Effects of probiotics on central nervous system functions in humans

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Contents

Abstract

Gut microbiota plays an important role in the gut-brain axis. Symbiosis of the gut microbiota maintains the physiological integrity of the host so as to ensure the normal functions of the gut and the brain. Probiotics have beneficial effects on both, physical and mental health, when administered in adequate amount. Thus, probiotics are considered as "psychobiotics", for their effects on central nervous system functions such as stress-related mental disorders and memory abilities, through the gut-brain axis. However, the efficacy of the probiotics on these central functions was in need to be systematically summarized. While there is a host of animal studies on microbiota, it has not yet been studied much how and where in the brain of humans they unfold their effects. Furthermore, antibiotics, having effects on commensal gut bacteria by eliminating and inhibiting them, have so far not been studied for their role in affecting brain functions.

In the current thesis, I performed two literature reviews and two experimental studies on central effects of pro- and antibiotics. The first review systematically analyzed previous research studying the effects of probiotics on central nervous system functions in both, animals and humans. The review concluded the most efficient probiotic interventions and evaluated the possibility of translating preclinical studies to clinical trials. In the second review, we aimed to evaluate the feasibility of a socio-psychological paradigm (Cyberball game) to be used in the following experimental studies with neuroimaging methods and manipulations of the GM. We examined the current neuroimaging literature employing the Cyberball game to induce social stress and feelings of exclusion. The review was intended to generate a framework describing neural processes during the stress.

Following the results of the two reviews, we conducted two clinical trials, to investigate effects of antibiotic rifaximin and probiotic *Bifidobacterium longum* 1714 on

neural activations during resting state and during the Cyberball game by using magnetoencephalography.

In both studies, the stress induced by the Cyberball game enhanced oscillatory brain activity in different areas and in different frequency bands. Both, rifaximin and probiotics had effects on specific neural oscillatory activities in response to the social stress – rifaximin improved subjects' relaxation status by reducing frontal and cingulate beta-1 band power, and *B.longum* 1714 enhanced emotion regulation process by increasing frontal and cingulate theta and alpha bands power. In addition, during the resting state, rifaximin favored individuals' relaxation status by increasing frontal alpha band power, and *B.longum* 1714 increased subject's arousal state by increasing theta band power in frontal and cingulate cortex and reducing the beta-3 band power in hippocampus and temporal cortex.

Rifaximin and *B.longum* 1714, both showed neural effects on the stress response through an "eubiotic" effect, which refers to a healthy balance of the micro-flora in the gastrointestinal tract. Our results provide evidence for gut microbiota alerting CNS functions. Both, reviews and experimental work give clues for further studies targeting the underlying mechanisms of interaction between gut microbiota and CNS function using neuroimaging in patients with psychiatric disorders or gastrointestinal diseases.

1 Introduction

The current dissertation describes a PhD project conduced at the MEG center of the University Hospital Tübingen. The whole project is part of a European training network – NeuroGut, investigating neural regulation of intestinal function. The topic of the project is on the gut-brain axis, and specially on the effects of probiotics on central nervous system functions in humans.

In the **Chapter 1**, I provided the scientific background and logic flow of the project. I started by introducing gut-brain axis, describing the gut microbiota and its important role in the gut-brain axis (**Chapter 1.1, 1.2, & 1.3**). Following, probiotics were introduced and discussed for their effects on the central functions through gut-brain axis (**Chapter 1.4**). The response to social stress was chosen as the central function to study potential effects of probiotics, for its close relation to other mental states (e.g. anxiety, depression levels) and cognitive abilities. Thus, stress and its role in the gut-brain axis was discussed (**Chapter 1.5**), and a social psychological paradigm (the Cyberbgall game) for inducing stress and the relevant humans' neural activities during this kind of stress was presented (**Chapter 1.6**). In **Chapter 1.7**, I provided current evidence supporting the usage of probiotics and antibiotics as a treatment strategy for stress regulation.

Later in Chapter **2, 3, 4, & 5**, summaries of **Study I, II, III & IV** were given. **Study I** was a systematic review summarizing the current efficient probiotic interventions on affecting central functions (both in animals and humans), and the manuscript was published in *Journal of Neurogastroenterology and Motility* in October 2016. **Study II** was a scoping review summarizing the neural processing under the social stress during the Cyberball game, and the manuscript was published in *Neuroscience and Biobehavioral Reviews* in May 2017. Study III represents experimental research on effects of antibiotic rifaximin on neural

responses to social stress during the Cyberball game. The manuscript is currently under review of *Neurotherapeutics*. Study IV was also an experiment on effects of probiotic *Bifidobacterium longum* 1714 on neural responses to social stress during the Cyberball game. The corresponding manuscript is in preparation.

In **Chapter 6 & 7**, attached are the published work **of Study I & II**. In **Chapter 8**, attached is the submitted work of **Study III**. In **Chapter 9**, attached is the manuscript draft of **Study IV**. Conclusions of the whole study and according indication were given in **Chapter 10**. **In Chapter 11**, I expressed my acknowledgement to the people I sincerely appreciate. Lastly, a list of references cited in this dissertation is provided in **Chapter 12**.

1.1 Gut-brain axis

There is bidirectional communication between the gut and the brain through different pathways, integrating neural, endocrine and immune systems (3). The communication has effects on the homeostasis in the gut and also on brain functions including cognitive and affective abilities. Thus disruption of the communication may cause on the one hand intestinal diseases and on the other hand mental disorders (4). Some patients with irritable bowel syndrome (IBS) or inflammatory bowel disorder have been found to have mental comorbidities such as anxiety, depression and autism, indicating the connection between the gut and brain (5).

Mechanisms by which the gut-brain axis exerts its effects involve the neural, immune and endocrine systems (6). The involved neural connections include the enteric, autonomic and central nervous systems. The enteric nervous system (ENS) does not only receive signals from the brain but also sends input to the brain via ascending neural fibers. The autonomic nervous system (ANS) consists of the sympathetic and parasympathetic nerves, co-operating with immune and endocrine system to communicate with the brain. The sympathetic system releases neurotransmitters like noradrenaline to inhibit intestinal motor and secretion. The parasympathetic system which is mediated by hypothalamic-pituitary-adrenal (HPA) axis is related to stress responses. The vagus nerve interfaces with parasympathetic system and has emotional effects as it is excessively activated during emotional stress. Several brain regions in the limbic system of the brain are connected to the ANS such as the hippocampus, the amygdala and the limbic cortex, and these regions also receive input from some other important regions such as prefrontal cortex, the anterior cingulate gyrus and so on. The mucosal immune system modulates cytokine production and thus interacts with the brain. Stress or mental state can influence gut functions. The activity of the HPA axis can affect the

level of cytokines in the gut. The GI tract produces hormones like gastrin and cholecystokinin that influence certain neurotransmitters such as serotonin (5-HT). Also, the gut microbiota is influential on tryptophan metabolites, which is a precursor of the 5-HT. Thus, the gut affects the brain also via the endocrine system. Overall, the gut-brain axis reveals bidirectional communication, integrating the host gut and brain activities.

1.2 Gut microbiota

Microbiota, as the ecological community of commensal, symbiotic and pathogenic microorganisms literally sharing our body space, host cells over 10 times the human cells (7). The majority of the microbiome lives in the human gastrointestinal (GI) tract and is composed of 10 to 100 trillion microorganisms containing 100 times as many genes as our genome (8). Bacteria are the most numerous class of microorganisms as compared to the other two domains of microorganisms (archaea and eukarya) in the GI. Bacteria consist of three divisions named *Phyla* – the *Bacteroides*, the *Firmicutes* and the *Actinobacteria* (9). The microbiota in the GI tract change though life: the initial inoculum in neonates depend on the way of delivery, early microbiota develops unstably but increase in diversity in the first 1-3 years of infancy, the microbial community becomes much more stable during adulthood yet individually very distinct according to different genetics, diet and environmental exposure etc. (10).

Although the gut microbiota (GM) in adults is a relatively stable community, it varies depending among individuals and changes due to altered environments (11). Besides the fundamental impact of host genotype, the GM can be modulated by diet, intake of antimicrobials and probiotics, and also fecal microbiota transplantation which is recently widely used for treatment of many disease (12). Keeping a healthy diet with low fat, low

calories and high fiber is getting more and more support as it helps stabilizing the GM and thus having beneficial effects on the hosts. Also, psychological stress can alter the GM though the gut-brain axis (13, 14), which will be discussed in more detail in the following **Chapter 1.5**.

1.3 Gut microbiota and gut-brain axis

GM is important, because of its role of modulating both, gut functions and brain functions, through the gut-brain axis. An increasing number of studies has found that GM influences the central nervous system (CNS) (3, 15). Multiple direct and indirect pathways exist, though which the GM influences the CNS through the gut-brain axis: the GM can influence activity of immune cells and alter levels of pro-inflammatory and anti-inflammatory cytokines which affect brain functions; tryptophan metabolism and neurotransmitter metabolism can be affected by the GM so as to influence the brain; short-chain fatty acids (SCFAs) are neuroactive bacterial metabolites and can also modulate brain activity and behaviors, etc. (13).

Many studies using germ-free (GF) animals have reported that the commensal microbes in the gut can affect behavior via immune, endocrine and/or neural systems. GF animals are maintained in a sterile environment to avoid postnatal colonization and can be used for comparing them with conventionally reared animals. Sudo's study was the first study to link the GM and behavior by showing increased HPA stress response and decreased brain-derived neurotrophic factor (BDNF) in the hippocampus of GF mice (16). Also, the GF mice had impaired memory function compared to mice with an intact intestinal microbiota, and had reduced preference for social novelty and increased repetitive self-grooming behavior (17, 18).

To study the role of the GM in affecting CNS functions, there are many methods to manipulate the GM. By means of exposing the GF mice to a natural environment, colonization them with specific microbiota, changing their diet or by means of infection, manipulation of the GM can be achieved.

Specifically, GM mice showed less anxiety-like behavior after 24 hours' exposure to the environment outside the sterile one, which suggested the importance of natural and intact composition of the GM for behavior (19). Also, GF mice showed less HPA stress response and less anxious behavior by reconstitution with *Bifidobacterium infantis* and enhanced HPA stress response after monoassociation with enteropathogenic *Escherichia coli* (16). The relationship between the behavioral phenotype of the GM mice and the corresponding brain regions has been described (6). The key regions consist of the striatum, a region integrating movement and emotional responses; the hippocampus, known for its functions of memory and spatial navigation; the amygdala, always considered as the "emotional brain", sending signals to the other key region for emotional regulation - hypothalamus.

Other methods for manipulating GM include changing diet and inducing infections. Mice on high-fat diet showed altered GM, and less burrowing behavior and disrupted memory compared to mice on a control diet. In addition, altered levels of BDNF and cytokines were also found, indicating the role of the GM in behavior through the immune system. In a study inducing infection with parasite *Trichuris muris* in mice, increased anxiety-like behavior was found. The altered behavior was also coupled with changes in the central nervous system biochemistry including decreased hippocampal BDNF mRNA and kynurenine, etc. (21). *Citrobacter rodentium* - infected mice also showed impaired memory function when exposed to acute stress (18).

Increasingly, studies have induced manipulations of GM using probiotics, described as gut bacteria, whose consumption has been advocated due to its beneficial effects on the hosts' health. Early studies using probiotics can be traced back 10 years. In humans, probiotic *L. casei* Shirota contained in milk has improved questionnaire-based mood and memory performance in healthy volunteers (22). In rats, *B. infantis* 35624 reduced depression-like behavior along with improved brain monoamines and metabolites (23, 24).

Compared to the animal studies, there are fewer studies in humans on the role of GM in central nervous system functions. One reason for the fewer studies in humans is the challenge to measure and manipulate the GM in human gut because of its diversity due to genetic and environmental differences among individual humans. Recently, however, researchers have found relations between the GM and behavior-related disorders such as Parkinson's disease and autism. Patients with Parkinson's disease had reduced abundance of *Prevotellaceae* in their feces. These changes of the GM composition could be related to the gastrointestinal dysfunction and motor symptoms in these patients via the ENS and the CNS (25). In individuals with autism, there were decreases in the *Bacteroidetes*/*Firmicutes* ratio and in the amount of *Bifidobacterium*, and increases in bacteria *Clostridium* and *Desulfovibrio* (26).

Nevertheless, maintaining a symbiosis of the GM is important for health, physically and mentally. In contrast, dysbiosis of the GM can disrupt the balance and may induce diseases. Probiotics as a means of stabilizing and improving the GM have lately received much attention due to its beneficial impact on CNS functions and mental health.

1.4 Psychobiotics and gut-brain axis

Briefly mentioned in the last chapter, probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (27, 28). Certain types of probiotics have been used to treat GI disorders, such as IBS (29, 30), and have also been studied in relation to treating visceral pain (31). Recently, probiotics have been described as "Psychobiotics" that "when ingested in adequate amounts have a positive mental health benefit" (32). They have been reported to influence the CNS by altering the GM composition. CNS functions mostly reported of being altered by probiotics are psychiatric disorders and memory abilities. Studies in animals and humans have suggested potential mechanisms underlining these probiotic effects. First, probiotics may directly alter CNS biochemistry, by affecting levels of BDNF, gaminobutyric acid (GABA), serotonin (5 hydroxytryptamine; 5 HT), and dopamine (DA), and thus influence mind and behavior (33-37). Both the vagus and the enteric nerves are involved in this gut-brain interaction and can be affected by certain probiotics (33, 34). The HPA stress response, which regulates mood and emotion has frequently been shown to be attenuated by probiotics via decreased corticosteroid (CORT) levels (38). The immune system can be influenced by probiotics, limiting pro-inflammatory cytokine production and inflammation, which, in turn, can affect the endocrine and nervous systems (23, 24). Probiotics manipulate GM by increasing microbiota diversity and beneficial bacteria compositions (39-41). Improved GM changes metabolites, such as short-chain fatty acids and tryptophan, which can indirectly improve CNS function (23, 42).

Anxiety

Anxiety has been characterized as emotional response to real or perceived imminent threat or the anticipation of future threat (43). Anxiety-like behaviors can be induced by experimental manipulation. Mice with chronic inflammation and infection showed enlarged anxiety-like behavior. High levels of anxiety could be reduced by *B.longum* NC3001 in two studies (21, 33). This strain also reduced enteric neurons excitability and BDNF expression. *L.helveticus* normalized the increased anxiety-like behavior of mice under chronic stress. Together with increased anti-inflammatory cytokine, decreased CORT and ACTH, increased brain 5-HT, NE and BDNF expression, *L.helveticus* has anxiolytic effects through the immune, the endocrine and the nervous systems (44). Healthy volunteers showed decreased subjective anxiety level after receiving probiotics *L. helveticus* and *B.longum* (45, 46). In patients with chronic fatigue syndrome, anxiety levels were decreased and fecal *Lactobacillus* and *Bifidobacteri*a were raised by *L. casei* Shirota (47).

Depression

Depression is a common psychiatric disorder that can be long-lasting and impair people's life (48). Depression is associated with stress experience and dysregulation of the immune system (49). Probiotic *L. plantarum* PS128 showed antidepressant effects in GF mice and mice with early life stress, and also changed cytokine levels (decreased inflammatory cytokine TNF-α and IL-6, increased anti-inflammatory cytokine IL-10) and metabolites of 5-HT and DA in the brain (35, 36). *L. rhamnosus* JB-1 showed vagus dependent effects of antidepressant effects and also altered GABA receptor expressions in the brain (34). In healthy volunteers, in addition to an anxiolytic effect, formulation of probiotics *L. helveticus* and *B.longum* also reduced depression compared to placebo (45, 46).

Autism

Autism spectrum disorder (ASD) describes a set of neurodevelopmental disorders characterized by defects in social interaction and communication (50). Probiotic *B. fragilis* NCT9343 has been shown to reduce ASD-related behaviors in animals. It also reduced intestinal permeability and tryptophan metabolites. Previous studies have reported that SCFA administration could result in some autistic-like behavior (51), however, *B. fragilis* did not show an effect on SCFA level. In humans, studies have examined effects of probiotic supplements in ASD. One study has reported that probiotics improved the ability of children to carry out orders and to concentrate (52). However, no effects on emotional response have been reported. Another study used questionnaires and obtained improvements in behavior after probiotic intake, i. e. showing lower scores of disruptive antisocial behavior and communication disturbances compared to placebo group (53). However, evidence for probiotics being able to alter behaviors in ASD is still limited, and further studies are needed (54).

Memory ability

Memory abilities, including spatial and non-spatial memory such as object recognition and emotional memory, have been improved by probiotics in animals in several studies (18, 40, 41, 44, 55-59). Some of the studies also found that probiotic increased BDNF and c-fos, two proteins that play important roles in the regulation of hippocampal-dependent memory (Liang, 2015; Smith 2014; Jeong, 2015). BDNF modulates synaptic plasticity of the hippocampus during neurogenesis, whereas c-fos is an immediate early gene that is required

for hippocampus-dependent long-term memory formation (60, 61). In humans, one study used scales to test different memory abilities (e.g. episodic memory, long-term memory), on which probiotics showed improving effects (22). A recent study using the Paired Associate Learning test from the Cambridge Neuropsychological Test, showed improvements of visuospatial memory performance following intake of *B.longum* 1714 (62). In short, animal studies have provided more convincing evidence for probiotic related memory effects than studies in humans. Clinical studies with standardized behavioral models are needed to test effects of probiotics on memory.

Stress response

One important central function is the response to stress, which is closely related to many psychiatric disorders (e.g. anxiety and depression) (65). In animals, probiotics of *L. rhamnosus* R0011and *L.helveticus* R0052 restored memory impairments and anxiety-like behavior induced by water avoidance stress as an acute stressor (18). Probiotic *B. infantis* 35624 reduced depression-like behavior caused by chronic stress such as maternal separation (24). In humans, cortisol levels as stress response have been measured and found to be reduced after probiotic treatments such as *Lactobacillus casei* Shirota and *B.longum* 1714 (63, 64). Due to the important role of the stress in brain functions, I expanded the discussion of the gut-brain axis to the role of stress in current paradigms for inducing stress, and to the regulation of stress responses by probiotics in **Chapter 1.5, 1.6 &1.7**. In **Chapter 8 & 9**, I have tested the effects of antibiotic rifaximin and probiotic *B.longum* 1714 on the stress response in healthy volunteers via the modulation of GM and its effects on the CNS. Stress was induced by a social psychology paradigm, called Cyberball game. I used

magnetoencephalography to measure the brain activations related to the processing of stress because of its fine spatial and high temporal resolution as neuroimaging method.

Cortical activity

In humans, clinical studies using neuroimaging methods have started to emerge, linking neural effects of probiotics with brain activity. The first study to demonstrate alterations in cortical activations by probiotics has used functional magnetic resonance imaging (fMRI) (8). A fermented dairy drink with probiotics affected the activity of brain regions controlling central processing of emotion and sensation – reduced activation in widely distributed areas including frontal and temporal cortices, parahippocampal gyrus, primary interoceptive and somatosensory cortices, in response to negative emotional stimuli (66). A recent study administrated *B.longum* NCC3001 to IBS patients and found that, in addition to an improvement in psychiatric comorbidity, brain activations to negative emotional stimuli were reduced in amygdala and fronto-limic regions (67). By now, findings of neural effects of probiotics largely come from animal studies, while evidence from human studies is much demanded for the potential applications in treatments and called for to be conducted with neuroimaging methods.

1.5 Stress and Gut-brain axis

Stress describes the effects of psychosocial and environmental factors on physical or mental well-being. Today almost everyone seems to be affected by the negative effects of stress (68, 69). Stress response, is considered as reactions that is elicited to counteract a possible threat and to adjust the organism to the stressor, which is individual specific (70).

There are two stress-responsive systems: the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (70, 71). Through these two systems, stress elicits emotional and behavioral reactions. When stress is uncontrollable and chronic, it can trigger mal-adaptive changes in the CNS and can give rise to structural and/or functional changes of the brain (72, 73). Specially, chronic stress can cause chronic sympathetic activation and leading to anxiety disorders and depression. The HPA axis and the SNS are involved in the onset, the development and the progression of anxiety and depression. In addition, memory abilities can be impaired by prolonged and high level of stress (74). Stress may raise corticosteroid secretion and inhibit hippocampal activity, which is a main brain area for memory and learning processes (75, 76).

Stress is also related to gut functions and is one of the factors for GI disorders. Stress has an impact on physiological gut functions including gut motility, secretion, visceral sensitivity and mucosal blood flow (77). IBS is a typical stress-sensitive disorder with an irritable bowl and irritable brain. Research has revealed that early life stress contributes to development of IBS symptoms (78, 79). Another consequence of stress on IBS is the induction of co-morbid psychiatric disorders (e.g. depression and/or anxiety) (5). To treat IBS, antidepressants such as selective serotonin reuptake inhibitors, have been utilized (80).

Furthermore, preclinical and clinical studies have shown that stress has a negative influence on the GM, which plays a crucial role in the gut-brain axis. Stress influences the composition and the function of the GM - stress is able to increase gut permeability and allow bacteria and bacterial antigens to cross the epithelial barrier; meanwhile this change also activates a mucosal immune response which in turn alters the composition of the GM (81, 82). Subsequently, alteration of the GM is related to a variety of GI and mental disorders

(83). Again, IBS as gut-brain axis disorder accompanying with anxiety and/or depression symptoms, has been demonstrated to have a disrupted GM (84).

By linking impacts of stress on both, CNS and gut functions, it seems that keeping a life style that avoides exaggerated stress may be good for the both, physical and mental health. However, given the fact that people suffer to certain degrees from stress and have different tolerances and responses to stress, one may consider the modulation of stress as a target to improve brain and gut functions. Due to the crucial role of GM for the processing of stress, improving GM to modulate stress responses is believed to be a beneficial strategy. Recently, microbiota-brain-gut axis has been discussed in different stress-related CNS disorders and has been demonstrated of being capable to regulate stress response (6, 85, 86).

As reviewed by our pervious study (see **Chapter 6**), probiotics reduce stress response through brain-gut interactions ("microbiome-gut-brain axis") and also attenuate the development of stress-induced disorders – both mental disorders or GI disorders (87). For example, a probiotic combination of *L. rahmnosus* and *L. helveticus* normalized memory dysfunction induced by an acute water avoidance stress (18); single probiotic strain of *B. infantis* prevented depression-like behavior caused by maternal separation – one of the best characterized animal models to induce chronic stress (24).

Following the discussion of the important role of stress in the gut-brain axis, and the strategy of using probiotics intervention as a manipulation of GM to modulate stress management, we next will discuss a standardized social psychology paradigm for inducing social stress in humans. The paradigm is presented as model to study effects of GM experimentally. This part summarizes the neural process of the social stress induced by the paradigm (presented fully in **Chapter 4**).

1.6 Social stress – Cyberball game

To study effects of probiotics neural and behavioral models can be translated from animal research to human studies, - however limitations also exist (see more details in **Chapter 6)**. Briefly, most of the standardized animal models for measuring anxiety- and depression- like behaviors are not translatable to humans, because measurements of anxiety and depression in humans rely primarily on psychology scales. Measurement of learning and memory abilities of humans are relatively more standardized compared to psychiatric conditions. For example, the Morris water maze for animals has a computerized version for humans: the object recognition tasks and fear conditioning are widely used in humans. Stress can be induced in a physical way as heat or cold stressor (88, 89), or in a psychological way as social stressor. The Trier Social stressor is widely used for inducing a stressful situation by letting participants give a public speech (90), which however, is difficult to be accomplished in a scanner.

In search of a stress paradigm that would allow the testing of social stress and psychobiotic effects on the stress response with neuroimaging methods in humans, we have identified the "Cyberball game" (CBG). This is a virtual online game that is often used to study social stress by social exclusion/rejection (91). Chronic experience of the social exclusion may also be regarded as a stressful situation, which may lead to the development of depression. Numerous studies using different methods such as fMRI and electroencephalography (EEG) have discovered widely distributed brain regions involved in social exclusion versus inclusion. However, various experimental designs have led to divergent results regarding the neural activations during this kind of social stress. Therefore, there was a need for a review aiming at the conclusive evaluation of the neural processes involved in the social stress/exclusion induced by the CBG.

Hence, we have conducted a scoping review on the current literature that has used neurophysiological measurements such as using fMRI and EEG to investigate the neuronal processes underlying the responses to social stress induced by the Cyberball game (see **Chapter 7**) (92). Considering methodologies and results of published work, we have identified a common spatio-temporal pattern of neural activations related to social stress/exclusion, and analyzed influential factors modulating neural activations (see more details in **Chapter 7**). Based on the results, we have developed our research hypotheses of how probiotics might influence subjects' neural activity. **In Study III** and **IV** (**see Chapter 8 & 9**), we have conducted two randomized, placebo-controlled, double-blinded trial, to test these hypotheses for the effects of administration of the antibiotic rifaximin and the probiotic *B.longum* 1714 on neural responses to the CBG.

1.7 Stress response regulation

Antibiotics

In **Chapter 1.3**, we have described usage of GF animals to study effects of the GM in the gut-brain axis. Recently, GM manipulations with antibiotics have been suggested as an alternatives and complementary strategy (93). Administrations of an antibiotic cocktail of bacitracin, neomycin and primaricin in mice reduced anxiety-like behavior and increased hippocampal BDNF mRNA expression (94). In another study, reduction of anxiety was also observed but along with reduced cognitive abilities and social deficits followed by antibiotic treatment (95). In this study brain BDNF levels also showed inconsistency to the last mentioned study– reduced in hippocampus. A mixed antibiotic treatment of bacitracin, neomycin, ampicillin and vancomycin impaired memory abilities of rats and was at the same

time associated with reduced BDNF and HPA activity - two brain molecules related to stress response (96).

Observations of behavioral changes in humans by antibiotics date 30 years back when patients treated for inflammations showed enlarged stress responses as becoming irritable and aggressive after taking amoxicillin-cavulanate. Patients revealed normal behavior after having stopped the mediation (97). However, another study reported a positive effect of amoxicillin: children with autism spectrum disorder showed an improvement of autistic symptoms, verbal skills and a reduction of repetitive behaviors after consumption of amoxicillin (98).

In our recent clinical trial in healthy volunteers, a locally acting broad-band antibiotic rifaximin, showed a stress-reducing effect along with altered brain activity during a resting-state recording and during the CBG acting as a social stressor (see more details in **Chapter 8**) (99). Rifaximin has poor bioavailability (<1% systemically absorbed), and little risk for provoking antibiotic resistance (100). Due to its property, it is used in healthy volunteers and also in patients without severe infection, e.g. in prevention of tropical diseases and traveler's diarrhea (101). A few trials have shown clinical efficacy in IBS (102, 103) and in small intestinal bacterial overgrowth (104, 105). Additionally, it is used to prevent a neuropsychiatric syndrome caused by the liver disease, hepatic encephalopathy (106). In a clinical trial, eight-weeks intake of rifaximin did enhance cognitive abilities in patients with liver cirrhosis, which altered brain activations during the relevant tasks (107). The neural effects of rifaximin were suggested to be mediated through gut-liver-brain axis by modulating gut bacteria, serum bilirubin and endotoxemia.

However, caution may be needed when coming to a conclusion of effects of antibiotics on the CNS, since the effects could be either positive or negative depending on

individuals' health conditions. Antibiotics appear to be effective on improving CNS functions in populations in which the disease has already started (98, 107). However, it needs to be noted that those who suffer from diseases and are in need for antibiotic treatments may have infections or altered GM already so their central functions may have been distorted by the infection. Any central effects by antibiotics would be blurred by indirectly acting mechanisms of the antibiotics. Antibiotics have been studied for their disturbing effect on stress-related central functions (e.g. anxiety, memory) in animals, due to its antimicrobial property. However, they have only rarely been investigated for their beneficial effects on central functions so far.

Probiotics

Probiotics have been much studied for their effects on stress response, stress-related central functions and as well as GI disorders (e.g. IBS) (refer again to **Chapter 1.4 & 6**). In animals, stress responses such as corticosterone levels, depressive behaviors and memory dysfunction were ameliorated by assumption of probiotics such as *L. casei* Shirota, *L. rhamnosus*, and *B.infantis* 35624 (18, 24, 108).

However, proofs for the beneficial effects of probiotics from human studies are limited due to the difficulty of translating animal models into clinic trials. For example, probiotics which were effective in rodents turned out to be ineffective in humans. Moreover, while changes in stress management in animals are quantified by physical behavioral parameters, in humans most often subjective psychological questionnaires are used with limited objectivity due to subjects' biases (81). In a study that targeting effects of *B.longum* 1714 on stress responses, not only behavioral effects were found, but also morning cortisol level (links to stress-related preparation) was decreased after intervention (62). The same

strain was used in our recent study (**Study IV**) which measured neural activation in response to a social stressor – the CBG (see **Chapter 9**). It has been indicated that *B.longum* 1714 plays a role in managing distress induced by a social stressor, by up-regulating processes of appraising the stressful events and down-regulating the negative emotions, indexed by increased frontal and cingulate theta and alpha neural oscillatory activities.

Since accumulating evidences from animal and human studies show that probiotics are capable of influencing stress responses by decreasing behavioral and endocrine components of stress, and by altering cortical activities in response to stressors, it seems promising to verify probiotics as a therapeutic strategy for mood and stress-related disorders.

2. Studies in effects of probiotics on CNS functions (study I)

Numerous studies have investigated the effects of probiotics on CNS functions, but so far a systematic summary of the most effective probiotic intervention has been lacking. Compared to animal studies which are better established and standardized, clinical studies in humans are still at their beginning and need further translation from animal studies.

The systematic review (study I) aimed to elaborate CNS functions that can be influenced by probiotics and summarize effective interventions including strain, dosage and duration, by analyzing previous and current studies in animals and humans; and to discuss the possibility of translating animal models to human studies.

In total, 38 studies were included: 25 in animals and 15 in humans (2 studies were conducted in both). Most studies used *Bifidobacterium* and *Lactobacillus*, with doses between 10^9 and 10^{10} colony- forming units for 2 weeks in animals and 4 weeks in humans. These probiotics showed efficacy in improving psychiatric disorder- related behaviors including anxiety, depression, autism spectrum disorder, obsessive-compulsive disorder, and memory abilities, including spatial and non-spatial memory. Since the translation of animal studies to humans has both, limitations and possibilities, I provide several suggestions of how to implement the translation of animal studies.

3. Functional neuroimaging studies in Cyberball game (study II)

Neuroimaging studies have investigated human brain responses to social stress induced by the social psychology paradigm – the CBG. Due to the inconsistency of the results there is a strong need to conclude the neuronal processes underlying social stress.

In a scoping review (study II), we aimed to identify a common spatio-temporal pattern of neural activations associated with the social stress/exclusion during the CBG, through mapping studies using neuropsychological measurements (e.g. fMRI, EEG).

In total, 42 studies were included and analyzed. An integrated framework describing neural activities under social exclusion in terms of both, temporal and spatial processes was provided. Regions of the insula, anterior cingulate cortex, temporal and prefrontal cortex were activated to social exclusion. These neural activities were pronounced at latencies ranging from 200 to 400 ms, and between 400 and 900 ms. Also, exclusion-related changes in neural oscillations revealed alpha power increase in frontal cortex and theta power increase in insula, subACC and fusiform face area (FFA).

The results of the review helped me designing my research studies (study II & study IV) on effects of antibiotic and probiotic interventions on neural responses to social stress by the CBG, and with interpreting the findings.

4. Effects of antibiotic rifaximin on central responses to social stress (study III)

As reviewed in our paper in study I (**chapter 6)**, probiotics appear to have beneficial effects in improving certain central functions (87), however, how strong the effects are, is still a matter of debate. A publication bias toward positive results cannot be excluded and negative results in relation the changes of central functions may have been unreported. Even for the clinical efficacy of probiotics in IBS, it has been concluded that there is limited evidence for positive effects of probiotics in IBS treatment (30). Mechanisms by which probiotics affect neural functions are incompletely understood, but there is clear evidence that they do affect the commensal GM by increasing diversity and/or competitive colonization. An "improved" GM influences metabolites such as short-chain fatty acids and thus improving then central functions. In order to see modulations of GM on neural response to social stress, we have tested firstly effects of an antibiotic prior to probiotic, since we expected to observe a larger effect of antibiotic as it may either kill or inhibit the growth of bacteria, thus influencing the GM.

In an explorative experiment (study III), we aimed to test the efficacy of rifaximin on the brain activities during a resting state not performing any tasks and during a social stress using magnetoencephalography (MEG). The study aimed to identify neural oscillatory responses to the social stress induced by the CBG and its modulation by rifaximin. For these purposes, we have conducted a randomized, double-blinded and placebo-controlled study with 16 healthy volunteers, whose brain activities were measured during resting state and during the social stress, before and after 7-days drug intervention with either rifaximin or placebo.

With MEG, we observed several brain regions with higher activations during social stress/exclusion in different oscillatory bands, which were associated with stress-related lower mood and increased subjective exclusion perception. Consumption of rifaximin for 7 days significantly increased alpha rhythm activity in the prefrontal and cingulate cortex during the resting state. Also during social stress, rifaximin decreased beta rhythm activity in the prefrontal and cingulate cortex during the social stress condition. The decrease of beta activity was correlated with increase of subjective exclusion perception.

Contrary to our hypothesis that rifaximin would increase the stress response it exhibited stress-reducing effects. Following the validation of using MEG to detect the neural stress response to the CBG and its modulation by manipulating GM, we moved forward to study the neural effects of probiotic in response to this social stressor.

5. Effects of Probiotic *B.longum* 1714 on central responses to social stress (study IV)

Probiotics have been been found to affect CNS functions. We have addressed the importance of stress response in the gut-brain axis and summarized effects of probiotics on the stress response in the previous studies (see **Chapter 1.5 & 1.7**). Recent research has moved forward to translate animal studies to clinical human studies and specially to utilize neuroimaging methods to detect brain activations altered by probiotics.

Based on evidence from studies in animals and humans, we have selected probiotic strain *Bifidobacterium longum* 1714 to study its neural effects on stress response. This strain had previously been demonstrated to modulate stress related behaviors in mice (59, 109). In humans, it showed effects of reducing stress responses to a cold stressor and improving cognitive abilities following treatment (64). It was interesting to investigate where and how probiotics affect cortical activations using neuroimaging method, both, during rest and in response to a stressful situation.

Since MEG was verified in our previous study (study III) on its capacity to detect neural oscillation activations during the social stress – CBG, in study IV, we conducted a similar clinical trial, by using the same paradigm, same neuroimaging technique and probiotic strain *B.longum* 1714. As a main study, 40 healthy participants were recruited and measured on their brain oscillatory activities with MEG during a resting state and during the CBG. Participants were examined both, before and after their intervention with either *B.longum* 1714 or placebo for 4 weeks. Also, their health status was assessed using the 36-item short-form health survey (SF36).

We observed that *B.longum* 1714 affected resting brain activities by increasing the frontal and cingulate theta band power and decreasing beta-3 band power in the hippocampus, fusiform, and temporal cortex. These neural activity changes were associated with subjective vitality changes indexed by SF36. For the neural activity change in response to the social stress, *B.longum* 1714 increased theta and alpha band power in the frontal and cingulate cortex, which were correlated with increased distress level during the stressor.

The results indicated *B.longum* 1714 enhanced participants' arousal status and reduced their mental fatigue. The strain also affected neural stress response by up-regulating emotion regulation.

6. Study I. Effect of probiotic on central nervous system functions in animals and humans: a systematic review

Author contributions

Paul Enck conceptualized the paper; Huiying Wang reviewed and evaluated the literature; and Huiying Wang, In-Seon Lee, Christoph Braun, and Paul Enck wrote the manuscript.

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Review

Effect of Probiotics on Central Nervous System Functions in Animals and Humans: A Systematic Review

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To systematically review the effects of probiotics on central nervous system function in animals and humans, to summarize effective interventions (species of probiotic, dose, duration), and to analyze the possibility of translating preclinical studies. Literature searches were conducted in Pubmed, Medline, Embase, and the Cochrane Library. Only randomized controlled trials were included. In total, 38 studies were included: 25 in animals and 15 in humans (2 studies were conducted in both). Most studies used *Bifidobacterium* (eg, *B. longum*, *B. breve*, and *B. infantis*) and *Lactobacillus* (eg, *L. helveticus*, and *L. rhamnosus*), with doses between 10^9 and 10^{10} colony-forming units for 2 weeks in animals and 4 weeks in humans. These probiotics showed efficacy in improving psychiatric disorder-related behaviors including anxiety, depression, autism spectrum disorder (ASD), obsessive-compulsive disorder, and memory abilities, including spatial and non-spatial memory. Because many of the basic science studies showed some efficacy of probiotics on central nervous system function, this background may guide and promote further preclinical and clinical studies. Translating animal studies to human studies has obvious limitations but also suggests possibilities. Here, we provide several suggestions for the translation of animal studies. More experimental designs with both behavioral and neuroimaging measures in healthy volunteers and patients are needed in the future.

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Key Words
Animals; Anxiety; Depression; Humans; Probiotics

Introduction

The microbiota, the ecological community of commensal, symbiotic, and pathogenic microorganisms literally sharing our body space, includes more than 10 times the number of host cells to human cells.[1] The majority of the microbiome lives in the gastrointestinal (GI) tract and is composed of 10 100 trillion microorganisms, containing 100 times as many genes as our genome.[2] Symbiosis of the gut microbiota (GM) can maintain a normal physiology in the host, while dysbiosis of the GM can shift the balance and may induce diseases.

