Prenatal establishment of the foal gut microbiota: a critique of the *in utero* colonisation hypothesis

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Abstract. Bacteria colonisation of the foal's gastrointestinal tract (GIT) is a critical developmental stage, effecting subsequent immunological and health outcomes. It has long been thought that the equine fetus develops in a sterile intrauterine environment and GIT colonisation commences at birth. Research now suggests that bacteria isolated from amniotic fluid are the initial colonisers of the fetal GIT, and exposure to the dam's microbiota and the external environment during birth provide supplementary colonisation. This *in utero* colonisation hypothesis has only recently been examined in the horse and microbiota were detected in the amniotic fluid and meconium of healthy equine pregnancies. This review highlights the possible colonisation routes of these bacteria into the fetal compartments and examines their likely origins from the existing maternal microbiome. However, the current data describing the amniotic microbiota of the horse are limited and there is a need for research to fill this gap. Understanding the significance of intrauterine microbes for foal GIT colonisation may provide strategies to improve neonatal health.

Additional keywords: amniotic fluid, gastrointestinal tract, meconium, sterile womb.

Introduction

Microbiota colonisation of the intestinal tract, during the early stages of neonatal life, plays a critical role in the immunological and physiological development of animals and humans (Schoster *et al*. 2013). In the horse, disrupted, imbalanced or incomplete colonisation has a major impact on foal health and can result in infectious gastrointestinal tract (GIT) diseases, which cause significant costs to the equine industry (Wohlfender *et al*. 2009). This typically results in neonatal losses, treatment expenses and a decrease of potential future earnings in the sale ring, on racetracks or in the breeding industry (Hong *et al*. 1993). The economic repercussions involved in attempting to improve the health of foals from GIT diseases is also considerable (Rose *et al*. 2018). Infections can result from prenatal exposure in the dam, or postnatally due to pathogenic organisms in the environment (Foote *et al*. 2012). The outcome of pathogen exposure is highly dependent on the immunological status of the neonate and juvenile animal (Torrazza and Neu 2011). Therefore, it is important to understand how and when these initial resident microbes commence colonisation of the GIT. This will allow the development of strategies to promote adequate immunological development. Moreover, it will permit delineation of the role of the microbiota in equine behaviour and temperament, an area of significant interest

in human health (Borre *et al*. 2014; Aatsinki *et al*. 2019; Zhuang *et al*. 2019), with important implications for farm animal behaviour and welfare (Kraimi *et al*. 2019).

The longstanding 'sterile womb paradigm' describes neonates from normal pregnancies being born devoid of microbiota, having developed in a sterile intrauterine environment including the placenta (Costa *et al*. 2016). The colonisation of the foal and the GIT is thought to commence at birth from exposure to the mother and the external environment (Aagaard *et al*. 2014). Research is now challenging this view, with the detection of microbial communities in the human placenta, amniotic fluid and neonatal meconium of apparently normal pregnancies (Cao *et al*. 2014; Wassenaar and Panigrahi 2014; Panelli *et al*. 2018). Traditionally, the presence of bacteria in the placenta was thought to be pathological, indicating an intrauterine infection; however, bacteria have also been discovered in humans in the absence of histological infection over the past few decades (Prince *et al*. 2015). Thus, there is increasing support for the hypothesis that the initial microbial colonisation commences *in utero*. The role that placental microbes play in establishing the neonate intestinal microbiota has not yet been established, and the timing and route of this initial colonisation remain unclear. However, it is well established that intestinal microbes play an

important role in the development of postnatal immunity, and this fundamental knowledge gap could be vital in further understanding the developmental origin of health and disease (Rehbinder *et al*. 2018). Moreover, it is likely that the bacteria in the amniotic fluid modulate the fetal immune system, thus preparing the neonate for the external environment outside the uterus (Hemberg *et al*. 2015). The focus of this review is to challenge traditional thinking on microbial colonisation of the neonatal GIT of the foal, by examining the *in utero* colonisation hypothesis, especially how the amniotic microbiota are established and their likely origins from the microbiota of the mare.

The traditional view of foal GIT colonisation

Foals, like all mammalian species, were thought to develop in a sterile intrauterine environment. This sterile womb paradigm was first suggested in 1885 by Theodor Escherich who declared human meconium to be free of viable bacteria. Further independent studies in 1927 and 1934 found the majority (62%, *n* = 150) of human meconium samples from healthy pregnancies to be free of both aerobic and anaerobic bacteria, apparently confirming the sterile placenta hypothesis (Burrage 1927; Hall and O'Toole 1934; Perez-Muñoz *et al*. 2017).

According to this traditional view, the foal's GIT is first colonised during birth, through exposure to the dam's vaginal, faecal and skin microbiota, as well as the immediate surrounding environment. Colonisation of the GIT is then further evolved by nursing and behaviours such as coprophagy (Charbonneau *et al*. 2016; Costa *et al*. 2016; Alipour *et al*. 2018; Costa and Weese 2018).

Studies that established the sterile womb paradigm employed traditional culture-based methods and microscopy (Burrage 1927; Hall and O'Toole 1934; Perez-Muñoz *et al*. 2017). After these early studies, microbiological research on human meconium stopped for over 30 years, until the sterile womb was challenged by Jiménez *et al*. (2008), who reported 100% (*n* = 21) of human meconium samples positive for bacterial growth. This sparked additional research, and recent studies have now employed modern sequencing technologies to further develop the idea that neither the placenta nor the fetal environment are sterile (Perez-Muñoz *et al*. 2017). The relevance and accuracy of both the culture-based and modern sequencing techniques are currently vigorously debated. The argument is advanced that positive samples from healthy pregnancies are the result of sampling contamination. However, later in this review, it will be established that microbiota detected in amniotic fluid and meconium do not appear to originate from the mother's reproductive tract or external environment, which would be the expected origin of contamination. Instead the evidence suggests an internal mother–fetus microbiological interaction. This human microbiome research implies that *in utero* colonisation of the fetal GIT is likely to occur in all mammalian species, including the horse.

