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The Influence of Antibiotics through the Microbiota-Gut-Brain Axis / Focussing on Cognitive Performance

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Abstract

This study explored the influence of gut microbiota, that was affected by antibiotics, on cognitive functioning through the microbiota-gut-brain axis. It hypothesized that antibiotics negatively impacts gut microbiota and that it would become visible as a dysbiosis, indicated by more bowel complaints and dysfunctional stool form. Also, it predicted that lower resting heart rate variability would be found in participants that used antibiotics. Furthermore, participants were expected to perform worse on the O-span task, a measure for working memory capacity or updating. Finally, low HRV was expected to relate to poor cognitive performance. The study included of total of 62 participants, of which 40 participants in the control condition (CC), and 22 participants in the antibiotics condition (AC). The AC included participants that had used antibiotics in the last three months, and the CC excluded participants that used antibiotics in the last 6 months. Results: There was no significant difference between the AC and CC on gut microbiota. Logically following, there was no difference found between the AC and CC on HRV and cognitive performance. Lastly, there was a negligible correlation found between HRV and cognitive performance. This might indicate that a different component of cognitive performance might be affected by vagal nerve activity. We suspect the lack of influence of antibiotics to be related to methodological limitations.

The Influence of Antibiotics On The Microbiota-Gut-Brain Axis, Focussing On Cognitive Performance

Antibiotics is a frequently prescribed drug, with an increase in use observed from 2000 to 2009 (Haeseker et al., 2012). Antibiotics is commonly prescribed without any counter measurements like probiotics. However, antibiotics may have a detrimental impact on the human gut microbiota and its reestablishment up to two years after treatment (Jernberg, Lö Fmark, Edlund, & Jansson, 2007). The gut microbiota is important in physical health and increasingly evident in mental health as it plays a role in the communication between the brain and the gateway to the body, the gut. Communication exists thanks to a complex interplay of several communication pathways, collectively referred to as the microbiota-gut-brain axis (Cussotto, Sandhu, Dinan, & Cryan, 2018; Fülling, Dinan, & Cryan, 2019). The gut microbiota can affect mental health, social behaviour and possibly cognition (Fülling et al., 2019; Steenbergen, Sellaro, van Hemert, Bosch, & Colzato, 2015). Although, these effects on mental wellbeing are usually explained by neuroendocrine pathways of the microbiota-gut-brain axis, microbiota is additionally able to influence the vagal pathway. Vagal nerve activity relates directly to cognition, with lower activity measured with heart rate variability, reflecting poorer performance (Hansen, Johnsen, & Thayer, 2003; Laborde, Mosley, & Thayer, 2017; Thayer, Hansen, Saus-Rose, & Helge Johnsen, 2009). Although the research on mental wellbeing is compelling, research directly looking at the influence of microbiota on cognitive performance is scarcely represented. This research addresses the lack of knowledge concerning the influence of antibiotics in humans, specifically focusing on cognition. If a more pronounced disadvantageous effect of antibiotics on mood and cognition can be found in humans, perhaps it is time to start structurally prescribing probiotics.

The Gut Microbiota and Antibiotics

The gut microbiota is believed to host over a thousand species and over seven thousand strains of bacteria, viruses or fungi, most of which are either harmless or beneficial (Ley, Peterson, & Gordon, 2006; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). The composition of microbiota and its resilience, vary greatly among individuals (Lozupone et al., 2012). The gut microbiota's functions include contributing to the immune system by protecting against pathogens and regulating nutrient absorption and fat distribution (Cryan & Dinan, 2012; Lozupone et al., 2012). The microbiota is fully developed at an age of three, but remains influenceable by many factors such as diet, exercise, stress and antibiotics (Lozupone et al., 2012; Ursell, Metcalf, Parfrey, & Knight, 2012).

Antibiotics instantly induce significant alterations to the gut microbiota by eradicating both pathogenic and healthy colonies of bacteria (Ursell et al., 2012). Within a week of completing an antibiotics treatment, the gut microbiota may nearly restore to its original condition. However, it is also possible that an alternative balance arises as some species can take up to two years to re-establish in the gut (Jernberg et al., 2007; Lozupone et al., 2012). Occasionally, an alternative balance results in a dysfunctional gut motility and secretion, called a dysbiosis (Bovenschen et al., 2006; Keeney, Yurist-Doutsch, Arrieta, & Finlay, 2014). This may become visible in side effects such as constipation, bloating, nausea or the most frequently, antibiotics associated diarrhoea (AAD) (Agamennone, Krul, Rijkers, & Kort, 2019). While some alternative balances are paired with side effects, other alternative balances perform the same basic function without suffering any symptoms (Keeney et al., 2014).

The Microbiota-Gut-Brain Axis

The microbiota-gut-brain axis is a collection of pathways that facilitates bidirectional communication between the microbiota, gut and the brain (Cryan & Dinan, 2012; Cussotto et al., 2018; Fülling et al., 2019). With this pathway, microbiota can have an impact on brain physiology, for example on myelination, blood-brain-barrier permeability, neuroinflammation and neurogenesis (Fülling et al., 2019). As such, microbiota's influence can be linked to mental health in the form of stress, anxiety, ruminative and aggressive thought and even social behaviour and possibly cognition (Fülling et al., 2019; Steenbergen et al., 2015). The microbiota-gut-brain axis comprises several pathways, including vagal nerve stimulation, microbial production of human neurotransmitters and interaction with the immune system (Bastiaanssen, Cowan, Claesson, Dinan, & Cryan, 2019; Cryan & Dinan, 2012). According to Bercik (2011), the dominance of the pathways depend on how the change in microbiota is induced, for example by infections, diet or probiotics. The most direct route of communication between the microbiota and the brain is perhaps through influencing the vagal nerve (Fülling, Dinan, & Cryan, 2019).