Recent studies have found a role for the GM in the gut-brain axis, which can alter minds and behaviors through the central ner-

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vous system (CNS).[3-8] Maintaining GM symbiosis is important for retaining healthy CNS functions. Sudo's study was the first linking the GM and CNS, showing an increased hypothalamic-pituitary-adrenal (HPA) stress response and decreased brain-derived neurotrophic factor (BDNF) levels in the hippocampus of germ-free (GF) mice.[9] Recently, researchers have found a relationship between the GM and CNS-related disorders in humans, such as Parkinson's disease and autism: overall diversity and individual genus abundances were associated with their symptoms.[10-12]

Probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host".[13,14] Some types of probiotics have been used to treat gastrointestinal disorders, such as irritable bowel syndrome (IBS).[15-18] Also, probiotics have been studied in relation to altering visceral pain responses.[19] Recently they have been reported to have an influence in the CNS by altering the GM composition.[3,8] Studies using probiotics to change CNS functions have increased over the last 10 years. The CNS functions mostly reported being altered by probiotics are psychiatric disorders and memory abilities. Studies in animals and humans have found, and reviews have summarized, potential mechanisms underlining these probiotic effects (Fig. 1). First, probiotics may directly alter CNS biochemistry, such as by affecting levels of BDNF, γ-aminobutyric acid (GABA), serotonin (5 hydroxytryptamine; 5 HT), and dopamine (DA), thus influencing mind and behavior.[20-24] Both the vagus and the enteric nerves are involved in this gut-brain interaction and can be affected by certain probiotics.[22,23] The HPA stress response, which regulates mood and emotion, has frequently been shown to be attenuated by probiotics, decreasing corticosteroid (CORT) levels.[25] The immune system can be influenced by probiotics, limiting pro-inflammatory cytokine production and inflammation, which, in turn, can affect the endocrine and nervous systems.[26,27] Probiotics manipulate GM by increasing microbiota diversity and beneficial bacteria compositions.[28-30] Improved GM changes metabolites, such as short-chain fatty acids and tryptophan, which can indirectly improve CNS function.[26,31]

While there are reviews describing effects of probiotics on CNS function, there has been no previous systematic review that analyzes all the current animal and human studies in the field and describes the most effective probiotic interventions. Furthermore, animal studies in this area outnumber human studies, because behavioral experiments in animals are better established and standardized, while clinical studies in humans on this topic started to increase a few years ago and need translation from preclinical studies. How-

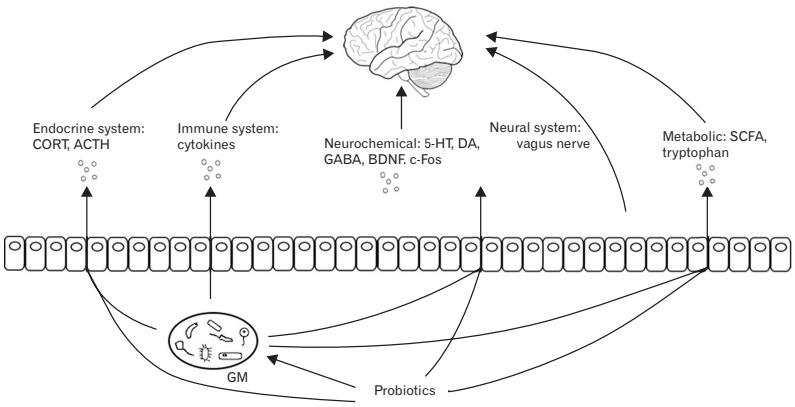

Figure 1. Mechanisms of probiotic effects on the central nervous system. Probiotics influence central nervous system (CNS) function through direct and indirect mechanisms. Probiotics affect the hypothalamic-pituitary-adrenal (HPA) axis, by altering corticosteroid (CORT) and/or adrenocorticotropic hormone (ACTH) levels. The immune system is influenced by limited pro-inflammatory cytokine production and inflammation, and this, in turn, has effects on the CNS. Probiotics can also directly alter CNS biochemistry, such as by affecting brain-derived neurotrophic factor (BDNF), c-Fos, γ-aminobutyric acid (GABA), 5 hydroxytryptamine (5-HT), and dopamine (DA) levels, thus influencing mind and behavior. The vagus and enteric nerves are also involved in this gut-brain interaction and are affected by certain probiotics. Probiotics manipulate the gut microbiota (GM) by increasing microbiota diversity and beneficial bacteria composition. An "improved" GM changes metabolites, such as short-chain fatty acids (SCFAs) and tryptophan, and so improves CNS function indirectly. The GM also interacts with the endocrine, immune, and neural systems.

ever, from preclinical animal models to clinical trials in humans, there is no direct translation. To bridge the gap between preclinical and clinical studies, a systematic review is needed to summarize effective probiotic interventions on CNS function. We first sought to describe CNS functions that can be influenced by probiotics. Second we provide information about probiotic interventions including strain, dosage, and duration. Furthermore, we analyze and discuss the potential translation of animal models to human studies.

Methods

Search Strategy

This systematic review was conducted according to guidelines of the "Cochrane Handbook for Systematic Reviews of Interventions", following the Preferred Items for Systematic Reviews and Meta-analysis guidelines.[32,33] Relevant studies were found by searches in the Pubmed, Medline, Embase, and Cochrane Library databases. Articles from 1950 to April 2016 were initially searched using the search terms "(probiotic OR gut microbiota) AND (behavior OR central nervous system)". Additional citations were sought using references in articles retrieved from searches. We only included articles written in English. The first search was undertaken by analyzing text words contained in the title and abstract, and of the key words describing the articles. The second search was conducted according to the citations from all identified reports and relevant review articles.

Study Selection

We included all animal and human studies using different strains of probiotics. In human studies reports of both healthy volunteers and patients were considered.

The abstracts of the retrieved papers were screened for matching the following criteria: (1) the study included a probiotic intervention and (2) the study tested CNS function. After exclusion of non-relevant studies, the remaining articles were screened for the following criteria: (1) the study was described as a randomized controlled study (RCT), (2) the study was described as double-blind if studied in human participants, (3) the study involved use of probiotics in single- and/or multi-strains and those that only used prebiotics or antibiotics were not included, and (4) the study included measures of behavioral experiments, neuropsychological measures (eg, electroencephalography, magnetoencephalography, and functional magnetic resonance imaging). Those that only involved neurophysiological measures were not included in the qualitative analysis but only in the Discussion (eg, measuring neurochemical level, HPA axis activity) because different studies tested quite divergent aspects of lower-level CNS activity.

Data Collection and Analysis

A data extraction and assessment form from the Cochrane Collaboration was used to further exclude inappropriate studies and to extract data we needed to analyze.[33] Double extraction of data was conducted: important data included the source of participants (animal species/strains, patient types), intervention groups (probiotic, placebo, or any other intervention), sample size, duration of interventions, and outcomes (behavioral changes as the primary outcome and lower-level changes [eg, biochemical changes] as secondary outcomes).

We assessed the quality of each study included using the Quality Assessment Tool for Quantitative Studies and the Quality Assessment Tool for Quantitative Studies Dictionary developed by The Effective Public Health Practice Project (EPHPP) (National Collaborating Centre for Methods and Tools [2008]).[34]

The inclusion of both animals and human studies resulted in great heterogeneity of participants, interventions, and CNS functions measured. The outcomes of each study were described. A qualitative synthesis of selected studies was made with the aim of coming to conclusions about which probiotic interventions, at which dose, and for how long, were more/most effective with regard to certain CNS functions. Because of the heterogeneity of designs a meta-analysis was deemed to be inappropriate.

Results

In total, 46 potentially relevant citations were obtained through the primary search strategy, which included animal and human studies, after excluding reviews (n = 98) (systematic reviews, narrative reviews, respective reviews, and systematic reviews and meta-analyses), other articles including comments, hypotheses, viewpoints, opinions, and editorials (n = 13), studies without the use of a probiotic (n = 33), and studies not focusing on CNS function (n = 16) (Fig. 2). Many articles concerned the potential, and demonstrated mechanisms of the effects, of GM and/or probiotics on CNS function. However, we only included studies that clearly described probiotics as interventions, and excluded studies only measuring GM composition when investigating CNS function. After the full screen, 8 more studies were excluded: four studies lacked a control arm or blinding, and 4 studies did not measure CNS functions at the behavioral level. In total, 38 studies remained for

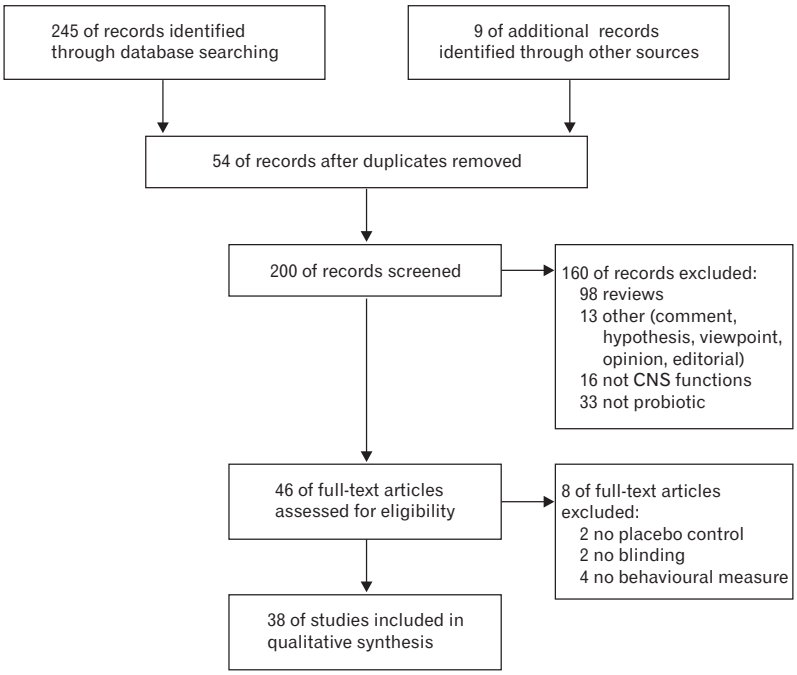

Figure 2. Preferred reporting items for systematic reviews and meta-analyses (PRISMA) scheme of retrieved literature. CNS, central nervous system.

the qualitative analysis. Of them, 15 were in humans and 25 were in animals (2 studies were conducted in humans and animals).

Animal Studies

Of the 25 studies, 19 provided explicit information in the Materials and Methods section on the numbers of animals allocated to each treatment group, and they accounted for all the animals in their results, while 6 studies provided specifics regarding treated animals only in the pertinent areas of the Results section. Results from the quality assessment tool for quantitative studies showed all studies were strong in global ratings for selection bias, study design, confounders, blinding, data collection method, and withdrawals and dropouts (no weak rating): 15 studies described assigning animals randomly to treatment groups or control, although these animal studies were supposed to be RCTs. Only four studies might have confounders because they used both male and female animals while the other studies only used single gender animals, excluding the effect of gender on the results. Only 13 studies reported that experimenters were blinded toward the interventions or exposure status of the participants (we included the other 12 studies that did not describe blinding, because animals were not aware of the research question or intervention, and we ignored potential effects of the experimenter). Data collection tools shown in all studies were considered valid and reliable; no study reported withdrawals or drop outs.

Although all of studies examined were on rodents (mice or rats), the selection of animals was heterogeneous in some respects: strains of animals and health conditions of the animals. Studies were also heterogeneous in the CNS functions they were looking at, the experimental models they used, the probiotics they used, and the dose and duration of the probiotic interventions. Due to the heterogeneity of the included studies, we only described the results based on the interventions and measurements of the CNS functions (Table 1).

Most of the studies (18/25) investigated the effects of a single strain of probiotics. In 18 studies using single-strain probiotics, seven used *Bifidobacterium*, eleven used *Lactobacillus*, and one used *Clostridium* (one used both *Bifidobacterium* and *Lactobacillus*). Almost all the studies found significant effects on measured CNS functions, except for one testing effect of *Bifidobacterium infantis* on depression-like behavior. The concentration of the effective probiotic interventions ranged from 10^7 to 10^{11} colony-forming units (CFU), with the most using 10^9 (14/25) or 10^{10} (6/25) CFU per animal per day. The duration of the probiotic treatments ranged

Table 1. Studies of Effects of Probiotics on Central Nervous System Functions in Animals

Study	Animal	CNS function	Probiotic (species, dosage [CFU/day], duration)	Outcome (behavioral level)	Secondary outcome
Liu et al,[20] 2016	ELS mice Naïve mice	Locomotor activity (open-field test) Anxiety (open-field test, elevated plus maze) Depression (sucrose-preference test, forced-swimming test)	*L. plantarum* PS128 10^9 28 days	Locomotor activity ↑ Anxiety (only in naïve mice) ↓ Depression (only in ELS mice) ↓	Corticosteroids (CORT) ↓ Cytokine: inflammatory cytokine TNF-α, IL-6 ↓, anti-inflammatory cytokine IL-10 ↑ Brain monoamines and metabolites: 5-HT ↑, 5-HIAA ↓, DA ↑, DOPAC ↓, HVA ↓
Liu et al,[21] 2016	GF mice	Locomotor activity (open-field test) Anxiety (elevated-plus maze) Depression (forced-swimming test)	*L. plantarum* PS128 10^9 16 days	Locomotor activity ↑ Anxiety ↓ Depression (-)	CORT: NA Brain monoamines and metabolites: DA ↑, HVA ↑, 5-HT ↑ and 5-HIAA ↑
Liang et al,[38] 2015	SPF CRS rats	Stress (chronic-restraint stress) Depression (sucrose-preference test) Anxiety (elevated-plus maze, open-field test) Non-spatial memory (object-recognition test, object-placement test)	*L. helveticus* NS8 10^9 Initial 21 days	Anxiety ↓ Depression ↓ Non-spatial memory ↑	CORT and ACTH ↓ Cytokines: IL-10 ↑ Brain monoamines: 5-HT ↑ and NE ↑ BDNF expression ↑
Wang et al,[30] 2015	Ampicillin-treated rats	Anxiety (elevate-plus maze) Spatial memory (Morris water maze)	*L. fermentum* N93 10^9 Initial 30 days	Anxiety ↓ Spatial memory ↑	CORT and ACTH ↓ Brain monoamines: MR ↑, NMDA ↑, GR: NA Brain BDNF: NA Colon inflammation: myeloperoxidase activity ↓ Fecal microbiota: *Bacteroides* ↑, *C. coccoides* ↓, *Firmicutes* ↓, *Lactobacillus* ↑
Smith et al,[29] 2014	RagI$^{-/-}$ mice	Stress response (water-avoidance stress) Anxiety (light/dark box test) Non-spatial memory (novel- object test)	*L. rhamnosus* R0011 + *L. helveticus* R0052 6×10^9 28 days	Anxiety ↓ Non-spatial memory ↑	CORT: NA Brain c-Fos expression ↑ Intestinal permeability ↓ Fecal microbiota: *Bacteroides* ↑, Enterobacteriaceae ↑, Firmicutes ↑
Luo et al,[42] 2014	Hyperammonemia rats	Anxiety (elevate-plus maze) Spatial memory (Morris water maze)	*L. helveticus* NS8 10^9 14 days	Anxiety ↓ Spatial memory ↑	Neuroinflammation: PGE2 ↓, IL-1β ↓ Brain monoamines: 5-HT ↓ Plasma kynurenine pathway: KYN/TRP ↑, KA/KYN ↓
Savignac et al,[36] 2014	Mice	Anxiety (defensive marble burying, elevated-plus maze, open field) depression (tail-suspension test, forced-swim test)	*B. longum* 1714/ *B. breve* 1205 10^9 Initial 21 days	*B. longum*: Anxiety ↓ Depression ↓ *B. breve*: Anxiety ↓	CORT: NA
Ohland et al,[37] 2013	Il-10 deficient mice	Anxiety (elevated Barnes Maze) Spatial memory (elevated Barnes maze)	*L. helveticus* R0052 10^9 21 days	Anxiety ↓ Spatial memory ↑	Colon inflammation ↓ Cytokines: NA CORT ↓ SCFA metabolites: NA

Table 1. Continued

Study	Animal	CNS function	Probiotic (species, dosage [CFU/day], duration)	Outcome (behavioral level)	Secondary outcome
Messaoudi et al,[39] 2011	Rats	Anxiety (conditioned defensive burying)	*B. longum* R0175 + *L. helveticus* R0052 10^9 14 days	Anxiety ↓	NA
Bravo et al,[23] 2011	Mice	Anxiety (open arms, elevated-plus maze, fear conditioning) Depression (forced-swim test)	*L. rhamnosus* JB-1 10^9 28 days	Anxiety ↓ Depression ↓	CORT ↓ GABA receptor expression influence depending on brain areas Probiotic effect via vagus nerve
Bercik et al,[22] 2011	Chronic colitis mice	Anxiety (step-down test)	*B. longum* NCC3001 10^{10} 14 days	Anxiety ↓	Colon inflammation: NA Brain BDNF expression: NA Enteric neurons excitability ↓ Probiotic effect via vagus nerve
Bercik et al,[35] 2010	T-muris infected mice	Anxiety (light/dark behavior, step-down test)	*B. longum* NCC3001 / *L. rhamnosus* NCC4007 10^{10} 10 days	Anxiety ↓ (*B. longum* only)	Colon inflammation ↓ Plasma cytokines: NA BDNF expression ↑ (only by *B. longum*) Tryptophan and kynurenine: NA No effect of vagotomy
Singh et al,[40] 2012	Rats	CFS and depression induced by forced-swim test (immobility period, post-swim fatigue time)	*L. acidophilus* as LAB or LAB FB 10^7 7 days	Depression ↓ (larger effect of LAB FB than LAB);	Brain oxido-nitrosative stress biomarker ↓ Cytokines: TNF-α ↓
Arseneautl-Bread et al,[41] 2012	MI rats	Post-MI depression (forced-swim test); social behavior (social interaction test); emotional memory (passive avoidance step-down test)	*B. longum* R0175 + *L. helveticus* R0052 10^9 14 days	Depression ↓ Social interaction ↑ Non-spatial memory ↑	Cytokines: pro-inflammatory cytokine IL-1β ↓ Intestinal barrier permeability ↓
Desbonnet et al,[27] 2010	MS rats	Depression (forced-swim test)	*B. infantis* 35624 10^{10} Initial 40 days	Depression ↓	CORT: NA Cytokines: IL-10 ↓ Tryptophan: NA Brain monoamines (-) Noradrenaline ↑
Desbonnet et al,[26] 2008	Rats	Depression (forced-swim test)	*B. infantis* 35624 10^{10} 14 days	No behavioral change	Cytokines: pro-inflammatory cytokines IL-6, IFN-γ ↓; anti-inflammatory cytokines IL-10 ↓ Tryptophan ↑ Brain monoamines and metabolites – 5-HIAA ↓, DOPAC ↓ Neuroendocrine: NA, CORT: NA , AVP: NA , CRF: NA
Liu etal,[44] 2015	VaD (vascular dementia) mice	Locomotor activity (open-field test) Spatial memory (Morris water maze)	*C. butyricum* WZMC1016 (CGMCC 9831) $10^6/10^7/10^8$ 42 days	Locomotor activity ↑ Spatial memory ↑	Morphological change of hippocampus ↓ BDNF expression ↑ Butyrate in feces and brain ↑ Fecal bacteria diversity ↑
Jeong et al,[47] 2015	Aged rats	Spatial memory (Y-maze, Morris water maze)	*L. plantarum* KY1032 + *L. curvatus* HY7601 10^{10} 48 days	Spatial memory ↑	Cytokines: pro-inflammatory cytokines NF-κB, ↓ BDNF ↑ Lipidemia ↓

Table 1. Continued

Study	Animal	CNS function	Probiotic (species, dosage [CFU/day], duration)	Outcome (behavioral level)	Secondary outcome
Savignac et al,[43] 2015	Mice	Non-spatial memory (object recognition, fear conditioning) Spatial memory (Barnes Maze)	*B. longum* 1714/ *B. breve* 1205 10^9 initial 21 days	*B. longum*: Non-spatial memory ↑ Spatial memory ↑ *B. breve*: Non-spatial memory ↑	Visceral sensitivity -colon distension: NA CORT: NA
Gareau et al,[45] 2011	*C. rodentium* infected mice	Memory dysfunction induced by water avoidance (novel-object test, T-maze);	*L. rhamnosus* R0011 + *L. helveticus* R0052 6×10^9 17 days	Non-spatial memory ↑	CORT ↓ Colon epithelial cell hyperplasia ↓ Cytokine: pro-inflammatory cytokines IFN γ ↓ Brain BDNF and c-Fos expression ↑ Microbiota: Firmicutes ↓, Enterobacteriaceae ↓, Eubacteria rectale ↓, *Lactobacillus* ↑
Davari et al,[46] 2013	Diabetic rats	Spatial memory (Morris water maze)	*L. acidophilus* 4356 + *B. lactis* 10140 + *L. fermentum* ATCC9338 2×10^{10} 56 days	Spatial memory ↑	Hippocampal long-term potentiation ↑ Serum glucose ↓ and insulin ↑ Oxidative stress biomarkers: SOD ↑, 8-OHdG ↓
Hsiao et al,[31] 2013	MIA mice	Autism spectrum disorder: Anxiety (open field, marble burying); Sensory gating (prepulse inhibition); Communicative behavior (ultrasonic vocalizations); Social interaction (3-chamber social test)	*B. fragilis* NCTC9343 10^{10} 6 days	Anxiety ↓ Sensory gating ↑ Communicative behavior ↑ Social interaction (-)	Intestinal permeability ↓ Tryptophan metabolites: indolepyruvate ↓ Microbiota: Lachnospiraceae ↓, Bacteroidales ↓
Kantak et al,[48] 2014	Male mice	obsessive-compulsive-disorder-like behavior (open field, marble burying, ultrasonic vocalizations, intermale aggression)	*L. rhamnosus* GG (ATCC 53103) 10^9 14, 28 days	Locomotor behavior ↓ Marble burying ↓ Ultrasonic vocalizations (-) Intermale aggression (-)	NA
D'Mello et al,[49] 2015	Male mice	Inflammation associated sickness behavior (social exploratory)	VSL#3 1.7×10^9 10 days	Social exploratory behavior in bile duct ligation treated mice ↑	Intestinal permeability: NA Cytokine: pro-inflammatory cytokine TNF-α ↓ Monocyte infiltration ↓ Microglial activation ↓ Fecal Microbiota (-)
Takada et al,[50] 2016	Male rats	Stress response to water avoidance stress	*L. casei* Shirota YIT 9029 3×10^9 14 days	NA	CORT ↓ c-Fos expression in the paraventricular nucleus ↓ Gastric vagal afferent activity ↑ Neuronal excitability of NTS ↑

ELS, early life stress; CORT, corticosterone; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-Hydroxyindoleacetic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; GF, germ free; NA, not applicable; SPF, specific pathogen free; CRS, chronic restraint stress; ACTH, adrenocorticotropic hormone; NE, norepinephrine; BDNF, brain-derived neurotrophic factor; MR, mineralocorticoid; NMDA, N-methyl-D-aspertate; GR, glucocorticoid; RagI-/-, RagI knockout; PGE2, prostaglandin E2; KYN, L-kynurenine; TRP, tryptophan; KA, kynurenic acid; SCFA, short-chain fatty acid; GABA, gamma-Aminobutyric acid; CFS, chronic fatigue syndrome; LAB, *Lactobacillus acidophilus*; FB, floating bead; MI, myocardial infarction; MS, maternal separation; AVP, arginine vasopressin; CRF, corticotrophin-releasing factor; VaD, vascular dementia; NF-κB, nuclear factor-kappa B; SOD, superoxide dismutase; 8-OHdG, 8-hydroxy-2'deoxyguanosine; MIA, maternal immune activation; VSL#3, a high-concentration probiotic preparation of 8 live freeze-dried bacterial (*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and Streptococcus thermophiles); NTS, nucleus tractus solitary; *B. breve*, *Bifidobacterium breve*; *B. fragilis*, *Bifidobacterium fragilis*; *B. infantis*, *Bifidobacterium infantis*; *B. lactis*, *Bifidobacterium lactis*; *B. longum*, *Bifidobacterium longum*; *C. butyricum*, *Clostridium butyricum*; *C. coccoides*, *Clostridium coccoides*; *C. rodentium*, *Citrobacter rodentium*; *L. acidophilus*, *Lactobacillus acidophilus*; *L. casei*, *Lactobacillus casei*; *L. curvatus*, *Lactobacillus curvatus*; *L. fermentum*, *Lactobacillus fermentum*; *L. helveticus*, *Lactobacillus helveticus*; *L. plantarum*, *Lactobacillus plantarum*; *L. rhamnosus*, *Lactobacillus rhamnosus*.

from 6 to 77 days, with the most frequent period being 14 days (7/25). Effects of different probiotics on different specific CNS functions in animals were analyzed and described in the following text.

Anxiety

Twelve studies tested anxiety-like behavior in animals (mice or rats). The anxiety-like behaviors were evaluated with the elevate plus/Barnes maze, light/dark box, defensive burying, open field/arms, fear conditioning, and step-down tests. Three of them used a single strain of *Bifidobacterium longum*, all with positive results, ie, the animals showed less anxious behavior.[22,35,36] Two studies using *Lactobacillus helveticus* also found reduced anxiety-like behaviors in immune-deficient mice and chronically restrained rats.[29,37,38] Two studies used *Lactobacillus rhamnosus* but only one showed reduced anxiety behaviors.[23,35] Two studies using *Lactobacillus plantarum* also found alleviated anxiety levels in mice after the intervention.[20,21] *Bifidobacterium breve* and *Lactobacillus fermentum* were used once each and showed anxiolytic effects.[30,36] Two studies using multi-strain probiotic combinations of *L. rhamnosus* + *L. helveticus* and *B. longum* + *L. helveticus* found reduced anxious behavior.[29,39]

Depression

Nine studies focused on depression and all reported positive results except one. Depression-like behaviors were measured with the tail-suspension, forced-swim, and sucrose-preference tests. *B. infantis* was used twice but only one study found reduced depression-like behavior. Each of the single strains of *B. longum*, *B. breve*, *L. rhamnosus*, and *L. helveticus* was also used once and all showed antidepressant effects.[23,26,27,36,40] Two studies tested *L. plantarum*, but it only had an effect in mice with the early life stress of maternal separation.[20,21] One study used a multi-strain combination of *B. longum* + *L. helveticus* and also showed positive effects.[41]

Cognitive function

Eleven studies tested cognitive function, and all showed the probiotics to be beneficial for memory performance. Spatial memory was tested with the Morris water maze and the Barnes maze tests; other non-spatial memory abilities were measured with the novel object, fear conditioning, passive avoidance step-down, and T-maze tests.

Single strains of *B. longum*, *B. breve*, and *L. helveticus* were effective on both spatial and non-spatial memories.[37,38,42,43] Single strains of *L. fermentum* and *Clostridium butyricum* improved spatial memory ability.[30,37,42,44] Multi-strain probiotics that were assessed to be effective with regard to non-spatial memory included combinations of *L. rhamnosus* + *L. helveticus*[29,45] and *B. longum* + *L. helveticus*,[41] and combinations of *Lactobacillus acidophilus* + *B. lactis* + *L. fermentum* and *L. plantarum* + *Lactobacillus curvatus* in spatial memory.[46,47]

Autism spectrum disorder and obsessive-compulsive disorder

Autism spectrum disorder-related behaviors were tested with the open field and marble- burying tests for anxiety, the pre-pulse inhibition test for sensorimotor, ultrasonic vocalization for communicative, and the three-chamber social test for social interaction behaviors. *Bifidobacterium fragilis* improved behaviors related to the ASD in maternal immune activation mice, including anxiety-like behavior, sensory gating and communicative behavior, but not social interaction behavior.[31]

Obsessive-compulsive disorder-related behaviors were also measured with the open field, marble burying, pre-pulse inhibition, ultrasonic vocalization and 3-chamber social tests. *L. rhamnosus* was found to be able to decrease obsessive-compulsive disorder-like behaviors in mice, but only locomotor ability and anxiety level. No effect was found in communicative or social interaction behaviors.[48] However, a recent study investigated sickness behavior using a social investigative behavior paradigm, and found VSL#3 improved sickness behavior with increased social exploratory behaviors.[49]

Stress response

Four studies involved stress induction to test behavioral response to stress. Stress was induced, with water avoidance stress as an acute stressor[29,45,50] and maternal separation as a chronic stressor.[27] Acute stress was used to induce anxiety, memory dysfunction and HPA response; chronic stress was used to induce depression.

Anxiety behavior was not successfully induced by water avoidance stress, while memory dysfunction was induced only in Gareau's study.[45] A probiotic combination of *L. rhamnosus* + *L. helveticus* prevented non-spatial memory dysfunction induced by acute stress.[45] One study only measured plasma corticosterone levels in response to acute stress and found a significant decrease due to *Lactobacillus casei* Shirota intervention.[50] For behavioral changes caused by chronic stress exposure, *B. infantis* normalized depression-like behavior induced by maternal separation.[27]

Mechanisms of action

In addition to outcomes on behavioral levels, we also collected data at the physiological level, exploring endocrine, immune, neural chemical, and metabolic changes due to probiotics. Most of the studies investigated serum corticosteroid levels and found they were decreased by various probiotics: *L. plantarum*, *L. helveticus*, *L. fermentum*, *L rhamnosus*, and *L. casei* Shirota.[20,23,30,37,44,45,50] Adrenocorticotropic hormone (ACTH) could also be decreased by *L. helveticus* and *L. fermentum*.[30,38] Colon inflammation was alleviated and cytokine levels were influenced: inflammatory cytokines such as IL-6 and TNF-α were decreased and anti-inflammatory cytokines such as IL 10 were increased.[20,30,35,37,38,40,42,45,47,49] These immune-effective probiotics were *L. plantarum*, *L. helveticus*, *L. fermentum*, *L. acidophilus*, *B. longum*, and *L. rhamnosus*. Brain monoamines, such as 5-HT and DA, could be increased by the probiotics *L. plantarum*, *L. helveticus*, and *B. infantis*, while their metabolites were reduced.[20,26,38] GABA receptor expression could be influenced by *L. rhamnosus*, depending on the brain area.[23] Brain BDNF and c-Fos mRNA expression increased after probiotic intervention with *L. helveticus*, *L. plantarum*, *L. rhamnosus*, *B. longum*, and *C. butyricum*, while c-Fos in the hypothalamus paraventricular nucleus was decreased by *L. casei* Shirota.[35,38,44,45,47] Two studies found effects of *L. rhamnosus* and *B. longum* that were mediated via the vagus nerve (ie, no effect in vagotomized mice),[22,23] and one study found *L. casei* Shirota to enhance gastric vagal afferent activity.[50] Enteric neuron excitability was inhibited by *B. longum*,[22] while visceral sensitivity by colon distension was unaffected.[43] One study found that a probiotic formulation of *B. longum* + *L. helveticus* reduced intestinal barrier permeability.[31,41] Probiotics *L. helveticus*, *B. infantis*, and *B. fragilis* influenced metabolites by enhancing serum tryptophan levels and inhibiting its metabolites.[26,31,42] Several studies conducted microbiota analyses on the fecal samples and found fecal microbiota were altered by probiotics: for example, *Bacteroides* and *Lactobacillus* were increased and *Firmicutes* decreased by *L. fermentum*.[29-31,44,45] More details are shown in Table 1.

Human Studies

In total, 15 human studies were included. All of the selected studies had strong ratings in the quality assessment tool for quantitative studies, although one of the studies did not describe the age and gender of the participants in each group.[51] Among the 15 studies, 8 used a single-strain probiotic (*L. casei*, *L. casei* subsp. *rhamnosus*, *L. casei* Shirota, *L. plantarum*, and *B. infantis*), of which 2 used probiotic containing milk, and the other 7 studies used multi-strain probiotics. Eight of the 15 studies found significant effects of the probiotic interventions. Doses of the effective interventions ranged from 10^7 to 3.63×10^{10}, and the duration of the treatments ranged from 20 days to 8 weeks. Doses around 10^9 (5/8) and 10^{10} (3/8) were used most often. Durations were most commonly 4 (6/15) and 8 (4/15) weeks. Due to the heterogeneity of the studies (eg, probiotic interventions and measurements of CNS functions), we only describe the results based on the different interventions (Table 2).

Psychiatric conditions

Here, we summarize the studies on depression, anxiety, and/or mood together, because in most of the studies, questionnaires that tested multiple psychiatric conditions were used. Fifteen studies tested healthy participants with respect to anxiety, depression, distress levels, mood state, and behavior problems.[39,51-62] The measurement tools included the General Health Questionnaire (GHQ), the Depression Anxiety and Stress Scale (DASS), the Leiden Index of Depression Sensitivity-Revised (LEIDS-r), the Positive and Negative Symptom Scale (PANSS), the State-Trait Anxiety Inventory (STAI), the Development Behavior Checklist (DBC), the Beck Depression Inventory (BDI), the Beck Anxiety Inventory (BAI), the Hopkins Symptom Checklist (HSCL-90), the Hospital Anxiety and Depression Scale (HADS), the Perceived Stress Scale (PSS), the Coping Checklist (CCL) (also used to counter the stress of daily life), and the questionnaire-based Profile of Mood State (POMS). Due to the different questionnaires, scales, and their combinations used in these studies, we only report here whether the probiotics treatment improved mental health/mood.

One study compared a probiotic capsule (containing *Lactobacillus casei*, *L. acidophilus*, *L. rhamnosus*, *Lactobacillus bulgaricus*, *B. breve*, *B. longum*, and *Streptococcus thermophilus*) and a probiotic yogurt (containing *B. lactis* and *L. acidophilus*) with the combination of conventional yogurt and a placebo capsule, and found the former two were more effective in alleviating distress, anxiety, and depression in petrochemical workers.[52] A recent study using multi-strain probiotics found improvement in the LEIDS-r score, which is predictive of depression.[53] Two studies by Messaoudi et al[39,61] found probiotic formulations of *B. longum* and *L. helveticus* could improve anxiety and depression in all participants, and also in those who had lower urinary free cortisol levels at baseline. One study using *L. casei* Shirota-containing milk improved mood only in the bottom third of the depressed distribution at baseline.[62] A study in chronic fatigue syndrome patients also used *L. casei* Shirota and found decreased anxiety levels following treatment.[51] One

Table 2. Studies of Probiotic Effects on Central Nervous System Functions in Humans

Study	Participants	Probiotic	Dosage (CFU/day) and duration	CNS function	Outcome	Secondary outcome
Takada et al,[50] 2016	140 healthy students	*L. casei* Shirota YIT 9029	1×10^9 8 weeks	STAI	No difference of STAI score	Change in salivary cortisol level before exam ↓ Decrease in physical symptoms ↓
Mohammadi et al,[52] 2015	70 petrochemical workers	probiotic yogurt (*L. acidophilus* LA5 + *B. lactis* Bb12) + placebo capsule; Conventional yogurt (*S. thermophilus* and *L. bulgaricus*.) + probiotic capsule (*L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus*)	probiotic yogurt: 10^7 in total Probiotic capsule: 3×10^3, 3×10^7, 7×10^9, 5×10^8, 2×10^{10}; 10^9, 3×10^8, respectively (2.88×10^{10} in total)/6 weeks	GHQ DASS HPA axis activity	Improvement of GHQ and DASS in probiotics yogurt and probiotics capsule groups; no difference in HPA axis activity	NA
Steenbergen et al,[53] 2015	40 healthy volunteers	Ecologic Barrier: *B. bifidum* W23, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *Lactococcus lactis* (W19 and W58).	5×10^9 4 weeks	LEIDS-r BDI BAI	Improvement of total score and item 'rumination' of LEIDS-r. No difference of scores of Beck Depression and Beck Anxiety Inventory	NA
Dickerson et al,[54] 2014	65 schizophrenia patients	L. rhamnosus GG (ATCC 53103+ *B. animalis* subsp. *lactis* Bb12	2×10^9 14 weeks	PANSS	No difference of toll score or positive, negative or general scores.	Severe difficulty in bowel movement ↓
Vaghef-Mehrabany et al,[55] 2014	46 patients with rheumatoid arthritis	*L. casei* 01	10^8 8 weeks	STAI	No difference of STAI score	Dietary: NA Cytokines: pro inflammatory cytokine TNF-α, IL-6, and IL-12 ↓, anti-inflammatory cytokine IL-10 ↑
Dapoigny et al,[56] 2012	50 IBS patients	*L. casei* subsp. *rhamnosus* LCR35	6×10^8 4 weeks	HADS	No difference in HADS score	IBS severity score: only clinically relevant decreased in subtype IBS-D ↓ Presence of *Lactobacillus* in feces: 85% of patients
Simrén et al,[57] 2010	74 IBS patients	Milk fermented with yoghurt bacteria *L. bulgaricus* + *S. thermophilus* and containing *L. paracasei* F19 + *L. acidophilus* La5 + *B. lactis* Bb12	2×10^{10} 8 weeks	HADS	No difference of HADS score	Diet: same among groups
Whorwell et al,[58] 2006	362 female IBS patients	*B. infantis* 35624	10^6, 10^8, 10^{10} 4 weeks	HADS	No difference in any of the dosages	IBS symptom: ↓ in 10^8 group

Table 2. Continued

Study	Participants	Probiotic	Dosage (CFU/day) and duration	CNS function	Outcome	Secondary outcome
Reale et al,[59] 2012	72 male smokers	*L. casei* Shirota	4×10^{10} 3 weeks	STAI	No difference in STAI score	Natural killer cell activity ↑ CD16+ cell ↑ BMI: NA Bowel function ↑
Parracho et al,[60] 2010	15 children (4-16Y) with ASD	*L. plantarum* WCFS1	4.5×10^{10} 3 weeks	DBC	No significant difference in DBC score	Bowel function: only different in stool consistency Fecal microbiota: *Lactobacillus* Lab 158 ↑, *Clostridium* E.rec482 ↓
Messaoudi et al,[39] 2011	55 healthy volunteers	*L. helveticus* R0052 + *B. longum* R0175	3×10^{9} 4 weeks	HSCL-90 HADS PSS CCL	Improvement of anxiety, depression and problem solving, and reduced UFC level in probiotics group	Median urinary free cortisol ↓
Messaoudi et al,[61] 2011	25 healthy volunteers (with lower UFC levels than median value at baseline	*L. helveticus* R0052 + *B. longum* R0175	3×10^{9} 4 weeks	HSCL-90 HADS PSS CCL	Improvement of anxiety and depression in probiotics group	NA
Rao et al,[51] 2009	35 CFS patients	*L. casei* Shirota	2.4×10^{10} 8 weeks	BDI BAI	Decreased anxiety symptoms in probiotic group	Fecal microbiota: aerobes ↑, anaerobes ↑ Bifidobacteria ↑, *Lactobacillus* ↑
Benton et al,[62] 2007	124 healthy volunteers	*L. casei* Shirota (containing milk)	6.5×10^{9} 10/20 days	Questionnaire-based POMS Episodic memory (Wechsler Memory Scale) Retrieval from long-term memory Verbal fluency Eating-associated behavior NART	Improved mood in the bottom third of the POMS depressed/elated distribution at baseline in probiotics group after 20 days Improved memory in probiotics group after 20 days	NA
Tillisch et al,[63] 2013	36 healthy females	FMPP (*B. lactis* I-2494 [DN-173 010], containing yogurt starters include *S. thermophilus* I-1630, *L. bulgaricus* I-1632 and I-1519) and *Lactococcus lactis subsp lactis* I-1631	*B. lactis* : 1.25×10^{10} *S. thermophilus* and *L. bulgaricus* : 1.2×10^{9} 4 weeks	Standard emotional faces-attention task for fMRI	Decreased activity to emotional faces in a large distributed network Changes in midbrain connectivity during resting state	NA

STAI, State-Trait Anxiety Inventory; NA, not applicable; GHQ, General Health Questionnaire; DASS, Depression Anxiety and Stress Scale; HPA, hypothalamic-pituitary-adrenal; BMI, body mass index; LEIDS-r, Leiden Index of Depression Sensitivity-Revised; BDI, Beck Depression Inventory; BAI, Beck Anxiety Inventory; PANSS, Positive and Negative Symptom Scale; IBS, irritable bowel syndrome; HADS, Hospital Anxiety and Depression Scale; ASD, autism spectrum disorder; IBS-D, diarrhea-predominant IBS; DBC, Development Behavior Checklist; HSCL-90, Hopkins Symptom Checklist; PSS, Perceived Stress Scale; CCL, Coping Checklist; UFC, urinary free cortisol; CFS, chronic fatigue syndrome; POMS, questionnaire-based Profile of Mood State; NART, National Adult Reading Rest; FMPP, fermented milk product with probiotic; fMRI, functional magnetic resonance imaging; *B. animalis*, *Bifidobacterium animalis*; *B. breve*, *Bifidobacterium breve*; *B. bifidum*, *Bifidobacterium bifidum*; *B. infantis*, *Bifidobacterium infantis*; *B. lactis*, *Bifidobacterium lactis*; *B. longum*, *Bifidobacterium longum*; *L. acidophilus*, *Lactobacillus acidophilus*; *L. brevis*, *Lactobacillus brevis*; *L. bulgaricus*, *Lactobacillus bulgaricus*; *L. casei*, *Lactobacillus casei*; *L. helveticus*, *Lactobacillus helveticus*; *L. paracasei*, *Lactobacillus paracasei*; *L. plantarum*, *Lactobacillus plantarum*; *L. rhamnosus*, *Lactobacillus rhamnosus*; *L. salivarius*, *Lactobacillus salivarius*; *S. thermophiles*, *Streptococcus thermophiles*.

recent study, also using *L. casei* Shirota, found decreased salivary cortisol levels in university students in response to stress, although no significant difference in STAI score was observed.[50]

However, other studies found no significant effect of their probiotic interventions. Patients with schizophrenia showed no change in PANSS score after treatment with *L. rhamnosus* for 14 weeks.[54] Patients with rheumatoid arthritis showed no change in anxiety levels, as assessed with the STAI after 8 weeks of *L. casei*.[55] Three studies conducted in IBS patients all looked into HADS scores before and after interventions, but found no effect of *L. casei* or fermented milk with *L. paracasei* and *L. acidophilus*.[56-58] In healthy male smokers, a 3-week intervention with *L. casei* showed no effect on STAI score.[59] In children with ASD, a 3-week intervention with *L. plantarum* did not change the DBC score.[60]

Memory and other cognitive abilities

The study of Benton et al[62] measured different memory and cognitive abilities in healthy participants, including episodic memory, tested with the Wechsler Memory Scale, retrieval from long-term memory, verbal fluency, eating-associated behavior, and premorbid intelligence, tested with the National Adult Reading Test. However, *L. casei* Shirota decreased memory abilities in all participants compared with the placebo, and had no effect on verbal fluency or eating-associated behavior.

