A metagenomic microbiome investigation in the deer mouse: A translational approach to obsessive-compulsive disorder

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Abstract

Obsessive-compulsive disorder (OCD)[1] is characterized by persistent, intrusive and often anxiety-provoking thoughts, i.e. obsessions and/or ritualistic behaviors (compulsions) that are expressed in an attempt to reduce the level of anxiety experienced. Although OCD is a common psychiatric illness that results in significant impairment in the normal functioning of patients, current pharmacotherapeutic strategies yield suboptimal results. Indeed, up to 40% of patients demonstrate treatment refractory symptomology to first-line intervention, i.e. chronic high-dose selective serotonin reuptake inhibitors (SSRIs)[2], while a further 40 – 60% of such cases also remain non-responsive to augmentation strategies. While previous efforts at developing effective treatment have generally been aimed at modulating brain neurotransmitter function within the cortico-striatal-thalamic-cortical (CSTC)[3] circuitry, recent research highlighted a possible role for dysbiosis, i.e. unstable changes in the gut microbiota, in psychiatric pathology. Although the relationship between dysbiosis and OCD is still largely unknown and taking into account that the exact neurobiological constructs underlying OCD have not yet been elucidated, investigations of the gut microbiota and its possible involvement in compulsive-like behavior, may provide valuable insight. In fact, the gut microbiota may potentially contribute to obsessive-compulsive pathology in meaningful ways, e.g. via modulation of immune responses in the central nervous system, alteration of neurotransmitter concentration and via indirect actions on the hypothalamus-pituitary axis (HPA)[4].

Therefore, the current project aimed to apply a validated animal model of naturalistically developing compulsive-like behavior, i.e. large nest building (LNB)[5] to investigate whether such behavior can potentially be associated with alterations in the gut microbiota compared to normal nest building (NNB)[6] controls. Further, as LNB has previously been shown to respond to chronic high-dose oral treatment with escitalopram, a clinically used SSRI, we wanted to establish whether the same treatment regimen would affect the composition of the gut microbiota differently in LNB, compared to NNB animal.

* * *

We demonstrate here that the composition of the gut microbiota in LNB animals is significantly different from that in the NNB cohort. As LNB transpires naturally over the course of development

[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitors
[3] cortico-striatal-thalamic cortical
[4] hypothalamus-pituitary-adrenal
[5] large nest building
[6] normal nest building

and given that animals included in this investigation have been randomly selected without litter bias, indicates that the difference observed in microbiota composition naturally parallels the differences observed in behavioral expression. Further, we found *Robinsoniella,* a gram-negative, spore-forming and non-motile bacterial genus to be more abundant in LNB animals. That *Robinsoniella* was found to be more abundant in LNB[1] compared to NNB[2] mice, may provide some valuable direction for continued exploration in studies relating to the underlying role of the GBA[3] in OCD[4]. However, in terms of causality, it needs to be determined whether an underlying neuropsychiatric construct that may be unique to LNB animals, i.e. alterations in neurotransmitter signaling or anxiogenic stress, elicited adaptive changes in the microbiota that is different from what is observed in the NNB cohort. It may well be possible that the microbiota composition of LNB animals can exert a bottom-up influence on the behavioral expression of LNB animals via nerve pathways or immunological signaling. This remains to be established in this model.

Although our findings pertaining to the response of the gut microbiota to escitalopram intervention are not statistically significant (Yano et al., 2015), the adaptation of the microbiota in LNB animals trended towards being more extensive compared to what was found in the NNB cohort. Considering that escitalopram is known to have antimicrobial effects, it is important to highlight that dysbiosis will result from changes in the inherent abundance of different gut microbiota strains. It may therefore be of potential value if future investigations consider the antimicrobial actions of SSRI[5] administration as a possible contributing factor to changes in central nervous system processes.

Taken together, the data presented here provide for the first time in an investigation of OC[6]-like behavior in animals evidence that altered microbial composition parallels the manifestation of a naturally developing compulsive-like phenotype. Further, we also highlight a possible association between adaptations in the microbiota composition and escitalopram intervention. Future investigations into a possible causal role of the gut microbiota in the etiology of compulsive phenotypes, are warranted. Specifically, the relationship between compulsive phenotypes, physiological and psychological stress, vagus nerve signaling and immune alterations on the one hand and adaptations in the microbiota of normal and compulsive-like deer mice on the other hand, needs further elucidation. Further, it would be valuable to characterize the behavioral response in LNB deer mice both in the presence and absence of microbiota to establish a clear mechanism for its potent

[1] large nest building
[2] normal nest building
[3] gut-brain axis
[4] obsessive-compulsive disorder
[5] selective serotonin reuptake inhibitor
[6] obsessive-compulsive

behavioral effect, as reported earlier. By providing a clearer roadmap for future investigation, such studies could possibly contribute to a better understanding of the neurobiology underlying OCD[1] that may ultimately lead to the development of better treatment.

Keywords: obsessive-compulsive disorder, microbiome, deer mouse, escitalopram, gut-brain axis, nest building, *Robinsoniella*

Solemn Declaration: I, Isabella M Scheepers (24161047), hereby solemnly declare that this dissertation is original work and that no part thereof has been copied from another source.

[1] obsessive-compulsive disorder

Congress proceedings and previous work

Congress proceedings

Data from the current investigation were presented at the following conferences:

IM Scheepers, BH Harvey, SMJ Hemmings, S Malan-Müller, PD Wolmarans (2018). *Associations between the gut-microbiome and large nest building: A translational approach to obsessive compulsive disorder*. Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST), Stellenbosch, South Africa, October 2018

IM Scheepers, BH Harvey, SMJ Hemmings, S Malan-Müller, PD Wolmarans (2018). *'n Ondersoek na die verwantskap tussen die intestinale mikrobioom en 'n kompulsiewe gedragsfenotipe in die hertmuismodel van obsessiewe-kompulsiewe siekte*. Studentesimposium in die Natuurwetenskappe, Arcadia, Pretoria, Suid-Afrika, Oktober 2018.

Previous work by the candidate

Previous work by the candidate pertaining to the deer mouse model of OCD (Addendum E):

De Wet Wolmarans, Isma Scheepers, Dan J Stein, Brain H Harvey (2018). *"Peromyscus maniculatus bairdii as a naturalistic mammalian model of obsessive-compulsive disorder: current status and future challenges".* Metabolic Brain Disease 33(2): 443-455.

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Table of Contents

1 Introduction

1.1 Dissertation layout and language

The current dissertation is compiled in article format, as prescribed and approved by North-West University. As such, the main body of the dissertation is presented as a single manuscript that will, following completion of the complete investigation in following studies, be submitted to international, peer reviewed neuroscience journals.

However, Chapter 1 provides a concise description of the project problem statement, study questions, aims, layout and expected outcomes. Chapter 2 comprises the literature background supporting the current project, while chapter 3 will report the detailed methodologies followed and findings of the investigation in the form of a scientific manuscript. This manuscript has been prepared according to the 'Instructions to Authors' provided by the journal in which this work is intended to be published (Cognitive, Affective, and Behavioral Neuroscience; CABN). Chapter 4 summarizes the key findings of the project and concludes the study as a whole. The addendums contain the 'Instructions to Authors' from CABN and letters of permission of co-authors for subjecting the manuscript for assessment purposes. Furthermore, one manuscript, co-authored by the candidate and that can be considered as a detailed 10-year review of the deer mouse model of OCD, is also provided. This is provided for the benefit of the reader only and is not subject to examination.

As Chapter 3 (manuscript) was prepared according to the guidelines of the American Psychological Association (APA) 6th ed., the referencing style of chapters 1 – 4 is applied in the same manner.

The dissertation is presented in US English as this was the prescribed language for the manuscript.

1.2 Problem statement

Obsessive-compulsive disorder (OCD)[1] affects more than 2% of the global population irrespective of sex. It significantly impacts the daily lives of patients by interfering with among others, interpersonal relationships, occupational and academic achievement (Rasmussen and Eisen, 1992, Okasha, 2002, El-Sayegh et al., 2003), and mental wellbeing. OCD is a multidimensional disorder that comprises several different symptom clusters that are all characterized by obsessions and/or compulsions (Pauls et al., 2014). Obsessions can be described as persistent unwanted thoughts, impulses and images that cause significant distress and anxiety (Association, 2013). Furthermore, compulsions can be described as either overt or covert neutralizing rituals performed in an attempt to suppress the level of anxiety experienced by the individual (Association, 2013). Although obsessions and compulsions are regarded as being seemingly senseless and time-consuming, patients often have very little control over its manifestation (Heyman et al., 2006), a dilemma complicated by the fact that the obsession-compulsion-relief cycle is subject to negative reinforcement. Indeed, that neutralizing compulsive rituals only provide brief respite of obsession-related anxiety, is problematic in that patients consistently engage in such behaviors in order to sustain a *perceived* level of control (Abramowitz and Jacoby, 2015).

Both highly selective serotonin reuptake inhibitors (SSRIs)[2], e.g. escitalopram, citalopram and fluvoxamine, as well as mainly serotonergic tricyclic antidepressants (TCAs)[3], e.g. clomipramine, display demonstrable efficacy in the treatment of OCD (Fineberg et al., 2007, Greist and Jefferson, 1998). However, a third of patients do not respond to such first line pharmacotherapy (Marazziti et al., 2016) and when neither the SSRIs nor serotonergic TCAs elicit a significant response, second-line therapy, often including augmentation treatment with low-dose dopaminergic antagonists (Eagle et al., 2014, da Rocha and Correa, 2011), can be initiated. Still, up to 50% of patients remain refractory to such second-line interventions as well (Marazziti et al., 2016), while low remission rates—less than 10% (Eisen et al., 1999)—and relapse following withdrawal of therapy is another major clinical challenge (Tollefson et al., 1994).

Recently, clinical investigations revealed a possible role for the human gut microbiome in what would otherwise be regarded as non-gut-related pathologies, notably also neuropsychiatric illnesses (Park et al., 2013, Bailey et al., 2011, Bailey and Coe, 1999, Claesson et al., 2011, Berrill, 2013). Therefore, and considering that the etiopathology of OCD is not yet fully elucidated and that patients respond only

[1] obsessive-compulsive disorder
[2] selective-serotonin reuptake inhibitors
[3] tricyclic antidepressants

sub-optimally to treatment (Atmaca, 2016), research has shifted its focus to novel targets of investigation, e.g. the microbiota-gut-brain axis (Wang and Kasper, 2014), which has recently been described. The human body consists of a multitude of human and microbial cells, the latter of which include the organisms that are located in the gut (Gill et al., 2006), i.e. gut microbiota. The human microbiome is not only a major contributor to the nutritional status of individuals, but also protects against invading pathogens (Kamada et al., 2013, Lawley and Walker, 2013, Sommer and Bäckhed, 2013). Moreover, the 'microbiota-gut-brain axis', existing in the form of neural, hormonal, and immunological signaling (Turna et al., 2016) and that involves the central (CNS)[1], autonomic (ANS)[2] and enteric (ENS)[3] nervous systems (Mayer, 2011, Carabotti et al., 2015) plays a significant role in linking such peripheral constructs to CNS functioning. This bidirectional communication system between the gut and the brain manifests via several unique direct and indirect mechanisms (Foster and Neufeld, 2013, Collins et al., 2012, Crumeyrolle-Arias et al., 2014) and are sensitive to changes in the normal functioning of the gut micriobiota (Goehler et al., 2005, Clarke et al., 2013). Indeed, when unstable imbalances in microbial composition, i.e. dysbiosis, are observed, concomitant non-gut-related pathologies may ensue (Rees, 2014).

As alluded to earlier, the etiopathological involvement of the gut microbiota in psychiatric illness has gained significant interest over the past decade. Although previous clinical research demonstrated a definite association between the gut and brain (Stilling et al., 2014, Park et al., 2013), limited data is available concerning such a possible relationship in OCD[4] (Turna et al., 2016). While the current pharmacotherapeutic interventions for OCD yield less than optimal response (Turna et al., 2016) and considering that the response of OC symptoms to SSRI[5] intervention is dose and time dependent, a noteworthy clinical challenge in the treatment of OCD remains the side-effect profile of high dose chronic SSRI treatment (Fineberg *et al.*, 2007) that includes a higher incidence of irritable bowel syndrome (Masand et al., 2006). Although research has yielded a substantial degree of insight into the neurobiology of OCD, for example in highlighting the role of the cortico-striatal-thalamic-cortical (CSTC)[6] circuitry in the pathogenesis of OCD (Okasha, 2002), very little is known about the possible influence of peripheral factors on its neurobiology and etiopathology. Therefore, taking into consideration the body of literature that established a bidirectional association between the gut microbiota and the brain both under normal and pathological circumstances (Stilling et al., 2014, Park

[1] central nervous system
[2] autonomic nervous system
[3] enteric nervous system
[4] obsessive-compulsive disorder
[5] selective serotonin reuptake inhibitors
[6] cortico-striatal-thalamic-cortical circuitry

et al., 2013), the current investigation will attempt to divulge more of this association in a robust animal model of OCD[1], *viz.* large nest building (LNB)[2] behavior in deer mice to investigate whether the gut microbiota may be regarded as a novel target for the investigation of putative new therapeutic interventions (Turna et al., 2016). Building on previous work performed in our laboratory during which we have characterized deer mouse *(Peromyscus maniculatus bairdii)* behavior as a naturalistic, non-induced rodent model in which to investigate compulsive-like manifestations (Wolmarans et al., 2013, Wolmarans et al., 2016a, Wolmarans et al., 2016b), the current research will employ the model to investigate whether LNB may be associated with altered gut microbial composition, and how such possible alterations will respond following chronic high dose oral escitalopram (50 mg/kg/day x 28 days) treatment. This is especially of relevance in the current work, as we have demonstrated previously that LNB is *completely reversed* following such intervention. As both LNB and the gut microbiota develop naturally over the course of time, it may indeed be possible that a unique relationship between aberrant nest building behavior and an altered microbial profile may exist.

1.3 Study questions

Considering the possibility that the gut microbiota may be regarded as a novel target for investigating new therapeutic strategies in the treatment of OCD (Turna et al., 2016), the following research questions will be addressed:

a) By employing aberrant LNB as a valid framework in which to investigate OC behavior in the deer mouse model of OCD (Wolmarans et al., 2016a), will LNB expressing deer mice present with a unique gut microbial composition, compared to normal nest building (NNB)[3] expressing control subjects?

b) Further, taking into account that chronic treatment with high dose oral escitalopram (50 mg/kg/day x 28 days in the deer mouse) has previously been shown to completely reverse the expression of LNB behavior to levels analogous to NNB (Wolmarans et al., 2016a), will such intervention be associated with an adaptive modification in the gut microbiota of LNB animals in such a way that it more closely resembles that observed in NNB animals? Furthermore, linking the therapeutic response previously observed in the model to possible changes in the gut microbiome and provide proof-of-concept for future continued investigations into the gut-brain relationship in compulsive phenotypes?

[1] obsessive-compulsive disorder
[2] large nest building
[3] normal nest building

An important note on the context of the current investigation

It is important to note that the current project forms part of a larger umbrella investigation that collectively investigates the role of the microbiome in LNB[1]-expressing animals. Although post-treatment nest building analyses as well as the influence of microbiota-altering techniques, e.g. co-habitation, also form part of the larger project, time constraints and practical challenges with animal numbers, as well as two unexpected animal deaths immediately post-treatment, prevented this investigation from addressing all of the original study objectives. Hence, the manuscript reported in this dissertation, is restricted to investigations of the baseline pre-treatment differences in the microbiome of NNB[2] vs LNB animals and to the response of such differences to chronic high dose oral escitalopram treatment. Therefore, the manuscript will not be submitted for publication yet, but will be prepared following completion of the larger project which will include both post-treatment nest building analyses and the effects of co-habitation on both the behavior and the microbiome of LNB, compared to NNB subjects.

1.4 Study aims and objectives

The current project will broadly aim to elucidate the nature of possible gut microbial correlates in an animal model of OCD[3] and OC[4] symptomology and how such possible associations may be modified with chronic high dose oral escitalopram treatment. Furthermore, we aim to apply this investigation as a first-of-its-kind foundational study in OCD to deliver a putative pre-clinical platform for future investigations relating to the treatment of refractory OCD in which associations between the gut and the brain form the core of focus of interest. This will be achieved by:

a. Characterizing nest building behavior in the deer mouse colony housed in the Vivarium of the NWU, Potchefstroom, and categorizing subjects into NNB and LNB cohorts, respectively;
b. Collecting baseline stool samples in treatment-naive animals of both behavioral cohorts and characterizing possible differences between the gut microbiota of NNB and LNB animals; and
c. Administering chronic drug treatment in the form of oral high dose escitalopram—50 mg/kg/day dissolved in the drinking water for 28 days—to animals of both behavioral cohorts and characterizing possible adaptive changes in the microbiome of said animals following such intervention.

[1] large nest building
[2] normal nest building
[3] obsessive-compulsive disorder
[4] obsessive-compulsive

1.5 Study layout and methodology

1.5.1 Study layout

To address the research questions asked in the current investigation, this project is divided into two main phases:

Phase 1 – Study objectives (a) and (b)

Considering that OCD[1] manifests in patients of both sexes and taking the ARRIVE[2] guidelines for research in animals into account (Kilkenny, 2010), the first phase of the investigation included 3 male and 3 female deer mice in both nesting cohorts, i.e. NNB[3] (*n* = 6) and LNB[4] (*n* = 6). However, as only 30% of deer mice express LNB behavior (Wolmarans et al., 2016a), a total of 18 deer mice (age 10 weeks at the onset of experiments) were initially screened for nest building behavior. Subsequently, baseline, treatment-naive fecal samples were collected from the 6 identified NNB and LNB animals respectively (Chapter 3, Manuscript A).

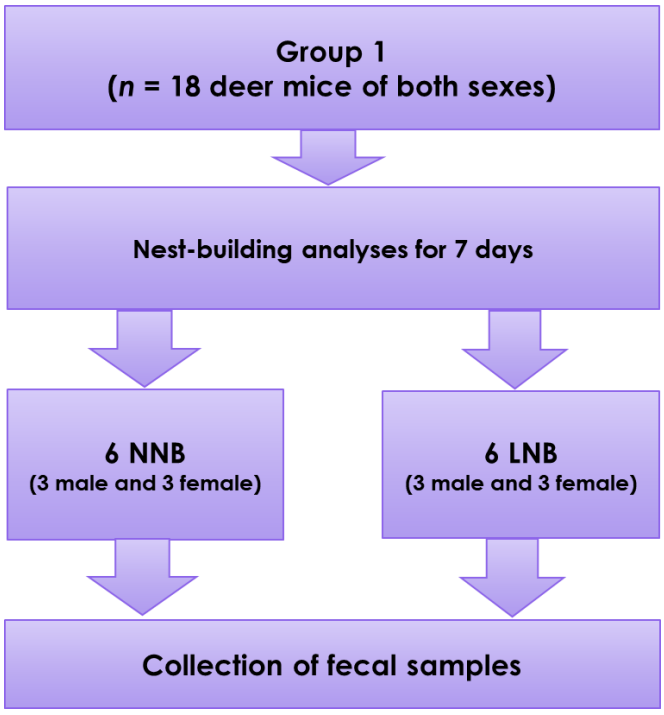

Figure 1-1 - Schematic representation of study objectives (a) and (b)

[1] obsessive-compulsive disorder
[2] Animal Research: Reporting of In Vivo Experiments
[3] normal nest building
[4] large nest building

Phase 2 – Study objective (c)

Last, to address study objective (c) separate groups of 5 NNB[1] and 5 LNB[2] deer mice (all female), were treated with either water or escitalopram (50 mg/kg/day) for a total of 28 days to establish whether the introduction of an OCD[3]-specific pharmacological intervention will modify the microbial content, as it has previously been shown to reverse LNB (Wolmarans et al., 2016a). Therefore, an initial number of 17 deer mice were initially screened for nesting behavior to ensure a yield of at least 5 LNB animals. In this group, fecal samples were collected both at baseline before treatment and immediately following treatment to establish the possible nature of drug-induced modifications in microbial composition.