The challenging hypothesis: *in utero* colonisation of the foal

Bacteria have now been detected in foal first pass meconium, suggesting that the GIT colonisation process may commence *in utero*, and is then supplemented by, instead of originating from, the exposure to further microbes during birth and later life (Jacquay *et al*. 2018; Quercia *et al*. 2019), as shown in Fig. 1. This hypothesis has been strongly debated, with very few studies questioning this theory in horses, although *in utero* colonisation is now gradually being accepted in human microbiome research (Aagaard *et al*. 2014; Collado *et al*. 2016; Chu *et al*. 2017). Fetal colonisation of the GIT plays a critical role in health development and disease prevention in young foals (Costa *et al*. 2016). Developing a mechanistic understanding of this process by establishing whether bacterial colonisation commences *in utero*, could fundamentally change our understanding of foal health and, subsequently, influence our management practises of both pregnant mares and newborn foals.

Foal development *in utero*

For *in utero* colonisation to be initiated, the amniotic fluid microbiota must interact with the developing fetus. Two stages of development must be established to determine the timing of initial colonisation. These include, first, the commencement and development of the fetal GIT and, second, the point in which amniotic fluid first enters the fetal GIT.

Fetal GIT development

The development of the foal's GIT commences *in utero* during gastrulation, beginning as early as Day 12 after fertilisation (Gaivão *et al*. 2014). Gastrulation is the first major morphogenetic change the embryo undergoes and is the stage at which simple cells of the conceptus are transitioned into more complex layers consisting of the major tissue endoderm, mesoderm and ectoderm. This process gives rise to the primitive intestine and it is during organogenesis that the inner endoderm develops into the complete fetal digestive tract and its associated organs, including the pancreas and liver. The primitive intestine of the fetus is well defined by Day 25 of gestation, and completion of organogenesis occurs after Day 40, but the GIT is still immature at this stage (Platt 1984; Franciolli *et al*. 2011). Although the primitive fetal GIT is developed, *in utero* colonisation is more likely to commence when the physiological process of fetal swallowing begins.

Fetal swallowing

Fetal swallowing is a key component of further GIT maturation and allows the passage of nutrient-rich amniotic fluid into the lumen of the primitive GIT. The commencement of fetal swallowing is not well defined in the horse; extrapolations can be made from human development to horses. First, for fetal swallowing to begin, organogenesis must be complete, which occurs after Day 40 of gestation (Platt 1984; Franciolli *et al*. 2011). Second, fetal swallowing of amniotic fluid contributes to the accumulation of meconium, and, in humans, it is known that meconium begins to form as early as the 12th week, coinciding with the end of the first trimester (Stinson *et al*. 2018; Woźniak *et al*. 2018). In horses, the transition from first to second trimester is ~14 weeks of gestation; therefore, it can be assumed that fetal swallowing
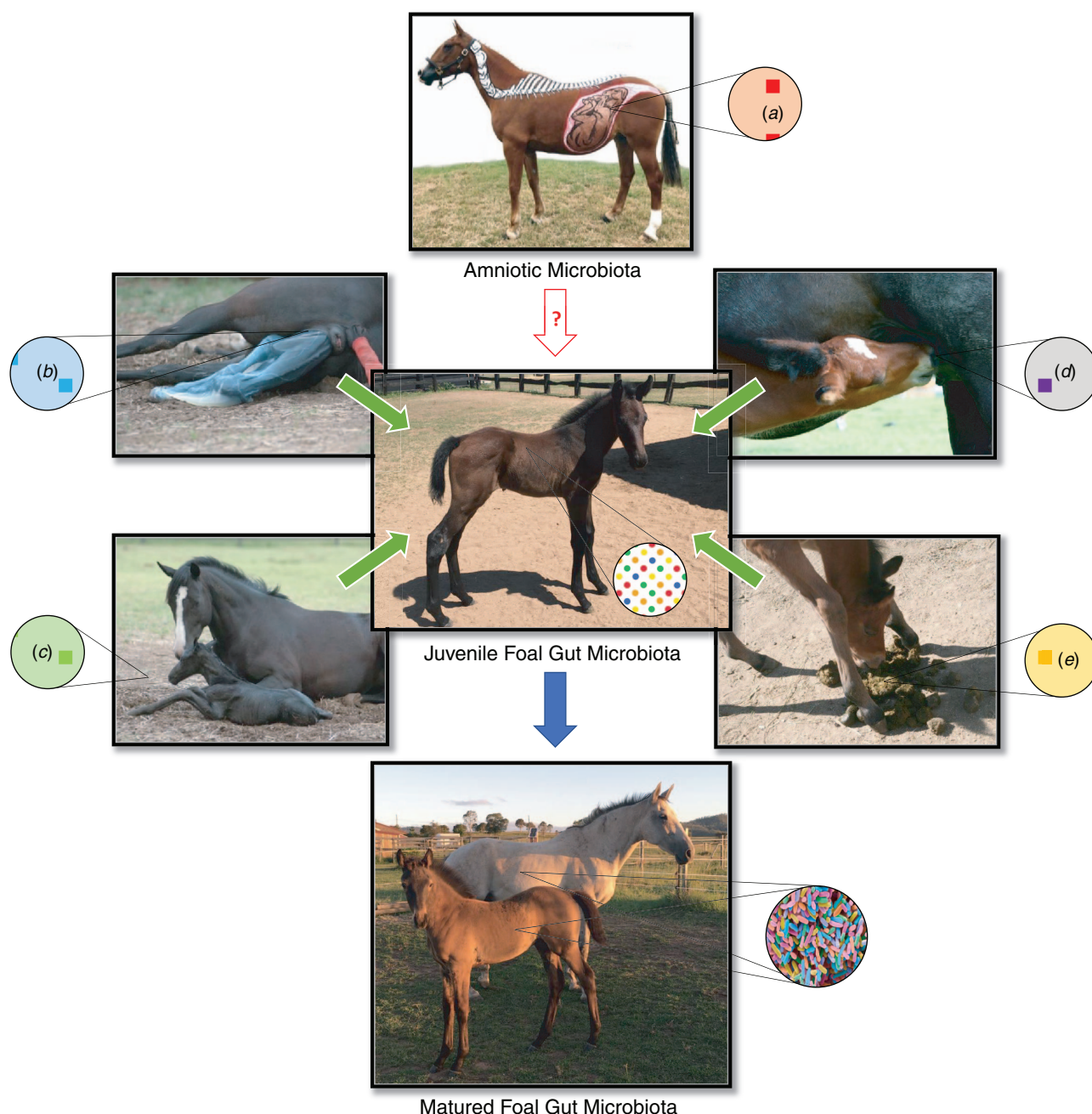
Fig. 1. *In utero* colonisation hypothesis. (*a*) Amniotic microbiota are proposed to commence foal gastrointestinal (GI) colonisation *in utero* with unique microbes, which is later supplemented by the currently known routes of colonisation. These include (*b*) during birth, through exposure to the dam's vaginal, faecal and skin microbiota, plus (*c*) the immediate surrounding environmental microbes. After birth, the GI tract (GIT) microbial community further evolves due to (*d*) suckling, nursing and (*e*) coprophagic behaviours. Thus, each pathway contributes uniquely to the establishment of the foal's juvenile GIT microbiota, which then continue to develop and mature, until it resembles that of the adult horse.

