Direct implications between prenatal alcohol consumption and gut microbiome in the neurodevelopment of mice offspring – focusing on foetal alcoholic spectrum disorders

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Running Head: Effect of prenatal alcohol consumption on gut microbiome and neurodevelopment

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Abstract

Disorders associated with substance abuse are a major public health crisis with few treatment options. According to World Health Organization (WHO) ethanol is the most widely used drug in the world, and it represents a risk factor for the advent of disease, disability, and eventually death. Foetal Alcoholic Spectrum Disorders (FASD) is a diagnostic term to describe the range of effects that can occur in an individual whose mother drank alcohol during pregnancy. These effects encompass both physical, mental, behavioural and further lifelong disabilities. Besides, ethanol can harm the gut microbiota. Gut microbiome is firstly acquired from the mother and it is crucial for intestinal homeostasis during hosts' lifetime. It is responsible for producing metabolites that benefits and protects the host from harm microbial colonization. Knowledge about the interactions between human gut microbes and the developing nervous system is still scarce. Nevertheless, animal models have shown that gut bacteria and microbial metabolites are strongly associated with Central Nervous System (CNS) homeostasis.

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Endotoxins such as Lipopolysaccharides (LPS) are hypothesized to have a major role in neurodegeneration, however, conclusions must be taken with care due to differences in sensitivity between humans and mice. In this review we focus on the role of gut microbiota on the neurodevelopment of mice when ethanol consumption is one of the major stressors during prenatal period. We detail the range of the endotoxin hypothesis in describing endotoxins' contribution to neurodegeneration and the influence that kynurenine pathway has on the process.

Introduction

Alcohol is a major threat to human health in modern society, accounting for 6% of total deaths each year (1). Several diseases caused by alcohol are defined by a spectrum of disorders, as for instance Alcoholic Liver Disease (ALD) and Foetal Alcoholic Syndrome Disorders (FASD). ALD progresses from asymptomatic liver steatosis to more severe features such as fibrosis, cirrhosis and alcoholic hepatitis. Moreover, the progression to acute disease is associated with dysbiosis caused by alcoholism (2). Foetal Alcohol Syndrome Disorders (FASD) represent a cluster of abnormalities, including growth deficiencies and neurological impairments. From that point, a collection of studies using animal models of prenatal alcoholic exposure (PAE) reported and confirmed a spectrum of deficits obtained by the offspring of alcoholic mothers (3).

The role of gastrointestinal tract in immune system homeostasis is central because it harbours around 70% of the lymphoid system (4). The gut harbours dense neuronal innervation exclusively inside which mediates the connection to the spinal cord and brain. One major neuronal connection is the vague nerve, capable of directly sense and send signals between various organs including the gut, and the brainstem. Vague nerve directs the connection between the brainstem and the gastrointestinal tract, which then links the muscular and mucosal layers along the gut directly to the brain. This signalling pathway affects feeding, anxiety, depressive and social behaviour (7). The gastrointestinal tract has a tremendous role due to the diversity of cell types enclosed and surrounding it, and due to exposure to external influencers such as dietary components and gut microbiota on the luminal side (7).

Chronic alcohol consumption can cause gut dysbiosis and damage gut barrier function. Damage in the gut barrier will increase permeability, allowing bacteria and bacterial products such as endotoxins into circulation which can lead to systemic

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inflammation and various other diseases (5, 9). Gut microbes, in particular those residing in the colon, play a dynamic interaction with the host, where molecules flow from one to another and vice-versa, inside the gut and beyond to other body sites. This dynamism affects offspring development during and after pregnancy. Thus, pregnancy and perinatal period represent a window of influence for the foetus as it is exposed to a broad collection of microbial-related signals and metabolites (6). The neonatal microbiome is generally acquired from the mother, and its development and maturation seem to be determined during the exchange happening before birth (8). The genetic landscape, nutrition routines of the mother, and the capacity of the 'mother-foetus system' to metabolize alcohol can increase or attenuate the risk and the action of alcohol on the foetus development (2). It is known the ability of alcohol to cross placental and blood brain barrier (BBB). Thus, alcohol presents an important toxic risk for the foetus by acting directly or by modulating target molecules and mechanisms present at different development stages. Nevertheless, different doses and thresholds influence the effect upon exposure, which in turn may explain the appearance of different FASD phenotypes (10).

Gut microbiome holds the promise to be targeted for translational research for substance use disorders. However, current knowledge is insufficient to elucidate basic questions related to gut microbiota composition in substances abuse situations compared with physiological states. Up until now, it was not found any clear microbial signature induced by all classes of drugs of abuse, alcohol included. However, preclinical and clinical evidence relating bacterial dysbiosis in response to drugs of abuse is growing (11).

Rodent models are crucial for researching topics about the mechanistic understanding of gut-brain signalling in cases of addiction and other psychiatric diseases. Additionally, the majority of data available about the role of gut microbiota on neurodevelopment and psychiatric disorders comes from animal models. Rodent models, such as germ-free mice allow a targeted manipulation of microbial gut content and thus a better interrogation for further translational in other organisms, such as humans (11). Studies on these models has been accomplished by researching antibiotic treatments, faecal transplantation, and gut colonization with selective microorganisms (12). Recent studies have linked the gut microbiota with social behaviour in mice in a modulative manner (13, 14, 15, 16).

In mammals, the main catabolic pathway is the kynurenine pathway (KP). In the KP there is described several neuroactive metabolites, such as kynurenic acid (KYNA),

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3-hydroxukynurenine (3-HK), and quinolinic acid (QA). KP is crucial for normal neurological function, and unbalanced levels of KYNA and QA may contribute to explain many neurological disorders' onset at some extent(17). In that sense, alterations on the normal KP function has been associated with schizophrenia, among other psychiatric disorders (18). Kynurenine (KYN) can be found in greater concentrations inside the brain than anywhere else in the body, including peripheral organs (19). Due to the polarity of KYNA and the lack of transporters, KYNA cannot cross BBB, and therefore it is stated that it must be formed locally in the brain from precursor KYN (20). In this paper we review the broad impact and modulatory role of the KP in neurodevelopment. Focusing on alcohol abuse settings, we analyse recent evidence relating the unbalanced offspring's gut microbiome and their further neurodevelopment.