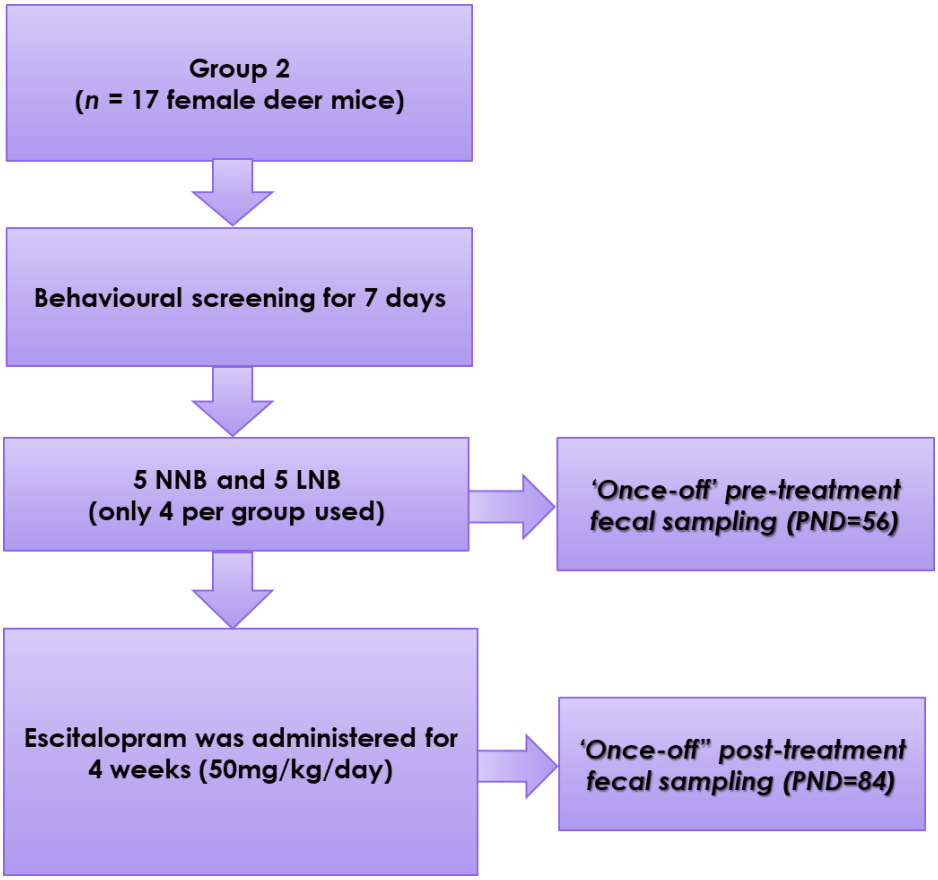

Figure 1-2 - Schematic representation of study objective (c)

[1] normal nest building
[2] large nest building
[3] obsessive-compulsive disorder

1.6 Predicted outcomes

Based on the literature reviewed, and that aberrant nest building represents an OC[1] phenotype in the deer mouse model of OCD[2] (Wolmarans et al., 2016a), we hypothesize NNB[3] and LNB[4] will present with unique gut microbial compositions. Moreover, we hypothesize that chronic treatment of animals expressing NNB and LNB behavior with high dose oral escitalopram (50 mg/kg/day x 28 days) will elicit adaptive changes in the microbiota of LNB animals to more closely resemble that observed in the NNB controls. As such, we hope to apply this investigation as a foundational platform for future studies which will focus on the possible involvement of 'dysbiosis' of the gut microbiota in OCD pathology. In fact, by demonstrating differences in microbial composition between the NNB and LNB cohorts and establishing that escitalopram elicits adaptive changes in the microbiota of LNB expressing animals, we will be able to provide putative evidence for the association of the gut-brain axis not only in the etiopathology of OCD, but also in the mechanisms underlying its response to SSRI[5] intervention.

[1] obsessive-compulsive
[2] obsessive-compulsive disorder
[3] normal nest building
[4] large nest building
[5] selective serotonin reuptake inhibitor

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2 Literature review

2.1 Obsessive-compulsive disorder in the clinical environment

2.1.1 Epidemiology and diagnosis

Obsessive-compulsive disorder (OCD)[1] is characterized by intrusive thoughts, i.e. obsessions, and persistent overt or covert behavioral repetitions, i.e. compulsions (Koch et al., 2014). The condition is debilitating and is diagnosed in up to 3% of the global population (Kessler et al., 2005, Bloch et al., 2008, Weissman, 1998, Zohar et al., 1992). Further, although OCD demonstrates equal prevalence in men and women, men often befall the illness at an earlier age (Jenike, 2004). Obsessions can involve any number of themes, including excessive doubt or feelings of guilt, perfectionism or exactness, thoughts of hurting oneself or another, and fears of losing objects (Heyman et al., 2006). Importantly, although seemingly delusional, obsessions are differentiated from delusions based on insight. Whereas delusional patients often demonstrate no insight into their symptoms, i.e. believing that the thoughts and ideas they experience and promulgate are accurate and true, OCD patients know that their obsessions are inappropriate, unfounded and irrational (Jenike, 2004). This knowledge is often associated with increased anxiety as patients struggle to come to terms with and suppress or prevent such obsessive intrusion. Thus, a functional relationship between obsessions and compulsions can be described in which compulsions are regarded as persistent behavioral routines borne from excessive attempts to neutralize obsession-related anxiety. As such, obsessions and compulsions have been shown to cluster together with respect to five main themes, *viz.* fears of contamination and cleaning rituals, fears of harm and checking compulsions, a need for symmetry and order associated with ordering compulsions, fears of losing objects and hoarding behavior, and intrusive inappropriate thoughts relating to sexual misconduct, religion, and violence. Although not resulting in overt compulsive behaviors, the latter lead to covert mental routines, e.g. praying (Markham et al., 2015). Further, the association between obsessions and compulsions is subject to negative reinforcement as compulsions provide only temporary relieve of anxiety and feelings of distress (Wu and Lewin, 2017). Importantly, an association between obsessions and compulsions is not a mandatory prerequisite for an accurate diagnosis of OCD, as some patients may experience only the one or the other (Association, 2013). That said, all humans experience OC[2]-like symptoms sometime during their lives and thus, whether diagnosed together or as separate symptoms, obsessions and compulsions are only described if they meet a set of criteria. First, symptoms must be time-consuming and be present for longer than 1hr/day. It must interfere significantly with the social, occupational and normal daily routines of

[1] obsessive-compulsive disorder
[2] obsessive-compulsive

patients, and it must not be the result of any other mental disorder or the use of substances (Association, 2013). Indeed, studies have shown that OCD[1] interferes with the normal functioning of patients to such an extent that it prevents them from living to their full potential (Piacentini et al., 2007, Ivarsson and Valderhaug, 2006, Storch et al., 2010). Especially the contamination/washing (C/W)[2] and safety/checking (S/C)[3] symptom clusters are problematic in this regard (Storch et al., 2010). Importantly, patients must realize the irrationality and futility of their symptoms and must attempt to engage in active thought processes directed at inhibiting said symptoms.

Concerning the general association between anxiety-provoking obsessions and compulsions, it is important to note that OCD is no longer classified as an anxiety disorder, but rather as the archetype disorder in a new diagnostic cluster, i.e. obsessive-compulsive and related disorders (Lissemore et al., 2015). The other conditions included in this group are body dysmorphic disorder, trichotillomania, excoriation (skin picking) and hoarding disorder. Although anxiety no longer forms a mandatory clinical characteristic for its diagnosis, it remains one of the key symptoms seen in patients with OCD. As such, debate about its clinical conceptualization still continues (Abramowitz and Jacoby, 2015).

2.1.2 Treatment

Considering that OCD is a severe and detrimental illness, safe and effective treatment is of essential value. Although no single treatment is overly successful, it has been shown that without therapeutic intervention, symptoms will persist (Skoog and Skoog, 1999). As is true for many other psychiatric conditions, including posttraumatic stress disorder, generalized anxiety disorder and major depression, OCD can be treated with both pharmacological and psychological, e.g. cognitive behavioral therapy (CBT)[4], interventions. While both approaches are associated with significant improvement in many sufferers, 30 – 40% of patients remain refractory to initial intervention (Atmaca, 2016).

Considering the pharmacological options, chronic high dose treatment with selective serotonin reuptake inhibitors (SSRIs)[5] is regarded as the first-line pharmacological treatment in both children and adults (Soomro et al., 2008). That serotonin reuptake inhibitors are used and found to be effective in the treatment of OCD (Fineberg and Gale, 2005, Fineberg et al., 2015, Varigonda et al., 2016), albeit only moderately so, implicates a role for serotonin in the neurobiology of OCD, which will be elaborated on in paragraph 2.2.2.3. With respect to OCD, treatment is administered in doses higher

[1] obsessive-compulsive disorder
[2] contamination/washing
[3] safety/checking
[4] cognitive-behavioral therapy
[5] selective serotonin reuptake inhibitors

than that used in the management of depression (Hollander et al., 2003a, Wheadon et al., 1993), while withdrawal within one year after initiation is associated with high relapse rates (Tollefson et al., 1994).

While both SSRIs[1] and serotonergic tricyclic antidepressants (TCAs)[2], e.g. clomipramine, are associated with analogous therapeutic outcomes, the more favorable side-effect profile of SSRIs ensures improved patient compliance. Indeed, it is the anticholinergic side effects of clomipramine, e.g. cardiotoxicity and significant sedation that are of great concern (Koran et al., 2007). That said, patients treated with SSRIs also report adverse treatment responses, including sexual dysfunction, headaches and insomnia that may ultimately result in treatment non-adherence (Fagiolini et al., 2012). Importantly, TCAs that primarily target noradrenergic reuptake, e.g. desipramine, are ineffective in the management of OCD[3] (Insel, 1985, Hoehn-Saric et al., 2000, Goodman et al., 1990).

In the case of patients remaining refractory to treatment following SSRI intervention, several strategies can be followed. First, SSRI treatment can be augmented with low dose dopamine-2 receptor antagonists, e.g. haloperidol or risperidone (da Rocha and Correa, 2011, Eagle et al., 2014, Bloch et al., 2006). Second, patients can be switched to another SSRI and third, higher doses of the same SSRI previously prescribed, may be used (Fineberg and Gale, 2005, Bloch et al., 2010, Stein et al., 2007).

Other strategies that can be used as either a first-line alternative to pharmacotherapy or in SSRI-refractory cases include CBT[4] (Wu and Lewin, 2017, Heyman et al., 2006, Frost and Steketee, 2002) and deep brain stimulation (DBS)[5] (Greenberg et al., 2010). Whereas DBS involves electrical stimulation of the brain areas involved in OCD, CBT comprises psychological interventions based on behavioral conditioning. In one such strategy, i.e. exposure and response prevention (ERP)[6], patients are exposed to contextual triggers, e.g. scenarios relating to contamination, while being prevented from engaging in compulsive washing rituals (McLean et al., 2015). Over time, individuals learn that no harm is done by such contamination, ultimately resulting in behavioral and impulse inhibition during future events (McLean et al., 2015).

[1] selective serotonin reuptake inhibitors
[2] tricyclic antidepressants
[3] obsessive-compulsive disorder
[4] cognitive behavioral therapy
[5] deep brain stimulation
[6] exposure and response prevention

2.2 The etiology and neurobiology of OCD

Although the etiology and neurobiology of OCD[1] is not yet fully elucidated, some advances in our understanding of a few features have been made. Importantly, OCD results from a complex interaction between environmental, genetic and neurobiological factors (Figure 2-1). In this section we will highlight some important aspects of these factors as they relate to the etiopathology of OCD.

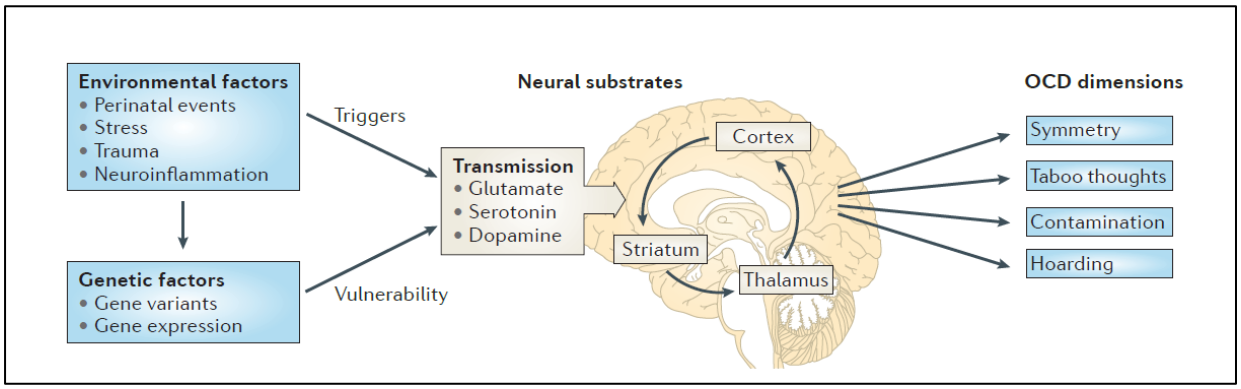

Figure 2-1 - An integrative view into the etiology and neurobiology of OCD (reproduced from (Pauls et al., 2014)

2.2.1 OCD as a neurodevelopmental disorder

Although not being classified as such per se, it has been proposed that OCD be considered as a neurodevelopmental disorder (Huyser et al., 2009, Rosenberg and Keshavan, 1998). In fact, a number of factors have been proposed to contribute to the development of OCD from a neurodevelopmental perspective. These will now briefly be summarized.

2.2.1.1 Early life adversity as a possible trigger for OCD

Childhood trauma, i.e. parental deprivation, neglect, abuse or exposure to threats can be considered as early life adversities that may have detrimental effects on brain circuitry, stress-responsivity, cognitive function and general health (Dube et al., 2009, Anda et al., 2008). Such early life interferences are important to consider in patients with OCD, considering that it has been shown to produce long term neurodevelopmental sequelae in some individuals (Lochner et al., 2002). Already from the time of birth, these events may trigger the etiopathological course of OCD. For instance, perinatal events (Geller et al., 2008), e.g. maternal use of harmful substances, tobacco and alcohol, as well as illness during pregnancy may alter the functional expression of OCD risk genes (Pauls et al., 2014). Further, such adversities are not only associated with childhood onset OCD, but have also been shown to increase the risk of childhood onset of other psychiatric illnesses, including autism (Glasson et al., 2004, Juul-Dam et al., 2001), schizophrenia (Cannon et al., 2000, Geddes and Lawrie, 1995,

[1] obsessive-compulsive disorder

Hultman et al., 1999, Jones et al., 1998, Sacker et al., 1995) and ADHD[1] (Knopik et al., 2005, Brookes et al., 2006, Linnet et al., 2003). Evidently, studies report a higher prevalence of OCD[2] in individuals who experienced childhood trauma compared to those who did not (Lochner et al., 2002).

2.2.1.2 Heritability

There is a strong genetic component to OCD, as indicated by a heritability rate of 20 – 80% in OCD patients (Bloch et al., 2010, Katerberg et al., 2010, Davis et al., 2013). As alluded to earlier, obsessions and compulsions cluster together with respect to five main themes or symptom dimensions. Interestingly, differences in heritability have been observed between these themes (Hanna et al., 2005), with a higher degree of heritability being shown within the symmetry and ordering subtype (Hanna et al., 2005, Katerberg et al., 2010, Davis et al., 2013). Further evidence for a genetic influence in OCD comes from family and twin based studies (Hanna et al., 2005, Hettema et al., 2001) and genetic segregation analyses (Nestadt et al., 2000, Cavallini et al., 1999, Alsobrook II et al., 1999, Hanna et al., 2002). Although no clear genetic correlate for OCD has been identified yet, it is the genes involved in glutamatergic signaling, e.g. glutamate ionotropic receptor NMDA[3] type subunit 2B (*GRIN2B);* (Arnold et al., 2009) and the primary neuronal glutamate transporter gene, solute carrier family 1 member 1 (*SLC1A1*)*;* (Wang et al., 2010, Arnold et al., 2006, Dickel et al., 2006, Wendland et al., 2009, Stewart et al., 2007) that are constantly highlighted in genome-wide association studies (Pauls et al., 2014, Pauls, 2008). A handful of studies have shown glutamatergic modulating agents, including topiramate (Van Ameringen et al., 2006, Ozkara et al., 2005, Hollander and Dell'Osso, 2006) and riluzole (Pittenger et al., 2008, Coric et al., 2005, Grant et al., 2007, Coric et al., 2003), to be effective in at least 50% of patients. However, as with most complex psychiatric conditions, OCD seems to result from a complex crosstalk between a multitude of genes and a wide range of socio-environmental triggers that makes it difficult to draw a conclusive picture of a so-called genetic roadmap (Pauls, 2008).

2.2.1.3 Infection and inflammation

Another piece of evidence supporting a neurodevelopmental theory of OCD is founded on the association between early-onset OCD and Group A streptococcal (GAS)[4] infections (Garvey et al., 1998). Indeed, sudden onset of OC[5] symptoms as well as exacerbation of OC symptomology following GAS[6] infections have been documented and defined as 'pediatric autoimmune neuropsychiatric

[1] attention deficit hyperactivity disorder
[2] obsessive-compulsive disorder
[3] *N*-methyl d-aspartate
[4] group-A streptococcal
[5] obsessive-compulsive
[6] group-A streptococcal

disorders associated with Streptococcal infections' (PANDAS)[1] (Garvey et al., 1998). Later, PANDAS was changed to 'pediatric acute-onset neuropsychiatric syndrome' (PANS)[2] to allow for the inclusion of other immune-related etiologies in abrupt childhood-onset psychiatric symptomology (Murphy et al., 2014). Broadly linked by aberrant immune function, several other similar cases have been reported following mycoplasma infections and Lyme's disease (Müller et al., 2004, Ercan et al., 2008, Schneider et al., 2002) and while several studies attempted to identify the exact organisms involved in said pathogenesis of OCD[3], results remain inconclusive (Teixeira et al., 2014, Swedo et al., 2012). Generally, the presentation of PANS only becomes overt within weeks or months following the infection, complicating the psychiatric diagnosis and treatment of these individuals (Cardoso, 2011). Furthermore, prophylactic treatment against GAS infections in OCD demonstrates only modest efficacy, indicating that infectious triggers, although often diagnosed, are not always contributing to the development of OCD (Perlmutter et al., 1999). Nevertheless, the link between OCD and infectious challenges may be found in aberrant immune responses. Indeed, in addition to the manifestation of PANS, recent investigations also reported associations between OCD and inflammation (Dantzer et al., 2008, Williams and Swedo, 2015, Köhler et al., 2014, Mitchell and Goldstein, 2014). Evidence indicate altered innate and adaptive immune-related functioning, including dysfunctional HPA[4]-axis involvement (Furtado and Katzman, 2015, Şimşek et al., 2016a), the presence of anti-neural antibodies directed at structures of the basal ganglia (Morer et al., 2006, Dale et al., 2005, Morer et al., 2008), and increased levels of pro-inflammatory cytokines (Gray and Bloch, 2012, Şimşek et al., 2016b, Rao et al., 2015) in patients with OCD (Rodríguez et al., 2017). Importantly, while PANS may be a direct consequence of neuroinflammation, the relationship between the manifestation of OCD and inflammation seems reciprocal, i.e. both top-down and bottom-up. This is for example demonstrated by reduced levels of neuroinflammatory markers following symptom attenuation after anti-OCD pharmacological intervention (Rodríguez et al., 2017). Thus, while inflammatory processes may in some cases be fundamental in the etiopathology of OCD, it may also be a consequence of altered neurobiological processes. The fact that the exact nature of inflammatory involvement in OCD in general is not yet understood, may explain why not all investigations agree. Indeed, some investigations failed to reveal any association between OCD and neuroinflammation (Fluitman et al., 2010a, Fluitman et al., 2010b, Denys et al., 2004, Denys et al., 2006). Nevertheless, that immune-modulating agents have shown promise in some investigations (Snider et al., 2005), and that

[1] pediatric autoimmune neuropsychiatric disorders associated with Streptococcal infections
[2] pediatric acute-onset neuropsychiatric syndrome
[3] obsessive-compulsive-disorder
[4] hypothalamus-pituitary-adrenal

associations between OCD[1] and markers of altered neuroinflammatory processes have also been reported, indicate that at least in some patients, OC[2] symptomology may be initiated, modulated and exacerbated by altered neuroinflammatory processes. To this end, it has been proposed that changes to the gut microbiota within the context of the gut-brain axis, may be a key aspect contributing to altered neuroinflammatory processes (refer to section 2.3 for detailed discussion).

