Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review

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Abstract

There is increasing evidence of a pivotal role of the gut microbiota (GUT-M) in key physiological functions in vertebrates. Many studies discuss functional implications of the GUT-M not only on immunity, growth, metabolism, but also on brain development and behavior. However, while the influence of the microbiota-gut-brain axis (MGBA) on behavior is documented in rodents and humans, data on farm animals are scarce. This review will first report the well-known influence of the MGBA on behavior in rodent and human and then describe its influence on emotion, memory, social and feeding behaviors in farm animals. This corpus of experiments suggests that a better understanding of the effects of the MGBA on behavior could have large implications in various fields of animal production. Specifically, animal welfare and health could be improved by selection, nutrition and management processes that take into account the role of the GUT-M in behavior.

Key words: Microbiota, microbiota-gut-brain axis, behavior, welfare, emotion, livestock

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Introduction

The gut microbiota (GUT-M) has received increased interest for several years because it is involved in many functions in humans and animals. The GUT-M is composed of bacteria, archaea, viruses and eukaryotes (including protozoa and fungi). The GUT-M has been demonstrated to influence immune function for years and to have wide impacts on health. Moreover, impairments of gut health can lead to many intestinal diseases and to dysbiosis, an unbalance in GUT-M, which facilitates many pathological states involving infections with pathogens or metabolic disorders [1-4]. The GUT-M has also a pivotal role in many extra-intestinal tissues and in various developmental processes and metabolism in host organs such as the liver, adipose tissue, bone, *etc* [5]. The brain is also a major target of the GUT-M because the microbiota produces metabolites and neurochemicals. At the same time, neurotransmitters like epinephrine and norepinephrine from the host influence the growth and virulence of bacteria [6]. The relationship between the GUT-M and the brain, so called microbiota-gut-brain axis (MGBA) includes influences upon brain development, neural processes (such as myelination or neurogenesis), pain processes, the hypothalamo-pituitary axis (HPA) and behavior [7]. The MGBA is also called microbiome-gut-brain axis by some authors, since the microbiome consists of not only the microbiota, but also microbiota genomes and products [8]. Although some methods used to investigate the MGBA have been recently criticized [9], there are more and more studies describing the influence of the GUT-M on the central nervous system (CNS) and the mechanisms involved in this interaction. The influence of the GUT-M on behavior is increasingly reported in rodents using germ-free animals (living in the absence of detectable living microorganisms) or in rodents and humans following the use of special diets affecting GUT-M composition, or microbiota transfer [10-16] using antibiotics or probiotics (live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host (see [17] for definitions). These studies demonstrate that there is increasing evidence that changes in the GUT-M

affect physiological and behavioral processes that are directly relevant to welfare such as stress, anxiety, changes in social behavior and memory. Whilst demonstrations of the influence of the GUT-M on behavior in farm animals remain scarce, manipulation of the microbiota in farm animals by supplying probiotics is common to improve production. Therefore, a critical examination of the influence of the GUT-M on behavior would be especially interesting from an animal welfare perspective.

This review aims at summarizing the influence of the MGBA on behavior in rodents and humans and to point out what has been observed in farm animals. Moreover, because the GUT-M varies according to host genetics and many external factors (Figure 1), we suggest that the GUT-M could be used to improve behavior and welfare on the farm.

I – The gut microbiota and its impact on brain development and behavior in rodent and human models

There is increasing evidence that the microbiota can influence host behavior. Most of the investigations on behavior have focused on the GUT-M in vertebrates where various routes of interaction between the GUT-M and the brain have been identified, including the immune and the enteroendocrine pathways, the enteric nervous system and the vagus nerve (Figure 2). Products of bacterial metabolism and structural components of bacterial cell walls influence a wide range of processes including host immune responses (e.g. cytokines) and activation of enteroendocrine cells which can affect the nervous system locally and systemically. The enteric nervous system is a major interface between the GUT-M and the host and it forms the most complex of intrinsic nerve circuits outside the CNS [18]. Through its neuronal networks and numerous neurotransmitters, it mirrors many aspects of the CNS and intimately interfaces with it *via* the autonomic nervous system. Although seldom recognized, the number of neurons in the enteric nervous system is comparable to

the number of neurons in the spinal cord, leading some authorities to refer to the enteric nervous system as the “second brain” or the “little brain”[18]. Moreover, research has demonstrated that 80 percent of the vagus nerve fibres carry information from the gut to the brain, rather than the other way round [19]. Thus, the vagus nerve is a major pathway of the MGBA as demonstrated by surgical sections that abolish the effect of the GUT-M on the brain and on behavior in mice [20-23]. Conversely, the brain modulates the physiology of the gut, the enteric immune system and the composition of the GUT-M. This influence can impair gut activity especially during host stress [24-26]. The GUT-M can additionally influence the behavior of host’s conspecifics through sensory cues even if they are not considered usually as constitutive of the MGBA [27]. These cues are mainly olfactory [28] but the GUT-M could even be related with visual cues in some cases: in pigeons for example [29], GUT-M composition is related to feather microbiota composition and the bacterial load on the plumage has been shown to influence the iridescent color of the feathers which is a fitness cue for the congeners.

1- Effects on anxiety-like behavior and stress responses

The question of the role of the GUT-M in anxiety-like behavior was raised following the pioneering study of Sudo et al. [30] which showed hyperactivity of the HPA axis under stress conditions in germ-free mice (without any microbiota) compared to specific pathogen-free mice. Other teams have subsequently confirmed the influence of the GUT-M on the development and regulation of the stress response system [13,14,16,31]. In addition, patients with gastro intestinal disorders such as irritable bowel syndrome (IBS) also have a deregulation of HPA axis activity [32,33]. Consequently, a link between the MGBA and anxiety-like behavior is not surprising and a significant modification of anxiety-like behavior has been observed in germ-free rodents compared with specific pathogen-free rodents in various tests [34-39]. These studies reveal the importance of the genetic background in the influence of the GUT-M on behavior. Indeed, the absence of GUT-M leads to increased anxiety-

like behavior in rodent strains genetically prone to exacerbate emotionality (F344 rats and BALB/c mice) [11,12] and provoked a reduction of anxiety-like behavior in moderately emotive strains (NMRI and Swiss mice) [37,38]. The germ-free rodent studies represent a large part of the literature on the MGBA concept. Nevertheless, the germ-free animal presents several important physiological alterations compared to a colonized one such as a reduction of the growth, alterations of the digestive functions or immune system impairments ([40] for review), thus it is not easy to demonstrate that the behavioral modifications observed in these animals are a direct consequence of the absence of GUT-M rather than of physiological changes. However, some authors have tried to reinforce the role of the presence of GUT-M in their studies by re-introducing standard microbiota into these germ-free animals and have observed a reversal of behavioral responses following bacterial colonization [37,39]. When it is not completely abolished, the GUT-M can be modified by the use of antibiotics. BALB/c mice treated with a mixture of nonabsorbable antimicrobials (bacitracin, neomycin and pimaricin) for seven days showed reduced anxiety-like behavior compared to controls in a light-dark box test [20]. Similarly, the low doses of penicillin in late pregnancy and early postnatal life induced long-term changes of microbiota composition and behavior. The antibiotic-treated mice exhibited impaired anxiety-like and social behaviors, and displayed a higher level of aggression in several tests, while concurrent *Lactobacillus rhamnosus* JB-1 probiotic supplementation prevented some of those alterations [41]. However, these results must be interpreted cautiously because antibiotic treatments are known to have neuroactive and neurotoxic potential. Regardless or in addition to their microbicidal effects, the antibiotics themselves may also influence enteric, peripheral and central nervous system functions [10].

Probiotics are live naturally occurring microorganisms which can improve health directly or indirectly by inhibiting growth and attachment of pathogens and favor the development of the intestinal epithelium and the immune responses. A probiotic can be used alone or in combination with other probiotics, a cocktail of microorganisms that may have different or common properties [42]. The exact mechanisms through which probiotics provide benefits are being studied and may differ

depending on the specific formulation. These mechanisms include modifications of the pH of the gastrointestinal tract, the provision of nutrients to the host, the production of antimicrobial or signaling molecules, competition with pathogens for ecological niches and available nutrients, promotion of the intestinal cell differentiation and turnover, increased mucus production and maturation of the immune system. Many studies in the literature suggest an anxiolytic effect of some probiotics. Mice treated with the probiotic *Lactobacillus rhamnosus* expressed reduced anxiety compared to control mice during the elevated plus maze [33] and a chronic administration of *Lactobacillus plantarum* leads to lower anxiety-like behavior in the open-field and elevated plus maze tests [43]. More demonstrative yet, Bercik et al. [44] showed that a daily gavage with the probiotic *Bifidobacterium longum* can normalize anxiety-like behavior in mice with infectious colitis in the step-down test and a supplementation with the probiotic *Lactobacillus helveticus* has led to a reduction of chronic stress-induced anxiety and depression in rats [45]. Messaoudi et al. [46] investigated the effect of a mixture of two probiotics (*Lactobacillus helveticus* and *Bifidobacterium longum*) on rodents and human volunteers. In both cases, a decrease in anxiety was revealed. Infection with pathogenic bacteria is another way to modify the composition of the GUT-M, which often leads to increased anxiety-like behavior in rodents. An infection of mice with *Campylobacter jejuni* or *Citrobacter rodentium* exacerbated anxiety-like behavior compared to control mice in different situations such as the elevated plus maze or the hole-board open field test [19,47,48]. Furthermore, the anxiogenic effects of these infections were not the result of an immunological response but appeared to be a direct action of bacteria on neural activation pathways [19,47]. However, one of the most striking experiment on the influence of the GUT-M on anxiety-like behavior is the study of Bercik et al. [20] who carried out a GUT-M transfer between a low (NIH Swiss) and a high (BALB/C) anxiety-like mouse strains presenting different microbial profiles based on denaturing gradient gel electrophoresis (DGGE). The germ-free BALB/c mice that received the GUT-M from the opposite mouse strain were less anxious than the controls BALB/c mice during the step-down test. In contrast, germ-free NIH Swiss mice responded more anxiously than controls during the same test. Therefore,

this experiment suggests that the GUT-M would be involved in the anxiety-like phenotype of these mice. Taken together, these findings suggest a significant influence of the MGBA on anxiety-like behavior.

2- Effects on memory

It is now increasingly recognized that the GUT-M communicates with the brain and acts on several brain structures such as the amygdala, the cortex and the hippocampus that all have a key role in memory processes [12,37,40]. Moreover, the relationship between anxiety and memory and learning has been widely demonstrated, suggesting an effect of the GUT-M on cognitive abilities [30,49,50]. This idea is supported by results obtained when comparing germ-free mice and specific pathogen-free mice in the novel object test and the T-maze test [15]. In both tests, the germ-free mice displayed memory deficits. Consistent with these findings, treatment with an antibiotic formulation resulted in a cecal composition shift with reduction of Firmicutes and Bacteroidetes and increase of Proteobacteria and Cyanobacteria and a decrease in memory capacities in mice subjected to novel object recognition test and social transmission of food preference test [51]. The influence of an antibiotic treatment on memory may nevertheless depend on the number of antibiotic products used and the sensitivity of the bacteria to this antibiotic. For example, in the Morris water maze the vancomycin antibiotic had no significant effect on murine memory despite a significant alteration of fecal microbiota [2]. The gut microbiota may also have different effects depending on the type of memory assessed. In a recent study, a treatment with an antibiotic mixture strongly disrupted microbial composition of mice and impaired novel object recognition but not spatial memory in the Barnes maze test [52]. Studies on probiotics supplementation agree that there are beneficial effects on memory performance in rodents [33,45,53-55]. Works conducted on pathogenic infections (with *E. coli* or *C. rodentium*) reported deleterious effects on memory in the mouse [15,56] and, in both cases, a treatment with probiotics attenuated these memory impairments. However, it is important

to emphasize that only the study of Smith et al. [54] performed a GUT-M composition analysis following probiotic administration and reported significant changes in the fecal microbiota of the mice. In humans also, improvement of emotional memory after probiotic administration has been associated with changes in GUT-M community composition [57].