1. Effect of alcohol consumption on mice gut microbiome

Infants acquire their microbiome most probably from their mothers during pregnancy, then it develops until relative maturation in the first three years of life (21). Prenatal environment and early postnatal colonization are crucial for a normal general and organ-specific development (12). Albeit the increasing amount of data, there is still no strong evidence about the gut microbiota influence in alcohol use disorder, with the remaining issues surrounding both physical and biological nuances still to be uncovered (22).

Preliminary studies indicate that the gut microbiome is dysregulated by the action of psychostimulants, alcohol, and opioids (11, 23, 24, 25). Chronic alcohol abuse has a prejudicial effect on the brain. It alters the composition and function of the gut microbiota (16, 27, 28, 29, 21), and cause liver lesions by the disruption of the intestinal barrier (26). In the case of alcohol use disorder (AUD), metagenomic studies on human patients showed that the gut microbiome had a higher proportion of functions related to alcohol metabolism (30). Even in the absence of alcohol exposure, AUD patients still have higher ethanol levels in faeces, confirming the presence of adapted microbes, an alcohol-dependent (AD) microbiota (16). Alcohol dependent (AD) microbiota defines the microbes present in the gut that produce alcohol. AD microbiota comprises species such as *Clostridium* spp., *Lactococcus* spp., *Turicibacter* spp., and *Akkermansia* spp., and they all share the functional ability to produce and/ or metabolize ethanol. Interestingly, when mice are not exposed to alcohol there still may be found increased portal vein ethanol

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concentration and this suggests that a higher colonization of AD microbiota occurs. It seems that the gut microbiome plays a role in the development of alcohol addiction.

A correlation was previously found between an increased intestinal permeability and physiological symptoms like depression, anxiety, and alcohol craving (31). Microbial changes also revealed correlation with altered intestinal permeability, however, germ-free mice reveal decreased depression and anxiety behaviours, and the same can be found in antibiotic-treated mice (32, 14, 33). Generally, in mice exposed with alcohol it was observed a decrease in *Proteobacteria* and specifically in *Enterobacteriaceae* (26).

Studies in humans showed another aggravated outcome of alcohol intake, the observed increase in *Enterobacteriaceae* abundance while *Lachnospiraceae* and *Ruminococcaceae* decrease. The problem is that the first produces dangerous endotoxins and the second produces beneficial short-chain fatty acids (SFCA) (38, 39). Alcohol consumption caused a significant decrease of *Bacteroidetes* (34, 5) and an increase in *Corynebacteriaceae* family and *Clostridiales*. Moreover, several studies reported that chronic alcohol consumption led to an overgrow of *Corynebacterium* genus members (36, 37). Not surprisingly, bacteria belonging to the orders *Bacteroidales*, *Clostridiales*, and *Enterobacteriales* were associated with protection against ALD and they were shared between resistant and faecal-microbiota transplantation (FMT)-protected mice. *Roseburia hominis*, a member of *Lachnospiraceae* was also associated with FMT-protected mice, however, some *Prevotella* groups (which belong to *Bacteroidales*) were associated with alcohol-sensitive mice (26). This suggests that different species and maybe even strains interact differently within the host. Despite their pathological relevance, *Bacteroides* species are important for the maintenance of a beneficial interaction with intestinal homeostasis. *Bacteroides* species may also control other competing pathogens by influencing the host immune system (35). A report characterized an alcohol-induced dysbiosis by changes in the relative abundances of certain species and found an increase in the abundance of *Bacteroides acidifaciens*, *Bacteroides eggerthii* and *Oscillospria* spp, both Gram-negative bacteria, and a decrease in *Bacteroides uniformis*, *Parabacteroides gordonii*, and *Akkermansia muciniphilla* (35). Similar patterns have been observed, where phylum *Firmicutes* and genus *Lactobacillus* were reduced, while *Enterococcus* (5), *Corynebacterium* and *Alcaligenes* spp. increased in the gut of mice fed with alcohol (35). However, caution must be considered because differences in experimental setup like alcohol diet models and duration of alcohol feeding may explain opposite changes in relative abundances of *Akkermansia* spp. In contrast to

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other reports, a recent study observed a slightly increase in the gut microbiota diversity after active and forced drinking in alcohol-withdrawal mice models, while a few commensal residents reduced in numbers (21). In the same study, it was observed an increase ratio *Firmicutes*/*Bacteroidetes* in the groups exposed to alcohol. However, the study could not explain the differences found in the gut microbiota observed between the active and forced drinking. For instance, despite increasing the overall diversity, some taxa abundances varied. Specifically, members of the *Allistipes*, *Odoribacter* groups (both Bacteroidetes) and *Bifidobacterium* (Firmicutes) changed their abundance level between active and forced drinking mice models (21). The discrepancies found in microbial composition alterations in gut microbiome studies when mice models are studied may be explained by the different alcohol administration regimen performed, as well other factors, such as subjacent liver disease (5, 35). Relevant is the fact that alcohol abuse induces gut dysbiosis in rodent models (11). Even in cases where faecal microbiota transplantation was performed, it did not affect alpha-diversity in the gut albeit avoiding the imbalance in the gut composition, namely by the depletion of *Bacteroides* (26). One possible causistic explanation to the protection to alcohol from alcohol-resistant mice is the overexpression of two defensins in the colon, Reg3β and Reg3γ (40). Both FMT and pectin-treated mice showed this overexpression.

In ALD settings, there is an association between alcohol intake and fungi overgrowth, namely *Candida* spp., promoting liver injury (41). Moreover, ALD stimulates a stronger host immune response in humans against fungi, and recent studies have been relating fungi presence with alcohol-induced diseases, like cirrhosis (42). In any case, the experimental setup, e.g. breeding facilities (43) also influences the gut microbiota composition, and some results can be explained by chance, additionally, alcohol-induced liver lesions are not observed in all cases (26).