2.2.2 Neurobiology

2.2.2.1 An overactive CSTC-circuitry in OCD

Considering that compulsions are directed at a specific goal or outcome, e.g. to lock a door, and that such behavior is subject to negative reinforcement, i.e. being expressed in return for a fleeting reduction in the level of anxiety experienced, abnormal regulation of goal-directed feedback processing has been proposed to underlie the symptomology of OCD (Gillan et al., 2011). Thus, it is not surprising that the brain areas implicated in OCD are, among others, those that mediate goal-directed behavior and reward feedback processing; here, the term 'reward' relates to adequate task completion. These brain areas include the prefrontal cortex, striatum and thalamic nuclei that communicate with each other via different pathways (Nambu, 2008, Evans et al., 2004, Husted et al., 2006, Van den Heuvel et al., 2011). The cortical-striatal-thalamic-cortical (CSTC)[3] circuitry (Figure 2-2) describes the functional organization of these structures (Stocco et al., 2010) which are organized in such a manner that the cortex innervates the striatum, which subsequently influences other parts of the basal ganglia to ultimately exert feedback via the thalamus to the cortex. Consisting of direct (behaviorally activating) and indirect (behaviorally inactivating) pathways, the CSTC circuitry is fundamental in the planning, execution and termination of complex motor behavior and reward-based learning – the two major processes that are hypothesized to be dysfunctional in patients with OCD (Stocco et al., 2010). Furthermore, it is believed that there is a bias in favor of the direct thalamus-activating pathway over the indirect thalamus-inhibiting pathway in the basal ganglia of OCD patients compared to healthy controls (Saxena and Rauch, 2000). This may not only manifest as an overactive orbitofrontal cortex (OFC)[4], but may also increase activity in both the caudate nucleus and the thalamus (Whiteside et al., 2004). The subsequent hyperactivity in the CSTC circuit as a whole is hypothesized to be central to the pathology of OCD. Central to the functioning of the CSTC circuitry,

[1] obsessive-compulsive disorder
[2] obsessive-compulsive
[3] cortico-striatal-thalamic-cortical
[4] orbito-frontal cortex

and in line with the proposed role of deficits in reward feedback, is dopaminergic and serotonergic signaling (please refer to paragraph 2.2.2.3).

Reduced cognitive flexibility and executive function deficits, both dependent on normal functioning of the orbitofrontal cortex, are often observed in patients with OCD[1] (Saxena and Rauch, 2000). In this regard, several neuroimaging studies provided substantial evidence for altered orbitofrontal functioning in OCD (Saxena et al., 1999, Saxena et al., 2001, Kwon et al., 2003). More specifically, hyperactivation of the frontal cortex during OC[2] symptom provocation is often observed in both clinical and preclinical investigations. For instance, metabolic hyperactivity in the OFC[3], anterior cingulate cortex (ACC)[4], thalamus, and the striatum (both the caudate and putamen) are associated with obsessive-compulsive symptoms (Maia et al., 2008, Menzies et al., 2008). Furthermore, these investigations collectively demonstrate that a direct correlation exists between the degree of CSTC[5]-activity and symptom severity and that such hyperactivity is reduced following successful treatment interventions (Hansen et al., 2002). Further, in preclinical models of compulsive behavior, repeated hyperactivation of the OFC has been shown to trigger and exacerbate excessive, compulsive-like grooming in mice (Ahmari et al., 2013). Central to the functioning of the CSTC-circuitry is the neurotransmitters glutamate, gamma-aminobutyric acid (GABA)[6], dopamine and serotonin. They will briefly be discussed in the following paragraphs.

[1] obsessive-compulsive disorder
[2] obsessive-compulsive
[3] orbito-frontal cortex
[4] anterior cingulate cortex
[5] cortico-striatal-thalamic-cortical
[6] gamma-aminobutyric acid

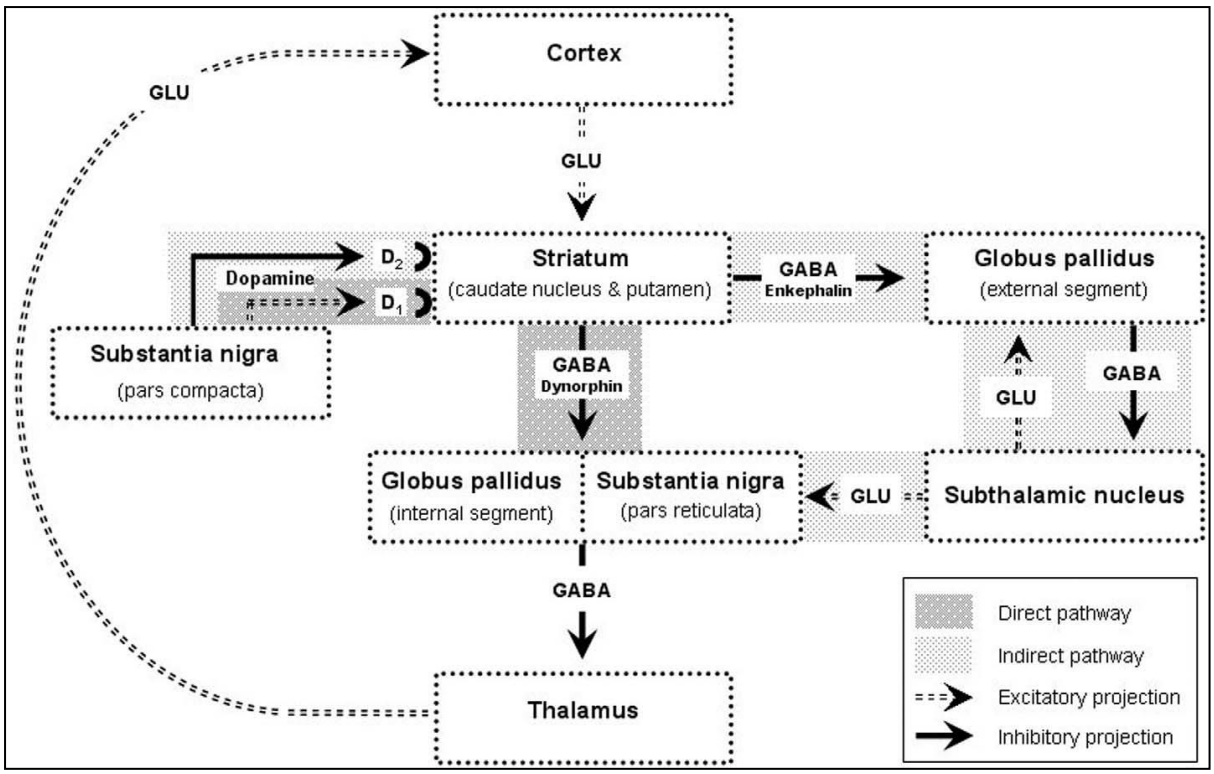

Figure 2-1 - The cortico-striatal-thalamic-cortical (CSTC) circuitry (reproduced from Tost et al., 2006)

2.2.2.2 A role for glutamate and GABA in OCD

Whereas glutamate can be regarded as the primary excitatory neurotransmitter in the brain (Pittenger et al., 2011), GABA[1] is the major inhibitory neurotransmitter (Petroff, 2002). Glutamate and GABA are fundamental role players in the normal functioning of the CSTC[2] circuitry. Nevertheless, treatment strategies aimed at manipulating glutamatergic and GABAergic neurotransmission generally fail to demonstrate ameliorative action. Thus, and considering that the primary focus of the current investigation involves the actions of serotonergic drugs, glutamate and GABA and the possible role they play in the neurobiology of OCD[3] will only briefly be summarized.

Given its role in excitatory signal propagation in the CSTC circuitry and that an overactive CSTC circuit has been proposed to underlie OCD, abnormalities in glutamatergic neurotransmission have been hypothesized to be involved in the pathophysiology of the condition (Pittenger et al., 2006, Carlsson, 2000, Rosenberg and Hanna, 2000, Rosenberg et al., 2000, Chakrabarty et al., 2005, Ting and Feng, 2008). Indeed, as alluded to earlier, studies have shown that glutamate-modulating drugs, e.g. riluzole, show promise in the treatment of refractory OCD in certain individuals (Pittenger et al., 2008, Pittenger, 2015). Further, in line with the literature reviewed above regarding the possible role of

[1] gamma-aminobutyric acid
[2] cortico-striatal thalamic cortical
[3] obsessive-compulsive disorder

neuroinflammation in OCD[1], correlations between elevated glutamate levels and autoimmune responses in the brain have been reported in OCD patients (Rotge et al., 2010). Indeed, it has been suggested that glutamate may act as a regulator of T-cell functioning via interactions with metabotropic glutamate receptors present on the surface of T-cells (Pacheco et al., 2007). Therefore, it is possible that bolstered glutamate release in OCD patients may contribute to enhanced cytokine production following infection-related activation of T-cells (Rotge et al., 2010). Further support for a link between compulsive behavior, excessive glutamate release and neuroinflammatory processes have been presented in pre-clinical data, demonstrating excessive glutamate release to be associated with neurotoxic modifications in microglial functioning and that such abnormalities are linked to repetitive and persistent behaviors in animal models (Frick and Pittenger, 2016).

The role of GABA[2], although necessary to inhibit signal propagation and the expression of voluntary motor actions, is less defined in the neurobiology of OCD. GABA tonically inhibits the relay of neural inputs via the basal ganglia to the thalamus (Kita, 2007). Under circumstances of behavioral activation, GABAergic functioning is disinhibited, resulting in the execution of motor behavior. This being true, numerous investigations have attempted to identify a possible role for GABAergic agents, e.g. the benzodiazepines, in the treatment of OCD; these remain ineffective (Baldwin et al., 2014, Katzman et al., 2014, Crockett et al., 2004, Hood, 2015, Hollander et al., 2003b, Bandelow et al., 2012). It therefore seems that, while GABA plays a regulatory role in the manifestation of both normal and aberrant behavior, and that its application in conditions relating to exclusive motor abnormalities, including muscle spasm (Lader, 2014, Rossiter, 2016) is valuable, its modification in the management of compulsions that may be founded on neuropsychological deficits, seems less useful. Moreover, it is likely that the anxiety experienced by OCD patients is borne from a different neuropsychological construct compared to patients with other forms of anxiety, e.g. generalized anxiety disorder, as the benzodiazepines, although not effective in OCD, demonstrate clinical efficacy in the latter group of conditions (Bandelow et al., 2015).

2.2.2.3 Performing a balancing act - serotoninergic and dopaminergic involvement in OCD

The earliest indication that serotonin may be involved in the pathogenesis of OCD came from pharmacological trials that demonstrated serotonin reuptake inhibitors to be effective in attenuating OCD symptoms to some extent (Soomro et al., 2008). However, not all patients respond to serotonergic interference, while a *causal* relationship between serotoninergic dysfunction and OCD[3] can also not be made based on an association between serotonergic intervention and treatment

[1] obsessive-compulsive disorder
[2] gamma-aminobutyric acid
[3] obsessive-compulsive disorder

response. Nevertheless, that SSRIs[1] remain the first line pharmacotherapeutic agents of choice, establishes at least some role for altered serotonergic functioning in most individuals with OCD[2] (Fineberg and Gale, 2005, Fineberg et al., 2012). That said studies aimed at elucidating the role of serotonergic dysfunction in OCD also yielded inconsistent results. For example, some investigations reported negative correlations between central serotonin transporter (SERT)[3] availability and OC[4] symptom severity (Reimold et al., 2007, Zitterl et al., 2008, Hesse et al., 2005, Pogarell et al., 2005, Hesse et al., 2011), while others failed to reveal any association (Van Der Wee et al., 2004). Also, whereas some previous reports revealed associations between OC symptoms and polymorphisms in genes involved in downstream serotonergic processes, the most recent genome wide association studies contradicted these reports, a discrepancy that probably has its origin in the differences in diagnostic and inclusion criteria used to select participants (Sinopoli et al., 2017). Further, many pharmacological strategies have been aimed at characterizing the neurobiological roles of specific serotonergic receptors in the manifestation of OCD. Although broad consensus exists that manipulation of serotonin receptors, most notably so the $5HT^5_{1A/B}$ and $5HT_{2A/B}$ subclasses, may modify the expression of symptoms, their role in the pathogenesis of OCD remains highly debated (Zohar et al., 1987, Zohar et al., 1992, Tucci et al., 2015, Tucci et al., 2014, Tsaltas et al., 2005, Hollander et al., 1991). Interestingly, while serotoninergic manipulation is also important in the treatment of many other psychiatric conditions, including major depression (Fournier et al., 2010), anxiety disorders (Bandelow et al., 2015, Bandelow et al., 2008) and schizophrenia (Mao et al., 2015), response rates in these conditions are equally suboptimal (Fournier et al., 2010, Helfer et al., 2016, Bandelow et al., 2008). Thus, attempting to shed more light on the neurobiological nature of serotonin involvement, research shifted instead to the role of serotonin in the regulation of broad cognitive processes, rather than focusing on disease-specific targets. This strategy proved to be more informative and has since highlighted the intricate role of serotonin as a regulatory neurotransmitter that in unison with dopamine, modulates and adjusts the motivational triggers driving rewarding-approach and punishing-avoidance feedback processing and action control (Cools et al., 2009). These findings informed our current understanding of the interactions between serotonin and dopamine and led research to conclude that dysregulated serotonin-dopamine interactions may alter the way in which individuals respond to their environment, thus presenting with aberrant symptomology (Den Ouden et al., 2015). Appraising the nature of this relationship from the perspective of OCD, the term

[1] selective serotonin reuptake inhibitors
[2] obsessive-compulsive disorder
[3] serotonin transporter
[4] obsessive-compulsive
[5] serotonin

'behavioral opponency' is appropriate to explain the serotonin-dopamine interactions. Two concepts must be highlighted here. First, dopamine is hypothesized to facilitate and promote reward-seeking behavior, whereas serotonin has been shown to act as the functional opponent of dopamine, inhibiting such behavior (Daw et al., 2002). Secondly, striatal dopaminergic signaling is elicited during the experience of reward, while suppression of dopamine release is observed during experiences of adverse events (Schultz, 2002). Further, these changes in dopaminergic signaling are thought to be responsible for reward- and punishment-related learning. With respect to OCD[1], reward could be conceptualized as task completion. For instance, as opposed to the neurobiological feedback processing in healthy individuals, when an OCD patient concerned about contamination engages in washing rituals, the absence of adequate feedback following task completion will elicit dopaminergic responses of equal magnitude during each hand washing cycle and the patient will persist to engage in reward-seeking behavior, i.e. constant hand-washing rituals (Cools et al., 2009). In healthy individuals, dopaminergic responses abate over time following presentation with the same outcome, thus not instigating and propagating reward-seeking behaviors. Considering that serotonin has been shown to curb the behavioral responses ensued by dopamine, it can be hypothesized that in patients with dysfunctional dopaminergic signaling, increased synaptic serotonin concentrations, as elicited by the administration of SSRIs[2], will dampen and regulate such dopaminergic responses. However, in the light of the high refractory rate observed in patients with OCD, recent findings by Figee et al. (2011) and Pinto et al. (2014) may explain the inconsistent therapeutic outcomes observed in OCD. First, different phenotypes of OCD have been linked with different underlying neurocognitive constructs in that patients with contamination OCD demonstrate deficits in reward anticipation and feedback processing. Second, patients with safety-related obsessions seems more cautious, less impulsive and are generally insensitive to processing punishing feedback. Thus, it is likely that differences in the manifestation of reward related engagement, and punishment related avoidance in patients with different OCD phenotypes, may contribute to differences in treatment response.

[1] obsessive-compulsive disorder
[2] selective-serotonin reuptake inhibitors

2.3 The gut microbiota and psychiatry: A new direction for future research

2.3.1 A concise overview of the gut microbiota

During the last few decades, our understanding of the gut microbiota and the role it fulfils in human physiological processes has changed significantly. To place a discussion of the relationship between the microbiota and human health and disease within the context of the current investigation, a few concepts and terms need clarification. The human body not only consists of many living human cells, but also contains the same magnitude of microbial cells, revealing a human to microbial cell ratio of close to 1:1 (Sender et al., 2016). Distinct microbial communities are found within different sites of the human body with approximately 10–100 trillion microbes inhabiting the human gastrointestinal tract (Gill et al., 2006) and this, together with the microbes (including bacteria, archaea, fungi and viruses; Kong, 2011) found *on* the human body, constitutes the human *microbiota*. The genes encoding the microbiota are known as the human *microbiome* (Clemente et al., 2012) and although it forms an integral part of the human body, its contribution to health and disease is still poorly defined (Eckburg et al., 2005). Further, an *enterotype* is a classification cluster of different bacterial ecosystems based on the dominance of certain bacterial phyla (Arumugam et al., 2011, Jeffery et al., 2012). The three most dominant human enterotypes are those characterized mainly by *Bacteriodes*, *Prevotella*, and *Ruminococcus* phyla(Arumugam et al., 2011). Different enterotypes have been shown to dominate during certain life stages or within specific populations (Arumugam et al., 2011).

Microbial colonization begins as early as “in-utero” (Koleva et al., 2015) and continues during the early stages of life (Perez et al., 2007) until reaching a stable state and composition by the age of 32 months (Breitbart et al., 2008, Adlerberth and Wold, 2009, Koenig et al., 2011); however, this process is most susceptible to change during the early stages of an individual’s development (Palmer et al., 2007). Together with the development and colonization of a healthy gut microbiota is the formation of a healthy mucosal intestinal barrier (Hooper et al., 2001). Considering the importance of the intestinal barrier to prevent pathogen invasion and subsequent infection, a healthy development of said barrier is essential. Importantly, a healthy functioning intestinal barrier is dependent on the colonization of the gut microbiota that ultimately results in its maturation and optimal functioning (Udall, 1981).