An alternative strategy for modifying the microbiota is to use dietary prebiotics. Prebiotics are fermentable oligosaccharides or polysaccharides that induce the growth of some gut bacteria that increase gut health. Unabsorbed or undigested carbohydrates are fermented by the gut microbiota in the large bowel, producing different end products like short-chain fatty acids (SCFAs) and lactic acid, which may have multiple effects. For example, it has been described that oral administration of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) affects behavior and specifically anxiety, depression-like behavior, cognition, and social behavior. These modifications are related to specific gene expression in the hippocampus and hypothalamus, gut microbiota composition, several SCFAs produced, and elevations in corticosterone and pro-inflammatory cytokine levels [6].

Modifications of the GUT-M through changes in raw materials of the diet appear also to influence cognitive abilities. An enrichment of beef in the diet of mice increases the microbial diversity in the colon and their memory scores in the hole-board apparatus [58]. A diet characterized by a high-fat composition also leads to differences in GUT-M composition and to memory impairments in the mouse and the rat [29,59,60].

However, studies are still needed to strengthen a causal relationship between GUT-M changes and memory abilities in these nutrition experiments.

3- Effects on social behavior

The MGBA seems to be also involved in other highly emotional behaviors such as social behavior. This behavior is impaired in germ-free rats in a test which consists of measuring behavior during an encounter with an unknown partner [36]. During the 2 minutes of the test, compared to specific

pathogen-free rats, the germ-free rats spend less time sniffing an unknown. These results are consistent with what Desbonnet et al. [24] found in a mouse model tested in the 3-chambered sociability test. The germ-free mice displayed social preference deficits by spending less time exploring a chamber containing a mouse than an empty chamber. In addition, when the germ-free mice are post-weaning colonized, their behavioral responses are reversed in the same test. However, this result could not be replicated in a subsequent study using the same mouse strain and the same 3-chambered test in which the authors observed opposite results [35]. Indeed, germ-free mice expressed greater social preference than specific pathogen-free mice. The authors assumed that the difference in the age of the germ-free mice between the two studies could be the explanation for the contradictory findings. They also mentioned the hyperactive behavioral responses of the mice in the Arentsen *et al.* work [35] and the differences in living conditions of the specific pathogen-free mice (isolators rearing in a study and not in the other). More recently, social behavior impairments and dysbiosis in the gut have also been reported in mouse offspring from mothers fed with a high-fat diet [61]. Interestingly, a probiotic (*Lactobacillus reuteri*) supplementation in the drinking water during 4 weeks led to the normalization of social behavior and this reversal of the social deficits involved the vagal pathway. In conclusion, all these data indicate that the GUT-M is required for a normal expression of social behavior in rodents. Moreover, differences of GUT-M composition have been revealed between autistic and control patients in an expanding volume of studies [42,62-64]. Similarly, altered GUT-M composition and social deficits have also been noted in a murine model of ASD [65,66]. These mice are characterized by disturbed anxiety-like and stereotyped behavior similar to those observed with germ-free mice [38]. An administration of probiotic *Bacteroides fragilis* has improved many of these behaviors including anxiety-like behavior (open-field exploration), communication deficits (ultrasonic vocalizations) and stereotyped behavior [65]. More interestingly, Sandler et al. [67] tested the effect of an antibiotic on 11 children with regressive-onset autism. Significant behavioral improvements were noticed during the treatment period and the behavioral improvements disappeared after the treatment. It has also been recently demonstrated that

Lactobacillus reuteri rescues social deficits in various mouse models for ASD based on genetic, environmental and idiopathic alterations [68].

4- Influence on feeding behavior

Fetissov [69] suggested that the bacteria-host communication influences the appetite-satiety balance in humans and rodents. First, bacterial components and metabolites have been shown to stimulate satiety pathways in the host in the short term through the stimulation of endocrine cells involved and the production of peptides related to feed intake [70,71]. Secondly, bacterial peptides use systemic routes and might act directly in the hypothalamus and so play a role in the long-term regulation of appetite. Moreover, the GUT-M appears to be involved in the expression of taste receptors in rodents [72,73].

It is now recognized that the MGBA is involved in many behavioral responses in humans and rodents and interventions with probiotics reinforce the theory of the influence of the GUT-M on behavior and the cognitive abilities. However, it is also important to note that the causal mechanisms by which the GUT-M and the brain communicate are not well described or understood and further investigations are needed to shed light on this microbiota-gut-brain axis communication.

II- The gut microbiota of farm animals

There is an increasing knowledge about the composition of GUT-M of farm animals (ruminants, horse, pig, rabbit, chicken, turkey, *etc*). Indeed, it is very important to characterize the GUT-M in farm animals so that it is possible to detect normal and abnormal changes. This knowledge should help to define and identify dysbiosis and to restore a healthy GUT-M. It should also help to predict susceptibility to infection and prevent welfare and health problems since GUT-M composition is

involved in the control of pathogen colonization [74,75]. However, understanding GUT-M composition is a complex issue since it varies along the digestive tract and there are also differences between lumen and mucosa, and even between the tip of the villi and the crypt. Moreover, GUT-M variations are induced by many factors related to the host and to the host environment.

1- Investigation of the *GUT-M in farm animals*

While the GUT-M is composed of bacteria, but also viruses, archea and eukaryotes and while bacteriophages have been shown to have an important role in bacteria composition, most studies only take into account the bacterial composition of the GUT-M. This is in line with methods available to measure this composition since there are more libraries of bacteria available for 16S rRNA gene sequencing than for viruses, archea and eukaryotes. Several methods are used to characterize the the GUT-M. The 16S rRNA gene sequencing directed by PCR, is commonly used to quantify GUT-M diversity and is effective in demonstrating the major phyla, families or genuses, but sometimes gives limited resolution. The table provides the characteristics of GUT-M bacteria in the main farm species (cow, sheep, horse, pig, rabbit, chicken, quail, duck) established by 16S rRNA gene sequencing. This table gives the composition at phylum level and sheds light on the large variation found within host species. Though not the main focus of this review, it is clear that accurate descriptions of the composition (at the genus or the species level) of the bacteria in different parts of the digestive tract greatly help us to understand the effects of host and external factors of modulation on the GUT-M ([71] in cow for example). Quantitative metagenomic shotgun sequencing also aims at investigating diversity directly from samples but can be technically challenging and is less frequently used. Other approaches look for GUT-M functionality by metatranscriptomics (RNA sequencing), metaproteomics (Mass spectrometry) or metabolomics (High resolution spectroscopy).

Each gut compartment hosts a microbiota with a particular composition and many studies investigated GUT-M composition along the digestive tract ([76] in pigs; [77] in horses; [78] in quail).

In horses for example, the composition of the GUT-M collected in the lumen is very different in caecum and colon compared to the upper compartments (stomach, jejunum and ileum) and is different from the GUT-M from mucosa [77]. In this example, data suggest that analysis from feces would be related to colonic segments only, but would not be related to upper compartments. Numerous studies use fecal samples to avoid animal sacrifice, which could be misleading.

Gut microbiota of the small intestine, caecum and colon in healthy adults is dominated by bacterial species belonging to two main phyla, Gram positive Firmicutes and Gram negative Bacteroidetes (Table). The small intestine is usually dominated by Firmicutes with major families including *Lactobacillaceae*, *Peptostreptococcaceae* or *Enterococcaceae*. Microbial complexity considerably increases in distal parts of intestinal tract, i.e. in the caecum and colon. It is important to remember, however, that the descriptions of the gut microbiota leave out many important factors such as host genetics, age or feed regime (see below) that may give rise to much greater variation. These factors may affect microbiota development and composition in the youngest animals and the differential development in early days of life.

2- *Variations in the GUT-M linked to the host*

The host genetics affects the GUT-M in numerous ways and this impact is related to inter and intra species differences in the GUT-M [79]. Domestication has also induced changes in GUT-M composition. For example, a metagenomic approach followed by a quantitative PCR showed that the GUT-M in wild Suidae (wild boars and Red river hogs) was characterized by a high abundance in *Bifidobacterium* which was not the case in domesticated Suidae characterized by abundance in *Lactobacillus* and *Enterobacteriaceae* as the major family [80]. It is important to note that diet was not controlled and thus confounded with genetics in this study. However, it has been demonstrated in domesticated pigs from the Pietrain strain that pig genome influences the GUT-M in the mid-colon and that the heritability of the load of some bacteria can even reach high values such as 0.32 to 0.57

[81]. Differences in the GUT-M related to host genetics have also been established between lines of the same species. With chicken lines selected on body weight, Zhao et al. [82] demonstrated that the host genotype and gender affected 68 out of 190 GUT-M species and that among them 15 belonged to *Lactobacillus*. Genetic selection on *Salmonella* carriage in chickens enabled the detection of Quantitative Trait Locis (QTLs) for both resistance to carrier state and resistance to *Salmonella* colonization [83,84]. Some bacterial families can be affected particularly by host genotype: in Pekin and Muscovy ducks for example, genotype affects *Lachnospiracecae*, *Bacteroidaceae* and *Desulfovibrionaceae* in the cecum, while overfeeding affects other families such as *Clostridiaceae*, *Lactobacillaceae*, *Streptococcaceae* and *Enterococcaceae [85]*. A divergent genetic selection on increased digestive efficiency in chickens was linked to changes in the GUT-M and has enabled the detection of QTLs related to the presence of some GUT-M bacteria [86]. In chickens, QTL for the presence of bacteria such as *Lactobacillus* and *L. crispatus* co-localize with QTLs for feeding behavior [87]. Host genetics would then influence both the behavioral phenotype and GUT-M composition. It is highly probable that behavior and the GUT-M influence each other as it has been demonstrated in stress processes where the brain influences gut peristaltis and GUT-M composition while the GUT-M interacts with CNS and the HPA axis [13].

The age of the host is also a major factor and the ontogeny of the GUT-M has been studied in many farm animals. The changes during early life have been described in several farm animals (chick: [88]; calf: [89,90]; piglet: [91]; foal: [92]). Microbial colonization is a complex process influenced by the host and many external factors, including maternal microbiota, birth process, early diet, perinatal stress and antibiotics use.

3- Variations of the GUT-M linked to the environment

The environment dramatically influences the newborn's GUT-M. In mammals, the contact of the newborn animal with its mother is physiologically indispensable and during parturition, the offspring is naturally inoculated with microbiota from the mother. However, in case of avian farm species, the young birds are industrially hatched, which means that eggs are disinfected and chicks reared without any contact with their mother or any older conspecific and the source of microbiota is thus limited to the environment. This way of husbandry is in sharp conflict with the natural conditions, where the mother bird represents the principal source of the GUT-M. Experimentally, young chicks reared in a sanitized environment with no contact with older conspecifics had profoundly different microbiota compared with chicks which were kept for 24 hours with the adult hen [93].

Other external factors such as infections can give rise to unbalance in the GUT-M. For example, early exposure to pathogenic bacteria can shape the overall microbiota composition in chicks infected with *Salmonella* Enteritidis inducing an expansion in the *Enterobacteriacecae* [94] and exposures to *Campylobacter jejuni* revealed that the shift of the GUT-M varies upon the age at which the chickens become colonized by this bacteria [95]. Parasitism can also influence GUT-M composition and the interplay between helminths and the bacterial populations is being elucidated. The various ways both populations influence each other are complex [96] and suggest that a better knowledge of the gut microbiota of nematodes themselves could lead to a better prevention of parasitic diseases [97].

Throughout life, housing conditions influence cecal microbiota in rabbits [98] and pigs [99] showing that environmental bacterial load influence the GUT-M. Breeding in different rearing systems can also influence GUT-M composition at the phylum level. For example, Bacteroidetes and Proteobacteria were more prevalent in chickens reared under free-range conditions than in cages, but this difference was manifested only in one of both lines [100]. Stocking density can influence crop and cecal microbiota composition in chickens [101]. Rearing conditions inducing stress can also influence the GUT-M. In horses for example, weaning and transport are stressful events and both can affect the GUT-M composition [102,103]. In Mach's experiment, foals' microbiota was modified during the first week after weaning until a relatively stable gut community was established at day 7

post-weaning. This modification can be partly explained by the nutritional change, however GUT-M composition after weaning was slightly modulated by the weaning method suggesting that the stress induced by the abrupt method has impacted the microbiota modification. An experiment in pigs has shown that even mild handling stressor such as single daily weighing is able to alter the GUT-M [104].