Microbiota and Vagal Nerve Activity

As mentioned before, the vagal nerve serves as a means for communicating changes in gut microbiota to the brain. An example of vagal nerve dependent communication is the beneficial influence of the bacterial strain L. rhamnoses (JB-1), often used as a probiotic, on GABA and mRNA expression in the hippocampus and amygdala in mice. These accommodating effects of probiotics were not found in mice that had undergone a vagotomy (Bravo et al., 2011). Similarly, in research with vagotomised mice by (Bercik, Park et al.,

2011), anxiety inducing and relieving effects were found due to colitis, that was induced chemically or through infections, and probiotics respectively. This was only true only for mice with functioning vagal nerves, irrespective of the neurotrophic factor BDNF and modulation of the immune system. Both examples illustrate the key role of the vagal nerve in communication between the microbiota and the brain. Contradicting result was found by (Bercik, Denou et al., 2011), who found that an effect on BDNF expression was found both in mice with vagotomy as well as in mice with an intact vagal nerve. This case, BDNF expression was influenced by an antimicrobial treatment. Thus, from this research it seems that bacterial strains in the form of probiotics and infections are dependent on vagal stimulation whilst antimicrobial treatment is not. This is however, an eluding topic with little research, especially on antibiotics. In research by Jang, Lee, Jang, Han, & Kim, (2018), mice that were treated with the antibiotic ampicillin, suffered a decreased microbiota richness with an alternate dominance of bacteria and increased levels of the endotoxin lipopolysaccharide. This resulted in increased anxiety-like behaviour. In this research, the pathway of the microbiota-gut-brain axis is mostly left untested and undiscussed. A study that specifically researches the influence of this endotoxin, that might be produced due to antibiotics, finds a vagal nerve dependent relationship and argues activation protects against inflammation (Huang, Wang, Jiang, Zhou, & Huang, 2010). Perhaps it is interesting to reconsider the mechanism behind antibiotics induced dysbiosis, by additionally analysing vagal nerve activity.

The Vagal Nerve

The vagal nerve is a bidirectionally functioning nerve, as it consists of efferent and afferent nerves between the viscera and the brain (Fülling, Dinan, & Cryan, 2019). The visceral efferent vagus fibers belong to the parasympathetic nervous system (PNS) and its postganglionic ends lie close to the sinus node, which determines heart rate (Brodal, 2010). Mucosal vagal afferents are a type of nerves that are involved in detecting changes of cytokines, gut peptides, nutrients and hormones. Through mucosal detection and communication, the gut microbiota can exert direct influence on the vagal nerve and thus the brain (Fülling, Dinan, & Cryan, 2019).

Heart rate variability; measuring vagal nerve activity. Vagal nerve activity, also vagal tone, can be measured with heart rate variability (HRV). HRV is the change in time interval between successive heart beats (Laborde et al., 2017). Vagal tone represents the contribution of the PNS to cardiac regulation and can thus be derived from HRV. The vagal

nerve activates when we breathe out and deactivates when we breathe in. In case of a high HRV, the vagal nerve can lower the high heart rate effectively and is therefore considered highly active. On the other hand, a low HRV reflects a less effective contribution of the vagal nerve, which is negatively related to physical and mental wellbeing (Thayer & Lane, 2009).

The Vagal Nerve and Cognition

This study explores the role of the vagal nerve in communication between gut microbiota and the brain and furthermore, the influence of microbiota in the brain. By separately looking at vagal nerve activity and its impact on the brain, the field of research broadens to cognition. The vagal nerve is linked to stress and cognition as it is a nerve that belongs to the PNS and stems from areas among which, the prefrontal cortex (Brodal, 2010; Laborde et al., 2017; Thayer & Lane, 2009). The prefrontal cortex plays a role in cognitive control, as activation enables the ability to hold sensory information, task contingencies and rules while distractions are present (Miller, 2000). Miyake distinguishes three different components in cognitive control: shifting, updating and inhibition. These components overlap but are distinctly different and can be measured separately (Miyake et al., 2000). Specifically updating is essential for working memory (WM), a mechanism where memories and novel information are combined and manipulated to deal with the situation at hand. For WM to function properly, a continued activation of the prefrontal cortex is necessary (Thayer & Hanson, 2009).

Previous research indeed relates vagal tone to cognitive performance (Hansen et al., 2003; Laborde, Furley, & Schempp, 2015; Thayer et al., 2009). In early research on WM, sustained attention and the influence of vagal tone, a homogenic group of male sailors are divided in groups of high and low resting HRV. The high HRV group showed more correct responses on the WM task as well as better reaction times and higher accuracy on the sustained attention task. In addition, these results were specifically found only in the trials that relied on executive functioning (Hansen et al., 2003). Based on this research by Hansen et al., (2003) and research alike, the neurovisceral model was introduced. With the neurovisceral model, an important relationship between HRV, prefrontal neural function and cognitive performance is suggested (Thayer et al., 2009). The neurovisceral model explains improved cognitive performance primarily with inhibitory control, but also through the influence of WM. In research conducted by Laborde, Furley and Schempp (2015), a higher resting vagal tone is specifically related to higher WM capacity or updating, as the means of measurement was the O-span task. The previously described research, although compelling,

only explains the influence of vagal nerve activity on cognition and does not include factors that might influence the vagal nerve, such as antibiotics. Therefore, this research explores the relation between microbiota, vagal nerve activity and cognition.