With respect to the bacterial composition of the gut microbiota, most of the human gut microbiota cluster within phyla *Bacteroidetes* and *Firmicutes* (Lozupone et al., 2012); however, members of the *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (Eckburg et al., 2005, Turnbaugh et al., 2006, Arumugam et al., 2011, Sommer and Bäckhed, 2013, Ley et al., 2005) are also present. The human microbiota, including the bacterial (Costello et al., 2009, Caporaso et al., 2011), viral (Reyes et al., 2010) and eukaryotic (Scanlan and Marchesi, 2008) constituents, is relatively stable in healthy adult

individuals (Rajilić-Stojanović et al., 2013, Faith et al., 2013, Sommer et al., 2017). That said, within- and between-individual variances are common, keeping in mind that a higher degree of variability is observed over time between different individuals than within the same individual (Eckburg et al., 2005). Several factors, e.g. diet (Wu et al., 2011), pharmacological interventions, i.e. antibiotics (O'sullivan et al., 2012, Jernberg et al., 2007, Dethlefsen et al., 2008), probiotics (Bravo et al., 2008, Bravo et al., 2011) and anti-psychotics (Davey et al., 2013, Bahr et al., 2015), as well as infections (Spiller and Garsed, 2009b, Spiller and Garsed, 2009a), can influence the overall composition of the human microbiota. Of these, diet or nutrition is believed to be the most influential factor (see paragraph 2.3.2.1; Flint et al., 2007, Bernstein and Shanahan, 2008).

2.3.2 Factors that influence the gut microbiome composition

2.3.2.1 Diet

Abrupt changes in diet can lead to significant alterations in the microbiome composition, which in turn may result in several beneficial or harmful consequences in the human body. For instance, drastic alterations in diet may alter the risk for disease, being either protective or detrimental, depending on the alteration introduced (Reid et al., 2011); this phenomenon has been directly linked to alterations in the microbiome (Wu et al., 2011). Indeed, *diet*-associated alterations in the microbiome have been linked to diabetes (Vijay-Kumar et al., 2010)—probably by enhancing energy harvest from food (Turnbaugh et al., 2006)—and impaired memory and learning (Li et al., 2009). Evidently, mice fed a meat-based diet showed a significant increase in α-diversity (microbial diversity within subjects) and richness in comparison to mice fed a standard pellet diet. Further, these changes were observed in parallel with improvements in memory consolidation and learning, which was ascribed to the higher levels of taurine obtained from a meat-based diet (Li et al., 2009). Also, David et al. (2014) confirmed a relationship between diet and alterations in the gut microbiota, evinced by data demonstrating that animal- and plant-based diets are associated with unique gut microbial compositions. Moreover, dietary changes not only influence gut microbes, but my also influence other organ systems, e.g. the pulmonary system (Trompette et al., 2014). Even of more relevance for the context of the current investigation, a comparison between European individuals consuming a westernized diet and African individuals on a rural, African diet, revealed the latter to protect against inflammation (De Filippo et al., 2010).

2.3.2.2 Physical or psychological stress

In humans, the most prevalent consequences of stress-related changes to the gut microbiota are associated with gastrointestinal pathology, i.e. inflammatory bowel disease (IBD)[1] and irritable bowel syndrome (IBS)[2] (Konturek et al., 2011). Therefore, while it is known that alterations in microbial composition can result in neuropsychiatric pathology (Foster and Neufeld, 2013), psychological stress in itself can elicit top-down alterations in the gut microbiome (Bailey, 2014). Several physiological functions and traits of the gastrointestinal system, e.g. gastrointestinal motility, secretion, permeability, gut barrier function, blood flow and visceral sensitivity are influenced by stress (Soderholm and Perdue, 2001, Konturek et al., 2011, Nakade et al., 2007). Gut permeability is of essential importance considering that an increase in permeability during times of stress, will allow immunomodulatory molecules to move into the systemic circulation (Meddings and Swain, 2000, Vanuytsel et al., 2014, Soderholm et al., 2002) and cause psychiatric pathology (Miller et al., 2011, Michopoulos et al., 2017). Some of these molecules include IL[3]-6, IL-10 and TNFα[4]. In fact, data from preclinical models have shown that stress caused by factors such as harsh light exposure, changes in sound, air ventilation, temperature, relative humidity, and human handling (Raff et al., 2011, Castelhano-Carlos and Baumans, 2009), can modulate the gut microbiome via immune-related pathways (Bailey, 2014).

2.3.2.3 Gender

When considering the microbiota and gender, studies have shown that gut microbial composition may indeed be gender-dependent (Kovacs et al., 2011, Fushuku and Fukuda, 2008, Gomez et al., 2015). Although no major differences in gut microbial composition are normally observed between males and females, greater abundance of the *Bacteriodes*- and *Prevotella* enterotypes have previously been observed in men compared to women (Mueller et al., 2006). This difference was related to the hormonal differences between men and women (Markle et al., 2013, Markle et al., 2014, Markle and Fish, 2014, Yurkovetskiy et al., 2013). On a functional level, sex differences in microbial composition were also observed in pre-clinical studies. Female mice have been shown to be more prone to microbiota-related and autoimmune diseases, for instance Type 1 Diabetes (Markle et al., 2013). Again, this observation was linked to the unique hormonal architecture in male vs. female mice (Markle et al., 2013), while sex hormone regulation in turn has been linked to proper functioning of the gut microbiota (Markle et al., 2013). Indeed, lower levels of testosterone were observed in germ-

[1] inflammatory bowel disease
[2] irritable bowel syndrome
[3] interleukin
[4] tumor necrosis factor alpha

free (GF)[1] mice compared to specific pathogen free (SPF)[2] mice (Markle et al., 2013). GF animals can be explained as animals completely free of any microbes, while gnotobiotic animals are raised with only a selected group of bacteria and SPF animals are only free of specific pathogens and contain a normal gut microbial composition. However, data have also indicated that *once challenged*, the male gut microbiota is more susceptible to injurious damage and that it triggers a greater inflammatory response compared to the microbiota of females (Homma et al., 2005).

2.3.2.4 Antibiotic treatment

Antibiotics can have detrimental effects on human health via modifications in the gut microbiota (Rashid et al., 2012, Sullivan et al., 2001) which is elicited by the suppression or even eradication of certain beneficial bacterial strains (Dethlefsen et al., 2008, Palmer et al., 2007, Eggesbø et al., 2011, De Filippo et al., 2010, Bech-Nielsen et al., 2012). One of the most common symptoms of antibiotic related microbiota changes is seen in the occurrence of antibiotic-associated diarrhea (Beaugerie and Petit, 2004). However, associations between antibiotic-related microbiome modifications and sudden manifestations of asthma and allergic hyper-reactivity in young patients (Marra et al., 2006, Prioult and Nagler-Anderson, 2005) have also been documented. Evidently, microbial composition will not be restored immediately following withdrawal of antibiotic treatment, the effects thereof in some instances still being observed 2 years post-treatment (Jernberg et al., 2007). In light of the use of probiotic supplementation during antibiotic therapy, it is interesting to note that probiotic intervention to restore the gut microbiota following the use of antibiotics, is not always effective (Suez et al., 2018, Zmora et al., 2018). That said, even though antibiotics have detrimental effects on human health by disturbing the both beneficial and harmful strains of intestinal bacteria (Bech-Nielsen et al., 2012), antibiotic treatment has also been associated with beneficial health effects, especially with respect to energy homeostasis, i.e. improved glucose tolerance, improved weight management and decreased levels of adipose inflammatory markers (Membrez et al., 2008). It was proposed that these effects are related to a decrease in inflammatory tone as well as a decrease in gastrointestinal permeability (Membrez et al., 2008, Cani et al., 2008b, Cani et al., 2008a).

2.3.3 Functions and adverse effects of the gut microbiota in human health and physiology

Broadly regarded, the functions and effects of the microbiota can primarily be considered as beneficial (Stecher and Hardt, 2011). Indeed, the human microbiota has been shown to protect against epithelial cell injury (Rakoff-Nahoum et al., 2004), contribute to processes of fat storage (Backhed et al., 2004)

[1] germ-free
[2] specific pathogen free

and immune system development (Ivanov et al., 2009, Atarashi et al., 2013) , stimulate intestinal angiogenesis, and to provide protection against pathogens (Stappenbeck et al., 2002). Furthermore, the microbiota influences and regulates a range of physiological parameters and processes in its host, including pH regulation, energy homeostasis, as well as intestinal epithelial cell proliferation and differentiation (Backhed et al., 2004, Rakoff-Nahoum et al., 2004, Samuel et al., 2008, Backhed et al., 2005, Ridaura and Belkaid, 2015). Furthermore, the microbiota is also a significant modulator of pharmacokinetic drug properties and can therefore influence the efficacy of pharmacotherapeutic treatment (Gonzalez et al., 2011); it has thus been proposed as an essential 'organ' that regulates and provides support to a range of physiological processes (Eckburg et al., 2005, Nicholson et al., 2012). Such effects are borne from either *symbiotic* or *commensal* relationships within the gut microbiota (Hooper and Gordon, 2001). Symbiotic relationships describe a beneficial relationship for only one of the involved partners but without harm to the other (Perret et al., 2000), whereas commensal relationships refer to a relationship where both partners will benefit equally from the relationship (Steinert et al., 2000). These functions will be briefly discussed in the following paragraphs.

2.3.3.1 The metabolic and protective functions of the gut microbiota

As alluded to in paragraph 2.3.3, microbial processes contribute to the nutritional status of the host (Backhed et al., 2005, Hooper and Gordon, 2001, Guarner and Malagelada, 2003, Kaakoush et al., 2015, El Aidy et al., 2015) by enhancing energy harvest (Dalby et al., 2017, Blaut, 2015), synthesizing essential vitamins, e.g. vitamins B_{12} and K as well as cofactors (Hill, 1997, Rowland et al., 2017, Conly et al., 1994), and promoting the absorption of essential minerals including magnesium, zinc and iron (Younes et al., 2001, Miyazawa et al., 1996, Zeng et al., 2017). Microbes are also responsible for the breakdown of complex lipids and polysaccharides and for the elimination of waste particles (Hooper and Gordon, 2001, Li et al., 2008, O'Hara and Shanahan, 2006). Apart from its functions in nutrition, the gut microbiota also prevents pathogen colonization of the gut. In cases where the gut microbiota is exposed to potentially harmful bacteria and other bacterial constituents, normal microbial homeostasis will often be maintained as the resident microbiota is able to inhibit invading pathogen overgrowth (Kamada et al., 2013, Lawley and Walker, 2013, Sommer and Bäckhed, 2013). Said protection is provided by the capacity of the resident microbiota to adjust its own level of expression that ultimately triggers a number of protective mechanisms such as depleting the nutrients competed for by the pathological invader, activating and modulating the adaptive immune system and directly inhibiting the invading pathogen via cell-cell mechanisms (Macia et al., 2012, Bäumler and Sperandio, 2016).

Taken together, the gut microbiota is essential for the fermentation and digestion of carbohydrates, the production of vitamins and to prevent colonization by pathogens (Clemente et al., 2012, Hooper and Macpherson, 2010, Kamada et al., 2013, Renz et al., 2012, Stecher and Hardt, 2011).

2.3.3.2 Immunological role

Normal development of the human immune system is partly dependent on the gut microbiota. The gastrointestinal tract is not only one of the largest and most complex organ systems in the human body (Clarke et al., 2010, Umesaki et al., 1999, Round and Mazmanian, 2009), but is also considered to be a major modulator of the immune system. In fact, 70 - 80% of the body's immune cells are found in close proximity to the gut microbiota, specifically in the gut-associated lymphoid tissue (Yamanaka et al., 2003). These immune cells are highly sensitive to changes in the gut microbial composition and when triggered, will result in lymphocyte accumulation and differentiation (Yamanaka et al., 2003). Depending on the nature of the trigger, these responses will either be beneficial or harmful to human health. Indeed, the importance of the gut microbiome in normal immune development has been demonstrated in preclinical models showing observable distinctions in immune maturation between germ-free mice and normal controls (Shanahan, 2009). These findings not only highlighted the importance of the environment in the normal development of a healthy microbiota, but also elucidated a clear and important role for the microbiota as an immune regulator. Although the functional characteristics of the gut associated immune system are determined by microbiota composition and therefore are subject to influence by the microbial manipulation (Brinkman et al., 2013), significant changes in both the microbiome and associated immune traits are less likely to transpire in young adulthood compared to childhood, mainly due to increased stability of the microbiome as we grow older (Brinkman et al., 2013).

The microbiome regulates the immune system via different mechanisms, including crosstalk and reactivity between toll-like receptors (TLRs[1]) and nucleotide-binding oligomerization domain (NOD)[2]-like receptors (NLRs)[3] found on and derived from immune cells and pathogen-associated molecular patterns (PAMPs)[4] found in bacteria (Hooper et al., 2012, Askarian et al., 2018). Studies have shown in vitro administration of certain carbohydrates, e.g. polysaccharide A, to induce innate and adaptive immune responses via changes in microbiota-TLR crosstalk (Wang et al., 2006). TLRs are present on most immune cells, notably also on those located in the gut, and are critical for activating pathogen-associated immune responses. As TLRs are relatively antigen non-specific, they can recognize broad

[1] toll-like receptors
[2] nucleotide-binding oligomerization domain
[3] NOD-like receptors
[4] pathogen-associated molecular patterns

classes of molecular patterns that are generally shared by most pathogens, but not by the host cells (Rakoff-Nahoum et al., 2004). In the case of pathogen invasion, TLRs, among other factors, are responsible for triggering inflammatory and immune responses, thereby bolstering the potential of an individual to combat and resist the consequences of infection. Additionally, TLRs[1] also have a number of non-immune related functions and are involved in maintaining epithelial homeostasis, protecting the epithelial layer and to repair mucosal damage (Rakoff-Nahoum et al., 2004). With respect to the current investigation, at least some pre-clinical evidence point to the possibility that TLRs may be involved in 'dysbiosis'-induced altered behavior (Rakoff-Nahoum et al., 2004). Whereas these interactions describe a bottom-up regulation of the immune system by the microbiota, a top-down mechanism for the regulation of the microbiome also exists in the form of interactions between Paneth cells and the microbiota. Paneth cells are an integral component of the gut-associated innate immune system and secrete several antimicrobial peptides and proteins following exposure to bacteria (Ayabe et al., 2000). A clinical example of the significance of such crosstalk is seen with the promotion of intestinal angiogenesis and subsequently increasing the gut's absorptive capacity (Stappenbeck et al., 2002).

As stated earlier, changes in the microbiota can trigger immune responses that may elicit either beneficial or harmful responses. Beneficial responses can be related to processes that protect against invading pathogens via adequate, but not excessive inflammatory responses as explained above, but also to anti-inflammatory responses. In fact, modifying the microbiota in animal models with LPS[2] administration, has been shown to bolster the release of anti-inflammatory cytokines, e.g. IL[3]-10 and transforming growth factor β (TGF- β)[4] (Ueda et al., 2010). To the contrary, harmful consequences can also be triggered following microbiota modification due to the excessive release of pro-inflammatory cytokines, e.g. IL-1 and IL-8 that can result in significant inflammatory responses (Ueda et al., 2010). These may have noteworthy consequences for human health as has been shown previously (Lyke et al., 2004).

2.3.3.3 'Dysbiosis' and human disease

The gut microbiota is malleable and ever-changing over the course of an individual's lifespan (De Filippo et al., 2010). To understand the role of the microbiota in the manifestation of clinical illness, be it psychiatric, peripheral or somatic, the concept of 'dysbiosis' must be understood. Whereas the microbiota will remain relatively stable throughout life (Costello et al., 2009), 'dysbiosis' refers to

[1] toll-like receptors
[2] lipopolysaccharide
[3] interleukin
[4] transforming growth factor beta

unstable imbalances, i.e. constantly adaptive imbalances between "protective" and "harmful" intestinal bacteria (Tamboli et al., 2004) that may contribute to a state of disease and/or influence its treatment (Clemente et al., 2012). Indeed, irrespective of the underlying cause of microbial modification, it has been shown that compositional variations in the microbiome can elicit such effects as a function of changes to the microbiota as a whole, or to specific taxa only (Virgin and Todd, 2011). 'Dysbiosis' in microbial composition can be considered as the basis of chronic bowel disorders, which are correlated with compositional changes in the gut microbiota (Morgan et al., 2012).

Imbalances in the gut microbiota may be associated with the predisposition of diabetes (Grasset et al., 2017), obesity (Kim et al., 2012a, Ley et al., 2006, Reinhardt et al., 2009), allergy (Penders et al., 2007, Fujimura and Lynch, 2015), IBD[1] (MacDonald and Monteleone, 2005), and chronic fatigue syndrome (Lakhan and Kirchgessner, 2010). Recently, an association between 'dysbiosis' and psychiatric pathology has been made clear by among others the high comorbidity rates of 'dysbiosis'-associated peripheral pathology, e.g. IBD, and neuropsychiatric illness, e.g. major depression (Park et al., 2013), generalized anxiety (Bercik et al., 2010), autism (Adams et al., 2011), and OCD[2] (Collins et al., 2012, Turna et al., 2016). Moreover, the influence of 'dysbiosis' on the gut-brain axis, can transpire on all levels of gut-brain communication, as summarized in section 2.3.3.3.

2.3.4 Intermicrobial communication and gene transfer

Inter-organism communication takes place between the different bacterial strains and microorganisms residing in the human gut, as well as between these and the host itself (Nicholson et al., 2012). Indeed, bacterial species can sense the presence of "self", i.e. organisms of the same species (intra-species), as well as other bacterial species (inter-species), by applying a mechanism known as quorum sensing, i.e. detecting and responding to changes in cell population density (Bassler and Miller, 2006, Miller and Bassler, 2001). Briefly, quorum sensing involves the secretion and detection of autoinducers, i.e. signaling molecules, that allows cells to sense and respond to one another. Further, a direct relationship exists between the measured auto-inducer concentration and the bacterial population density. Mechanistically viewed, quorum sensing influences the bacterial population by adjusting patterns of genetic expression to yield functional clusters of bacteria in a manner that will benefit the larger community (Shukla and Bhathena, 2016). In other words, by facilitating cell differentiation, intercellular communication and the exchange of diffusible signals

[1] inflammatory bowel disease
[2] obsessive-compulsive disorder

between the bacteria and its host, it allows the commensal community to function as a multicellular organism (Bassler and Miller, 2006).

Major functions of quorum sensing within a bacterial community are to control population virulence (Chan et al., 2015, Lee and Song, 2005, Darkoh et al., 2015), motility, biofilm formation, and sporulation (Sepulchre et al., 2007). In practical terms, changes in bacterial gene expression related to taxa-specific behaviors, i.e. virulence and sporulation (Sepulchre et al., 2007) may result in either beneficial or detrimental effects within the host (Lupp and Ruby, 2005). For example, quorum sensing can result in the inhibition of toxin release (Laughton et al., 2006, Li et al., 2011, Medellin-Peña et al., 2007), production of nutrients and vitamins (Goodman et al., 2009, Lebeer et al., 2008, Strozzi and Mogna, 2008, van Reenen and Dicks, 2011) and in modulating mucosal signaling pathways (Hord, 2008). Thus, functional quorum sensing is essential for a healthy gut microbiota and it is believed that dysfunctional processes of quorum sensing are partially underlying microbiome 'dysbiosis' (Gao et al., 2018, Breton et al., 2013).

