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Impact of the gut microbiota on the neuroendocrine and behavioural responses to stress in rodents

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Abstract – The gastro-intestinal tract hosts a complex microbial ecosystem, the gut microbiota, whose collective genome coding capacity exceeds that of the host genome. The gut microbiota is nowadays regarded as a full organ, likely to contribute to the development of pathologies when its dynamic balance is disrupted (dysbiosis). In the last decade, evidence emerged that the gut microbiota influences brain development and function. In particular, comparisons between germ-free and conventional laboratory rodents showed that the absence of the gut microbiota exacerbates the hypothalamic pituitary adrenal (HPA) system reactivity to stress and alters the anxiety-like behaviour. Furthermore, the dysfunctions observed in germ-free animals can be corrected if the gut microbiota is restored in early life but not in adulthood, suggesting a critical period for microbiota imprinting on the responsiveness to stress. The modes of action are still to be deciphered. They may involve transport of neuroactive bacterial metabolites to the brain through the bloodstream, stimulation of the vagus nerve or of entero-endocrine cells, or modulation of the immune system and, consequently, of the inflammatory status. The discovery that the gut microbiota regulates the neuroendocrine and behavioural responses to stress paves the way for the hypothesis that gut microbiota dysbioses could contribute to the pathophysiology of anxiety-related disorders. In this regard, treatments of anxiety-prone rodent strains with probiotics or antibiotics aimed at modifying their gut microbiota have shown an anxiolytic-like activity. Clinical trials are now needed to know if results obtained in preclinical studies can translate to humans.

Keywords: Gut-brain axis / germ-free / probiotic / hypothalamic pituitary adrenal axis / anxiety

Résumé – Effet du microbiote intestinal sur les réponses neuroendocrinienne et comportementale au stress. Le tractus gastro-intestinal héberge une communauté microbienne complexe appelée microbiote, dont le potentiel génétique excède celui de l'hôte en richesse et diversité. Le microbiote intestinal est considéré aujourd'hui comme un véritable organe, susceptible de contribuer au développement de pathologies si son équilibre est rompu (on parle alors de dysbiose). Au cours de la dernière décennie, des travaux ont commencé à mettre en évidence que le microbiote intestinal influençait le développement et le fonctionnement du cerveau. En particulier, des comparaisons entre rongeurs axéniques et conventionnels ont montré que l'absence de microbiote intestinal intensifiait la réponse au stress de l'axe corticotrope et modifiait le niveau d'anxiété. Ces anomalies ne peuvent être corrigées que si la restauration du microbiote chez les animaux axéniques intervient avant l'âge adulte. Ceci suggère l'existence d'une période critique du développement au cours de laquelle le microbiote influence la maturation des structures cérébrales impliquées dans la réponse au stress. Les mécanismes d'action ne sont pas encore complètement élucidés. Pourraient intervenir des métabolites microbiens neuro-actifs, atteignant le cerveau par voie sanguine, une stimulation des afférences intestinales du nerf vague, une stimulation des cellules endocrines de la paroi intestinale, ou une modulation du système immunitaire et, par conséquent, du statut inflammatoire de l'organisme. La découverte que le microbiote intestinal régule les réponses neuroendocrinienne et comportementale au stress conduit à l'hypothèse que des dysbioses du microbiote pourraient contribuer à la physiopathologie des troubles anxieux ou des troubles de l'humeur ayant une composante anxieuse. À cet égard, la modulation du microbiote intestinal avec des probiotiques ou des antibiotiques chez des lignées de rongeurs prédisposées à l'anxiété a un effet de type anxiolytique. Des essais cliniques sont maintenant nécessaires pour déterminer si ces résultats précliniques sont transposables à l'Homme.

Mots clés : Axe intestin-cerveau / axénie / probiotique / axe corticotrope / anxiété

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1 Introduction

The gastro-intestinal tract is inhabited with 10^{14} bacterial cells, a figure ten times greater than the total of eukaryotic cells in the human body. This gut microbiota consists of around 1000 bacterial species and 7000 strains, whose collective genome coding capacity is thought to be 150 times greater than that of the host genome. The gut microbiota is regarded nowadays as a full organ exchanging molecular signals with the intestinal wall, and hence communicating with the whole body (Delzenne *et al.*, 2011; Grenham *et al.*, 2011; Lozupone *et al.*, 2012).