commences between Day 40 and Day 100 of gestation. Amniotic fluid volume increases in the mare from Week 8 of gestation (Whitwell and Jeffcott 1975), further indicating that it is likely that fetal swallowing and *in utero* GIT colonisation commence during this time frame.

The process of fetal swallowing plays a significant role in the growth and development of the fetus. It has been demonstrated in pigs, sheep and rabbits that preventing amniotic fluid swallowing by surgical oesophageal ligation leads to inhibition of gastric development and acid secretion (Mulvihill *et al.* 1986; Trahair and Sangild 2000; Sangild *et al.* 2002; Cellini *et al.* 2006). However, infusion of amniotic fluid at the same rate returns gastric weight, gastric acid secretion, and serum gastrin concentrations back to normal (Mulvihill

et al. 1986). Although this has yet to be demonstrated in horses, the same relationships are likely to occur in the equine fetus. If amniotic microbiota are initially introduced to the fetal GIT at the commencement of fetal swallowing, it is possible that they influence the growth and development of the fetus.

As mentioned previously, fetal swallowing of amniotic fluid is considered a major contributor to the formation and accumulation of meconium in the fetal GIT throughout pregnancy (Costa *et al*. 2016). The *in utero* colonisation hypothesis has been developed from the recent detection of bacteria in foal meconium and their suggested amniotic fluid origin (Jacquay *et al*. 2018; Quercia *et al*. 2019), further supporting the idea that fetal swallowing commences *in utero* GIT colonisation.

Amniotic microbiota

The presence of bacteria in the amniotic fluid and placenta were thought to be associated only with infection and pathology. The investigation into the presence of amniotic microbiota in healthy pregnancies was prompted after the detection of non-pathogenic bacteria in the meconium of newborn foals that appeared to originate from the amniotic fluid (Hong *et al*. 1993; Foote *et al*. 2012; Wassenaar and Panigrahi 2014; Hemberg *et al*. 2015; Quercia *et al*. 2019). This novel area of research in equine science has now detected bacteria and other microbes in amniotic fluid from healthy equine pregnancies, indicating that microorganisms may indeed exist in the amniotic fluid, thereby negating the concept of a sterile womb (Hemberg *et al*. 2015; Quercia *et al*. 2019).

Hemberg *et al*. (2015) examined 46 amniotic fluid samples from foaling Standardbred mares on one stud farm in Sweden. Bacterial growth was found in amniotic fluid samples collected at parturition from 26 pregnancies, resulting in both healthy and unhealthy foals, as defined by the foal's ability to stand unaided within 2 h (normal) or requiring more than 2 h respectively. The group requiring more than 2 h to stand also had more complications during foaling and postpartum than did the healthy group. Pure cultures were isolated from 33% of the amniotic fluid samples, mixed growth of no more than two different bacteria were isolated from 24% of the samples, and 43% of the samples were negative for bacterial growth. Of the positive cultures, coagulase-negative staphylococci were the bacteria most frequently isolated, occurring in 54% of all positive samples. Other bacteria isolated from the amniotic fluid included haemolytic streptococci, and isolates identified as belonging to *Pantoea* spp., *Micrococcus* spp., *Escherichia coli* and *Acinetobacter* spp. However, the main limitation of these findings was the use of culture-dependent analysis, as this approach would have highlighted the dominant bacteria capable of growing under the selective culture conditions, thus biasing and limiting in-depth analysis of the whole microbiota present. Interestingly, there were no significant differences between the types of bacterial species detected in the amniotic fluid samples collected from the healthy and unhealthy foal groups (Hemberg *et al*. 2015).

Recently, Quercia *et al*. (2019) utilised next generation sequencing-based techniques to characterise the microbiota present in equine amniotic fluid samples, and in foal meconium samples collected from 13 Standardbred mare–foal pairs. Subsequent bioinformatics showed that bacterial sequences assigned to 32 unique operational taxonomic units (OTUs), were detected in both the amniotic fluid and meconium samples, indicating a strong microbial similarity between the two sample types (Quercia *et al*. 2019). It agrees with the similarity found in humans by Ardissone *et al*. (2014), with over 50% of bacteria identified in meconium also being detected in amniotic fluid. The source of the microbiota in the horse is yet to be elucidated; however, the OTUs identified in both the amniotic fluid and foal meconium samples were related to well known opportunistic bacteria, as well as other mare microbiota that are resident to the skin, oral cavity and the gut. For example, Quercia *et al*. (2019) reported OTUs from bacterial species belonging to the genera *Pseudomonas*, *Sphingomonas*, *Aerococcus* and *Streptococcus*, all of which are regarded as opportunistic bacteria. OTUs were also related to *Staphylococcus* and *Acinetobacter*, which are genera that have been identified as part of the mare's skin microbiota, and some OTUs were identified to *Delftia*, a genus that has been detected as a component of the mare's oral microbial community. Microbes belonging to the phylum Bacteroidetes, and the genera *Akkermansia* and *Faecalibacterium*, were identified from the mare's distal GIT (Quercia *et al*. 2019).