Another way the microbiota adapts to an ever-changing environment, is by means of gene transfer (Ochman et al., 2000). Transfer of genes takes place between different microbial species, between commensal microbial communities and pathogens, or between different pathogens (van Reenen and Dicks, 2011). This process allows bacteria not only to evolve through mutation and rapid replication, but also by acquiring new deoxyribonucleic acid (DNA)[1] that can be incorporated into its genome. Gene-transfer transpires via several mechanisms. First, *natural transformation* involves the uptake and incorporation of extracellular environmental DNA by bacteria that developed the competence to do so. Such intact or fragmented DNA constantly reaches the external environment from decaying cells (Lorenz and Wackernagel, 1994, De Vries et al., 2004). Second, *conjugative transfer*, especially between bacteria and small genetic entities such as plasmids, involves organism-to-organism transfer of DNA via pore formation (El Yacoubi et al., 2007). Last, *transduction* describes the process by which bacterial DNA is transferred between cells by means of bacteriophages, i.e. bacteria-infecting viruses (Brabban et al., 2005, Zinder and Lederberg, 1952). Successful gene transfer, either between bacteria or between bacteria and organisms of different kingdoms (yeast, plants, viruses) (Davison, 1999, Gebhard and Smalla, 1998), is dependent on several factors (Van den Eede et al., 2004). These factors include *inter alia*, the mechanism of transfer, the availability and persistence of stable extracellular environmental DNA, and the ability of the host to take up and recombine foreign DNA fragments (Van den Eede et al., 2004). In this regard, commensal bacteria can act as reservoirs for transferred genes that may encode antibiotic resistance as is true for commensal *Escherichia coli* (Singh et al., 2005,

[1] deoxyribonucleic acid

Ochman et al., 2000, Sommer et al., 2010). Indeed, this evolutionary ability of bacteria is of great concern considering the current antibiotic resistance epidemic (Ammor et al., 2007, Salyers et al., 2004, Zhou et al., 2005). However, although gene-transfer may be regarded as a novel therapeutic approach in the treatment of microbiota-associated pathologies, the process as it occurs naturally seems to be benefit the survival of the bacteria only, while literature on the therapeutic potential for its hosts, is lacking.

2.3.5 The microbiota-gut-brain axis

Originally, the idea of the gut-brain axis evolved from findings which directly linked differences in microbial composition with altered behavior and cognition (Stilling et al., 2014), seen for example in patients with comorbid IBD[1] and depression, anxiety, and stress (Park et al., 2013, Bailey et al., 2011, Bailey and Coe, 1999, Claesson et al., 2011, Berrill, 2013). In another investigation, altered social behavior was also observed to be concomitant with 'dysbiosis' in microbial composition (Desbonnet et al., 2010). Therefore, the question was asked how changes in the microbiota can influence these and other non-gut-related processes in the human body and, following numerous investigations, research concluded that the gut-brain axis involves a complex framework of communication that includes cell-cell signaling and between-system signaling that involves several organ systems, including the central nervous system. Based on these findings, the microbiota has also been described as a collection of *"mind-altering bugs"* (Cryan and Dinan, 2012). In this section, we will divulge how the gut and brain communicates and how alterations to the microbiota may contribute to dysfunctional communication processes.

2.3.5.1 Nervous system connections as the foundation of gut-brain crosstalk

The limbic system, consisting of the limbic cortex, amygdala and hippocampus, is responsible for a myriad of functions, including fear and/or arousal processing, sensory and motor functions, as well as memory and spatial navigation. Further, the limbic system also receives input from other brain regions involved in these functions, i.e. the prefrontal cortex and the striatum. Importantly, the limbic system is extensively innervated by the autonomic nervous system, a connection that forms the primary neuronal bridge between the brain and the periphery (Rajmohan and Mohandas, 2007). Responsible for the bidirectional neuronal communication between the gut and the brain, the autonomic nervous system consists of both the parasympathetic (vagal) and sympathetic nerves, the former of which is the major pathway responsible for brain-periphery communication. Following

[1] inflammatory bowel disease

excitation or activation, the parasympathetic and sympathetic systems will have an excitatory or an inhibitory effect on the gut respectively.

THE VAGAL NERVE

Ascending vagal nerves constitute a direct link between the gut and the central nervous system (CNS)[1] and are especially involved in anxiety-like behavior. Further, the vagal nervous system is also able to recognize and respond to changes in the gut microbial composition (Goehler et al., 2005), a process known to result in behavioral alterations in both humans and preclinical models (Bercik et al., 2011). In fact, in vagotomized mice, in which the vagal nerve has been removed to reduce acid secretion in the GIT, probiotic supplementation with *Bifidobacterium longum* failed to result in anxiolytic-like effects, as opposed to its effects in non-vagotomized subjects (Bercik et al., 2011). Also, in another study of vagotomized vs. non-vagotomized mice, long-term probiotic treatment with *Lactobacillus rhamnosus* resulted in anti-depressant and anxiolytic-like responses linked with limbic specific alterations in GABA[2] concentration in non-vagotomized mice only (Bravo et al., 2011). These findings show probiotics to be a possible valuable adjunctive therapy when treating psychiatric diseases. Anxiety and depression respond to the same first line treatment, providing some evidence for the same neurobiological constructs to be involved in the pathophysiology thereof (Nutt et al., 2002). Possible mechanisms of how the vagal nerve senses changes in the gut microbiota and transfer those to altering central nervous system functioning, are still being investigated (Suarez et al., 2018). It is known however, that cholinergic signaling in the CNS broadly modulates catecholaminergic signaling (Olofsson et al., 2012), possibly providing some explanation for its effects following changes in bottom-up vagal neurotransmission in response to microbiota modification.

NEUROTRANSMITTERS AND THEIR PRECURSORS

The gut microbiota is involved in the production and release of several neurotransmitters, *viz*. serotonin—90% of the total body concentration—(*Streptococcus, Escherichia, Enterococcus*), dopamine—50% of total body concentration—(*Bacillus, Serratia*), GABA (*Lactobacillus, Bifidobacterium*), norepinephrine (*Escherichia, Bacillus, Saccharomyces*), and acetylcholine (*Lactobacillus)* (Collins et al., 2012). Strains of *Lactobacilli* and *Bifidobacterium* produce GABA (Collins et al., 2012), while oral administration *of Bifidobacterium infantis* increases levels of the serotonin precursor, tryptophan (Desbonnet et al., 2008). Both GABA and serotonin are critical components of the CSTC circuit implicated in the neurobiology of OCD[3]. Moreover, these organisms are sensitive to

[1] central nervous system
[2] gamma-aminobutyric acid
[3] obsessive-compulsive disorder

changes in said neurotransmitters in the host; for example, in the event of a stress response, fight-or-flight associated noradrenalin release will result in altered noradrenalin-related gene expression in the gut microbiota as well as altered conjugative transfer between enteric bacteria (Peterson et al., 2011). Despite these relationships being known, their functional significance within the field of psychiatry is still under investigation. Whether microbe-associated changes in neurotransmitter release can be directly linked to altered CNS[1] concentrations, or if it elicits indirect responses in the brain via changes in vagal nerve activity, is still unknown.

Considering that the first line pharmacotherapeutic treatment of OCD[2] and other CNS conditions with SSRIs depends on modifications in serotoninergic signaling, several investigations aimed to elucidate the bidirectional relationship between alterations in gut serotonin concentrations and psychiatric disease (Clarke et al., 2013). In addition to its own contribution to serotonin release, the microbiota also promotes the biosynthesis of serotonin by enterochromaffin-like cells (ECs)[3] (Yano et al., 2015). In fact, as referred to earlier, the gut not only produces and utilizes more than 90% of the body's overall serotonin concentrations, but also serves as a reservoir for the majority of total-body serotonin (Gershon and Tack, 2007). Moreover, the gut microbiota is essential for the metabolism of tryptophan, the precursor of serotonin (Lyte, 2013), while reduced levels of serotonin and its metabolite, 5-hydroxyindolacetic acid (5-HIAA)[4], were observed in antibiotic treated mice (Desbonnet et al., 2015). That said, a direct association between changes in the gut microbial composition and altered striatal serotonin metabolism has also been demonstrated in GF mice, in which a higher serotonergic turnover rate was observed compared to conventional animals (Heijtz et al., 2011). Given that serotonin plays an integral part in the pathophysiology of OCD, it can be hypothesized that by modulating the central serotonergic neurotransmission, the gut microbiota may influence the underlying pathology of OC[5] behavior (Okasha, 2002), constituting proof-of-concept for investigations into possible microbiome-OCD relationships.

THE ROLE OF THE HPA-AXIS AND THE IMMUNE SYSTEM IN GUT-BRAIN CROSS TALK

Given that obsessions and compulsions can cause significant distress, a causal relationship between OCD and stress-related altered microbiota composition has been proposed (Turna et al., 2016). Furthermore, stress-related microbiota modification and 'dysbiosis' have also been proposed to complicate the prognosis of psychiatric illness by exacerbating the clinical severity of comorbid anxiety

[1] central nervous system
[2] obsessive-compulsive disorder
[3] enterochromaffin-like cells
[4] 5-hydroxyindolacetic acid
[5] obsessive-compulsive

and depression (De Palma et al., 2014). As such, it has previously been proposed that in some cases, rather than being the result of stress itself, OCD[1] may ensue because of stress on the microbiota (Rees, 2014), the link of which can possibly be found in the immune system. Over the past two decades, a number of advances have been made with respect to our understanding of trauma and stress on brain function. In fact, a vast body of research now confirms pathophysiological relationships between OC[2] symptom manifestation and markers of altered immune response (da Rocha and Correa, 2011, Furtado and Katzman, 2015, Gray and Bloch, 2012, Rodríguez et al., 2017, Garvey et al., 1998).

As alluded to earlier, positive associations between obsessive-compulsive symptoms onset and experiences of trauma and stress have been documented (Findley et al., 2003, Toro et al., 1992, Rosso et al., 2012). Indeed, up to 37% of patients describing their onset of OCD symptoms noted that these symptoms were preceded by non-traumatic stressful life events, including experiences related to health, education and bereavement (Real et al., 2011). Further, associations between birth-related complications, e.g. prolonged labor, and OC symptom manifestation later in life, have been demonstrated. Trauma, both in early childhood (Briggs and Price, 2009, Carpenter and Chung, 2011) as well as later in life (Frydman et al., 2014, Lafleur et al., 2011), especially in women (Maina et al., 1999), has been shown to exacerbate OC symptomology. Despite its apparent link to stress, data on HPA[3] axis functioning in OCD are inconsistent. Increased baseline activity of the HPA axis in OCD has been illustrated by studies measuring cortisol at multiple points throughout the day or by 24-hr urinary free cortisol collection (Gustafsson et al., 2008, Kluge et al., 2007). Similarly, elevated cerebrospinal fluid (CSF)[4] levels of corticotrophin releasing hormone (CRH)[5] and adrenocorticotrophic hormone (ACTH)[6] have also been reported in OCD (Kluge et al., 2007, Monteleone et al., 1997, Catapano et al., 1992). That said, other studies do not confirm elevated HPA axis activity in patients with OCD (Chappell et al., 1996, Bailly et al., 1994). However, more is known about the role of aberrant immune responses per se, in the etiopathology of OCD. Briefly, a relative shift to an inflammatory as opposed to an anti-inflammatory state (Furtado and Katzman, 2015, Gray and Bloch, 2012, Rao et al., 2015) has been proposed. These alterations are believed to contribute to psychiatric pathology in a number of ways. First, peripherally produced pro-inflammatory cytokines (Miller et al., 2009) as well as T helper 17 cells (Th17) (Dileepan et al., 2016) have been shown to enter the central nervous system where they mediate inflammatory responses in the basal ganglia and the anterior cingulate cortex, both

[1] obsessive-compulsive disorder
[2] obsessive-compulsive
[3] hypothalamus-pituitary-adrenal
[4] cerebrospinal fluid
[5] corticotrophin releasing hormone
[6] adrenocorticotrophic hormone

regions of the brain that are closely involved in OC symptom presentation. Second, neurotransmitter availability is compromised, contributing to the deficits in serotonergic activity that are believed to play a major role in the neurobiology of OCD[1] (Fernandez and Gaspar, 2012). Third, antineuronal antibodies produced by the humoral immune system are generated against the basal ganglia, contributing to neurodegeneration and oxidative stress (Morer et al., 2008, Dale et al., 2005). Last, symptom exacerbation in OCD has been linked to dysregulated activation of pro-inflammatory microglia that contribute to increased synaptic glutamate release, thereby inducing neurotoxicity (Frick et al., 2013, Prinz and Priller, 2014). While the PANS[2] phenomenon is currently being investigated within the context of clinical psychopathology (see paragraph 2.2.1.3), it is possible that compositional differences in the microbiota may underlie the unique manifestation of immune-associated neuropsychiatric responses of affected individuals. Indeed, the gut-brain relationship has more recently attracted significant interest with respect to its ability to activate the immune system via cytokine release (Turna et al., 2016). Moreover, whereas altered immune function can lead to microbial modification (Vijay-Kumar et al., 2010), microbial alterations in turn can result in aberrant behavior and elicit abnormal brain development (Heijtz et al., 2011).

In summary, 'dysbiosis' can contribute to the activation of the HPA[3] axis and also trigger a pro-inflammatory state (Montiel-Castro et al., 2013), both of which have previously been associated with OCD (Furtado and Katzman, 2015, Şimşek et al., 2016a). Considering evidence demonstrating that a dysregulated immune system is associated with at least some cases of OCD (Frick and Pittenger, 2016, Bhattacharyya et al., 2009), the clinical significance of such microbiota-immune system interactions must be considered in future investigations (Figure 2-3).

[1] obsessive-compulsive disorder
[2] pediatric acute onset neuropsychiatric syndrome
[3] hypothalamus-pituitary-adrenal

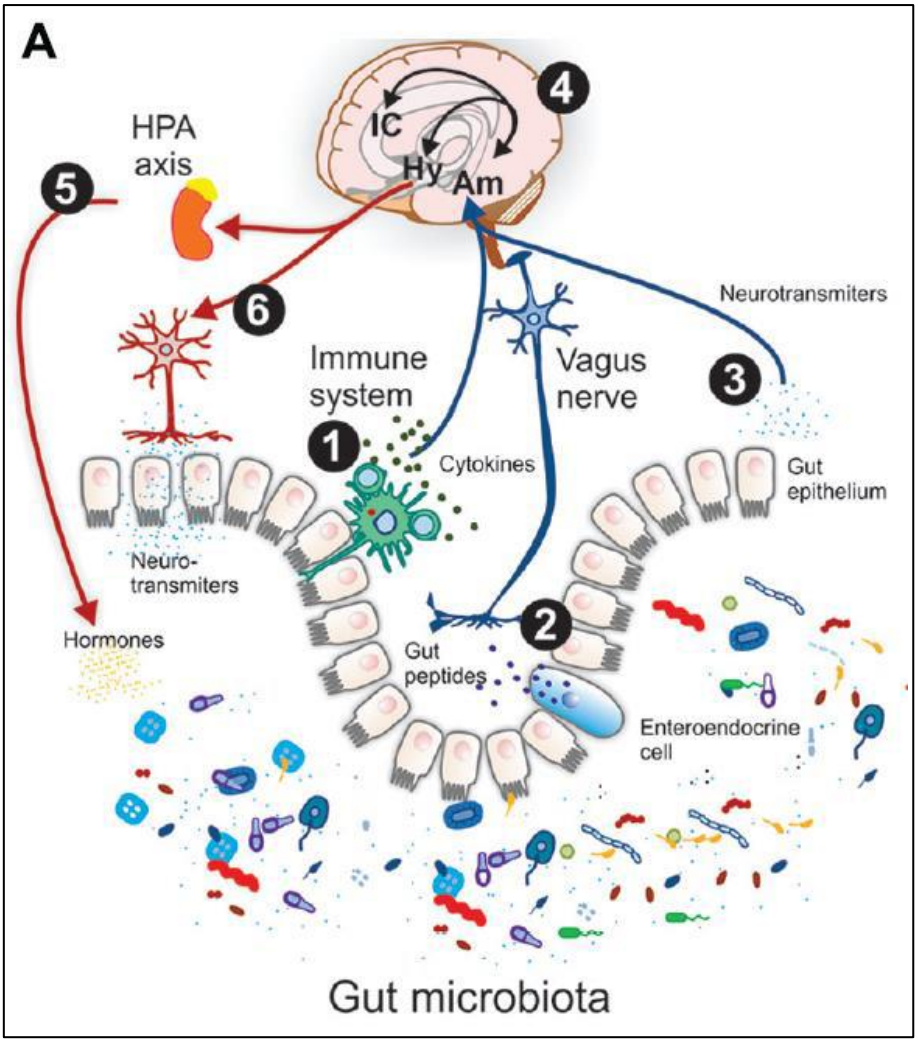

Figure 2-3 - The microbiota-gut-brain axis and the involved direct and indirect pathways of communication. Communication between the gut and brain is elicited via 1) the immune system, 2) the vagal nerve, 3) neurotransmitters or their precursors, 4) involvement of a discrete neural network, including the amygdala (Am), insular cortex (IC) and hypothalamic activation (Hy), 5) HPA-axis activity, and 6) sympathetic activation. (reproduced from Montiel-Castro et al. 2013)

Considering the above, a substantial body of research proposes a role for altered gut microbiota composition in the modulation of said crosstalk mechanisms. Moreover, evidence exists that such modifications may result in aberrant neurocognitive processes, thereby playing a role in the etiopathology of psychiatric illness (Fond et al., 2015, Kennedy et al., 2012). Fundamental findings from this body of literature that are of relevance for the current investigation, will now be summarized.

2.3.5.2 Methods of modification of the gut microbial composition in pre-clinical models

While microbial modification in humans are mostly brought about by probiotic supplementation, several methods of modification have been used in preclinical models to alter gut microbiota. These include cohabitation (Bercik et al., 2011), fecal transplantation (Collins et al., 2013), the use of pre- and probiotics, and diet alterations (Davey et al., 2013, Bahr et al., 2015).

COHABITATION

Cohabitation or cohousing can be described as animals living in close proximity to one another, thereby resulting in such subjects, confounding factors aside, presenting with near-identical microbial compositions (Song et al., 2013). Cohabitation is not to be confused with cross-fostering, which describes the switching of pups between moms of different litters, within the first 48 hours after birth. As opposed to cohabitation from an older age, cross-fostering can elicit permanent changes in the microbiota (Daft et al., 2015). When considering cohabitation as a method of microbial modification, it is important to note that the initial maternal environment will have a dominant effect on the microbial composition (Friswell et al., 2010). In fact, cohabitation of isogenic mice, i.e. having the same or a similar genome, but from different environments since weaning resulted in near analogous microbiome presentation (Orcutt et al., 1987).

In line with findings linking alterations in the gut microbiota to changes in immune responses, gut associated immunity has also been influenced by cohabitating mice of different genotypes. Interestingly, such alterations were associated with differences in responses to disease triggers or treatment (Brinkman et al., 2013). Importantly, cohabitation can be of value when microbial manipulation is considered as a treatment intervention, especially in rodent models, as it is less invasive compared to fecal transplants and is associated with natural changes in the microbiome, as opposed to the relatively non-individualized approaches, e.g. probiotic supplementation (Ridaura et al., 2013). An aspect of consideration when considering cohabitation will be coprophagy, the occurrence of rodents eating another's feces, leading to microbiota carry over. However, an important consideration is that, while within-species microbial modification in the gut microbiota have been observed as a result of cohabitation, it is important to emphasize that the microbial composition of specific mouse strains is often resistant to inter-species cohabitation (Campbell et al., 2012).