The gut microbiota is present all along the gastro-intestinal tract. However its main density is in the colon with around 10^{11} bacterial cells/g of content, which live in a slightly acidic (pH: 5.7 to 6.9) and anoxic (*Eh*: −100 to −200 mV) environment. The great advances made in the last fifteen years in culture-independent techniques, including quantitative PCR, fluorescent *in situ* hybridization (FISH), genetic fingerprinting and sequencing based methods have revolutionized our knowledge of the taxonomic diversity, community structure and metabolic capability of the gut microbiota (Delzenne *et al.*, 2011; Grenham *et al.*, 2011; Lozupone *et al.*, 2012; Weinstock, 2012). The gut microbiota is dominated by two phylotypes, *Bacteroidetes* and *Firmicutes*, with *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* phyla present in relatively low abundance (Lozupone *et al.*, 2012). However, despite the consistency of these main components, their relative proportions and the species and strains present vary markedly across individuals. Many factors, including the genetic background, the physiological characteristics, the age and the dietary habits of the host, drive these interindividual differences (Lozupone *et al.*, 2012). This has been elegantly illustrated by a gut microbiota transfer experiment between mouse and zebrafish. When germ-free mice were colonised with zebrafish microbiota and germ-free zebrafish with mouse microbiota, the host reshaped the transplanted microbiota in both cases (Rawls *et al.*, 2006). This observation indicates that the physiological characteristics, habitats and diet exert a key influence on the gut microbiota community structure but the underlying mechanisms remain to be identified.

At birth, the gut of the neonate is sterile. Colonization commences when delivery exposes the infant to maternal and environmental micro-organisms. The initial infant microbiota has primarily a maternal signature; first colonizers are enterobacteria, enterococci, staphylococci, bifidobacteria, *Bacteroides*, *Clostridium*, and arise from the maternal faecal microbiota and, to a lesser extent, from the maternal vaginal microbiota. Then the diversity of the gut microbiota increases as a result of dietary changes (weaning) and environment, and reaches a complex adult-like community structure at around 3 years of age. As a developing ecosystem, the infant gut microbiota is vulnerable and factors such as the delivery mode, breast-feeding *vs.* bottle-feeding or antibiotic treatments may influence the colonization, and hence the community structure and functional capabilities of the gut microbiota in later life (Campeotto *et al.*, 2007; Delzenne *et al.*, 2011; Favier *et al.*, 2002; Grenham *et al.*, 2011; Lozupone *et al.*, 2012). The gut microbiota is sensitive to disturbances in adulthood, too. Acute events such as infections, antibiotics or dietary changes often induce an unstable state, which, in most cases, tends to revert to the pre-disturbance healthy stable state, once the disturbing factor has faded. Sometimes, however, the transient unstable state moves to a degraded stable state, called dysbiosis, which can trigger the development of pathologies. For example, an irritable bowel syndrome can develop on the occasion of an acute gastro-intestinal infection and persist thereafter for years (Dupont, 2011; Lozupone *et al.*, 2012).

