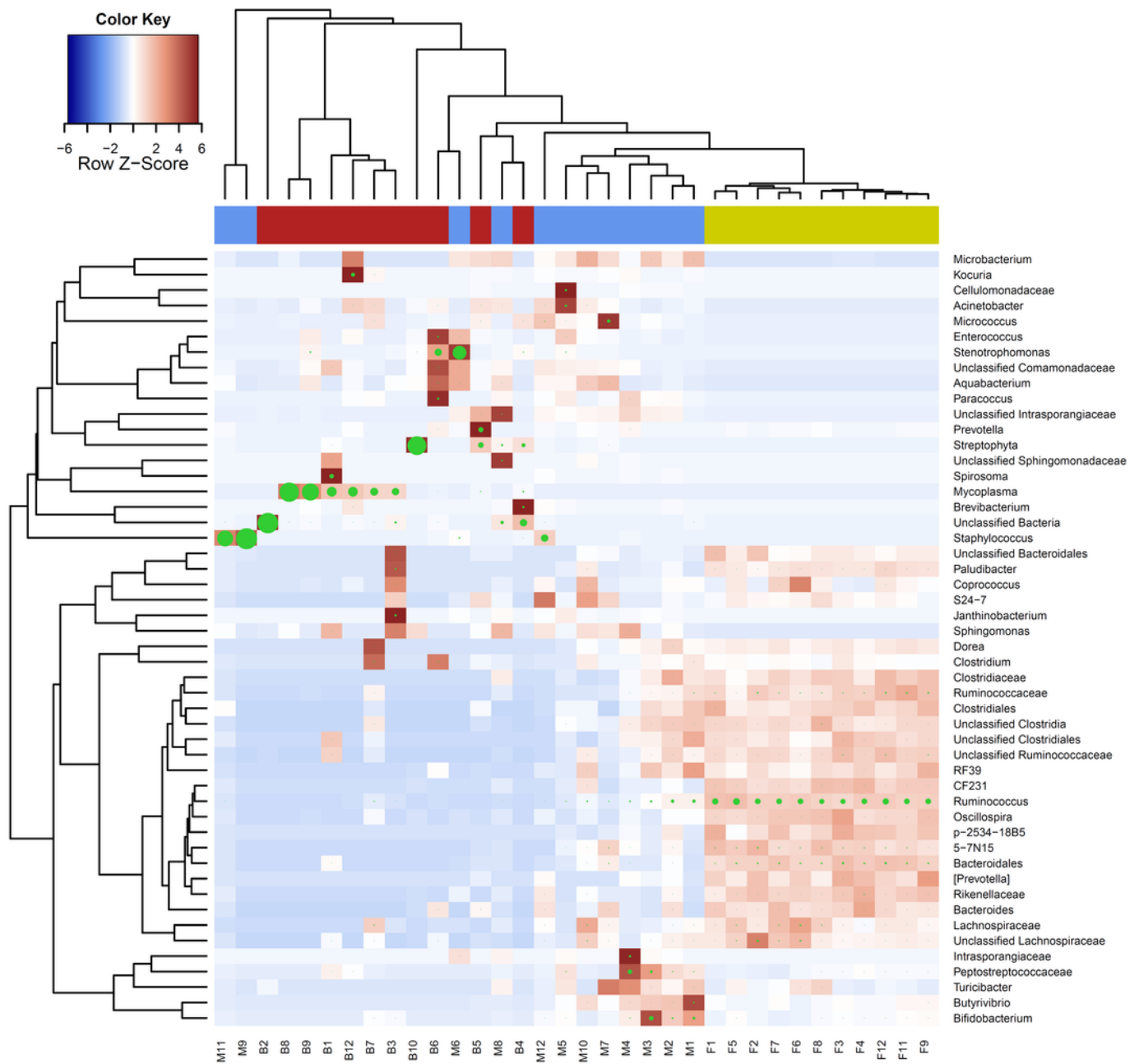
Curves indicate Chao1 index, a measure of community diversity, at each sampling depth, as shown on the X-axis. Error bars indicate SEM



2

Cluster analysis of bacterial composition at genus level

Heatmap showing hierarchical clustering of analysis of bacterial composition profiles for the 50 most abundant genus level taxa as a proportion of total sequences for each sample. Coloured bar beneath upper dendrogram indicates sample environment; fecal (yellow), milk cell (blue), white blood cell (red). Taxa are indicated by row labels and individual animal and sample environment indicated by column labels; F (fecal), M (milk cell), B (white blood cell), and numbers representing animal identification. Heatmap colour (blue to dark red) signify relative prevalence of each taxa across samples and green circles show absolute proportions for each taxa within a sample, with circle size proportional to sequence abundance.