Neuroimaging study

There was only one neuroimaging study, using functional magnetic resonance imaging (fMRI), investigating the change in brain activity to emotional stimuli and basal brain activation after intake of a fermented milk product with probiotic (FMPP) containing *B. lactis* with yogurt starters, *S. thermophilus*, *L. bulgaricus*, and *Lactococcus lactis* subsp. *lactis*.[63] The FMPP decreased activity of a large distributed network including affective, viscerosensory, and somatosensory cortices to emotional faces, and changed midbrain connectivity during the resting state.

Mechanisms of action

Two studies found reduced cortisol levels in saliva and urine after probiotic interventions with *L. casei* Shirota and multi-strain *L. helveticus* + *B. longum*, respectively.[39,50] The immune system could be improved by the probiotic *L. casei*, with evidence of reduced pro-inflammatory cytokines (TNF-α, IL-6, and IL-12), increased regulatory cytokines (eg, IL-10), and increased natural killer cell activity in smokers.[55,59] Only one study in humans investigated metabolites of the tryptophan pathway but did not find any significant change by probiotics.[52] Many of the human studies looked at bowel function, and they did find reduced difficulties in bowel movement, IBS severity, and symptoms in patients.[54,56,58,60] Microbiota analysis helped to confirm that fecal microbiota were altered by probiotic intervention: *Lactobacillus* was increased and *Clostridium* decreased by *L. plantarum*, whereas *Bifidobacteria* and *Lactobacillus* were increased by *L. casei* Shirota.[51,60] More details are shown in Table 2.

Summarizing all the studies in animals and humans that focused on CNS functions, including psychiatry conditions (anxiety and depression) and memory abilities, *Bifidobacterium* and *Lactobacillus* were the probiotics used most frequently. Doses ranged from 10^7 to 4×10^{10} CFU per day and most studies used 10^9 and 10^{10} CFU in animals and 3×10^9 CFU in humans. The duration of intake ranged from 1 week to 6 months with the most frequent durations of 2 weeks in animals and 4 weeks in humans.

Discussion

The number of studies using probiotics to improve central nervous system function has increased over the past 10 years, though with a focus on effects in animals. Researchers have used various strains of probiotics and studied various CNS functions. Summarizing the divergent findings motivated us to perform a systematic review of this research area. Previously, there was no systematic review or meta-analysis of the effects of probiotics on CNS function in animals and humans. To date, there are a few reviews on probiotic effects on infantile colic, which may reflect peripheral nervous system action, and one recent study reviewing only human studies.[64-66] Similarly, among the 56 RCTs so far which tested probiotics in adults with IBS that have or have not shown effects on peripheral (bowel) functions,[15] none have investigated whether any CNS effect was affected or improved. We identified studies applying probiotics as single- or multiple-strain preparations in animals and humans. Because of the diversity of the interventions and the CNS functions tested in these studies, we did not perform a meta-analysis.

Effects of Probiotics

Combining all the studies in animals and humans, probiotics appear to have a positive effect in improving central nervous system function. However, a publication bias toward positive results cannot be excluded. Based on currently available studies, we can see that most of the studies used *Bifidobacterium* and *Lactobacillus* preparations, and most of them were effective in improving specific CNS functions. Again, however, negative results in relation to other functions may have been unreported, even in otherwise positive

studies. Doses of 10^9 and 10^{10} CFU have been used in most studies showing an effect on behaviors. An intake of the probiotic for 2 weeks in animals and 4 weeks in humans is apparently sufficient to elicit measurable effects.

B. longum, *B. breve*, *B. infantis*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, and *L. casei* were the most commonly used preparations in these studies, as single- or multi-strain preparations, all of which were able to improve anxiety, depression, and memory related behaviors, based on animal models. All of these probiotics are regarded as "good" bacteria, presumably inhibiting the growth of harmful bacteria or pathogens and/or improving the immune system.[67-69] These probiotics were also found to reduce the symptoms of gastrointestinal disease, such as irritable bowel syndrome.[15,16,58,70] Probiotics may play an important role in gut-brain axis communication, thereby benefiting both the brain and the gut.

While some studies found no significant effect of probiotic intervention, the evidence is inadequate to conclude that the interventions were ineffective because some difficulties and/or weak points exist. For example, schizophrenia as a severe mental illness, and being closely related to a genetic disposition, may be a case in which probiotics can hardly be expected to have a significant effect on changing symptoms.[54] Probiotic doses in some studies were below the supposed effective dosages (at least 10^9 CFU), such as 10^8 CFU in the study in rheumatoid arthritis patients and 10^6, 5×10^7, and 10^8 CFU in IBS patients.[55-58] Also, in two studies, one in male smokers and one in children with ASD, the intervention periods were 3 weeks, shorter than the effective period, which seems to be 4 weeks, that can make a measurable effect.[59,60]

Also, caution is warranted when drawing conclusions from the human studies that used psychological questionnaires and/or scales rather than behavioral or neuropsychological experiments, resulting in subjective biases. The clinical efficacy of probiotic interventions and guidelines for their administration in diseases such as diarrhea, allergies, IBS, and inflammatory bowel disease has been addressed in previous reviews.[15,71,72] More studies investigating probiotic effects in mental disorders are needed.

Mechanisms of Action of Probiotic Effects

The current state-of-the-art suggests several mechanisms: the endocrine system, immune system, action of enteric neurons, and commensal bacteria (or their metabolic activity). This evidence has come primarily from preclinical studies, while a few clinical studies have analyzed cortisol and cytokine levels in saliva and plasma. The HPA axis activity has been linked closely to mood disorders and memory abilities.[73,74] Many probiotics reduced HPA axis activity by decreasing CORT and/or ACTH levels, including most of the *Lactobacillus* strains tested: *L. plantarum*, *L. helveticus*, *L. fermentum*, *L. rhamnosus*, and *L. casei*.[19,22,29,36,39,43,44,49] However, single strains of *Bifidobacterium* such as *B. infantis*, *B. longum*, and *B. breve* had no effect on CORT levels.[27,29,36] BDNF is the key for neurogenesis and synaptic plasticity, which structurally support CNS function.[75,76] *Lactobacillus* (*L. plantarum*, *L. helveticus*, *L. fermentum*), *Bifidobacterium* (*B. longum*), and *C. butyricum* increased brain BDNF.[34,37,43,46] Neuronal activation can be indicated by the nuclear localization of c-Fos; the effect of c Fos in the CNS depends on its location. The combination of *L. rhamnosus* + *L. helveticus* improved c Fos expression in the hippocampus and improved memory ability, while *L. casei* decreased it in the paraventricular nucleus of hypothalamus region and alleviated stress responses.[45,50] Neurotransmitters 5-HT, DA, and GABA are closely related to psychiatric conditions, and were influenced directly by many strains of probiotic (*L. plantarum*, *L. helveticus*, *L. fermentum*, *L. rhamnosus*, and *B. infantis*). The vagus nerve has been proposed as a pathway of probiotic effects because neurochemical and behavioral changes due to *L. rhamnosus* and *B. longum* were not seen in vagotomized animals. Direct evidence for the role of the vagus nerve also comes from studies showing that gastric vagal afferent activity was enhanced by *L. casei*. The excitability of the enteric nervous system, which is connected to the vagus nerve, has been shown to be modulated by *B. longum*.[22]

Probiotics also alter CNS function indirectly through several other pathways. *L. helveticus*, *B. infantis*, and *B. fragilis* enhanced serum tryptophan (precursor of 5-HT) levels and reduced its metabolites. Most of the probiotics tested affected the immune system by decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines. Another important pathway through which probiotics may modulate CNS function is intestinal barrier permeability, which is essential for maintaining the immune and nervous systems. Increased intestinal barrier permeability is associated with psychiatric disorders, such as depression and autism, while it can be restored by probiotic formulations of *B. longum* and *L. helveticus*, along with improved CNS function.[31,41,77]

According to the data reviewed, different probiotics exhibited several common effects; however, these effects were strain-dependent and occurred via different pathways at a lower level of the CNS. Thus, more studies are needed for clarify which probiotics target which central biochemical substances and behaviors. In clinical applications, interventions with a probiotics cocktail may have greater effects, because different probiotics may create their effects at the same time through different pathways. However, as yet, such

an approach currently lacks clinical evidence.[15]

Translation of Animal Studies

There are many animal studies about the gut microbiome-brain interaction using germ-free, specific pathogen free (SPF), or gnotobiotic animals, colonization with specific microbiota, probiotic intake, and infections to deliberately alter the GM and to manipulate CNS function.[9,29,30,35,37,45,78-82] In humans, we are not able to adapt most of these models for ethical reasons. Ten of the animal studies included in our review used probiotics in animals whose health state had been disturbed by various manipulations, which included antibiotic treatment, gene knockout, inflammation, infection, maternal immune activation, hyperammonemia, and diabetic induction, and depression induced by myocardial infarction. All of the manipulations were aimed at inducing changes in CNS function, including anxiety and depression-like behavior, memory impairment, or ASD-like behaviors. In humans, interfering with the participants' healthy state is not an option. What is possible is to explore the GM composition, correlating it to certain behaviors, and using probiotics to manipulate the GM-brain interaction. It is also possible to temporarily affect single functions, such as the stress response at the central level or the GM composition by varying the food or drugs used. As yet, evidence from studies using probiotics is confined to animal studies. Validity estimates of probiotic intervention from human studies are still missing and thus need to be carried out.

The translation of behavioral models from animals to humans has both possibilities and limitations. The tests used to measure anxiety in animals, such as the elevate plus/Barnes maze, light/dark box, defensive burying, open field/arms, and step-down, have no equivalents in humans, and neither do tests such as the forced swim and maternal separation for inducing depression and negative mood. In human studies, measurements of anxiety and depression rely primarily on scales such as the HADS, which has accuracy issues due to subjectivity and emotional bias from the participants/patients. Moreover, it remains questionable whether the behavioral tests used in animals do, in fact, adequately reflect the assumed CNS dysfunction (anxiety, depression) in humans and, more specifically, in patients. This leads to a demand for appropriate behavioral tests of anxiety and depression not only for patients with psychiatric disorders but also for the healthy population, and for adequate behavioral measures in animals that match these functions and dysfunctions in humans.

In addition to behavioral measurements, neuroimaging methods may provide insights as to what is altered in the brain that causes behavioral changes after the consumption of probiotics. An emotional faces attention task used in the fMRI study of Tillisch et al[63] is one example: the brain response to emotional stimuli that may be related to psychiatric conditions was changed after a 4-week intake of probiotics.

Learning and memory abilities can be tested via numerous paradigms in humans. For spatial memory, there are computerized versions of the Morris water maze (VMWT) and the Blue Velvet Arena (BVA), which is also a variant of the Morris water maze for humans.[83-86] For non-spatial memory, object recognition tasks and fear conditioning have been used widely in humans. These memory tasks can be conducted in combination with neuroimaging experiments, such as fMRI and magnetoencephalography (MEG) experiments.

There are also several ways to experimentally induce stress in humans. The Trier Social Stress Test, developed 20 years ago, during which participants are asked to play a role in a job interview, or in performing a public speech, can effectively increase the HPA axis and sympathetic-adrenal-medullary activity.[87] Social stress can also be induced using the Cyberball paradigm, during which stress comes from social exclusion and/or ostracism.[88,89] Noise as a stressor is easy to manipulate in a laboratory environment by exposing participants to unpleasant sounds so as to induce psychological stress. Cognitive tasks can also stimulate stress responses with the advantage of being able to study the stress level by measuring task performance.[90] Other and more physical stressors include cold pressor tasks,[91] heat pain,[92] and the CO_2 challenge test, inducing stress/panic in participants by inhaling carbon dioxide-enriched air.[93] All of these have also been shown to be compatible with brain imaging studies.

Conclusions and Indications

We reviewed the effect of probiotics on the central nervous system in randomized controlled trials in animals and humans, and analyzed the possibility of translating animal models to human studies because few human studies have been conducted to date. According to the qualitative analyses of current studies, we can provisionally draw the conclusion that *B. longum*, *B. breve*, *B. infantis*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, and *L. casei* were most effective in improving CNS function, including psychiatric disease-associated functions (anxiety, depression, mood, stress response) and memory abilities. Doses between 10^9 and 10^{10} CFU and durations of 2 weeks in animals and 4 weeks in humans have shown sufficient effects. Also, translations of animal studies to human studies may be applicable. Human studies can be conducted using the same probiotics and similar experimental paradigms in the emotional and

neurocognitive domains. More experimental designs in humans should be developed, and more neuroimaging studies should be conducted rather than using only psychological questionnaires or scales. In addition to studies in healthy populations, clinical studies in patients with mental diseases would be worthwhile, because those with gastrointestinal disorders and psychiatric comorbidities, in general, appear to benefit from probiotic interventions.

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References

1. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837-848.
2. Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. Science 2006;312:1355-1359.
3. Farmer AD, Randall HA, Aziz Q. It's a gut feeling: how the gut microbiota affects the state of mind. J Physiol 2014;592:2981-2988.
4. Keightley PC, Koloski NA, Talley NJ. Pathways in gut-brain communication: evidence for distinct gut-to-brain and brain-to-gut syndromes. Aust N Z J Psychiatry 2015;49:207-214.
5. Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. Biol Psychiatry 2013;74:720-726.
6. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 2012;10:735-742.
7. Cryan JF, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 2011;23:187-192.
8. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 2012;13:701-712.
9. Sudo N, Chida Y, Aiba Y, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 2004;558(Pt 1):263-275.
10. Tomova A, Husarova V, Lakatosova S, et al. Gastrointestinal microbiota in children with autism in Slovakia. Physiol Behav 2015;138:179-187.
11. Kang DW, Park JG, Ilhan ZE, et al. Reduced incidence of Prevotella and other fermenters in intestinal microflora of autistic children. PLoS One 2013;8:e68322.
12. Scheperjans F, Aho V, Pereira PA, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord 2015;30:350-358.
13. Rijkers GT, de Vos WM, Brummer RJ, Morelli L, Corthier G, Marteau P. Health benefits and health claims of probiotics: bridging science and marketing. Br J Nutr 2011;106:1291-1296.
14. Lilly DM, Stillwell RH. Probiotics: growth-promoting factors produced by microorganisms. Science 1965;147:747-748.
15. Mazurak N, Broelz E, Storr M, Enck P. Probiotic therapy of the irritable bowel syndrome: why is the evidence still poor and what can be done about it? J Neurogastroenterol Motil 2015;21:471-485.
16. Ford AC, Quigley EM, Lacy BE, et al. Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic idiopathic constipation: systematic review and meta-analysis. Am J Gastroenterol 2014;109:1547-1561.
17. Lee BJ, Bak YT. Irritable bowel syndrome, gut microbiota and probiotics. J Neurogastroenterol Motil 2011;17:252-266.
18. DuPont HL. Biologic properties and clinical uses of rifaximin. Expert Opin Pharmacother 2011;12:293-302.
19. Chichlowski M, Rudolph C. Visceral pain and gastrointestinal microbiome. J Neurogastroenterol Motil 2015;21:172-181.
20. Liu YW, Liu WH, Wu CC, et al. Psychotropic effects of *Lactobacillus plantarum* PS128 in early life-stressed and naïve adult mice. Brain Res 2016;1631:1-12.
21. Liu WH, Chuang HL, Huang YT, et al. Alteration of behavior and monoamine levels attributable to *Lactobacillus plantarum* PS128 in germ-free mice. Behav Brain Res 2016;298:202-209.
22. Bercik P, Park AJ, Sinclair D, et al. The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil 2011;23:1132-1139.
23. Bravo JA, Forsythe P, Chew MV, et al. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci USA 2011;108:16050-16055.
24. O'Sullivan E, Barrett E, Grenham S, et al. BDNF expression in the hippocampus of maternally separated rats: does *Bifidobacterium breve* 6330 alter BDNF levels? Benef Microbes 2011;2:199-207.
25. Ait-Belgnaoui A, Durand H, Cartier C, et al. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 2012;37:1885-1895.
26. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 2008;43:164-174.
27. Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. Neuroscience 2010;170:1179-1188.
28. Kwok LY, Wang L, Zhang J, Guo Z, Zhang H. A pilot study on the effect of *Lactobacillus casei* Zhang on intestinal microbiota parameters in Chinese subjects of different age. Benef Microbes 2014;5:295-304.
29. Smith CJ, Emge JR, Berzins K, et al. Probiotics normalize the gut-brain-microbiota axis in immunodeficient mice. Am J Physiol Gastrointest

Liver Physiol 2014;307:G793-G802.
30. Wang T, Hu X, Liang S, et al. *Lactobacillus fermentum* NS9 restores the antibiotic induced physiological and psychological abnormalities in rats. Benef Microbes 2015;6:707-717.
31. Hsiao EY, McBride SW, Hsien S, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 2013;155:1451-1463.
32. Moher D, Shamseer L, Clarke M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Syst Rev 2015;4:2046-4053.
33. Higgins JPT, Green S. Cochrane handbook for systematic reviews of interventions. Version 5.1.0. The Cochrane Collaboration, 2011. Available from URL: http://handbook.cochrane.org/ (accessed 22 Sep 2016).
34. National Collaborating Centre for Methods and Tools (2008). Quality assessment tool for quantitative studies. Hamilton: MaMaster University (updated 13 April 2010). Available from URL: http://www.nccmt.ca/resources/search/14 (accessed 22 Sep 2016).
35. Bercik P, Verdu EF, Foster JA, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology 2010;139:2102-2112, e1.
36. Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterol Motil 2014;26:1615-1627.
37. Ohland CL, Kish L, Bell H, et al. Effects of *Lactobacillus helveticus* on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. Psychoneuroendocrinology 2013;38:1738-1747.
38. Liang S, Wang T, Hu X, et al. Administration of *Lactobacillus helveticus* NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. Neuroscience 2015;310:561-577.
39. Messaoudi M, Lalonde R, Violle N, et al. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 2011;105:755-764.
40. Singh PK, Chopra K, Kuhad A, Kaur IP. Role of *Lactobacillus acidophilus* loaded floating beads in chronic fatigue syndrome: behavioral and biochemical evidences. Neurogastroenterol Motil 2012;24:366-e170.
41. Arseneault-Bréard J, Rondeau I, Gilbert K, et al. Combination of *Lactobacillus helveticus R0052* and *Bifidobacterium longum R0175* reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. Br J Nutr 2012;107:1793-1799.
42. Luo J, Wang T, Liang S, Hu X, Li W, Jin F. Ingestion of *Lactobacillus* strain reduces anxiety and improves cognitive function in the hyperammonemia rat. Sci China Life Sci 2014;57:327-335.
43. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive processes in an anxious mouse strain. Behav Brain Res 2015;287:59-72.
44. Liu J, Sun J, Wang F, et al. Neuroprotective Effects of *Clostridium butyricum* against Vascular Dementia in Mice via Metabolic Butyrate. Biomed Res Int 2015;2015:412946.
45. Gareau MG, Wine E, Rodrigues DM, et al. Bacterial infection causes stress-induced memory dysfunction in mice. Gut 2011;60:307-317.
46. Davari S, Talaei SA, Alaei H, Salami M. Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome-gut-brain axis. Neuroscience 2013;240:287-296.
47. Jeong JJ, Kim KA, Ahn YT, et al. Probiotic mixture KF attenuates age-dependent memory deficit and lipidemia in Fischer 344 rats. J Microbiol Biotechnol 2015;25:1532-1536.
48. Kantak PA, Bobrow DN, Nyby JG. Obsessive-compulsive-like behaviors in house mice are attenuated by a probiotic (*Lactobacillus* rhamnosus GG). Behav Pharmacol 2014;25:71-79.
49. D'Mello C, Ronaghan N, Zaheer R, et al. Probiotics improve inflammation-associated sickness behavior by altering communication between the peripheral immune system and the brain. J Neurosci 2015;35:10821-10830.
50. Takada M, Nishida K, Kataoka-Kato A, et al. Probiotic *Lactobacillus casei* strain Shirota relieves stress-associated symptoms by modulating the gut-brain interaction in human and animal models. Neurogastroenterol Motil 2016;28:1027-1036.
51. Rao AV, Bested AC, Beaulne TM, et al. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. Gut Pathog 2009;1:6.
52. Mohammadi AA, Jazayeri S, Khosravi-Darani K, et al. The effects of probiotics on mental health and hypothalamic-pituitary-adrenal axis: a randomized, double-blind, placebo-controlled trial in petrochemical workers. Nutr Neurosci Published Online First: 16 Apr 2015. doi: 10.1179/1476830515Y.0000000023.
53. Steenbergen L, Sellaro R, van Hemert S, Bosch JA, Colzato LS. A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. Brain Behav Immun 2015;48:258-264.
54. Dickerson FB, Stallings C, Origoni A, et al. Effect of probiotic supplementation on schizophrenia symptoms and association with gastrointestinal functioning: a randomized, placebo-controlled trial. Prim Care Companion CNS Disord Published Online First: 13 Feb 2014. doi: 10.4088/PCC.13m01579.
55. Vaghef-Mehrabany E, Alipour B, Homayouni-Rad A, Sharif SK, Asghari-Jafarabadi M, Zavvari S. Probiotic supplementation improves inflammatory status in patients with rheumatoid arthritis. Nutrition 2014;30:430-435.
56. Dapoigny M, Piche T, Ducrotte P, Lunaud B, Cardot JM, Bernalier-Donadille A. Efficacy and safety profile of LCR35 complete freeze-dried culture in irritable bowel syndrome: a randomized, double-blind study. World J Gastroenterol 2012;18:2067-2075.
57. Simrén M, Ohman L, Olsson J, et al. Clinical trial: the effects of a fermented milk containing three probiotic bacteria in patients with irritable bowel syndrome - a randomized, double-blind, controlled study. Aliment Pharmacol Ther 2010;31:218-227.
58. Whorwell PJ, Altringer L, Morel J, et al. Efficacy of an encapsulated probiotic *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. Am J Gastroenterol 2006;101:1581-1590.
59. Reale M, Boscolo P, Bellante V, et al. Daily intake of *Lactobacillus casei* Shirota increases natural killer cell activity in smokers. Br J Nutr 2012;108:308-314.
60. Parracho HMRT, Gibson GR, Knott F, Bosscher D, Kleerebezem M, McCartney AL. A double blind, placebo controlled, crossover designed

probiotic feeding study in children diagnosed with autistic spectrum disorders. International Journal of Probiotics and Prebiotics 2010;5:69.
61. Messaoudi M, Violle N, Bisson JF, Desor D, Javelot H, Rougeot C. Beneficial psychological effects of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in healthy human volunteers. Gut Microbes 2011;2:256-261.
62. Benton D, Williams C, Brown A. Impact of consuming a milk drink containing a probiotic on mood and cognition. Eur J Clin Nutr 2007;61:355-361.
63. Tillisch K, Labus J, Kilpatrick L, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 2013;144:1394-1401, e1-e4.
64. Sung V, Collett S, de Gooyer T, Hiscock H, Tang M, Wake M. Probiotics to prevent or treat excessive infant crying: systematic review and meta-analysis. JAMA Pediatr 2013;167:1150-1157.
65. Romijn AR, Rucklidge JJ. Systematic review of evidence to support the theory of psychobiotics. Nutr Rev 2015;73:675-693.
66. Anabrees J, Indrio F, Paes B, AlFaleh K. Probiotics for infantile colic: a systematic review. BMC Pediatr 2013;13:186.
67. Groeger D, O'Mahony L, Murphy EF, et al. *Bifidobacterium infantis* 35624 modulates host inflammatory processes beyond the gut. Gut Microbes 2013;4:325-339.
68. Lomasney KW, Cryan JF, Hyland NP. Converging effects of a *Bifidobacterium* and *Lactobacillus* probiotic strain on mouse intestinal physiology. Am J Physiol Gastrointest Liver Physiol 2014;307:G241-G247.
69. Saulnier DM, Ringel Y, Heyman MB, et al. The intestinal microbiome, probiotics and prebiotics in neurogastroenterology. Gut Microbes 2013;4:17-27.
70. O'Mahony L, McCarthy J, Kelly P, et al. *Lactobacillus* and *Bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 2005;128:541-551.
71. McFarland LV. From yaks to yogurt: the history, development, and current use of probiotics. Clin Infect Dis 2015;60(suppl 2):S85-S90.
72. Floch MH, Walker WA, Sanders ME, et al. Recommendations for probiotic use--2015 update: proceedings and consensus opinion. J Clin Gastroenterol 2015;49(suppl 1):S69-S73.
73. Dedovic K, Ngiam J. The cortisol awakening response and major depression: examining the evidence. Neuropsychiatr Dis Treat 2015;11:1181-1189.
74. Osborne DM, Pearson-Leary J, McNay EC. The neuroenergetics of stress hormones in the hippocampus and implications for memory. Front Neurosci 2015;9:164.
75. Mariga A, Mitre M, Chao MV. Consequences of brain-derived neurotrophic factor withdrawal in CNS neurons and implications in disease. Neurobiol Dis Published Online First: 22 Mar 2016. doi: 10.1016/j.nbd.2016.03.009.
76. Tsai SJ. Attention-deficit hyperactivity disorder may be associated with decreased central brain-derived neurotrophic factor activity: clinical and therapeutic implications. Med Hypotheses 2007;68:896-899.
77. Julio-Pieper M, Bravo JA, Aliaga E, Gotteland M. Review article: intestinal barrier dysfunction and central nervous system disorders – a controversial association. Aliment Pharmacol Ther 2014;40:1187-1201.
78. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. Mol Psychiatry 2014;19:146-148.
79. Diaz Heijtz R, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci USA 2011;108:3047-3052.
80. Lyte M, Li W, Opitz N, Gaykema RP, Goehler LE. Induction of anxiety-like behavior in mice during the initial stages of infection with the agent of murine colonic hyperplasia *Citrobacter rodentium*. Physiol Behav 2006;89:350-357.
81. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 2011;23:255-264, e119.
82. Nishino R, Mikami K, Takahashi H, et al. Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol Motil 2013;25:521-528.
83. Astur RS, Ortiz ML, Sutherland RJ. A characterization of performance by men and women in a virtual Morris water task: a large and reliable sex difference. Behav Brain Res 1998;93:185-190.
84. Astur RS, Taylor LB, Mamelak AN, Philpott L, Sutherland RJ. Humans with hippocampus damage display severe spatial memory impairments in a virtual Morris water task. Behav Brain Res 2002;132:77-84.
85. Kalová E, Vlcek K, Jarolímová E, Bures J. Allothetic orientation and sequential ordering of places is impaired in early stages of Alzheimer's disease: corresponding results in real space tests and computer tests. Behav Brain Res 2005;159:175-186.
86. Laczó J, Andel R, Vyhnalek M, et al. Human analogue of the morris water maze for testing subjects at risk of Alzheimer's disease. Neurodegener Dis 2010;7:148-152.
87. Kirschbaum C, Pirke KM, Hellhammer DH. The 'Trier Social Stress Test' – a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 1993;28:76-81.
88. Sebastian C, Viding E, Williams KD, Blakemore SJ. Social brain development and the affective consequences of ostracism in adolescence. Brain Cogn 2010;72:134-145.
89. Williams KD, Jarvis B. Cyberball: a program for use in research on interpersonal ostracism and acceptance. Behav Res Methods 2006;38:174-180.
90. Allen AP, Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. Neurosci Biobehav Rev 2014;38:94-124.
91. Schwabe L, Haddad L, Schachinger H. HPA axis activation by a socially evaluated cold-pressor test. Psychoneuroendocrinology 2008;33:890-895.
92. Cian C, Barraud PA, Melin B, Raphel C. Effects of fluid ingestion on cognitive function after heat stress or exercise-induced dehydration. Int J Psychophysiol 2001;42:243-251.
93. Vickers K, Jafarpour S, Mofidi A, Rafat B, Woznica A. The 35% carbon dioxide test in stress and panic research: overview of effects and integration of findings. Clin Psychol Rev 2012;32:153-164.

7. Study II. How the brain reacts to social stress (exclusion) – a scoping review

Author contributions

Paul Enck conceptualized the paper; Huiying Wang reviewed and evaluated the literature; and Huiying Wang, Christoph Braun, and Paul Enck wrote the manuscript.

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Review article

How the brain reacts to social stress (exclusion) – A scoping review

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ABSTRACT

Objectives: The Cyberball paradigm is often used to study social stress by exclusion/rejection. We aimed to review the existing neuroimaging literatures in order to provide an overview of the neurophysiological mechanisms of social exclusion.
Method: Literature search was conducted to identify neurophysiological studies that investigated effects of social exclusion on neural activity using the Cyberball game and the relevant influential factors on these effects.
Results: In total, 42 studies using different neuroimaging methods were considered. Regions of the insula, anterior cingulate cortex, temporal and prefrontal cortex were activated to social exclusion. These neural activities were pronounced at latencies ranging from 200 to 400 ms, and between 400 and 900 ms. Influential factors were identified and categorized as intrinsic and extrinsic factors.
Conclusion: An integrated framework describing neural activities under social exclusion in terms of both, temporal and spatial processes is provided. Furthermore, the summary of influential intrinsic and extrinsic factors may help us to understand the diversity of the processes and may guide clinical therapy of stress related disorders.

1. Introduction

A peer relationship is very important in adolescence and adults. Ostracism and rejection by peers or by significant ones cause social pain (Eisenberger, 2012; Eisenberger et al., 2003). Chronic experience of the social exclusion may also be regarded as a stressful situation, which may lead to development of depression.

A number of studies have used a standardized paradigm called 'Cyberball game' to study ostracism (Williams and Jarvis, 2006). In this game, participants believe that they are playing an online ball-tossing game with two players, while these two players are actually programmed. Participants are socially rejected in the so called 'exclusion condition', by receiving the ball only a few times and less often than the other players. The 'exclusion condition' is always compared with the 'inclusion condition', during which participants receive the ball equally often as the other players. This paradigm has been proven reliable and valid to induce feelings of rejection in adults and adolescents (Eisenberger et al., 2003; Williams and Jarvis, 2006). According to a post-game questionnaire with a 'Need Threat Scale' and 'Mood Scale', participants indicated lower levels of belonging, self-esteem, control and meaningful existence, and also higher distress after completing the exclusion block as compared to the inclusion block (Williams and Jarvis, 2006; Zadro et al., 2004). A recent published meta-analysis of Cyberball studies showed a fairly large average ostracism effect (Hartgerink et al., 2015).

Technical development in neuroimaging methods allows researchers to investigate how the human brain responds to the exclusion situation during its occurrence rather than after the game, and to explore the mechanisms that underlie the subjective feelings reported after the game. Studies using different methods such as functional magnetic resonance imaging (fMRI) and electroencephalography (EEG) have discovered widely distributed brain regions involved in social exclusion versus inclusion. Regions such as cingulate cortex (the anterior and posterior parts) and insula are activated, which are related to affects and emotions (Bolling et al., 2011a; Masten et al., 2009). Being excluded, these neural activations appear to represent negative emotions of sadness and distress. The activation of the prefrontal cortex (PFC) has been taken as index of attention control and emotion regulation (Sebastian et al., 2011). As yet, one quantitative meta-analysis aimed to explore if the pain matrix was activated by social rejection during Cyberball, by analyzing 12 fMRI studies with a total of 244 participants (Cacioppo et al., 2013). However, the authors stated

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that the evidences from the included studies were not sufficient to reach an unambiguous conclusion. While numbers of studies have provided information about brain activations during social exclusion/rejection, so far results have only been pooled partly, ignoring studies using EEG to a large part. Although fMRI studies have given us a clear insight in specific locations of functional brain regions, they have limitations in measuring precisely the temporal sequence of when neural activations occur.

Therefore, currently we conduct a scoping review on relevant literatures that have conducted neuropsychological measurements such as using fMRI and EEG to investigate the neuronal processes underlying responses to social stress induced by the Cyberball game. Through mapping included studies according to their methodologies and results, we aim to identify a common spatio-temporal pattern of neural activations related to social exclusion and to analyze influential factors modulating neural activations.

2. Method

The current scoping review was guided by the methodological framework proposed by Arksey and O'Malley (2005). The framework outlines a five-stages approach that includes identifying the research question; searching for relevant studies; selecting studies; charting data; and collating, summarizing, and reporting the results.

2.1. Identifying the research questions (stage 1)

We focused on following main research questions in our scoping review: How were the neural activities of social exclusion measured in currently existing literatures? What are the common features of the neural activities across studies? Which factors influence these neural activations?

2.2. Identifying relevant studies (stage 2)

Relevant studies were found by a search in the PUBMED databases. Articles from 1950 to April 2016 were initially identified using the search string "Cyberball". We only included articles written in English. The first search was undertaken by analyzing titles, abstracts, and the key words describing the articles, to identify studies that met our inclusion criteria. A second search was conducted using citations from all identified reports and relevant review articles.

2.3. Selection of studies (stage 3)

Search results were screened on the basis of the titles and abstracts before full texts were assessed. The abstracts of the retrieved papers were screened for matching the following criteria: (1) the study included participants aged from adolescents to adults; (2) participants can be in different health status such as healthy control (HC), autism spectrum disorder (ASD) and borderline personality disorder (BPD), etc.; (3) the study used methods recording functional brain activity, including fMRI, positron emission tomography (PET), transcranial magnetic stimulation (TMS), magnetoencephalography (MEG), EEG, etc.; (4) the study measured neural responses during the Cyberball game and compared a social rejection/exclusion condition (exclusion block and/or exclusive throw) with an inclusion condition (inclusion block and/or inclusive throw). Those that only analyzed effects of factors on neural response to social exclusion without comparing exclusion with inclusion conditions were not included in our study. All studies that did not meet our criteria were regarded as irrelevant studies.

2.4. Charting data (stage 4)

The following data of the eligible articles were extracted for assessment: first author, year of publication, country focus, type of measurement method, population characteristics, experimental comparisons, and the primary outcomes measures of interests.

2.5. Collating, summarizing, and reporting results (stage 5)

We synthesized the data according our predefined research questions. The study characteristics were summarized and shown according to country, study method (fMRI/EEG/TMS), study design (within/between-subject design), and experimental comparison. Then, main features of each study results were summarized for each study method respectively.

3. Result

3.1. Study selection and characteristics

The literature search yielded an initial total of 123 citations, 4 of which were identified through other sources. After a first screen of the titles and abstracts of these articles, 52 studies were considered eligible for our review, after excluding 71 studies only had behavioral measurement. The full versions of these articles were reviewed and 10 studies were excluded due to absence of a comparison of neural responses between conditions of exclusion and inclusion. In total, 42 studies remained for data analysis (Fig. 1).

Table 1 illustrates main characterizes of the included studies. Among these studies, neural recording methods focused on EEG (n = 13) (Catassi et al., 2013; Cristofori et al., 2015; Crowley et al., 2009, 2010; Gutz et al., 2011, 2015; Kawamoto et al., 2013; McPartland et al., 2011; Sreekrishnan et al., 2014; Themanson et al., 2013; van Noordt et al., 2015; Weschke and Niedeggen, 2013, 2015), fMRI (n = 28) (Bolling et al., 2011a, 2011b, 2011c, 2015; Bonenberger et al., 2015; Cascio et al., 2015; Cristofori et al., 2015; Domsalla et al., 2014; Eisenberger et al., 2003, 2007a, 2007b; Gonzalez et al., 2015; Luo et al., 2016; Masten et al., 2011a, 2011b, 2011c; Maurage et al., 2012; Moor et al., 2012; Nishiyama et al., 2015; Onoda et al., 2010; Preller et al., 2016; Puetz et al., 2014; Rudolph et al., 2016; Sebastian et al., 2011; van Harmelen et al., 2014; Will et al., 2015, 2016; Wudarczyk et al., 2015), and rTMS (n = 1) (Fitzgibbon et al., 2016) in these studies. Nearly half of the studies were conducted in the USA,

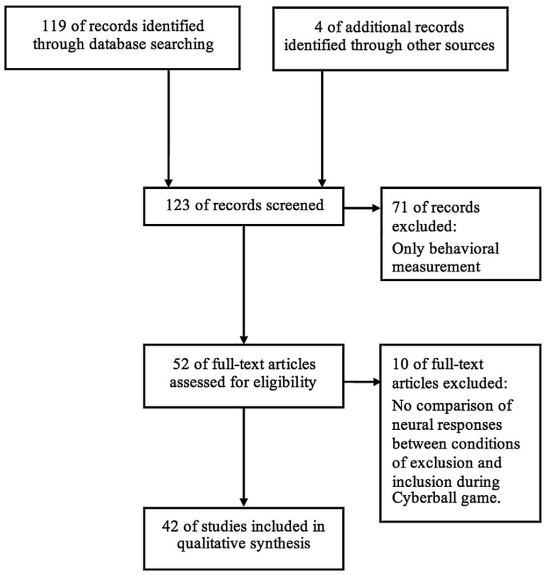

Fig. 1. PRISMA scheme of retrieved literature.

Table 1
Study characteristics.