Advances in the knowledge of the gut microbiota have spurred research on the connections between this "forgotten organ" (O'Hara and Shanahan, 2006) and its host. Effects of the gut microbiota that had been known for years have been re-examined; they include fermentation of indigestible food fractions (*e.g.* dietary fibre, resistant starch, oligosaccharides), anaerobic metabolism of proteins and peptides, vitamin synthesis, biotransformation of biliary salts and xenobiotics, development and metabolism of the intestinal epithelium, maturation and functioning of the immune system (Delzenne *et al.*, 2011; Hooper *et al.*, 2001; Maynard *et al.*, 2012). In parallel, new functions have been discovered such as the regulation of the energy metabolism (Delzenne *et al.*, 2011; Tremaroli and Bäckhed, 2012) or of the intestinal angiogenesis (Stappenbeck *et al.*, 2002). Lately, evidence has emerged that the gut microbiota takes part in the crosstalk between the gut and the brain, and hence influences brain development and function (Cryan and Dinan, 2012; Grenham *et al.*, 2011). Studies comparing germ-free laboratory rodents with their conventionally colonized counterparts have revealed the influence of the gut microbiota on brain core functions such as the neurotransmission systems, the synaptogenesis or the production of neurotrophins. Impairments of the permeability of the blood-brain barrier, or of memory and cognition have also been reported in germ-free animals (Braniste *et al.*, 2014; Cryan and Dinan, 2012; Grenham *et al.*, 2011). In this review, we will focus on the effects of the gut microbiota on the neuroendocrine and behavioural response to stress as these are, to date, the most documented and consistent data (Foster and Neufeld, 2013; Luna and Foster, 2015).

2 The gut microbiota regulates the neuroendocrine and behavioural responses to stress

2.1 Germ-free rodents have a hyper-responsive hypothalamic pituitary adrenal (HPA) system

To test the influence of the gut microbiota on the reactivity of the hypothalamic pituitary adrenal (HPA) system, we applied an acute stress to germ-free (GF) and conventional, specific pathogen-free (SPF), F344 rats. The experiment consisted in subjecting the animals to an open-field test. Briefly, each rat was placed for 6 min in a rectangular open arena, which was strongly illuminated at the centre; openness and excessive light are aversives that cause a stress to the animals. Age-matched GF and SPF rats that were not subjected to the open-field test served as non-stressed counterparts and corticosterone was measured in blood collected just after the test in