The Gut Microbiota and Cognitive Function

Research on the influence of microbiota on cognition diverts to memory and learning and often relates back to stress or mental wellbeing. Cussotto and collegues (2018) argue in a meta-review that differences in cognitive performance directly relate to gut microbiota and indirectly through the stress evoked by microbiota. Allen and colleagues' (2016) research suggests that a single type of psychobiotics, that is microorganisms specifically beneficial for mental health, can alleviate the acute stress response and long-term daily self-reported stress responses in healthy adults. Additionally, they find a subtle increase in visuo-spatial memory performance on the associative memory task. As this improvement is paired with EEG-patterns of prefrontal cortex activity, the researchers suggest that specifically top-down processing enabled by WM is affected by the probiotic, rather than associative memory performance (Allen et al., 2016; Hales, Israel, Swann, & Brewer, 2009). Desbonnet and colleagues (2015) illustrate that antibiotics treatment in mice negatively influences cognitive performance. This effect is established using the novel object recognition task and measures both working memory, anxiety and attention (Silvers, Harrod, Mactutus, & Booze, 2007). Thus, some research suggests manipulated microbiota can directly impact cognition, possibly WM components.

What becomes evident from the literature is the growing importance of the microbiota-gut-brain axis and the influence microbiota has beyond our physical wellbeing. One of the environmental factors that can alter microbiota composition is antibiotics. When such alteration of microbiota occurs, the vagal nerve pathway, one of the microbiota-gut-brain axis pathways, might exert different signalling. Finally, the effect on human behaviour of stress, anxiety or depression-related behaviour is more pronounced with pathways such as the neuroendocrine pathway at work. As the vagal pathway is linked to WM and may be influenced by microbiota, it is interesting to further explore this pathway. To test this, the following research question is presented:

RQ. Can gut microbiota, when affected by antibiotics, influence cognitive performance through vagal nerve dependent communication?

H_1: Antibiotics use in the last three months is associated with a dysfunctional bowel as indicated by more complaints measured with the Bowel Complaints questionnaire and/or type 1-2 and 6-8 on the Bristol Stool Form Scale.

Antibiotics instantly induce significant alterations of the gut microbiota by indiscriminately eradicating colonies of bacteria (Ursell et al., 2012), with some bacteria taking up to two years to re-establish (Jernberg et al., 2007). AAD is the most frequent observed effect and indicator of gut dysbiosis (Agamennone et al., 2019). Additionally, constipation and symptoms such as bloating and nausea occur (Bovenschen et al., 2006; Keeney et al., 2014). Only when symptoms occur which signal towards a dysbiosis, an altered microbiota might be detrimental to functioning and wellbeing.

H_2: Antibiotics use in the last three months is associated with a lower resting HRV as indicated by lower RMSSD values.

In various cases, communication of the gut microbiota towards the brain is vagal dependent (Bercik, Denou, et al., 2011; Bravo et al., 2011; Pinto-Sanchez et al., 2017). In the case of antibiotics, sometimes the influence might not be dependent on the vagal nerve (Bercik, P., et al., 2011). However, other times, an endotoxin that is dependent on vagal nerve communication suggests otherwise (Huang et al., 2010; Jang et al., 2018). Lower HRV resting tone, which can be measured with RMSSD, relates to poorer cognitive performance (Hansen et al., 2003; Laborde et al., 2017; Thayer et al., 2009).

H_{3a}: Antibiotics use in the last three months is associated with poorer WM performance as indicated by fewer words correct not ordered on the O-span task.

H_{3b}: Antibiotics use in the last three months is more strongly associated with poorer WM performance as indicated by fewer words correct ordered on the O-span task.

Previous hypotheses served as manipulation checks for this hypothesis. Namely, we expect antibiotics to have a detrimental effect on gut function, this to have an impact on vagal nerve activity, and this in turn on cognitive performance. Additionally, the effect is expected to be more pronounced when WM performance is measured with words correct ordered because the strain on WM capacity is higher than the measurement words correct not ordered. Specifically, participants have to remember the words and place them in the right order, instead of merely remembering the words.

H_4: Higher WM performance, as indicated by more words correct ordered and words correct not *ordered, is related to higher resting HRV as indicated by higher RMSSD values.*

Higher HRV resting tone which can be measured with RMSSD, relates to better cognitive performance (Hansen et al., 2003; Laborde et al., 2017; Thayer et al., 2009), including WM capacity or updating.

Methods

Design

This research used a between-subjects design. Precisely, scores were obtained from participants in two groups: participants who used antibiotics in the last three months, the antibiotics condition (AC), and participants who did not use antibiotics in the last six months, the control condition (CC). This grouping variable measured on nominal level, is the main independent variable and is used to test the effect of antibiotics on gut microbiota, HRV and updating.

Scores on the O-span task were the main dependent variables and consist of words correct not ordered and words correct ordered. These variables are measured on an interval level and were used to compare WM capacity of participants in the AC and CC. The O-span task was automated and counterbalanced. Specifically, the screen displayed operation-word sequences of varying lengths in different orders to participants. The Bristol Stool Form Scale (BSFS) was measured on ordinal level and the Bowel Complaints questionnaire (BCQ) score was transformed into a sum score on interval level. Both were dependent variables used to assess the microbial richness of participants in the AC and CC. Root mean square of successive differences (RMSSD) was the parameter that was used for participants' HRV. It was measured on a ratio level. In the second hypothesis, RMSSD was the dependent variable to see if lower HRV is found in AC compared to CC. In the last hypotheses, RMSSD was the independent variable and words correct ordered, and secondarily words correct not ordered, were the dependent variables. This hypothesis was included to assess if it was indeed the vagal pathway that was utilized and excludes the possibility that alternative pathways were at work.

Participants

Participants were recruited from Leiden University through an internal study participation platform, with flyers and Facebook groups and from personal network. Participants were selected based on the inclusion criteria: antibiotics-use in the last three months without remaining symptoms or no antibiotics-use in the last six months. All participants additionally fit the criteria: 18-35 years old, Dutch-speaking, no history of depression and no medication use expect for antibiotics or hormonal contraceptives.