Study characteristics	All (n = 42) (Ref.)	EEG (n = 13) (Ref.)	fMRI (n = 28) (Ref.)	TMS (n = 1) (Ref.)
Country focus				
• USA	• 20	• 6 (Crowley et al., 2009, 2010; Sreekrishnan et al., 2014; van Noordt et al., 2015; Themanson et al., 2013; McPartland et al., 2011)	• 14 (Masten et al., 2009, 2011a, 2011b, 2011c; Bolling et al., 2011a, 2011b, 2011c, 2015; Cascio et al., 2015; Rudolph et al., 2016; Eisenberger et al., 2003, 2007a, 2007b; Gonzalez et al., 2015)	• 0
• EU	• 17	• 6 (Gutz et al., 2011, 2015; Weschke and Niedeggen, 2013, 2015; Cristofori et al., 2015, 2013)	• 11 (Moor et al., 2012; Puetz et al., 2014; Will et al., 2015, 2016; Domsalla et al., 2014; van Harmelen et al., 2014; Maurage et al., 2012; Bonenberger et al., 2015; Wudarczyk et al., 2015; Preller et al., 2016; Sebastian et al., 2011)	• 0
• Asia	• 4	• 1 (Kawamoto et al., 2013)	• 3 (Nishiyama et al., 2015; Onoda et al., 2010; Luo et al., 2016)	• 0
• Australia	• 1	• 0	• 0	• 1 (Fitzgibbon et al., 2016)
Study design				
• Within subject	• 23	• 11 (Crowley et al., 2009, 2010; Sreekrishnan et al., 2014; van Noordt et al., 2015; Themanson et al., 2013; Gutz et al., 2011; Weschke and Niedeggen, 2013, 2015; Cristofori et al., 2013, 2015; Kawamoto et al., 2013)	• 12 (Masten et al., 2009, 2011b, 2011c; Bolling et al., 2011a, 2011c, 2015; Cascio et al., 2015; Eisenberger et al., 2003, 2007a; Gonzalez et al., 2015; Will et al., 2015; Nishiyama et al., 2015)	• 0
• Between subject	• 19	• 2	• 16	• 1
– Gene	– 3	– 0	– 3 (Eisenberger et al., 2007b; Bonenberger et al., 2015; Luo et al., 2016)	– 0
– Disease	– 6	– 2 (McPartland et al., 2011; Gutz et al., 2015)	– 4 (Masten et al., 2011a; Bolling et al., 2011b; Domsalla et al., 2014; Maurage et al., 2012)	– 0
– Psychological state	– 1	– 0	– 1 (Onoda et al., 2010)	– 0
– development	– 2	– 0	– 2 (Moor et al., 2012; Sebastian et al., 2011)	– 0
– Life experience	– 4	– 0	– 4 (Rudolph et al., 2016; Puetz et al., 2014; Will et al., 2016; van Harmelen et al., 2014)	– 0
– Intervention	– 3	– 0	– 2 (Wudarczyk et al., 2015; Preller et al., 2016)	– 1 (Fitzgibbon et al., 2016)
Experimental comparisons				
• Comparison between blocks	• 26	• 0	• 25 (Masten et al., 2009, 2011a, 2011b; Masten et al., 2011c; Bolling et al., 2011a, 2011b, 2011c, 2015; Cascio et al., 2015; Rudolph et al., 2016; Eisenberger et al., 2003, 2007a, 2007b; Gonzalez et al., 2015; Puetz et al., 2014; Domsalla et al., 2014; van Harmelen et al., 2014; Maurage et al., 2012; Bonenberger et al., 2015; Wudarczyk et al., 2015; Preller et al., 2016; Sebastian et al., 2011; Nishiyama et al., 2015; Onoda et al., 2010; Luo et al., 2016)	• 1 (Fitzgibbon et al., 2016)
• Comparison between trials	• 16	• 13	• 3	• 0
– 'Not my run' vs. 'exclusion' after throwing	– 7	– 5 (Crowley et al., 2009, 2010; van Noordt et al., 2015; McPartland et al., 2011; Kawamoto et al., 2013)	– 2 (Moor et al., 2012; Will et al., 2016)	
– 'My run' vs. 'exclusion' after throwing	– 2	– 1 (Sreekrishnan et al., 2014)	– 1 (Will et al., 2015)	
– 'Inclusionary throw' vs. 'exclusionary throw' after throwing	– 3	– 3 (Themanson et al., 2013; Cristofori et al., 2013, 2015)	– 0	
– 'Other' vs. 'self' after receiving	– 4	– 4 (Gutz et al., 2011, 2015; Weschke and Niedeggen, 2013, 2015)	– 0	
Neural data analysis				
• Whole-brain analysis	• 28	• 0	• 28	
• Region of interest analysis	• 16	• 0	• 16 (Masten et al., 2011a, 2011b; Bolling et al., 2011a, 2011b, 2011c, 2015; Cascio et al., 2015; Rudolph et al., 2016; Moor et al., 2012; Will et al., 2015, 2016; van Harmelen et al., 2014; Wudarczyk et al., 2015; Preller et al., 2016; Nishiyama et al., 2015; Luo et al., 2016)	
• ERP	• 10	• 10 (Crowley et al., 2009, 2010; Sreekrishnan et al., 2014; Themanson et al., 2013; McPartland et al., 2011; Gutz et al., 2011, 2015; Weschke and Niedeggen, 2013, 2015; Kawamoto et al., 2013)		
• Neural oscillations	• 4	• 4 (van Noordt et al., 2015; Cristofori et al., 2013, 2015; Kawamoto et al., 2013)		

Table 2
Convergent results from the EEG studies.

Stage	Neural index (Ref.)	Sample size	Influential factor
Early stage (200–300 ms)	Frontal P2 (Sreekrishnan et al., 2014; McPartland et al., 2011)	96	ASD
	Frontal-central and parietal N2 (Themanson et al., 2013; Weschke and Niedeggen, 2013)	55	
Early stage (300–400 ms)	Occipital-parietal P3 (Crowley et al., 2010; Themanson et al., 2013; Gutz et al., 2011, 2015; Weschke and Niedeggen, 2013, 2015; Kawamoto et al., 2013)	232	BPD
Late stage (400–900 ms)	Frontal and occipital-parietal LSW (Crowley et al., 2009, 2010; Sreekrishnan et al., 2014; McPartland et al., 2011)	157	Na.
Whole time window (0–2 s)	Frontal alpha wave (van Noordt et al., 2015; Kawamoto et al., 2013)	52	Na.
	Insula, subACC, and FFA theta wave (van Noordt et al., 2015; Cristofori et al., 2013, 2015)	63	Na.

Abbreviations: P2 (known as the P200), a positive waveform component that peaks at about 200 ms after the stimulus onset; N2 (N200), is a negative-going wave that peaks at 200–350 ms post-stimulus; P3 (P300), a positive-going wave with a latency of 250–500 ms; LSW (late slow wave), a component with peak latency at 400–900 ms; alpha wave, neural oscillations in the frequency range of 8–15 Hz; theta wave, neural oscillations in the frequency range of 4–7 Hz; ASD, autism spectrum disorder; BPD, borderline personality disorder.

and 40.5% of the studies from Europe. Only 3 studies were conducted in Asia and 1 in Australia. Over half (54.8%) of the experiments used a within subject design, to only study the effects of social exclusion. The other 45.2% of the experiments used a between subject design, to explore factors on neural activations of social exclusion. These 19 between-subject studies investigated intrinsic factors such as genes (n = 3) (μ-opioid receptor (OPRM1), the serotonin transporter promoter receptor (5-HTTLPR), and Monoamine Oxidase A (MAOA)), diseases (n = 6) (social anxiety disorder (SAD), autism spectrum disorder (ASD), borderline personality disorder (BPD), and alcohol-dependence (AD)), psychological states (n = 1) (self-esteem) and development (n = 2), and extrinsic factors of life experience (n = 4) (chronic rejection, early life separation, victimization, and childhood emotional maltreatment) and interventions (n = 3) (chemical anxiety cue, the serotonin 2A/1A receptor (5-HT 2A/1A) stimulation, TMS).

To observe effects of social exclusion, different experimental comparisons were made. All EEG studies compared exclusion to inclusion between trials: five studies compared trials in which the other two players played with each other during exclusion with inclusion blocks, which were labeled as 'exclusion' and 'not my run' respectively; one study compared trials in which the participant threw the ball to either of the other players during inclusion block with trials in which the other players threw to each other during exclusion block; three studies compared trials in which the other players threw to the participant, with trials in which they did not throw to the participant in all blocks; four studies compared trials in which the other players received the ball with trials in which the participant received it. Most of (25/28) the MRI studies compared blocks of inclusion with exclusion, and only 3 of them compared between trials in each condition.

Among the included EEG studies, 10 studies examined neural activity with event-related potential (ERP) analysis comparing social exclusion with inclusion (Crowley et al., 2009, 2010; Gutz et al., 2011, 2015; Kawamoto et al., 2013; McPartland et al., 2011; Sreekrishnan et al., 2014; Themanson et al., 2013; Weschke and Niedeggen, 2013, 2015), 1 of which also applied source analyses to estimate neural generators of the ERPs (Crowley et al., 2010). Also, 4 studies used time-frequency analyses to investigate neural oscillations under social exclusion compared to inclusion (Cristofori et al., 2013, 2015; Kawamoto et al., 2013; van Noordt et al., 2015). Among the fMRI studies, all of them carried out whole-brain analyses in contrast of social exclusion versus inclusion. In addition, 16 studies defined several region of interest (ROI) analyses to address region-specific hypotheses: ACC, MCC, insula, PFC, OFC, STG, IFG, MFG, hippocampus (Bolling et al., 2011a, 2011b, 2011c, 2015; Cascio et al., 2015; Luo et al., 2016; Masten et al., 2011a, 2011b; Moor et al., 2012; Nishiyama et al., 2015; Preller et al., 2016; Rudolph et al., 2016; van Harmelen et al., 2014; Will et al., 2015, 2016; Wudarczyk et al., 2015).

3.2. Neural activities of social exclusion

Data collected from the included studies were capable to support us to build a conceptual framework, which provided temporal information of when and what neural processes occurred and spatial information of which brain regions were activated.

3.2.1. EEG studies

EEG studies have observed different event-related potentials (ERPs) and neural oscillations that were pronounced during the exclusion condition compared to inclusion. These neural indexes were summarized according to different stages. The total sample size among all EEG studies for each neural index was calculated (Table 2). An early stage ranging from 200 to 300 ms was identified as frontal P2 and frontal and parietal N2 (McPartland et al., 2011; Sreekrishnan et al., 2014; Themanson et al., 2013; Weschke and Niedeggen, 2013). However, in patients with ASD the frontal P2 was attenuated (McPartland et al., 2011). Occipital-parietal P3 indexed early stage effects ranging from 300 to 400 ms (Crowley et al., 2010; Gutz et al., 2011, 2015; Kawamoto et al., 2013; McPartland et al., 2011; Themanson et al., 2013; Weschke and Niedeggen, 2013, 2015). For this marker, patients with BPD revealed pronounced activity even during the inclusion condition (Crowley et al., 2010; Gutz et al., 2015; Kawamoto et al., 2013). Late stage effects ranging from 400 to 900 ms were indexed by the late slow wave (LSW) in frontal and occipital-parietal cortex (Crowley et al., 2009, 2010; McPartland et al., 2011; Sreekrishnan et al., 2014). Exclusion-related changes in neural oscillations revealed alpha power increase in frontal cortex and theta power increase in insula, subACC and fusiform face area (FFA) as compared to the inclusion condition (Catassi et al., 2013; Cristofori et al., 2015; Kawamoto et al., 2013; van Noordt et al., 2015).

3.2.2. fMRI studies

Twenty-eight fMRI studies have found various brain regions activated during social exclusion and variability among different populations. Sample size through all studies for each of the main areas identified was calculated and the corresponding influential factors that were suggested to modulate the neural response due to social exclusion were also summarized (Table 3).

The cingulate cortex was mostly reported: 24 out of 28 studies found the anterior cingulate cortex (ACC) activation (24/28) (ventral, dorsal and subgenual) (Bolling et al., 2011a, 2011b, 2011c, 2015; Bonenberger et al., 2015; Eisenberger et al., 2003, 2007b; Gonzalez et al., 2015; Luo et al., 2016; Masten et al., 2009, 2011a, 2011b, 2011c; Maurage et al., 2012; Nishiyama et al., 2015; Onoda et al., 2010; Preller et al., 2016; Puetz et al., 2014; Rudolph et al., 2016; Sebastian et al., 2011; van Harmelen et al., 2014; Will et al., 2015, 2016; Wudarczyk et al., 2015), and 6 of them found the posterior cingulate cortex (PCC) activations (Bolling et al., 2011a, 2011b, 2011c, 2015; Maurage et al., 2012; Preller et al., 2016). ACC activation was affected by several factors: people with different MAOA gene polymorphisms showed different levels of ACC activation; adolescents with ASD showed more pronounced ACC activation than HC; early life separation lowered ACC activation compared to normally developed individuals; chronic rejec-

Table 3
Convergent results from the fMRI studies.

Main brain regions (Ref.)	Total sample size	Influential factor
ACC (Masten et al., 2009, 2011a, 2011b, 2011c; Bolling et al., 2011a, 2011b, 2011c, 2015; Rudolph et al., 2016; Eisenberger et al., 2003, 2007b; Gonzalez et al., 2015; Puetz et al., 2014; Will et al., 2015, 2016; van Harmelen et al., 2014; Maurage et al., 2012; Bonenberger et al., 2015; Wudarczyk et al., 2015; Preller et al., 2016; Sebastian et al., 2011; Nishiyama et al., 2015; Onoda et al., 2010; Luo et al., 2016)	749	MAOA ASD ELS Chronic rejection Self-esteem 5-HT 2A/1A stimulation
PCC (Bolling et al., 2011a, 2011b, 2011c, 2015; Maurage et al., 2012; Preller et al., 2016)	128	Na.
PFC (Masten et al., 2009, 2011a, 2011b, 2011c; Bolling et al., 2011a, 2011b, 2011c, 2015; Eisenberger et al., 2003; Gonzalez et al., 2015; Puetz et al., 2014; Will et al., 2015, 2016; van Harmelen et al., 2014; Maurage et al., 2012; Sebastian et al., 2011; Onoda et al., 2010; Luo et al., 2016)	548	5-HTTLPR ASD AD Early life separation Self-esteem
Insula (Masten et al., 2009, 2011a, 2011c; Bolling et al., 2011a, 2011b, 2011c, 2015; Rudolph et al., 2016; Eisenberger et al., 2003; Moor et al., 2012; Puetz et al., 2014; Domsalla et al., 2014; Maurage et al., 2012; Bonenberger et al., 2015; Nishiyama et al., 2015; Onoda et al., 2010; Luo et al., 2016)	487	ASD AD Early life separation Self-esteem
Temporal lobe (Bolling et al., 2011a, 2011b, 2011c, 2015; Maurage et al., 2012; Luo et al., 2016)	149	Na.

tion enhanced ACC activation; people with lower trait self-esteem had higher ACC activation; pharmacological stimulation with 5-HT 2A/1A receptor agonist decreased ACC activation (Eisenberger et al., 2007b; Masten et al., 2011a; Onoda et al., 2010; Preller et al., 2016; Puetz et al., 2014; Will et al., 2016).

Over half of the fMRI studies (18/28) localized PFC during the exclusion condition, including anterior, ventral medial, dorsal medial, and ventrolateral PFC (Bolling et al., 2011a, 2011b, 2011c, 2015; Cristofori et al., 2015; Eisenberger et al., 2003; Gonzalez et al., 2015; Luo et al., 2016; Masten et al., 2011a, 2011b, 2011c; Maurage et al., 2012; Onoda et al., 2010; Puetz et al., 2014; Sebastian et al., 2011; van Harmelen et al., 2014; Will et al., 2015, 2016). PFC activation was also affected by different factors. Gene polymorphisms of 5-HTTLPR influenced the level of activation; HC with lower self-esteem showed increased PFC activation; alcohol-dependent individuals and people with early life separation had decreased PFC activation (Luo et al., 2016; Masten et al., 2011a; Maurage et al., 2012; Onoda et al., 2010; Puetz et al., 2014).

Insula (the anterior and posterior) activation was observed in 17 studies (Bolling et al., 2011a, 2011b, 2011c, 2015; Bonenberger et al., 2015; Cristofori et al., 2015; Domsalla et al., 2014; Eisenberger et al., 2003; Luo et al., 2016; Masten et al., 2011a, 2011c; Maurage et al., 2012; Moor et al., 2012; Nishiyama et al., 2015; Onoda et al., 2010; Puetz et al., 2014; Rudolph et al., 2016). Insula activation was increased in ASD patients, alcohol-dependent and low self-esteemed individuals, while decreased in those with early life separation (Masten et al., 2011a; Maurage et al., 2012; Onoda et al., 2010; Puetz et al., 2014). The temporal lobe (inferior/middle/superior temporal gyrus, superior temporal sulci) (6/28) was activated by social exclusion as well (Bolling et al., 2011a, 2011b, 2011c, 2015; Luo et al., 2016; Maurage et al., 2012), and its activation was inhibited under chemosensory anxiety cues (Wudarczyk et al., 2015).

Seven studies have analyzed functional connectivity between the activated brain regions during social exclusion (Bolling et al., 2011a, 2011b, 2011c; Luo et al., 2016; Maurage et al., 2012; Onoda et al., 2010; Puetz et al., 2014). Most of these studies (5/7) found connectivity between ACC and PFC (Bolling et al., 2011a, 2011c; Maurage et al., 2012; Onoda et al., 2010; Puetz et al., 2014). This ACC-PFC connectivity was reduced in alcohol-dependent individuals (Maurage et al., 2012), and also, but higher, in people with high self-esteem (Maurage et al., 2012; Onoda et al., 2010). Other areas connected to ACC were middle temporal gyrus (MTG), the right interior parietal lobule (IPL), precuneus, and temporoparietal junction (Bolling et al., 2011a; Puetz et al., 2014). Connectivity between dorsal and rostral ACC was also observed, but differentiated between people with different 5-HTTLPR genotypes (Luo et al., 2016). There was also increased connectivity between the right insula and the paracentral lobule for exclusion as compared to inclusion (Bolling et al., 2011b).

3.2.3. TMS study

One recent study applied – in healthy adults – low-frequency rTMS (1 Hz) at the vlPFC, which has been found activated during exclusion in other studies (Fitzgibbon et al., 2016). However, there was no effect of rTMS on subjective scores rating social exclusion, except for a positive correlation between greater aversive impact scores to social exclusion with personal distress in the rTMS group.

4. Discussion

To our knowledge, this review is the first approach summarizing all of the current brain imaging data of social exclusion induced by the Cyberball game, besides one systematic review which had only included fMRI studies (Cacioppo et al., 2013), and one recent narrative review, which provided a summary of neural processes during social exclusion in their theoretical framework considering rather few studies (Kawamoto et al., 2015). In their framework, they described neural processes during social exclusion as intrapersonal processes, which included detection, appraisal and regulation. ERP N2 and dACC activations were assumed to be involved in the detection processes, P3 and activities of dACC and AI were related to appraisal, and frontal slow wave and vlPFC activation corresponded to regulation processes.

In our review, we have focused on neural activity during social exclusion, which is defined as intrapersonal processes by Kawamoto et al. (2015). We here provide a modified framework by analyzing currently existing neuroimaging studies utilizing the Cyberball paradigm, and summarize influential factors of the neural processes. In our framework, we propose two stages of neural processing of exclusion related information (Fig. 2). The temporal dynamics has been defined based on results from the EEG studies: early stage neural processing are indexed by P2, N2 and P3 and reflect modulation of attention, emotion, arousal and appraisal. Later stage activities, indexed by slow waves lasting from 400 ms to 900 ms, are regarded as cognitive processes of emotion regulation. Due to lack of a fair spatial resolution of EEG technique, the corresponding localizations of the neural processes in each stage were identified by analyzing the results from the included fMRI studies in addition to summarizing the results from EEG studies. Also, we have summarized the influential factors that appeared to alter the neural response to the social exclusion, consisting of intrinsic and

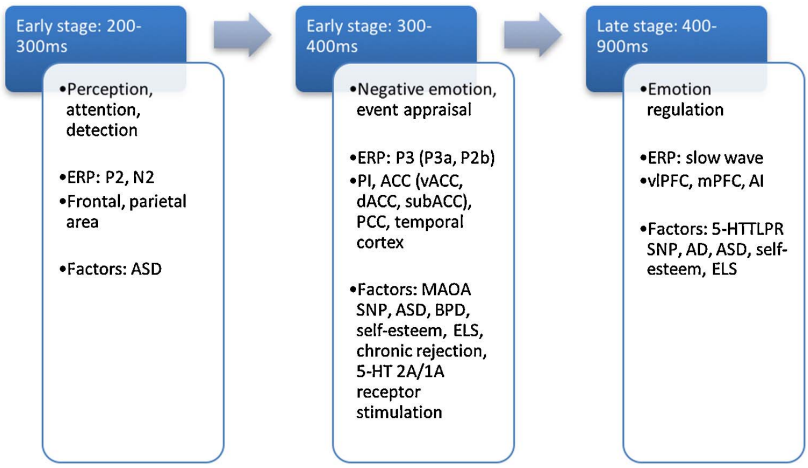

Fig. 2. Key processing during social exclusion and corresponding brain regions.

extrinsic factors. These factors have been integrated into our framework, indicating their effects on different neural processes.

As the results from the EEG studies, the frontal P2 is more pronounced during social exclusion. P2 indicates perception and processing of salient stimuli, for which attention is needed (Key et al., 2005; Luck and Hillyard, 1994; Sreekrishnan et al., 2014). At the same time, frontal-parietal N2 reflects neural alarm activation for conflict monitoring, in response to the exclusion event (Themanson et al., 2013; Weschke and Niedeggen, 2013). Also, the frontal theta activity in the 200–400 ms time range may reflect behavioral conflict and/or violation of expectation to receive the ball and realization of not receiving it (Cavanagh et al., 2013; van Noordt et al., 2015). In the early stage interval from 300 to 400 ms, negative emotions are induced by the exclusion event, followed by appraisal of the event. The P3 complex is enhanced during social exclusion, with P3a and P3b indicating differential neural processes. P3a (the first part of the P3 complex) is related to negative mood induced by ostracism (Gutz et al., 2011; Weschke and Niedeggen, 2013). P3a indicates the contributions of the ACC and posterior insula, which may correspond to the fMRI studies showing the ACC activations related to emotion and PI related to a more visceral pain response to social exclusion (Bolling et al., 2011b; Craig, 2009; Gutz et al., 2011; Polich, 2007; Volpe et al., 2007; Weschke and Niedeggen, 2015). The ventral part of ACC reflects a primary emotional reaction to exclusion, which is innate and spontaneous (Bolling et al., 2011c; Gonzalez et al., 2015; Somerville et al., 2006). Some studies have stated that dACC reflects a secondary and more cognitive process, which is associated with conflict monitoring, emotional awareness, and reward-based decision making (Bolling et al., 2011c; Cascio et al., 2015; Gonzalez et al., 2015; Jarcho et al., 2011; McRae et al., 2008; Somerville et al., 2006; van Veen et al., 2009; Weissman et al., 2003). Greater activity in subACC, mostly described as a neural marker or predictor of depression, reflects higher negative emotions induced by the exclusion event, since it is connected to other limbic structures (Brody et al., 1999; Cascio et al., 2015; Greicius et al., 2007; Haas et al., 2007; Keedwell et al., 2009; Masten et al., 2011b; Sebastian et al., 2011). P3b indexes stimulus evaluation and categorization started, which is presumed to be a posterior network, including the temporal cortex and PCC (Bledowski et al., 2004; Gutz et al., 2011; Weschke and Niedeggen, 2013, 2015; Wronka et al., 2012).

Cognitive processes of affect-regulation occur after 400 ms, and are regarded as a late stage of neural processing (Sreekrishnan et al., 2014). According to the EEG studies, the late positive slow wave (400–900 ms) reflects emotional regulation (Crowley et al., 2009; Kross et al., 2011; Ochsner et al., 2004; Phan et al., 2005). The negative slow wave in frontal cortex reflects engagement of evaluative processes, when anticipating various arousing stimuli (Baas et al., 2002; Crowley et al., 2010). The frontal theta modulation at later processing stage is closely linked to distress and anxiety, much like the frontal slow-wave activity reported in other studies. It is suggested that the frontal theta activity is related to higher and more stable levels of anxiety and neurochemical changes in dopaminergic systems (Cavanagh and Shackman, 2015; Themanson et al., 2013), and enhanced in persons with a history of depression during processing of negative valence outcomes (Cavanagh et al., 2011). Data from the fMRI studies allow us to localize the sub-regions of the frontal cortex to be involved in the late stage. Several studies have revealed that the vlPFC is activated during social exclusion and negatively correlates with activities in the insula and subACC, reflecting a function of emotion regulation (Eisenberger et al., 2003; Gonzalez et al., 2015; Lorenz et al., 2003; Luo et al., 2016; Masten et al., 2009). The rTMS study has also reported an effect of brain stimulation at vlPFC on negative behavioral outcome of social exclusion, which however, depends on individual traits (Fitzgibbon et al., 2016). The mPFC is also involved, suggested to respond during self-evaluation triggered by social exclusion (Kelley et al., 2002; Macrae et al., 2004), and to regulate negative affect via connections with ACC and amygdala (Buckholtz et al., 2008; Sebastian et al., 2011). Additionally, differing from the posterior insula, the anterior insula has functions of subjective awareness and cognitive control, because of connectivity to the middle and dorsal ACC (Bolling et al., 2011b; Cascio et al., 2015; Craig, 2009; Deen et al., 2011; Dosenbach et al., 2007).

4.1. Functional connectivity

Functional connectivity has been found between main activated regions during social exclusion, indicating a network of brain regions involved in processing social exclusion and stress (Fig. 3).

Enhanced connectivity between PFC (mPFC and vlPFC) and ACC (vACC and dACC) is supposed to be involved in regulating intra- and inter-individual functioning (Bolling et al., 2011a; Onoda et al., 2010). This frontal-cingulate connectivity is negative (increase of frontal activity accompanies with decreased cingulate activity), as frontal activity is associated with regulation and inhibition of negative mood, by disrupting ACC activity (Maurage et al., 2012; Onoda et al., 2010). Positive frontal-cingulate functional activity in individuals with low self-esteem and alcohol dependence reveals a failure on the part of the PFC to suppress ACC functioning (Puetz et al., 2014).

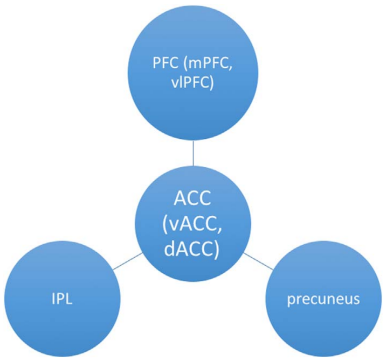

Fig. 3. Main functional connectivity during social exclusion.

A larger network in response to social exclusion is the connectivity of vACC to mPFC, IPL and precuneus – three regions from the central components of the default mode network (DMN) (Buckner et al., 2008). This connectivity could result from engagement of reflective processing during social exclusion, when the participants are no longer playing the game and might begin to question the motives of other players, or to ruminate generally on the situation (Bolling et al., 2011c). Therefore, this pattern might come up during the later stage and be inversely related to emotional processing since participants start to become disengaged. The connectivity between vACC and DMN during rest has been found stronger in people with depression compared to controls, highlighting a potential relationship between vACC-DMN connectivity, negative emotion, and rumination (Greicius et al., 2007).

4.2. Influential factors

The studies with between-subject designs have addressed factors that influence neural activity of social exclusion among populations. We summarized these factors as two categories: the intrinsic factors of gene, disease (disorder), psychological state and development; and the extrinsic factors of life experience and interventions. The impacts of these factors were analyzed and integrated in our framework of neural processes of social exclusion.

Neural process of attention and perception in the early stage was influenced by the ASD. Children with ASD had decreased engagement of attentional resources to social cues, indexed by a reduced amplitude of P2 for rejecting events (Dawson et al., 2004; McPartland et al., 2011).

In the following, emotions arousal and event appraisal in the early stage were affected by many intrinsic and extrinsic factors. The influential intrinsic factors include psychiatric disorders, psychological trait and also genes. Children with ASD had attenuated vACC activity suggesting a hypo emotion arousal due to social exclusion (Bolling et al., 2011b). In contrast, patients with BPD had pronounced parietal P3b ERPs which however were not distinctive between inclusion and exclusion condition, suggesting negative perception of even inclusionary event in this group (Gutz et al., 2015). People with low self-esteem showed higher activation in the ventral and dorsal parts of ACC than those with higher self-esteem suggesting higher emotion arousal and deeper evaluation in the former group (Onoda et al., 2010). Genetically, Single-Nucleotide Polymorphisms (SNPs) of MAOA gene, which encodes Monoamine Oxidase was influential to the dACC activation to the social exclusion (Eisenberger et al., 2007b).

Extrinsic factors of previous life experience and drug interventions influenced appraisal of social exclusion by altering activations of the dACC. Children with chronic rejection and early life separation all felt more distress due to exclusion compared to controls, but with differential dACC activities – higher in chronically rejected and decreased in early life separation (Puetz et al., 2014; Will et al., 2016). Chronic rejection and early life separation are both, life stress and a cause of alterations in neural processes of social exclusion in later life, and may increase the risk for developing mental disorders such as depression and anxiety. Further studies need to clarify the function of dACC during social exclusion. Intervention of the 5-HT 2A/1A receptor stimulation reduced the distress induced by the social exclusion and modulated the neural processes by decreasing the dACC activation (Preller et al., 2016). Other studies also have shown that Acetaminophen reduced the dACC and AI activations, and the 3,4-methylenedioxymethamphetamine alleviated perception of social exclusion by reducing participants' subjective report of negative mood (Dewall et al., 2010; Frye et al., 2014). Evidences of drug interventions influencing effects of social stress may guide clinical therapy of stress-related disorders, such as depression and anxiety, etc.

The late stage of the neural processes is essential since it regulates the negative emotion induced during the early stage. Ontogenetic development helps people to acquire strategies to regulate negative emotion induced by social stress, indicated by higher activation of vlPFC in adults compared to adolescents (Sebastian et al., 2011). Patients with ASD have difficulties in making critical distinctions based on social context at late processing stages, as indexed by the absence of differential late slow wave (McPartland et al., 2011). Alcohol-dependent individuals had decreased vPFC and the middle frontal gyrus activities, indicating an impaired regulation of the exclusion feeling (Maurage et al., 2012). People with low self-esteem also showed higher activation in the PFC, indicated more emotion regulation which might have been induced by higher early processes of the emotional arousal and appraisal (Onoda et al., 2010). Differential activation of the PFC among individuals with different SNPs of (5-HTTLPR), which is involved in many mental conditions such as alcoholism, depression and obsessive-compulsive disorder, suggested a neural mechanism of 5-HTTLPR on the social cognition (Luo et al., 2016). Reduced activation of PFC in children with early life stress suggested an negative effect of previous rejection experience on processing and deficient regulation of the negative affect during the current exclusion event (Puetz et al., 2014).

5. Conclusion

A host of studies have applied the Cyberball paradigm to induce neural responses to social exclusion/rejection and addressed the neural signature of it. Despite sparse interpretations of the results, converging data have pointed out the main brain areas of the neural activations, and according to a systematic analysis, time frames of the activities can be identified. Relevant influential factors have been also investigated and categorized as intrinsic and extrinsic factors in our study. However, several aspects in this topic are still missing and remain to be clarified.

On one hand, specific functions of sub regions in each activated region are controversial, for example the dorsal ACC. Most of the studies suggested the dACC was related to a more cognitive process as concluded in our study (Jarcho et al., 2011; McRae et al., 2008; Somerville et al., 2006; van Veen et al., 2009; Weissman et al., 2003), while others argued that dACC activation was related to social exclusion perception and associated affective distress, acting as complementary processes of a neural alarm system (Eisenberger et al., 2003; Maurage et al., 2012). This controversy also exists in subgenual ACC, which is mostly associated negative emotion, however, it was found positively related to vPFC activity and suggested to be important in regulating negative affect (Masten et al., 2009).

On the other hand, supplement of the theoretical framework of the neural processes under social exclusion is needed. Our theoretical frame was based on a systematic review of both EEG and fMRI studies, however, this still needs to be finally testified by experimental studies. For instance, the neural processes of perception and attention has been defined as happening from 200 to 300 ms with indexes of P2 and N2,

but the corresponding localizations are rather obscure due to limitation of a fairly spatial resolution of EEG and temporal resolution of fMRI. Therefore, an improvement of both high temporal and spatial resolution of neural activity of the social exclusion is needed and may be able to be achieved by technique of a combined fMRI-EEG and/or MEG.

In sum, we have reviewed a multitude of brain data on social exclusion/rejection induced by the Cyberball paradigm. Through mapping the studies using fMRI and EEG, we have integrated the results into a theoretical framework of neural processes during this social stress, including the influential factors as well. We hope this review could guide further studies in this topic to help the understanding of the social exclusion more comprehensively. Exploring the potential influential factors may also benefit development of psychological and clinical therapy for those suffer from stress-related disorders.

Conflicts of interest

The authors declare no potential conflicts of interest.

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References

Arksey, H., O'Malley, L., 2005. Scoping studies: towards a methodological framework. Int. J. Soc. Res. Methodol. 8, 19–32.
Baas, J.M., Kenemans, J.L., Bocker, K.B., Verbaten, M.N., 2002. Threat-induced cortical processing and startle potentiation. Neuroreport 13, 133–137.
Bledowski, C., Prvulovic, D., Hoechstetter, K., Scherg, M., Wibral, M., et al., 2004. Localizing P300 generators in visual target and distractor processing: a combined event-related potential and functional magnetic resonance imaging study. J. Neurosci. 24, 9353–9360.
Bolling, D.Z., Pelphrey, K.A., Vander Wyk, B.C., 2015. Unlike adults, children and adolescents show predominantly increased neural activation to social exclusion by members of the opposite gender. Soc. Neurosci. 15, 1–12.
Bolling, D.Z., Pitskel, N.B., Deen, B., Crowley, M.J., Mayes, L.C., Pelphrey, K.A., 2011a. Development of neural systems for processing social exclusion from childhood to adolescence. Dev. Sci. 14, 1431–1444.
Bolling, D.Z., Pitskel, N.B., Deen, B., Crowley, M.J., McPartland, J.C., et al., 2011b. Enhanced neural responses to rule violation in children with autism: a comparison to social exclusion. Dev. Cogn. Neurosci. 1, 280–294.
Bolling, D.Z., Pitskel, N.B., Deen, B., Crowley, M.J., McPartland, J.C., et al., 2011c. Dissociable brain mechanisms for processing social exclusion and rule violation. Neuroimage 54, 2462–2471.
Bonenberger, M., Plener, P.L., Groschwitz, R.C., Gron, G., Abler, B., 2015. Polymorphism in the micro-opioid receptor gene (OPRM1) modulates neural processing of physical pain, social rejection and error processing. Exp. Brain Res. 233, 2517–2526.
Brody, A.L., Saxena, S., Silverman, D.H., Alborzian, S., Fairbanks, L.A., et al., 1999. Brain metabolic changes in major depressive disorder from pre- to post-treatment with paroxetine. Psychiatry Res. 91, 127–139.
Buckholtz, J.W., Callicott, J.H., Kolachana, B., Hariri, A.R., Goldberg, T.E., et al., 2008. Genetic variation in MAOA modulates ventromedial prefrontal circuitry mediating individual differences in human personality. Mol. Psychiatry 13, 313–324.
Buckner, R.L., Andrews-Hanna, J.R., Schacter, D.L., 2008. The brain's default network: anatomy, function, and relevance to disease. Ann. N.Y. Acad. Sci. 1124, 1–38.
Cacioppo, S., Frum, C., Asp, E., Weiss, R.M., Lewis, J.W., Cacioppo, J.T., 2013. A quantitative meta-analysis of functional imaging studies of social rejection. Sci. Rep. 3, 2027.
Cascio, C.N., Konrath, S.H., Falk, E.B., 2015. Narcissists' social pain seen only in the brain. Soc. Cogn. Affect. Neurosci. 10, 335–341.
Catassi, C., Bai, J.C., Bonaz, B., Bouma, G., Calabro, A., et al., 2013. Non-Celiac Gluten sensitivity: the new frontier of gluten related disorders. Nutrients 5, 3839–3853.
Cavanagh, J.F., Bismark, A.J., Frank, M.J., Allen, J.J., 2011. Larger error signals in major depression are associated with better avoidance learning. Front. Psychol. 2, 331.
Cavanagh, J.F., Eisenberg, I., Guitart-Masip, M., Huys, Q., Frank, M.J., 2013. Frontal theta overrides pavlovian learning biases. J. Neurosci. 33, 8541–8548.
Cavanagh, J.F., Shackman, A.J., 2015. Frontal midline theta reflects anxiety and cognitive control: meta-analytic evidence. J. Physiol. Paris 109, 3–15.
Craig, A.D., 2009. How do you feel – now? The anterior insula and human awareness. Nat. Rev. Neurosci. 10, 59–70.
Cristofori, I., Harquel, S., Isnard, J., Mauguiere, F., Sirigu, A., 2015. Monetary reward suppresses anterior insula activity during social pain. Soc. Cogn. Affect. Neurosci. 10, 1668–1676.
Cristofori, I., Moretti, L., Harquel, S., Posada, A., Deiana, G., et al., 2013. Theta signal as the neural signature of social exclusion. Cereb. Cortex 23, 2437–2447.
Crowley, M.J., Wu, J., McCarty, E.R., David, D.H., Bailey, C.A., Mayes, L.C., 2009. Exclusion and micro-rejection: event-related potential response predicts mitigated distress. Neuroreport 20, 1518–1522.
Crowley, M.J., Wu, J., Molfese, P.J., Mayes, L.C., 2010. Social exclusion in middle childhood: rejection events, slow-wave neural activity, and ostracism distress. Soc. Neurosci. 5, 483–495.
Dawson, G., Toth, K., Abbott, R., Osterling, J., Munson, J., et al., 2004. Early social attention impairments in autism: social orienting, joint attention, and attention to distress. Dev. Psychol. 40, 271–283.
Deen, B., Pitskel, N.B., Pelphrey, K.A., 2011. Three systems of insular functional connectivity identified with cluster analysis. Cereb. Cortex 21, 1498–1506.
Dewall, C.N., Macdonald, G., Webster, G.D., Masten, C.L., Baumeister, R.F., et al., 2010. Acetaminophen reduces social pain: behavioral and neural evidence. Psychol. Sci. 21, 931–937.
Domsalla, M., Koppe, G., Niedtfeld, I., Vollstadt-Klein, S., Schmahl, C., et al., 2014. Cerebral processing of social rejection in patients with borderline personality disorder. Soc. Cogn. Affect. Neurosci. 9, 1789–1797.
Dosenbach, N.U., Fair, D.A., Miezin, F.M., Cohen, A.L., Wenger, K.K., et al., 2007. Distinct brain networks for adaptive and stable task control in humans. Proc. Natl. Acad. Sci. U. S. A. 104, 11073–11078.
Eisenberger, N.I., 2012. The pain of social disconnection: examining the shared neural underpinnings of physical and social pain. Nat. Rev. Neurosci. 13, 421–434.
Eisenberger, N.I., Lieberman, M.D., Williams, K.D., 2003. Does rejection hurt? An FMRI study of social exclusion. Science (New York, NY) 302, 290–292.
Eisenberger, N.I., Taylor, S.E., Gable, S.L., Hilmert, C.J., Lieberman, M.D., 2007a. Neural pathways link social support to attenuated neuroendocrine stress responses. Neuroimage 35, 1601–1612.
Eisenberger, N.I., Way, B.M., Taylor, S.E., Welch, W.T., Lieberman, M.D., 2007b. Understanding genetic risk for aggression: clues from the brain's response to social exclusion. Biol. Psychiatry 61, 1100–1108.
Fitzgibbon, B.M., Kirkovski, M., Bailey, N.W., Thomson, R.H., Eisenberger, N., et al., 2016. Low-frequency brain stimulation to the left dorsolateral prefrontal cortex increases the negative impact of social exclusion among those high in personal distress. Soc. Neurosci. 1–5.
Frye, C.G., Wardle, M.C., Norman, G.J., de Wit, H., 2014. MDMA decreases the effects of simulated social rejection. Pharmacol. Biochem. Behav. 117, 1–6.
Gonzalez, M.Z., Beckes, L., Chango, J., Allen, J.P., Coan, J.A., 2015. Adolescent neighborhood quality predicts adult dACC response to social exclusion. Soc. Cogn. Affect. Neurosci. 10, 921–928.
Greicius, M.D., Flores, B.H., Menon, V., Glover, G.H., Solvason, H.B., et al., 2007. Resting-state functional connectivity in major depression: abnormally increased contributions from subgenual cingulate cortex and thalamus. Biol. Psychiatry 62, 429–437.
Gutz, L., Kupper, C., Renneberg, B., Niedeggen, M., 2011. Processing social participation: an event-related brain potential study. Neuroreport 22, 453–458.
Gutz, L., Renneberg, B., Roepke, S., Niedeggen, M., 2015. Neural processing of social participation in borderline personality disorder and social anxiety disorder. J. Abnorm. Psychol. 124, 421–431.
Haas, B.W., Omura, K., Constable, R.T., Canli, T., 2007. Emotional conflict and neuroticism: personality-dependent activation in the amygdala and subgenual anterior cingulate. Behav. Neurosci. 121, 249–256.
Hartgerink, C.H., van Beest, I., Wicherts, J.M., Williams, K.D., 2015. The ordinal effects of ostracism: a meta-analysis of 120 Cyberball studies. PLOS ONE 10, e0127002.
Jarcho, J.M., Berkman, E.T., Lieberman, M.D., 2011. The neural basis of rationalization: cognitive dissonance reduction during decision-making. Soc. Cogn. Affect. Neurosci. 6, 460–467.
Kawamoto, T., Nittono, H., Ura, M., 2013. Cognitive, affective, and motivational changes during ostracism: an ERP, EMG, and EEG study using a computerized Cyberball task. Neurosci. J. 304674.
Kawamoto, T., Ura, M., Nittono, H., 2015. Intrapersonal and interpersonal processes of social exclusion. Front. Neurosci. 9, 62.
Keedwell, P., Drapier, D., Surguladze, S., Giampietro, V., Brammer, M., Phillips, M., 2009. Neural markers of symptomatic improvement during antidepressant therapy in severe depression: subgenual cingulate and visual cortical responses to sad, but not happy, facial stimuli are correlated with changes in symptom score. J. Psychopharmacol. (Oxf., Engl.) 23, 775–788.
Kelley, W.M., Macrae, C.N., Wyland, C.L., Caglar, S., Inati, S., Heatherton, T.F., 2002. Finding the self? An event-related fMRI study. J. Cogn. Neurosci. 14, 785–794.
Key, A.P., Dove, G.O., Maguire, M.J., 2005. Linking brainwaves to the brain: an ERP primer. Dev. Neuropsychol. 27, 183–215.
Kross, E., Berman, M.G., Mischel, W., Smith, E.E., Wager, T.D., 2011. Social rejection shares somatosensory representations with physical pain. Proc. Natl. Acad. Sci. U. S. A. 108, 6270–6275.
Lorenz, J., Minoshima, S., Casey, K.L., 2003. Keeping pain out of mind: the role of the dorsolateral prefrontal cortex in pain modulation. Brain: J. Neurol. 126, 1079–1091.
Luck, S.J., Hillyard, S.A., 1994. Electrophysiological correlates of feature analysis during visual search. Psychophysiology 31, 291–308.
Luo, S., Yu, D., Han, S., 2016. Genetic and neural correlates of romantic relationship satisfaction. Soc. Cogn. Affect. Neurosci. 11, 337–348.
Macrae, C.N., Moran, J.M., Heatherton, T.F., Banfield, J.F., Kelley, W.M., 2004. Medial prefrontal activity predicts memory for self. Cereb. Cortex 14, 647–654.
Masten, C.L., Colich, N.L., Rudie, J.D., Bookheimer, S.Y., Eisenberger, N.I., Dapretto, M., 2011a. An fMRI investigation of responses to peer rejection in adolescents with autism spectrum disorders. Dev. Cogn. Neurosci. 1, 260–270.
Masten, C.L., Eisenberger, N.I., Borofsky, L.A., McNealy, K., Pfeifer, J.H., Dapretto, M., 2011b. Subgenual anterior cingulate responses to peer rejection: a marker of