Previous reports noted that antibiotic treatment in mice after alcohol consumption reduced gut bacterial number, consequently reducing endotoxin levels, and diminished liver inflammation (44, 45). The reduction of neuroinflammation derived by antibiotic treatment clearly revealed the relevance of the gut microbiota abundance and pathogen-associated molecular patterns (PAMPs) in the gut-brain axis in many contexts, such as alcohol consumption (46). In the gut-brain axis context, chronic-to-acute models of alcohol consumption showed that alcohol causes major prejudice in brain and gut, namely causing neuroinflammation in CNS and cytokines overexpression in the small intestine. The relevance of the gut microbiota load on these processes is shown by the decrease in

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inflammatory levels during antibiotic treatment. However, antibiotic treatment also increased the levels of inflammasome components and cytokines processed by the inflammasome found in CNS and gut, which may be contradictory (46).

2. Gut microbiota-derived metabolites and brain

More than a century ago, Elie Metchnikoff was the first scientist to launch the hypothesis of interconnectedness between gut microbes and neurological disease (7). Recently, there was an increase number of evidence suggesting that gut microbiota, particular bacterial species, and gut microbial-related metabolites impact neurological states, behaviour, cognition and neurodegeneration (47). Microorganisms can produce molecules that can be detected in many sites inside host's body, including the brain (48).

Microorganisms-associated molecular patterns (MAMPs) are well-conserved components of microbial cells, and they are involved in the maintenance of structural integrity and basic function of microorganisms. MAMPs are complex and diverse molecules such as nucleotides, lipids, carbohydrates and peptides (49). Additionally, they are necessary for the correct immune and neurodevelopment. However, if present above threshold, MAMPs can promote acute and chronic inflammation involved in neurological disorders. Among cellular surface component MAMPs, peptidoglycan and lipopolysaccharide (LPS) seem to be sufficient to modify brain development and function. Regarding peptidoglycan, recent studies reported that it can translocate to the brain and affect its development by altering gene expression and social behaviour (50). LPS can barely cross the BBB but it can be detected in the brain surroundings alongside its specific receptor (51). Studies where LPS was injected in mice showed that it can induce cognitive impairment (52), and serious depressive-like behaviours, among others (7). Moreover, LPS can also affects foetal brain development (53).

The gut microbiota-derived signals can also stimulate neuroinflammation in alcohol acute-on-chronic presentations (46). Additionally, alcohol affects metabolites composition, such as the production of serotonin and bile acids, among other bioactive metabolites (54). The current hypothesis is that alcohol explains the elevated levels of toxic bacterial by-products in the circulatory system. This happens because alcohol consumption triggers the oxidative stress in the intestine thus disrupting the epithelial barrier, causing damage in the gut tight junction proteins (11). Then, gut bacteria can

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translocate to the circulatory system and there they produce toxic by-products such as LPS and peptidoglycan (PGN) (55). Further complications can happen when LPS (also generally termed endotoxin) reach the liver in acute alcohol consumption. Such bacterial components can translocate into circulation from the intestinal lumen as consequence of prolonged alcohol consumption (56). In the liver, the metabolism starts, and a cascade of inflammatory responses is initiated. Endotoxins, residual alcohol and other alcohol-derived metabolites go beyond liver and reach systemic circulation and other organs like the peripheral immune system and the central nervous system (CNS) promoting more dramatic damage (46).

2.1. The kynurenine pathway

Tryptophan metabolism is essential for normal neurological behaviour. The gut microbiota can metabolize tryptophan into indole derivatives, tryptamine and kynurenine metabolites. All these compounds can exert neuroactive effects on the host (7). Many of compounds derived from the tryptophan metabolism in the gut can cross BBB and directly affect the brain.

One of the most known metabolites originated from the tryptophan is serotonin (5-hydroxytryptamine, 5-HT). Serotonin is a neurotransmitter strongly associated with both emotional and cognitive functions. KYN, KYNA, and QA are biologically relevant metabolites produced in the KP. These compounds have roles in various immune and neurological modulatory functions. Among these functions, KYNA and QA plays a role in neuromodulation, while KYNA is a neuroprotective compound, serving as an antagonist to all ionotropic glutamate receptors (63, 64). However, the antagonist effect of KYNA is not completely resolved (65, 66, 67). KYNA can be produced in many ways inside the body like epithelial (68), red blood (69), skeletal muscle (70), human peripheral blood mononuclear (71), and pancreatic islet cells (72), as well as fibroblasts (73). KYNA can indirectly influence brain development because it is an inhibitor of both N-methyl-D-aspartate (NMDA) and α7 nicotinic acetylcholine receptors which are critical for the process (105, 106). Since KYNA concentration in ileum wall is reduced, it is suggested that the higher levels of KYNA found in the gut are originated in the gut microbiota. However, it can also be originated from external sources like broccoli, or basil (107, 108). Moreover, some evidence reported that KYNA regulates the growth and therefore the composition of the gut microbiota in a selectively way (109). From the gut, KYNA goes

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to the bloodstream rapidly (107). *Escherichia coli* (80) *Pseudomonas* spp., and *Aerococcus* spp., are among the species with active roles in the KYNA metabolism (110, 111).

Four kynurenine aminotransferases (KATs I-IV) are described in mammals (74, 75, 76, 77, 78). The presence of KATS is widespread in the body, and their activity was already described in the liver, kidney, small intestine, dermal fibroblasts, and brain (73, 74, 80). In the presence of reactive oxygen species (ROS), KATs can skip the co-substrate and produce KYNA directly. KATs produce KYNA under physiological conditions. KATs uses 2-oxoacid as a co-substrate to catalyse the irreversible transamination of 1-KYN, forming KYNA (81, 82).

Similar to serotonin, KYN plays a role in brain function and damage where neuroinflammation is triggered (112, 113). KYN is mostly captured from the periphery, and about 40% is produced locally in the CNS, being then able to cross BBB (85). KYN metabolites in particular, act on memory, anxiety-like and stress-like behaviours through neuronal glutamate receptors recognition (79). In the KP, KYN can undergo two routes. In the rate-limiting route, KYN generates 3-HK, which further generates QA, which is a NMDA receptor agonist. Alternatively, KYN generates KYNA, a NMDA antagonist. Comparing QA to KYNA, the first can act as a neurotoxic compound and the second has a neuroprotective effect. Under physiological state, KYN generates KYNA. However, under neuroinflammation, the majority of KYN is converted to QA, triggering a cascade of neurotoxic reactions (92). Increased QA/KYNA ratio induces the activation of NMDA, which in turn activates neurotoxicity by higher glutamatergic activity. Above normal neurotoxicity exposure increases the chances of damage in memory and the appearance of mood disorders, as showed in rats (114).