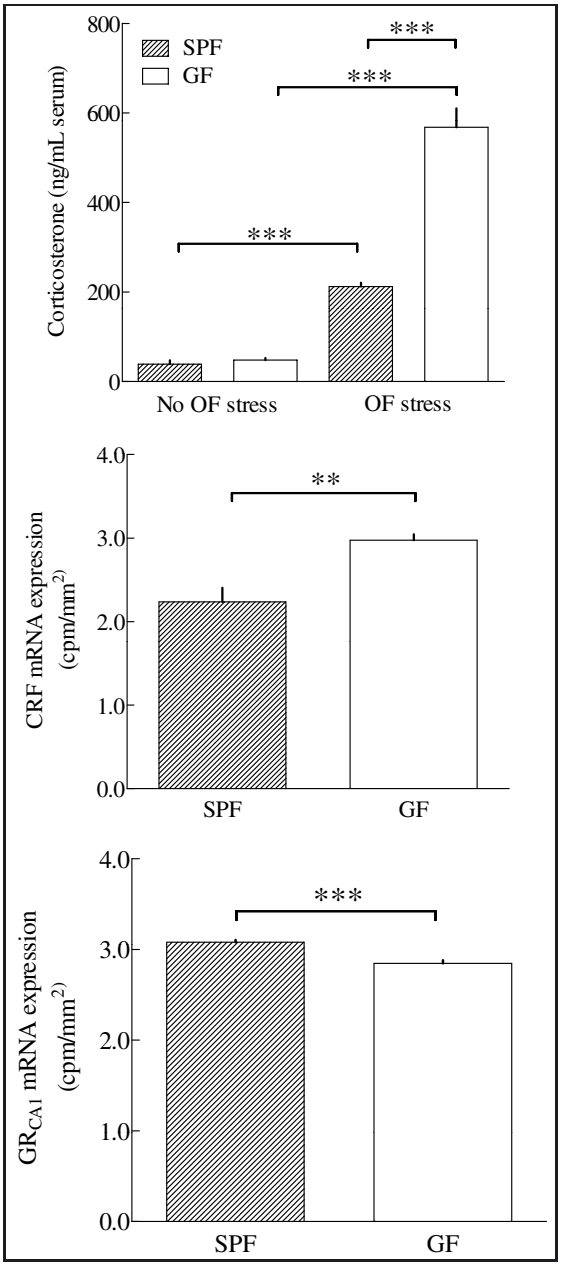


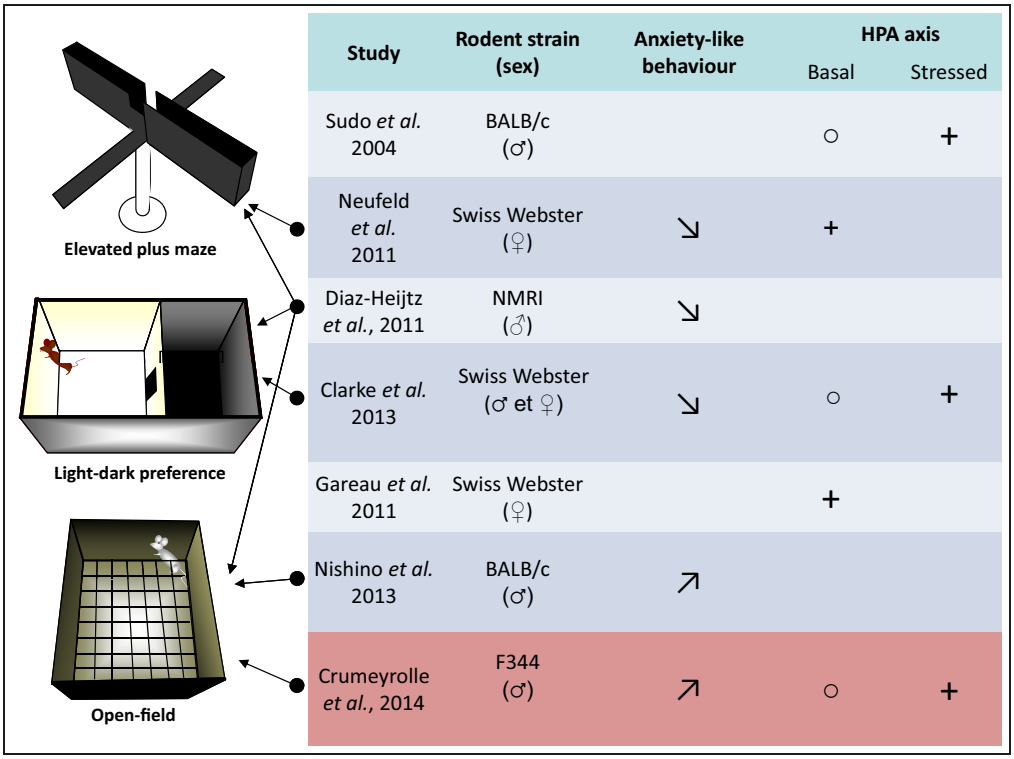
Fig. 1. Comparison of the hypothalamic pituitary adrenal (HPA) system reactivity between germ-free (GF) and conventional (SPF) F344 rats after an open-field (OF) stress. A greater elevation of systemic corticosterone occurred in GF OF-stressed rats than in the SPF counterparts. This was accompanied by a greater corticotropin releasing factor (CRF) mRNA expression level in the hypothalamus and a lower glucocorticoid receptor (GR) mRNA expression level in the hippocampal CA_1 area. Data are means with sem (n = 8 to 12 rats/group). ** $p < 0.01$, *** $p < 0.001$.

both stressed and non-stressed animals. Stressed GF rats exhibited a serum corticosterone concentration almost 3 times greater than stressed SPF rats, whereas no difference was observed between non-stressed GF and SPF rats. As shown in Figure 1, the dramatic difference in stressed rats was accompanied by an increase in the expression of the gene encoding the corticotropin releasing factor (CRF) in the hypothalamus, and by a decrease in the expression of the gene encoding the glucocorticoid receptor (GR) in the hippocampus of the GF rats (Crumeyrolle-Arias *et al.*, 2014). These data indicate definitely that the gut microbiota regulates the HPA system. They are consistent with the hyper-secretion of corticosterone in response to an acute restraint stress initially described in BALB/c GF mice by Sudo *et al.* (2004) and confirmed by Clarke *et al.* (2013) in Swiss GF mice, using a novel environment stress. Other experiments with Swiss mice showed also an increase of the baseline serum corticosterone concentration in GF animals compared with conventional counterparts (Gareau *et al.*, 2011; Neufeld *et al.*, 2011). Overall, all these studies show that the absence of the gut microbiota fundamentally intensifies the HPA axis activity (Fig. 2).