Exclusion criteria were: people who use antibiotics for a chronic condition, suffer from clinical forms of depression, or use other medication.

From a group of 68 participants, 6 participants were excluded from the analysis. Participants were often excluded because they did not adhere to the criteria. Additionally, two participants dropped out during the Monsell Task-Switching Task, as they were not able to pass the practice phase. The excluded participants fit the age group of our participants and were students, but often used some form of medication, in two occasions for mental or attentional problems. Our final sample included 62 participants (10 males, 52 females), with 22 participants in the AC and 40 participants in the CC. The age range was 18 – 28 years with a mean of 22 years. The conditions AC (3 males, 19 females, mean age = 22) and CC (7 males, 33 females, mean age = 22.1) did not differ significantly from one another. Participants received four credits or €15 compensation. Prior to the commence of the study, an application for ethical approval was filed which described the design of the study, the participant groups, the purpose and so on, to make sure the study was conducted in accordance with the applicable laws and guidelines. The ethical committee of the Psychology at Leiden University approved this study.

Procedure

General procedure. Participants started by reading the information letter and the informed consent. They were then instructed to fill in the background information form, which included questions about the duration and type (pill/injection) of antibiotics. Afterwards, they were instructed to wear a polar band, following which they filled in the BCQ (Bovenschen et al., 2006), Defecation Frequency, BSFS (Vandeputte et al., 2016), Positive Negative Affect Scale (PANAS), Difficulty in Emotion Regulation Scale (DERS), the Persistence, Perseveration and Perfectionism Questionnaire (PPPQ-22) and the Regulatory Focus Questionnaire (RFQ) in this order. The PANAS, DERS, PPPQ-22 and RFQ were not relevant and therefore not analysed in this research.

Afterwards, HRV was measured during a 5-minute recording. This recording took place after the questionnaires, so participants got used to the test environment and had been sitting down for a while. Afterwards, participants performed three computer tasks, starting with the Monsell Task-switching task (Monsell, 2003), then the global-local task (Modigliani, Bernstein, & Govorkov, 2001) and finishing with the Automated version of the O-span task (Unsworth, Heitz, Schrock, & Engle, 2005). As this research paper focussed on the

component updating, closely related to WM capacity, only the O-span task was analysed. Participants finally read the debriefing letter and received the compensation (credits/money).

Location. The study took place at the FSW (Faculty of Social Sciences) lab SB08.

Duration. The typical duration of the study was 105 minutes, varying from 90 minutes to 120 minutes.

General instructions. During the test session, participants were informed about the order and approximate length of the questionnaires. Participants were informed that their answers were processed anonymously and were asked to answer truthfully. Participants were then asked to sit still without using their phone for the 5-minute HRV measurement. After the measurement, participants were informed about the order and duration of the following computer tasks. They were additionally informed that all tasks have one or more practice rounds before the start of the test session. Finally, participants were instructed to read instructions carefully and to ask any remaining questions.

Informed consent. An informed consent was obtained from the participants prior their participation to the study. It included information on the recording and anonymously analysing of (physiological) data and the participant's right to withdraw.

Debriefing. A debriefing letter thanked participants for their involvement and provided information on the purpose (research the relationship between antibiotics-use, microbiota richness and cognitive performance) and expectations (antibiotics might result in poorer cognitive flexibility due to a reduced microbiota) of the study. It additionally asked the participants to keep this information in confidence.

Apparatus, Equipment and Software

Computers with the software Qualtrics and E-prime were used. Qualtrics was used for the questionnaires BCQ, BSFS (and excluded from this paper the Defecation Frequency, PANAS, DERS, PPPQ, RFQ). E-prime was used for the Automated version of the O-span task (and excluded from this paper the Monsell Task-switching task). The smartphone application Elite HRV was used in combination with a polar band to measure HRV. The polar band is a Bluetooth 4.0 heart rate monitor that needs to be strapped around the chest to produce an accurate measurement.

Automated Version of the O-span Task

This research used an adapted automated version of the O-span task (Unsworth, Heitz, Schrock, & Engle, 2005). The tasks's purpose is to solve simple mathematical questions, the operations, while remembering random word for recall. The task contains 60 operation-word

pairs which are presented in 15 trials of two to six pairs. The task is counterbalanced as the trials are presented in different orders to participants. To increase the strain on WM, participants are required to read the operation-word pair aloud and write down the recalled words on a piece of paper, instead of merely selecting the words on the screen.

The main dependent variable was the number of correct words written down in the right position (words correct ordered) (interval). It most clearly measured WM capacity in our sample as it is more difficult to achieve, that is, puts the most strain on WM. The second dependent variable was the correct words in a different position (words correct not ordered), which puts less strain on WM.

Questionnaires

The Bristol Stool Form Scale. The BSFS measures stool form and thus gives an indication of the intestinal colon transit time and the microbial richness (Lewis & Heaton, 1997; Vandeputte et al., 2016). Transit time has a central importance in evaluating gut physiology and disease and is measured more accurately by stool form than defecation frequency (Lewis & Heaton, 1997). As the BSFS additionally reflects microbial richness, this research focused on the BSFS and left the Defecation Frequency questionnaire out of the analysis. The BSFS is used in the clinical setting, for example, to identify constipation and diarrhoea in patients with functional bowel disorder (Longstreth et al., 2006; Vandeputte et al., 2016). This questionnaire was added to give an indication of the gut function and microbiota's richness and was used to evaluate if possible dysbiosis occurred due to antibiotics.