3

Bar chart of bacterial composition at family level.

Stacked bar chart of the 100 most abundant family level taxa found in each of the faecal (n=11), milk cell (n=12), and white blood cell (n=11) environments. Bars show mean bacterial proportions for each environment.

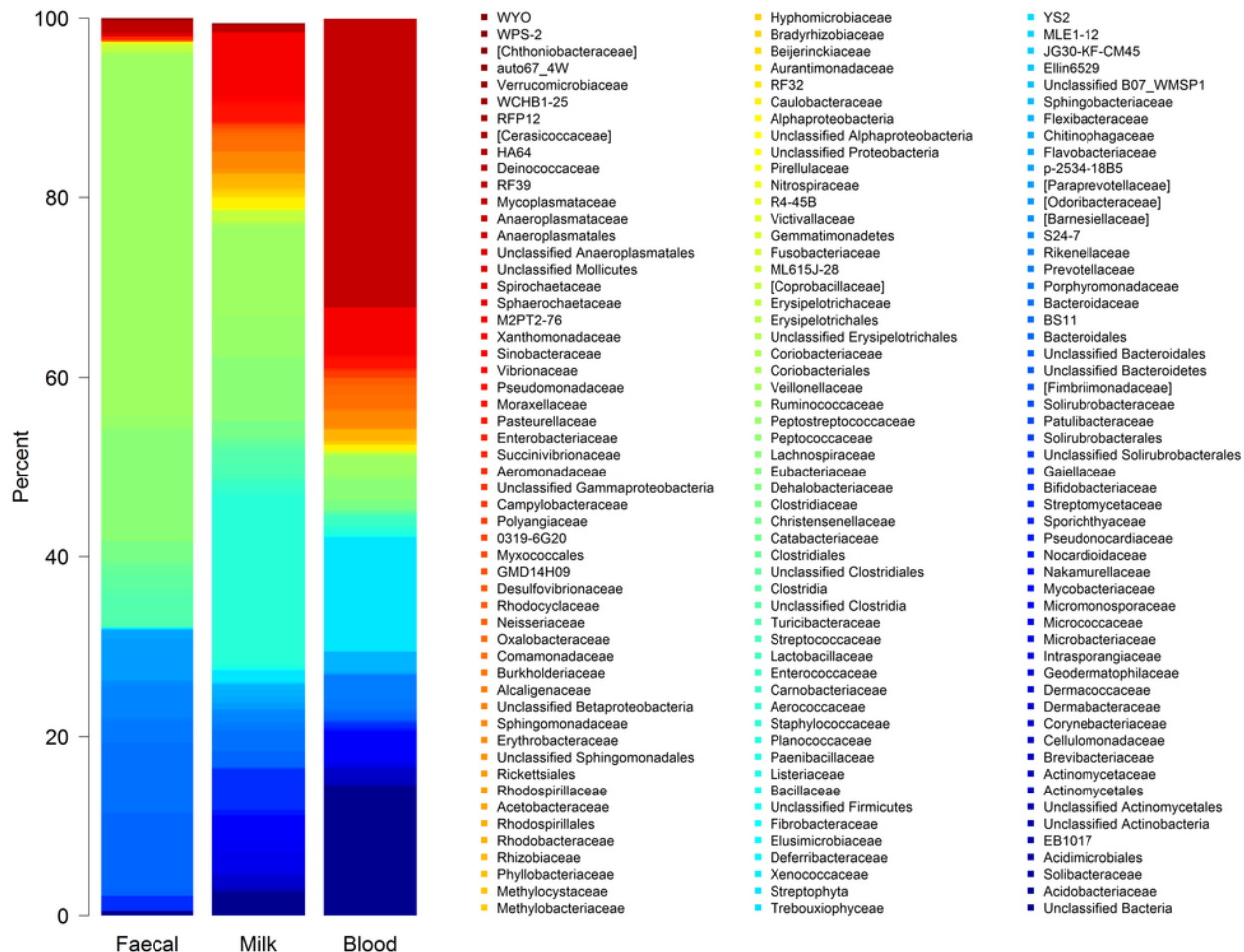


Figure 4 (on next page)

OTU network

OTU network linking samples and OTUs found in each sample. Sample types indicated by coloured shapes; fecal (yellow circle), milk cell (blue square), and white blood cell (red diamond). OTUs are shown by white dots and lines join OTU with sample which that OTU is found in. Lines are coloured according to sample type the OTUs are found in; fecal (yellow), milk cell (blue) and, white blood cell (red). Network arranged using a force directed layout where samples that share more OTUs are placed closer together.