Up until now there were described three rate-limiting enzymes in the KP. They are tryptophan 2,3-dioxygenase (TDO), indolamine 2,3-dioxygenase (IDO)1, and IDO2 (80). Shifting from physiological to inflammatory conditions, tryptophan will be mostly directed to the generation of KYN by the activations of indoleamine-2,3-dioxygenase (IDO), and further metabolized to KYNA, decreasing serotonin concentration. Proinflammatory cytokines activate IDO which in turn catalyses the generation of KYN from tryptophan throughout the neuroregulatory metabolism, that includes an active role in progression of neurological damage (88, 89, 90, 91). A great number of proinflammatory cytokines can activate IDO enzymes IDO1, IDO2, and TDO, such as TNF-α, IL-1β, and IL-6 (92, 91). The result is the unbalance of the ratio 5-HT/KYNA,

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leading towards potential detrimental effects on the organism, since KYNA is a neurotoxic metabolite (57, 58). IDO is thus relevant because its increased activation induces changes in the tryptophan metabolism in prejudice to the organism (59). However, the effect is coined to IDO1 and not to IDO2.

KYN (L-kynurenine) is critical in the pathway because it is the bioprecursor of KYNA and 3-HK. KYNA formation happens as a result of the irreversible transamination of KYN, and 3-HK is catalysed by kynurenine 3-monooxygenase (KMO) via a different route inside the pathway. KYNA and 3-HK have neuroactive properties, such as neuroinhibitory and proexcitotoxic features, respectively (60, 61, 62). Thus, KP is likely to be a good target for analysing drug abuse, seeking and relapsing behaviours (83, 84).

A report observed that tryptophan catabolism was impacted following binge-on-chronic alcohol consumption. Namely, KYNA and xanthurenic acid (XA) levels significantly decreased after alcohol consumption (35). Despite the evidence linking IDO1 directly to abnormal brain function, IDO2 and TDO may still play a role in the brain. On the other hand, it was not reported any change on the activation of TDO during alcohol consumption in mice (59). IDO1, IDO2 and TDO can be present in other areas of the brain and act on the observed neurological impaired phenotypes. Additionally, these enzymes may act differently accordingly to subclinical settings of depression (93). Since KYNA is assumedly uncapable of crossing BBB (20), its presence inside the brain suggested that peripheric TRP or KYN synthesize KYNA inside the brain, as it seems to be the case in a number of cases of neurological disease settings, like amyotrophic lateral sclerosis (ALS) (94).

Placenta connects the foetus with the mother, allowing nutrient transport via the blood circulation link between them. Interestingly, placenta expresses KP including KATs and KMO (95, 96, 97), and recent studies in mice revealed that KYNA and 3-HK are also present in this tissue (98, 99). Although placenta can convert maternal tryptophan to serotonin in the first period of pregnancy, placenta also transforms tryptophan to kynurenine (96). Besides its major role in foetal growth and development, placenta may be the source of KP metabolites found in the foetus, specifically those found in the foetus brain. Due to chemical similarities between KYNA and 3-HK and the fact that both share the same transporters, it has been assumed that 3-HK enters the foetus via the same maternal circulation mechanisms as KYNA. Despite sharing the same transporter – the large neutral amino acid transporter (20) – 3-HK can cross BBB and KYNA cannot. There is little clue about how the KP affect pregnancy and about how it is synthesized and

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regulated during that period, but KYNA may play a role later in life, specifically in terms of neurological diseases' progression. In a normal scenario, mammals' brain levels of KYNA and 3-HK are higher before birth, even though KAT I, KAT II and KMO are already present in the foetal brain (100, 101, 102, 103).

A study observed that peripheral KYNA cannot cross the placenta, because when KYNA was given to the mother, the KYNA levels in the foetal plasma and brain remained unaltered (18). However, if the mother was administered with KYN, the KYN levels of the placenta, and the foetus plasma and brain increased rapidly. This evidence shows that KYN can be transported from mother to foetus through the placenta *in vivo*, which seems to be a normal occurrence during pregnancy. Interestingly, KYNA generated by the mother (or in this case administered) cannot cross the placenta, as the same study reported. However, KYNA can be synthesized from KYN in the foetal mouse brain (104).

In settings of alcohol addiction, a study observed interruption in the alcohol seeking and relapse in rats when KMO was inhibited (115), suggesting a peripheral aversion mechanism to alcohol, shifting the KP pathway towards KYNA production (116). The alcohol aversion was induced by KYNA and 3-HK by aldehyde dehydrogenase (ALDH) inhibition, restoring physiological levels of acetaldehyde (117). However, the aversion mechanism is not clear, since KMO inhibition does not hinder KYNA generation in alcohol-consumption mouse model (59). Many factors may explain the incongruences, like the inherent metabolic differences between rats and mice, or experimental setup. Alcohol consumption inverts the physiological ratios of TRP/KYN, TRP/5-HT, and 5-HIAA/5-HT indicating that serotonin is no longer being produced and KYN is then formed in the brain of alcoholised mice (59). Accordingly, in depression-related animal models, lower tryptophan levels and abnormal IDO expression are commonly found (118).

Gut microbiota is a main factor contributing to plasmatic TRP availability, acting in its regulation (121). After microbiota depletion there is a decrease in TRP concentration in the brain independently of ethanol exposure in rats (119). The increased KYN levels found in the brain in cases of chronic ethanol consumption is related with the gut microbiota since antibiotic treatment avoided the brain accumulation of KYN (22). However, higher levels of KYN found in the brain and in brain periphery after voluntary chronic ethanol intake is not levelled. Among the factors explaining this asymmetry is the gut microbiota, which only influences KYN brain concentration but not peripherical level (22). Of note is the incapacity of C57BL/6J mouse model to recapitulate any positive

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association between anxiety-like behaviours and the gut microbiota or brain KYN concentration (22). Additionally, in the last 15 days of the chronic ethanol intake experiment it was not observed any increase in the plasma KYN levels opposing the changes in the KYN brain levels when bacterial were removed. The observation suggests that the gut microbiota may not even be involved. However, there are alternative explanations for this, as it could result of tissue-dependent alterations in the activity of KYN-degrading enzymes by ethanol and antibiotic treatment (120). There is the assumption that microorganisms have the ability to metabolize KYNA, however it is not certain how biochemically extended is the reaction (110, 111).