Participants were asked to judge what their defecation looked like in the past 24 hours and to which of the provided drawing and description it matched most accurately. Normal stool forms are defined as those types that are least likely to evoke symptoms: categories 3, 4 and 5. In this research, these categories are therefore considered to reflect a healthy gut microbiota composition. Categories 1, 2 and 8 represent hard stools with long transit time, in other words (mild) constipation. Categories 6 and 7 represent soft and unstructured stool with fast transit time with watery diarrhoea being the worst form. Softer stool types are an indication of decreased gut microbiota and are often associated with antibiotics use (Blake, Raker, & Whelan, 2016; Vandeputte et al., 2016). Both constipation and diarrhoea are related to functional dysbiosis (Chassard et al., 2012) and in this research therefore classified as such. By allocating the different types of stool forms to healthy or dysfunctional, a categorical variable for subsequent analysis remains.

The Bowel Complaints Questionnaire. The Bowel Complaints Questionnaire (BCQ) was used in addition to the BSFS, to evaluate bowel function in the AC and CC. The gastrointestinal (GI) questionnaire by Bovenschen and colleagues (2006) is understandable and has high predictability of actual GI symptoms, or bowel complaints (Bovenschen et al., 2006; Tielemans et al., 2013). The BCQ uses a 7-point Likert scale, and although it has good reproducibility, symptom severity is rated consistently lower than interview reported ratings. To increase the objectiveness of the questionnaire, a 4-point symptom frequency scale (no / yes, monthly / yes, weekly / yes, daily) was used instead. The questionnaire had 20 questions using this frequency scale and each question asked to rate a specific GI symptom. These questions combined were the main variable. The questionnaire used in our study had three additional questions on binary (yes / no), about the impact of gut dysfunction on life. As bowel complaints were the main interest, next to the stool form, these questions were used as a second variable.

HRV measurement. HRV values were measured in a 5-minute recording in which participants did not engage in any activities, as Thayer found that resting vagal nerve activity relates to cognitive performance (Colzato, Jongkees, De Wit, Van Der Molen, & Steenbergen, 2018; Thayer et al., 2009). In the study by (Laborde et al., 2015), the tonic resting state of HRV was the only significant parameter in predicting WM updating. HRV that was measured during the task or reactivity HRV were additionally recorded but had no significant predictive value. In this research we therefore included only the resting HRV to test our hypotheses. HRV analysis can be performed in the time-domain and one of the parameters is the root mean square of successive differences (RMSSD). This parameter is also one of the main HRV parameters (Laborde et al., 2017). The RMSSD was used to reflect participants' HRV because it reflects vagal tone (Thayer & Lane, 2000) but is less susceptible to respiratory influences (Hill, Siebenbrock, Sollers, & Thayer, 2009) in comparison to other variables that reflect vagal tone, such as high-frequency (HF) HRV (Laborde et al., 2017).

Analysis

Data analysis was conducted using IBM SPSS 26. Questionnaire responses were imported from Qualtrics. O-span task data was imported from E-run 2.0 and from Excel, as the recalled words were hand written and entered into an Excel file. These responses were combined into the variables words correct ordered and words correct not ordered. After data was gathered in SPSS, the data was screened on missing values and errors. In order to check normality and outliers, exploratory analyses of continues variables were conducted. This

included a boxplot to detect and remove outliers and a histogram, Q-Q plot and Kolmogorov-Smirnov test to assess normality. The first hypothesis served as a manipulation check as it assessed whether the manipulation of the AC or CC, had influence on gut microbiota. The BSFS and BCQ measured intestinal colon transit time and bowel complaints, respectively, and were used to represent the gut microbiota. The BCQ score was transformed into a sum score subsequent to the analysis, a Mann-Whitney U Test. For the analysis of the BSFS a Chi-square test for independence (with Yates Continuity Correction) was performed. For the second hypothesis that assesses HRV by comparing RMSSD scores between the AC and CC, an independent samples t-test was conducted. For the third hypothesis, an independent samples t-test was conducted to test whether people who have used antibiotics perform less on the O-span task. Both independent samples t-tests were two-tailed. The last hypothesis, to check whether HRV values and O-span task performance influence one another, a Pearson correlation was used.

Results

Data Screening

Data from the final group of sixty-two participants was screened. The frequencies of the categorical variables were checked. One missing value was found on the BSFS. As the participant's response was present for all other variables, the participant was excluded only when the response for the specific analysis was missing (BSFS) but included in the other analyses (BCQ, RMSSD, O-span) by using the option Exclude cases pairwise. No errors or missing values were found in the continues variables, that included BCQ, RMSSD, words correct ordered and not ordered. Our final n was therefore 62 participants, with 22 participants in the antibiotics condition (and 21 for the Bristol Stool Form Scale) and 40 participants in the control condition.

Quality Assurance

In order to check normality and outliers, exploratory analyses of the relevant continuous variables were examined. The exploratory analyses included tests of normality, histograms, normal Q-Q plots and boxplots. Significance on the Shapiro Wilk's test and skewness found for the BCQ and RMSSD values, indicated no normal distributions. RMSSD values in the AC and CC were positively skewed and two outliers were found within the CC. Considering there was nothing odd about the participants except for their RMSSD value, there was no reason to exclude them. However, the quality of their HRV measurements was only fair as opposed to good, meaning result may be skewed. When evaluating the entire sample of

62 participants, a total of 14 cases had fair or even poor HRV measurement quality. Poor quality means that results are likely skewed. To reduce the effect of skewness due to poor measurement quality, RMSSD values were transformed. As the data was positively skewed, RMSSD values were computed using square root. Subsequent analysis, including a Shapiro-Wilk's test ($p > .05$) and a visual inspection of diagrams, illustrated a normal distribution of RMSSD values after transformation, with a skewness of −0.15 (SE = 0.491) and a kurtosis of −1.12 (SE=0.953) for the AC and a skewness of −0.06 (SE= 0.374) and a kurtosis of −0.19 (SE= 0,733) for the CC.