Another very important external modulation of the GUT-M is given by the feed which may drastically influence GUT-M composition and activity. Such influences are being increasingly studied since diets, or the water bacterial load, may induce unbalance in the GUT-M and lead to pathological states. Such unbalance can lead to dysbiosis and then enteritis, or to other diseases targeting some other organs such as lungs, since unbalance gives rise to inflammation of the gut wall and facilitate bacteria leakage across the epithelial wall. This modulation by the diet has mainly been investigated in farm animals and reviewed in many animal species [105] for review in horses; [106] in chicken; [107] in piglets, [85,108] in ducks, *etc*). Most of these studies compare diets based on high fiber with diets containing raw materials providing high energy levels. Other nutritional means used to modify the GUT-M are the provision of prebiotics or probiotics. Prebiotics are fermentable oligosaccharides or polysaccharides that induce the growth of some gut bacteria that increase gut health while, as previously mentioned, probiotics are microorganisms which improve animal health directly or indirectly by producing substrates that stimulate growth of commensals, inhibit growth of pathogens, favor the development of the intestinal epithelium and the immune responses. Probiotics are largely used in animal nutrition to improve gut health, increase feed efficiency and milk quality [42,109] and it has been demonstrated in piglets that they can influence serotonin and dopamine concentrations in the hypothalamus [110]. They are also use to prevent the effects of stressful events such as transportation in horses for example [111] but this improvement is not always related to a change in the GUT-M as mentioned by a meta-analysis carried out in calves [109]. Lactic acid bacteria are commonly used as probiotics, and their impact on gut health, immunity and the prevention of the establishment of pathogenic bacteria has been increasingly studied.

Farm animal GUT-M can thus vary with a wide range of factors each of which have many different consequences but the results on behavior are weakly documented and rarely taken into account. Furthermore, only few studies have used GUT-M manipulations to disentangle effects of nutritional or environmental factors and GUT-M effects.

III- Effect of the microbiota-gut-brain axis on behavior in farm animals

There is emerging evidence that the GUT-M is able to influence behavior in farm animals as has been shown in rodents and humans. Colonization of farm animals with a pathogen was known to induce sickness behavior for a long time, but recent studies demonstrate that the influence of the MGBA is not limited to the area of disease and can also occur in healthy animals. Studies based on germ-free animals, provisions of probiotics or prebiotics, diet modifications, demonstrated that changes in the GUT-M are related with changes in many behavioral patterns. Because of the size of farm animals, this influence of the MGBA has been established mainly with studies using probiotics while very few studies on germ-free animals are available since these animals must be kept in isolators.

1- Effects on emotional reactivity and anxiety-like behavior

A recent experiment with germ-free birds demonstrated that the absence of GUT-M reduces emotional reactivity in Japanese quail in fear and social perturbation situations without major influence on growth [112]. The authors used germ-free quail chicks that were kept germ-free or inoculated with a dilution of GUT-M from adults of the same line. Quail chicks were reared and tested in isolators in order to avoid contamination. Germ-free quails spent less time in tonic immobility, were less reactive during the social separation test and were less neophobic in a novel object test than inoculated quail chicks. The use of a GUT-M transfer has also demonstrated the

influence of microbiota on emotional reactivity in this species [113]. The authors used genetic lines of quails that have been selected for either a high fearfulness (E+) or a low fearfulness (E-). Germ-free quail chicks from the E+ line were inoculated with feces from either a E+ quail or from a E- quail and were reared in different isolators. Quails that received feces from the E- line expressed a lower emotional reactivity during the second week of age than the quails colonized by feces from the E+ line. This result was reversed two weeks later. These behavioral differences can be related to GUT-M differences and modifications over time and they could be the consequence of the resilience of the GUT-M to recover its equilibrium present in the E+ host, which is in part driven by the host genotype. Abdel-Azeem et al. [114] showed that the administration of the probiotic *Bacillus amyloliquefaciens* helped to reduce distress calls in turkeys and the supplementation of the diet with a probiotic (*Pediococcus acidilactici*) reduced emotional reactivity in quails [115].

In horses, the relationship between the GUT-M and behavior has been suggested by correlations obtained in fistulated horses submitted to behavioral tests before and after a nutritional change [116]. The modification of the diet from a fibrous diet with 100% hay to a diet with increased energy (57% hay and 43% barley) induced significant increases of colonic total anaerobic bacteria, lactate-utilizing bacteria and amylolytic bacteria concentrations. After this transition, the horses were submitted to a sociability test where behavior was analyzed when an unfamiliar horse was introduced into the adjacent stall and to a neophobia test assessed from the reaction to the presence of a novel object placed near a feeder in a test arena. The time spent in vigilance during the sociability test tended to positively correlate with cecal and colonic amylolytic bacteria concentrations while the time spent in vigilance during novel object test was correlated with caecal lactate-utilizing and colonic amylolytic bacteria concentrations.

2- Effects on memory

As in rodents, probiotics have been shown to enhance memory in quail: supplemented birds made fewer errors in a test where they had to remember the cup they had previously visited among eight rewarded cups [115]. In Yucatan pigs, differences in the maternal diet during gestation and lactation have been used to modify microbiota activity in the sows and their offspring [117]. Sows were either fed a standard diet or a Western diet enriched in energy, sugar and fat. SCFAs used to measure microbiota activity were decreased in sows fed the Western diet and in their piglets. Piglets from sows fed the Western diet, i.e with reduced GUT-M activity, had higher working memory in a hole board test where they had to learn where were the bowls that contained chocolate-coated peanuts among unrewarded bowls.

3- Effects on social behavior

Using probiotics, it has been shown that spores of *Bacillus amyloquefaciens* decrease aggression in turkeys [114]. However, the most promising information was obtained for feather pecking behavior in hens. Gentle feather pecking is considered as a normal social exploratory behavior and consists in a soft pecking while severe feather pecking is an intense pecking and pulling out feathers which can induce pain in the victim. This injurious behavior considered as an abnormal behavior have been recently supposed to be associated with the MGBA. Indeed, it has been shown that divergently selected lines of hens for severe feather pecking also differ in hens' GUT-M [118] and in immunity [119]. Nevertheless, it is still not possible to decide conclusively whether differences in feather pecking induced difference in the GUT-M or whether differences in the GUT-M induced difference in behavior via the MGBA [120]. The latter explanation agrees with data about GUT-M metabolites such as total SCFAs and biogenic amines since both were also different between these lines [121] and SCFAs have been shown to be involved in the MGBA and influence social behavior. Differences in the gene expression of two genes (ABCB1 and TNSF15) involved in inflammatory bowel disease (IBD) are also been reported between birds expressing feather pecking or not [122]. Moreover, the serotonin whose

synthesis depends on various bacterial families in the GUT-M [49,50,123,124] is also involved in feather pecking behavior in hens [120]. Ingestion of feathers could lead to an increase of gut wall stimulation and therefore an impaired serotonin signalling [125]. These data would then be in agreement with an influence of GUT-M activity on the development of feather pecking through the MGBA. Brunberg et al. [125] proposed to investigate if the differences in GUT-M composition are already present in the young chick before the development of feather pecking behavior in order to characterize the main direction of the microbiota-gut-brain interactions in this model.

4- Effects on feeding behavior

Gut pathogens may induce illnesses states that are commonly accompanied by reduction in feed intake but some other influences of the GUT-M on feeding behavior can be found in farm animals. In turkeys, spores of *Bacillus amyloquefaciens* have been shown to increase feeding frequency and duration [114]. The genetic lines of chickens divergently selected on feed efficiency we previously mentioned differ in feeding behavior and a QTL for feeding behavior co-localizes with QTLs for some bacteria from the GUT-M [87]. This co-localization suggests an influence of these bacteria on eating behavior but this influence still need to be strengthened by experiments using GUT-M manipulation.

Changes in feeding behavior induced by the MGBA are suspected in ruminants when they are affected by acidosis which occurs with high-energy low-fiber diets. Eating behavior can be modified with rumen liquor transplantation when cows are affected by acidosis [126] and even if pain alleviation or inflammation reduction can also explain the effect on eating behavior, this veterinary practice suggests that rumen microbiota influences appetite in such pathological state. In cows affected by subacute acidosis, ruminal GUT-M is modified [127] and feeding behavior is affected with a reduced feed intake and a reduced duration of rumination. *Saccharomyces cerevisiae*, a probiotic commonly used in ruminants, has a protective effect on physiological changes induced by acidosis such as reduction of the ruminal pH, changes in volatile fatty acids [42,128] and it has been shown to

induce also behavioral changes such as reduction of the minimum interval between meals and tendency for longer time spent ruminating [129].

This limited information about the influence of the MGBA on behavior in farm animals suggests that it can have large influences that have not been properly appreciated. These influences of the GUT-M on behavior can be added to its influence on health *via* its role in the immune response and tends to put the GUT-M as a pivotal actor for welfare state achievement [130].

IV- Prospective of the microbiota-gut-brain axis concept in the welfare of farm animals

The concept of the MGBA leads us to reconsider many factors that can influence behavior and health in farm animals. The influence of the MGBA will have to be taken into account in future and that may drastically change genetic selection, infection detection, nutrition and management processes. Furthermore, the improvement of gastrointestinal functionality is of the utmost importance because it positively influences health and welfare of animals, but also performance by preventing loss in feed efficiency and the use of antibiotics.

1 Selecting the host GUT-M

Even if a recent article demonstrated that the human GUT-M is shaped more by environmental factors than by human genome [131], we should not underestimate the influence of the host genetics on the colonization of the gut by the microbiota. Several studies have demonstrated that the host genome influences the composition of the GUT-M. For example, a study from twins has identified many microbial taxa whose abundances were influenced by host genetics [132] and associations between host single nucleotide polymorphisms and bacterial taxa have been described [133]. The host gut is able to select the microbiota it encounters and only part of the bacteria present

in the gut are able to develop in it. This explains why different genetic lines reared in similar conditions and fed the same diets have different GUT-M compositions. Selection for different genotypes could then lead to differences in GUT-M and consequently in behavior, immunity and feed efficiency [134]. As previously mentioned, selection for increased feed efficiency has led to differences in GUT-M in chickens and several QTLs are related to these differences in GUT-M composition and co-localize with loci involved in feeding behavior [87]. Moreover, these lines divergently selected for feed efficiency also differ in emotional reactivity. It appears then that these differences in behavior may have been driven by the effect of selection on the host genes involved in behavior, but also on the genes involved in GUT-M carriage.

A better understanding of the relationship between the host genome, the GUT-M and deleterious behaviors would be of great interest for animal welfare. A comprehensive link between the GUT-M and feather pecking could lead to alternative strategies for selection against this damaging behavior. As previously indicated, many rearing situations can induce stress and are related with changes in the GUT-M. It appears then that when stressful situations cannot be avoided, selection for resilient GUT-M would help reducing anxiety-like and depressive-like behaviors.

2 Improving behavior via nutrition and the GUT-M

The MGBA concept should have large consequences in livestock nutrition. Diet composition (use of prebiotics or probiotics or raw materials) is already carefully checked to favor a good GUT-M and gut health. However, it appears with the MGBA that diet composition will also have to be designed for desired behaviors or to ensure a “good” neurobiological development when more data are available. Supplementation with pre- or probiotics would be useful before or during stressful events such as manipulation or transport, to avoid the activation of the HPA axis and anxiety-like behaviors. The provision of various amino acids modifies GUT-M composition but the consequences on behavior are poorly documented. In chickens, provision of tryptophan has been shown to modify the GUT-M [135]

and to reduce serum corticosterone, serotonin and heat shock protein 70. These results can be related with other studies demonstrating that tryptophan metabolism into serotonin is involved in feather pecking behavior [136] and that its supplementation can reduce gentle feather pecking behavior in this species [137]. Moreover, a better understanding of the roles of GUT-M in feeding behavior, especially in modulation of appetite and satiety, could have large consequences on animal nutrition. Animal nutrition is presently based on our knowledge of needs and the ability of various diets to fulfil these needs but if it is considered that the GUT-M also modulates appetite and satiety as shown in rodents and humans, this could have large consequences on feed preferences and intake if it is established in farm animal. In future, nutritional rules for farm animals could be improved by increased knowledge about the way bacterial growth modulates the digestive cues related to satiety and taste, and about peptides produced by bacteria that could be involved in the hypothalamic regulation of appetite. A better understanding of appetite regulation would help managing feed intake, feed frustration and anorexia related to disease states.