FECAL TRANSPLANTATION

Fecal transplantation or fecal microbiota transplantation (FMT)[1] is the process of transplanting feces from a healthy donor to a recipient, usually with the aim to restore the intestinal microbial balance of the recipient (Xu et al., 2015). As opposed to cohabitation, which is not possible in humans, fecal transplantation has already been trialed in clinical samples (Taur et al., 2018, Bakken et al., 2011). The method is a relatively simple and valuable means of microbial modification. Indeed, FMT from a normal to an affected subject has been shown to be prophylactic for certain IBS-associated pathological traits, while the opposite was true in the other direction, i.e. whereby IBS[2]-traits have

[1] fecal microbiota transplantation
[2] irritable bowel syndrome

been carried over from an affected to a healthy subject (De Palma et al., 2017). The same was shown for adiposity-traits in non-Western diet- vs. Western diet-fed mice (Khoruts et al., 2010, Fond et al., 2015, Turnbaugh et al., 2009). FMT[1] presents with several advantages as a putative clinical intervention in psychiatric illness (Evrensel and Ceylan, 2016). The method is cost-effective and potentially valuable in terms of reproducibility (Evrensel and Ceylan, 2016). Currently, fecal transplantation represents an exciting novel method for the treatment of inflammatory and other diseases (Hota and Poutanen, 2018), including psychiatric conditions (Pamer, 2014). CNS[2] disorders that have demonstrated at least some response to FMT include Parkinson's disease, chronic fatigue syndrome (CFS)[3] and autism (Bercik et al., 2011, Collins et al., 2013).

PROBIOTIC TREATMENT

Probiotic treatment involves the administration of 'beneficial' living bacterial strains, with the aim to restore the microbial balance (Stilling et al., 2014). In clinical settings, the use of probiotics to restore the microbial balance during and following antibiotic treatment, is common practice (Shanahan, 2009). However, while some research debate its efficacy (Suez et al., 2018, Benton et al., 2007), it has been shown that the timing of intervention plays a major role in determining the therapeutic outcome (Obermeier et al., 2003). Probiotics may have some advantage over other techniques, e.g. cohabitation and FMT, in that the abundance of specific strains to be administered can be adjusted according to specific outcomes. For example, it has been shown that a single immunomodulatory polysaccharide derived from *Bacteroides fragilis* is of benefit in correcting both mucosal and systemic immune defects in GF[4] mice (Mazmanian et al., 2005). Thus, given the description of a gut-brain axis that may function via immune mechanisms and that probiotics have shown immunomodulatory properties, such interventions may provide a novel therapeutic avenue for investigating in psychiatric illness. Further, several pre-clinical studies have shown that probiotics resulted in behavioral modifications. In animal models of depression (Messaoudi et al., 2011, Gilbert et al., 2013) and anxiety (Bercik et al., 2010, Bercik et al., 2011, Bravo et al., 2011, Messaoudi et al., 2011, Ohland et al., 2013, Distrutti et al., 2013, Desbonnet et al., 2010) mice treated with probiotics have shown behavioral changes akin to anti-depressant and anxiolytic effects, respectively. It has further been shown that the anti-depressant-like effects of probiotics seemed to be mediated via GABA[5] receptor signaling (Bravo et al., 2011). Although a paucity of clinical data exists, at least some studies indicate

[1] fecal microbiota transplantation
[2] central nervous system
[3] chronic fatigue syndrome
[4] germ-free
[5] gamma-aminobutyric acid

neurobiological changes in humans, akin to that described in animals, e.g. altered CNS[1] GABA receptor expression in anxiety patients treated with probiotics (O'Mahony et al., 2009). Very little data on probiotic interventions in OCD[2] exists in humans. Nevertheless, in the quinpirole sensitization rat model of OCD, changes in several communities of gut bacteria (predominantly *Lachnospiraceae* and *Ruminococcaceae*) were associated with the induction of compulsive checking behavior; the authors suggested that changes in these microbes may serve to support the energy use requirements of compulsive checking and OCD (Jung et al., 2018). Also, in a study by Kantak et al. (2014), pharmacologically induced OC-like behavior in BALB/cJ mice has been shown to attenuate following both fluoxetine and treatment with *Lactobacillus rhamnosus*. Furthermore, the therapeutic outcome was comparable between the groups (Kantak et al., 2014).

Collectively, research pertaining to the use of probiotics in psychiatric illness came to describe such interventions as psychobiotics (Dinan et al., 2013). In fact, that their therapeutic effects are not only mediated by changes in neurotransmitter release, but also by altering brain activity in regions which are essential for central processing of emotion and sensation (McKernan et al., 2010), necessitates their investigation as potentially highly effective psychotropic drugs (O'Mahony et al., 2009, Bailey and Coe, 1999, Forsythe et al., 2010, Ohland et al., 2013, Umesaki et al., 1999, Misra and Mohanty, 2017). As such, clinical validation of probiotics as a unique and novel class of psychiatric treatment is currently underway.

DIETARY MODIFICATION

As mentioned earlier (paragraph 2.3.2.1), microbial composition can also be altered with dietary modification (Ohland et al., 2013). Further, the importance of diet is evidenced by an individual's dependence on maternal breast milk for the normal development and functionality of the gut microbiota and the immune system (Walker et al., 2015). Also, during the ingestion of specific food groups and nutrients, certain biologically relevant molecules, e.g. tryptophan, will be released in the gut (O'mahony et al., 2015). This is of essential value in the current study considering that OCD and other psychiatric illnesses, i.e. anxiety and depression, are for instance associated with imbalances in serotonergic functioning. Interestingly, meat-rich diets result in significant expansion in bacterial diversity, in parallel with decreased anxiety-like behavior and improved working and reference memory (Li et al., 2009). These findings give us a clear indication for the involvement of diet in psychiatry through the gut-brain axis. For a more detailed discussion of these and other diet-related effects, please refer to paragraph 2.3.2.1.

[1] central nervous system
[2] obsessive-compulsive disorder

PHARMACOLOGICAL TREATMENT

Initially, it was believed that the weight-gaining effects of atypical antipsychotics could be explained by their effects on the hunger and satiety centers in the brain (Baptista, 1999). However, later it was demonstrated that such drugs influence gut microbial composition (Bahr et al., 2015) and, while normal mice treated with olanzapine gained significant weight over the course of treatment, GF[1] mice gained significantly less (Morgan et al., 2014). Interestingly, and keeping the current project in mind, while risperidone influenced microbial composition significantly, treatment with neither anti-psychotics nor SSRIs[2] showed any effect on the microbiota compared to placebo (Bahr et al., 2015). Further, given that antipsychotics not only modify microbial composition, but also elicit drug-related ameliorative effects in psychosis (Davey et al., 2013, Bahr et al., 2015), it has been postulated that the combined neurobiological and microbial effect is responsible for the favorable treatment outcome seen in patients with psychosis (Collins et al., 2012).

Taken together, the findings discussed in this section demonstrate that modification of the gut microbiota may be a potential novel and useful therapeutic target for the treatment of psychiatric illness (Wang and Kasper, 2014). As such, research that can assess the efficacy of gut microbiota modification as a possible augmentation strategy in patients with severe, debilitating psychiatric symptomology, is essential.

2.3.5.3 A narrow window of opportunity for intervening with microbiota modification in psychiatry

Research indicates that treatment strategies aimed at microbial modification are generally more successful during the early stages of development. For instance, it has been argued that microbial modification will be of no value as a potential therapeutic intervention in adults with immune-allergic disorders as the effects later in life, when the immunological basis has already been laid, will be negligible (Shanahan, 2009). Indeed, this argument is supported by data demonstrating inconsistency and inefficiency in the use of probiotic treatment in adults with IBD[3] (Hedin et al., 2007). Also in mice, a narrow window of opportunity for microbial modification has been documented during the early stages of development, i.e. one week post-partum. However, a second window of therapeutic benefit, *viz.* 4 weeks of age but before weaning, has been identified (Gensollen et al., 2016). Importantly though, when the window of opportunity for change has passed, e.g. when mice reach adulthood, this window will possibly be closed (Sudo et al., 2004). These findings are in line with observations that successful microbiota-associated behavioral changes coincide with critical periods of brain

[1] germ-free
[2] selective-serotonin reuptake inhibitors
[3] inflammatory bowel disease

development (Bercik et al., 2011, Heijtz et al., 2011, Sudo et al., 2004). In one study, GF[1] mice presenting with behavioral abnormalities were treated with probiotics at different stages of development. However, behavioral changes were only observed in the groups treated early in life (Neufeld et al., 2011). Furthermore, while differences in behavior have previously been seen in adult GF mice after colonization with normal mice, concomitant changes in neurochemistry remained insignificant, probably due to the window of opportunity having closed within this specific framework (Clarke et al., 2013). Therefore, *prospective* clinical research is necessary to determine the effects of early life microbiota-targeting interventions on psychiatric outcomes later in life. Although such research is problematic given that it would be difficult to determine whether patients would progress to psychiatric illness later in life in the absence of such interventions, large cohort studies may shed some light on the therapeutic potential of microbial modification during early life in preventing psychiatric manifestations later in life.

2.3.6 Translational insights into investigations of the gut microbiota in animal models

Animal models are used as valuable tools to gain insight into the human body's physiology, neurobiology and only recently, the microbiome. GF, gnotobiotic and SPF[2] animal models, especially in mice, have been extensively studied with the aim to elucidate the role of the gut microbiota in various pathologies. In this paragraph, we will briefly look at the different types of models used in investigation of the microbiome with a short summary of their advantages and shortfalls.

In general, animal models used to study the gut microbiota are differentiated based on the methods by which microbial colonization are controlled, allowing selective exposure to specific combinations of bacterial strains, irrespective of the modification technique used. For example, as mentioned earlier while GF animals are completely free of any microbes, gnotobiotic animals are raised with only a selected group of bacteria. SPF animals are only free of specific pathogens and contain a normal gut microbial composition. Studies comparing these animal models have been of essential value in providing novel insights into the gut microbiota, especially the specific effects of individual bacterial strains on health and disease. Collectively, these techniques provide a comprehensive framework for investigating the role of the GBA[3] in psychiatry, which is crucial for our understanding of how peripheral interventions may be of therapeutic benefit in patients suffering from mental illness. Importantly, GF, gnotobiotic and SPF techniques have different applications and each present research with unique advantages when studying gut-brain interactions. For example, while reduced

[1] germ-free
[2] specific pathogen free
[3] gut-brain axis

NMDA[1] mRNA[2] expression, increased brain-derived neurotrophic factor (BDNF)[3] mRNA expression and decreased in $5HT^4_{1A}$ receptor mRNA expression were seen in GF compared to SPF mice (Neufeld et al., 2011), increased levels of corticosterone in parallel with anxiolytic behavioral responses were also seen in GF, but not other mouse models (Neufeld et al., 2011). Whether these findings are mouse species specific, need to be determined. However, it seems that GF mice in general are, based on their bolstered baseline anxiety levels, the ideal frameworks in which to study the effects of selective modifications of the microbiota in anxiety.

That said, the use of SPF techniques provides several logical advantages over the use of GF mice. First, they allow for the investigation of the natural developmental effects of a healthy microbiome on brain development. Second, they can be used to determine the baseline differences in the normal gut colonizing microbiomes of animals of different behavioral phenotypes, enabling researchers to search for specific microbial targets that may underlie, or that may be affected by the development of psychiatric symptomology. On the other hand, gnotobiotic mice colonized with particular strains of microbes, can be of value to investigate specific neurodevelopmental effects of said strains. However, where they lack in translation, is in ignoring the potential effects of the complex interactions between different microbial strains in modifying disease progress later in life. It is also near impossible to study the bidirectional relationships in the gut-brain axis, as these models only allow for a bottom-up approach to be followed.

In the current investigation, we aim to study the baseline differences in the gut microbial composition between animals that naturally present with normal and OC[5]-like behavior, *viz.* large nest building, respectively. Therefore, the SPF technique will be applied.

[1] N-methyl-D-aspartate
[2] messenger ribonucleic acid
[3] brain-derived neurotrophic factor
[4] serotonin
[5] obsessive-compulsive

2.4 Key concepts of the deer mouse model of OCD

The current investigation will build on previous work performed in our laboratory concerning the deer mouse *(Peromyscus maniculatus bairdii)* model of OCD[1]. With respect to face value, deer mice housed in captivity present with non-induced aberrant behaviors across different behavioral phenotypes that resemble symptom heterogeneous compulsive-like behavior. Not only do these behaviors manifest in some individuals only, but they are also characteristic of both sexes, thereby baring some resemblance to the clinical epidemiology of OCD. OC[2]-like behavioral phenotypes that have been characterized in deer mice include high motor stereotypy (Wolmarans et al., 2013), LNB[3] (Wolmarans et al., 2016a) and HMB[4] behavior (Wolmarans et al., 2016b). Importantly, these may, but do not necessarily present in the same individuals and occur to different degrees within individuals of the larger population. Further, in line with clinical evidence (Rosa et al., 2012, Kim et al., 2012b, Berrocal et al., 2006), it has been demonstrated that high stereotypical deer mice present with altered sociability in the presence of non-stereotypical controls, whereby stereotypical subjects will group together, or be marginalized by animals of the non-stereotypical cohort. Considering the predictive validity of the model, high stereotypy and LNB favorably respond to chronic, but not sub-chronic, high dose oral escitalopram treatment, *viz.* 50 mg/kg/day for 28 days; however, HMB remains treatment refractory, possibly representing a treatment-resistant compulsive-like phenotype (Wolmarans et al., 2016a, Wolmarans et al., 2016b, Wolmarans et al., 2013). The model is also founded on robust construct validity following findings that high stereotypical deer mice present with a significant decrease in striatal serotonin transporter (SERT)[5] expression compared to their non-stereotypical controls. The latter results are in line with that demonstrated in patients with OCD (Hesse et al., 2005, Reimold et al., 2007, Zitterl et al., 2008) and implicates a role for hyposerotonergic signaling in high stereotypical animals. Considering also that deer mouse behavior is irresponsive to noradrenergic interference (Korff et al., 2009), as well as that altered cyclic adenosine monophosphate (cAMP)[6]-phosphodiesterase (PDE)[7]-4 signaling underlies high stereotypical behavior, the model provides a robust framework in which to study the underlying neurobiology and symptom manifestation of OCD in a natural pre-clinical model. Hence, the current investigation will investigate possible associations

[1] obsessive-compulsive disorder
[2] obsessive-compulsive
[3] large nest building
[4] high marble burying
[5] serotonin transporter
[6] cyclic adenosine monophosphate
[7] phosphodiesterase

between OC[1]-like LNB[2] behavior and changes in microbial composition, and its response to high dose oral escitalopram interference.

For a complete review of the deer mouse model and its validity, please refer to Addendum E for theoretical overview of the model co-written by the candidate.

2.5 Summary

Treatment response in psychiatric disease, notably also in OCD[3], is a significant challenge that increases the burden on the mental health system (Atmaca, 2016). Following from this literature review, the importance of a healthy microbial balance in mental health is evident. Evidently, a functionally balanced gut microbiota is not only important to prevent and moderate gastrointestinal disorders, but also in psychiatry. Further, we underline the value of a healthy gut-brain relationship, as aberrancies in this cross-talk are associated with significant mental disease risks. That said, the relationship between the gut and the CNS[4] is not yet fully elucidated. In fact, research are now only in the beginning stages of what may potentially be the next breakthrough avenue in the treatment of a range of human conditions. Therefore, further investigation into the functions of the GBA[5] can be regarded as essential. However, in order to understand and elucidate the potential of microbiota-targeting interventions, a thorough understanding of the developmental effects of the microbiome in naturally occurring neuropsychiatric phenotypes is necessary. As such, the current investigation will employ a validated and robust animal model of naturally developing OC-like phenotypes, to shed more light on this question.

[1] obsessive-compulsive
[2] large nest building
[3] obsessive-compulsive disorder
[4] central nervous system
[5] gut-brain axis

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Naturalistic compulsive-like behavior in the deer mouse (Peromyscus maniculatus bairdii) is associated with alterations in the gut microbiome

* * *

Author Contributions:

- *Isma Scheepers* designed the investigation in consultation with *De Wet Wolmarans and Stefanie Malan-Müller*, performed all behavioral, pharmacological and DNA extraction experiments and assisted with statistical analyses. She also wrote the first version of the manuscript, and edited the manuscript following input from the co-authors.

- *Thomaz Bastiaanssen* performed all statistical analyses reported in this article in consultation with *Isma Scheepers* and *Stefanie Malan-Müller.* He also provided significant input into the statistical and experimental methodology and in the interpretation of the microbiome results.

- *Brian H Harvey* was study-assistant supervisor, funded the project and assisted in the interpretation of results.

- *John Cryan, Gerald Clarke* and *Kieran Rea* provided significant and valuable input into the study design and highlighted key issues of the data reported here and how to address them.

- *Leonard Santana* is professor and senior lecturer at the Department of Statistics, NWU, Potchefstroom. He performed all initial statistical analyses with *Isma Scheepers* and trained and assisted *Isma Scheepers* to program the statistical software 'R'.

- *Rencia van der Sluis*, senior lecturer in the Department of Biochemistry, NWU Potchefstroom, assisted with all methods related to and that resulted in the extraction of microbial DNA.

- *De Wet Wolmarans* and *Stefanie Malan-Müller* acted as supervisor and co-supervisor of this study, respectively. They have conceptualized and designed this work and were instrumental in every phase of this investigation. They also revised the first version of this dissertation, including this article.

* * *

Important Information:

- The manuscript has been drafted according to the standard framework and technical criteria relating to the reporting of microbiota data. Although a number of concepts and descriptions may seem unusual, these are in line with microbiome methods and literature.

- As per the instructions to the author, figures and legends are provided at the end of the manuscript.

- All co-authors not directly involved as supervisors or co-supervisors in the current study, provided consent for the paper to be assessed as part of the MSc. thesis of *Isma Scheepers* (Addendum A).

Naturalistic compulsive-like behaviour in the deer mouse *(Peromyscus maniculatus bairdii)* is associated with alterations in the gut microbiome

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ABSTRACT

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric illness that significantly impacts the lives of affected people. Current pharmacotherapeutic interventions yield suboptimal responses and a need for novel treatment strategies exists. Recently, the role for the gut-brain axis (GBA) in psychiatric illness emerged as a potential target for therapeutic exploitation. However, studies concerning the role of the GBA in OCD are limited. Compulsive nest building in rodents has seen to be in unison with OCD. In an attempt to investigate whether a naturally occurring obsessive-compulsive-like phenotype in a rodent model may be associated with perturbations in the gut microbiome, this investigation characterized the gut microbiota in large (LNB) and normal (NNB) nest building deer mice (n = 6 per group; one fecal sample analyzed per animal in each cohort). Furthermore, as the selective serotonin reuptake inhibitor (SSRI) and anti-OCD drug, escitalopram, has been shown to abrogate LNB, we also investigated whether its administration (50 mg/kg/day x 28 days) affects the gut microbiota of LNB animals differently compared to that of the controls (n = 4 per group; one fecal sample analyzed per animal before and after treatment, respectively). Our results reveals the microbial composition of LNB animals to be distinctly different compared to controls, with *Robinsoniella* being more abundant in the OC-phenotype (q < 0.2, effect size ≈ 1.8). Furthermore, although not statistically significant, escitalopram tended to modify the microbiota of LNB to a greater extent compared to controls. These findings provide proof-of-concept for continued investigation of the GBA in the deer mouse model of OCD.