2.2 Germ-free rodents have an impaired behavioural response to stress

In the open-field test described above, as well as in a social interaction test, the absence of the gut microbiota enhanced the anxiety-like behaviour (Fig. 2). In the social interaction test, the GF F344 rats were more reluctant to social interaction at the beginning of the encounter with an unknown partner; in the open-field test, they hesitated for a longer time before investigating the arena, spent more time in the corners, and avoided the stressful highly-lit centre area (Crumeyrolle-Arias *et al.*, 2014). In contrast with these findings in rats, the absence of the gut microbiota in mice resulted in the majority of the studies in an anxiolytic effect, using other behavioural tasks such as the elevated plus maze or light-dark preference test (Clarke *et al.*, 2013; Diaz-Heijtz *et al.*, 2011; Neufeld *et al.*, 2011) (Fig. 2). Only in one study (Nishino *et al.*, 2013) did GF mice show a greater anxiety-like behaviour than their SPF counterparts, when subjected to the open-field or the marble-burying test (Fig. 2). Such discrepancies could result from methodological differences but the genetic background also plays an important role in the anxious behaviour (Griebel *et al.*, 2000). Interestingly, the F344 rat strain we used, as well as the BALB/c mouse strain chosen by Nishino *et al.* (2013) are both sensitive to stress, while the mice strains chosen in the other studies (Swiss, NMRI) are reported to be moderately emotive. Accordingly, we hypothesize that the gut microbiota has a buffering effect on the behavioural response to an acute stress. Presence of the gut microbiota tones down anxiety-like behaviour in strains genetically prone to anxiety but tones up the same behaviour in strains genetically less prone to anxiety (Crumeyrolle-Arias *et al.*, 2014) (Fig. 3). This hypothesis is consistent with the results of a gut microbiota transfer experiment, which showed a decrease in anxiety-like behaviour in the step-down test after inoculation of anxiety-prone BALB/c GF mice with a microbiota originating from anxiety-resistant Swiss mice and, conversely, an increase of this behaviour after inoculation of Swiss GF mice with BALB/c mice microbiota (Bercik *et al.*, 2011a).

The latter experiment showed that the genetics-associated anxiety-like phenotype can be modulated by colonizing the gut of GF mice in adulthood. However, other findings suggest the existence of a critical period in infancy and adolescence,



Study	Rodent strain (sex)	Anxiety-like behaviour	HPA axis	
			Basal	Stressed
Sudo *et al.* 2004	BALB/c (♂)		○	+
Neufeld *et al.* 2011	Swiss Webster (♀)	↘	+	
Diaz-Heijtz *et al.*, 2011	NMRI (♂)	↘		
Clarke *et al.* 2013	Swiss Webster (♂ et ♀)	↘	○	+
Gareau *et al.* 2011	Swiss Webster (♀)		+	
Nishino *et al.* 2013	BALB/c (♂)	↗		
Crumeyrolle *et al.*, 2014	F344 (♂)	↗	○	+

Fig. 2. Summary of the impact of the absence of the gut microbiota on anxiety-like behaviour and hypothalamic pituitary adrenal (HPA) system reactivity in rodents. All studies consistently report that germ-free life exacerbates the HPA system reactivity. On the contrary, depending on the behavioural task and on the rodent strain, germ-free life increases or decreases the anxiety-like behaviour.