Quercia *et al*. (2019) also compared the bacteria of amniotic fluid and meconium with those of the mare's distal GIT. Among all three environments, six OTUs were shared, namely, *Escherichia coli*, *Enterococcus faecalis*, *Streptococcus equinus*, *Agathobacter ruminis*, *Paraclostridium bifermentas* and *Acinetobacter iwoffii*. No OTUs were exclusively shared between amniotic fluid and mare faeces, whereas 75 OTUs were shared between meconium and the mare's distal GIT samples, while 32 OTUs were common between meconium and amniotic fluid samples. These results suggest internal transmission routes for the microbes, where the mare's microbiota are vertically transmitted to the fetus. The shared OTUs of the meconium with both the amniotic fluid and mare's distal GIT further suggest that each of these sites provides a specific contribution to the overall meconium microbial community (Quercia *et al*. 2019). These colonisation routes have also been demonstrated in humans (Charbonneau *et al*. 2016; Pelzer *et al*. 2017).

One limitation of the study by Quercia *et al*. (2019) is the inability to differentiate between live and dead bacteria due to the sole use of next generation sequencing, with no culture for viability assessment. However, the culture of live bacteria by Hemberg *et al*. (2015) confirmed that at least some bacteria present in amniotic fluid are viable. Whether all bacterial DNA detected in amniotic fluid and meconium comes from live cells remains to be determined. Moreover, the source of these bacteria can be questioned. Research in humans and mice has observed distinct similarities between the amniotic microbiota and the mother's oral microbiota (Fardini *et al*. 2010; Ardissone *et al*. 2014). Although Quercia *et al*. (2019) demonstrated similarities among the amniotic, meconium

and the mare's faecal microbiota, other locations of the dam, such as the oral cavity, were not examined. Since the dam's oral microbiota would be contributing to the GIT, and, subsequently, the faecal microbiomes, it could also be influencing the amniotic microbiota. However, this hypothesis has not yet been explored in the horse.

These early studies have provided evidence of non-pathological prenatal exposure of the equine fetus to microbes; however, there are some limitations. These findings are limited in scope due to the small number of samples and the alternate culture-dependent and culture-independent analytical methods used. Furthermore, as meconium composition is considered as a record of changes occurring throughout pregnancy (Woźniak *et al.* 2018), it can be questioned whether sampling just one section of the meconium would provide an accurate analysis of the entire microbiota and the potential GIT colonisation process. Variations between distal versus proximal samples of the meconium could indicate the origin of bacteria in the foal's GIT. It could potentially demonstrate whether the bacteria enter from swallowed amniotic fluid and colonise the GIT, or from the external environment starting at birth. Nonetheless, the presence of live bacteria and components of bacterial DNA in the amniotic fluid and the meconium casts serious doubt on the application of the sterile womb paradigm to equine pregnancy. This is an area that needs additional research to further explore the relationship between amniotic and meconium microbiota, and to determine how microbes first enter the fetal compartments and, in turn, influence the development of the foal from prenatal into neonatal life.

Proposed routes of colonisation

How microbes enter the fetal compartments is not entirely clear. To our knowledge, no research has focused on the colonisation routes in horses, but parallels can be drawn from studies examining humans and other animal models. Microbial taxa that have been found in the placenta, both pathogenic and non-pathogenic, most frequently originate from the mother, and three different colonisation routes have been proposed (Charbonneau *et al.* 2016; Pelzer *et al.* 2017), as demonstrated for the horse in Fig. 2. First, the ascension of microorganisms from the vagina and cervix to the uterus. Second, the haematogenous spread from the oral cavity to the placenta, and, third, maternal dendritic cells sampling bacteria from the intestinal lumen, which are internalised, and then transported to the placenta (Pararas *et al.* 2006; Moreno and Franasiak 2017; Pelzer *et al.* 2017). Introduction of bacteria can also occur iatrogenically during invasive procedures, such as amniocentesis or percutaneous fetal blood sampling (Pararas *et al.* 2006). This section will focus on the natural ascension and transfer from mother to offspring of such microorganisms, rather than those introduced via iatrogenic routes, which would have minimal relevance in the horse, since amniocentesis are very rarely being performed in the mare (Schmidt *et al.* 1991).
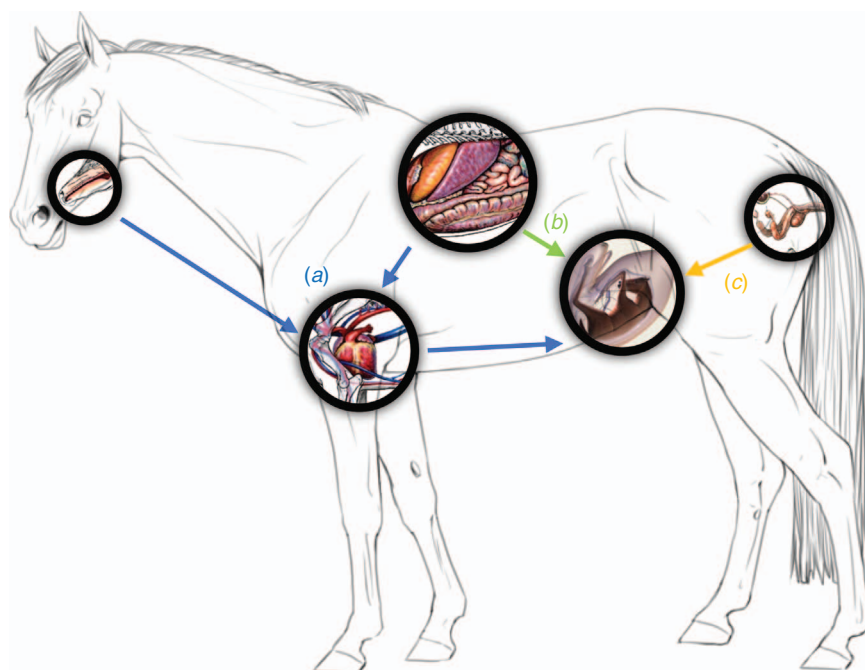

Fig. 2.   Proposed routes of fetal compartment colonisation. (*a*) Haematogenous and trans-placental transfer from the distal sites of the dam to placenta, including the oral cavity and gastrointestinal tract (GIT). (*b*) Dendritic cell sampling from GIT and internalising and transporting to placenta. (*c*) Ascension from lower genital tract through the cervix into the uterus and through the cervical star area into the placenta.