From a practical point of view, provision of pre- or probiotics in addition to the diet is the easiest way to influence the GUT-M *via* nutrition. Prebiotics and probiotics can have complementary effects, however there are expensive contrary to the modifications of the feed composition. For poultry, probiotics could be fed at the hatchery in order to improve gut colonization. *In ovo* injection of prebiotics or a combination of pre-and probiotic at the 12^{th} day of the embryonic development has been shown to influence host transcription and appears to stimulate the proliferation of the embryonic GUT-M [138,139]. We need more studies to quantify the long-term effect on health and behavior of such provision of pre- or probiotics at the hatchery. An exciting new perspective on GUT-M - host symbiosis comes from the finding that pioneer colonizers, the first bacteria to reach the neonatal gut, will impact the future health since they can directly influence the development of the intestine and the nutrient matrix it provides for sequential implantation of future microorganisms [140].

In mammals, the GUT-M can even be orientated before birth since the maternal diet can influence GUT-M composition in the offspring. As previously mentioned in rodents [141], the maternal diet can influence GUT-M activity in the offspring and this modulation can influence social behavior. In piglets, GUT-M activity (measured by quantitative analysis of SCFAs) is reduced and responses to reward are modified when sows are fed with a high-sugar and fat diet during pregnancy [117]. Such demonstrations suggest that nutrition of breeders may be able to modulate behavior in the offspring and that this has to be investigated in farm animals.

3- *Improving management practices through the GUT-M*

Many husbandry situations can give rise to stress states during animal rearing and this state may modify GUT-M composition which can reinforce the negative effects of stress. Based on these interactions described among the MGBA, it appears that protecting a balanced GUT-M would help in the management of stress [13,142] and this would help preventing infection [24]. We saw that most of studies focusing on behavior used probiotics that were able to decrease stress cues [114,115] and to modify behavior and prevent various diseases such as acidosis in ruminants [128,143]. Nutritional transition focusing specific GUT-M changes could also help reducing stress since we saw in horses that these GUT-M changes due to increased diet energy are related to behavioral stress response related to particular bacteria [116].

A better knowledge about the MGBA of the farm animal would also help to detect silent infections and then modify the management of many diseases. Changes in behavior are commonly used to detect illness. Inflammatory states are commonly associated with changes in a reduction of comfort and feeding behavior and in motivation for social interactions. However, some pathogens do not induce illness cues at animal level and this asymptomatic carrier state prevents the detection of such infections. The existence of the MGBA suggests that changes in behavior could happen, even if the host does not express classical sickness behavior commonly associated with disease. This would

explain why the presence of Campylobacter, a bacterium that is involved in a foodborne toxi-infection in human, can be detected by automated behavioral analysis of poultry flocks [144] while no clinical cue can be detected in chickens carrying this bacterium. Another example is given with chickens that have been infected by *Salmonella* Enteritidis and that are also considered as asymptomatic carriers. While no clinical cue can be detected in each infected chick, changes in behavior occur during the weeks and sometimes the days following infection: reduction in feeding [145], in inter-individual distances and in running bouts [146].

4- Needs for improved tools to use the MGBA

This enhanced understanding requires improved methods. The use of germ-free animals (mainly rodents but also chicks) has been critical to our understanding of how the GUT-M can influence health, disease, and behavior especially when coupled with mono-association (inoculation with a single bacterial strain), defined microbiota, or humanized microbiota strategies. To circumvent some of the physiological disadvantages of germ-free and mono-associated mice (poor barrier effect, maturation of immune response and intestine development) while still maintaining a controlled microbiota, mice reconstituted with defined microbiota were established. Schaedler initiated these studies by defining key cultivable bacteria, which were experimentally inoculated to germ-free mice in various “cocktails” of aerobes and anaerobes [147,148]. The cocktail was refined and standardized resulting in “altered Schaedler's flora” (ASF) that is now most commonly used in gnotobiotic research and companies [149]. The ASF community offers significant advantages to study homeostatic as well as disease-related interactions by taking advantage of a well-defined, limited community of microorganisms. Now, it would be interesting to develop such cocktails of bacteria for each farm animal species to go further in the MGBA studies.

Additionally, moving forward, we face a number of challenges in each animal model. For example, the vast majority of intestinal microorganisms remain uncultivable. Can novel culture methods or

creative strategies to eliminate selectively targeted agents be developed? How to include other GUT-M members like viruses, protozoa and fungi in the MGBA analyzes? How do we avoid microbiota drift to optimize reproducibility among studies? Can microbiota be banked adequately for future studies? Facing these issues is a great challenge to improve our knowledge about the MGBA in farm animals.

Conclusion

Thanks to the many ways of manipulating the GUT-M (germ-free, antibiotics, probiotics, diet, microbiota transfer), it is increasingly recognized that the microorganisms colonizing the host's digestive tract can directly or indirectly act on the nervous system and influence host behavior. The majority of studies on the subject have used rodent or human models and it seems that the GUT-M can influence emotional behavior, memory capacities, social and feeding behavior also in poultry, pig, horse and ruminants. However, germ-free animals reared and kept in isolators are poor models for farm animals and it will be a big step to apply results to the farm environment. Many studies are correlational and the presence of specific microorganisms is not controlled experimentally while investigations with microbiota reconstitutions that reverse behavioral changes and investigate mechanisms are still lacking. Many methodological issues have to be faced to get a better knowledge about the variations of the GUT-M, the role it can play in the MGBA of the farm animal and how it could help reducing certain deleterious behaviors and increasing behavioral adaptation *via* genetic selection, nutrition, stress management and detection of silent infections. In summary, it is necessary to take this MGBA concept into account in an applied interest to farming conditions since it can have large consequences in animal welfare.

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Table and figure captions

Table: Taxonomic profiles of major gut bacterial communities at the phylum level in farm animals using 16 rRNA gene pyrosequencing (Percentage of sequences assigned), based on [89], [150], [77], [151], [98], [95], [78], [85].

Figure 1: Gut microbiota as a key actor for animal welfare

Figure 2: Influence of the microbiota-gut-brain axis on behavior

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Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review

Highlights

- The microbiota-gut-brain axis (MGBA) has a major role on rodent and human behavior.
- The MGBA can also influence behavior and memory capacities in several farm animals.
- The MGBA can help for genetic selection, stress management or infection detection.
- Taking into account the MGBA could widely improve farm animal welfare and health.

Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review

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Abstract

There is increasing evidence of a pivotal role of the gut microbiota (GUT-M) in key physiological functions in vertebrates. Many studies discuss functional implications of the GUT-M not only on immunity, growth, metabolism, but also on brain development and behavior. However, while the influence of the microbiota-gut-brain axis (MGBA) on behavior is documented in rodents and humans, data on farm animals are scarce. This review will first report the well-known influence of the MGBA on behavior in rodent and human and then describe its influence on emotion, memory, social and feeding behaviors in farm animals. This corpus of experiments suggests that a better understanding of the effects of the MGBA on behavior could have large implications in various fields of animal production. Specifically, animal welfare and health could be improved by selection, nutrition and management processes that take into account the role of the GUT-M in behavior.

Key words: Microbiota, microbiota-gut-brain axis, behavior, welfare, emotion, livestock

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Introduction

The gut microbiota (GUT-M) has received increased interest for several years because it is involved in many functions in humans and animals. The GUT-M is composed of bacteria, archaea, viruses and eukaryotes (including protozoa and fungi). The GUT-M has been demonstrated to influence immune function for years and to have wide impacts on health. Moreover, impairments of gut health can lead to many intestinal diseases and to dysbiosis, an unbalance in GUT-M, which facilitates many pathological states involving infections with pathogens or metabolic disorders [1-4]. The GUT-M has also a pivotal role in many extra-intestinal tissues and in various developmental processes and metabolism in host organs such as the liver, adipose tissue, bone, *etc* [5]. The brain is also a major target of the GUT-M because the microbiota produces metabolites and neurochemicals. At the same time, neurotransmitters like epinephrine and norepinephrine from the host influence the growth and virulence of bacteria [6]. The relationship between the GUT-M and the brain, so called microbiota-gut-brain axis (MGBA) includes influences upon brain development, neural processes (such as myelination or neurogenesis), pain processes, the hypothalamo-pituitary axis (HPA) and behavior [7]. The MGBA is also called microbiome-gut-brain axis by some authors, since the microbiome consists of not only the microbiota, but also microbiota genomes and products [8]. Although some methods used to investigate the MGBA have been recently criticized [9], there are more and more studies describing the influence of the GUT-M on the central nervous system (CNS) and the mechanisms involved in this interaction. The influence of the GUT-M on behavior is increasingly reported in rodents using germ-free animals (living in the absence of detectable living microorganisms) or in rodents and humans following the use of special diets affecting GUT-M composition, or microbiota transfer [10-16] using antibiotics or probiotics (live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host (see [17] for definitions). These studies demonstrate that there is increasing evidence that changes in the GUT-M

affect physiological and behavioral processes that are directly relevant to welfare such as stress, anxiety, changes in social behavior and memory. Whilst demonstrations of the influence of the GUT-M on behavior in farm animals remain scarce, manipulation of the microbiota in farm animals by supplying probiotics is common to improve production. Therefore, a critical examination of the influence of the GUT-M on behavior would be especially interesting from an animal welfare perspective.

This review aims at summarizing the influence of the MGBA on behavior in rodents and humans and to point out what has been observed in farm animals. Moreover, because the GUT-M varies according to host genetics and many external factors (Figure 1), we suggest that the GUT-M could be used to improve behavior and welfare on the farm.

I – The gut microbiota and its impact on brain development and behavior in rodent and human models

There is increasing evidence that the microbiota can influence host behavior. Most of the investigations on behavior have focused on the GUT-M in vertebrates where various routes of interaction between the GUT-M and the brain have been identified, including the immune and the enteroendocrine pathways, the enteric nervous system and the vagus nerve (Figure 2). Products of bacterial metabolism and structural components of bacterial cell walls influence a wide range of processes including host immune responses (e.g. cytokines) and activation of enteroendocrine cells which can affect the nervous system locally and systemically. The enteric nervous system is a major interface between the GUT-M and the host and it forms the most complex of intrinsic nerve circuits outside the CNS [18]. Through its neuronal networks and numerous neurotransmitters, it mirrors many aspects of the CNS and intimately interfaces with it *via* the autonomic nervous system. Although seldom recognized, the number of neurons in the enteric nervous system is comparable to

the number of neurons in the spinal cord, leading some authorities to refer to the enteric nervous system as the “second brain” or the “little brain”[18]. Moreover, research has demonstrated that 80 percent of the vagus nerve fibres carry information from the gut to the brain, rather than the other way round [19]. Thus, the vagus nerve is a major pathway of the MGBA as demonstrated by surgical sections that abolish the effect of the GUT-M on the brain and on behavior in mice [20-23]. Conversely, the brain modulates the physiology of the gut, the enteric immune system and the composition of the GUT-M. This influence can impair gut activity especially during host stress [24-26]. The GUT-M can additionally influence the behavior of host’s conspecifics through sensory cues even if they are not considered usually as constitutive of the MGBA [27]. These cues are mainly olfactory [28] but the GUT-M could even be related with visual cues in some cases: in pigeons for example [29], GUT-M composition is related to feather microbiota composition and the bacterial load on the plumage has been shown to influence the iridescent color of the feathers which is a fitness cue for the congeners.