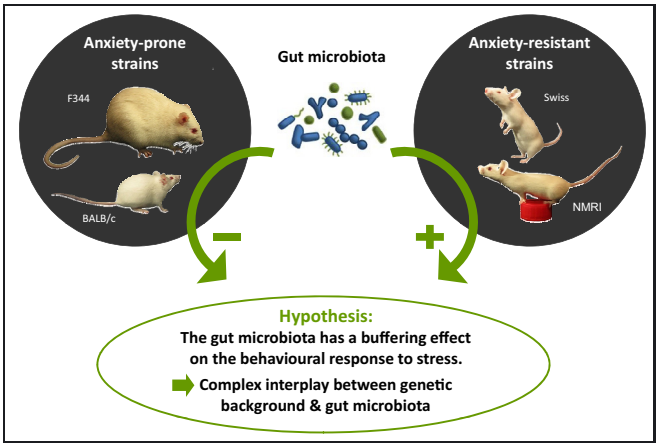


Fig. 3. We hypothesize that the gut microbiota has a buffering effect on the behavioural response to stress. The gut microbiota moderates the anxiety-like behaviour in strains genetically prone to anxiety (F344 rat, BALB/c mouse). On the contrary, it enhances the anxiety-like behaviour in strains genetically resistant to anxiety (Swiss mouse, NMRI mouse).

during which the gut microbiota may imprint on several functions of the central nervous system, including the reactivity to stress. Indeed, if the maladaptive neuroendocrine or behavioural response to an acute stress in GF mice can be corrected by gut colonization with faecal bacteria from SPF mice up to the age of six weeks, this normalization is no longer observed when the gut microbiota restoration is performed in adulthood (Clarke *et al.*, 2013; Sudo *et al.*, 2004).

2.3 Transient alterations of the gut microbiota modify the anxiety-like behaviour

Germ-free animals are invaluable models for proof of concept studies aimed at demonstrating the role of the gut microbiota in physiological processes, but they are not reflective of real life scenarios. In this regard, other approaches using for example antibiotics or probiotics to perturb the gut microbiota of conventionally-raised animals are more valid. As the organism of conventional animals, including their central nervous system, was exposed to the gut microbiota imprinting since early life, it could develop normally. Furthermore, transient alterations of the gut microbiota due to antibiotic treatments or to the consumption of probiotic bacteria are events occurring in humans; therefore, the conclusions drawn from this type of studies are more likely to translate to humans.

Several studies investigating the effect of a disturbed gut microbiota on the anxiety-like behaviour have been reported in the literature. For example, the administration to BALB/c SPF mice of a mixture of non-absorbable antibiotics in drinking water for 7 days markedly altered the composition of the gut microbiota. In this anxiety-prone mouse strain, the gut microbiota disruption resulted into a strong anxiolytic-like effect in the step-down and light-dark preference tests.

Both antibiotic-induced changes in gut microbiota and behaviour were reversible as initial phenotypes were recovered two weeks after the last antibiotic administration. Furthermore, the lack of effect of the antibiotic treatment in BALB/c GF mice definitely ascertained that the gut microbiota disruption was responsible for the behavioural changes observed in the SPF mice (Bercik *et al.*, 2011a).

Other groups have made use of probiotics. Again in BALB/c SPF mice, a daily gavage for 28 days with the probiotic *Lactobacillus rhamnosus* JB-1 reduced stress-induced plasma corticosterone concentration and anxiety-like behaviour in the elevated plus-maze test (Bravo *et al.*, 2011). A more recent study from the same group, using different probiotic strains (*Bifidobacterium longum* 1714 and *Bifidobacterium breve* 1205) and different behavioural tasks (open-field, elevated plus maze and marble burying tests) confirmed the anxiolytic-like activity of probiotics in the anxiety-prone BALB/c mouse strain (Savignac *et al.*, 2014). Similar findings were obtained in rats. A probiotic combination (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) administered by gavage during 7 days to Wistar rats significantly reduced anxiety-like behaviour in the conditioned defensive burying test (Messaoudi *et al.*, 2011).