O-span task performance was measured with the two variables, words correct not ordered and words correct ordered. A Shapiro-Wilk's test ($p > .05$) and a visual inspection of diagrams found no normal distribution of words correct not ordered scores, with a skewness of 0.82 (SE = 0.491) and a kurtosis of −0.40 (SE=0.95) for the AC and a skewness of −1.15 (SE= 0.374) and a kurtosis of −1.1 (SE= 0.73) for the CC. As normal distribution was not found, the non-parametric Mann-Whitney U Test was used to test the hypothesis. For words correct ordered scores, a Shapiro-Wilk's test ($p > .05$) and a visual inspection of diagrams found normal distribution, with a skewness of 0.61 (SE = 0.491) and a kurtosis of −0.01 (SE = 0.95) for the AC and a skewness of −1.01 (SE = 0.37) and a kurtosis of −1.15 (SE = 0.73) for the CC. As normal distribution was found, the parametric T-test was used for further analysis.

Data views

All relevant variables are described in the table below. The mean, standard deviation and range are provided for the AC in table 1 and CC in table 2.

Table 1. Relevant values of the important variables for the antibiotics condition.

	words correct ordered	words correct not ordered	RMSSD (computed)	BCQ	BSFS
N	22	22	22	22	21
Value missing	0	0	0	0	1
Mean	37.05	44.50	7.65	30.27	3.71
Median	36.50	43.50	7.88	27.00	3.00
Mode	31	38a	4.51a	20a	3
Std. Deviation	8.85	5.90	2.12	8.20	1.55
Variance	78.24	34.83	4.51	67.16	2.41
Minimum	20	34	4.51	20	2
Maximum	55	56	11.58	47	8

a. Multiple modes exist. The smallest value is shown.

Table 2. Relevant values of the important variables for the control condition.

	words correct ordered	words correct not ordered	RMSSD (computed)	BCQ	BSFS
N	40	40	40	40	40
Value missing	0	0	0	0	0
Mean	41.33	46.90	7.38	27.73	4.20
Median	45.00	47.50	7.30	27	4
Mode	46a	57	5.82	22	3
Std. Deviation	11.09	8.670	2.29	6.59	1.60
Variance	122.99	75.17	5.26	43.44	2.57
Minimum	17	26	2.55	20	2
Maximum	60	60	12.40	47	8

Manipulation Checks

For the BCQ score, consisting of 20 questions about specific symptoms that are all indicators of a dysfunctional gut, we have made a sum score. We have done this to include as much information about bowel complaints as possible. The BCQ sum score was not normally distributed, therefore a non-parametric test was used. A Mann-Whitney U Test revealed no significant difference in bowel complaints levels of people who used antibiotics (*Md* = 6, n= 22) and people who did not (*Md* = 5.5, n= 40), *U* = 376, *z* = −.943, *p* = .345, *r* = .119. Similarly, no significant difference in presence of symptoms was found between the AC (*Md* = 0, n= 22) and CC (*Md* = 0, n= 40), *U* = 416.5, *z* = −.455, *p* = .649, *r* = -0.058. The value 0, in this case, stands for negative answers to all three questions regarding the impact of gut function on daily functioning.

For the BSFS, a categorical variable, a Chi-square test for independence (with Yates Continuity Correction) indicated no significant association between antibiotics use and gut microbiota, healthy or dysbiotic, χ^2 (1, *n* = 61) = .000, *p* = 1.000, *phi* = −0.017.

Confirmatory Tests

Hypothesis 2: Antibiotics use in the last three months is associated with a lower resting HRV as indicated by lower RMSSD values.

The second hypothesis predicts that antibiotics-use is associated with lower resting HRV values, as indicated by lower RMSSD values. After transformation, a normal distribution of

RMSSD values was found. The independent samples t-test was used to compare the RMSSD values of people who used antibiotics and people who did not. There was no significant difference in values for the AC (M = 7.65, SD = 2.12) and CC (M = 7.38, SD = 2.29; t (58) = 0.45, p = .66). The magnitude of the differences in the means (mean difference = 0.26, 95% CI: −0.92 to 1.45) was small (eta squared = .003).

The third hypothesis measures WM performance as indicated by the O-span task. The O-span task has two dependent variables, words correct not ordered and words correct ordered. The hypotheses were: antibiotics-use is associated with fewer words correct not ordered and words correct ordered. Since no normal distribution was found for words correct not ordered, a non-parametric test was used. A Mann-Whitney U Test revealed no significant difference in the correct words not ordered on the O-span task of people who used antibiotics (Md = 27.45, n = 22) and people who did not (Md = 33.73, n = 40), U =351, z = −1.31, p = .19, r = .17 (small effect).

An independent samples t-test was conducted to compare the O-span scores, correct ordered, for people who used antibiotics and people who did not. There was no significant difference in scores for people who used antibiotics (M = 37.05, SD = 8.85) and people who did not (M = 41.89, SD = 10.99; t (58) = −1.76, p = .08). The magnitude of the differences in the means (mean difference = −4.85, 95% CI: −10.35 to 0.65) was small (eta squared = .05).

Finally, the fourth hypothesis expects that higher resting HRV is related to better O-span task performance. Namely, we expect antibiotics to have a detrimental effect on gut function, this to have an impact on vagal nerve activity, and this in turn on cognitive performance. To find out whether such relationship exists, a Pearson correlation was performed and described in table 3.