1- Effects on anxiety-like behavior and stress responses

The question of the role of the GUT-M in anxiety-like behavior was raised following the pioneering study of Sudo et al. [30] which showed hyperactivity of the HPA axis under stress conditions in germ-free mice (without any microbiota) compared to specific pathogen-free mice. Other teams have subsequently confirmed the influence of the GUT-M on the development and regulation of the stress response system [13,14,16,31]. In addition, patients with gastro intestinal disorders such as irritable bowel syndrome (IBS) also have a deregulation of HPA axis activity [32,33]. Consequently, a link between the MGBA and anxiety-like behavior is not surprising and a significant modification of anxiety-like behavior has been observed in germ-free rodents compared with specific pathogen-free rodents in various tests [34-39]. These studies reveal the importance of the genetic background in the influence of the GUT-M on behavior. Indeed, the absence of GUT-M leads to increased anxiety-

like behavior in rodent strains genetically prone to exacerbate emotionality (F344 rats and BALB/c mice) [11,12] and provoked a reduction of anxiety-like behavior in moderately emotive strains (NMRI and Swiss mice) [37,38]. The germ-free rodent studies represent a large part of the literature on the MGBA concept. Nevertheless, the germ-free animal presents several important physiological alterations compared to a colonized one such as a reduction of the growth, alterations of the digestive functions or immune system impairments ([40] for review), thus it is not easy to demonstrate that the behavioral modifications observed in these animals are a direct consequence of the absence of GUT-M rather than of physiological changes. However, some authors have tried to reinforce the role of the presence of GUT-M in their studies by re-introducing standard microbiota into these germ-free animals and have observed a reversal of behavioral responses following bacterial colonization [37,39]. When it is not completely abolished, the GUT-M can be modified by the use of antibiotics. BALB/c mice treated with a mixture of nonabsorbable antimicrobials (bacitracin, neomycin and pimaricin) for seven days showed reduced anxiety-like behavior compared to controls in a light-dark box test [20]. Similarly, the low doses of penicillin in late pregnancy and early postnatal life induced long-term changes of microbiota composition and behavior. The antibiotic-treated mice exhibited impaired anxiety-like and social behaviors, and displayed a higher level of aggression in several tests, while concurrent *Lactobacillus rhamnosus* JB-1 probiotic supplementation prevented some of those alterations [41]. However, these results must be interpreted cautiously because antibiotic treatments are known to have neuroactive and neurotoxic potential. Regardless or in addition to their microbicidal effects, the antibiotics themselves may also influence enteric, peripheral and central nervous system functions [10].

Probiotics are live naturally occurring microorganisms which can improve health directly or indirectly by inhibiting growth and attachment of pathogens and favor the development of the intestinal epithelium and the immune responses. A probiotic can be used alone or in combination with other probiotics, a cocktail of microorganisms that may have different or common properties [42]. The exact mechanisms through which probiotics provide benefits are being studied and may differ

depending on the specific formulation. These mechanisms include modifications of the pH of the gastrointestinal tract, the provision of nutrients to the host, the production of antimicrobial or signaling molecules, competition with pathogens for ecological niches and available nutrients, promotion of the intestinal cell differentiation and turnover, increased mucus production and maturation of the immune system. Many studies in the literature suggest an anxiolytic effect of some probiotics. Mice treated with the probiotic *Lactobacillus rhamnosus* expressed reduced anxiety compared to control mice during the elevated plus maze [33] and a chronic administration of *Lactobacillus plantarum* leads to lower anxiety-like behavior in the open-field and elevated plus maze tests [43]. More demonstrative yet, Bercik et al. [44] showed that a daily gavage with the probiotic *Bifidobacterium longum* can normalize anxiety-like behavior in mice with infectious colitis in the step-down test and a supplementation with the probiotic *Lactobacillus helveticus* has led to a reduction of chronic stress-induced anxiety and depression in rats [45]. Messaoudi et al. [46] investigated the effect of a mixture of two probiotics (*Lactobacillus helveticus* and *Bifidobacterium longum*) on rodents and human volunteers. In both cases, a decrease in anxiety was revealed. Infection with pathogenic bacteria is another way to modify the composition of the GUT-M, which often leads to increased anxiety-like behavior in rodents. An infection of mice with *Campylobacter jejuni* or *Citrobacter rodentium* exacerbated anxiety-like behavior compared to control mice in different situations such as the elevated plus maze or the hole-board open field test [19,47,48]. Furthermore, the anxiogenic effects of these infections were not the result of an immunological response but appeared to be a direct action of bacteria on neural activation pathways [19,47]. However, one of the most striking experiment on the influence of the GUT-M on anxiety-like behavior is the study of Bercik et al. [20] who carried out a GUT-M transfer between a low (NIH Swiss) and a high (BALB/C) anxiety-like mouse strains presenting different microbial profiles based on denaturing gradient gel electrophoresis (DGGE). The germ-free BALB/c mice that received the GUT-M from the opposite mouse strain were less anxious than the controls BALB/c mice during the step-down test. In contrast, germ-free NIH Swiss mice responded more anxiously than controls during the same test. Therefore,

this experiment suggests that the GUT-M would be involved in the anxiety-like phenotype of these mice. Taken together, these findings suggest a significant influence of the MGBA on anxiety-like behavior.

2- Effects on memory

It is now increasingly recognized that the GUT-M communicates with the brain and acts on several brain structures such as the amygdala, the cortex and the hippocampus that all have a key role in memory processes [12,37,40]. Moreover, the relationship between anxiety and memory and learning has been widely demonstrated, suggesting an effect of the GUT-M on cognitive abilities [30,49,50]. This idea is supported by results obtained when comparing germ-free mice and specific pathogen-free mice in the novel object test and the T-maze test [15]. In both tests, the germ-free mice displayed memory deficits. Consistent with these findings, treatment with an antibiotic formulation resulted in a cecal composition shift with reduction of Firmicutes and Bacteroidetes and increase of Proteobacteria and Cyanobacteria and a decrease in memory capacities in mice subjected to novel object recognition test and social transmission of food preference test [51]. The influence of an antibiotic treatment on memory may nevertheless depend on the number of antibiotic products used and the sensitivity of the bacteria to this antibiotic. For example, in the Morris water maze the vancomycin antibiotic had no significant effect on murine memory despite a significant alteration of fecal microbiota [2]. The gut microbiota may also have different effects depending on the type of memory assessed. In a recent study, a treatment with an antibiotic mixture strongly disrupted microbial composition of mice and impaired novel object recognition but not spatial memory in the Barnes maze test [52]. Studies on probiotics supplementation agree that there are beneficial effects on memory performance in rodents [33,45,53-55]. Works conducted on pathogenic infections (with *E. coli* or *C. rodentium*) reported deleterious effects on memory in the mouse [15,56] and, in both cases, a treatment with probiotics attenuated these memory impairments. However, it is important

to emphasize that only the study of Smith et al. [54] performed a GUT-M composition analysis following probiotic administration and reported significant changes in the fecal microbiota of the mice. In humans also, improvement of emotional memory after probiotic administration has been associated with changes in GUT-M community composition [57].

An alternative strategy for modifying the microbiota is to use dietary prebiotics. Prebiotics are fermentable oligosaccharides or polysaccharides that induce the growth of some gut bacteria that increase gut health. Unabsorbed or undigested carbohydrates are fermented by the gut microbiota in the large bowel, producing different end products like short-chain fatty acids (SCFAs) and lactic acid, which may have multiple effects. For example, it has been described that oral administration of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) affects behavior and specifically anxiety, depression-like behavior, cognition, and social behavior. These modifications are related to specific gene expression in the hippocampus and hypothalamus, gut microbiota composition, several SCFAs produced, and elevations in corticosterone and pro-inflammatory cytokine levels [6].

Modifications of the GUT-M through changes in raw materials of the diet appear also to influence cognitive abilities. An enrichment of beef in the diet of mice increases the microbial diversity in the colon and their memory scores in the hole-board apparatus [58]. A diet characterized by a high-fat composition also leads to differences in GUT-M composition and to memory impairments in the mouse and the rat [29,59,60].

However, studies are still needed to strengthen a causal relationship between GUT-M changes and memory abilities in these nutrition experiments.

3- Effects on social behavior

The MGBA seems to be also involved in other highly emotional behaviors such as social behavior. This behavior is impaired in germ-free rats in a test which consists of measuring behavior during an encounter with an unknown partner [36]. During the 2 minutes of the test, compared to specific

pathogen-free rats, the germ-free rats spend less time sniffing an unknown. These results are consistent with what Desbonnet et al. [24] found in a mouse model tested in the 3-chambered sociability test. The germ-free mice displayed social preference deficits by spending less time exploring a chamber containing a mouse than an empty chamber. In addition, when the germ-free mice are post-weaning colonized, their behavioral responses are reversed in the same test. However, this result could not be replicated in a subsequent study using the same mouse strain and the same 3-chambered test in which the authors observed opposite results [35]. Indeed, germ-free mice expressed greater social preference than specific pathogen-free mice. The authors assumed that the difference in the age of the germ-free mice between the two studies could be the explanation for the contradictory findings. They also mentioned the hyperactive behavioral responses of the mice in the Arentsen *et al.* work [35] and the differences in living conditions of the specific pathogen-free mice (isolators rearing in a study and not in the other). More recently, social behavior impairments and dysbiosis in the gut have also been reported in mouse offspring from mothers fed with a high-fat diet [61]. Interestingly, a probiotic (*Lactobacillus reuteri*) supplementation in the drinking water during 4 weeks led to the normalization of social behavior and this reversal of the social deficits involved the vagal pathway. In conclusion, all these data indicate that the GUT-M is required for a normal expression of social behavior in rodents. Moreover, differences of GUT-M composition have been revealed between autistic and control patients in an expanding volume of studies [42,62-64]. Similarly, altered GUT-M composition and social deficits have also been noted in a murine model of ASD [65,66]. These mice are characterized by disturbed anxiety-like and stereotyped behavior similar to those observed with germ-free mice [38]. An administration of probiotic *Bacteroides fragilis* has improved many of these behaviors including anxiety-like behavior (open-field exploration), communication deficits (ultrasonic vocalizations) and stereotyped behavior [65]. More interestingly, Sandler et al. [67] tested the effect of an antibiotic on 11 children with regressive-onset autism. Significant behavioral improvements were noticed during the treatment period and the behavioral improvements disappeared after the treatment. It has also been recently demonstrated that

Lactobacillus reuteri rescues social deficits in various mouse models for ASD based on genetic, environmental and idiopathic alterations [68].

4- Influence on feeding behavior

Fetissov [69] suggested that the bacteria-host communication influences the appetite-satiety balance in humans and rodents. First, bacterial components and metabolites have been shown to stimulate satiety pathways in the host in the short term through the stimulation of endocrine cells involved and the production of peptides related to feed intake [70,71]. Secondly, bacterial peptides use systemic routes and might act directly in the hypothalamus and so play a role in the long-term regulation of appetite. Moreover, the GUT-M appears to be involved in the expression of taste receptors in rodents [72,73].

It is now recognized that the MGBA is involved in many behavioral responses in humans and rodents and interventions with probiotics reinforce the theory of the influence of the GUT-M on behavior and the cognitive abilities. However, it is also important to note that the causal mechanisms by which the GUT-M and the brain communicate are not well described or understood and further investigations are needed to shed light on this microbiota-gut-brain axis communication.

II- The gut microbiota of farm animals

There is an increasing knowledge about the composition of GUT-M of farm animals (ruminants, horse, pig, rabbit, chicken, turkey, *etc*). Indeed, it is very important to characterize the GUT-M in farm animals so that it is possible to detect normal and abnormal changes. This knowledge should help to define and identify dysbiosis and to restore a healthy GUT-M. It should also help to predict susceptibility to infection and prevent welfare and health problems since GUT-M composition is

involved in the control of pathogen colonization [74,75]. However, understanding GUT-M composition is a complex issue since it varies along the digestive tract and there are also differences between lumen and mucosa, and even between the tip of the villi and the crypt. Moreover, GUT-M variations are induced by many factors related to the host and to the host environment.