The first proposed route, for colonisation of normal amniotic fluid, is ascension of bacteria from the genital tract through the cervix and into the placenta. This route has been well defined for the invasion of pathogens causing intrauterine infections, namely placentitis. The most common pathogens implicated include *Streptococcus equi* subspecies *zooepiddemicus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Hong *et al*. 1993; Foote *et al*. 2012). The bacteria then infect various locations including the fetal membranes, umbilical cord, placenta, amniotic fluid and, finally, the fetus (Hitti *et al*. 2001; Fardini *et al*. 2010). It is currently unknown whether, in the absence of infection, non-pathogenic bacteria can ascend from the genital tract of the mare into the fetal compartments.

The second proposed route of colonisation is the haematogenous transfer of microbiota from distal locations in the dam, including the oral cavity and the GIT, to the placenta. This idea originated when an increasing number of human studies reported intrauterine infections caused by bacterial species that were not found in the lower genital tract, but instead are commensal species originating from the oral cavity, suggestive of an haematogenous transfer mechanism (Fardini *et al*. 2010; Han *et al*. 2010). Some of these species include *Fusobacterium nucleatum* and *Bergeyella*, *Eikenella*, *Capnocytophaga* spp., and *Porphyromonas gingivalis* (Fardini *et al*. 2010; Mesa *et al*. 2013). *Fusobacterium nucleatum* and *Bergeyella* spp. were identified to be associated with preterm birth, with the same clonal species being found in the subgingival plaque of the mother, but not in the supragingival plaque, or vaginal, or rectal, microbiota (Fardini *et al*. 2010; Han *et al*. 2010). Other epidemiologic studies have also established a link among oral microbiota, periodontal disease and preterm birth, with Aagaard *et al*. (2015) demonstrating that the placental microbiota are more homologous with those of the oral cavity, as opposed to the vagina. Mice have been used as human models to confirm that a diverse group of oral bacteria can translocate to the placenta and the meconium of the newborn (Jiménez *et al*. 2008; Fardini *et al*. 2010). Further studies have suggested that haematogenous transfer routes are involved with bacterial transmission during pregnancy (Fardini *et al*. 2010; Mesa *et al*. 2013; Cao *et al*. 2014; Aagaard *et al*. 2015). For example, Gram-negative periodontal pathogens, including their products such as lipopolysaccharides, have been shown to enter the circulatory system and produce low-grade bacteraemia, that could then translocate to the placenta or uterus to influence birth outcomes (Mesa *et al*. 2013). Another study was able to isolate microorganisms belonging to the genera *Enterococcus*, *Streptococcus*, *Staphylococcus* and *Propionibacterium* from umbilical cord blood, which also supports a haematogenous transfer of bacteria from distal maternal locations to the fetus (Cao *et al*. 2014). Similar results have been reported for cattle, with their calves' umbilical blood harbouring key uterine pathogens such as *Bacteroides* spp., *Fusobacterium* spp. and *Prophyromonas* spp. Presence of uterine pathogens in faeces and blood indicates the feasibility of spread of haematogenous bacteria from the gut to the uterus. Ascending contamination of the uterus cannot be discarded

as an important route, as the vagina also harbours the main uterine pathogens. However, other uterine pathogens such as *Prevotella* spp., *Helcococcus* spp., *Filifactor* spp., *Campylobacter* spp. and *Arcanobacterium* spp. were not found as part of the cow's core vaginal microbiome, which may also indicate a haematogenous transfer route (Jeon *et al*. 2017). It has been suggested that the mechanism by which microbiota move through the circulatory system could be facilitated by other bacteria such as *Fusobacterium nucleatum*. These have the ability to bind to vascular endothelium and alter permeability, functioning as an 'enabler' for other bacteria, such as *Escherichia coli*, to move through the circulatory system (Wassenaar and Panigrahi 2014). Studies in mice and cattle have also shown that bacteria can be transported to extra-intestinal sites by mononuclear cells (Jeon *et al*. 2017). Detailed mechanisms of haematogenous transfer of non-pathogenic microbiota from distal locations of the mare to the placenta and fetal compartments have not been described in the horse.

Dendritic cell sampling is the third proposed route through which bacterial translocation from the GIT to the placenta can occur. Transfer across the GIT epithelium is restricted by a well established barrier; however, dendritic cells can penetrate this barrier and enter the intestinal lumen via their intraepithelial dendrite extensions. This allows the dendritic cells to internalise bacteria from the GIT and shuttle them across the epithelial barrier to the periphery (Foti and Ricciardi-Castagnoli 2005; Nicoletti *et al*. 2009). *In vitro* and *in vivo* studies in mice have examined the ability of dendritic cells to sample both non-pathogenic *Escherichia coli* and pathogenic *Salmonella* (Rescigno *et al*. 2001; Wallace *et al*. 2016). It was determined that, in both cases, dendritic cells take up the bacteria directly, although, in the presence of pathogens, their numbers doubled. It was concluded that dendritic cells have the ability to penetrate and sample the GIT lumen, and to facilitate transfer of both pathogenic and non-pathogenic bacteria to peripheral sites through the lymphatic system (Rescigno *et al*. 2001; Wallace *et al*. 2016). Once pathogenic bacteria are in the lymphatic system, an efficient immune response is initiated. Dendritic cells play a key role in immunological regulation but are a new concept in the *in utero* colonisation hypothesis; therefore, their ability to transfer non-pathogenic bacteria to the equine placenta has not yet been explored.