adolescents' risk for depression. Dev. Psychopathol. 23, 283–292.
Masten, C.L., Eisenberger, N.I., Borofsky, L.A., Pfeifer, J.H., McNealy, K., et al., 2009. Neural correlates of social exclusion during adolescence: understanding the distress of peer rejection. Soc. Cogn. Affect. Neurosci. 4, 143–157.
Masten, C.L., Telzer, E.H., Eisenberger, N.I., 2011c. An FMRI investigation of attributing negative social treatment to racial discrimination. J. Cogn. Neurosci. 23, 1042–1051.
Maurage, P., Joassin, F., Philippot, P., Heeren, A., Vermeulen, N., et al., 2012. Disrupted regulation of social exclusion in alcohol-dependence: an fMRI study. Neuropsychopharmacology 37, 2067–2075.
McPartland, J.C., Crowley, M.J., Perszyk, D.R., Naples, A., Mukerji, C.E., et al., 2011. Temporal dynamics reveal atypical brain response to social exclusion in autism. Dev. Cogn. Neurosci. 1, 271–279.
McRae, K., Reiman, E.M., Fort, C.L., Chen, K., Lane, R.D., 2008. Association between trait emotional awareness and dorsal anterior cingulate activity during emotion is arousal-dependent. Neuroimage 41, 648–655.
Moor, B.G., Guroglu, B., Op de Macks, Z.A., Rombouts, S.A., Van der Molen, M.W., Crone, E.A., 2012. Social exclusion and punishment of excluders: neural correlates and developmental trajectories. Neuroimage 59, 708–717.
Nishiyama, Y., Okamoto, Y., Kunisato, Y., Okada, G., Yoshimura, S., et al., 2015. fMRI study of social anxiety during social ostracism with and without emotional support. PLOS ONE 10.
Ochsner, K.N., Ray, R.D., Cooper, J.C., Robertson, E.R., Chopra, S., et al., 2004. For better or for worse: neural systems supporting the cognitive down- and up-regulation of negative emotion. Neuroimage 23, 483–499.
Onoda, K., Okamoto, Y., Nakashima, K., Nittono, H., Yoshimura, S., et al., 2010. Does low self-esteem enhance social pain? The relationship between trait self-esteem and anterior cingulate cortex activation induced by ostracism. Soc. Cogn. Affect. Neurosci. 5, 385–391.
Phan, K.L., Fitzgerald, D.A., Nathan, P.J., Moore, G.J., Uhde, T.W., Tancer, M.E., 2005. Neural substrates for voluntary suppression of negative affect: a functional magnetic resonance imaging study. Biol. Psychiatry 57, 210–219.
Polich, J., 2007. Updating P300: an integrative theory of P3a and P3b. Clin. Neurophysiol. 118, 2128–2148.
Preller, K.H., Pokorny, T., Hock, A., Kraehenmann, R., Stampfli, P., et al., 2016. Effects of serotonin 2A/1A receptor stimulation on social exclusion processing. Proc. Natl. Acad. Sci. U. S. A.
Puetz, V.B., Kohn, N., Dahmen, B., Zvyagintsev, M., Schuppen, A., et al., 2014. Neural response to social rejection in children with early separation experiences. J. Am. Acad. Child Adolesc. Psychiatry 53, 1328e8–1337e8.
Rudolph, K.D., Miernicki, M.E., Troop-Gordon, W., Davis, M.M., Telzer, E.H., 2016. Adding insult to injury: neural sensitivity to social exclusion is associated with internalizing symptoms in chronically peer-victimized girls. Soc. Cogn. Affect. Neurosci. 11, 829–842.
Sebastian, C.L., Tan, G.C., Roiser, J.P., Viding, E., Dumontheil, I., Blakemore, S.J., 2011. Developmental influences on the neural bases of responses to social rejection: implications of social neuroscience for education. Neuroimage 57, 686–694.
Somerville, L.H., Heatherton, T.F., Kelley, W.M., 2006. Anterior cingulate cortex responds differentially to expectancy violation and social rejection. Nat. Neurosci. 9, 1007–1008.
Sreekrishnan, A., Herrera, T.A., Wu, J., Borelli, J.L., White, L.O., et al., 2014. Kin rejection: social signals, neural response and perceived distress during social exclusion. Dev. Sci. 17, 1029–1041.
Themanson, J.R., Khatcherian, S.M., Ball, A.B., Rosen, P.J., 2013. An event-related examination of neural activity during social interactions. Soc. Cogn. Affect. Neurosci. 8, 727–733.
van Harmelen, A.L., Hauber, K., Gunther Moor, B., Spinhoven, P., Boon, A.E., et al., 2014. Childhood emotional maltreatment severity is associated with dorsal medial prefrontal cortex responsivity to social exclusion in young adults. PLOS ONE 9.
van Noordt, S.J., White, L.O., Wu, J., Mayes, L.C., Crowley, M.J., 2015. Social exclusion modulates event-related frontal theta and tracks ostracism distress in children. Neuroimage 118, 248–255.
van Veen, V., Krug, M.K., Schooler, J.W., Carter, C.S., 2009. Neural activity predicts attitude change in cognitive dissonance. Nat. Neurosci. 12, 1469–1474.
Volpe, U., Mucci, A., Bucci, P., Merlotti, E., Galderisi, S., Maj, M., 2007. The cortical generators of P3a and P3b: a LORETA study. Brain Res. Bull. 73, 220–230.
Weissman, D.H., Giesbrecht, B., Song, A.W., Mangun, G.R., Woldorff, M.G., 2003. Conflict monitoring in the human anterior cingulate cortex during selective attention to global and local object features. Neuroimage 19, 1361–1368.
Weschke, S., Niedeggen, M., 2013. The effect of the physical presence of co-players on perceived ostracism and event-related brain potentials in the Cyberball paradigm. PLOS ONE 8, e71928.
Weschke, S., Niedeggen, M., 2015. ERP effects and perceived exclusion in the Cyberball paradigm: correlates of expectancy violation? Brain Res. 1624, 265–274.
Will, G.J., Crone, E.A., Guroglu, B., 2015. Acting on social exclusion: neural correlates of punishment and forgiveness of excluders. Soc. Cogn. Affect. Neurosci. 10, 209–218.
Will, G.J., van Lier, P.A., Crone, E.A., Guroglu, B., 2016. Chronic childhood peer rejection is associated with heightened neural responses to social exclusion during adolescence. J. Abnorm. Child Psychol. 44, 43–55.
Williams, K.D., Jarvis, B., 2006. Cyberball: a program for use in research on interpersonal ostracism and acceptance. Behav. Res. Methods 38, 174–180.
Wronka, E., Kaiser, J., Coenen, A.M., 2012. Neural generators of the auditory evoked potential components P3a and P3b. Acta Neurobiol. Exp. (Warsz.) 72, 51–64.
Wudarczyk, O.A., Kohn, N., Bergs, R., Gur, R.E., Turetsky, B., et al., 2015. Chemosensory anxiety cues moderate the experience of social exclusion – an fMRI investigation with Cyberball. Front. Psychol. 6, 1475.
Zadro, L., Williams, K.D., Richardson, R., 2004. How low can you go? Ostracism by a computer is sufficient to lower self-reported levels of belonging, control, self-esteem, and meaningful existence. J. Exp. Soc. Psychol. 40, 560–567.

8. Study III. Effects of rifaximin on central responses to social stress – a pilot experiment (under review)

Author contribution

PE is responsible for the integrity of the work - the inception of the study and publication of the work. HW contributed to the design of the study, data collection and analysis, drafting of the manuscript, and critical revisions of the manuscript. CB and PE contributed to the design of the study, data analysis, critical revisions of the manuscript. All authors approved the final version of the manuscript.

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Effects of rifaximin on central responses to social stress – a pilot experiment

Original research

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Abstract

Background & Aims: Probiotics that promote the gut microbiota have been reported to reduce stress responses, and improve memory and mood. Whether and how antibiotics that eliminate or inhibit pathogenic and commensal gut bacteria, also affect central nervous system functions in humans is so far unknown.

Methods: In a double-blinded randomised study, 16 healthy volunteers (27.00±1.60 years; nine males) received either rifaximin (600mg/day) (a poorly absorbable antibiotic) or placebo for 7 days. Before and after the drug intervention, brain activities during rest and during a social stressor inducing feelings of exclusion (Cyberball game) were measured using magnetoencephalography (MEG).

Results: Social exclusion significantly affected (p<0.001) mood and increased exclusion perception. MEG showed brain regions with higher activations during exclusion as compared to inclusion, in different frequency bands. Seven days of rifaximin increased prefrontal and right cingulate alpha power during resting state. Low beta power showed an interaction of intervention (rifaximin, placebo) x condition (inclusion, exclusion) during the Cyberball game in bilateral prefrontal and left anterior cingulate cortex. Only in the rifaximin group, a decrease (p=0.004) in power was seen comparing exclusion to inclusion; the reduced beta-1 power was negatively correlated with a change in the subjective exclusion perception score.

Conclusion: Social stress affecting brain functioning in a specific manner, is modulated by rifaximin. Contrary to our hypothesis that antibiotics have advert effects on mood, the antibiotic exhibited stress-reducing effects similar to reported effects of probiotics (Supported by NeuroGUT, a EU 7th Framework Programme ITN no. 607652; ClinicalTrials.gov identifier number NCT02793193).

Keywords: Gut-brain axis, antibiotic, stress, Cyberball, MEG

1 Introduction

The gut-brain axis is essential for communication between the enteric nervous system (ENS) and central nervous system (CNS) functions. The commensal gut microbiota therefore, may play an important role on this axis through neural, immune and endocrine pathways. Previous studies have found altered gut microbiota (GM) composition not only to affect ENS functions (1), but can also change CNS functions in animals and humans (2-4). Among the most frequently investigated CNS functions are social functions, learning and memory functions, and stress responsiveness in animals, yet few data are available in humans (5-8).

While many tools are available to manipulate the microbiota in animals (germ-free or specifically colonized animals, antibiotic elimination of the microbiota, fecal microbiota tranfer between animals and from humans to animals, etc.) (9-12), few allow similar approaches in humans. As we have recently summarized (13), probiotics (and to a lesser degree prebiotics) have been used to affect CNS functions in animals and humans, with a variety of different bacterial strains but predominantly *Lactobacillus* and *Bifidobacterium*.

While some of these were available as nutrient supplements even before their therapeutic benefit became evident, others have been developed specifically for the purpose to act as therapeutic agents in intestinal (e.g. irritable bowel syndrome, IBS; inflammatory bowel diseases, IBD) and extra-intestinal disorders (atopy, diabetes). More recently, disturbed CNS functions e.g. of neurological (autism spectrum disorder, ASD) or psychiatric nature (mood disorders) have come into focus (14-16), and first studies have evaluated the effects of probiotics on related CNS functions in healthy volunteers, while studies in respective patient populations are still scarce (17).

The mechanisms by which probiotics affect central functions are incompletely understood, but include - among others - direct effects on the commensal gut microbiota (via increased diversity and/or competitive colonization), enhancement of their metabolic functions, or stimulation of the enteric neural or immune system, all of which could directly or indirectly stimulate the gut-brain axis and elicit such central effects.

Despite its wide use in every-day medicine, antibiotics have rarely been investigated for their effects on central functions, presumably for two reasons: their use in healthy subjects is limited by their ability to induce antibiotic resistance that may be detrimental in case antibiotics are needed for treatment of acute bacterial infections; and patients that are in need for antibiotics are suffering from acute infections, and any central effect seen may as well be a consequence of the acute disease rather than antibiotics-induced CNS consequences of manipulating the gut microbiota. While antibiotics-induced peripheral consequences, e.g. diarrhea and irritable bowel syndrome-type symptoms are well established, long-term CNS consequences of antibiotic use have not been investigated so far.

Rifaximin is a locally (intestinally) acting broad-band antibiotic with poor bioavailability (<1% systemic absorption), thus with minimal risk for provoking antibiotic resistance (18). This specific property allows its use both in healthy volunteers as well as in patients without severe infection, e.g. in traveler's diarrhea (19). A few trials have shown clinical efficacy in IBS (20, 21) and in small intestinal bacterial overgrowth (22, 23), but here the mechanism of action is less unclear.

Most animal work of "psychobiotics" relate to their effects on standardized stress paradigms, specifically social stressors such as open-field (14) maternal separation (24), and defensive burying test (14, 25). In search for a stress paradigm that would allow the testing of social stress with neuroimaging methods in humans, we have identified the "Cyberball game"

(CBG), which is an virtual game that is often used to study social stress by exclusion/rejection (26). Different from other human stress tasks, such as the Trier Social Stress test (27), the CBG can be employed easily in a brain scanner, thus allowing direct evaluation of associated neurobiological processes, to compensate for the lack of direct physiological stress-markers in human research, as compared to animal studies.

During this CBG, participants play a computer-simulated ball tossing game with two players whose behaviors are programmed. Participants feel distressed during the period when the other players barely throw a ball to him/her - a so-called exclusion or rejection (ostracism) condition, compared to the condition when all three players have the same chance to receive and throw the ball. In addition to the subjective reports of distress during the exclusion condition, studies also showed physiological changes such as raised cortisol level, higher skin conductance, and increased facial temperature (28-33).

Various experimental set-ups of the CBG tests have been used in the past. A systematics review of neuroimaging studies summarized these setups and showed its reliability to induce social stress in healthy volunteers (34). Accordingly, regions of the insula, anterior cingulate cortex (ACC), temporal and prefrontal cortex (PFC) were activated to social exclusion (35-37). Neural oscillations are thought to play a key role in processing neural information, and different types of oscillatory activities are being studied for their functions. Exclusion-induced changes in neural oscillations such as alpha and theta frequency bands have also been reported in these areas (38-40).

In order to investigate the involvement of brain areas and their modulation by rifaximin, magnetoencephalography (MEG), as a functional neuroimaging technique with fine spatial resolution and high temporal resolution was used to study the effects of CBG (41). In order to test the efficacy of MEG identifying neural response to the social stressor,

and the potential antibiotic effect of rifaximin on the stress effect, we conducted an exploratory experiment in healthy volunteers with a short-term (7 days) intake of rifaximin. Because of a putative antibiotic effect of rifaximin on the commensal microbiota, we hypothesized that rifaximin would, in contrast to known probiotic effects on CNS functions (13), increase the stress response following social exclusion.

2 Materials and Method

2.1 Participants

Sixteen volunteers participated in the study. All participants met our inclusion criteria: 1) non-smoker for at least 3 months, 2) with a body mass index of 18-30, 3) without any chronic allergies, 4) willing to discontinue the consumption of probiotic and prebiotic-containing foods or potentially immune-enhancing dietary supplements, 5) receiving no immune-suppressing intervention and not having any immunosuppressive illness within the last year, 6) receiving no antibiotic therapy within the last 2 months, 7) having no psychiatric or gastrointestinal disorder, 8) having no non-removable metal parts in the body. Informed consent was obtained from all participants prior to joining the study. The protocol has been approved by the Ethics Board of the University of Tübingen Medical School (No. 503/2015BO1, approved on 26.08.2015), and registered at ClinicalTrials.gov (identifier number NCT02793193).

2.2 Study design

Our pilot study was a randomized, double-blinded, and parallel-group design, in which the participants visited our laboratory twice for MEG measurements: at baseline and one day after the end of drug intake. The intervention and the control group took the

antibiotic rifaximin (3 x 200mg/d) or placebo pills, respectively for 7 days; drugs and placebos were provided by the university hospital pharmacy. The randomization scheme was unblinded after completion of the experiment and the data evaluation.

During the intervention period, participants were instructed to avoid the consumption of food containing probiotics/prebiotics, or potentially immune-enhancing dietary supplements. This was supported by providing them with a list of "prohibited" foods (**Appendix 1**).

2.3 Questionnaires

To survey participants' health status, the 36-item short-form health survey (SF-36) was used (42). The SF-36 includes eight concepts: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions. Participants were asked to finish the SF-36 during each of the two visits.

After each of the inclusion and exclusion blocks (see below), participants needed to complete two questionnaires to assess their acute level of distress. We employed the self-report measures of the Need Threat Scale (NTS), Mood questionnaire (MQ) and the subjective 'exclusion perception' (SEP) on a scale rating between 1-5 (**Appendix 2**); all these scales are validated standard for the CBG (26, 43). The NTS was designed to measure the feelings and emotional consequences of social rejection and higher scores related to higher distress level. Its four items comprised self-esteem, belonging, meaningful existence and control, and combined ratings have been used as a measure of social distress in previous studies. The MQ was used to assess mood, using questions (are you feeling bad/good, happy/sad, pleasant/angry, and friendly/unfriendly). The SEP was to record participants'

feeling of being included/ostracized by asking them to rate two statements ('I was ignored' and 'I was excluded').

2.4 Cyberball game

In the CBG, the participants were asked to play a ball tossing game with two other virtual players programmed by the experimenter. They were made believe that the two players were real and were playing the game. To minimize gender effects, male participants played with 2 female players, and female participants played with 2 male players. During the game, the other players were depicted as cartoon characters with their photos aside and names below. The volunteering participant was represented by a cartoon in the middle lower part of the screen and could throw the ball to either of the other two players on the left or right, by pressing the left or right button on the response box (**Figure 1**).

The CBG consisted of 4 blocks: inclusion - exclusion - inclusion – exclusion conditions; this order was maintained for all volunteers. In each inclusion blocks, there were 108 trials, during 1/3 (36 trials) of which the participant received the ball from the other players (for another 1/3 the ball was played to one of the other players, and for the other 1/3 between the other two players). The order of the ball throwing to the participant was pseudo-randomized. The 1/3 of trials in the inclusion block when the virtual players threw the ball to each other and not to the participant, were called "not my turn" events.

To equalize the numbers of analyzed trials when the virtual players threw the ball to each other and not to the participant, we set 47 total trials in each exclusion block. The participants received the ball 3 times (trials 14, 25 or 26, and 39 or 40 or 41 in block 2 and 4, respectively) to maintain their attention. The first 5 exclusion trials, the 3 trials of the participant receiving the ball, and the 3 trials of participant throwing the ball were not

analyzed but discarded. The remaining 36 so-called "rejection" events were used for comparison with the 36 "not my turn" events in the inclusion block. Visual stimuli of these trials did not differ in two conditions, so any difference of brain activities was supposed to be due to the participants' inner state. The trial began with the ball being presented in the cartoon for 500-2000 ms randomly to imitate a real life situation. Then the ball was moving for 2000 ms before reaching the target player (**Figure 1**). After each of the inclusion and exclusion blocks, participants completed the Need Threat Scale, the Mood Questionnaire, and the exclusion perception question.

Figure 1. Example of a trial in the Cyberball game.

2.5 Magnetoencephalography recording

Brain magnetic fields were measured with a 275-channel whole-head magnetoencephalograph. Participants were studied in supine position. During each recording session, 5 minutes resting state was recorded prior to recording while playing the CBG. During the resting state, participants were instructed to move as little as possible and to be awake, while keeping their eyes closed. During the CBG, task instructions were projected onto a screen in front of the participants via a video projector and a mirror system. Participants were asked to fixate the screen and hold a response box to get ready for the task. Participants were also instructed to move as little as possible. MEG signals were sampled at a rate of 585.94 Hz with an anti-aliasing filter set to 292.97 Hz.

In order to overlay the brain activity derived from MEG on anatomical scans, high-resolution (1 mm, isotropic) T1-weighted structural MR images were acquired using an

MPRAGE sequence with a 3-T MR scanner (University Hospital Tübingen, Germany) for each participant, but at a separate occasion.

2.6 Data analysis

2.6.1 Data analysis: questionnaires

To test the intervention-related changes in participants' health status scored by SF-36, changes from before to after the 7-days intervention were computed by subtracting the baseline assessment from the corresponding post-intervention values. To control the intervention-related changes on the NTS, the MQ and SEP during the CBG, changes after each intervention were computed for each condition.

Data were analyzed using SPSS 21 (IBM, Armonk, NY, USA). Changes in SF-36 were entered into a two-independent-sample Mann-Whitney U test of intervention (rifaximin vs. placebo), as this data was not normally distributed. The changes in NTS, MQ and SEP were entered in a 2 x 2 ANOVA of intervention as a between-subject factor (rifaximin vs. placebo) x condition as a within-subject factor (exclusion vs. inclusion). Where significant main effects or interaction were observed, pairwise comparisons were used to assess each time point with a Bonferroni adjusted threshold ($\alpha = 0.025$).

2.6.2 Data analysis: MEG data

Preprocessing

Analysis of the MEG data was carried out using Matlab (Mathworks, Natick, USA) and the open-source toolboxes Fieldtrip (44). The resting state dataset were cut into time windows of 2s. Data in this time window were filtered using a 4Hz high pass frequency filter. Non-physiological jumps in the MEG signal and trials with jump and muscle artifacts were

excluded by an automatic rejection algorithm. Any trial in each channel whose variance exceeded 10^{-25} T^2 were excluded from further analysis. The continuously recorded dataset of the CBG was segmented with respect to stimulus onset (when one player threw the ball) and baseline adjusted using a [-1000: 2000] ms trial interval. Trials in which the other players threw the ball towards each other during the inclusion blocks were defined as 'inclusion' condition, and those during exclusion blocks were defined as 'exclusion' conditions.

Time-frequency analysis

The time-frequency analysis used the multitaper windowed fast fourier transform 'MTMFFT' implemented in Fieldtrip. The 'multitaper method' (MTM) is based on Slepian sequences as tapers. The frequency of interest ranged from 4 to 30Hz with step of 2 Hz and the smoothing window is +/-3 Hz:

Source analysis

Using the time-frequency determined by the analysis described above, oscillatory sources of theta, alpha, beta-1, beta-2 and beta-3 bands (6, 11, 16, 21, and 26 Hz) were localized using beamformer techniques. We applied the Dynamical Imaging of Coherent Sources (DICS) method (45). In order to estimate the individual source activity, each participant's brain recorded as T1-MR image was divided in a regular three dimensional grid with a 1 cm resolution and a spatial inverse filter was computed from both conditions and both visits, as common filter. Then we applied this common filter to each condition and each visit separately in order to obtain the respective source power. The MEG data in each condition was coregistrated with the individual structural MR images respectively.

Source statistics

For testing effects of stress induced by the CBG, the source power in each frequency band from all the participants at the baseline visit were entered in a paired-samples T-test comparing exclusion with inclusion condition. For analyzing the effect of rifaximin, we performed source-level statistics for the data obtained from the resting-state condition and the CBG, respectively. For resting-state, changes in the source power in each frequency band was computed by subtracting the baseline from the post-intervention. The changes of the source power were entered into an independent T-test with intervention (rifaximin vs. placebo) as between factor. For the CBG, changes in the source power were computed by subtracting the baseline from the post-intervention in each condition in each frequency band. Changes of source power were entered in a two-way ANOVA of interventions (rifaximin vs. placebo) x conditions (exclusion vs. inclusion). The statistical analysis was done separately for each frequency band. To localize significant activations, the cluster-based permutation method for multiple comparisons (corrected) was used with a significance level of alpha of 0.05.

2.6.3 Correlation between behavioral and MEG data

To correlate the neural activity and subjective stress reports, for each condition source power in the clusters that differed significantly between conditions during the first visit was averaged. The averaged source power was correlated with the scores of NTS, MQ and SEP for each condition separately, using Pearson correlations. These correlations were considered significant at a corrected threshold of $p < .05$.

To correlate the change in neural activity with change in the subjective reports by rifaximin, correlations were done for the resting state task and the CBG, respectively. For the resting state task, averaged source power was calculated for clusters that differed significantly between both visits. The averaged source power was correlated with changes in

health status, for each group separately. For the CBG, for each condition and each intervention, source power in the clusters that differed significantly between both visits was averaged. The averaged source power was correlated with changes in the scores of NTS, MQ and SEP in each condition and each group, using Pearson correlations. These correlations were considered significant at a corrected threshold of $p < .05$.

3. Results

Sixteen healthy participants met the inclusion criteria of the study and completed the experiment (9 males; age: 27.00 ± 1.60 years age; BMI: 22.21 ± 0.48). Eight participants completed the intervention with rifaximin (6 males, age: 26.50 ± 1.05; BMI: 22.48 ± 0.58) and eight with placebo (3 males, mean age: 27.50 ± 3.12; BMI: 21.94 ± 0.81).

3.1 Stress effect by the Cyberball game

There was a significant difference between inclusion and exclusion in the global score of the NTS ($t_{15} = 5.06$, $p < 0.001$). In the exclusion condition, participants reported higher scores of the global NTS score. The score of the MQ is significantly lower in the exclusion condition compared to inclusion condition ($t_{15} = -5.40$, $p < 0.001$). Also, in the SEP participants revealed a significantly higher score of exclusion perception ($t_{15} = 13.64$, $p < 0.001$).

Source statistics of MEG power in each frequency band showed brain regions that had significantly higher activations during the exclusion compared to the inclusion condition (**Figure 2**): in the theta frequency band (6 Hz), the left fusiform gyrus, the right inferior and superior parietal lobule, the right thalamus and the left middle occipital gyrus ($p = 0.008$); in the alpha frequency band (11 Hz), the bilateral posterior cingulate gyrus, the bilateral inferior and superior parietal lobule, the left hippocampus and parahippocampal gyrus and the left

fusiform gyrus (p = 0.002); in the beta 1 frequency band (16 Hz), the bilateral inferior and middle temporal lobule, the bilateral fusiform gyrus, the bilateral hippocampus, the left inferior and middle occipital cortex (p = 0.01); in the beta 2 frequency band (21 Hz), the right cingulate gyrus, the bilateral fusiform gyrus, the bilateral hippocampus, the left occipital cortex, the right parahippocampal gyrus (p = 0.008); in the beta 3 frequency band (26 Hz), the left posterior cingulate gyrus, the bilateral fusiform gyrus, the bilateral hippocampus and parahippocampal gyrus, the bilateral inferior, middle, superior temporal lobule, and the bilateral thalamus (p = 0.01).

Figure 2

To investigate whether the neural activity during the CBG was correlated to the subjective report of the social stress, we correlated the averaged power changes in each cluster for each frequency band and scores of NTS, MQ and SEP. No significant correlation was found.

3.2 Intervention effect by rifaximin

3.2.1 Physical and psychological health status

Comparing the scores of the questionnaires between the rifaximin and the placebo group at baseline, no group differences were found for any score of the SF36-item survey. After intervention, only the difference in “Emotional well-being”, a sub-item of the SF36-item survey, was significant between groups (U = 11, p = 0.02). There was a significantly higher increase of “Emotional well-being” in the rifaximin group (Median = 11.13) than the placebo group (Median = 5.88).

3.2.2 Effects of Rifaximin on resting-state MEG

We tested the effects of the intervention on the source power in each frequency band during resting state. An independent T-test showed a significant cluster of increased power in the alpha band (11 Hz) in the right superior and inferior frontal cortex, the bilateral middle cingulate gyrus extending to the left insula in rifaximin group as compared to the placebo group (**Figure 3**, $p = 0.05$).

Figure 3

3.2.3 Resting-state and health status

To investigate whether the increase of neural activity during resting-state was correlated to the participants' health state, we correlated the averaged power changes in the significantly activated cluster with the changes in SF-36 scores. However, no correlation was found, neither across both groups nor within each group.

3.2.4 Rifaximin effect on neural response to the Cyberball game

To test effects of interventions and conditions on the neural response to the CBG, a two-way ANOVA test was carried out. No significant main effect of Intervention or Condition was found. Only in beta-1 band (16H), a significant interaction of Interventions and Conditions was found in the left inferior and superior and the bilateral middle frontal cortex, and the left anterior cingulate gyrus (**Figure 4**; $p < 0.05$). As a post hoc analysis, we compared brain activity changes between two conditions (exclusion vs. inclusions) for each intervention group separately. Only in the rifaximin group, a decrease in beta-1 power, in the bilteral inferior, supperior and middle frontal cortex, the bilateral anterior, middle and posterior cingulate gyrus, the bilateral parietal and postcentral cortex, could be demonstrated for exclusion as compared to inclusion (**Figure 5**; $p = 0.002$). A summary of frequency bands

and neuroanatomical areas found to be related with social stress, rifaximin intervention and their interaction is provided (**Table 1**).

Figure 4, Figure 5

Table 1

3.2.5 Neural activity and subjective report of the social exclusion

A correlation between neural activity change and subjective report changes of social exclusion was tested only in case of a significant difference of neural activity changes between intervention groups. Therefore, we only tested correlations between neural activity changes in the cluster with changes of the subjective report of NTS, MQ and SEP for each condition during the CBG, respectively. Only in the rifaximin group, a significant correlation was obtained between SEP score changes and beta-1 power changes (**Figure 6;** $r = 0.86$, $p = 0.006$)

Figure 6

4 Discussion

Using MEG, we observed an effect of stress, induced by the CBG, on the neural oscillations in different frequency bands and at distributed brain areas. Daily intervention of 600 mg rifaximin for one week significantly increased the frontal and cingulate alpha power during resting state, and decreased prefrontal and cingulate low beta power during social exclusion compared to inclusion. These neural changes were only observed in the group that

consumed rifaximin, indicating that one-week intervention of rifaximin did have effects on CNS functions in healthy volunteers.

4.1 Stress effect

Numerous studies have used fMRI and electroencephalogram (EEG) to measure neural response to the CBG, while our study is the first one using MEG that has found that social exclusion produced larger neural oscillatory activities in various frequency bands in certain brain areas. The fusiform facial area was more activated in all the frequency bands from theta, alpha to beta waves. This finding is consistent with previous studies, suggesting an enhanced processing of the other players' faces and learning of the social value of their faces according to the unpleasant/negative social exclusive event. Activations of the parietal area were found in low frequency band – theta and alpha waves, and temporal activations in beta bands. In a recent scoping review of neuroimaging data of CBG, the summarized key neural processing of social exclusion pointed out that parietal activity was involved in an early stage process including perception and attention (34). Hereby, we may suggest that theta and alpha oscillations play a crucial role in perception and attention of the exclusion event. Temporal activations in beta bands may reflect an emotional arousal and event appraisal processes (35, 46). Interestingly, in addition to the cingulate gyrus which has been reported by many previous studies, we have also obtained significant activations of hippocampus and parahippocampal gyrus in alpha and beta bands, indicating stronger memory consolidation and retrial during the social exclusion.

Our results reveal different neural oscillations as compared to previous studies that mainly showed increased power in theta band in the frontal cortex, insula, subgenual ACC during exclusion as compared to the inclusion condition (38-40, 47). Absence of frontal areas

and insula activation in response to the social exclusion may be due to the limited sample size of the current trial, and needs further investigation.

4.2 Rifaximin effect

Rifaximin has been approved for the use in humans to treat acute and chronic gastrointestinal infections and disorders. However, few studies have been done to investigate its role in the gut-brain axis, while probiotics have been more commonly addressed for its positive effects on both, gut and brain functions (13, 48-50). Therefore, it is worthwhile to conduct clinical research to test the influence of rifaximin on the brain according to its safety and efficiency on preventing traveler diarrhea and treating gastrointestinal disorders (51). So far, there are only few studies that have investigated the central effect of rifaximin. Several cognition functions, including working memory in patients with liver cirrhosis, have been reported to improve with rifaximin (52, 53). In the study by Ahluwalia et al., eight-week intake of rifaximin enhanced working memory and inhibitory control with altered brain activities in patients with liver cirrhosis (52). Despite the lack of a placebo control group in this study, the patients showed raised activations of subcortical regions (e.g. thalamus, caudate insula and hippocampus) and operculum, and enhanced functional connectivity between these regions during the working memory task; and decrease in frontal-parietal activations during the inhibitory control task. The author suggested the central effects of rifaximin to be mediated by the gut-liver-brain axis by modulating gut bacteria, serum bilirubin and systemic endotoxemia.

While previous studies have found central effects of rifaximin only in patients, any improvement in well-being might be mediated by the alleviation of symptoms of the disease from which patients were suffering. Our study is the first one exploring direct effects of rifaximin on the CNS in healthy individuals. Our preliminary results showed that rifaximin

increased volunteers' emotional well-being and alpha oscillation in the frontal and cingulate cortex during the resting state. When experiencing social stress during CBG, the rifaximin group had a decrease of low beta power in the prefrontal cortex and the anterior cingulate cortex after intervention period.

From our results, one could conclude that rifaximin is beneficial in improving mental health by relieving stress, both, in resting conditions and in stressful situations. Finding that volunteers taking rifaximin became less nervous, calmer and happier after the intervention (as is reflected by the respective SF36 items), an improvement of emotional well-being was achieved. Larger frontal and cingulate alpha power that was observed for the rifaximin group during resting-EEG has often been observed when participants were more relaxed, e.g. as effect of music therapy leading to reduced anxiety levels (54, 55).

During socially stressful situations as induced by the exclusion condition in the CBG, a decreased frontal and cingulate low beta power was found for rifaximin group. Also, beta-1 band power reduction was negatively correlated to the change of SEP after rifaximin intervention, showing that as the participants perceived more exclusion, there was higher reduction of the beta-1 power in the PFC and ACC. Frontal beta power has been reported to occur in mental fatigue and appears negatively related to mental stress level (56). In a study investigating central effects of the phosphatidylserine supplement, which can decrease perceived stress and improve mood, a reduced frontal beta-1 power by phosphatidylserine after an induction of stress has been reported (57). In that study, the author suggested the decreased beta-1 power at the frontal region was associated with a more relaxed state. Similarly, a moderate massage also decreased the beta activity in the brain as well as participants' stress level (58, 59). As activations in the PFC and ACC have been reported involved in emotion regulation during social exclusion (34, 46), the reduced beta-1 power

band in these areas may be related to higher demands for emotion regulation of the perceived exclusion as well as to reduce mental stress.

Additional evidences can be provided by two fMRI studies investigating alteration of neural processes of emotional stimuli by probiotics intervention. One study using fermented milk product with probiotic, reported a brain activity shift from arousal-based resting-state network to a regulatory network, and reduced neural activities in affective and viscerosensory cortices to emotional stimuli after 4 weeks' intervention (60). A recent study in IBS patients showed *Bifidobaterium longum* NCC3001 also reduced neural responses in amygdala and fronto-limbic regions to fearful stimuli (17). Changed activations in amygdala and fronto-limbic have indicated modulated hypothalamic-pituitary axis activity and emotion regulation due to the stimuli (61, 62). According to these convergent evidences, the reduced power in the frontal and cingulate regions in beta power found in our study may indicate less mental fatigue and a higher level of ongoing mental regulation during the stressful event. In summary, rifaximin may have effects of improving relaxation while reducing anxiety levels and stress responses by modulating central processing of emotion.