2.2. Lipopolysaccharides (LPS)

Endotoxin is a lipopolysaccharide that consists of mostly the outer membrane of gram-negative bacteria, and it is found during bacterial infection in different body sites, such as intestine and skin. During bacterial infection, gut inflammation, and neurodegenerative disease, endotoxin levels raise in blood plasma. Despite individual variances, endotoxin is widespread in many organisms, and its concentration correlates with neurodegeneration. Recently, the endotoxin hypothesis of neurodegeneration was developed, which associates the raising of endotoxin levels to neurodegeneration (122). Endotoxin hypothesis of neurodegeneration tries to explain the causative role of endotoxin in several neurodegenerative diseases, such as Parkinson's disease (PD), ALS and fronto-temporal dementia (FTD). Endotoxins can cross mucosal membranes, and it happens in the gut and lungs, for instance. Irrespectively of where endotoxins are found in the body, intestinal permeability plays a crucial role in the endotoxin circulation and concentration (123, 124). If endotoxin hypothesis is correct, all sorts of neurological diseases and impairments may be reduced by tackling endotoxin levels or neuroinflammation it can trigger. However, the hypothesis may be hard to test since not all endotoxins are the same. LPS toxicity has species', even strain's, environmental nuances. For instance, differences were found in LPS-related host response from *E. coli* and *Bacteroides dorei* colonization. Where the LPS from *E. coli* triggers a strong inflammatory response, *B. dorei* does not, and its LPS can indirectly inhibit the host against *E. coli* inflammation (122, 126, 127).

Maternal influence on the foetus can also be seen in terms of LPS exposure. Prenatal and early postnatal exposure to mother LPS can induce a variety of neurological

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symptoms which can be persistent until adulthood (122). Studies revealed that increased endotoxin levels in blood of humans trigger systemic inflammation and activation of microglia in the brain. In rodents, microglia are also activated, brain synapses and neurons are lost, and memory is impaired (122). One important fact is that humans are much more sensitive to endotoxins when compared to mice (148). LPS has been reported as sufficient to cause neuronal loss in brains of rodents (44, 46). Dose and timing can alter the effect of endotoxin in organisms, which lead to priming and tolerance responses, meaning increased or decreased consequent reactions to inflammation, respectively (136, 137). LPS signalling is clinically relevant to alcohol-induced liver pathology (46). Different patterns of ethanol consumption show different effects. As an example, the chronic alcohol intake allows gut microbial translocation and higher levels of LPS in blood, as well it is associated with a decrease in tight junction protein expression. However, these observations were not found when the binge behaviour was tested (22). Interestingly, another report concluded that tight junctions in mice are not relevant for the alcohol sensitivity (26). Previous results suggest that chronic ethanol intake is responsible for increasing the nitrosative stress. Nitrosative stress activates MMP-9, increasing the chances of gut permeability by decreasing tight junction expression. In a case where gut barrier is disrupted, bacteria can translocate, releasing LPS in plasma (22).

Toll-like receptor 4 (TLR4) is critical for recognition of bacterial endotoxin, being the endotoxin major pattern recognition receptor (138). TLR4 is also a relevant pattern recognition receptor in alcohol induced neuroinflammation (46). Microglia express TLR4 and react to alcohol consumption by sensing and responding to it, besides being involved in many immune signalling pathways (139, 140, 141, 142). There is little evidence supporting the LPS cross BBB (143), however other evidence suggested that TLR4 influences alcohol-induced neuroinflammation (46). Albeit LPS cannot cross BBB, a recent study showed that it can induce BBB opening in an aging 5XFAD mouse model of compound delivery, which was accompanied by weight loss and increased lethality (144). BBB higher permeability will result in more neuroinflammation and neurodegeneration states through the translocation of plasma components to the brain. The presence of endotoxins can turn on BBB permeability, although how endotoxins enters the brain is still poorly understood (145, 122, 146). Only high concentrations of endotoxin can affect BBB, and low or medium doses barely affects the brain (143). This suggest that peripheral LPS plays a critical role in inducing inflammation by activating

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peripheral nerves; or activating BBB with further cytokines' release or immune cells recruitment into the brain; or activating circumventricular organs.

There is a chance that bacterial signals can be sufficient to induce gut and brain inflammation and further organ damage. LPS thus emits a signal that crosses BBB via interaction with juxta-cerebrovascular cells. However, acute alcohol consumption can disrupt BBB and then the direct role of LPS on triggering neuroinflammation is observed (147). Increase of inflammatory cytokines and activated immune cells in the liver and brain may be triggered by other bacterial-derived or intestinal signals and lead to brain and multi-organ inflammation in other body sites (46).

Studies have shown that administration of bacterial inoculum or LPS results in depressive and anxiety-like phenotypes linked to the brain indolamine 2,3-dioxygensae (IDO) in mice (129, 120, 130, 131). As expected, IDO antagonists such as 1-methyl-l-tryprophan and miR-874-3p prevented those phenotypes (128). Moreover, inflammatory elicitor LPS can also stimulate the presence of IDO1 by increasing the expression of inflammatory mediators (135).

Gut and hepatic portal vein are the routes of endotoxin entering in the body. Liver can clear gut-derived endotoxins and peripheral blood endotoxins almost entirely (132, 133). Interestingly, blood endotoxin levels occurring in cirrhosis are similar to those found in Alzheimer's (134). However, further investigation is necessary to understand this similarity, since blood ammonia and cytokine levels also increased in such disorders.