These three proposed routes have not been defined in the horse as mechanisms for non-pathogenic bacteria to transfer into the placenta and fetal compartments. Preliminary studies on the equine amniotic microbiome have shown similarities to the dam's GIT microbiota (Quercia *et al*. 2019). Therefore, it is possible that haematogenous transfer and dendritic cell sampling also occur in the horse and transfer maternal bacteria to the placenta and fetal fluids. More studies are required to define the mechanisms underpinning vertical transmission of bacteria to the fetal gut of the foal *in utero*.

The microbiota of the dam

There is no doubt that during birth, the foal is exposed to a considerable number of bacteria. However, as the *in utero*

colonisation hypothesis implies that bacteria are also being relocated internally from a variety of the dam's microbial sites to create an amniotic microbiota during pregnancy, it is important to understand what microbiota occur normally in the mare. Some similarities between the bacteria in amniotic fluid and the dam's microbiomes have been identified (Table 1); however, the current amniotic data are too limited to justify any statistical analysis and infer relevance. It is important to note that the normal composition of the equine microbiome can vary among regions, ages and management systems, and there are inter-horse variations (Costa and Weese 2018), which would likely have an impact on the comparisons to the amniotic microbiome. Here, we focus on the oral, distal GIT and reproductive tract microbiota of the mare, as these are the origin of the proposed routes of colonisation.

Oral microbiota

There are limited data on the oral microbiome of the healthy horse, with most studies having focused on disease such as periodontitis. Kennedy *et al.* (2016) identified 179 microbial family groups in the oral cavity of the horse; however, only 7% of these were associated with the healthy oral microbiome. The most discriminative bacteria identified from the healthy oral microbiota belonged to the phyla Proteobacteria and Firmicutes (Table 1; Kennedy *et al.* 2016). These results were supported by Gao *et al.* (2016), who identified additional phyla in the healthy equine subgingival microbiome, including Actinobacteria, Bacteroidetes, Chloroflexi, Fusobacteria, Spirochaetes, Synergistetes, Tenericutes and Saccharibacteria. These same phyla have been identified in the human oral microbiome (Gao *et al.* 2016), which is noteworthy, because human oral bacteria, such as *Bergeyella* spp. from the phylum Bacteroidetes, and *Fusobacterium nucleatum*, have been identified in placental infections causing adverse pregnancy outcomes, indicating the possible haematogenous oral-placenta route of colonisation proposed earlier (Fardini *et al.* 2010; Pelzer *et al.* 2017).

Comparing these bacteria identified in the oral cavity (Meyer *et al.* 2010; Gao *et al.* 2016; Kennedy *et al.* 2016) with those detected in amniotic fluid and meconium by Quercia *et al.* (2019; as outlined in Table 1), only one family was shared between the oral cavity and meconium, namely, *Xanthomonadaceae*. Although this result would indicate no relationship between oral and amniotic microbiomes, the data were collected from one study that has not been replicated. That study also included only bacteria with the highest abundance from each site; therefore, it is possible that oral bacteria may appear in the placental fluids at a lower abundance than the other sites. Alternatively, oral bacteria may play a role only in adverse pregnancy outcomes and not be present in the normal amniotic microbiota. This could be supported by the presence of *Actinobacillus* spp. in the equine oral cavity, which has previously been described as a pathogen causing abortion in the mare, but has not been isolated from normal pregnancies in recent studies (Layman *et al.* 2014; Quercia *et al.* 2019). At this stage in equine amniotic-microbiota research, it is unclear whether oral bacteria form part of the normal inhabitants in the fetal compartments.

Distal GIT microbiome

The GIT harbours a complex microbial community consisting of archaea, bacteria, fungi, parasites and viruses (Costa *et al.* 2016; Costa and Weese 2018). The intestinal microbiota are recognised for their essential role in maintaining host health, by providing protection against overgrowth of pathogenic organisms, contributing to development of the intestinal epithelium, breaking down complex feed particles, and modulating the local immune system and metabolic functions (Costa and Weese 2012, 2018; Schoster *et al.* 2013; Wallace *et al.* 2016). A properly functioning GIT, along with a balanced intestinal microbiota, is fundamental for maintenance of host health and performance, and the ecological system must respond to changes so as to maintain gut homeostasis (Martin *et al.* 2017). Disruption of the normal microbial community is associated with problems including colitis, laminitis and diarrhoea in young foals. These GIT diseases are leading causes of morbidity and mortality (Costa and Weese 2012; Costa *et al.* 2016). The resident equine GIT microbiota have been predominantly evaluated using faecal samples, due to ease of non-invasive collection. However, faeces are representative only of the distal part of the GIT tract, and while having shown microbial similarities to that of the caecum, faecal microbes are a poor indicator of the composition of the microbiota present in the small intestine (Schoster *et al.* 2013; Costa and Weese 2018). Therefore, the following discussion will focus on the distal GIT or hindgut microbiota.

Consistent with the majority of mammalian species, the equine distal gut consists predominantly of Firmicutes (Table 1; Willing *et al.* 2009; Kuhl *et al.* 2011; Schoster *et al.* 2013; Costa and Weese 2018). Bacteroidetes and Verrucomicrobia are the second-most abundant phyla, and-with the development of next generation sequencing, bacteria of the class Clostridia, which includes several *Clostridium* species, have also been recognised as dominant species present in the distal intestinal tract of the adult horse (Costa and Weese 2018). The amniotic and meconium microbiota also include Firmicutes, with distinct similarities to the distal GIT of the adult horse (Table 1). Comparing the results from Quercia *et al.* (2019) with previously published literature, *Escherichia coli* and the family Ruminococcaceae are present both in the distal GIT and the amniotic fluid. In addition, the genera *Enterococcus*, *Streptococcus* and *Staphylococcus* are present in the amniotic fluid and meconium as well as in the distal GIT (Willing *et al.* 2009; Kuhl *et al.* 2011; Schoster *et al.* 2013).