Table 3. *Pearson Product-moment correlations between O-span measures "words correct ordered" and "words correct not ordered" and the HRV measurement "RMSSD".*

	words correct ordered	words correct not ordered	RMSSD
words correct ordered	1		
words correct not ordered	.890**	1	
RMSSD	.043	.070	1

** $p < .001$ (2-tailed).

The relationship between HRV (as measured by RMSSD) and WM capacity was investigated using Pearson product-moment correlation coefficient. Words correct ordered was the primary indicator of WM capacity. Preliminary analyses were performed to ensure no violation of the

assumption of normality, linearity and homoscedasticity. There was a very weak, positive correlation between the RMSSD and words correct ordered, $r = .04$, $n = 62$, $p < .60$, with high levels of HRV associated with high O-span task score. Similar weak correlation was found when using the variable words correct not ordered as indicator of WM capacity.

Discussion

The aim of the current study was to explore whether antibiotics-use has an influence on microbiota and in turn on cognition, specifically through vagal nerve dependent communication. As mentioned in the introduction, antibiotics target both pathogenic and healthy colonies of bacteria and when side effects of alternative stabilization of microbiota occur (Bovenschen et al., 2006; Keeney et al., 2014; Lozupone et al., 2012), it might negatively influence us through the microbiota-gut-brain axis (Cryan & Dinan, 2012; Fülling et al., 2019). Through this pathway, microbiota can affect brain physiology and can thus be linked to mental health, social behaviour and possibly cognition (Fülling et al., 2019; Steenbergen et al., 2015). Studies manipulating the microbiota of vagotomised mice, suggest the importance of the vagal nerve in communication to the brain and ultimately, behaviour (Bercik, 2011; Bravo et al., 2011; O'Leary et al., 2018; Steenbergen et al., 2015). Therefore, the focus of this research lies on the vagal nerve pathway. Various research relates vagal nerve activity to cognition, in some cases specifically to WM capacity, by illustrating that higher resting HRV relates to better cognitive performance (Hansen et al., 2003; Laborde et al., 2015; Thayer et al., 2009). Studies researching the effect of manipulating microbiota, can in some cases be linked to WM capacity, but finds no conclusive responsible pathway of the microbiota-gut-brain axis (Desbonnet et al., 2015; Silvers et al., 2007). This research questioned "Can gut microbiota, when affected by antibiotics, influence cognitive performance through vagal nerve dependent communication?" By doing this, the research aimed to explore various uncertainties: whether antibiotics negatively impacts gut microbiota, if microbiota affects vagal nerve activity and if so, whether cognitive performance suffers.

Implications of the results

The first hypothesis served as a manipulation check and predicted that antibiotics-use is associated with a dysbiotic gut microbiota as indicated by more bowel complaints and dysfunctional stool form. No significant differences were found in normal or dysfunctional stool forms and bowel complaints between participants in the antibiotics condition (AC) and participants in the control condition (CC). As such, it is unlikely that antibiotics-use is

associated with a dysbiosis of gut microbiota. Furthermore, it is unlikely that the vagal nerve and cognitive performance were affected by antibiotics.

The second hypothesis predicts that antibiotics-use is associated with lower resting HRV, thus reflecting vagal nerve activity. No significant difference in resting HRV was found between the AC and the CC. These results can imply two things. Assuming that our first hypothesis accurately portrays the limited influence of antibiotics on gut microbiota, a non-significant impact on the vagal nerve follows logically. If gut microbiota is impacted but not measured with our proxies on the other hand, it might mean that a different microbiota-gut-brain axis pathway is utilized.

Moving on, to explore the link between antibiotics and cognition, the third hypothesis describes that antibiotics-use is associated with poorer O-span task performance (fewer words correct ordered and words correct not ordered). Again, no significant differences between either O-span task measures were found between the AC and the CC. This means that cognitive performance, specifically WM capacity or updating, is not positively nor negatively influenced by antibiotics-use. Within our theoretical framework, this is a logical finding since we find that antibiotics have no influence on gut microbiota to begin with.

The fourth hypothesis investigates whether vagal nerve activity relates to O-span task performance. This hypothesis was initially added to confirm that it is indeed the vagal nerve pathway that is responsible for communication between the microbiota and the brain. However, antibiotics does not seem to impact microbiota or cognitive performance in this research. Regardless of the impact of antibiotics on the vagal nerve, resting vagal nerve activity is expected to relate to cognitive performance (Laborde, Furley, & Schempp, 2015; Thayer, Hansen, Saus-Rose, & Helge Johnsen, 2009). Negligible positive correlations are found between HRV values and O-span task performance. This might mean that there is no correlation between vagal nerve activity and WM capacity, which would be contradictory to the previously mentioned research.

Limitations of this study

The discrepancies found between our results and our predictions, might be explained by several methodological limitations but might also address some uncertainties within the scientific literature.

In this research we assume that bowel complaints, measured with the BCQ, and dysfunctional stool form, measured with the BSFS, reflect a dysbiosis of the gut microbiota. However, these questionnaire’s are not commonly used to reflect a gut dysbiosis. Rather, the

BFSF is used in clinical settings to determine if, for example, Irritable Bowel Syndrome (IBS) patients suffer from diarrhoea (Blake et al., 2016). Also, the BSQ merely determines whether bowel complaints are present. As we find no difference between the AC and CC, one alternative interpretation is that that stool form (BSFS) and bowel complaints (BCQ) do not accurately measure the influence of antibiotics on microbiota. Participants were asked about their stool form in the last 24 hours to interpret their microbiota at that moment. However, this might not accurately represent an effect of antibiotics. With a dysfunctional microbiota composition, as might be the case for patients with IBS, stool form can be widely fluctuating, ranging from diarrhoea or constipation (Chassard et al., 2012). Thus, information on the latest stool bowel function might appear normal when overall function is not.