In a PD onset, both gut dysfunction and dysbiosis (149) act as a trigger for the remaining symptoms and disease-phenotypes (150). Patients will then have an increased gut permeability which gives room to the translocation of LPS though LPS binding protein (LBP), causing an elevation of blood endotoxins (151). Similar to what is observed in gut permeability of PD patients, when peripheral endotoxin is administered to mice, gut permeability increases caused by the overexpression of α-synuclein present in neurons located in the large intestine (152).

Although endotoxin levels increase in neurodegenerative diseases and part of endotoxin role in neurodegeneration had been uncovered, there is still insufficient data to assume that endotoxin is sufficient to trigger neurodegenerative processes. Nevertheless, targeting endotoxins may be a route to tackling neurodegeneration. Examples of such approaches are selective gut microbiome changes, where species which hold more toxic LPS are deprecated; reduce the chances of gut permeability; or to avoid increased blood endotoxin levels (122).

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3. Neurodevelopment and behavioural impact of alcohol intake and gut dysbiosis

The most vulnerable periods for CNS development is the first half and the the last two-thirds of the first gestational trimester, and for brain growth, the third trimester. Rodent models showed that the acceleration of brain growth occurs in the early postnatal period (153, 154). During these periods, alcohol can have increasingly teratogenic effects. In the perinatal period, gut colonization and neuronal organization are very dynamic, representing a crucial window for the normal neurodevelopment of the organism. There is evidence of early neurodevelopment window where the influence of the gut microbiota is clear (155). Studies using germ-free mice have been published to test hypotheses related to the co-evolution of the gut microbiota and the host organism, several examining the association between the gut microbiota and the structure and function of CNS.

Neurogenesis and neural activity are among the neurodevelopmental processes affected by the gut microbiome (156, 157, 158, 159). They are affected by the aberrational pattern levels of neurotrophic factors, neuropeptides and neurotransmitters (156, 157, 160, 161). Moreover, the gut microbiome can further impact host neurological conditions through changes in signalling pathways. One such case is the exaggerated glucocorticoid response explained by the hypothalamic-pituitary-adrenal dysregulation in germ-free and antibiotic treated mice. Ultimately, this dysregulation is associated with observed behavioural settings related to social activity (162, 163, 164), anxiety (157, 165), cognitive function and depression (165, 166, 160, 161, 164, 167, 168). Surprisingly, germ-free mice did not reveal equivalent protection for alcohol-induced liver damage as antibiotic treatment mice (169). This contradiction can be explained by the pivotal role gut microbiota may have during the early development, exerting a beneficial influence in the immunologic responses to alcohol intake.

Alcohol can largely contribute to the breakdown of the intestinal barrier integrity by inducing inflammatory signalling, causing gut bacterial dysbiosis (40), disturbing luminal homeostasis (170, 171), increasing enterocyte cellular stress, and by shifting structural proteins regulation (172). Alcohol addiction impact several neurological processes, like myelination, neurotransmission, inflammation, as well metabolic alterations related to behaviour, for instance depression and abnormal sociability. Alcohol exerts influence on TLR, both directly, by affecting correct TLR signal transduction (180,

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181), and indirectly, by recognizing endogenous (182, 183) and exogenous danger signals (184). Studies reported that absence of TLR in knockout and knockdown experiments in mice resulted in inflammatory protection in liver (185) and brain (186, 187, 188) after alcohol intake. Same results were obtained when antibiotics were presented to mice aiming to reduce bacterial LPS (an exogenous signal), suggesting that gut microbiota has influence on triggering tissue inflammation in the liver and brain when alcohol is consumed.

Leclercq et al performed preclinical studies and showed that there was a link between leaky gut (increased intestinal permeability) and social behaviour impairments in humans (16). This link goes along with the known relation between gut microbiome and social behaviour in multiple animal models, including mice (189). The connection between gut dysbiosis and alcohol addiction is that the former is also associated with relevant metabolic alterations and neurological processes. Besides, alcohol can easily cross the BBB and morphological changes in microglia and proinflammatory gene expression are examples of neuroinflammation events caused by persistent alcohol intake. As it seems gut bacteria is relevant in anxiety-like behaviours associated with alcohol withdrawal where the intestinal permeability plays a crucial role (173, 174). The presence of bacteria in mesenteric lymph nodes is indicative of disrupted intestinal permeability and it happens right after chronic ethanol consumption is interrupted. None of these events were observed during the binge alcohol consumption (22). Moreover, there is a relation between gut alterations and impaired sociability in AUD (16).

Studies showed that transplanting gut microbiota from AUD patients to mice prevents the neuroprotective effect of β-hydroxybutyrate (BHB) (16). This happens because certain bacterial genera start to produce ethanol and a reduction of lipolysis is found. These two events are associated with a lower hepatic synthesis of BHB. Inoculation of dysbiotic alcohol-dependent microbiota from specific alcohol-dependent patients leads to a reduced synthesis of BHB (16). This reduced BHB availability may be the first cause of alterations in brain functions and behaviour in mice. The fact is that the role of gut microbiota in metabolic and behavioural disorders is evident as seen by the fact that microbiota transplantations from human to mice can recapitulate these disorders' phenotypes. Faecal-microbiota transplantation studies with humanized gut microbiota in mice or from other mice remain speculative if they are kept in a preclinical setting. However, recent evidence from FMT has shown that alterations on gut microbiota is sufficient to explain worsen neurological and psychological symptoms in a range of

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diseases, such as multiple sclerosis (175), Parkinson's (176), Alzheimer's (177), depression (178), schizophrenia (179), and others (7).

3.1. Foetal Alcoholic Spectrum Disorders

Foetal alcohol spectrum disorder (FASD) is a term now with 20 years and its consensus definition is: "an umbrella term describing the range of effects that can occur in an individual whose mother drank alcohol during pregnancy (…) These effects include physical, mental, behavioural, and/ or learning disabilities with possible lifelong implications (…) The term FASD encompasses all other diagnostic terms, such as Foetal Alcohol Syndrome (FAS), and is not intended for use as a clinical diagnosis.". This definition was revised in 2016 in order to make FASD into a diagnostic term. However, to diagnose FASD it is required a multidisciplinary team with physical and neurodevelopment expertise. FASD has now two diagnostic categories: "i) FASD with sentinel facial features and evidence of impairment in three or more identified neurodevelopmental domains, with PAE either confirmed or unknow, and; ii) FASD without sentinel facial features, with evidence of impairment in three or more identified neurodevelopmental domains, and confirmed PAE". A third category is also specified in a way that describes children "at risk for neurodevelopmental disorder and FASD associated with PAE" (2).