These similarities suggest that microbes from the distal GIT of the dam could be a significant source of the amniotic fluid microbiota. Moreover, the proximal GIT microbiota could also be a source of microbes, but its composition is unknown. In addition, the bacteria detected in the distal GIT are essentially a snapshot in time, and many factors can affect the microbiota present, including age, diet, environmental conditions, disease and the use of supplements such as

Table 1.　Comparison of microbiota detected in the mare across five anatomical locations

*GIT, gastro-intestinal tract. [1]Gao et al. (2016); [2]Kennedy et al. (2016); [3]Meyer et al. (2010); [4]Costa and Weese (2018); [5]Kuhl et al. (2011); [6]Martin et al. (2017); [7]Schoster (2018); [8]Schoster et al. (2013); [9]Willing et al. (2009); [10]Quercia et al. (2019); [11]Hemberg et al. (2015); [12]Pugh (2018); [13]Fraga et al. (2008); and [14]Hoyles et al. (2013)

Microbiota detected	Location				
	Mouth and oesophagus	Distal GIT*	Vagina	Amniotic fluid	Foal meconium
Phylum Actinobacteria	+[1,2]	+[4,5]	+[14]	+[10,11]	+[10]
Class Actinobacteria	+[2]		+[14]	+[10,11]	+[10]
Order Actinomycetales	+[2]		+[14]		
Family Corynebacteriaceae			+[14]		
Corynebacterium spp.			+[14]		
Family Microbacteriaceae				+[10,11]	
Frigoribacterium spp.				+[10]	
Family Micrococcaceae	+[2]				+[10]
Arthrobacter spp.					+[10]
Rothia spp.	+[2]				
Family Mycobacteriaceae	+[2]				
Mycobacterium spp.	+[2]				
Family Nocardioidaceae	+[2]				
Nocardioides spp.	+[2]				
Family Sanguibacteraceae				+[10]	+[10]
Sanguibacter spp.				+[10]	+[10]
Phylum Bacteroides	+[1,3]	+[4,6,9]		+[10]	+[10]
Class Bacteroidetes	+[3]			+[10]	
Order Bacteroidales	+[3]				
Family Prevotellaceae	+[3]				
Prevotella spp.	+[3]				
Class Flavobacteria				+[10]	
Order Flavobacteriales				+[10]	
Family Flavobacteriaceae				+[10]	
Wautersiella spp.				+[10]	
Class Sphingobacteria				+[10]	+[10]
Order Sphingobacteriales				+[10]	+[10]
Family Sphingobacteriaceae				+[10]	+[10]
Pedobacter spp.				+[10]	
Phylum Firmicutes	+[1,2,3]	+[4,5,7,8,9]	+[12,13]	+[10,11]	+[10]
Class Bacilli	+[2,3]	+[5]		+[10,11]	+[10]
Order Bacillales	+[2,3]	+[5]		+[10,11]	+[10]
Family Staphylococcaceae	+[3]	+[5]		+[10,11]	+[10]
Staphylococcus spp.	+[3]	+[5]		+[10,11]	+[10]
Family Not assigned	+[2]				
Gemella spp.	+[2]				
Order Lactobacillales	+[3]	+[5,8,9]	+[13]	+[10,11]	+[10]
Family Aerococcaceae				+[10]	+[10]
Aerococcus spp.					+[10]
Facklamia spp.				+[10]	+[10]
Family Carnobacteriaceae					+[10]
Carnobacterium spp.					+[10]
Family Enterococcaceae	+[3]	+[5]	+[13]	+[10]	+[10]
Enterococcus spp.	+[3]	+[5]	+[13]	+[10]	+[10]
Family Lactobacillaceae	+[2]	+[9]	+[13]		
Lactobacillus spp.	+[2]	+[9]	+[13]		
Family Streptococcaceae	+[3]	+[5,8,9]		+[10,11]	+[10]
Streptococcus spp.	+[3]	+[5,8,9]		+[10,11]	+[10]
Class Clostridia		+[4,7,8,9]	+[12]	+[10]	
Order Clostridiales		+[4,7,8,9]	+[12]	+[10]	
Family Colostridiaceae		+[4,9]	+[12]		
Family Lachnospiraceae		+[7]			
Family Ruminococcaceae		+[7,8]		+[10]	

Table 1.   (*continued*)

Microbiota detected	Mouth and oesophagus	Distal GIT*	Location Vagina	Amniotic fluid	Foal meconium
Class Negativicutes	+[3]				
Order Veillonellales	+[3]				
Family Veillonellaceae	+[3]				
Veillonella spp.	+[3]				
Phylum Proteobacteria	+[1,2,3]	+[4,5,9]		+[10,11]	+[10,11]
Class Alphaproteobacteria	+[2]			+[10]	+[10]
Order Rhizobiales					+[10]
Family Rhizobiaceae					+[10]
Agrobacterium spp.					+[10]
Order Rhodobacterales	+[2]				
Family Rhodobacteraceae	+[2]				
Order Rhodospirillales				+[10]	
Family Acetobacteraceae				+[10]	
Gluconacetobacter spp.				+[10]	
Class Betaproteobacteria	+[2,3]			+[10]	
Order Burkholderiales	+[2,3]			+[10]	
Family Burkholderiaceae	+[2]				
Lautropia spp.	+[2]				
Family Comamonadaceae				+[10]	
Comamonas spp.				+[10]	
Family Oxalobacteraceae	+[2]			+[10]	
Janthinobacterium spp.				+[10]	
Undibacterium spp.	+[2]				
Class Gammaproteobacteria	+[2,3]	+[5,9]		+[10,11]	+[10,11]
Order Enterobacterales	+[3]	+[5,9]		+[10,11]	+[10,11]
Family Enterobacteriaceae	+[3]	+[5,9]			+[11]
Escherichia spp.	+[3]	+[5,9]			+[11]
Escherichia coli	+[3]	+[5,9]			+[11]
Family Erwiniaceae	+[3]			+[10,11]	+[10]
Erwinia spp.				+[10]	+[10]
Pantoea spp.	+[3]			+[11]	
Family Morganellaceae	+[3]				
Proteus spp.	+[3]				
Order Pasteurellales	+[2,3]				
Family Pasteurellaceae	+[2,3]				
Actinobacillus spp.	+[2,3]				
Order Pseudomonadales	+[2,3]			+[10,11]	+[10]
Family Moraxellaceae	+[2,3]			+[10,11]	+[10]
Acinetobacter spp.	+[3]			+[10,11]	+[10]
Enhydrobacter spp.	+[2]				
Moraxella spp.	+[2]				
Psychrobacter spp.				+[10]	+[10]
Family Pseudomonadaceae				+[10]	+[10]
Pseudomonas spp.				+[10]	+[10]
Order Xanthomonadales	+[2]			+[10]	+[10]
Family Xanthomonadaceae	+[2]			+[10]	+[10]
Stenotrophomonas spp.				+[10]	+[10]
Phylum Fusobacteria	+[1,3]		+[12]		
Phylum Spirochaetes	+[1]	+[4,9]			
Phylum Chloroflexi	+[1]				
Phylum Synergistetes	+[1]				
Phylum Tenericutes	+[1]				
Phylum Candidatus Saccharibacteria	+[1]				
Phylum Verrucomicrobia		+[4]			
Phylum Fibrobacteres	+[4,8]				