KEYWORDS

OCD, gut-brain axis, deer mouse, gut microbiota, escitalopram, 16S rRNA, *Robinsoniella*

INTRODUCTION

Obsessive compulsive disorder (OCD) is a multidimensional psychiatric disorder that is characterized by intrusive, often anxiogenic thoughts, i.e. obsessions and/or ritualistic behaviors (compulsions) that are often expressed in an attempt to reduce the level of anxiety caused by the obsessive thoughts (Pauls et al., 2014, Wu and Lewin, 2017; APA, 2013). Obsessive-compulsive (OC) symptoms affect the lives of affected patients on several levels and can have a detrimental impact on their quality of life (Schwartzman et al., 2017, Kugler et al., 2013). The condition is phenotypically heterogeneous and symptoms cluster together with respect to five main themes, fears of contamination and cleaning rituals, fears of harm and checking compulsions, a need for symmetry and order associated with ordering compulsions, intrusive inappropriate thoughts relating to sexual misconduct, religion, and violence, and fears of losing objects and collecting behavior (Leckman et al., 2010). With respect to collecting compulsions, hoarding disorder has now been reclassified as a unique disorder within the obsessive-compulsive and related disorders category in the most recent edition of the DSM-V (APA, 2013).

Chronic high-dose selective serotonin reuptake inhibitors (SSRIs) are currently recommended as first line pharmacotherapy for OCD (Soomro et al., 2008), while increasing the dose of the current SSRI used, switching to another SSRI, or SSRI-antipsychotic augmentation strategies are all clinically employed for the effective treatment of refractory OCD (da Rocha and Correa, 2011, Eagle et al., 2014, Bloch et al., 2006). Nevertheless, whereas two-thirds of patients respond to first line pharmacotherapy, only half of the SSRI-resistant individuals respond to augmentation strategies (Marazziti et al., 2016). Therefore, better understanding of the neurobiological and pathophysiological processes underlying OCD is needed in order to develop novel and more effective pharmacotherapeutic interventions.

During the past decade, the gut-brain axis (GBA) and its involvement in psychiatric disease have gained significant interest (Mayer et al., 2015). Communication between the gut and the brain takes place on a number of functional levels, including via neural and immunological signaling (Turna et al., 2016). The vagus nerve, for example, constitutes a direct link between the gut and the brain and has the ability to recognize and adapt to changes in microbial composition and to communicate these changes to the brain (Sudo et al., 2004), via adaptive changes in central nervous system neurotransmitter receptor expression (Bravo et al., 2011). Whereas this effect has been shown with respect to GABAergic signaling following *Lactobacillus rhamnosus* administration in healthy rodents (Bravo et al., 2011), it is also true for other neurotransmitters, e.g. serotonin (Kannampalli et al., 2014) and dopamine (Kannampalli et al., 2014).

The gut microbiota is regarded as one of the major immunomodulatory factors in the human body (Masand et al., 2006, Kantak et al., 2014; Peterson et al., 2015). Functional modifications in the immune system may be instrumental in central nervous system functioning, which may manifest as OC symptomology (Turna et al., 2016). Indeed, unstable changes in the gut microbiota are known to increase the permeability of the gastrointestinal epithelium to allow passage of immunomodulatory components to the systemic circulation (Tremellen and Pearce, 2012, Leclercq et al., 2014). However, a top-down modulation of the gut microbiota is also possible. As is true for anxiety, OCD is associated with significant levels of psychological stress (Fluitman et al., 2010, Kluge et al., 2007, Wu and Lewin, 2017). In turn, stress has been shown to alter gut microbiota composition, leading to significant pathological sequelae, in some cases causing the up flaring or exacerbation of i.e. irritable bowel syndrome and (Konturek et al., 2011, Soderholm and Perdue, 2001, Nakade et al., 2007). In light of this, several investigations into microbiota manipulation in animal models of psychiatric illness have been performed. For example, probiotics have shown demonstrable promise in models of depression (Desbonnet et al., 2008, Messaoudi et al., 2011a, Bravo et al., 2011, Desbonnet et al., 2010, Savignac et al., 2014, Singh et al., 2012), anxiety (Messaoudi et al., 2011a, Bravo et al., 2011, Luo et al., 2014, Bercik et al., 2011, Bercik et al., 2010, Savignac et al., 2014) and also OCD (Kantak et al., 2014). Further, clinical investigations have also provided promising data, describing improvements in cognitive flexibility in anxious and chronically fatigued patients (Steenbergen et al., 2015, Rao et al., 2009, Messaoudi et al., 2011a, Messaoudi et al., 2011b).

To study possible associations between naturally occurring compulsive-like behavior and alterations in the gut microbiome, the deer mouse model of OCD (Wolmarans et al., 2016, Wolmarans et al., 2013, Wolmarans et al., 2017), was employed. Briefly, roughly 30% of laboratory housed deer mice of both sexes express aberrantly large nest building behavior (LNB). Such behavior manifests by the age of 8 weeks and is persistent and repetitive over the course of several trials. Further, as all animals are housed and maintained under the same circumstances, LNB serves no apparent functional purpose (Wolmarans et al., 2016), and hence is OCD-like in its presentation. We have also previously shown LNB to be highly responsive to chronic high-dose (50 mg/kg/day) oral treatment with the SSRI, escitalopram (Wolmarans et al., 2016). LNB therefore develops without any prior intervention may be reversed with alterations in serotonergic signaling. This investigation thus attempted to establish whether the natural development of LNB behavior is associated with differences in the gut micriobiota compared to what is seen in normal nest building (NNB) subjects. Further, we wanted to establish how the microbiota of LNB vs NNB animals will respond to intervention with an SSRI previously shown to abrogate the expression of LNB.

MATERIALS AND METHODS

Experimental layout

Study objective 1: To characterize the gut microbiota of NNB and LNB animals

The first phase employed six (three male and three female) deer mice of each nesting cohort, i.e. NNB and LNB to determine the baseline characteristics of the gut microbiota. However, considering that only 30% of deer mice express LNB behavior, 20 deer mice were initially screened for nest building behavior (see *'Animals'*; Wolmarans et al., 2016). A single fresh fecal sample was collected from each individually housed mouse on the day following the last nest building screen (see *'Sample collection and DNA extraction'*)

Study objective 2: To determine the response of the gut microbiota of NNB and LNB animals to chronic escitalopram

Following a single pre-treatment fecal sampling of a separate group of 4 LNB and 4 NNB deer mice, all animals in this phase of the investigation were treated with escitalopram (50 mg/kg/day x 28 days) for a total of 28 days (Wolmarans et al., 2016). Thereafter, another single post-treatment fecal sampling was performed for each animal. Only female animals were employed during this phase of the study, as they were to be used in further investigations relating to gut microbial modification by means of litter-dam cross-fostering (data not included in this report). Thus, an initial group of 17 female deer mice was screened for NB behavior to ensure a yield of at least 4 NNB and 4 LNB females.

Animals

Deer mice of both sexes were initially obtained from the deer mouse colony of the North-West University (NWU), Potchefstroom, South Africa (ethical approval number: NWU-00284-17-S5; AnimCare Research Ethics Committee, NHREC Registration Number: AREC-130913-015). The original breeding pairs were established using animals obtained from the *Peromyscus* Genetic Stock Centre at the University of South Carolina, USA. As explained above, a total of 37 deer mice (10 weeks of age at the onset of investigation) of both sexes were initially screened for LNB. However, only 20 animals were ultimately selected for LNB and NNB behavior. The rest of the subjects were included in unrelated investigations. Animals were bred according to standard out-bred protocol and housed and maintained in the specific-pathogen-free (SPF) area of the Vivarium at the North-West University, Potchefstroom, South Africa. Breeding pairs were randomly allocated without prior knowledge of nest building profiles. After weaning, offspring were housed in same-sex cages until one week prior to the onset of the first nest building analysis to prevent biased modification of microbial composition, from

which point they were allocated individually to cages. Further, from this day onward, all experimental analyses were conducted in the same cages. Animals were kept on a 12-hour light/dark cycle (06h00/18h00), and food and water were provided ad lib. All mice received food from the same batch of pelleted rodent chow throughout the study. Cages were cleaned and new bedding material added once a week on the same day.

Nest building analysis

Nest building behavior was quantified as described previously (Wolmarans et al., 2016). Briefly, nesting behavior was assessed in each animal for 7 consecutive 24-hour periods. An excess of pre-weighed, sterile non-scented cotton wool was introduced in the roof of each home cage every day between 15h00 and 16h00. As mice generally build their nests just before dawn (Jirkof, 2014), the remaining cotton wool was only removed and weighed between 13h00 and 14h00 on the following day. Each day, built nests were removed, discarded and additional pre-weighed cotton wool supplied. Animals did not have access to any other form of nesting material, and food and water (or escitalopram solution) were supplied as normal. Daily nesting scores were expressed in grams of cotton wool utilized with a cumulative nesting score determined after one week (Wolmarans et al., 2016). As nest building is a natural behavior expressed by all rodents (Smithers, 1984), only animals that consistently built large nests over the course of 7 days were included in the LNB cohort. This was determined by plotting the total nesting scores against the coefficients of variance with respect to daily nesting behavior, where LNB was defined as nesting behavior that clustered within the upper quarter of the nesting score distribution, while demonstrating the lowest degree of variance. Likewise, NNB animals were identified as those individuals that built the smallest nests consistently over the course of 7 days (Wolmarans et al., 2016).

DNA analyses

Sample collection and DNA extraction

Fresh fecal samples were collected during the first hour of the dark (wake) cycle with a sterilized tweezer, transferred to 1.5ml Eppendorf™ Safe-Lock tubes (Hong et al., 2010) and immediately flash frozen in liquid nitrogen. Samples were kept frozen at -80 degrees centigrade until the extraction of DNA (Carroll et al., 2010). A QIAamp® PowerFecal® DNA kit (QIAGEN, Valencia, CA, USA) was used to extract the microbial DNA from fecal samples, as per the manufacturer's instructions (see Addendum B; QIAamp® PowerFecal® DNA Kit Handbook). The protocol ensures maximal cell lysis of bacterial cell wall components. The Thermo-Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer was used to assess the quality and quantity of the extracted microbial DNA.

DNA sequencing

Paired-end sequencing of the V3 to V4 hypervariable regions using custom primers, i.e. regions that demonstrate considerable sequence diversity (approximately 300bp), of the 16S rRNA was performed by Macrogen® Inc. (South-Korea) on a MiSeq (Illumina) platform. The Herculase II Fusion DNA Polymerase Nextera XT Index Kit® V2 was used according to the 16S Metagenomic Sequencing Library, Preparation Part #15044223, Rev. B protocol. After performing quality control (see Addendum B; Raw Data Report, Macrogen®), samples proceeded to library construction by random fragmentation and 5' and 3' adapter ligation. Adapter-ligated fragments were then gel purified and PCR amplified. For cluster generation, bridge-amplification was applied and synthesis (SBS) technology used for sequencing (for the complete Illumina® Next generation sequencing (NGS) workflow, see Addendum B; Raw Data Report, Macrogen®). The V3 to V4 hypervariable region was used as high between-genus variability of these regions can be used to distinguish between closely related bacteria. The V3 to V4 region is also used in clinical studies for taxonomic classification (Group, 2012). However, these regions only recently gained interest in mouse microbiome studies (Shin et al., 2016, Kozich et al., 2013).

Statistical analysis

First, quality control of raw *fastq* sequencing files were performed using *fastqc* and *multiqc* programs, respectively (Ewels et al., 2016), enabling the identification of biases and trends already early in the process of data analysis (Ewels et al., 2016). Second, we used a Divisive Amplicon Denoising Algorithm (DADA) 2 (version 1.8) (Callahan et al., 2016) in R studio (R version 3.4.3; R-studio version 1.1.456) for the construction of an amplicon sequence variant (ASV) table. This is considered as a higher resolution analogue of the more traditional operational taxonomic unit (OTU) tables used (Callahan et al., 2016). The DADA 2 workflow consisted of the following steps: inspecting the read quality profiles, filtering and trimming low-quality reads, identifying error rates, dereplication (eliminating redundant comparisons), sample inference, merging paired reads, constructing an ASV table, removing chimera's and assigning taxonomy, using the Ribosomal Database Project (RDP) as a reference database (Cole et al., 2005). The *phyloseq* package in R was used to further analyze the microbiome data, determining α- and β-diversity using Bray-Curtis distance, observing most abundant taxa and constructing ASV tables with rarefied reads used to perform principle component analyses (PCA). The Aitchison distance was used for PCA, rather than Bray Curtis distance known to be used for principal coordinate analysis (PCoA) as recent studies have shown this method not only to be more robust for compositional data (Gloor et al., 2017) but also in revealing insights into the qualitative aspects of data and the relationships between groups (Aitchison and Greenacre, 2002). This was done to identify

patterns in relative abundance for each ASV using the *Aldex* package in R and R studio (RStudio Team; 2016). To test for statistically significant differences in the relative abundance of ASVs between groups, we used permutational multivariate analysis of variance (PERMANOVA). This compared the microbiome composition between groups, with *p*-values < 0.05 regarded as significant using the *Aldex* package. First, PERMANOVA was done to test for statistically significant differences between untreated NNB and LNB animals, and secondly to test for significant differences between the pre- and post-treatment microbiota composition. The Wilcoxon signed-rank test was performed at both the $p < 0.05$ and $p < 0.1$ levels to determine if any statistically significant differences existed within (α-diversity—using Chao 1, Shannon, Simpson, Observed and Fisher distance matrices) and between each individual's diversity (β-diversity—using Bray-Curtis distance). Furthermore, to identify differentially abundant genera, the *pairwise_DA_wrapper* function from the *Tjazi* package was used. This is considered as a high power t-test, to determine effect sizes and q values for the prediction of false discovery rates at a threshold of $p < 0.2$ (Personal communication, Thomaz Bastiaanssen; Schirmer et al., 2016). All analyses were performed on ASV level. ASV counts per sample, ASV taxonomical assignments, R scripts and metadata are available for respective groups (Addendum B)

RESULTS

Objective 1

All samples were rarefied beforehand resulting in 20 334 reads per sample (supplementary data; Addendum B). To assess for differences in microbial composition in nest building behavior between the NNB (three males and three females) and LNB (three males and three females) animals, a total of 12 fecal samples were analyzed. A total of 62 genera were detected (top-20 genera illustrated in supplementary data, Addendum B). Wilcoxon's signed rank test revealed no differences in α- and β-diversity between NNB and LNB animals (supplementary data; Addendum B). A subsequent PCA of the Aitchison distance was generated from the analysis of 62 ASVs and revealed a clear separation in the microbial clustering of NNB and LNB cohorts (**Figure 1**). PC1 and PC2 accounts for 19.42% and 15.89% of the variance observed, respectively and PERMANOVA revealed this distinction to be statistically significant (PERMANOVA; $p < 0.05$). Moreover, a differentially abundant genus, *Robinsoniella*, was seen to be more abundant in the LNB cohort (**Figures 2a and 2b**; false-discovery rate [FDR] q ~ 0.1, effect size ~ 1.75).

Objective 2

All reads were rarefied which resulted in 21 824 reads per sample for the comparison of gut microbial composition profiles between pre- and post-treated LNB and NNB animals (supplementary data;

Addendum B). To test whether the microbial composition changed as a function of treatment (four NNB and four LNB), a total of 16 fecal samples were analyzed. Based on the same diversity indices used for Objective 1, neither the α- or β-diversities were significantly different between pre- vs post-treatment. A PCA of the Aitchison distance was generated using 67 ASVs, where PC1 and PC2 account for 19.23% and 14.15% of the total variance, respectively (**Figure 3**). However, PERMANOVA failed to reveal a significant difference between the pre- and post-treatment microbial composition within the behavioral groups (PERMANOVA $p < 0.05$). Further, Wilcoxon's signed-rank test also failed to reveal a statistical difference between the composition of treated NNB and LNB animals ($p < 0.05$). That said, the microbial composition of the LNB animals trended toward a greater change with respect to the Aitchison distance moved as a function of treatment (**Figure 4**).

DISCUSSION

The major findings of the present work are 1) although no significant differences in α- diversity exists between NNB and LNB animals, a clear separation in terms of gut microbiota composition (as reflected by the *beta* diversity) exists between the two cohorts, 2) *Robinsoniella* is more abundant in LNB than in NNB animals, and 3) the gut microbiota of LNB animals changes more profoundly with escitalopram treatment compared to that observed in the NNB cohort.

The neurobiological and pathophysiological processes underlying OCD are not yet fully elucidated, with current treatment options also yielding suboptimal results (Atmaca, 2016). During the past decade, our understanding of the GBA has expanded significantly (Sherwin et al., 2018). Indeed, the GBA plays a significant role in the etiopathology of a number of psychiatric illnesses, including depression (Park et al., 2013) and anxiety (Bercik et al., 2010), while modification of the gut microbiota in these conditions yielded promising results (Burokas et al., 2017). In fact, following publication of the first case report (Garvey et al., 1998), research has divulged an etiological association between changes in immune functioning and OC symptomology. Hence, in this investigation we attempted to elucidate possible associations between a naturally developing compulsive-like phenotype, i.e. LNB in the deer mouse, and alterations in the gut microbiota. Further, given that the first-line pharmacotherapeutic intervention for OCD, i.e. chronic high-dose escitalopram (Fineberg and Gale, 2005, Varigonda et al., 2016, Fineberg et al., 2015), also reverses LNB behavior in deer mice (Wolmarans et al., 2016), we sought to establish whether the same treatment regimen will elicit unique compositional adaptations in the baseline microbiota of LNB vs NNB animals.

Our finding that the composition of the gut microbiota in LNB animals is significantly different from that in the NNB cohort is a noteworthy observation. Taking into account that LNB transpires naturally

over the course of development and given that animals included in this investigation have been randomly selected without litter bias, indicates that the difference observed in microbiota composition parallels the differences observed in behavioral expression, probably following an association that is naturalistic. Although no significant differences were observed in α-diversity, β-diversity differed between the two cohorts (see results; data not illustrated), *Robinsoniella,* a gram-negative, spore-forming and non-motile bacterial genus from the family *Lachnospiraceae* (phylum *Firmicutes*), was more abundant in LNB compared to NNB animals (**Figure 2a and 2b**). Interestingly, most organisms of the human gut microbiota cluster within the phyla *Bacteroidetes* and *Firmicutes* (Lozupone et al., 2012). However, *Robinsoniella* was only recently isolated from human clinical samples (Cotta et al., 2009, Gomez et al., 2015), with only one species, i.e. *Robinsoniella peoriensis*, thus far identified. That *Robinsoniella* was found to be more abundant in LNB compared to NNB mice may provide some valuable direction for continued exploration in studies relating to the underlying role of the GBA in OCD. Considering the proposed role of immune alterations and OCD (Swedo et al., 2012, Dantzer et al., 2008, Williams and Swedo, 2015, Köhler et al., 2014, Mitchell and Goldstein, 2014) and that *Robinsoniella* has previously been associated with exacerbated immune responses (Shen et al., 2010), this study provides proof-of-concept for further investigations into its role—or the factors that contribute to its abundance—in the manifestation of OC-like behavior in the deer mouse model.

In terms of causality, further work is needed to determine whether the observed changes in this genus in LNB mice are associated with neurobiological changes that typically underlie OCD, i.e. alterations in serotonergic signaling or anxiogenic stress, and which are not observed in the NNB cohort. It may also be possible that the microbiota composition of LNB animals can exert a bottom-up influence on the behavioral expression of LNB animals (Heijtz et al., 2011) via nerve pathways or immunological signaling (Rees, 2014). This remains to be established.