Rifaximin is known because of its benefits in modulating the gastrointestinal functions and treating IBS. Moreover, it has been shown that rifaximin is effective to prevent travelers' diarrhea (51). Furthermore, rifaximin has been found in some studies to promote beneficial bacteria in the gut such as *Bifidobacteria* and *Lactobacilli* (63-65). Rifaximin induced changes of CNS functions might be mediated by rifaximin-induced altered gut GM composition or diversity that lead to changes of metabolites such as short-chain fatty acids and tryptophan, which in turn can influence the CNS (14, 66). Similar to probiotics, rifaximin influences immune system mucosal inflammation by reducing level of certain interleukins and tumor necrosis factor α (65). Thereby, the improved immune function could affect the

endocrine and nervous system (67). As yet, probiotics and not antibiotics, have been regarded as having positive effects on gastrointestinal and increasingly also on central functions by modulating the gut microbiota through the gut-brain axis. However, our findings indicate that, also certain antibiotics such as rifaximin may act on the CNS by modulating the GM in a similar way as probiotics.

The present study had some limitations that need discussion. First, as a pilot study that explored whether modulation of GM might have any effect on CNS functions we did not collect stool samples for microbiome analysis, which could give more insight of the mechanism of action of rifaximin. Second, the stress response induced by the CBG was testified by subjective measures of distress, but neither physiological nor hormonal stress responses were measured and correlated with the neural responses. Third, no power calculation was performed because of the exploratory character of the trial, which was supposed to generate a hypothesis that was planned to be tested in a future study, but the serendipity of the finding motivated us to report it separately. For the same reason, no sex differences were expected on the pilot data. Additionally, the limited sample size may have concealed further behavioral and/or neural changes induced by the social stress and/or the rifaximin intervention. The present study provides evidence that it is worthwhile to compare effects of rifaximin and probiotics as well as their combination on central nervous system functions. Further research should in particular study the effect of rifaxmin comprehensively by correlating the microbiological, physiological, psychological, and neural responses due to stress and intervention effects.

5 Conclusion

Using MEG we were able to identify a neural signature of social stress and its modulation due to rifaximin. Oscillatory neuromagnetic activity in different frequency bands and brain areas reflected aspects of neural processes during social exclusion. One-week of rifaximin intervention influenced the prefrontal and cingulate alpha oscillation in the resting state and the prefrontal and cingulate low beta oscillation as a response to social stress. To our knowledge, our study is the first one exploring the central effect of an antibiotic in healthy volunteers. Further studies investigating this effect in a larger population are expected to confirm these findings and might highlight even more subtle effects on brain activities and social well-being. Including peripheral physiological parameters in the study and testing patients with functional gastrointestinal disorders appear to be promising to elucidate the pathways and mechanisms how GM affects brain functions.

6 Conflicts of interest

The authors declare no potential conflicts of interest.

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References

1. Hyland NP, Cryan JF. Microbe-host interactions: Influence of the gut microbiota on the enteric nervous system. Dev Biol. 2016;417(2):182-7.

2. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci. 2012;13(10):701-12.

3. Cryan JF, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil. 2011;23(3):187-92.

4. Cryan JF, Dinan TG. More than a gut feeling: the microbiota regulates neurodevelopment and behavior. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2015;40(1):241-2.

5. Proctor C, Thiennimitr P, Chattipakorn N, Chattipakorn SC. Diet, gut microbiota and cognition. Metab Brain Dis. 2017;32(1):1-17.

6. Gareau MG. Microbiota-gut-brain axis and cognitive function. Adv Exp Med Biol. 2014;817:357-71.

7. O'Mahony SM, Felice VD, Nally K, Savignac HM, Claesson MJ, Scully P, et al. Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. Neuroscience. 2014;277:885-901.

8. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. Molecular psychiatry. 2014;19(2):146-8.

9. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol. 2004;558(Pt 1):263-75.

10. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil. 2011;23(3):255-64, e119.

11. Wang T, Hu X, Liang S, Li W, Wu X, Wang L, et al. Lactobacillus fermentum NS9 restores the antibiotic induced physiological and psychological abnormalities in rats. Benef Microbes. 2015;6(5):707-17.

12. Ferrere G, Wrzosek L, Cailleux F, Turpin W, Puchois V, Spatz M, et al. Fecal microbiota manipulation prevents dysbiosis and alcohol-induced liver injury in mice. J Hepatol. 2017;66(4):806-15.

13. Wang H, Lee IS, Braun C, Enck P. Effect of probiotics on central nervous system functions in animals and humans - a systematic review. J Neurogastroenterol Motil. 2016.

14. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451-63.

15. Kantak PA, Bobrow DN, Nyby JG. Obsessive-compulsive-like behaviors in house mice are attenuated by a probiotic (Lactobacillus rhamnosus GG). Behav Pharmacol. 2014;25(1):71-9.

16. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A. 2011;108(38):16050-5.

17. Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, et al. Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome. Gastroenterology. 2017;153(2):448-59 e8.

18. DuPont HL. Biologic properties and clinical uses of rifaximin. Expert Opin Pharmacother. 2011;12(2):293-302.

19. DuPont HL, Jiang ZD, Okhuysen PC, Ericsson CD, de la Cabada FJ, Ke S, et al. A randomized, double-blind, placebo-controlled trial of rifaximin to prevent travelers' diarrhea. Ann Intern Med. 2005;142(10):805-12.

20. Pimentel M, Morales W, Chua K, Barlow G, Weitsman S, Kim G, et al. Effects of rifaximin treatment and retreatment in nonconstipated IBS subjects. Dig Dis Sci. 2011;56(7):2067-72.

21. Pimentel M, Park S, Mirocha J, Kane SV, Kong Y. The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome: a randomized trial. Ann Intern Med. 2006;145(8):557-63.

22. Gatta L, Scarpignato C. Systematic review with meta-analysis: rifaximin is effective and safe for the treatment of small intestine bacterial overgrowth. Aliment Pharmacol Ther. 2017;45(5):604-16.

23. Scarpellini E, Gabrielli M, Lauritano CE, Lupascu A, Merra G, Cammarota G, et al. High dosage rifaximin for the treatment of small intestinal bacterial overgrowth. Aliment Pharmacol Ther. 2007;25(7):781-6.

24. Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the Probiotic Bifidobacterium Infantis in the Maternal Separation Model of Depression. Neuroscience. 2010;170(4):1179-88.

25. Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterol Motil. 2014;26(11):1615-27.

26. Williams KD, Jarvis B. Cyberball: a program for use in research on interpersonal ostracism and acceptance. Behavior research methods. 2006;38(1):174-80.

27. Allen AP, Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. Neurosci Biobehav Rev. 2014;38:94-124.

28. McQuaid RJ, McInnis OA, Matheson K, Anisman H. Distress of ostracism: oxytocin receptor gene polymorphism confers sensitivity to social exclusion. Soc Cogn Affect Neurosci. 2015;10(8):1153-9.

29. Beekman JB, Stock ML, Marcus T. Need to Belong, Not Rejection Sensitivity, Moderates Cortisol Response, Self-Reported Stress, and Negative Affect Following Social Exclusion. J Soc Psychol. 2016;156(2):131-8.

30. Blackhart GC, Eckel LA, Tice DM. Salivary cortisol in response to acute social rejection and acceptance by peers. Biol Psychol. 2007;75(3):267-76.

31. Stroud LR, Salovey P, Epel ES. Sex differences in stress responses: social rejection versus achievement stress. Biol Psychiatry. 2002;52(4):318-27.

32. Kelly M, McDonald S, Rushby J. All alone with sweaty palms--physiological arousal and ostracism. International journal of psychophysiology : official journal of the International Organization of Psychophysiology. 2012;83(3):309-14.

33. Paolini D, Alparone FR, Cardone D, van Beest I, Merla A. "The face of ostracism": The impact of the social categorization on the thermal facial responses of the target and the observer. Acta psychologica. 2016;163:65-73.

34. Wang H, Braun C, Enck P. How the brain reacts to social stress (exclusion) - A scoping review. Neurosci Biobehav Rev. 2017;80:80-8.

35. Bolling DZ, Pelphrey KA, Vander Wyk BC. Unlike adults, children and adolescents show predominantly increased neural activation to social exclusion by members of the opposite gender. Soc Neurosci. 2016;11(5):475-86.

36. Eisenberger NI, Taylor SE, Gable SL, Hilmert CJ, Lieberman MD. Neural pathways link social support to attenuated neuroendocrine stress responses. Neuroimage. 2007;35(4):1601-12.

37. Maurage P, Joassin F, Philippot P, Heeren A, Vermeulen N, Mahau P, et al. Disrupted regulation of social exclusion in alcohol-dependence: an fMRI study. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2012;37(9):2067-75.

38. Cristofori I, Harquel S, Isnard J, Mauguiere F, Sirigu A. Monetary reward suppresses anterior insula activity during social pain. Soc Cogn Affect Neurosci. 2015;10(12):1668-76.

39. Kawamoto T, Nittono H, Ura M. Cognitive, Affective, and Motivational Changes during Ostracism: An ERP, EMG, and EEG Study Using a Computerized Cyberball Task. Neuroscience journal. 2013;2013:304674.

40. van Noordt SJ, White LO, Wu J, Mayes LC, Crowley MJ. Social exclusion modulates event-related frontal theta and tracks ostracism distress in children. Neuroimage. 2015;118:248-55.

41. Cohen D. Magnetoencephalography: detection of the brain's electrical activity with a superconducting magnetometer. Science (New York, NY). 1972;175(4022):664-6.

42. Ware JE, Jr., Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care. 1992;30(6):473-83.

43. Sebastian C, Viding E, Williams KD, Blakemore SJ. Social brain development and the affective consequences of ostracism in adolescence. Brain Cogn. 2010;72(1):134-45.

44. Oostenveld R, Fries P, Maris E, Schoffelen JM. FieldTrip: Open source software for advanced analysis of MEG, EEG, and invasive electrophysiological data. Comput Intell Neurosci. 2011;2011:156869.

45. Gross J, Kujala J, Hamalainen M, Timmermann L, Schnitzler A, Salmelin R. Dynamic imaging of coherent sources: Studying neural interactions in the human brain. Proc Natl Acad Sci U S A. 2001;98(2):694-9.

46. Bolling DZ, Pitskel NB, Deen B, Crowley MJ, Mayes LC, Pelphrey KA. Development of neural systems for processing social exclusion from childhood to adolescence. Dev Sci. 2011;14(6):1431-44.

47. Cristofori I, Moretti L, Harquel S, Posada A, Deiana G, Isnard J, et al. Theta signal as the neural signature of social exclusion. Cerebral cortex (New York, NY : 1991). 2013;23(10):2437-47.

48. Romijn AR, Rucklidge JJ. Systematic review of evidence to support the theory of psychobiotics. Nutr Rev. 2015;73(10):675-93.

49. Mazurak N, Broelz E, Storr M, Enck P. Probiotic Therapy of the Irritable Bowel Syndrome: Why Is the Evidence Still Poor and What Can Be Done About It? J Neurogastroenterol Motil. 2015;21(4):471-85.

50. Ford AC, Quigley EM, Lacy BE, Lembo AJ, Saito YA, Schiller LR, et al. Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic idiopathic constipation: systematic review and meta-analysis. Am J Gastroenterol. 2014;109(10):1547-61; quiz 6, 62.

51. Ng QX, Ho CYX, Shin D, Venkatanarayanan N, Chan HW. A meta-analysis of the use of rifaximin to prevent travellers' diarrhoea. J Travel Med. 2017;24(5).

52. Ahluwalia V, Wade JB, Heuman DM, Hammeke TA, Sanyal AJ, Sterling RK, et al. Enhancement of functional connectivity, working memory and inhibitory control on multi-modal brain MR imaging with Rifaximin in Cirrhosis: implications for the gut-liver-brain axis. Metab Brain Dis. 2014;29(4):1017-25.

53. Bajaj JS, Heuman DM, Sanyal AJ, Hylemon PB, Sterling RK, Stravitz RT, et al. Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. PLoS One. 2013;8(4):e60042.

54. Nunez PL, Wingeier BM, Silberstein RB. Spatial-temporal structures of human alpha rhythms: theory, microcurrent sources, multiscale measurements, and global binding of local networks. Hum Brain Mapp. 2001;13(3):125-64.

55. Fachner J, Gold C, Erkkila J. Music therapy modulates fronto-temporal activity in rest-EEG in depressed clients. Brain Topogr. 2013;26(2):338-54.

56. Tanaka M, Ishii A, Watanabe Y. Neural effects of mental fatigue caused by continuous attention load: a magnetoencephalography study. Brain Res. 2014;1561:60-6.

57. Baumeister J, Barthel T, Geiss KR, Weiss M. Influence of phosphatidylserine on cognitive performance and cortical activity after induced stress. Nutr Neurosci. 2008;11(3):103-10.

58. Diego MA, Field T, Sanders C, Hernandez-Reif M. Massage therapy of moderate and light pressure and vibrator effects on EEG and heart rate. Int J Neurosci. 2004;114(1):31-44.

59. Field T, Ironson G, Scafidi F, Nawrocki T, Goncalves A, Burman I, et al. Massage therapy reduces anxiety and enhances EEG pattern of alertness and math computations. Int J Neurosci. 1996;86(3-4):197-205.

60. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology. 2013;144(7):1394-401, 401 e1-4.

61. Arnsten AF, Rubia K. Neurobiological circuits regulating attention, cognitive control, motivation, and emotion: disruptions in neurodevelopmental psychiatric disorders. J Am Acad Child Adolesc Psychiatry. 2012;51(4):356-67.

62. Eack SM, Wojtalik JA, Barb SM, Newhill CE, Keshavan MS, Phillips ML. Fronto-Limbic Brain Dysfunction during the Regulation of Emotion in Schizophrenia. PLoS One. 2016;11(3):e0149297.

63. Maccaferri S, Vitali B, Klinder A, Kolida S, Ndagijimana M, Laghi L, et al. Rifaximin modulates the colonic microbiota of patients with Crohn's disease: an in vitro approach using a continuous culture colonic model system. J Antimicrob Chemother. 2010;65(12):2556-65.

64. Ponziani FR, Scaldaferri F, Petito V, Paroni Sterbini F, Pecere S, Lopetuso LR, et al. The Role of Antibiotics in Gut Microbiota Modulation: The Eubiotic Effects of Rifaximin. Dig Dis. 2016;34(3):269-78.

65. Xu D, Gao J, Gillilland M, 3rd, Wu X, Song I, Kao JY, et al. Rifaximin alters intestinal bacteria and prevents stress-induced gut inflammation and visceral hyperalgesia in rats. Gastroenterology. 2014;146(2):484-96 e4.

66. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. J Psychiatr Res. 2008;43(2):164-74.

67. Powell N, Walker MM, Talley NJ. The mucosal immune system: master regulator of bidirectional gut-brain communications. Nat Rev Gastroenterol Hepatol. 2017;14(3):143-59.

Table 1. Summarized frequency bands and neuroanatomical areas found to be associated with social stress, the application of rifaximin, and interaction of both.

Comparison	Frequency band	Brain region	Hemisphere	*P* vale
Stress effect : Exclusion vs.	6 Hz	Fusiform gyrus	Left	0.008
		Inferior parietal lobule	Right	
		Superior parietal lobule	Right	
		Thalamus	Right	
		Middle occipital gyrus	Left	
	11 Hz	Posterior cingulate gyrus	Bilateral	0.002
		Inferior parietal lobule	Bilateral	
		Superior parietal lobule	Bilateral	
		Hippocampus	Left	
		Parahippocampal gyrus	Left	
		Fusiform gyrus	Left	
	16Hz	Inferior temporal cortex	Bilateral	0.01
		Middle temporal cortex	Bilateral	
		Fusiform cortex	Bilateral	
		Hippocampus	Bilateral	
		Inferior occipital cortex	Left	
	21 Hz	Cingulate gyrus	Right	0.008
		Fusiform cortex	Bilateral	
		Hippocampus	Bilateral	
		Parahippocampal gyrus	Right	

inclusion		Occipital cortex	Left	
	26 Hz	Posterior cingulate gyrus	Left	0.01
		Fusiform gyrus	Bilateral	
		Hippocampus	Bilateral	
		Parahippocampal gyrus	Bilateral	
		Interior temporal lobule	Bilateral	
		Middle temporal lobule	Bilateral	
		Superior temporal lobule	Bilateral	
		Thalamus	Bilateral	
Rifaximin effect on resting state: rifaximin vs. placebo	11 Hz	Inferior frontal gyrus	Left	0.05
		Middle frontal gyrus	Left	
		Superior frontal gyrus	Bilateral	
		Middle cingulate gyrus	Right	
		Insula	Left	
Rifaximin effect * stress effect on the Cyberball game: (Rifaximin vs. placebo) * (exclusion vs. inclusion)	16 Hz	Inferior frontal gyrus	Left	0.05
		Superior frontal gyrus	Left	
		Middle frontal gyrus	Middle	
		Anterior cingulate gyrus	Left	

Legend of figures

Figure 1. Schematic outline of a trial in the Cyberball game.

Figure 2. Activation in different frequency bands during exclusion vs. inclusion condition. A. Theta frequency band (6 Hz): the left fusiform gyrus, he right inferior and superior parietal lobule, the right thalamus and the left middle occipital gyrus ($p = 0.008$); B. Alpha frequency band (11 Hz): the bilateral posterior cingulate gyrus, the bilateral inferior and superior parietal lobule, the left hippocampus and parahippocampal gyrus and the left fusiform gyrus ($p = 0.002$); C. Beta 1 frequency band (16 Hz): the bilateral inferior and middle temporal lobule, the bilateral fusiform gyrus, the bilateral hippocampus, the left inferior and middle occipital cortex ($p = 0.01$); D. Beta 2 frequency band (21 Hz): the right cingulate gyrus, the bilateral fusiform gyrus, the bilateral hippocampus, the left occipital cortex, the right parahippocampal gyrus ($p = 0.008$); E. Beta 3 frequency band (26 Hz): the left posterior cingulate gyrus, the bilateral fusiform gyrus, the bilateral hippocampus and parahippocampal gyrus, the bilateral inferior, middle, superior temporal lobule, and the bilateral thalamus ($p = 0.01$).

Figure 3. Difference of changed brain activity during resting-state comparing rifaximin vs. placebo. A cluster including the left inferior and middle frontal cortex, the bilateral superior frontal cortex, the right middle cingulate gyrus and the left insula showed significantly more increased power in alpha band (11 Hz), $p < 0.05$.

Figure 4. Difference of neural activity change in 16 Hz during the Cyberball game by interaction of interventions and conditions effects. A cluster including the left inferior and superior and the bilateral middle frontal cortex, and the left anterior cingulate gyrus, showed a reduced neural activity change in beta-1 band (16 Hz) comparing exclusion vs. inclusion and rifaximin vs. placebo, $p < 0.05$.

Figure 5. Difference of neural activity change in 16 Hz during the Cyberball game comparing exclusion vs. inclusion conditions in the rifaximin group. Regions of the bilteral inferior, supperior and middle frontal cortex, the bilateral anterior, middle and posterior cingulate gyrus, the bilateral parietal and postcentral cortex, showed a reduced neural activity change in beta-1 band (16 Hz) comparing exclusion vs. inclusion conditions, $p < 0.002$.

Figure 6. Correlation between change of neural activity with the change of SEP in the rifaximin group. Change of the averaged beta-1 band (16 Hz) power in the activated cluster during exclusion correlated negatively with the change of SEP ($r = -0.86$, $p = 0.02$, n = 8).

Table 1. Summarized frequency bands and neuroanatomical areas found to be associated with social stress, the application of rifaximin, and interaction of both.

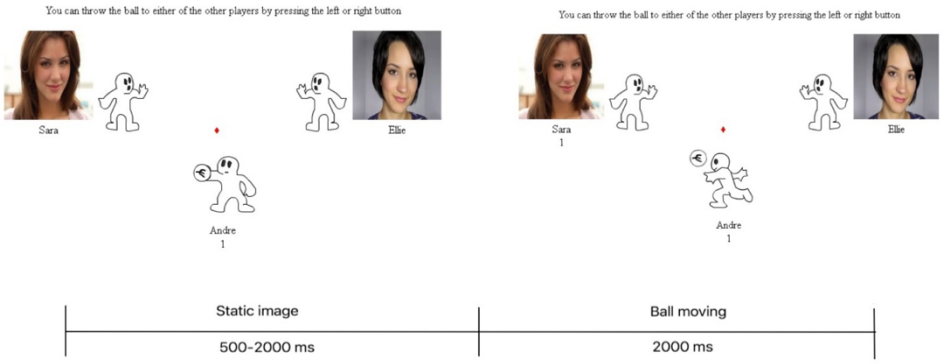

Figure 1

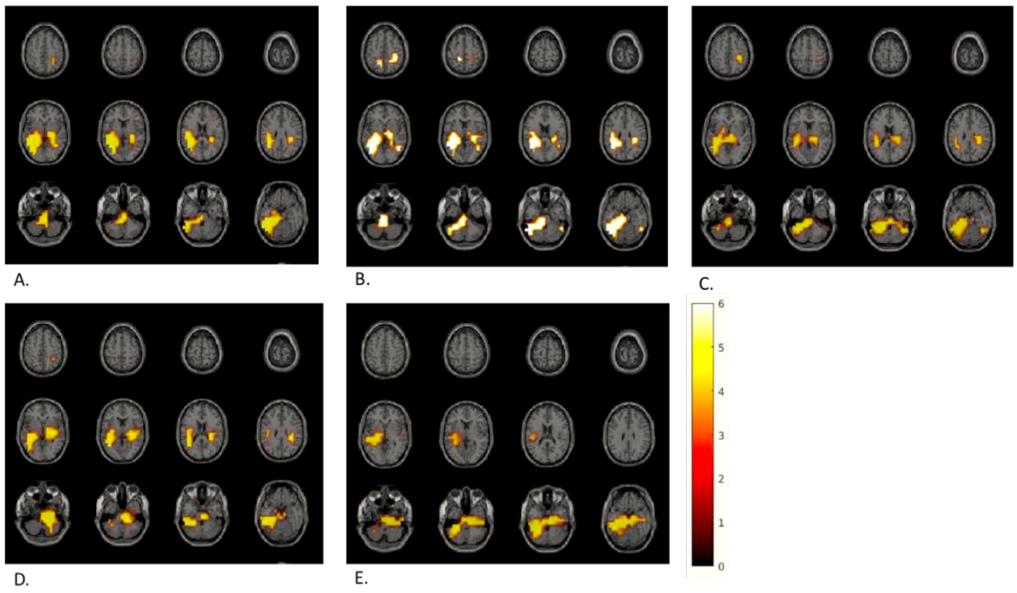

Figure 2.

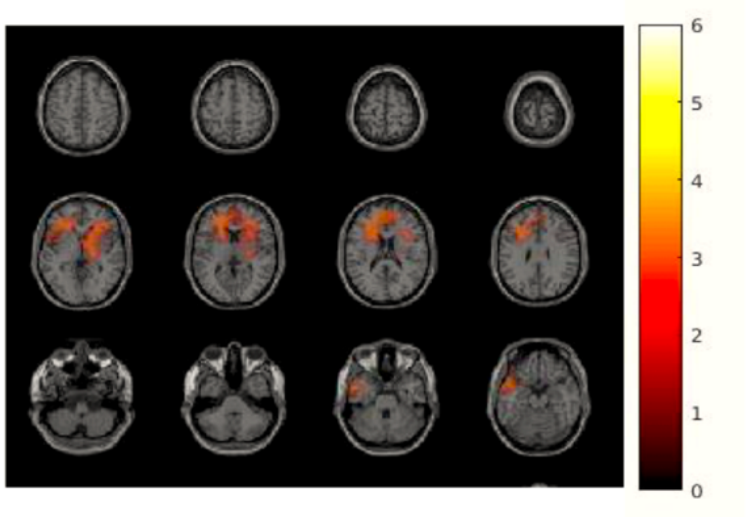

Figure 3.

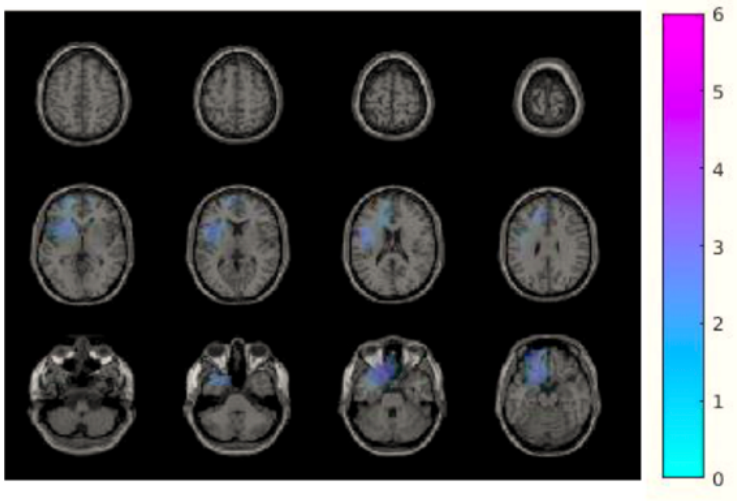

Figure 4.

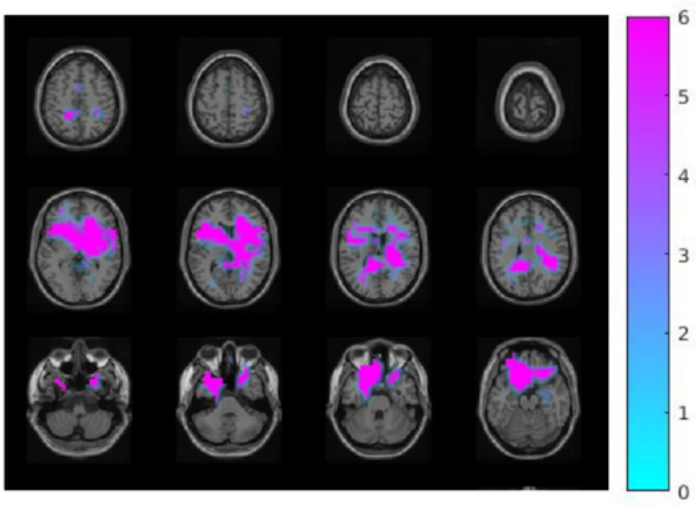

Figure 5.

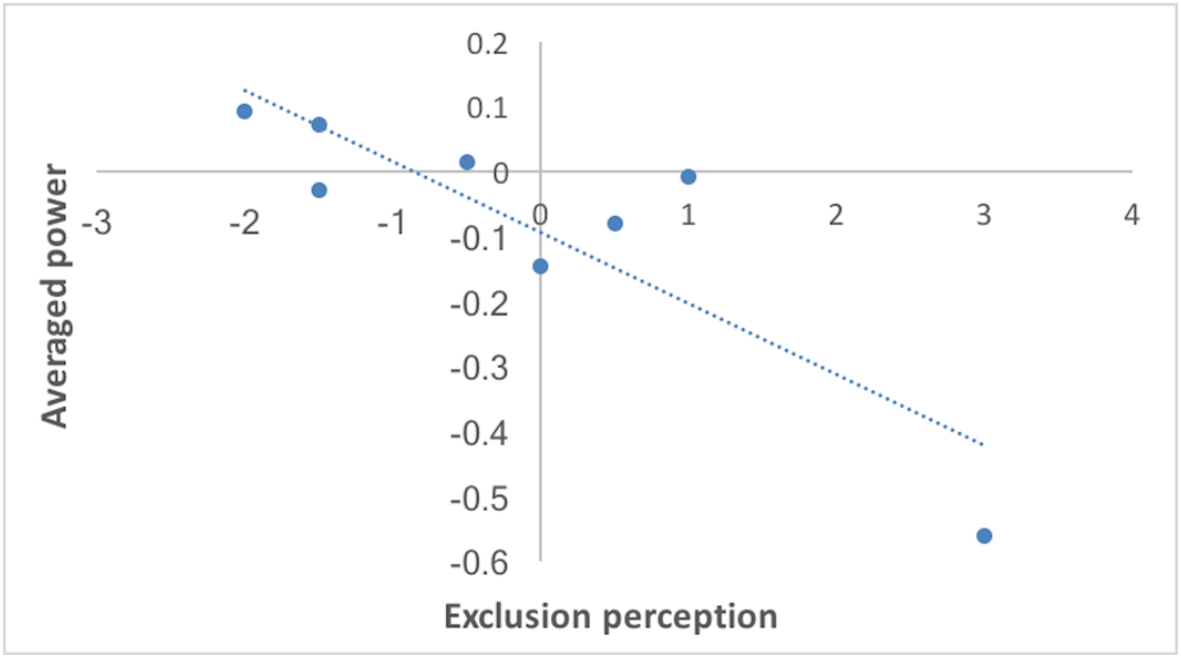

Figure 6

Appendix 1. Probiotic/prebiotic containing food not to eat during intervention

Probiotic richly containing food:

Yogurt containing probiotics (eg. Dannon Activia, Yakult, or any other brands you know)

Goat's milk

Soy milk

Kefir

Sauerkraut

Pickles

Kimchi

Umeboshi plums

Tempeh

Dark chocolate

Microalgae

Natto

Poi (kind of mashing cooked taro plant)

Miso soup

Kombucha Tea

Prebiotic richly containing food:

Raw Chicory root

Raw Jerusalem artichoke

Raw Dandelion greens

Raw garlic

Raw leek

Raw onion

Appendix 2: Assessment of Need Threat Scale, Mood Questionnaire and Exclusion Perception.

All items need to be rated on a scale from 1 ('not at all') to 5 ('very much'). (R) = reversed scored.

Need

Belonging:

1. I felt disconnected with one or more players.
2. I felt rejected by other players.
3. I felt like an outsider.
4. I felt belonged to the group. (R)
5. The other players interacted with me a lot. (R)

Self-esteem:

6. I felt good about myself. (R)
7. My self-esteem was high. (R)
8. I felt I was liked. (R)
9. I felt insecure.
10. I felt satisfied. (R)

Meaningful existence:

11. I felt invisible.
12. I felt meaningless.
13. I felt non-existent.
14. I felt important. (R)
15. I felt useful. (R)

Control:

16. I felt powerful. (R)
17. I felt I had control over the course of the game. (R)
18. I felt I had the ability to significantly alter events. (R)
19. I felt I was unable to influence the actions of others.
20. I felt the other players decided everything.

Mood

During the game I felt:

1. Good (R)
2. Bad
3. Happy (R)
4. Sad
5. Pleasant (R)
6. Angry

7. Friendly (R)
8. Unfriendly

Exclusion perception

1. I was ignored.
2. I was excluded

9. Study IV. Psychobiotic *Bifidobacterium longum* 1714 modulates brain activity of healthy volunteers during social stress (in preparation)

Author contribution

PE is responsible for the integrity of the work - the inception of the study and publication of the work. HW contributed to the design of the study, data collection and analysis, drafting of the manuscript, and critical revisions of the manuscript. CB and PE contributed to the design of the study, data analysis, critical revisions of the manuscript. All authors approved the final version of the manuscript.

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Psychobiotic *Bifidobacterium longum* 1714 modulates brain activity of healthy volunteers during social stress

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Abstract

Background/aims: Probiotics have been described as "psychobiotics" with beneficial effects on mental status or health. Preclinical and clinical studies have found probiotics to reduce stress responses and associated behaviors, and to improve cognitive abilities. It has become increasingly interesting to use neuroimaging methods to identify where and how psychobiotics affect cortical activation.

Methods: In a randomized, double-blinded, placebo-controlled trial, we studied effects of *Bifidobacterium longum* 1714 on neural responses to social stress, induced by the "Cyberball game", a standardized social psychology stress paradigm. Forty healthy volunteers received either *B.longum* 1714 or placebo for four weeks. Their health status was assessed using the 36-item short-form health survey (SF36), and brain activation during resting state and social stress using magnetoencephalography.

Results: *B. longum* 1714 affected resting state neural oscillations with an increase in theta band in the frontal and cingulate cortex ($p < 0.05$) and a decrease in beta-3 band in the hippocampus, fusiform, and temporal cortex ($p < 0.05$), both of which were associated with subjective vitality changes, as measured by SF36. All groups showed increased distress to social stress after 4-weeks intervention. Only the group received probiotics but not the placebo group showed neural activity changes following social stress, with increased theta and alpha band power in the frontal and cingulate cortex and supramarginal gyrus ($p = 0.03$; 0.04).

Conclusion: Our results indicate that *B.longum* 1714 affects resting state neural oscillations in theta and beta band that may be related to enhanced arousal and reduced mental fatigue. The probiotic strain modulated neural oscillations of theta and alpha bands during social stress, indicative of counter-regulation of negative emotions.

Keywords: Gut-brain axis, psychobiotic, stress, cyberball game, MEG

1 Introduction

Probiotics have been described as “psychobiotics", a family of bacteria that, ingested in appropriate quantities, have a positive mental health benefit (1). Studies assessing the effects of psychobiotics on central nervous system (CNS) functions include preclinical studies conducted in rodent animals, and translational studies in healthy volunteers, psychiatric patients, and patients with irritable bowel syndrome (2-5). The effects have been summarized in many reviews as anxiolytic, antidepressant and memory improving (6, 7).

Studies on psychobiotics have been put forward to neuroscience investigations, most of which are preclinical in nature. However, clinical studies using neuroimaging methods are less abundant but have started to emerge, which provide insight into modulations of CNS functions in humans. The first study to demonstrate neural effects of probiotics in humans was using functional magnetic resonance imaging (fMRI) (8). Brain activations to emotional faces were altered by a fermented dairy drink with probiotics in comparison to placebo. A more recent study in patients with irritable bower syndrome (IBS) also revealed improvement in psychiatric comorbidity and reduction in limbic activity to negative emotional stimuli (3).

Stress is a crucial factor for many mental disorders such as anxiety and depression. Administration of *Lactobacillus rhamnosus* JB-1 attenuated behavioral deficits induced by a chronic psychosocial stressor in mice, and prevented related immunoregulatory alterations (9). In healthy humans, studies have directly measured stress responses such as cortisol levels and stress perception, and showed effects of probiotics on modulating these responses. For example, Takada et al. used academic examinations as a stressor in students and found that *Lactobacillus casei* Shirota suppressed stress-induced increases in saliva cortisol and physical symptoms (10). Allen et al. reported attenuated cortisol output and subjective stress to a cold pressor (11). Despite the increasing number of studies, it is still unclear and interesting to

investigate where and how probiotics affect cortical activations in response to a stressful situation.

Social stress induced by social exclusion/rejection is considered as a chronic stressor that is able to induced emotional, behavioral and physiological changes (12). Numerous studies have utilized a standardized paradigm, called "Cyberball Game" (CBG), to study the effects of social stress and ostracism (exclusion), and the corresponding neural response (13). The CBG is "an online ball-tossing game that participants believe they are playing with two or three players" (13). Participants feel more distressed in periods when the other players most of the time only play with each other and do not involve them in the game, compared to the periods in which all players have the same chance to receive and throw the ball. Physiological changes of cortisol levels and skin conductance have been observed during such social stress situations (14-17). According to a scoping review summarizing neuroimaging studies that have used MRI and electroencephalography (EEG) during the CBG, regions of the prefrontal cortex (PFC), anterior cingulate cortex (ACC) and temporal cortex are involved in neural processing of this type of social stressor (18). Also, social stress during the CBG was consistently associated with altered neural oscillations in certain brain areas, such as increase in alpha band power in frontal cortex and theta band power in the ACC, insula and fusiform areas (19-22).

In a recent study, we have verified the efficacy of magnetoencephalography (MEG), a neuroimaging technique with fine spatial resolution and high temporal resolution (23, 24), to measure neuromagnetic oscillations during social stress induced by the CBG (25). In this study we tested effects of a locally (intestinally) acting antibiotic (rifaximin) on the stress effect, and found that 7-days of rifaximin intake alleviated the stress response by reducing low beta band power in the PFC and ACC. Also, frontal and cingulate alpha band during

resting state was reduced after rifaximin intake, reflecting a raised relaxation status. Rifaximin has been reported to relieve IBS symptoms (26), treat small bacterial overgrowth (27), and prevent travelers' diarrhea (28). Studies in animals and humans have revealed that rifaximin reduces toxic intestinal bacterial growth and promotes beneficial bacteria in the gut such as *Bifidobacteria* and *Lactobacilli* (29-31), which may be responsible to mediate its "eubiotic" (25) neural effect on the stress response (30).

In the current trial, we tested whether *Bifidobacterium longum* 1714 affected healthy volunteers' health status and neural activities during a resting state measurement. We also investigated whether *B.longum* 1714 – in comparison to placebo – was able to alter neural oscillations associated with the CBG-induced social stressor. We selected this specific strain as our test organism, as it had previously been demonstrated to modulate stress related behaviors in animals (32, 33). Supporting evidence is also provided by a clinical study that showed reduced stress responses and improved cognitive activity following treatment (11). We hypothesized that the probiotic strain *B.longum* 1714 would show effects on resting brain activity and on neurophysiological responses to CBG induced social stress. In particular, we assumed that during the resting state, *B.longum* 1714 would show similar effects as rifaximin, causing an increase of alpha band power associated with higher level of relaxation. During the CBG, stress-related neural oscillations of the theta and alpha band power would be changed after probiotic treatment; similarly to the effects of rifaxmin, beta band power would also be reduced by *B.longum* 1714.

2 Materials and methods

2.1 Participants

Based on previously published data (11), we estimated that - with a power of 0.95 for a 2 x 2 repeated measure ANOVA - a minimum sample size of 34 was required to demonstrate an effect size $f = 0.2$ at $\alpha = 0.05$ in a parallel-group designed study. The study was completed with 40 healthy volunteers, after having recruited initially sixty-one participants. Eighteen participants were excluded because they did not meet the inclusion criteria, and three of them could not be included in the final analysis because of the intake of an antibiotic during the intervention period (see **Figure 1** for detailed trial profile). Criteria for inclusion were: 1) non-smoker for at least 3 months, 2) a body mass index (BMI) of 18-30, 3) no chronic allergies, 4) willing to discontinue their normal consumption of probiotics and prebiotic-containing foods or potentially immune-enhancing dietary supplements, 5) receiving no immune-suppressing intervention and not having any immunosuppressive illness within the last year, 6) receiving no antibiotic therapy within the last 2 months, 7) having no chronic psychiatric or gastrointestinal disorder, 8) and having no non-removable metal parts in the body. Informed consent was obtained from all participants prior to joining the study. The protocol had been approved by the Ethics Board of the University of Tübingen Medical School (No. 503/2015BO1, as of August 26, 2015), and was registered at ClinicalTrials.gov (identifier No. NCT02793193).

Figure 1.

2.2 Design

A randomized, double-blinded, and parallel-group design was employed. Participants were screened for the irritable bowel syndrome and psychiatric disorders using the Rome III criteria (34) and the Patient Health Questionnaire (PHQ) (35). Demographic and baseline

psychological information was also recorded. After screening, participants were randomly allocated in different intervention groups and took either probiotic or placebo for 4 weeks (28 days). The probiotic and placebo preparations in equally looking sachets were provided by Alimentary Health Ltd, Cork, Ireland. The randomization scheme was only unblinded after completion of the experiment and complete data evaluation. At baseline and one day after the intervention period, participants visited our lab for the MEG measurements. In addition, they visited the lab for acquiring structural MR images on a different day, regardless of their intervention schedule.