probiotics (Willing *et al*. 2009; Schoster 2018; De La Torre *et al*. 2019). Therefore, comparisons among studies should be considered with caution. Future studies that examine the origin of the equine amniotic microbiome could minimise these variables by comparing amniotic fluid and the dam's distal GIT from samples collected at the same time point. Furthermore, using such an approach throughout a mare's pregnancy would be beneficial for determining whether changes in the GIT microbiome are reflected in the amniotic or meconium microbiota at birth.

Reproductive tract microbiota

Bacteria in the equine uterus have previously been associated with inflammation and endometritis. It is now thought that the mare may establish and support a normal moderately diverse uterine microbiome sourced from vaginal microbiota during puberty and mating (Heil *et al*. 2018; Pugh 2018). The composition of the normal uterine microbiome appears similar to populations found on the external cervical os, which can be explained by the open cervix, and communication between the uterine lumen and cranial vagina during oestrus. Heil *et al*. (2018) reported on the beta diversity, and although over 5400 OTUs were detected, the taxonomical identification of these sequences remains unclear. It has been documented that the normal vaginal microbiota of the horse consist of the genera *Lactobacillus*, *Enterococcus*, *Coryneobacterium* and *Fusobacterium*, as well as the family Clostridiaceae, suggesting that these may also form part of the normal uterine microbiota (Table 1) (Fraga *et al*. 2008; Hoyles *et al*. 2013; Pugh 2018).

In contrast, the pathogenic bacteria causing endometritis in horses have been well defined. Microorganisms that have been associated with this disease include *Streptococcus zooepidemicus*, α-haemolytic *Streptococcus* spp., *Pseudomonas aeruginosa*, *Pseudomonas* spp., *Corynebacterium* spp., *Staphylococcus* spp., *Escherichia coli*, *Klebsiella* spp., and some yeasts and fungi (Diel de Amorim *et al*. 2016). Interestingly, some of these bacteria that have previously been defined as pathogens causing endometritis, have now been detected in the amniotic fluid from healthy pregnancies of mares, including *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp. and *E. coli* (Quercia *et al*. 2019). Such findings suggest that these microorganisms may, instead, be opportunistic pathogens that can cause pathology under altered conditions, but are usually part of the normal uterine microbiome. The fact that under some conditions they do cause inflammation and pathology suggests that it may be the stability of the microbial community that contributes to a healthy pregnancy, whereas disruption of the resident microbiota allows for organisms to colonise and proliferate rapidly, causing dysbiosis (Pugh 2018). This suggests that an imbalance in the microbial community is the cause of complications, rather than is the presence of individual microorganisms.

Supporting this idea, *Clostridium* spp. has been observed in the upper and lower reproductive tract of the mare, and a reduction in its population appears to result in the overgrowth of other organisms such as *Fusobacterium* spp. It has been suggested that some organisms belonging to the class Clostridia exert a protective effect in the uterus during pregnancy, although this has not been explored in detail (Pugh 2018). Since the resident uterine microbiota in the mare are not well defined, no clear comparisons of their role in establishing the amniotic microbiota can be made at this time. It appears that a stable community can help maintain or support a healthy pregnancy, but it is unclear whether these microbes can infiltrate a healthy pregnancy and contribute to the amniotic microbiota.

Conclusions

The long-standing paradigm that a healthy pregnancy is dependent on a sterile placental environment is currently vigorously debated. Few anatomical sites can be described as naturally sterile; therefore, the observation that the placenta harbours a unique microbiota is not surprising, especially since it is the first organ to nourish and maintain the fetus. The presence of microbes in the healthy placenta, umbilical cord and meconium of humans has sparked interest that fetal microbial contact *in utero* is a normal phenomenon occurring across all mammalian species, including the horse.

Bacteria have now been detected in the amniotic fluid and meconium from healthy equine pregnancies and it is our contention that these bacteria commence colonisation of the GIT *in utero*. This, in turn, will have an impact on the fetal immune system, preparing the foal for the environment outside the uterus. However, this is a new area of research and it is not well defined what bacteria are present in the normal amniotic microbiome of the horse and what role they play during pregnancy and fetal development.

The equine placenta and fetal fluids have vital roles in growth and development of the fetus. Further, the amniotic fluid that surrounds the fetus is the first fluid to enter the fetal GIT and significantly contributes to the development and accumulation of meconium. Meconium in the foal documents the changes occurring within the amniotic fluid throughout pregnancy, and since amniotic fluid can be used as an indicator of fetal health, the meconium is also regarded as useful for evaluating the neonatal foal. It is currently unknown whether microbial changes occurring throughout gestation can also be detected in the meconium of the foal.

It is not known when and how bacteria enter the amniotic fluid during pregnancy, but the species detected show similarities to maternal microbiomes, suggesting that the amniotic microbiome is established from internal sites within the mare. Determining what bacteria compose the amniotic microbiome, when they enter the fetal compartments and colonise the amniotic fluid and their potential origins will provide crucial information as to how they influence the development of the foal from fetal to neonatal life. The significance of intrauterine microbes for foal GIT colonisation remains to be elucidated but could be key in improving neonatal health and development.

Conflicts of interest

The authors declare no conflicts of interest. WLB and GB are members of the Editorial Board of *Animal Production Science* but had no role in the peer review and acceptance of this paper.

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