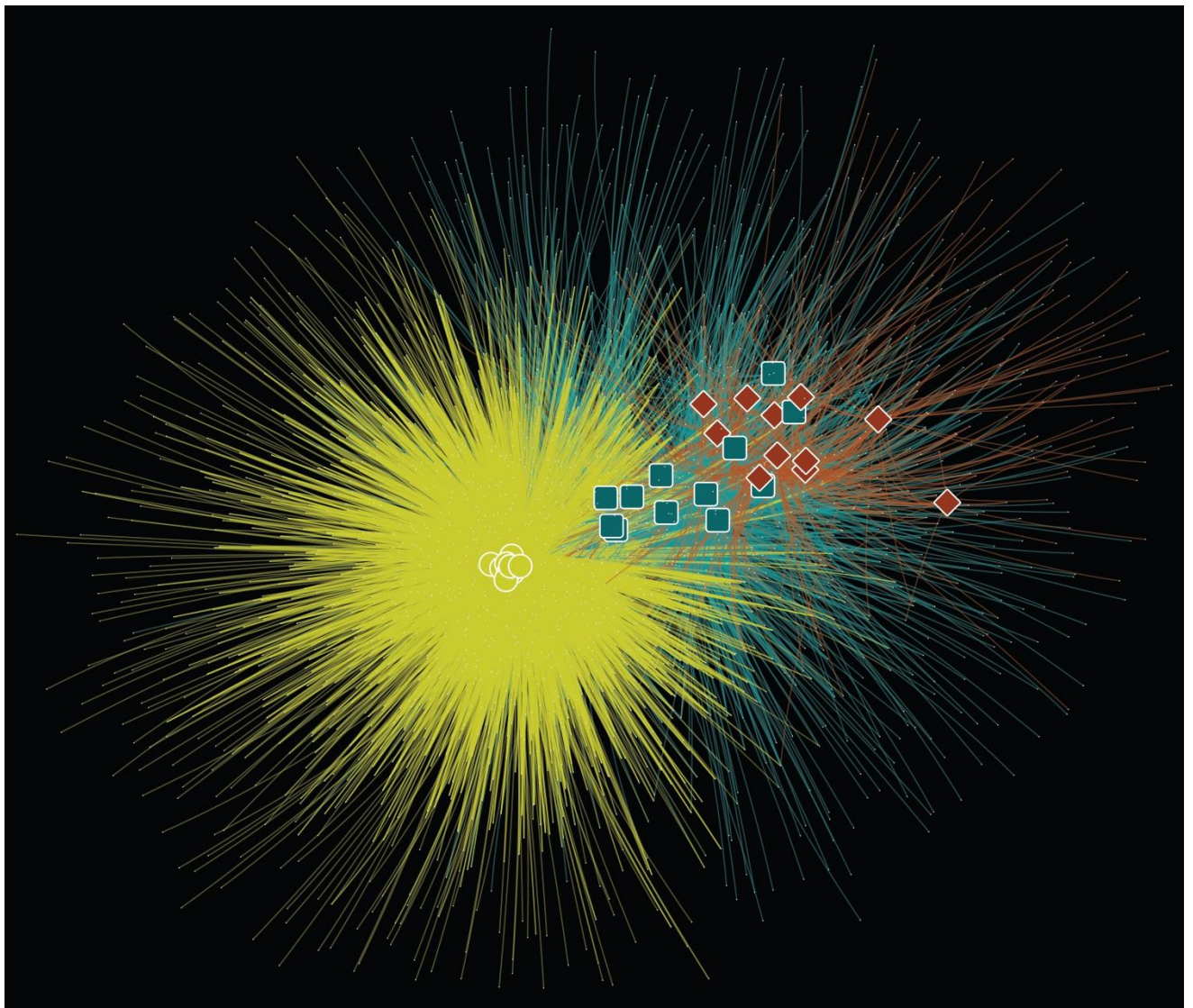


Table 1(on next page)

Bacterial OTUs (bacterial signatures) found in the three biological samples from one or more sampled animal.