During the intervention period, participants were instructed to avoid consumption of food containing probiotics/prebiotics, or potentially immune-enhancing dietary supplements. This was supported by providing them with a list of "prohibited" foods (**Appendix 1**).

2.3 Materials

Each probiotic sachet contained 20 g of 10^9 colony-forming units *B.longum 1714* strain with maltodextrin and magnesium stearate; each placebo sachet contained only 20 g of maltodextrin and magnesium stearate. Participants were instructed to consume one sachet every morning with food within 15 minutes, by mixing the content into 50 ml of water.

2.4 Questionnaires

To record participant's health status, the 36-item short-form health survey (SF36) was used (36). The SF36 includes eight subscales: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/vitality, and general health perceptions. Participants were asked to finish the SF36 at the beginning of each of the two visits.

After each block of the CBG (see below), participants needed to complete three questionnaires to assess their acute level of distress. We employed the self-report measures of the Need Threat Scale (NTS), the Mood Questionnaire (MQ) and the Subjective 'Exclusion Perception' (SEP) (**Appendix 2**); all these scales are validated standards for the CBG (13, 37). The NTS was designed to measure the feelings and emotional consequences of social rejection, and higher scores related to higher distress level. Its four items (rated between 1 and 5 for "weak" to "strong") comprised self-esteem, belonging, meaningful existence and control, and combined ratings have been used as a measure of social distress in previous studies. The MQ was used to assess mood, using 8 questions (are you feeling bad, good, happy, sad, pleasant, angry, friendly and unfriendly), all rated between 1 and 5. The SEP was to record participants' feeling of being included/ostracized by asking them to rate two statements ('I was ignored' and 'I was excluded') between 1 and 5.

2.5 Cyberball game

In the CBG, the participants were asked to play a ball tossing game with two other virtual players programmed by the experimenter. They were made believe that the two players were real and were playing the game. To minimize gender effects, male participants played with 2 female players, and female participants played with 2 male players. During the game, the other players were depicted as cartoon characters with their photos aside and names below. The volunteering participant was represented by a cartoon in the middle lower part of the screen and could throw the ball to either of the other two players on the left or right, by pressing the left or right button on the response box (**Figure 2**).

Figure 2.

The CBG consisted of 4 blocks: inclusion - exclusion - inclusion – exclusion conditions; this order was maintained for all volunteers. In each inclusion block, there were 108 trials, during 1/3 (36 trials) of which the participant received the ball from the other players (for another 1/3 the ball was played to one of the other players, and for the other 1/3 between the other two players). The order of the ball throwing to the participant was pseudo-randomized. The 1/3 of trials in the inclusion block when the virtual players threw the ball to each other and not to the participant, were called "not my turn" events.

To equalize the numbers of analysed trials when the virtual players threw the ball to each other and not to the participant, we set 47 total trials in each exclusion block. The participants received the ball 3 times (trials 14, 25, or 26, and 39, 40, or 41 in block 2 and 4, respectively) to maintain their attention. The first 5 exclusion trials, the 3 trials the participant receiving the ball, and the 3 trials the participant was throwing the ball were not analysed but discarded. The remaining 36 so-called "rejection" events were used for comparison with the 36 "not my turn" events in the inclusion block. Visual stimuli of these trials did not differ in two conditions, so any difference of brain activities was supposed to be due to the participants' inner state. The trial began with the ball being presented in the cartoon for 500-2000 ms randomly to imitate a real life situation. Then the ball was moving for 2000 ms before reaching the target player (**Figure 2**). After each of the inclusion and exclusion blocks, participants completed the NTS, the MQ, and the SEP.

2.6 Magnetoencephalography recording

Brain magnetic fields were measured with a 275-channel whole-head magnetoencephalograph (CTF Omega, Port Coquitlam, Canada). Participants were studied in supine position. During each recording session, 5 minutes resting state was recorded prior to recording while playing the CBG. During the resting state, participants were instructed to

move as little as possible and to be awake, while keeping their eyes closed. During the CBG, task instructions were projected onto a screen in front of the participants via a video projector and a mirror system. Participants were asked to fixate the screen and hold a response box to get ready for the task. Participants were also instructed to move as little as possible. MEG signals were sampled at a rate of 585.94 Hz with an anti-aliasing filter set to 292.97 Hz.

In order to overlay the brain activity derived from MEG on anatomical scans, high-resolution (1 mm, isotropic) T1-weighted structural MR images were acquired using an MPRAGE sequence with a Siemens MAGNETOM Trio 3T scanner (Siemens AG, Erlangen, Germany) (12-channel array head coil) for each participant, but at a separate occasion.

2.7 Data Analysis

2.7.1 Data analysis: questionnaires

Data analysis was conducted using SPSS 21 (IBM, Armonk, NY, USA). To examine whether there was a significant difference in health status between groups at baseline, scores of SF36 during the first visit were entered into a non-parametric two-independent-sample Mann-Whitney U test of *Intervention* as between factor (*B.longum 1714* vs. Placebo), as parametric assumptions of these data were violated. To test the intervention-related changes in participants' health status scored by SF36, changes from before to after the 4-weeks intervention were computed by subtracting the baseline assessment from the corresponding post-intervention values. Non-parametric two-independent-sample Mann-Whitney U test was used to examine the change of SF36 between *Intervention* (*B.longum 1714* vs Placebo).

To examine whether subjective ratings for the CBG were different between groups at baseline, scores of NTS, MQ, and SEP acquired during the first visit were entered into an independent T-test with *Intervention* as between factor (*B.longum 1714* vs. Placebo). To

control the intervention-related changes of the NTS, the MQ and SEP during the CBG, changes after each intervention were computed for each condition and entered into a 2 x 2 repeated measure ANOVA with *Intervention* as a between-factor (*B.longum 1714* vs. Placebo) x *Condition* as a within-factor (exclusion vs. inclusion). Where significant main effects or interaction were observed, pairwise post-hoc comparisons were used with a Bonferroni adjusted threshold ($\alpha = 0.025$). Mean data are reported as $M \pm SD$.

2.7.2 Data analysis: MEG - data

Preprocessing

Analysis of the MEG data was carried out using Matlab (Mathworks, Natick, USA) and the open-source toolboxes Fieldtrip (38). The resting state dataset were cut into time windows of 2 s. Data in this time window were filtered using a 4 Hz high pass frequency filter. Non-physiological jumps in the MEG signal and trials with jump and muscle artifacts were excluded by an automatic rejection algorithm that excluded all trial in which the variance exceeded 10^{-25} in any channel.

The continuously recorded dataset during the CBG was segmented in epochs of 3 s with 1 s of pre-stimulus interval time-locked to the moment at which the players started to throw the ball). Trials in which one of the virtual players threw the ball towards the other virtual player during the inclusion blocks were defined as ‘inclusion’ condition, and those during exclusion blocks were defined as ‘exclusion’ condition.

Time-frequency analysis

The time-frequency analysis used the multitaper windowed fast fourier transform ‘MTMFFT’ implemented in Fieldtrip. The 'multitaper method' (MTM) is based on Slepian

sequences as tapers. The frequency of interest ranged from 4 to 30Hz with step of 2 Hz. The frequency smoothing window is +/-3 Hz:

Source analysis

Using the time-frequency determined by the analysis described above, oscillatory sources of theta, alpha, beta-1, beta-2 and beta-3 bands (6, 11, 16, 21, and 26 Hz) were localized using beamformer techniques. We applied the Dynamical Imaging of Coherent Sources (DICS) method (39). In order to estimate the individual source activity, each participant's brain recorded as T1-MR image was divided in a regular three dimensional grid with a 1 cm resolution. A spatial inverse filter was computed from both conditions and both visits, as common filter. The common filter was applied to each condition and each visit separately in order to obtain the respective source power. The MEG data in each condition were coregistered with the respective individual structural MR images respectively.

Source statistics

We performed source-level statistics to assess effects of intervention on the data obtained from the resting-state condition and the CBG, respectively. To check if there was any difference between groups at baseline prior to any intervention, resting-state at baseline source power was compared with an independent T-test with *Intervention* (*B.longum* 1714 vs. placebo) as between factor. Then, intervention-induced changes in source power were computed in each frequency band by subtracting the baseline from the post-intervention. The changes of the source power were entered into an independent T-test with *Intervention* (*B.longum* 1714 vs. placebo) as between factor. For the CBG, source power at baseline in each frequency band was also tested with an independent T-test with *Interventions* (*B.longum* 1714 vs. placebo) as between factor to check if brain activations showed differences between groups. Subsequently, changes in the source power after intervention were computed by

subtracting the baseline values from the post-intervention for each condition in each frequency band. Changes of source power were entered in a two-way ANOVA of *interventions* (*B.longum* 1714 vs. placebo) x *conditions* (exclusion vs. inclusion). The statistical analysis was done separately for each frequency band. To localize significant activations, the cluster-based permutation method for multiple comparisons was used with a significance level of alpha of 0.05.

2.7.3 Correlation between questionnaire and MEG data

To investigate the relationship between changes in neural activity and changes in the subjective reports induced by *B.longum 1714*, correlations analyses were carried out for both, the resting state recording and the CBG, respectively. For the resting state recording, averaged source power within clusters was calculated for the clusters differing significantly between both visits. The averaged source power was correlated with changes in health status (SF36) for each group separately. For the CBG, for each condition and each intervention, source power within the clusters that differed significantly between both visits was averaged for each condition and each group. The averaged source power was correlated with changes in the scores of NTS, MQ and SEP separately for each condition and each group, using Pearson correlations.

3 Result

A total of 40 participants were included in the analysis with half of them receiving *B.longum* 1714 intervention. Sex of participants was matched between groups. Age and BMI of participants were not significantly different between groups (See **Table 1** for details).

3.1 Effects on subjective ratings

At baseline, no significant differences of SF36 scores or CBG subjective scores (NTS, MQ, and SEP) were seen between groups (also see **Table 1**).

A non-parametric two-independent-sample test was conducted to compare changes of SF36 scores after intervention between groups. No significant difference was found.

An ANOVA (*Intervention* x *Condition*) testing changes of the NTS, MQ and SEP revealed significant main effects of *condition* on NTS (F (1, 33) = 5.91, p = 0.02) and on SEP (F (1, 36) = 5.61, p = 0.02). Participants in all groups reported increased scores of NTS (exclusion: M = 5.20 ± 2.37; inclusion: M = -4.32 ± 1.85) and SEP (exclusion: M = 0.94 ± 0.42; inclusion: M = - 0.30 ± 0.16) in the exclusion compared to inclusion condition, after four weeks of intervention (**Figure 3**).

Figure 3.

3.2 MEG data

3.2.1 Resting state

At baseline, no group difference of source power during resting state was observed in any frequency band. After the intervention, an increased theta band (6Hz) power was found in one cluster, including regions of bilateral inferior, middle and superior frontal cortex (IFC, MFC and SFC), and the bilateral anterior and middle cingulate cortex (ACC and MCC), comparing *B.longum* 1714 with the placebo group ($p < 0.05$; **Figure 4A**). Also, a reduced beta-2 band (26Hz) power was obtained in a cluster, consisting of the bilateral fusiform gyrus (FFG), the bilateral hippocampus (HIPP), the left inferior and superior temporal and bilateral

middle temporal cortex (ITC, STC, and MTC), the left cerebellum (CBL), comparing both groups ($p < 0.05$; **Figure 4B**).

Figure 4.

To investigate whether the changes of neural activity during resting state were correlated to changes of the participants' health state, we correlated the changes of averaged power within activated clusters (shown above) in theta and beta-2 bands, with changes in SF36 scores. In both groups, a significant positive correlation was obtained between changes of the SF36 subscale "Energy/Vitality" with changes of theta band power in the cluster ($r = 0.33$, $p = 0.04$; **Figure 5A**).

Group-specific correlations, revealed that only in the *B.longum* 1714 group ,changes of SF36 score for 'Energy/Vitality' positively correlated with changes of averaged theta band power ($r = 0.61$, $p = 0.007$), and negatively correlated with changes of beta-3 band power in the activated clusters during the resting state, respectively ($r = -0.50$, $p = 0.04$; **Figure 5B**).

3.2.2 Cyberball Task

At baseline, source power in each frequency band during the CBG showed no significant difference between groups. After the 4-week intervention, main effects of *B.longum* 1714 were seen for changes of source power in theta (6Hz) band and alpha band (11Hz). Theta band showed an increased power in one cluster, consisting of the right IFC and the bilateral MFC and SFC, the left ACC the bilateral MCC, and the right supramarginal gyrus (SMG), comparing *B.longum* 1714 with placebo in both conditions inclusion and exclusion ($p = 0.03$; **Figure 6A**). Alpha band also showed an increased power in the cluster, including regions of the right IFC the bilateral MFC and SFC, the bilateral ACC and MCC,

and the right SMG, for *B.longum* 1714 compared to placebo in both conditions (p = 0.04; **Figure 6B**). No main effect of condition or interaction of intervention and condition were observed.

Figure 6.

Correlation analysis between changes of averaged power within activated clusters (as shown above) and changes of NTS, MQ and SE ratings revealed an association between the neural activity changes and the subjective effects: Only with *B.longum* 1714 and only during the exclusion condition, NTS changes positively correlated with changes of the theta band power (r = 0.62, p = 0.008) and alpha band power (r = 0.54, p = 0.03; **Figure 7**).

Figure 7.

Summaries of frequency bands and neuroanatomical areas found to be related to *B.longum* 1714 intervention, and associations of neural activity changes and subjective effects are provided in **Table 2 and Table 3**, respectively.

4 Discussion

The current study used a functional neuroimaging method, MEG, to show that 4-weeks intake of the probiotic strain *B.longum* 1714 had significant effects on neural activities in both, the resting state and the response to a social stressor in healthy participants. Comparing *B.longum* 1714 with placebo, the neural activities during resting state showed increased power in theta band in the frontal and cingulate cortex, and decreased power in beta-3 band in the fusiform cortex, hippocampus, temporal cortex and cerebellum. While no

changes in SF36 (general health) scores were found with *B.longum* 1714, alterations in the resting brain activity were associated with the SF36 scale "Energy/Vitality", which asked for energy and fatigue experienced during the preceding 4 weeks. In response to the social stressor CBG, all groups showed higher distress levels and reported higher exclusion perception after the intervention period. However, only in *B.longum* 1714 group, neural responses during the CBG showed increases in theta and alpha bands power in the frontal and temporal cortex and supramarginal gyrus for both CBG conditions (inclusion and exclusion conditions). The increased neural activities associated with higher subjective distress occurred in the probiotic group only for exclusion condition.

Specifically, during the resting state, after the probiotic intervention, theta band power - in the frontal and cingulate cortex - was higher and beta-3 band power - in the fusiform cortex, hippocampus, temporal cortex and cerebellum - was lower. Although no significant change of SF36 was noted following the 4-week probiotic intake, the increase of theta band and the decrease of beta-3 band power found for the resting state were associated with an increase of perceived energy levels, as assessed by the SF36. Contrary to our hypothesis of larger alpha band power in the frontal and cingulate cortex, which is supposed to indicate improved relaxation, the increase of the theta and the decrease of the beta-3 band power can instead be seen as improvement of energy status. In the literature, increased power in theta band (whole brain, especially prominent in the frontal regions) on a resting-state EEG was observed after consumption of a major source of metabolic energy – glucose, and went along with improved attention and arousal (40, 41). A decrease in theta band power in cingulate cortex was correlated with subjective level of fatigue in one MEG study (42). Similarly, an increase in beta band power could be caused by mental fatigue (43), and inversely, reduced beta power could index increased alertness and arousal, accompanied with decreased anxiety and stress (44, 45). It has been well studied that probiotics can alter metabolism of short-

chain fatty acids and vitamin thus increasing ATP production and providing energy (46). Based on these findings, we may speculate that the effects of *B.longum* 1714 are due to enhanced participants' quality of life and especially their energy status and decreasing fatigue. This enhanced vitality/energy status is also reflected by participants' altered resting neural activity. Considering the effects of probiotics on individuals' energy balance appears to be an attractive, yet admittedly speculative, explanation that nicely links subjective ratings of individual states and neurophysiological findings of resting state activity in our study. Measures of metabolic changes may help to elucidate these and other putative mechanism of action of *B.longum* 1714 in the future.

All participants reported higher distress level during the exclusion condition compared to the inclusion condition after 4-weeks of intervention. The increased level of distress was observed regardless of the type of intervention (probiotic, placebo). Social exclusion/rejection from others can occur in daily life as a chronic stressor, thus repetition of this stress may reinforce the feeling of being excluded and consolidate the memory of the stressful event. This general increase of distress ratings was not observed in our last study testing the effects of rifaximin (25) in the same paradigm, probably due to the shorter period of the intervention. While in the previous study rifaximin was administered for 1 week, in the current study the intervention lasted for 4 weeks.

After administration of *B.longum* 1714, theta and alpha bands power were increased in the frontal and cingulate cortex (ACC and MCC). In the CBG increased alpha and theta power was observed in SMG during all conditions when participants observed the other players throwing the ball to each other. Although both groups reported higher subjective distress, changes in neural processing of social stress was changed only following *B.longum* 1714 and not placebo. Similarly, the correlation of changes of subjective distress with neural

activities was only seen with *B.longum* 1714. Thus, our data support the notion that *B.longum* 1714 plays a role in managing stress responses by modulating the relevant neural processes; *B. longum* 1714 affecting individuals' neurophysiology is a novel findings and was previously only reported for behavioral data in animals and humans (11, 32, 33).

As hypothesized, *B.longum* 1714 altered theta and alpha band power of stress-related neural oscillations in frontal and cingulate cortex. However, unlike rifaximin in our previous study, *B.longum* 1714 did not influence beta band activity in the CBG (25). Our current results are consistent with previous studies using EEG revealing that ostracism distress is related to pronounced alpha band power by the stressor at the frontal cortex and theta band power in the ACC, FFA and insula (19, 20, 22). Previously reported ACC activations were related to processing of negative emotions and event appraisal due to the social stress/exclusion, and PFC activation was related to emotional regulation (18, 47-49). Theta band in ACC and insula was described as a marker of social pain in the context of the CBG and was also found during a cold pressor test and physical pain (50, 51). In our study, the increased theta band activity could be associated with induced negative emotions and relevant appraisal processes elicited by the exclusion event (19, 20, 22). Similar changes of theta band activity induced by *B.longum* 1714 during the CBG were also observed in the resting state data of the current study, which were attributed to increased arousal conditions. Enhanced arousal levels in the CBG might be related to more intense responses to the social stressor.

In previous EEG studies (21, 22), alpha band increase in frontal brain areas was involved in the neural processes during social stress, indexing either a high level of distress or the correlation of stress management/regulation. Lower frontal alpha power was found in anxiety, which might be considered as a specific mode of stress (52, 53). With respect to our

study, one might speculate that the increased alpha activity indexes inhibition of limbic activity and thereby counter-regulate negative emotions and stress. Taking this argument further, we may suggest that in contrast to placebo, *B. longum* 1714 reduced participants' stress response and enabled them to manage the increased distress level by upregulating processes appraising stressful events and downregulating negative emotions.

Comparing the *B.longum* 1714 group with the placebo group, a significant alleviation of perceived stress during the CBG, as assessed by subjective ratings, was missing. This may be because that stress-reducing effect of the probiotic strain was not strong enough at the behavioral level to counteract the enhanced stress effect over time. Alternative explanations would be that – despite their validation in CBG studies – the assessment tools were not sensitive enough for our study in volunteers, the implementation of the CBG was not optimal, or the effect size induced by the 4-week probiotic intake. Nevertheless, we believe that probiotic *B.longum* 1714 acts on a neural level managing social stress, consistent with previous studies. The strain *B.longum* 1714 has been shown in preclinical and clinical studies to reduce stress, anxiety and depression-like behaviors and, most strikingly also improve memory performance (11, 32, 33).

Other studies - in line with our data - showed neural modulation by different psychobiotic strains, and provided some clues of the potential mechanisms involved. Preclinical studies have reported a rise of serotonin and dopamine levels, two crucial neurotransmitters regulating mood and emotions, in brain regions of mice such as prefrontal cortex after interventions with *L.planatraum* and *L.helveticus* (54-56). Another study showed *L. rhamnosus* (JB-1) modulated GABAergic system and reduced stress-related psychiatry-like behaviors in mice. Some of the effects have been found to be mediated by the vagus nerve (2). However, effects of the same strain were not found in a clinical study in healthy

volunteers, indicating possibly the challenge of translating preclinical studies into clinical relevance (57).

Two studies using fMRI measuring brain activations to negative emotional stimuli showed reduced activations in regions such as the insula, somatosensory, amygdala and fronto-limbic areas, which are associated with neural processing of stress and emotion (3, 8). In our recent study, rifaximin had effects on the neural response to the social stress implying negative emotions, but on different brain activities - reduced frontal and cingulate beta band power (25). Therefore, although affecting neural response to emotional processing like probiotics, rifaximin may act through different neural pathways than probiotics. Although the central mechanisms by which rifaximin affects stress management are not well understood, one may speculate on an "eubiotic" effect of probiotics promoting beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* (30, 31). Therefore, although such "psychobiotic" effects appear to be strain-dependent, results of existing studies help to understand the possible pathways of the strain we used in the current trial. Effects of different strains on diverse neural oscillations in response to stress are recommended to obtain a detailed picture of the effects of probiotics on brain functioning.

5 Conclusion

As a putative psychobiotic, *B.longum* 1714 showed effects on influencing resting neural activities associated with reduced mental fatigue, and on neural responses to social stress induced by an virtual online game. Our results have provided new evidence for this “psychobiotic” strain to affect central functions through activation of certain brain regions. The understanding of neural oscillations activated by stress and their modulation by

psychobiotics is still at its beginning. Studies that examine the mechanisms of this psychobiotics along the gut-brain axis and to compare their effects on other central nervous system functions with other interventions are warranted. In order to get the full picture of the effects of *B.longum* 1714 on the brain future research should consider also its effects on other central nervous system functions, for example pain sensitivity, mood, memory abilities, both in healthy controls and in patients with psychiatric (depression, anxiety), neurologic (neurodegenerative), and gastrointestinal disorders such as IBS.

6 Conflicts of interest

Eileen Murphy is Research Director at Alimentary Health. Alimentary Health provided the placebo and probiotic containing the *B. longum* 1714 strain, but had no further influence on the design of the study, the data collection, and the data evaluation. The authors declare no potential conflicts of interest.

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Reference

1. Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. Biol Psychiatry. 2013;74(10):720-6.

2. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A. 2011;108(38):16050-5.

3. Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, et al. Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome. Gastroenterology. 2017;153(2):448-59.e8.

4. Rao AV, Bested AC, Beaulne TM, Katzman MA, Iorio C, Berardi JM, et al. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. Gut Pathog. 2009;1(1):6.

5. Steenbergen L, Sellaro R, van Hemert S, Bosch JA, Colzato LS. A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. Brain Behav Immun. 2015;48:258-64.

6. Romijn AR, Rucklidge JJ. Systematic review of evidence to support the theory of psychobiotics. Nutr Rev. 2015;73(10):675-93.

7. Wang H, Lee IS, Braun C, Enck P. Effect of Probiotics on Central Nervous System Functions in Animals and Humans: A Systematic Review. J Neurogastroenterol Motil. 2016;22(4):589-605.

8. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology. 2013;144(7):1394-401, 401 e1-4.

9. Bharwani A, Mian MF, Surette MG, Bienenstock J, Forsythe P. Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. BMC Med. 2017;15(1):7.

10. Takada M, Nishida K, Kataoka-Kato A, Gondo Y, Ishikawa H, Suda K, et al. Probiotic Lactobacillus casei strain Shirota relieves stress-associated symptoms by modulating the gut-brain interaction in human and animal models. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2016;28(7):1027-36.

11. Allen AP, Hutch W, Borre YE, Kennedy PJ, Temko A, Boylan G, et al. Bifidobacterium longum 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. Translational psychiatry. 2016;6(11):e939.

12. Krieger N. Theories for social epidemiology in the 21st century: an ecosocial perspective. Int J Epidemiol. 2001;30(4):668-77.

13. Williams KD, Jarvis B. Cyberball: a program for use in research on interpersonal ostracism and acceptance. Behavior research methods. 2006;38(1):174-80.

14. Beekman JB, Stock ML, Marcus T. Need to Belong, Not Rejection Sensitivity, Moderates Cortisol Response, Self-Reported Stress, and Negative Affect Following Social Exclusion. The Journal of social psychology. 2016;156(2):131-8.

15. Blackhart GC, Eckel LA, Tice DM. Salivary cortisol in response to acute social rejection and acceptance by peers. Biol Psychol. 2007;75(3):267-76.

16. Kelly M, McDonald S, Rushby J. All alone with sweaty palms--physiological arousal and ostracism. International journal of psychophysiology : official journal of the International Organization of Psychophysiology. 2012;83(3):309-14.

17. McQuaid RJ, McInnis OA, Matheson K, Anisman H. Distress of ostracism: oxytocin receptor gene polymorphism confers sensitivity to social exclusion. Soc Cogn Affect Neurosci. 2015;10(8):1153-9.

18. Wang H, Braun C, Enck P. How the brain reacts to social stress (exclusion) - A scoping review. Neuroscience and biobehavioral reviews. 2017;80:80-8.

19. Cristofori I, Harquel S, Isnard J, Mauguiere F, Sirigu A. Monetary reward suppresses anterior insula activity during social pain. Soc Cogn Affect Neurosci. 2015;10(12):1668-76.

20. Cristofori I, Moretti L, Harquel S, Posada A, Deiana G, Isnard J, et al. Theta signal as the neural signature of social exclusion. Cerebral cortex (New York, NY : 1991). 2013;23(10):2437-47.

21. Kawamoto T, Nittono H, Ura M. Cognitive, Affective, and Motivational Changes during Ostracism: An ERP, EMG, and EEG Study Using a Computerized Cyberball Task. Neuroscience journal. 2013;2013:304674.

22. van Noordt SJ, White LO, Wu J, Mayes LC, Crowley MJ. Social exclusion modulates event-related frontal theta and tracks ostracism distress in children. Neuroimage. 2015;118:248-55.

23. Cohen D, Cuffin BN. Demonstration of useful differences between magnetoencephalogram and electroencephalogram. Electroencephalography and clinical neurophysiology. 1983;56(1):38-51.

24. Hall EL, Robson SE, Morris PG, Brookes MJ. The relationship between MEG and fMRI. Neuroimage. 2014;102:80-91.

25. Wang H, Braun C, Enck P. Effects of rifaximin on central responses to social stress - a pilot experiment. Neurotherapeutics. Submitted.

26. Li J, Zhu W, Liu W, Wu Y, Wu B. Rifaximin for Irritable Bowel Syndrome: A Meta-Analysis of Randomized Placebo-Controlled Trials. Medicine (Baltimore). 2016;95(4):e2534.

27. Gatta L, Scarpignato C. Systematic review with meta-analysis: rifaximin is effective and safe for the treatment of small intestine bacterial overgrowth. Aliment Pharm Ther. 2017;45(5):604-16.

28. Ng QX, Ho CYX, Shin D, Venkatanarayanan N, Chan HW. A meta-analysis of the use of rifaximin to prevent travellers' diarrhoea. Journal of travel medicine. 2017;24(5).

29. Maccaferri S, Vitali B, Klinder A, Kolida S, Ndagijimana M, Laghi L, et al. Rifaximin modulates the colonic microbiota of patients with Crohn's disease: an in vitro approach using a continuous culture colonic model system. The Journal of antimicrobial chemotherapy. 2010;65(12):2556-65.

30. Ponziani FR, Scaldaferri F, Petito V, Paroni Sterbini F, Pecere S, Lopetuso LR, et al. The Role of Antibiotics in Gut Microbiota Modulation: The Eubiotic Effects of Rifaximin. Dig Dis. 2016;34(3):269-78.

31. Xu D, Gao J, Gillilland M, 3rd, Wu X, Song I, Kao JY, et al. Rifaximin alters intestinal bacteria and prevents stress-induced gut inflammation and visceral hyperalgesia in rats. Gastroenterology. 2014;146(2):484-96 e4.

32. Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2014;26(11):1615-27.

33. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive processes in an anxious mouse strain. Behav Brain Res. 2015;287:59-72.

34. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology. 2006;130(5):1480-91.

35. Kroenke K, Spitzer RL, Williams JB, Lowe B. The Patient Health Questionnaire Somatic, Anxiety, and Depressive Symptom Scales: a systematic review. General hospital psychiatry. 2010;32(4):345-59.

36. Ware JE, Jr., Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care. 1992;30(6):473-83.

37. Sebastian C, Viding E, Williams KD, Blakemore SJ. Social brain development and the affective consequences of ostracism in adolescence. Brain Cogn. 2010;72(1):134-45.

38. Oostenveld R, Fries P, Maris E, Schoffelen JM. FieldTrip: Open source software for advanced analysis of MEG, EEG, and invasive electrophysiological data. Comput Intell Neurosci. 2011;2011:156869.

39. Gross J, Kujala J, Hamalainen M, Timmermann L, Schnitzler A, Salmelin R. Dynamic imaging of coherent sources: Studying neural interactions in the human brain. Proc Natl Acad Sci U S A. 2001;98(2):694-9.

40. An YJ, Jung KY, Kim SM, Lee C, Kim DW. Effects of blood glucose levels on resting-state EEG and attention in healthy volunteers. J Clin Neurophysiol. 2015;32(1):51-6.

41. Wang C, Szabo JS, Dykman RA. Effects of a carbohydrate supplement upon resting brain activity. Integrative physiological and behavioral science : the official journal of the Pavlovian Society. 2004;39(2):126-38.

42. Ishii A, Tanaka M, Watanabe Y. The neural mechanisms underlying the decision to rest in the presence of fatigue: a magnetoencephalography study. PLoS One. 2014;9(10):e109740.

43. Tanaka M, Ishii A, Watanabe Y. Neural effects of mental fatigue caused by continuous attention load: a magnetoencephalography study. Brain Res. 2014;1561:60-6.

44. Diego MA, Field T, Sanders C, Hernandez-Reif M. Massage therapy of moderate and light pressure and vibrator effects on EEG and heart rate. Int J Neurosci. 2004;114(1):31-44.

45. Field T, Ironson G, Scafidi F, Nawrocki T, Goncalves A, Burman I, et al. Massage therapy reduces anxiety and enhances EEG pattern of alertness and math computations. Int J Neurosci. 1996;86(3-4):197-205.

46. LeBlanc JG, Chain F, Martin R, Bermudez-Humaran LG, Courau S, Langella P. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microb Cell Fact. 2017;16(1):79.

47. Bolling DZ, Pelphrey KA, Vander Wyk BC. Unlike adults, children and adolescents show predominantly increased neural activation to social exclusion by members of the opposite gender. Social neuroscience. 2016;11(5):475-86.

48. Luo S, Yu D, Han S. Genetic and neural correlates of romantic relationship satisfaction. Soc Cogn Affect Neurosci. 2016;11(2):337-48.

49. Preller KH, Pokorny T, Hock A, Kraehenmann R, Stampfli P, Seifritz E, et al. Effects of serotonin 2A/1A receptor stimulation on social exclusion processing. Proc Natl Acad Sci U S A. 2016;113(18):5119-24.

50. Chang PF, Arendt-Nielsen L, Chen AC. Dynamic changes and spatial correlation of EEG activities during cold pressor test in man. Brain Res Bull. 2002;57(5):667-75.

51. Chen AC, Dworkin SF, Haug J, Gehrig J. Topographic brain measures of human pain and pain responsivity. Pain. 1989;37(2):129-41.

52. Luijcks R, Vossen CJ, Hermens HJ, van Os J, Lousberg R. The Influence of Perceived Stress on Cortical Reactivity: A Proof-Of-Principle Study. PLoS One. 2015;10(6):e0129220.

53. Roozendaal B, McEwen BS, Chattarji S. Stress, memory and the amygdala. Nat Rev Neurosci. 2009;10(6):423-33.

54. Liang S, Wang T, Hu X, Luo J, Li W, Wu X, et al. Administration of Lactobacillus helveticus NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. Neuroscience. 2015;310:561-77.

55. Liu WH, Chuang HL, Huang YT, Wu CC, Chou GT, Wang S, et al. Alteration of behavior and monoamine levels attributable to Lactobacillus plantarum PS128 in germ-free mice. Behav Brain Res. 2016;298(Pt B):202-9.

56. Liu YW, Liu WH, Wu CC, Juan YC, Wu YC, Tsai HP, et al. Psychotropic effects of Lactobacillus plantarum PS128 in early life-stressed and naive adult mice. Brain Res. 2016;1631:1-12.

57. Kelly JR, Allen AP, Temko A, Hutch W, Kennedy PJ, Farid N, et al. Lost in translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects. Brain Behav Immun. 2017;61:50-9.

Table 1. Demographic and baseline information.

	***B.longum* 1714**	**Placebo**	***P* value**
Sex			
Male	N=7	N=7	n/a
Female	N=13	N=13	
Birth delivery	N=2	N=2	n/a
Caesarean section	N=18	N=18	
Vaginal delivery			
Age	31.00±2.28	33.00±2.83	ns.
BMI	23.00±0.68	22.00±0.55	ns.
SF36			
Physical functioning	96.84±1.03	97.63±0.80	
Role limitations due to physical health	100.00±0.00	100.00±0.00	
Role limitations due to emotion problems	100.00±0.00	95.00±5.00	ns.
Energy/Vitality	72.25±2.42	75.25±2.94	
Emotional well-being	85.78±1.22	84.42±1.75	
Social functioning	100.00±0.00	94.74±2.20	
Pain	88.75±2.67	89.75±2.80	
General health	82.50±2.31	87.37±2.40	
Cyberball game			
NTS			
-Inclusion	-28.30±3.55	-36.90±2.28	
-Exclusion	14.33±4.55	26.90±3.48	
MQ			
-Inclusion	13.80±1.47	18.03±0.99	ns.
-Exclusion	2.38±2.25	-5.48±2.17	
SEP			
-Inclusion	-4.68±0.28	-5.30±0.21	
-Exclusion	0.15±0.92	2.23±0.61	

Abbreviations: BMI, body mass index; SF36, 36-item short-form health survey NTS, Need Threat Scale; MQ, mood questionnaire; SEP, subjective exclusion perception; ns. not significant.

Table 2. Summarized neuroanatomical areas and frequency bands of changed neural activities influenced by effect of condition, intervention and/or interaction of condition and intervention.

Comparison	Frequency band	Brain region	Hemisphere	*P* vale
Intervention effect on resting state: *B.longum* group vs. Placebe group	Theta ↑	IFC	B	<0.05
		MFC	B	
		SFC	B	
		ACC	B	
		MCC	B	
	Beta-3 ↓	FFG	B	<0.05
		HIPP	B	
		ITC	L	
		MTC	B	
		STC	L	
		CBL	L	
Intervention effect on the Cyberball game for all conditions: *B.longum* group vs. Placebo group	Theta ↑	IFC	R	0.03
		MFC	B	
		SFC	B	
		ACC	L	
		MCC	B	
		SMG	R	
	Alpha ↑	IFC	R	0.04
		MFC	B	
		SFC	B	
		ACC	B	
		MCC	B	
		SMG	R	

Abbreviations: IFC, inferior frontal cortex; MFC, middle frontal cortex; ACC, anterior cingulate cortex; MCC, middle cingulate cortex; FFG, fusiform gyrus; HIPP, hippocampus; ITC, inferior temporal cortex; STC, superior temporal cortex; MTC, middle temporal cortex; CBL, cerebellum; SMG, supramarginal gyrus; B, bilateral; L, left; R, right.

Table 3. Summarized correlations of changes of averaged power in activated areas with changes of subjective results. The changes of neural activation during resting state was correlated with changes of SF36, and the changes of the neural activation during Cyberball game was correlated with changes of the NTS, respectively for each of the two conditions of exclusion and inclusion.

Groups	Subjective item	Resting state MEG		Functional MEG during Cyberball	
		Theta band power change	Beta-3 band power change	Theta band power change	Alpha band power change
Both groups	SF36-Energy/ Vitality	$r = 0.33$ $p = 0.04$	-	-	-
***B.longum* group**	SF36-Energy/ Vitality	$r = 0.61$ $p = 0.007$	$r = -0.50$ $p = 0.04$	-	-
	NTS			$r = 0.62$ $p = 0.008$	$r = 0.54$ $p = 0.03$

Abbreviations: SF36, 36-item short-form health survey; NTS, Need Threat Scale; MEG, magnetoencephalography.

Figure legend

Figure 1. The CONSORT flow diagram of the clinical trial

Figure 2. Schematic outline of a trial in the Cyberball game.

Figure 3. Main effects of condition on NTS and SEP. Participants in all groups reported increased scores of NTS and SEP in the exclusion condition compared to inclusion condition, after 4 weeks' intervention.

Figure 4. Difference of neural activity change during resting-state comparing *B.longum* 1714 vs. placebo. A. After the intervention, an increased theta band (6Hz) power was obtained in a cluster including regions of bilateral IFC, MFC, and the bilateral ACC and MCC, comparing *B.longum* 1714 with the placebo group ($p < 0.05$). **B.** After the intervention, reduced beta-2 band (26Hz) power was obtained in a cluster, consisting of the bilateral FFG and HIPP, left ITC and STC, bilateral MTC and left CBL, comparing *B.longum* 1714 with the placebo group ($p < 0.05$).

Figure 5. Correlation between neural activity change and SF36 change. A. In all group, a positive correlation was obtained between changes of SF36 item "Energy/Vitality" with changes of theta band power in the cluster ($r = 0.33$, $p = 0.04$). **B.** In only *B.longum* 1714 group, changes of SF36 item 'Energy/Vitality' positively correlated with change of averaged theta band power ($r = 0.61$, $p = 0.007$), and negatively correlated with change of beta-3 band power in the activated clusters during the resting state, respectively ($r = -0.50$, $p = 0.04$).

Figure 6. Difference of neural activity change during the CBG comparing B.longum 1714 vs. placebo. A. Theta band showed an increased power in a cluster, consisting of the right IFC and the bilateral MFC and SFC, the left ACC the bilateral MCC, and the right supramarginal gyrus (SMG), comparing *B.longum* 1714 group and the placebo group in both conditions ($p = 0.03$). **B.** Alpha band power also showed an increased power in cluster, including regions of the right IFC the bilateral MFC and SFC, the bilateral ACC and MCC, and the right SMG, comparing *B.longum* 1714 group and the placebo group in both conditions ($p = 0.04$). No main effect of condition or interaction of intervention and condition were observed.

Figure 7. Correlation between neural activity change during the CBG and subjective score changes. Only in *B.longum* 1714 group and only during the exclusion condition, NTS changes positively correlated with changes of the theta band power ($r = 0.62$, $p = 0.008$) and alpha band power ($r = 0.54$, $p = 0.03$).