3 How does the gut microbiota communicate with the brain to influence the anxiety-like behaviour?

It is clear that there is communication between the gut microbiota and the brain. How such communication occurs is not yet fully understood but multiple mechanisms could be involved (Fig. 4). First, bacterial cells could signal the host through their structural molecules, such as cell wall components (*e.g.* lipo-polysaccharides in Gram negative bacteria) or flagellum proteins (*e.g.* flagellin). They also produce a vast array of metabolites, including short-chain fatty acids released from carbohydrate fermentation (*e.g.* butyrate), amino-acid derivatives (*e.g.* ammonia and phenolic compounds), biliary salt metabolites, secondary metabolites produced from dietary phytochemicals or xenobiotics (*e.g.* polyphenol derivatives), and even, in some bacterial species, neurotransmitter-like molecules (*e.g.* acetylcholine). Some of these molecules can be absorbed through the intestinal epithelium and finally be found in the systemic circulation (Wikoff *et al.*, 2009), whereupon they can cross the blood-brain barrier and act on the brain tissues. Another key direct pathway is the activation of the parasympathetic arm of the autonomic nervous system (vagus nerve) and of the enteric nervous system. Finally, the gut microbiota can also activate the immune system, thus influencing the balance of the circulating levels of pro-inflammatory and anti-inflammatory cytokines that directly affect brain function, or trigger the production of neuropeptides by the entero-endocrine cells present in the intestinal epithelium (Cryan and Dinan, 2012).

A gut microbiota dysbiosis can lead to an excessive production of bacterial metabolites that become toxic for the organism. This is the case in the hepatic encephalopathy, in which ammonia, indole derivatives or short-chain fatty acids

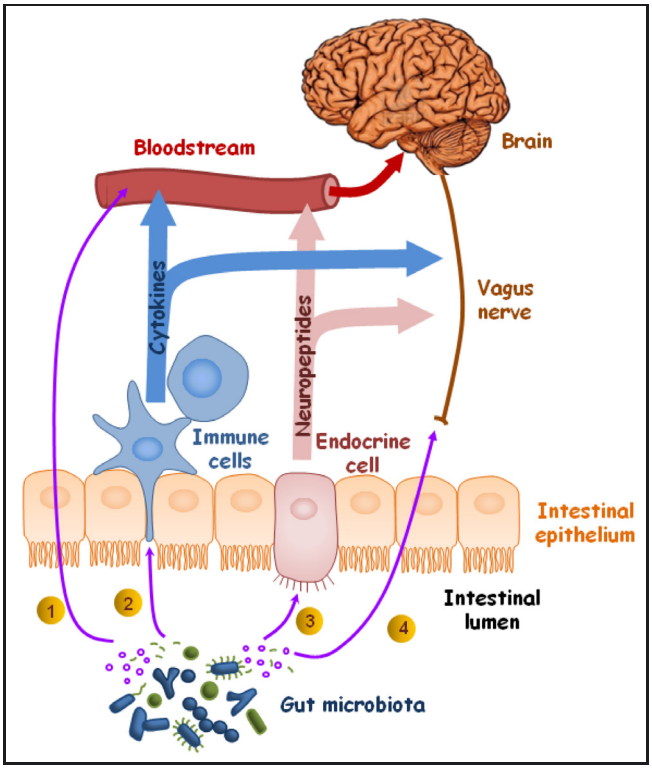


Fig. 4. Pathways involved in communication between the gut microbiota and the brain. Effective bacterial molecules can be metabolites released in the gut lumen (*e.g.* fermentation products or neurotransmitter-like molecules, here in green) or cell structural components (*e.g.* lipo-polysaccharides of the cell wall, here in purple). They can reach the brain *via* the systemic circulation (1), signal the immune system and trigger the production of cytokines (2), signal the entero-endocrine cells and trigger the production of neuropeptides (3), or activate the vagal and enteric nervous system afferent fibers (4).