Perhaps microbiota is negatively impacted, but not to an extent that it is visible in more symptoms or dysfunctional stool form. By using the BSFS and BSQ, rather than techniques such as culturing bacteria or ribosomal RNA targeted polymerase chain reaction and sequencing to establish a difference in gut microbiota composition, information about specific bacteria populations is left behind (Bercik, Denou, et al., 2011). This can be relevant to better understand what consequences antibiotics have, and when this negatively affects brain physiology. For example, increase of an endotoxin can influence the vagal nerve. Alternatively, certain bacterial strains with a larger presence after antibiotics, might stimulate the production of, for example, certain neurotransmitters or peptides which can alter vagal nerve signalling (Fülling et al., 2019). Closer observation could be necessary to observe when these endotoxins, neurotransmitters or peptides are present after antibiotics treatment and might be of influence.

Furthermore, since we do not measure an effect of antibiotics on HRV or on cognitive performance, a logical assumption would be that antibiotics does not result in a dysbiosis and thus we find no effect. This lack of effect of antibiotics might alternatively be explained by the period of time between the antibiotics treatment and the test moment. In (Bercik, Denou et al., 2011), tests with vagotomised mice were performed on the fourth and tenth day after finishing a two-day treatment. A difference in microbiota composition along with a difference in exploratory and apprehensive behaviour was found in the first two weeks after treatment. Two weeks after the antimicrobial treatment, difference in gut microbiota between the groups faded and so did the difference in behaviour. In our research, after initially aiming to recruit participants that used antibiotics in the last month, a period of up to three months was adapted. Perhaps, a shorter period between antibiotics treatment and testing, would lead to

more profound differences in this research too. However, this prompts a logistical challenge. Additionally, participants that were classified in the control condition, did not use antibiotics in the last six months. In research by Jernberg, Lö Fmark, Edlund, & Jansson (2007) the diversity of microbiota is reduced mostly in the first week, but a long term effect is observed for up to two years. Arguably, participants in our antibiotics condition and control condition might both suffer long term effects and might not be sufficiently distinct. In this analysis an influence of antibiotics is investigated by interpreting differences between AC and CC, therefore it is important that these groups are distinct. In future research on the influence of antibiotics on human performance, it is important to clearly divide both conditions. For the AC, it can be helpful to aim to test participants that have taken antibiotics up until two weeks before the research, whilst the CC should not have taken antibiotics for the last two years. When short-term effect of antibiotics on cognitive performance are found, it can be interesting to include participants with longer periods between antibiotics-use and testing, to investigate long term antibiotics impact. Antibiotics-use does not affect HRV nor cognitive performance in this research, which could be explained by the notion that antibiotics influence is not properly represented.

In comparison to animal studies or studies with probiotics interventions, it is a challenge to create similar test conditions for human studies with antibiotics. Probiotics interventions allow for random selection and assignment with a probiotics intervention and placebo. With antibiotics on the other hand, only people who had antibiotics prescribed to them, can participate due to ethical limitations. The selection and assignment are therefore not truly random. Another challenge that arises, is finding enough participants who used antibiotics. In our case, 22 participants with antibiotics-use in the last three months were found, whereas we aimed for 40 participants. Ideally, each condition contains 30 participants. When this is not the case, the chance that a non-significant result is found when in fact a significant effect is present, increases (Faber & Fonseca, 2014). As described earlier, it might be beneficial to recruit participants that have used antibiotics more recently, within the past two weeks. This however, increases the challenge of finding enough participants further.

A possibility is that the resting vagal tone is not influenced by an antibiotics induced change of microbiota. Research with mice underlies vagal dependency with probiotics, but this is not directly the case for antibiotics (Bercik, Denou, et al., 2011; Bercik, Park, et al., 2011; Bravo et al., 2011). More research needs to be conducted to find out what influences vagal tone at rest. It is clear that there is a genetic component to vagal tone (Thayer & Lane,

2009). In this research, the increase in cognitive performance that is observed, only relates to a resting vagal tone, but does not explain what influences this vagal tone (Hansen et al., 2003). Research that relates gut microbiota to cognitive performance, and thus could be related to influence of vagal tone, does not explicitly research the pathway at work but sometimes points towards the influence of few neuromodulators. This research predicted that vagal tone is influenced by microbiota. However, we were not able to establish a link due to the fact that no effect of antibiotics was found. In the future, it might be more helpful to firstly establish a link between antibiotics-use, microbiota and vagal nerve activity.

Similarly, it is hard to draw conclusions about the influence of antibiotics-use on cognitive performance, since we believe antibiotics-use was not properly represented or measured. However, it is also possible that antibiotics does not impact cognition, or not specifically WM or updating. For example, in previous research on mice, WM performance is measured with a test that additionally measures anxiety and attention, which makes it hard to draw conclusions about the exact influence (Desbonnet et al., 2015; Silvers et al., 2007). We did expect to find a correlation between HRV and cognitive performance, since this is observed in the literature (Hansen et al., 2003; Laborde et al., 2017; Thayer et al., 2009). It can be possible that the cognitive performance component that we measured, working memory or updating, does not specifically relate to HRV. Afterall, research such as the neurovisceral model by (Thayer & Lane, 2009) points to the cognitive component inhibition and suggests that working memory might also be affected.

This research did not support the original hypotheses. The results did not illustrate that antibiotics negatively impacted stool form or bowel complaints that represented dysfunctional gut microbiota. Within our research, it is likely that no impact of antibiotics was found due to methodological limitations. Logically following, between the AC and CC no differences in vagal nerve activity were observed. It is hard to draw a conclusion about the vagal nerve pathway under influence of antibiotics, given no impact of antibiotics were found. Furthermore, no differences were found in performance on the O-span task. Finally, the last hypothesis predicted that higher resting HRV values relate to higher working memory capacity. A negligible correlation is found between cognitive performance and HRV. This result suggests that cognitive performance might be influenced, but not specifically working memory or updating. Future research needs to focus on the relation between antibiotics and vagal nerve activity, vagal nerve activity and cognitive performance and then explore what relations might exist.

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