The effects of PAE on neurogenesis go beyond all stages of gestation and last throughout life, namely in specific areas of the brain and the detrimental effects occur on processes such as neurogenesis, differentiation and synaptogenesis (153, 190, 191, 192). Dosage, duration and timing of PAE, mother genetic setting, nutrition and metabolism, epigenetic factors (193, 194), foetal exposure to stress (195, 196, 197), and mother capacity to metabolize alcohol (198, 199) are all possible causes of FASD. There is a possible association between PAE and neurodevelopmental disorders, as the evidence show that, in PAE, on first-to-third trimester mice models there was observed delayed developmental features, such as genitive geotaxis, auditory startle, cliff aversion and air righting (200). Similar observations were made in children with FASD where brain maturation is impaired, besides their smaller brain and basal ganglia sizes (201). There is a possible association between such small volume of the basal ganglia and abnormal neurobehavioral phenotypes, such as impaired visuospatial abilities and executive functioning (202, 203).

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Additionally, mice PAE models showed affected hypothalamus, hippocampus, and cerebellum, namely pro-inflammatory signalling and abnormalities at the neuronal development level. The same brain regions are also affected in individuals presenting FASD (204, 205, 206, 207). Microglial cell death is a common consequence of alcohol (208). The importance of microglia for normal brain development is seen for instance in processes related to brain synaptic function (209). Alcohol can directly activate microglia through mediation from TLR2 and TLR4 signalling. Afterwards, ROS and NO production and a cascade of cytokine levels are triggered (180). Interestingly, pro-inflammatory microglia that had been activated by alcohol has also a role in facilitating the clearance of developing hypothalamic and Purkinje neurons after PAE-related apoptosis (208, 210).

In summary, altered brain development and behavioural changes are observed consistently as result of inflammation and microglial activation in third trimester PAE mice models. These prejudicial effects in the foetal brain during development are similar to those observed in individuals with FASD. Moreover, acute alcohol exposure during third trimester specifically can cause reduced brain volumes (microcephaly), significantly loss of GABAergic and pyramidal neurons in the cortex, and behavioural effects like increased hyperactivity later on in life (212).

Using a mouse model of FASD it was observed alcohol-induced neurotoxicity associated with a prolonged activation of astrocytes and higher levels of pro-inflammatory mediators such as TLR4, NF-κB, and interleukin-1β (IL-1β) in many brain regions (235). To prove this assumption, a TLR4 double knockout mouse model was generated and it did not show any damage induced by alcohol during development (236). Expression of tumour necrosis factor-alpha (TNF-α) and IL-1β is increased in the cerebral cortex and cerebellum (231, 186). Simultaneously, microglial cell proliferation also occurs (232, 233). Both events are triggered by increased and prolonged alcohol consumption. Therefore, such state of microglia and astrocytes activation will result in a higher level of neuronal cell death (234).

Overall, the effects of prenatal alcohol on the neurodevelopment can be observed in a myriad of neurological processes, for instance involving microglia and astrocytes, promoting above normal levels of inflammatory response during the CNS development (211).

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4. Discussion

Alcohol consumption has a wide range of effects throughout the body, from the gastrointestinal tract to the CNS. Alcohol addiction patients show emotional and cognitive impairments, like depression-related symptoms, as well as a significantly higher expression level of proinflammatory cytokines in the serum and brain (59). Preclinical and clinical literature confirm the strong negative effect of alcohol on the organism (11). However, the diagnostic and clinical definition of alcohol damage to multiple end organs is not easily performed. This occurs due to the individual physiological setting of the patient and the unpredictable onset of the upcoming disease. Compared with clinical, preclinical studies using animal models have the advantage of controlling the experimental variables, such as nutrition, genetic landscape, health status, exposure to other drug or stressors, dose, duration and gestational presentation time of alcohol intake. Moreover, mice models can be used to study more severe phenotypes resulting from transgenic lines in order to answer more mechanistic questions within the FASD outcomes (213).

There is no clear gut microbiome signature associated with alcohol abuse disorders. However, in the alcohol-resistant mice and in the FMT-transplanted mice group, *Lachnospiraceae*, *Ruminococcaceae* and some members of *Enterobacteriales* were markedly present (26). In mice sensitive to alcohol, *Bacteroides* taxon is underrepresented. Alcohol-sensitive mice had lower *Bacteroides* presence and alcohol-resistant mice harboured *Helicobacter and Flexispira*. Thus, it is suggested that specific groups of bacteria can have an influence in the sensitivity to alcohol (26). To go further in the taxonomic discrimination of the microbes present or absent in alcohol use settings, more discriminative molecular techniques must be performed, as it is exemplified by the strain specific traits observed in the *Prevotella* OTUs coined to different alcohol-resistant phenotypes in mice (26).

Depending on the mouse model used, alcohol intake may vary, since the preference for drinking alcohol voluntarily is not the same. For instance, C57BL/6 mouse model prefers alcohol over water and are most used when the goal is to investigate acute alcohol drinking (214). Interestingly, sexual differences on alcohol-mediated phenotypes cannot be ruled out. In a study of acute alcohol intake using a PAE gestational model, males showed an increase of anxiety-like behaviours and females showed the opposite (215). To account for experimental differences, factors such as mouse research model selection, and pattern and duration of ethanol exposure may explain the different results

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when the relationships between the microbiota-gut-brain axis and the consequences of ethanol abuse are investigated (22).