1- Investigation of the *GUT-M in farm animals*

While the GUT-M is composed of bacteria, but also viruses, archea and eukaryotes and while bacteriophages have been shown to have an important role in bacteria composition, most studies only take into account the bacterial composition of the GUT-M. This is in line with methods available to measure this composition since there are more libraries of bacteria available for 16S rRNA gene sequencing than for viruses, archea and eukaryotes. Several methods are used to characterize the the GUT-M. The 16S rRNA gene sequencing directed by PCR, is commonly used to quantify GUT-M diversity and is effective in demonstrating the major phyla, families or genuses, but sometimes gives limited resolution. The table provides the characteristics of GUT-M bacteria in the main farm species (cow, sheep, horse, pig, rabbit, chicken, quail, duck) established by 16S rRNA gene sequencing. This table gives the composition at phylum level and sheds light on the large variation found within host species. Though not the main focus of this review, it is clear that accurate descriptions of the composition (at the genus or the species level) of the bacteria in different parts of the digestive tract greatly help us to understand the effects of host and external factors of modulation on the GUT-M ([71] in cow for example). Quantitative metagenomic shotgun sequencing also aims at investigating diversity directly from samples but can be technically challenging and is less frequently used. Other approaches look for GUT-M functionality by metatranscriptomics (RNA sequencing), metaproteomics (Mass spectrometry) or metabolomics (High resolution spectroscopy).

Each gut compartment hosts a microbiota with a particular composition and many studies investigated GUT-M composition along the digestive tract ([76] in pigs; [77] in horses; [78] in quail).

In horses for example, the composition of the GUT-M collected in the lumen is very different in caecum and colon compared to the upper compartments (stomach, jejunum and ileum) and is different from the GUT-M from mucosa [77]. In this example, data suggest that analysis from feces would be related to colonic segments only, but would not be related to upper compartments. Numerous studies use fecal samples to avoid animal sacrifice, which could be misleading.

Gut microbiota of the small intestine, caecum and colon in healthy adults is dominated by bacterial species belonging to two main phyla, Gram positive Firmicutes and Gram negative Bacteroidetes (Table). The small intestine is usually dominated by Firmicutes with major families including *Lactobacillaceae*, *Peptostreptococcaceae* or *Enterococcaceae*. Microbial complexity considerably increases in distal parts of intestinal tract, i.e. in the caecum and colon. It is important to remember, however, that the descriptions of the gut microbiota leave out many important factors such as host genetics, age or feed regime (see below) that may give rise to much greater variation. These factors may affect microbiota development and composition in the youngest animals and the differential development in early days of life.

2- *Variations in the GUT-M linked to the host*

The host genetics affects the GUT-M in numerous ways and this impact is related to inter and intra species differences in the GUT-M [79]. Domestication has also induced changes in GUT-M composition. For example, a metagenomic approach followed by a quantitative PCR showed that the GUT-M in wild Suidae (wild boars and Red river hogs) was characterized by a high abundance in *Bifidobacterium* which was not the case in domesticated Suidae characterized by abundance in *Lactobacillus* and *Enterobacteriaceae* as the major family [80]. It is important to note that diet was not controlled and thus confounded with genetics in this study. However, it has been demonstrated in domesticated pigs from the Pietrain strain that pig genome influences the GUT-M in the mid-colon and that the heritability of the load of some bacteria can even reach high values such as 0.32 to 0.57

[81]. Differences in the GUT-M related to host genetics have also been established between lines of the same species. With chicken lines selected on body weight, Zhao et al. [82] demonstrated that the host genotype and gender affected 68 out of 190 GUT-M species and that among them 15 belonged to *Lactobacillus*. Genetic selection on *Salmonella* carriage in chickens enabled the detection of Quantitative Trait Locis (QTLs) for both resistance to carrier state and resistance to *Salmonella* colonization [83,84]. Some bacterial families can be affected particularly by host genotype: in Pekin and Muscovy ducks for example, genotype affects *Lachnospiracecae*, *Bacteroidaceae* and *Desulfovibrionaceae* in the cecum, while overfeeding affects other families such as *Clostridiaceae*, *Lactobacillaceae*, *Streptococcaceae* and *Enterococcaceae [85]*. A divergent genetic selection on increased digestive efficiency in chickens was linked to changes in the GUT-M and has enabled the detection of QTLs related to the presence of some GUT-M bacteria [86]. In chickens, QTL for the presence of bacteria such as *Lactobacillus* and *L. crispatus* co-localize with QTLs for feeding behavior [87]. Host genetics would then influence both the behavioral phenotype and GUT-M composition. It is highly probable that behavior and the GUT-M influence each other as it has been demonstrated in stress processes where the brain influences gut peristaltis and GUT-M composition while the GUT-M interacts with CNS and the HPA axis [13].

The age of the host is also a major factor and the ontogeny of the GUT-M has been studied in many farm animals. The changes during early life have been described in several farm animals (chick: [88]; calf: [89,90]; piglet: [91]; foal: [92]). Microbial colonization is a complex process influenced by the host and many external factors, including maternal microbiota, birth process, early diet, perinatal stress and antibiotics use.

3- Variations of the GUT-M linked to the environment

The environment dramatically influences the newborn's GUT-M. In mammals, the contact of the newborn animal with its mother is physiologically indispensable and during parturition, the offspring is naturally inoculated with microbiota from the mother. However, in case of avian farm species, the young birds are industrially hatched, which means that eggs are disinfected and chicks reared without any contact with their mother or any older conspecific and the source of microbiota is thus limited to the environment. This way of husbandry is in sharp conflict with the natural conditions, where the mother bird represents the principal source of the GUT-M. Experimentally, young chicks reared in a sanitized environment with no contact with older conspecifics had profoundly different microbiota compared with chicks which were kept for 24 hours with the adult hen [93].

Other external factors such as infections can give rise to unbalance in the GUT-M. For example, early exposure to pathogenic bacteria can shape the overall microbiota composition in chicks infected with *Salmonella* Enteritidis inducing an expansion in the *Enterobacteriacecae* [94] and exposures to *Campylobacter jejuni* revealed that the shift of the GUT-M varies upon the age at which the chickens become colonized by this bacteria [95]. Parasitism can also influence GUT-M composition and the interplay between helminths and the bacterial populations is being elucidated. The various ways both populations influence each other are complex [96] and suggest that a better knowledge of the gut microbiota of nematodes themselves could lead to a better prevention of parasitic diseases [97].

Throughout life, housing conditions influence cecal microbiota in rabbits [98] and pigs [99] showing that environmental bacterial load influence the GUT-M. Breeding in different rearing systems can also influence GUT-M composition at the phylum level. For example, Bacteroidetes and Proteobacteria were more prevalent in chickens reared under free-range conditions than in cages, but this difference was manifested only in one of both lines [100]. Stocking density can influence crop and cecal microbiota composition in chickens [101]. Rearing conditions inducing stress can also influence the GUT-M. In horses for example, weaning and transport are stressful events and both can affect the GUT-M composition [102,103]. In Mach's experiment, foals' microbiota was modified during the first week after weaning until a relatively stable gut community was established at day 7

post-weaning. This modification can be partly explained by the nutritional change, however GUT-M composition after weaning was slightly modulated by the weaning method suggesting that the stress induced by the abrupt method has impacted the microbiota modification. An experiment in pigs has shown that even mild handling stressor such as single daily weighing is able to alter the GUT-M [104].

Another very important external modulation of the GUT-M is given by the feed which may drastically influence GUT-M composition and activity. Such influences are being increasingly studied since diets, or the water bacterial load, may induce unbalance in the GUT-M and lead to pathological states. Such unbalance can lead to dysbiosis and then enteritis, or to other diseases targeting some other organs such as lungs, since unbalance gives rise to inflammation of the gut wall and facilitate bacteria leakage across the epithelial wall. This modulation by the diet has mainly been investigated in farm animals and reviewed in many animal species [105] for review in horses; [106] in chicken; [107] in piglets, [85,108] in ducks, *etc*). Most of these studies compare diets based on high fiber with diets containing raw materials providing high energy levels. Other nutritional means used to modify the GUT-M are the provision of prebiotics or probiotics. Prebiotics are fermentable oligosaccharides or polysaccharides that induce the growth of some gut bacteria that increase gut health while, as previously mentioned, probiotics are microorganisms which improve animal health directly or indirectly by producing substrates that stimulate growth of commensals, inhibit growth of pathogens, favor the development of the intestinal epithelium and the immune responses. Probiotics are largely used in animal nutrition to improve gut health, increase feed efficiency and milk quality [42,109] and it has been demonstrated in piglets that they can influence serotonin and dopamine concentrations in the hypothalamus [110]. They are also use to prevent the effects of stressful events such as transportation in horses for example [111] but this improvement is not always related to a change in the GUT-M as mentioned by a meta-analysis carried out in calves [109]. Lactic acid bacteria are commonly used as probiotics, and their impact on gut health, immunity and the prevention of the establishment of pathogenic bacteria has been increasingly studied.

Farm animal GUT-M can thus vary with a wide range of factors each of which have many different consequences but the results on behavior are weakly documented and rarely taken into account. Furthermore, only few studies have used GUT-M manipulations to disentangle effects of nutritional or environmental factors and GUT-M effects.

III- Effect of the microbiota-gut-brain axis on behavior in farm animals

There is emerging evidence that the GUT-M is able to influence behavior in farm animals as has been shown in rodents and humans. Colonization of farm animals with a pathogen was known to induce sickness behavior for a long time, but recent studies demonstrate that the influence of the MGBA is not limited to the area of disease and can also occur in healthy animals. Studies based on germ-free animals, provisions of probiotics or prebiotics, diet modifications, demonstrated that changes in the GUT-M are related with changes in many behavioral patterns. Because of the size of farm animals, this influence of the MGBA has been established mainly with studies using probiotics while very few studies on germ-free animals are available since these animals must be kept in isolators.

1- Effects on emotional reactivity and anxiety-like behavior

A recent experiment with germ-free birds demonstrated that the absence of GUT-M reduces emotional reactivity in Japanese quail in fear and social perturbation situations without major influence on growth [112]. The authors used germ-free quail chicks that were kept germ-free or inoculated with a dilution of GUT-M from adults of the same line. Quail chicks were reared and tested in isolators in order to avoid contamination. Germ-free quails spent less time in tonic immobility, were less reactive during the social separation test and were less neophobic in a novel object test than inoculated quail chicks. The use of a GUT-M transfer has also demonstrated the

influence of microbiota on emotional reactivity in this species [113]. The authors used genetic lines of quails that have been selected for either a high fearfulness (E+) or a low fearfulness (E-). Germ-free quail chicks from the E+ line were inoculated with feces from either a E+ quail or from a E- quail and were reared in different isolators. Quails that received feces from the E- line expressed a lower emotional reactivity during the second week of age than the quails colonized by feces from the E+ line. This result was reversed two weeks later. These behavioral differences can be related to GUT-M differences and modifications over time and they could be the consequence of the resilience of the GUT-M to recover its equilibrium present in the E+ host, which is in part driven by the host genotype. Abdel-Azeem et al. [114] showed that the administration of the probiotic *Bacillus amyloliquefaciens* helped to reduce distress calls in turkeys and the supplementation of the diet with a probiotic (*Pediococcus acidilactici*) reduced emotional reactivity in quails [115].

In horses, the relationship between the GUT-M and behavior has been suggested by correlations obtained in fistulated horses submitted to behavioral tests before and after a nutritional change [116]. The modification of the diet from a fibrous diet with 100% hay to a diet with increased energy (57% hay and 43% barley) induced significant increases of colonic total anaerobic bacteria, lactate-utilizing bacteria and amylolytic bacteria concentrations. After this transition, the horses were submitted to a sociability test where behavior was analyzed when an unfamiliar horse was introduced into the adjacent stall and to a neophobia test assessed from the reaction to the presence of a novel object placed near a feeder in a test arena. The time spent in vigilance during the sociability test tended to positively correlate with cecal and colonic amylolytic bacteria concentrations while the time spent in vigilance during novel object test was correlated with caecal lactate-utilizing and colonic amylolytic bacteria concentrations.