accumulate in the systemic circulation, cross the blood-brain barrier and contribute to neuropsychological dysfunctions, including anxiety, cognitive impairment, mental confusion, and sedation (Frederick, 2009; Moroni *et al.*, 1998). Other examples of a direct action of bacterial metabolites on the brain are related to behavioural abnormalities in neurodevelopmental disorders. In studying the relationships between gut microbiota dysbiosis and behaviour in a mouse model known to display features of the autism spectrum disorder, Hsiao *et al.* (2013) identified in these animals a serum metabolite of bacterial origin, the 4-ethylphenylsulfate (4-EPS), whose concentration was 46-fold higher than in naive mice. Daily systemic administration of 4-EPS to naive mice for three weeks induced in an open-field test an anxiety-like behaviour similar to that observed in the diseased mice. Interestingly, 4-EPS is chemically related to *p*-cresol (4-methylphenol), an end-product of bacterial tyrosine metabolism, which has been reported to be a possible urinary biomarker for autism (Altieri *et al.*, 2011). The involvement of the autonomic nervous system in the communication between the gut microbiota and the brain was demonstrated by vagotomy experiments. In their study on the anxiolytic-like activity of the probiotic *L. rhamnosus* JB-1 in

anxiety-prone BALB/c mice subjected to an open-field test, Bravo *et al.* (2011) showed that a subdiaphragmatic vagotomy nullified the probiotic effect. This was also the case in another study using a different probiotic (*B. longum* NCC3001) and a different behavioural task (step-down test) (Bercik *et al.*, 2011b). However, the mechanisms through which vagal afferents became activated by the gut microbiota are still unclear. In addition, in some instances, it has been so far impossible to unravel the mechanisms explaining the behavioural changes induced by a gut microbiota modification. In their study on the anxiolytic-like activity of oral antibiotics in the anxiety-prone BALB/c mouse strain, Bercik *et al.* (2011a) found that this activity was independent of the vagal integrity and of the intestinal neurotransmitter and cytokine levels. Clearly, there is currently a need for further mechanistic studies to explain the relative contributions of the different putative pathways through which the gut microbiota and the brain communicate.

4 Translation from preclinical studies to humans

Although most of the informations about the effect of the gut microbiota on the neuroendocrine and behavioural responses to stress derive from experimental studies in animals, there is a growing body of clinical studies assessing this issue in humans. An interesting example is the study by Messaoudi *et al.* (2011) combining a preclinical study in rats and a clinical study in healthy humans. The preclinical study showed an anxiolytic-like activity of the probiotic combination *L. helveticus* R0052 and *B. longum* R0175. In the clinical study, which was double-blind and placebo-controlled, daily consumption of the probiotic combination for one month reduced psychological distress as measured with questionnaires assessing mood and anxiety levels self-perception. In another double-blind, placebo-controlled study, the consumption for three weeks of a milk drink containing the probiotic *Lactobacillus casei* Shirota improved mood in the one third of the healthy volunteers whose mood was initially poor (Benton *et al.*, 2007). Similar findings were obtained recently with a multispecies probiotic formulation (*Bifidobacterium bifidum* W23, *Bifidobacterium animalis* subsp. *lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *L. casei* W56, *Lactobacillus salivarius* W24, and *Lactococcus lactis* W19 and W58) consumed during four weeks by healthy subject (Steenbergen *et al.*, 2015). Generally, mechanisms of action still need to be identified. In the study by Messaoudi *et al.* (2011), the probiotic consumption reduced the urinary concentration of free cortisol, providing a potential mechanism to explain the mood improvement. A functional magnetic resonance imaging study revealed that a four-week intake of a multispecies probiotic formulation (*B. animalis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactococcus lactis* subsp. *lactis*) affected the activity of the brain structures that control processing of emotion and sensation (Tillisch *et al.*, 2013). It is yet to be determined if the probiotics used in those clinical trials acted *per se* or through a gut microbiota modification, and which communication pathways between gut and brain were involved. Nevertheless, there are now sufficient preclinical studies and clinical trials in healthy humans to justify to undertake clinical studies in humans suffering from anxiety-related disorders. In this regard, it is noticeable that differences in gut microbiota composition have been recently shown in depressed patients *vs.* healthy controls (Jiang *et al.*, 2015; Naseribafrouei *et al.*, 2014), and that one study reported an improvement in the symptoms of depressive patients when an antibiotic, minocycline, was given orally for 6 weeks in addition to the antidepressant treatment (Miyaoka *et al.*, 2012).

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Disclosure

The authors declare no conflict of interest.

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