CONSORT
TRANSPARENT REPORTING of TRIALS

CONSORT 2010 Flow Diagram

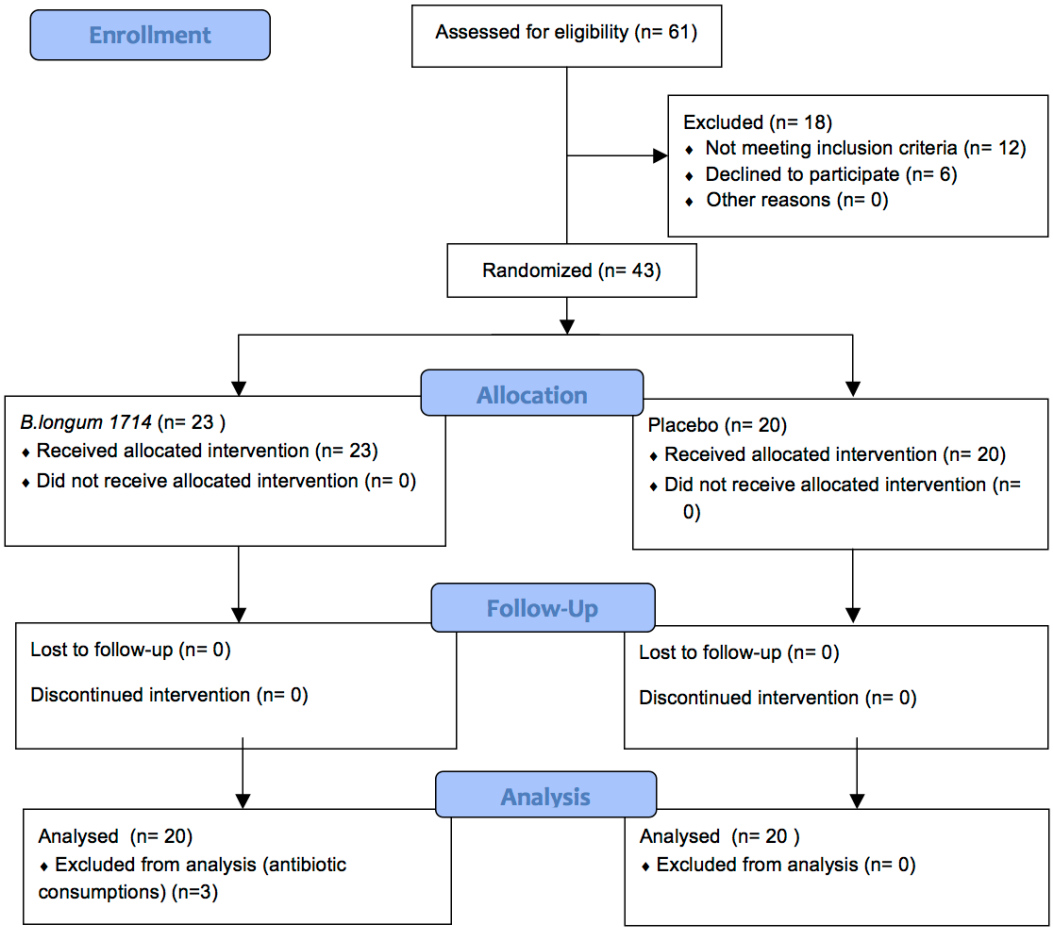

Figure 1.

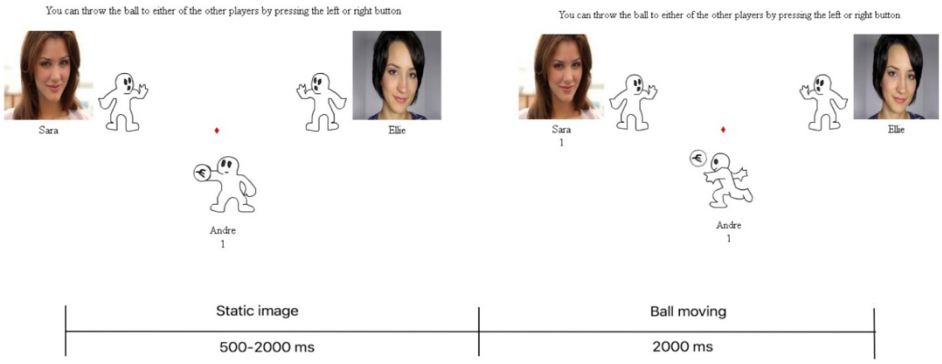
You can throw the ball to either of the other players by pressing the left or right button
Sara
Ellie
Andre
1
You can throw the ball to either of the other players by pressing the left or right button
Sara
1
Ellie
Andre
1
Static image
500-2000 ms
Ball moving
2000 ms

Figure 2.

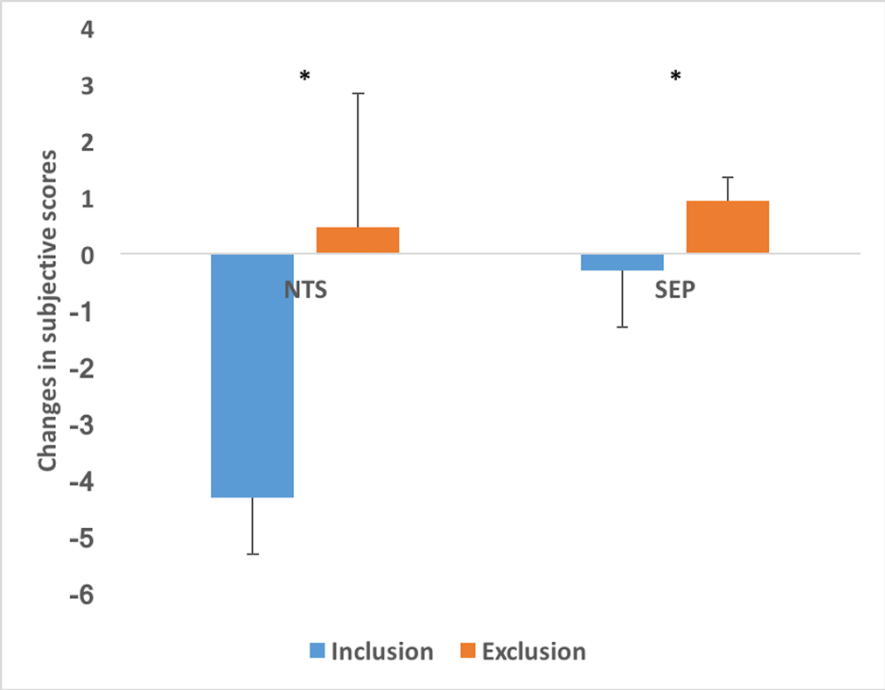
4
3
2
1
0
-1
-2
-3
-4
-5
-6
Changes in subjective scores
*
*
NTS
SEP
Inclusion
Exclusion

Figure 3.

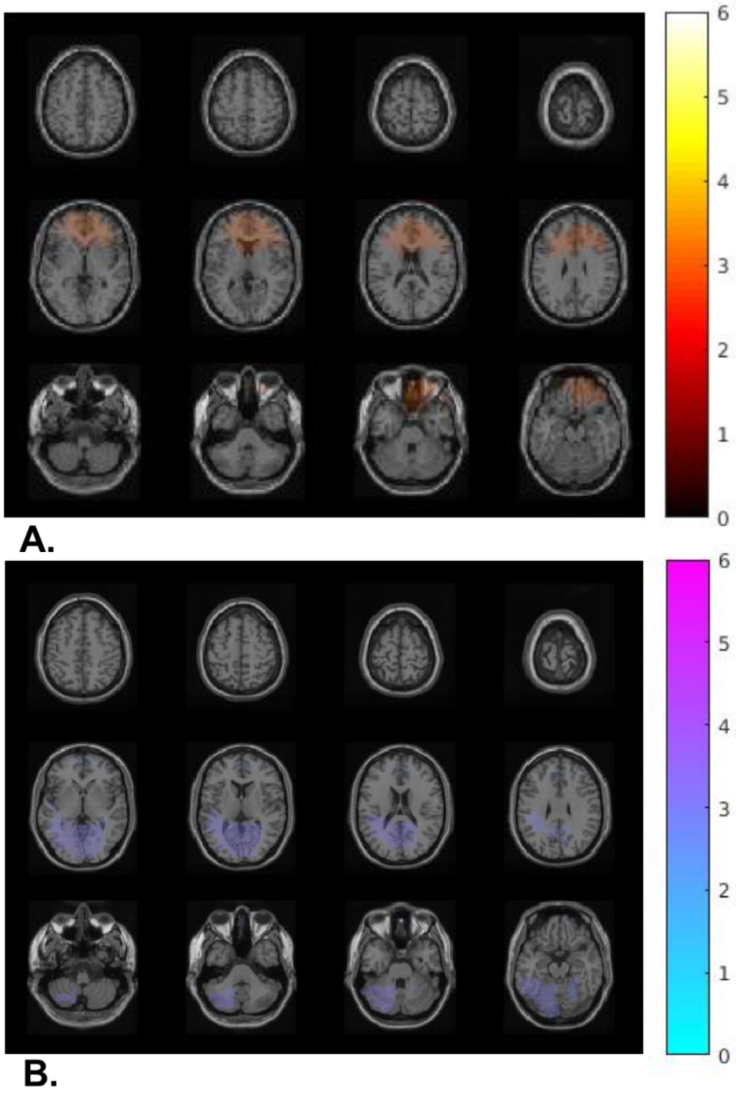

Figure 4.

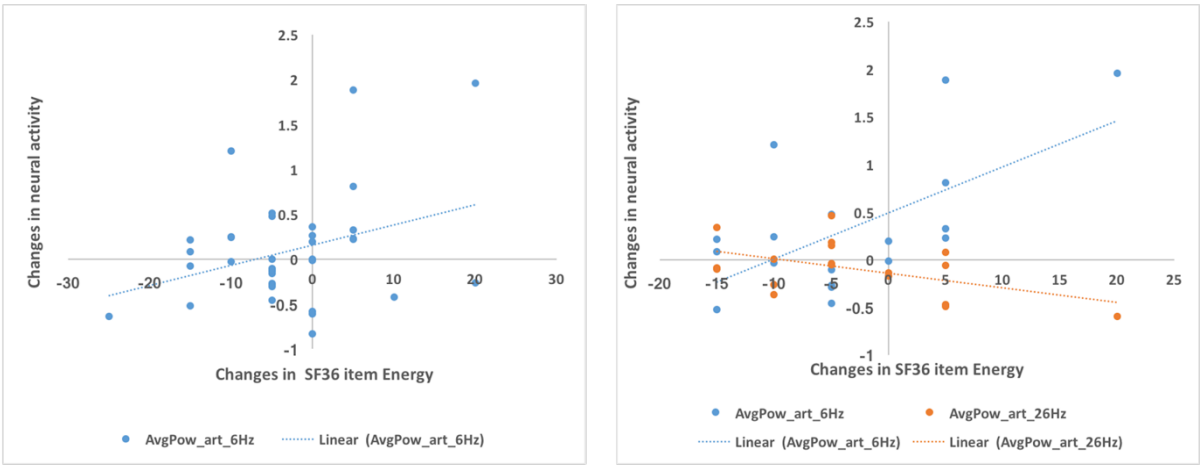
Changes in neural activity
Changes in SF36 item Energy
AvgPow_art_6Hz
Linear (AvgPow_art_6Hz)
A.
Changes in neural activity
Changes in SF36 item Energy
AvgPow_art_6Hz
AvgPow_art_26Hz
Linear (AvgPow_art_6Hz)
Linear (AvgPow_art_26Hz)
B.

Figure 5.

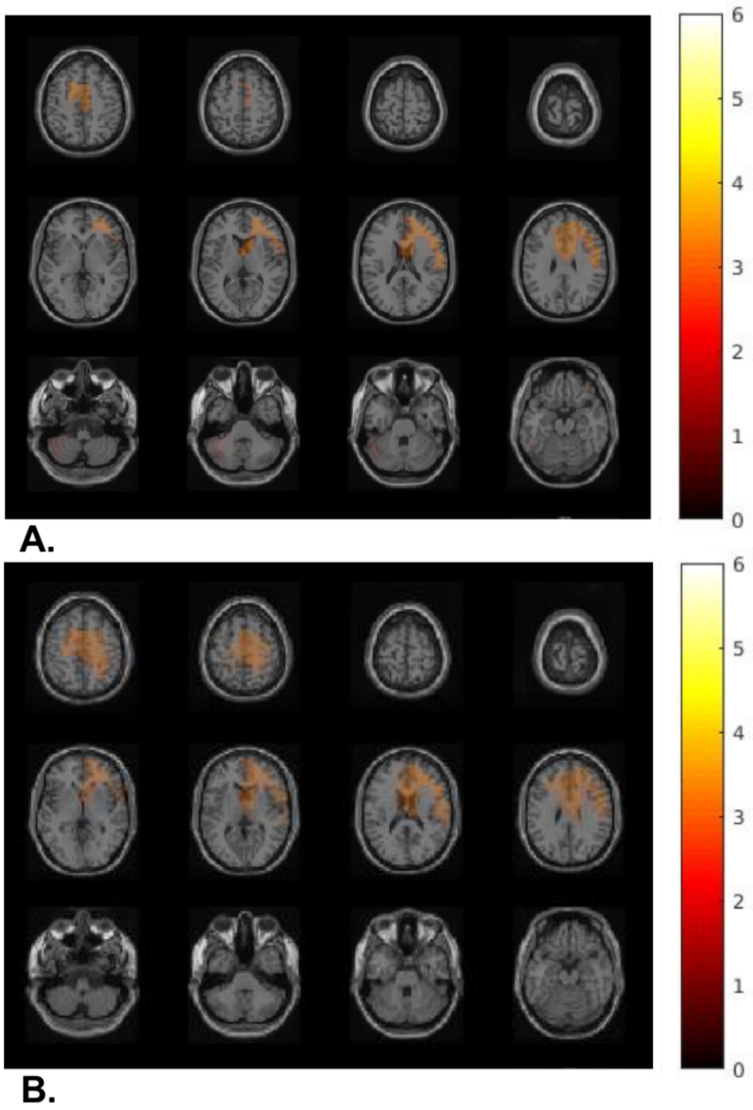

Figure 6.

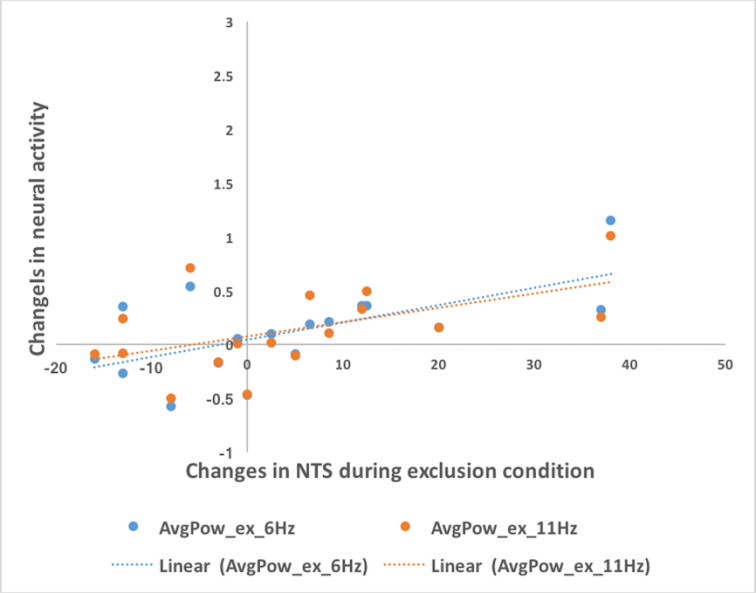
Changels in neural activity
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-20
-10
0
10
20
30
40
50
Changes in NTS during exclusion condition
AvgPow_ex_6Hz
AvgPow_ex_11Hz
Linear (AvgPow_ex_6Hz)
Linear (AvgPow_ex_11Hz)

Figure 7.

Appendix 1. Probiotic/prebiotic containing food not to eat during intervention (same as Page 95)

Appendix 2. Assessment of Need Threat Scale, Mood Questionnaire and Exclusion Perception (same as Page 96)

10. Conclusion and indication

According to the results in study I, II, III and IV, we conclude: 1) probiotics, also considered psychobiotics, play a role in gut-brain axis and have effects on central nervous system functions. *Bifidobacterium* and *Lactobacillus* are most effective in improving psychiatric disease associated functions (anxiety, depression, mood and stress response). 2) While the animal models are mostly standardized, developing experiment models in clinical trials to study effects of probiotics on CNS functions are applicable and in need. 3) Stress response is a crucial element influencing other CNS functions, and also mediates gut-brain interaction. There are paradigms to study stress response in humans, but feasibility of adapting these paradigms to neuroimaging experiments need to be verified. 4) The Cyberball game is a widely used social psychological paradigm that can induce stress. It involves different stages of neural processes in certain brain regions, which are influenced by many factors such as psychological health conditions and early life experience. 5) The neural signature of social stress by the CBG can be identified by MEG, showing oscillatory neuromagnetic activities in different frequency bands ranging from theta to beta oscillations. 6) Rifaximin as an antibiotic, can modulate brain activity associated with improved the relaxation status during a resting state and during the social stress. 7) Probiotic *B.longum* 1714 can alter resting neural activity associated with mental vitality and neural response during social stress by enhancing emotion regulation.

Indicated by the results of our studies, future studies could be carried out in the following aspects: 1) specification of the effects of a single probiotic strain, 2) development of human models in GM-gut-brain axis, 3) correlation of microbiome composition and physiological parameters with cortical activities, 4) clinical studies in patients with mental disorders and GI disorders, e.g. IBS.

According to the systematic review in study I, different types of probiotics treatments in either single strain or multiple strains, were applied in the previous studies. Although some of the treatments showed influences on more or less common CNS functions, they appeared to go through diverse pathways – either by altering immunomodulating cytokine levels, or neurotransmitter metabolisms, or via the vagus nerve (110). Also, it happened that the same strain showed effects in different situations, e.g. *L. plantarum* PS128 had general anxiety reducing effects in mice under different experiment set ups while antidepressant effect only in mice that experienced early stress but not in GF mice (35, 36). Therefore, more studies need to be conducted to explore the specific effects of single probiotics on changes of biochemical substances and behavior. In addition, combinations of multi-strains of probiotics have been reported to have larger/greater health benefits (111). Although the relevant evidences are yet limited, it would be interesting to test interaction effects of multiple probiotic strains and also their efficacy on CNS functions.

Human studies in this field are less abundant compared to animal studies, and most of them utilized only psychological questionnaires or scales, rather than measuring behavioral or neural changes by probiotics during an experimental task. Possible experimental setups have been described in study I, and thus will not be expanded here again. Worthy to note that, multimodel neuroimaging technique such as EEG-fMRI may allow an ongoing investigations of neural effects of probiotics/antibiotics in both, high temporal and spatial resolutions (112). Nevertheless, studies are needed to bridge the gap between preclinical and clinical studies.

As considered as “eubiotics”, rifaximin and probiotics *B.longum* 1714 may promote the growth of certain bacteria and produce a favorable gut microbiota environment so as to foster the their beneficial effects on the brain (1). A further comprehensive analysis of the

correlation of GM composition, physiological changes, cortical activities and behavioral output would be helpful to understand the effects of these eubiotics in the gut-brain axis.

Effects of probiotics and rifaximin have both been tested for their effects on relieving IBS symptoms (113, 114). However, although probiotics have also been investigated for effects on psychiatric comorbidities of IBS, no convincing evidence has been demonstrrated (115-117). Only a recent fMRI study has reported an alleviated depression symptom and reduced limbic brain activations to emotional stimuli in IBS patients (67). More similar studies using neuroimaging methods in patients with psychiatric and GI disorders are required. Also, antibiotic rifaximin may be a promising candidate as well to study its neural functions in clinics.

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Hereby, I would like to thank Prof. Dr. Paul Enck and Prof. Dr. Christoph Braun, for their supervisions on my study and supports to my life in Germany. Paul inspired me with his open mind to design my PhD projects, provided me with many opportunities to attend international and national academic meetings, and trained me as a researcher rather than only a student. Christoph supervised me day to day – I feel greatly thankful to him for his appearance whenever I needed him. I learned much from him on neuroscience theories and programming. Also, he is not only a supervisor but also a good friend indeed.

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12. References

1. Ponziani FR, Scaldaferri F, Petito V, Paroni Sterbini F, Pecere S, Lopetuso LR, et al. The Role of Antibiotics in Gut Microbiota Modulation: The Eubiotic Effects of Rifaximin. Dig Dis. 2016;34(3):269-78.

2. Zoppi G. [Probioticss, prebiotics,synbiotics and eubiotics]. Pediatr Med Chir. 1998;20(1):13-7.

3. Cryan JF, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil. 2011;23(3):187-92.

4. Mayer EA. Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci. 2011;12(8):453-66.

5. Fadgyas-Stanculete M, Buga AM, Popa-Wagner A, Dumitrascu DL. The relationship between irritable bowel syndrome and psychiatric disorders: from molecular changes to clinical manifestations. Journal of molecular psychiatry. 2014;2(1):4.

6. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol. 2012;10(11):735-42.

7. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124(4):837-48.

8. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355-9.

9. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308(5728):1635-8.

10. Dominguez-Bello MG, Blaser MJ, Ley RE, Knight R. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. Gastroenterology. 2011;140(6):1713-9.

11. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647-51.

12. Walsh CJ, Guinane CM, O'Toole PW, Cotter PD. Beneficial modulation of the gut microbiota. FEBS Lett. 2014;588(22):4120-30.

13. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci. 2012;13(10):701-12.

14. Farmer AD, Randall HA, Aziz Q. It's a gut feeling: How the gut microbiota affects the state of mind. J Physiol-London. 2014;592(14):2981-8.

15. Keightley PC, Koloski NA, Talley NJ. Pathways in gut-brain communication: evidence for distinct gut-to-brain and brain-to-gut syndromes. Aust N Z J Psychiatry. 2015;49(3):207-14.

16. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol. 2004;558(Pt 1):263-75.

17. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. Mol Psychiatr. 2014;19(2):146-8.

18. Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, et al. Bacterial infection causes stress-induced memory dysfunction in mice. Gut. 2011;60(3):307-17.

19. Nishino R, Mikami K, Takahashi H, Tomonaga S, Furuse M, Hiramoto T, et al. Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol Motil. 2013;25(6):521-8.

20. Jorgensen BP, Hansen JT, Krych L, Larsen C, Klein AB, Nielsen DS, et al. A Possible Link between Food and Mood: Dietary Impact on Gut Microbiota and Behavior in BALB/c Mice. Plos One. 2014;9(8).

21. Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology. 2010;139(6):2102-12 e1.

22. Benton D, Williams C, Brown A. Impact of consuming a milk drink containing a probiotic on mood and cognition. Eur J Clin Nutr. 2007;61(3):355-61.

23. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. Journal of psychiatric research. 2008;43(2):164-74.

24. Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. Neuroscience. 2010;170(4):1179-88.

25. Scheperjans F, Aho V, Pereira PA, Koskinen K, Paulin L, Pekkonen E, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord. 2015;30(3):350-8.

26. Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, et al. Gastrointestinal microbiota in children with autism in Slovakia. Physiol Behav. 2015;138:179-87.

27. Lilly DM, Stillwell RH. PROBIOTICS: GROWTH-PROMOTING FACTORS PRODUCED BY MICROORGANISMS. Science. 1965;147(3659):747-8.

28. Rijkers GT, de Vos WM, Brummer RJ, Morelli L, Corthier G, Marteau P. Health benefits and health claims of probiotics: bridging science and marketing. Br J Nutr. 2011;106(9):1291-6.

29. Ford AC, Quigley EMM, Lacy BE, Lembo AJ, Saito YA, Schiller LR, et al. Efficacy of Prebiotics, Probiotics, and Synbiotics in Irritable Bowel Syndrome and Chronic Idiopathic

Constipation: Systematic Review and Meta-analysis. Am J Gastroenterol. 2014;109(10):1547-61.

30. Mazurak N, Broelz E, Storr M, Enck P. Probiotic Therapy of the Irritable Bowel Syndrome: Why Is the Evidence Still Poor and What Can Be Done About It? J Neurogastroenterol. 2015;21(4):471-85.

31. Chichlowski M, Rudolph C. Visceral pain and gastrointestinal microbiome. J Neurogastroenterol Motil. 2015;21(2):172-81.

32. Dinan TG, Stanton C, Cryan JF. Psychobiotics: A Novel Class of Psychotropic. Biol Psychiat. 2013;74(10):720-6.

33. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, et al. The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil. 2011;23(12):1132-9.

34. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A. 2011;108(38):16050-5.

35. Liu WH, Chuang HL, Huang YT, Wu CC, Chou GT, Wang S, et al. Alteration of behavior and monoamine levels attributable to Lactobacillus plantarum PS128 in germ-free mice. Behavioural brain research. 2016;298(Pt B):202-9.

36. Liu YW, Liu WH, Wu CC, Juan YC, Wu YC, Tsai HP, et al. Psychotropic effects of Lactobacillus plantarum PS128 in early life-stressed and naive adult mice. Brain Res. 2016;1631:1-12.

37. O'Sullivan E, Barrett E, Grenham S, Fitzgerald P, Stanton C, Ross RP, et al. BDNF expression in the hippocampus of maternally separated rats: does Bifidobacterium breve 6330 alter BDNF levels? Benef Microbes. 2011;2(3):199-207.

38. Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, et al. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology. 2012;37(11):1885-95.

39. Kwok LY, Wang L, Zhang J, Guo Z, Zhang H. A pilot study on the effect of Lactobacillus casei Zhang on intestinal microbiota parameters in Chinese subjects of different age. Benef Microbes. 2014;5(3):295-304.

40. Smith CJ, Emge JR, Berzins K, Lung L, Khamishon R, Shah P, et al. Probiotics normalize the gut-brain-microbiota axis in immunodeficient mice. Am J Physiol Gastrointest Liver Physiol. 2014;307(8):G793-802.

41. Wang T, Hu X, Liang S, Li W, Wu X, Wang L, et al. Lactobacillus fermentum NS9 restores the antibiotic induced physiological and psychological abnormalities in rats. Benef Microbes. 2015;6(5):707-17.

42. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451-63.

43. Crocq MA. A history of anxiety: from Hippocrates to DSM. Dialogues Clin Neurosci. 2015;17(3):319-25.

44. Liang S, Wang T, Hu X, Luo J, Li W, Wu X, et al. Administration of Lactobacillus helveticus NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. Neuroscience. 2015;310:561-77.

45. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, et al. Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. Br J Nutr. 2011;105(5):755-64.

46. Messaoudi M, Violle N, Bisson JF, Desor D, Javelot H, Rougeot C. Beneficial psychological effects of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in healthy human volunteers. Gut Microbes. 2011;2(4):256-61.

47. Rao AV, Bested AC, Beaulne TM, Katzman MA, Iorio C, Berardi JM, et al. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. Gut Pathog. 2009;1(1):6.

48. Vilagut G, Forero CG, Barbaglia G, Alonso J. Screening for Depression in the General Population with the Center for Epidemiologic Studies Depression (CES-D): A Systematic Review with Meta-Analysis. PLoS One. 2016;11(5):e0155431.

49. Schmidt PJ. Mood, depression, and reproductive hormones in the menopausal transition. Am J Med. 2005;118 Suppl 12B:54-8.

50. Ivanov HY, Stoyanova VK, Popov NT, Vachev TI. Autism Spectrum Disorder - A Complex Genetic Disorder. Folia Med (Plovdiv). 2015;57(1):19-28.

51. Thomas RH, Meeking MM, Mepham JR, Tichenoff L, Possmayer F, Liu SY, et al. The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. J Neuroinflamm. 2012;9.

52. Kaluzna-Czaplinska J, Blaszczyk S. The level of arabinitol in autistic children after probiotic therapy. Nutrition. 2012;28(2):124-6.

53. Parracho HMRTG, G.R.; Knott, F.; Bosscher, D., Kleerebezem, M.; McCartney, A.L. . A double-blind, placebo-controlled, crossover-designed probiotic feeding study in children diagnosed with autistic spectrum disorders. International Journal of Probiotics and Prebiotics. 2010;5(2):69-74.

54. Srinivasjois R, Rao S, Patole S. Probiotic supplementation in children with autism spectrum disorder. Arch Dis Child. 2015;100(5):505-6.

55. Arseneault-Breard J, Rondeau I, Gilbert K, Girard SA, Tompkins TA, Godbout R, et al. Combination of Lactobacillus helveticus R0052 and Bifidobacterium longum R0175

reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. Br J Nutr. 2012;107(12):1793-9.

56. Jeong JJ, Kim KA, Ahn YT, Sim JH, Woo JY, Huh CS, et al. Probiotic Mixture KF Attenuates Age-Dependent Memory Deficit and Lipidemia in Fischer 344 Rats. J Microbiol Biotechnol. 2015;25(9):1532-6.

57. Liu J, Sun J, Wang F, Yu X, Ling Z, Li H, et al. Neuroprotective Effects of Clostridium butyricum against Vascular Dementia in Mice via Metabolic Butyrate. Biomed Res Int. 2015;2015:412946.

58. Ohland CL, Kish L, Bell H, Thiesen A, Hotte N, Pankiv E, et al. Effects of Lactobacillus helveticus on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. Psychoneuroendocrinology. 2013;38(9):1738-47.

59. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive processes in an anxious mouse strain. Behav Brain Res. 2015;287:59-72.

60. Cowansage KK, LeDoux JE, Monfils MH. Brain-derived neurotrophic factor: a dynamic gatekeeper of neural plasticity. Curr Mol Pharmacol. 2010;3(1):12-29.

61. Mizuno K, Giese KP. Hippocampus-dependent memory formation: Do memory type-specific mechanisms exist? J Pharmacol Sci. 2005;98(3):191-7.

62. Allen AP, Hutch W, Borre YE, Kennedy PJ, Temko A, Boylan G, et al. Bifidobacterium longum 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. Transl Psychiat. 2016;6.

63. Takada M, Nishida K, Kataoka-Kato A, Gondo Y, Ishikawa H, Suda K, et al. Probiotic Lactobacillus casei strain Shirota relieves stress-associated symptoms by modulating the gut-brain interaction in human and animal models. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2016;28(7):1027-36.

64. Allen AP, Hutch W, Borre YE, Kennedy PJ, Temko A, Boylan G, et al. Bifidobacterium longum 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. Translational psychiatry. 2016;6(11):e939.

65. Esch T, Stefano GB, Fricchione GL, Benson H. The role of stress in neurodegenerative diseases and mental disorders. Neuroendocrinol Lett. 2002;23(3):199-208.

66. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology. 2013;144(7):1394-401, 401 e1-4.

67. Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, et al. Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome. Gastroenterology. 2017;153(2):448-59 e8.

68. Jones M. Stress: Myth, theory and research. Psychol Health. 2003;18(2):275-6.

69. Esch T. Health in stress: Change in the stress concept and its significance for prevention, health and life style. Gesundheitswesen. 2002;64(2):73-81.

70. McCarty R, Gold PE. Catecholamines, stress, and disease: a psychobiological perspective. Psychosom Med. 1996;58(6):590-7.

71. Negrao AB, Deuster PA, Gold PW, Singh A, Chrousos GP. Individual reactivity and physiology of the stress response. Biomed Pharmacother. 2000;54(3):122-8.

72. McEwen BS. Brain on stress: How the social environment gets under the skin (vol 109, pg 17180, 2012). P Natl Acad Sci USA. 2013;110(4):1561-.

73. Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci. 2009;10(6):434-45.

74. McEwen BS, Sapolsky RM. Stress and cognitive function. Curr Opin Neurobiol. 1995;5(2):205-16.

75. Garcia R. Stress, hippocampal plasticity, and spatial learning. Synapse. 2001;40(3):180-3.

76. McEwen BS. Stress and hippocampal plasticity. Annu Rev Neurosci. 1999;22:105-22.

77. Konturek PC, Brzozowski T, Konturek SJ. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. J Physiol Pharmacol. 2011;62(6):591-9.

78. Camilleri M, Di Lorenzo C. Brain-gut axis: from basic understanding to treatment of IBS and related disorders. J Pediatr Gastroenterol Nutr. 2012;54(4):446-53.

79. Sperber AD, Drossman DA. Irritable bowel syndrome: a multidimensional disorder cannot be understood or treated from a unidimensional perspective. Ther Adv Gastroenter. 2012;5(6):387-93.

80. Wall GC, Bryant GA, Bottenberg MM, Maki ED, Miesner AR. Irritable bowel syndrome: a concise review of current treatment concepts. World J Gastroenterol. 2014;20(27):8796-806.

81. Dinan TG, Cryan JF. Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology. 2012;37(9):1369-78.

82. Lyte M, Vulchanova L, Brown DR. Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell Tissue Res. 2011;343(1):23-32.

83. Moloney RD, Desbonnet L, Clarke G, Dinan TG, Cryan JF. The microbiome: stress, health and disease. Mamm Genome. 2014;25(1-2):49-74.

84. Rajilic-Stojanovic M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, et al. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology. 2011;141(5):1792-801.

85. Bravo JA, Julio-Pieper M, Forsythe P, Kunze W, Dinan TG, Bienenstock J, et al. Communication between gastrointestinal bacteria and the nervous system. Curr Opin Pharmacol. 2012;12(6):667-72.

86. Scott LV, Clarke G, Dinan TG. The Brain-Gut Axis: A Target for Treating Stress-Related Disorders. Mod Tr Pharmacopsych. 2013;28:90-9.

87. Wang H, Lee IS, Braun C, Enck P. Effect of Probiotics on Central Nervous System Functions in Animals and Humans: A Systematic Review. J Neurogastroenterol Motil. 2016;22(4):589-605.

88. Cian C, Barraud PA, Melin B, Raphel C. Effects of fluid ingestion on cognitive function after heat stress or exercise-induced dehydration. International journal of psychophysiology : official journal of the International Organization of Psychophysiology. 2001;42(3):243-51.

89. Schwabe L, Haddad L, Schachinger H. HPA axis activation by a socially evaluated cold-pressor test. Psychoneuroendocrinology. 2008;33(6):890-5.

90. Kirschbaum C, Pirke KM, Hellhammer DH. The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology. 1993;28(1-2):76-81.

91. Williams KD, Jarvis B. Cyberball: a program for use in research on interpersonal ostracism and acceptance. Behav Res Methods. 2006;38(1):174-80.

92. Wang H, Braun C, Enck P. How the brain reacts to social stress (exclusion) - A scoping review. Neuroscience and biobehavioral reviews. 2017;80:80-8.

93. Luczynski P, Neufeld KAM, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. Int J Neuropsychoph. 2016;19(8).

94. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology. 2011;141(2):599-609, e1-3.

95. Desbonnet L, Clarke G, Traplin A, O'Sullivan O, Crispie F, Moloney RD, et al. Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. Brain Behav Immun. 2015;48:165-73.

96. Frohlich EE, Farzi A, Mayerhofer R, Reichmann F, Jacan A, Wagner B, et al. Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. Brain Behav Immun. 2016;56:140-55.

97. Macknin ML. Behavioral changes after amoxicillin-clavulanate. Pediatr Infect Dis J. 1987;6(9):873-4.

98. Kuhn M, Grave S, Bransfield R, Harris S. Long term antibiotic therapy may be an effective treatment for children co-morbid with Lyme disease and Autism Spectrum Disorder. Med Hypotheses. 2012;78(5):606-15.

99. Wang H, Braun C, Enck P. Effects of rifaximin on central responses to social stress - a pilot experiment. Neurotherapeutics. Submitted.

100. DuPont HL. Biologic properties and clinical uses of rifaximin. Expert Opin Pharmacother. 2011;12(2):293-302.

101. DuPont HL. Therapeutic Effects and Mechanisms of Action of Rifaximin in Gastrointestinal Diseases. Mayo Clin Proc. 2015;90(8):1116-24.

102. Pimentel M, Morales W, Chua K, Barlow G, Weitsman S, Kim G, et al. Effects of rifaximin treatment and retreatment in nonconstipated IBS subjects. Dig Dis Sci. 2011;56(7):2067-72.

103. Pimentel M, Park S, Mirocha J, Kane SV, Kong Y. The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome: a randomized trial. Ann Intern Med. 2006;145(8):557-63.

104. Gatta L, Scarpignato C. Systematic review with meta-analysis: rifaximin is effective and safe for the treatment of small intestine bacterial overgrowth. Aliment Pharmacol Ther. 2017;45(5):604-16.

105. Scarpellini E, Gabrielli M, Lauritano CE, Lupascu A, Merra G, Cammarota G, et al. High dosage rifaximin for the treatment of small intestinal bacterial overgrowth. Aliment Pharmacol Ther. 2007;25(7):781-6.

106. Kimer N, Krag A, Moller S, Bendtsen F, Gluud LL. Systematic review with meta-analysis: the effects of rifaximin in hepatic encephalopathy. Aliment Pharmacol Ther. 2014;40(2):123-32.

107. Ahluwalia V, Wade JB, Heuman DM, Hammeke TA, Sanyal AJ, Sterling RK, et al. Enhancement of functional connectivity, working memory and inhibitory control on multi-modal brain MR imaging with Rifaximin in Cirrhosis: implications for the gut-liver-brain axis. Metab Brain Dis. 2014;29(4):1017-25.

108. Takada M, Nishida K, Kataoka-Kato A, Gondo Y, Ishikawa H, Suda K, et al. Probiotic Lactobacillus casei strain Shirota relieves stress-associated symptoms by modulating the gut-brain interaction in human and animal models. Neurogastroenterol Motil. 2016.

109. Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterol Motil. 2014;26(11):1615-27.

110. Cerdo T, Ruiz A, Suarez A, Campoy C. Probiotic, Prebiotic, and Brain Development. Nutrients. 2017;9(11).

111. Chapman CMC, Gibson GR, Rowland I. Health benefits of probiotics: are mixtures more effective than single strains? Eur J Nutr. 2011;50(1):1-17.

112. Lemieux L, Salek-Haddadi A, Josephs O, Allen P, Toms N, Scott C, et al. Event-related fMRI with simultaneous and continuous EEG: description of the method and initial case report. NeuroImage. 2001;14(3):780-7.

113. Ford AC, Quigley EM, Lacy BE, Lembo AJ, Saito YA, Schiller LR, et al. Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic idiopathic constipation: systematic review and meta-analysis. Am J Gastroenterol. 2014;109(10):1547-61; quiz 6, 62.

114. Menees SB, Maneerattannaporn M, Kim HM, Chey WD. The efficacy and safety of rifaximin for the irritable bowel syndrome: a systematic review and meta-analysis. Am J Gastroenterol. 2012;107(1):28-35; quiz 6.

115. Dapoigny M, Piche T, Ducrotte P, Lunaud B, Cardot JM, Bernalier-Donadille A. Efficacy and safety profile of LCR35 complete freeze-dried culture in irritable bowel syndrome: a randomized, double-blind study. World J Gastroenterol. 2012;18(17):2067-75.

116. Simren M, Ohman L, Olsson J, Svensson U, Ohlson K, Posserud I, et al. Clinical trial: the effects of a fermented milk containing three probiotic bacteria in patients with irritable bowel syndrome - a randomized, double-blind, controlled study. Aliment Pharmacol Ther. 2010;31(2):218-27.

117. Whorwell PJ, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, et al. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. Am J Gastroenterol. 2006;101(7):1581-90.