2- Effects on memory

As in rodents, probiotics have been shown to enhance memory in quail: supplemented birds made fewer errors in a test where they had to remember the cup they had previously visited among eight rewarded cups [115]. In Yucatan pigs, differences in the maternal diet during gestation and lactation have been used to modify microbiota activity in the sows and their offspring [117]. Sows were either fed a standard diet or a Western diet enriched in energy, sugar and fat. SCFAs used to measure microbiota activity were decreased in sows fed the Western diet and in their piglets. Piglets from sows fed the Western diet, i.e with reduced GUT-M activity, had higher working memory in a hole board test where they had to learn where were the bowls that contained chocolate-coated peanuts among unrewarded bowls.

3- Effects on social behavior

Using probiotics, it has been shown that spores of *Bacillus amyloquefaciens* decrease aggression in turkeys [114]. However, the most promising information was obtained for feather pecking behavior in hens. Gentle feather pecking is considered as a normal social exploratory behavior and consists in a soft pecking while severe feather pecking is an intense pecking and pulling out feathers which can induce pain in the victim. This injurious behavior considered as an abnormal behavior have been recently supposed to be associated with the MGBA. Indeed, it has been shown that divergently selected lines of hens for severe feather pecking also differ in hens' GUT-M [118] and in immunity [119]. Nevertheless, it is still not possible to decide conclusively whether differences in feather pecking induced difference in the GUT-M or whether differences in the GUT-M induced difference in behavior via the MGBA [120]. The latter explanation agrees with data about GUT-M metabolites such as total SCFAs and biogenic amines since both were also different between these lines [121] and SCFAs have been shown to be involved in the MGBA and influence social behavior. Differences in the gene expression of two genes (ABCB1 and TNSF15) involved in inflammatory bowel disease (IBD) are also been reported between birds expressing feather pecking or not [122]. Moreover, the serotonin whose

synthesis depends on various bacterial families in the GUT-M [49,50,123,124] is also involved in feather pecking behavior in hens [120]. Ingestion of feathers could lead to an increase of gut wall stimulation and therefore an impaired serotonin signalling [125]. These data would then be in agreement with an influence of GUT-M activity on the development of feather pecking through the MGBA. Brunberg et al. [125] proposed to investigate if the differences in GUT-M composition are already present in the young chick before the development of feather pecking behavior in order to characterize the main direction of the microbiota-gut-brain interactions in this model.

4- Effects on feeding behavior

Gut pathogens may induce illnesses states that are commonly accompanied by reduction in feed intake but some other influences of the GUT-M on feeding behavior can be found in farm animals.

In turkeys, spores of *Bacillus amyloquefaciens* have been shown to increase feeding frequency and duration [114]. The genetic lines of chickens divergently selected on feed efficiency we previously mentioned differ in feeding behavior and a QTL for feeding behavior co-localizes with QTLs for some bacteria from the GUT-M [87]. This co-localization suggests an influence of these bacteria on eating behavior but this influence still need to be strengthened by experiments using GUT-M manipulation.

Changes in feeding behavior induced by the MGBA are suspected in ruminants when they are affected by acidosis which occurs with high-energy low-fiber diets. Eating behavior can be modified with rumen liquor transplantation when cows are affected by acidosis [126] and even if pain alleviation or inflammation reduction can also explain the effect on eating behavior, this veterinary practice suggests that rumen microbiota influences appetite in such pathological state. In cows affected by subacute acidosis, ruminal GUT-M is modified [127] and feeding behavior is affected with a reduced feed intake and a reduced duration of rumination. *Saccharomyces cerevisiae*, a probiotic commonly used in ruminants, has a protective effect on physiological changes induced by acidosis such as reduction of the ruminal pH, changes in volatile fatty acids [42,128] and it has been shown to

induce also behavioral changes such as reduction of the minimum interval between meals and tendency for longer time spent ruminating [129].

This limited information about the influence of the MGBA on behavior in farm animals suggests that it can have large influences that have not been properly appreciated. These influences of the GUT-M on behavior can be added to its influence on health *via* its role in the immune response and tends to put the GUT-M as a pivotal actor for welfare state achievement [130].

IV- Prospective of the microbiota-gut-brain axis concept in the welfare of farm animals

The concept of the MGBA leads us to reconsider many factors that can influence behavior and health in farm animals. The influence of the MGBA will have to be taken into account in future and that may drastically change genetic selection, infection detection, nutrition and management processes. Furthermore, the improvement of gastrointestinal functionality is of the utmost importance because it positively influences health and welfare of animals, but also performance by preventing loss in feed efficiency and the use of antibiotics.

1 Selecting the host GUT-M

Even if a recent article demonstrated that the human GUT-M is shaped more by environmental factors than by human genome [131], we should not underestimate the influence of the host genetics on the colonization of the gut by the microbiota. Several studies have demonstrated that the host genome influences the composition of the GUT-M. For example, a study from twins has identified many microbial taxa whose abundances were influenced by host genetics [132] and associations between host single nucleotide polymorphisms and bacterial taxa have been described [133]. The host gut is able to select the microbiota it encounters and only part of the bacteria present

in the gut are able to develop in it. This explains why different genetic lines reared in similar conditions and fed the same diets have different GUT-M compositions. Selection for different genotypes could then lead to differences in GUT-M and consequently in behavior, immunity and feed efficiency [134]. As previously mentioned, selection for increased feed efficiency has led to differences in GUT-M in chickens and several QTLs are related to these differences in GUT-M composition and co-localize with loci involved in feeding behavior [87]. Moreover, these lines divergently selected for feed efficiency also differ in emotional reactivity. It appears then that these differences in behavior may have been driven by the effect of selection on the host genes involved in behavior, but also on the genes involved in GUT-M carriage.

A better understanding of the relationship between the host genome, the GUT-M and deleterious behaviors would be of great interest for animal welfare. A comprehensive link between the GUT-M and feather pecking could lead to alternative strategies for selection against this damaging behavior. As previously indicated, many rearing situations can induce stress and are related with changes in the GUT-M. It appears then that when stressful situations cannot be avoided, selection for resilient GUT-M would help reducing anxiety-like and depressive-like behaviors.

2 Improving behavior via nutrition and the GUT-M

The MGBA concept should have large consequences in livestock nutrition. Diet composition (use of prebiotics or probiotics or raw materials) is already carefully checked to favor a good GUT-M and gut health. However, it appears with the MGBA that diet composition will also have to be designed for desired behaviors or to ensure a “good” neurobiological development when more data are available. Supplementation with pre- or probiotics would be useful before or during stressful events such as manipulation or transport, to avoid the activation of the HPA axis and anxiety-like behaviors. The provision of various amino acids modifies GUT-M composition but the consequences on behavior are poorly documented. In chickens, provision of tryptophan has been shown to modify the GUT-M [135]

and to reduce serum corticosterone, serotonin and heat shock protein 70. These results can be related with other studies demonstrating that tryptophan metabolism into serotonin is involved in feather pecking behavior [136] and that its supplementation can reduce gentle feather pecking behavior in this species [137]. Moreover, a better understanding of the roles of GUT-M in feeding behavior, especially in modulation of appetite and satiety, could have large consequences on animal nutrition. Animal nutrition is presently based on our knowledge of needs and the ability of various diets to fulfil these needs but if it is considered that the GUT-M also modulates appetite and satiety as shown in rodents and humans, this could have large consequences on feed preferences and intake if it is established in farm animal. In future, nutritional rules for farm animals could be improved by increased knowledge about the way bacterial growth modulates the digestive cues related to satiety and taste, and about peptides produced by bacteria that could be involved in the hypothalamic regulation of appetite. A better understanding of appetite regulation would help managing feed intake, feed frustration and anorexia related to disease states.

From a practical point of view, provision of pre- or probiotics in addition to the diet is the easiest way to influence the GUT-M *via* nutrition. Prebiotics and probiotics can have complementary effects, however there are expensive contrary to the modifications of the feed composition. For poultry, probiotics could be fed at the hatchery in order to improve gut colonization. *In ovo* injection of prebiotics or a combination of pre-and probiotic at the 12^{th} day of the embryonic development has been shown to influence host transcription and appears to stimulate the proliferation of the embryonic GUT-M [138,139]. We need more studies to quantify the long-term effect on health and behavior of such provision of pre- or probiotics at the hatchery. An exciting new perspective on GUT-M - host symbiosis comes from the finding that pioneer colonizers, the first bacteria to reach the neonatal gut, will impact the future health since they can directly influence the development of the intestine and the nutrient matrix it provides for sequential implantation of future microorganisms [140].

In mammals, the GUT-M can even be orientated before birth since the maternal diet can influence GUT-M composition in the offspring. As previously mentioned in rodents [141], the maternal diet can influence GUT-M activity in the offspring and this modulation can influence social behavior. In piglets, GUT-M activity (measured by quantitative analysis of SCFAs) is reduced and responses to reward are modified when sows are fed with a high-sugar and fat diet during pregnancy [117]. Such demonstrations suggest that nutrition of breeders may be able to modulate behavior in the offspring and that this has to be investigated in farm animals.

3- *Improving management practices through the GUT-M*

Many husbandry situations can give rise to stress states during animal rearing and this state may modify GUT-M composition which can reinforce the negative effects of stress. Based on these interactions described among the MGBA, it appears that protecting a balanced GUT-M would help in the management of stress [13,142] and this would help preventing infection [24]. We saw that most of studies focusing on behavior used probiotics that were able to decrease stress cues [114,115] and to modify behavior and prevent various diseases such as acidosis in ruminants [128,143]. Nutritional transition focusing specific GUT-M changes could also help reducing stress since we saw in horses that these GUT-M changes due to increased diet energy are related to behavioral stress response related to particular bacteria [116].

A better knowledge about the MGBA of the farm animal would also help to detect silent infections and then modify the management of many diseases. Changes in behavior are commonly used to detect illness. Inflammatory states are commonly associated with changes in a reduction of comfort and feeding behavior and in motivation for social interactions. However, some pathogens do not induce illness cues at animal level and this asymptomatic carrier state prevents the detection of such infections. The existence of the MGBA suggests that changes in behavior could happen, even if the host does not express classical sickness behavior commonly associated with disease. This would

explain why the presence of Campylobacter, a bacterium that is involved in a foodborne toxi-infection in human, can be detected by automated behavioral analysis of poultry flocks [144] while no clinical cue can be detected in chickens carrying this bacterium. Another example is given with chickens that have been infected by *Salmonella* Enteritidis and that are also considered as asymptomatic carriers. While no clinical cue can be detected in each infected chick, changes in behavior occur during the weeks and sometimes the days following infection: reduction in feeding [145], in inter-individual distances and in running bouts [146].

4- Needs for improved tools to use the MGBA

This enhanced understanding requires improved methods. The use of germ-free animals (mainly rodents but also chicks) has been critical to our understanding of how the GUT-M can influence health, disease, and behavior especially when coupled with mono-association (inoculation with a single bacterial strain), defined microbiota, or humanized microbiota strategies. To circumvent some of the physiological disadvantages of germ-free and mono-associated mice (poor barrier effect, maturation of immune response and intestine development) while still maintaining a controlled microbiota, mice reconstituted with defined microbiota were established. Schaedler initiated these studies by defining key cultivable bacteria, which were experimentally inoculated to germ-free mice in various “cocktails” of aerobes and anaerobes [147,148]. The cocktail was refined and standardized resulting in “altered Schaedler's flora” (ASF) that is now most commonly used in gnotobiotic research and companies [149]. The ASF community offers significant advantages to study homeostatic as well as disease-related interactions by taking advantage of a well-defined, limited community of microorganisms. Now, it would be interesting to develop such cocktails of bacteria for each farm animal species to go further in the MGBA studies.

Additionally, moving forward, we face a number of challenges in each animal model. For example, the vast majority of intestinal microorganisms remain uncultivable. Can novel culture methods or

creative strategies to eliminate selectively targeted agents be developed? How to include other GUT-M members like viruses, protozoa and fungi in the MGBA analyzes? How do we avoid microbiota drift to optimize reproducibility among studies? Can microbiota be banked adequately for future studies? Facing these issues is a great challenge to improve our knowledge about the MGBA in farm animals.