Profiling was performed by pyrosequencing of bacterial DNA.

OTU ID	Phylum	Classification	Animal ID
1759, 851, 1942	<i>Firmicutes</i>	<i>Ruminococcus</i>	3,5,7,9,12
251, 1805	<i>Firmicutes</i>	<i>Peptostreptococcaceae</i>	1,5,7,8,12
2052	<i>Actinobacteria</i>	<i>Bifidobacterium</i>	3,5,7,8
1954, 980, 1511, 883	<i>Firmicutes</i>	<i>Lachnospiraceae</i>	3,5,7
119, 792	<i>Firmicutes</i>	<i>Ruminococcaceae</i>	1,2,7
187, 259	<i>Bacteroidetes</i>	<i>Bacteroidales</i>	3,5
813	<i>Bacteroidetes</i>	<i>Paludibacter</i>	3,5
476, 478	<i>Firmicutes</i>	<i>Sarcina</i>	5,12
1681	<i>Actinobacteria</i>	<i>Agrococcus jenensis</i>	5
2183	<i>Actinobacteria</i>	<i>Microbacterium</i>	8
1151	<i>Actinobacteria</i>	<i>Nakamurellaceae</i>	8
589	<i>Bacteroidetes</i>	<i>Bacteroidaceae</i> 5-7N15	5
1188	<i>Bacteroidetes</i>	<i>Parabacteroides</i>	1
2149	<i>Bacteroidetes</i>	<i>Bacteroidales</i> S24-7	3
67	<i>Cyanobacteria</i>	<i>Streptophyta</i>	12
939	<i>Firmicutes</i>	<i>Blautia</i>	7
1983	<i>Firmicutes</i>	<i>Clostridia</i>	7
1533	<i>Firmicutes</i>	<i>Clostridiaceae</i>	7
1219	<i>Firmicutes</i>	<i>Coproccoccus</i>	12
1053	<i>Firmicutes</i>	<i>Turicibacter</i>	2
806	<i>Planctomycetes</i>	<i>Pirellulaceae</i>	5
1369	<i>Proteobacteria</i>	<i>Agrobacterium</i>	3
1834	<i>Proteobacteria</i>	<i>Escherichia</i>	7
238	<i>Tenericutes</i>	<i>Mollicutes</i> RF39	5

Table 2 (on next page)

Abundance of bacterial taxa.

Profiling was performed by pyrosequencing of bacterial DNA. Taxonomic names listed correspond to the highest level identified (0.8 confidence) for each group of bacterial sequences detected. Phylum indicated by letter in parentheses: F = Firmicutes, B = Bacteroidetes, T = Tenericutes, C = Cyanobacteria, P = Proteobacteria, A = Actinobacteria.

Rank	Feces	White blood cells	Milk somatic cells
1	(F) <i>Ruminococcus</i> (28.6%)	(T) <i>Mycoplasma</i> (33.9%)	(F) <i>Staphylococcus</i> (27.6%)
2	(B) <i>Bacteroidales</i> (8.2%)	(C) <i>Streptophyta</i> (24.1%)	(F) <i>Ruminococcus</i> (7.2%)
3	(F) <i>Ruminococcaceae</i> (6.5%)	Unclassified bacteria (13.4%)	(F) <i>Peptostreptococcaceae</i> (6.5%)
4	(B) <i>Bacteroidaceae</i> 5-7N15 (6.0%)	(B) <i>Prevotella</i> (4.5%)	(A) <i>Bifidobacterium</i> (5.6%)
5	(F) Unclassified <i>Lachnospiraceae</i> (4.0%)	(P) <i>Stenotrophomonas</i> (3.3%)	(F) <i>Butyrivibrio</i> (2.3%)
6	(F) <i>Lachnospiraceae</i> (3.8%)	(P) <i>Acinetobacter</i> (1.1%)	(P) <i>Stenotrophomonas</i> (2.1%)
7	(B) <i>Paraprevotellaceae</i> CF231 (3.5%)	(A) <i>Micrococcus</i> (0.8%)	(F) <i>Ruminococcaceae</i> (2.0%)
8	(B) <i>Rikenellaceae</i> (3.4%)	(A) <i>Kocuria</i> (0.7%)	(A) <i>Intrasporangiaceae</i> (1.8%)