In summary, PAE models suggest that alcohol exposure during gestation affect immensely the brain, producing brain abnormalities associated with aberrant cognitive function (216). Although, PAE models are selected depending on the time of exposure: first trimester acute exposure is more prone to study craniofacial malformations; third trimester is best for questioning learning deficits and exhibition of anxiety- and depressive-like behaviours. Thus, accordingly to evidence observed in PAE mice models and clinical studies, early gestation exposure to alcohol seem to promote abnormal facial phenotypes leading to more severe disease settings, however, late exposure may trigger behavioural impairments. Gaps in the knowledge of FASD are related to the wide scope of outcomes promoted by prenatal alcohol exposure, namely the interaction between neurodevelopment and behaviour. Moreover, few studies cover other morbidities that occur simultaneously with FASD. Additionally, for the development of better therapeutics on substance use disorders it is needed a better understanding of the interplay that occurs within the gut microbiome.

Dysbiosis is a loosely and increasingly controversial term used to describe changes in gut microbial composition. It assumes an imbalance or abrupt alteration of microflora that can further cause detrimental effects on the host (218). One of the problems of the term is that it is not associated with the lack of commensal microorganisms that have a positive impact for the host – termed ‘eubiotic’ state – for example by producing short-chain fatty acids (219). Overcoming both terms may need a different approach to the gut microbiota research, shifting from its composition to its microbial function, which comprise host-microbe and microbe-microbe interactions. For ALD, a study concluded that controlling gut dysbiosis can prevent liver damage (26). However, other factors must be considered, such as the innate sensitivity of mice to alcohol. Direct contact between gut bacteria and epithelial cells happens when protective mucus layer in the colon is altered or absent. In such cases bacteria translocate into the epithelial cells by penetrating the crypts, thus triggering an inflammatory response (217).

There is a clear association between gut microbiota composition and their metabolites and host homeostasis, metabolism and immunity (35). The participation of the gut microbiota in the brain and small intestine is significant, since antibiotic treatment reducing the bacterial load in the gut also decreases the expression of alcohol-induced proinflammatory cytokines (46). A recent report observed for the first time that acute-on-

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chronic alcohol consumption triggers neuroinflammation and increases the expression of proinflammatory cytokines in small intestine (46). The critical role between gut microbiome and the gut-brain axis in an alcohol intake scenario is highlighted by the avoidance of elevated levels of proinflammatory cytokine expression in the CNS and small intestine exerted by antibiotic treatment in mice.

Serotonin is responsible for the appearance of feelings associated with good mood, like calmness, relaxation, and contentment. This leads to the monoamine hypothesis of depression and anxiety, where the absence, reduction or abnormal levels of 5-HT raises the chance of such disorders, and this is exactly what happens when alcohol is consumed in increased concentrations and during persistent periods of time (228). The connection between KYNA levels in the blood and neurological disorders is becoming significantly apparent, since it was found lower KYNA levels in patients with schizophrenia (220), Alzheimer's (221, 222), and migraine (223). The lower KYNA blood level may indicate that TRP ,or even KYN, were transferred through BBB and they would synthetize KYNA locally in the brain (80). Thus, a neurodevelopmental hypothesis of schizophrenia (224) is gaining strength as the observations of increased levels of KYNA in the foetal rat brain may explain cognitive impairments later in life, as well an above normal level of KYNA in the adult brain (225, 226, 227). However, plenty of questions remain unanswered related to the dynamics of KP on the mother-foetus relationship during prenatal period. More studies are needed to elucidate, for instance, how maternal KYN and subsequent metabolites enter the foetus, and what is happening to KYN in the placenta while exposed to externally levels of the compound.

Relevant roles should be attributed to each enzyme and metabolite derived from the KP. As for IDO1, it is shown to have a role in inducing alcohol drinking addiction, depressive-like behaviours, as well as memory impairments in mice exposed to alcohol (59). IDO1's role is based on the privileged direction route to produce KYN from TRP, instead of serotonin. Further studies may elucidate the alcohol effect on behaviour by testing different antagonists and agonists, and the underlying genetic background around IDO enzymes. However, the aversion mechanisms are not clear, since KMO inhibition does not hinder KYNA generation in alcohol-consumption mouse model. Many factors may explain these incongruences, like the inherent metabolic differences between rats and mice, or experimental setup (229). Next research goals should focus on the effect of direct treatment with KP metabolites on the foetal neurodevelopment.

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Further understanding is needed to clarify what are the impact of gut bacteria and bacterial-derived metabolites on the initiation of organ damage. Bacterial metabolites, such endotoxins, may have a direct role on the uprising of host inflammation level, and this is supported by the evidence showing the release of bacteria and LPS into the systemic circulation after alcohol intake (230, 56, 174, 170). Levels of serum endotoxin and cytokine are increased in chronic and binge alcohol consumption cases, further contributing to life-long cognitive impairments, (174). Although, key questions of the endotoxin hypothesis of neurodegeneration are still looking for answers (122). Understanding the general and disease-specific role of plasma endotoxins in neurodegeneration, and the effect of increase/ decrease endotoxin levels have on neurodegeneration will help the development of better treatments.

The main future challenges are related to the observation of neurodevelopment long-lasting effects caused by the interplay between host and its resident microbes. The prenatal period remains the main window of opportunity where major interactions happen involving microbial colonization and nervous system, among others. One remaining question to be elucidated is what is the causative event that explains alcohol craving and consumption. Whether gut microbiome and immunomodulatory metabolites' changes can be cause or consequence of alcohol-seeking behaviour and subsequent consumption needs more research as well.

Further clinical evidence is needed to support wider and stronger associations between human and mouse biological and physiological similarities. Final conclusions, based on causal inferences, must be taken with caution due to inherent inter-species differences between humans and mice (7).

Gut-brain axis research field has seen mostly studies pointing out correlations among gut metabolomics' profiles, whereas mechanistic approaches about the casual effects of microbial-produced metabolites have on the brain and subsequently phenotypes are scarce (7). The identification and characterization of microbial species and communities' roles on neurological states is necessary to uncover causality links in the gut microbiome-brain research. New knowledge relating gut metabolites to their brain effect in a particular neurological state will pave the way for better hypotheses generation aiming a better understanding on disease aetiology. Eventually, it can also contribute to the development of improved treatments, such as the deployment of probiotics based on empiric evidence rather than the limited treatment efficacy and limited number of probiotic strains commercially available at the moment.

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Conflict of interest

The authors declare no conflict of interest.

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