Conclusion

Thanks to the many ways of manipulating the GUT-M (germ-free, antibiotics, probiotics, diet, microbiota transfer), it is increasingly recognized that the microorganisms colonizing the host's digestive tract can directly or indirectly act on the nervous system and influence host behavior. The majority of studies on the subject have used rodent or human models and it seems that the GUT-M can influence emotional behavior, memory capacities, social and feeding behavior also in poultry, pig, horse and ruminants. However, germ-free animals reared and kept in isolators are poor models for farm animals and it will be a big step to apply results to the farm environment. Many studies are correlational and the presence of specific microorganisms is not controlled experimentally while investigations with microbiota reconstitutions that reverse behavioral changes and investigate mechanisms are still lacking. Many methodological issues have to be faced to get a better knowledge about the variations of the GUT-M, the role it can play in the MGBA of the farm animal and how it could help reducing certain deleterious behaviors and increasing behavioral adaptation *via* genetic selection, nutrition, stress management and detection of silent infections. In summary, it is necessary to take this MGBA concept into account in an applied interest to farming conditions since it can have large consequences in animal welfare.

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Table and figure captions

Table: Taxonomic profiles of major gut bacterial communities at the phylum level in farm animals using 16 rRNA gene pyrosequencing (Percentage of sequences assigned), based on [89], [150], [77], [151], [98], [95], [78], [85].

Figure 1: Gut microbiota as a key actor for animal welfare

Figure 2: Influence of the microbiota-gut-brain axis on behavior

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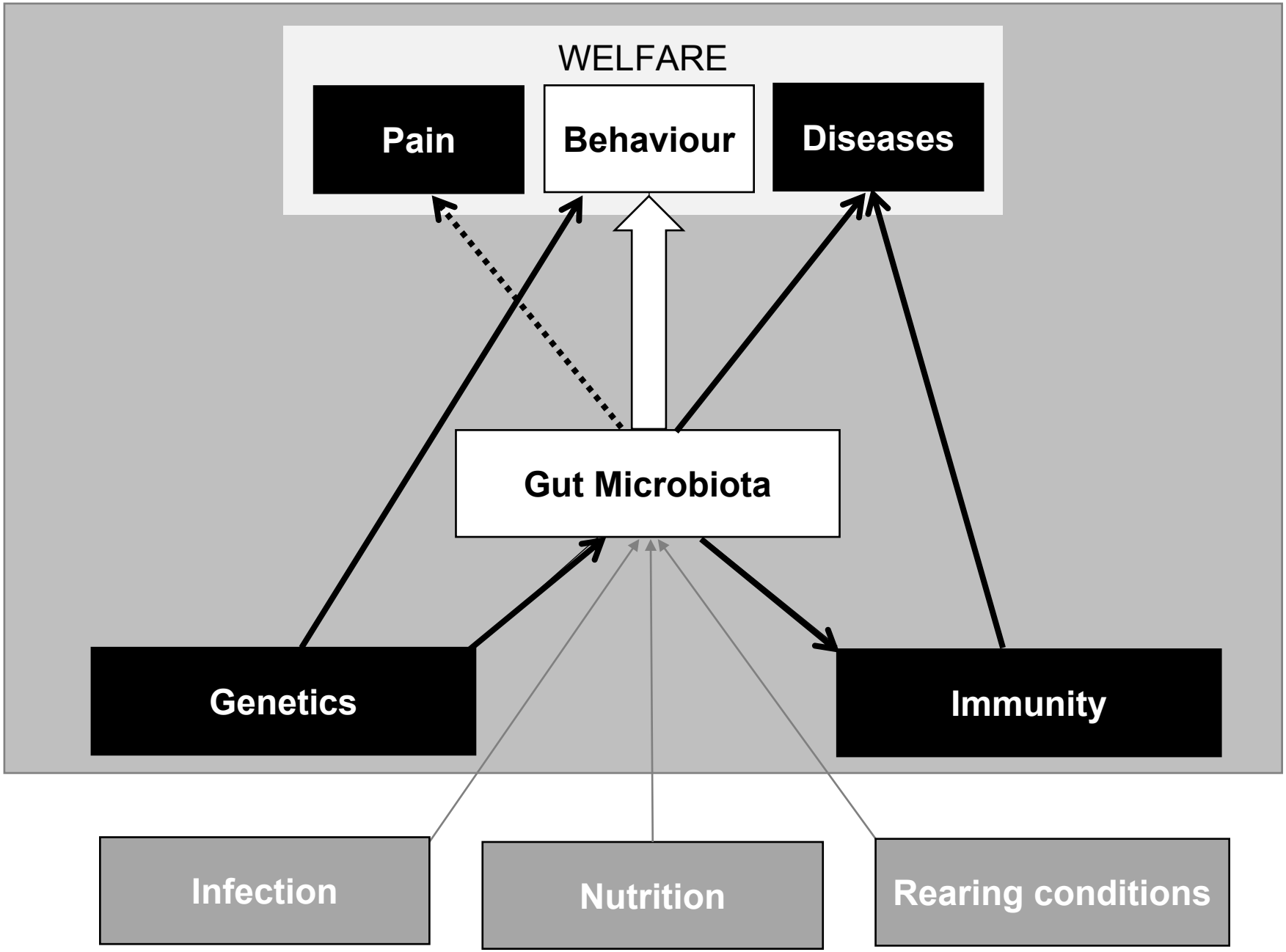
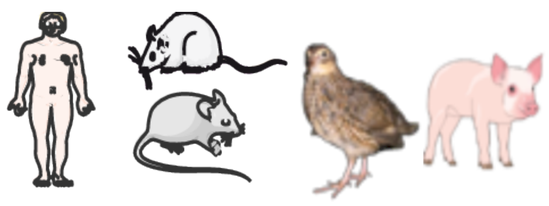


Figure 1: Gut microbiota as a key actor for animal welfare

Anxiety-like behavior

✖ − ✚ ▲ ▮

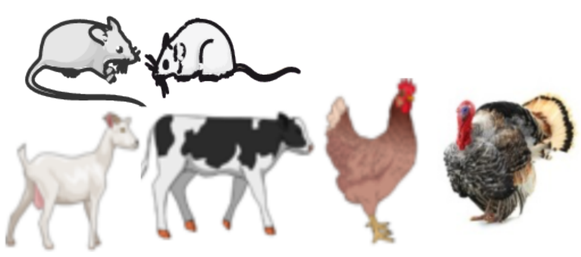


Memory capacities

✖ − ✚ ▲ ●

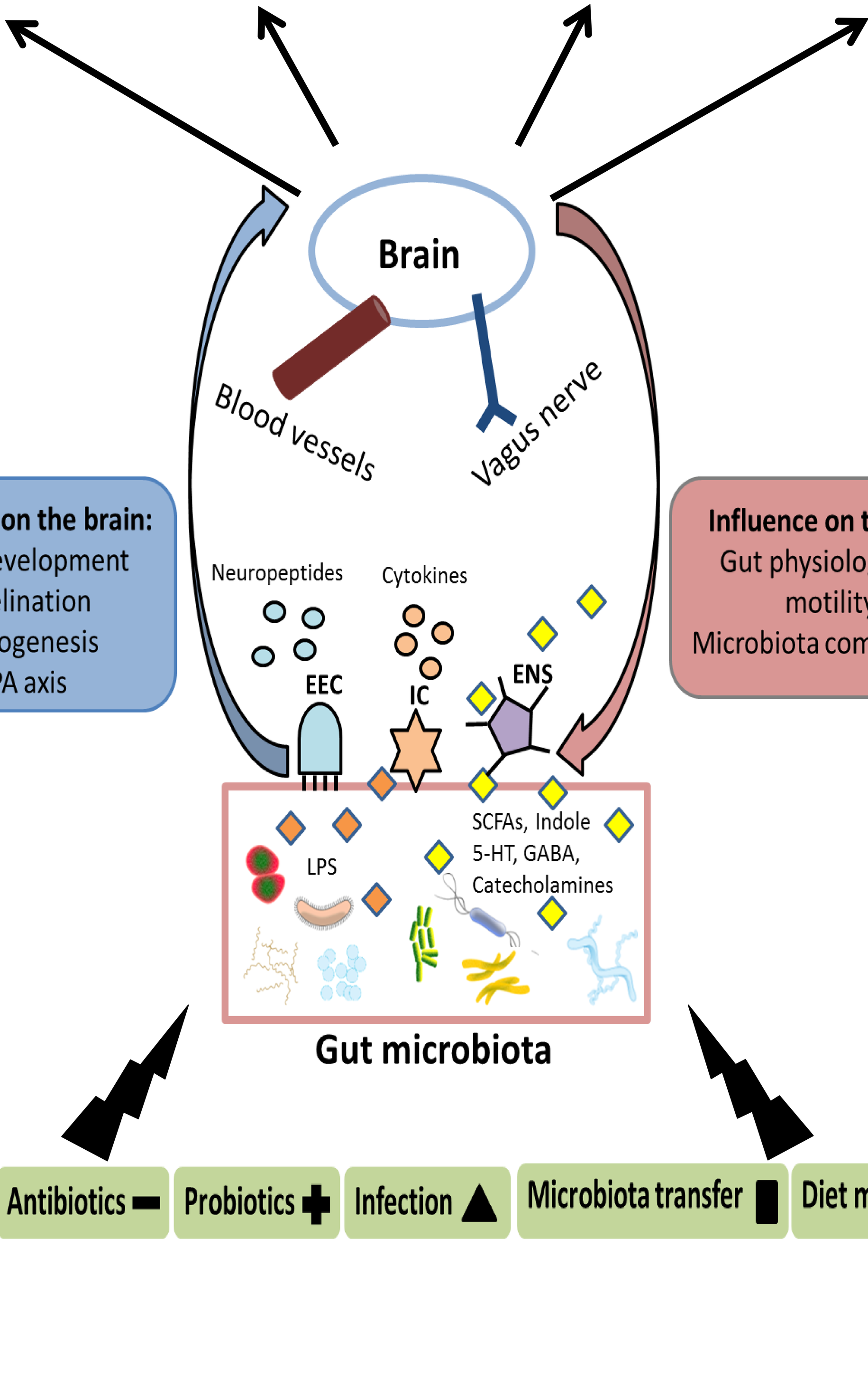
Social behavior

✖ ✚ ●



Feeding behavior

− ✚ ▲ ●



Influence on the brain:
Brain development
Myelination
Neurogenesis
HPA axis

Influence on the gut:
Gut physiology and motility
Microbiota composition

Germ-free ✖ | Antibiotics − | Probiotics ✚ | Infection ▲ | Microbiota transfer ▮ | Diet modification ●

Figure 2: Influence of the MGBA on behavior.
Different strategies can be used to modify the gut microbiota composition (indicated at the bottom of the figure: germ-free animals, antibiotic, probiotic, pathogen infection, microbiota transfer, dietary modification). The gut microbiota composed of viruses, archaea and bacteria can act directly or indirectly on the brain via cell structural components (lipo-polysaccharides = LPS) or with the release of microbial metabolites (short-chain fatty acids = SCFAs, neurotransmitters, catecholamines, indole ...), that can be absorbed by the intestinal epithelium, then released into the bloodstream and cross the blood-brain barrier; use the immune pathway and the production of pro-inflammatory cytokines by immune cells (IC); stimulate the enteric nervous system (ENS) and its sensory neurons or induce the secretion of neuropeptides by entero-endocrine cells (EEC). All these molecules can reach the brain via the blood circulation or the activation of vagal afferent fibers. In addition to the effects on brain development, myelination, neurogenesis or HPA axis activity, the consequences of the MGBA have been investigated on the anxiety-like behavior in human, rodent, turkey, quail and horse; on memory capacities in human, rodent, quail and pig; on social behavior in human, rodent, chicken, turkey and quail; on feeding behavior in rodent, goat, cow, chicken and turkey. The bi-directional communication of this MGBA also involve effects of the nervous system on gut microbiota motility, physiology and composition.