Although the role of serotonin in psychiatric illness is well-studied (Marazziti, 2017), very little is known regarding the contribution of peripherally derived serotonin on brain functioning. In fact, nearly 50% of the total serotonin concentration in the human body is located in the gut, being produced by the gut microbiota (Yano et al., 2015). Changes in the microbiota within- and between the two nesting cohorts did not differ pre- and post-treatment, but this is in line with previous reports (Bahr et al. 2015). However, adaptation of the microbiota in LNB animals trended towards being more extensive compared to that in the NNB cohort. An explanation of this effect will be speculative, however a few points must be highlighted. Very recently, citalopram, the racemic mixture of R- and S-(es)-citalopram has been shown to increase the intestinal permeability in the ileum (Cussotto et al.,

2018) while showing demonstrable antimicrobial activity against *Escherichia coli* and *Lactobacillus rhamnosus* (Cussotto et al., 2018). These findings are congruent with previous data that have demonstrated the antimicrobial potential of SSRI administration against mostly gram positive bacteria such as *Staphylococcus* and *Enterococcus* (Ayaz et al., 2015a, Munoz-Bellido et al., 2000, Ayaz et al., 2015b). That said, the antimicrobial potency of escitalopram against these strains seems to be lower compared to that of another SSRI, fluoxetine (Cussotto et al., 2018, Bohnert et al., 2011). This effect can possibly be ascribed to the potent ability of fluoxetine in inhibition of drug-eliminating efflux pumps in bacterial cell walls (Bohnert et al., 2011). Considering that unstable changes in otherwise stable microbiota will result from changes in the inherent abundance of different gut microbiota strains and that such changes have been linked to psychiatric pathology (Mangolia et al., 2016), future investigations into the antimicrobial actions of SSRI administration and whether this may be a contributing factor to changes in central nervous system processes, are needed. Specifically, it may be possible that changes in the abundance of some strains may allow adaptations in others that in turn provide systemic benefit to the host (Rastall et al., 2005).

CONCLUSION

Taken together, the data presented here provides for the first time in an investigation of OC-like behavior in animals that differences in microbial composition parallels the manifestation of a naturally developing compulsive-like phenotype. Further, we also highlight a possible association between changes in the microbiota composition and escitalopram intervention. Future investigations into a possible causal role of the gut microbiota in the etiology of compulsive phenotypes are warranted. Specifically, the relationship between compulsive phenotypes, physiological and psychological stress, and immune alterations on the one hand and adaptations in the microbiota of normal and compulsive-like deer mice on the other hand, needs further elucidation. Further, it would be valuable to characterize the behavioral response in LNB deer mice both in the presence and absence of microbiota, using GF and SPF mice, to establish a clear mechanism for its potent behavioral effect, as reported earlier (Neufeld et al., 2011). Such studies could possibly contribute to a better understanding of the neurobiology underlying OCD and may ultimately lead to the development of better treatment.

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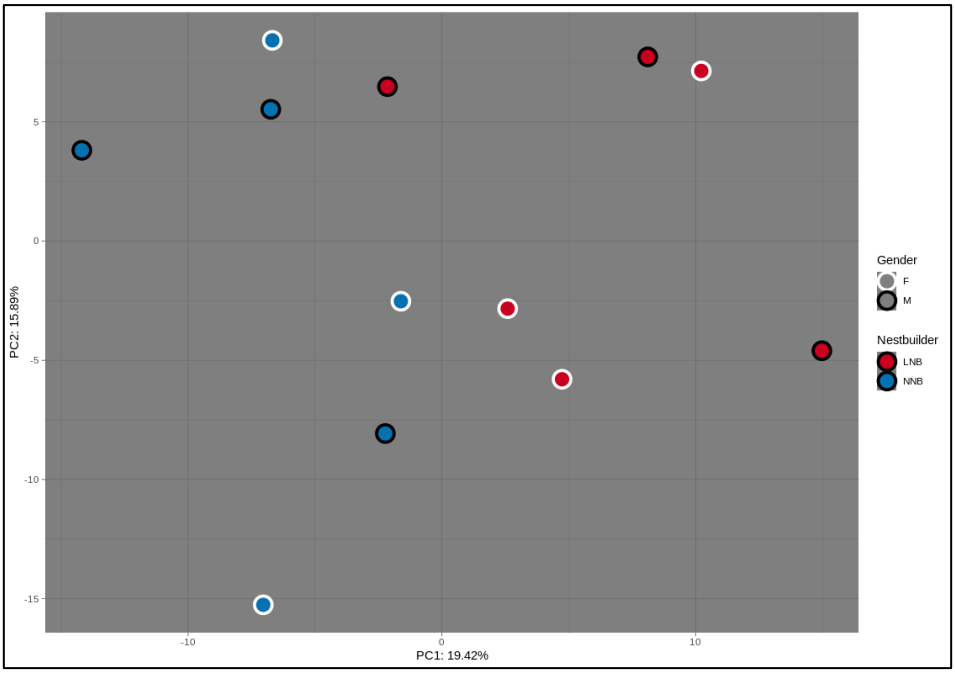

Figure 1 - PCA comparing the microbial composition of NNB and LNB animals with the Aitchison distance (PERMANOVA $p < 0.05$).

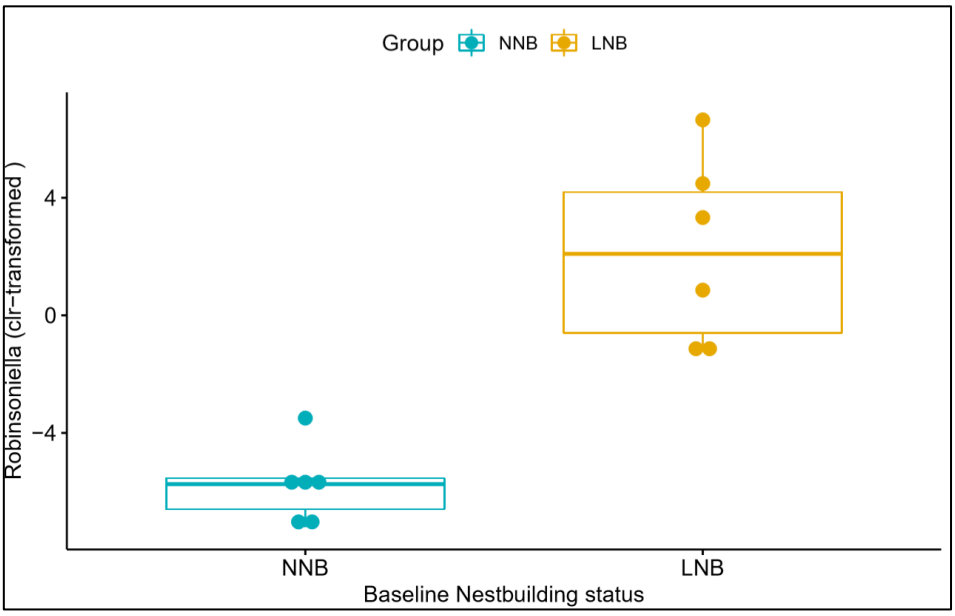

Figure 2a – A comparison of the relative abundance of Robinsoniella in NNB and LNB animals. Median, 25th and 75th percentile displayed. Whiskers are indicative of the minimum and maximum Robinsoniella abundance (crl.transformed) ($q < 0.2$ and effect size ≈ 1.8)

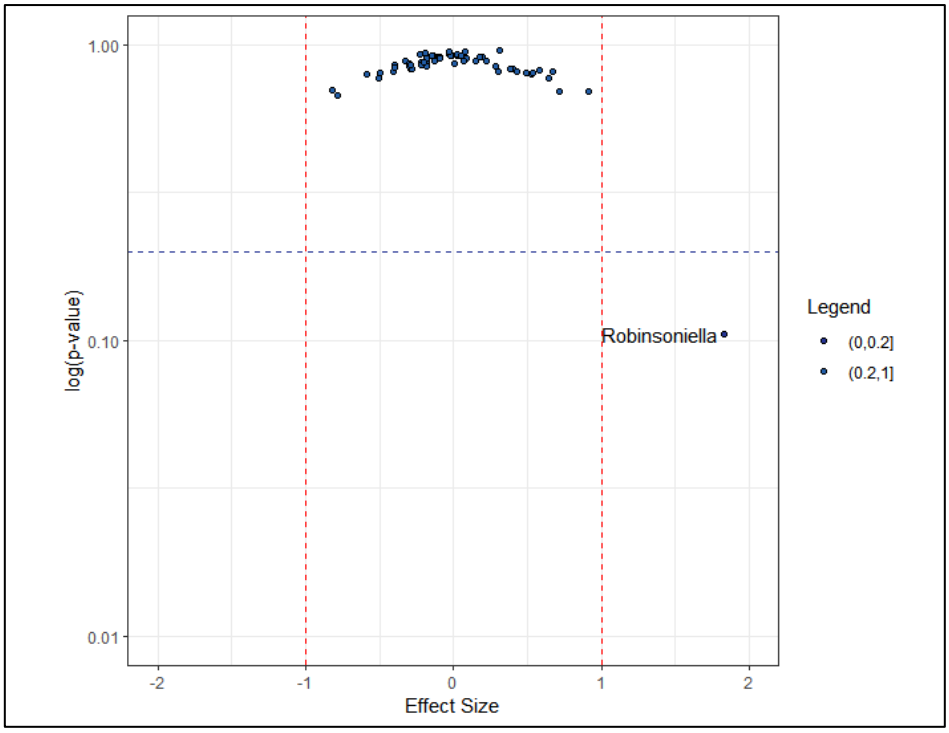

Figure 2b - Volcano plot of the effect size related to Robinsoniella abundance (e. value > 1)

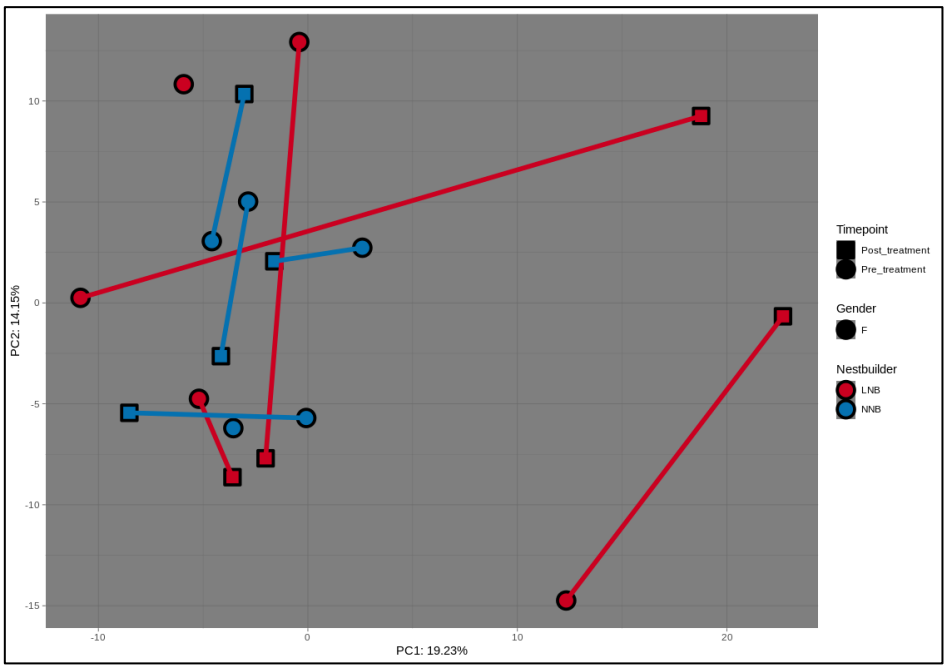

Figure 3 - PCA comparing the microbial composition between the pre- post-treatment samples (4 NNB and 4 LNB) with the Aitchison distance (PERMANOVA p > 0.05)

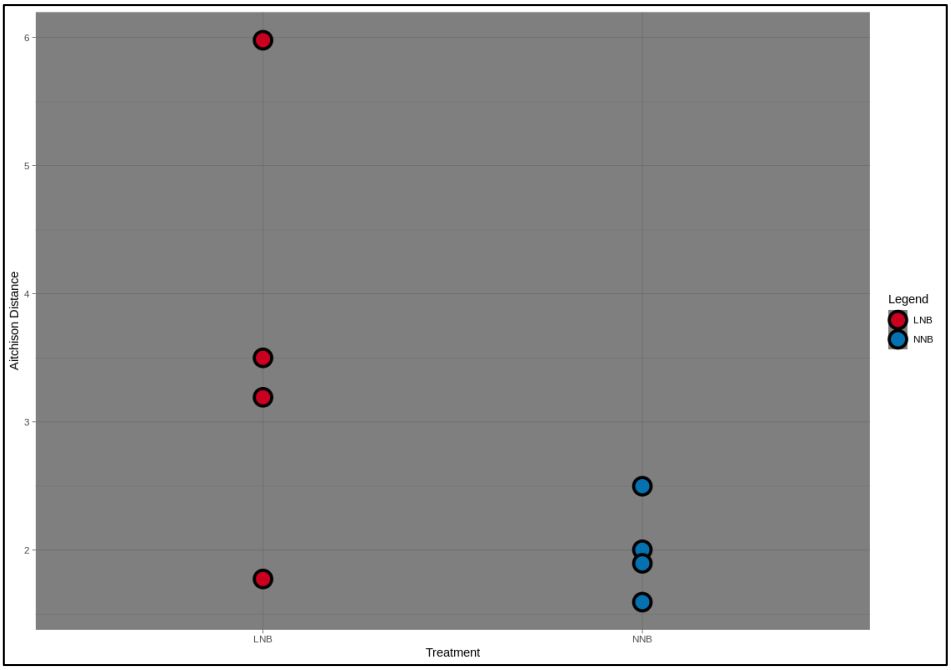

Figure 4 - Comparison of the Aitchison distance traveled between NNB and LNB animals following treatment with chronic escitalopram ($p<0.05$)

4 Conclusion

The main findings of this project provided valuable and noteworthy evidence that naturally expressed compulsive-like behavior in the deer mouse is associated with alterations in the gut microbiota. More specifically, 1) although no significant differences in α-diversity were shown, β-diversity significantly differed between NNB[1] and LNB[2] animals, 2) *Robinsoniella* is more abundant in LNB than in NNB animals, and 3) although not statistically significant, the gut microbiota of LNB animals adapts more profoundly as a function of treatment, compared to that observed in the NNB cohort.

* * *

The neurobiological and pathophysiological processes underlying OCD[3] is not yet fully elucidated with current treatment options also yielding suboptimal results (Atmaca, 2016). During the past decade, our understanding of the GBA[4] was significantly expanded (Sherwin et al., 2018). Indeed, the GBA plays a significant role in the etiopathology of a number of psychiatric illnesses, including depression (Park et al., 2013) and anxiety (Bercik et al., 2010), while modification of the gut microbiota in these conditions also delivered promising results (Burokas et al., 2017). While data pertaining to the exact involvement of the GBA in OCD is limited, it is for example known that pediatric OCD may transpire without any prior history of psychiatric illness during and following acute peripheral infections and that such symptoms dissipate after successful treatment of the infective state (Pavone et al., 2018). In this regard, dysbiosis, i.e. time-dependent unstable imbalances in the microbiota within a certain individual, is known to increase the permeability of the gastrointestinal epithelium to allow passage of immunomodulatory components to the systemic circulation (Tremellen and Pearce, 2012, Leclercq et al., 2014). However, a top-down modulation of the gut microbiota is also possible in that stress itself may alter the gut microbiota composition, leading to significant pathological sequelae, i.e. irritable bowel syndrome and inflammatory bowel disease (Konturek et al., 2011, Soderholm and Perdue, 2001, Nakade et al., 2007).

* * *

Our finding that the composition of the gut microbiota in LNB animals is significantly different from that in the NNB cohort is noteworthy. Since LNB transpires naturally over the course of development, it indicates that the difference observed in microbiota composition naturally parallels the differences observed in behavioral expression. Further, *Robinsoniella,* a gram-negative, spore-forming and non-

[1] normal nest building
[2] large nest building
[3] obsessive-compulsive-disorder
[4] gut-brain axis

motile bacterial genus from the family *Lachnospiraceae* (phylum *Firmicutes*), was more abundant in LNB[1] compared to NNB[2] animals. This may provide some valuable direction for continued exploration in studies relating to the underlying role of the GBA[3] in OCD[4], especially as *Robinsoniella* has previously been associated with significant immunomodulation (Shen et al., 2010). However, in terms of causality, it needs to be determined whether an underlying neuropsychiatric construct that may be unique to LNB animals, i.e. alterations in neurotransmitter signaling or anxiogenic stress, elicited adaptations in the microbiota of this cohort. It may also be possible that the microbiota composition of LNB animals can exert a bottom-up influence on the behavioral expression of LNB animals (Heijtz et al., 2011) via nerve pathways or immunological signaling (Rees, 2014).

* * *

In terms of the response of the gut microbiota to serotonergic intervention, our findings are also informative. While the statistical insignificance of our findings are in line with previous reports (Bahr et al., 2015), i.e. that changes in the microbiota within- and between the two nesting cohorts did not seem different after treatment, the adaptation of the microbiota in LNB animals trend towards being more extensive compared to what was found in the NNB cohort. Recently, citalopram has been shown to increase the intestinal permeability in the ileum (Cussotto et al., 2018) while showing demonstrable antimicrobial activity against *Escherichia coli* and *Lactobacillus rhamnosus* (Cussotto et al., 2018). These findings are congruent with previous data that have demonstrated the antimicrobial potential of SSRI[5] administration against mostly gram positive bacteria such as *Staphylococcus* and *Enterococcus* (Ayaz et al., 2015a, Ayaz et al., 2015b, Munoz-Bellido et al., 2000). That said, the antimicrobial potency of escitalopram against these strains seem to be lower compared to that of another SSRI, fluoxetine (Cussotto et al., 2018, Bohnert et al., 2011) Considering that 'dysbiosis' will result from changes in the inherent abundance of different gut microbiota strains and that 'dysbiosis' has been linked to psychiatric pathology (Mangiola et al., 2016), it may be of potential value if future investigations consider that the antimicrobial actions of SSRI administration may be a contributing factor to changes in central nervous system processes. More specifically, it may be possible that changes in the abundance of some strains, may allow adaptations in others that may result in potential systemic benefit to the host (Rastall et al., 2005).

[1] large nest building
[2] normal nest building
[3] gut-brain axis
[4] obsessive-compulsive disorder
[5] selective serotonin reuptake inhibitor

* * *

Taken together, the data presented here provides for the first time in an investigation of OC[1]-like behavior in animals that altered microbial composition parallels the manifestation of a naturally developing compulsive-like phenotype. Further, we also highlight a possible association between adaptations in the microbiota composition and escitalopram intervention.

4.1 Shortcomings and future studies

Although the current study delivered a preliminary proof-of-concept implicating a role for alterations in the gut microbiota in OCD[2], some shortcomings and key points to consider for future studies have to be discussed.

Although our sample sizes were based on previous findings (Turnbaugh et al., 2009), future studies will benefit not only from larger sample sizes within each behavioral cohort, but also from a sex specific approach. Our sample sizes of 3 animals per sex per behavioral cohort were not sufficient to reveal between-sex differences, should they have existed. As such, we propose that 16 deer mice per behavioral cohort should be employed that include 8 animals of both sexes. Furthermore, as different compulsive-like phenotypes may potentially be borne from different neurophysiological constructs, we propose future studies to delineate between possible unique associations of high marble burying and spontaneous stereotypy in deer mice, both of which have been validated as compulsive-like traits (Wolmarans et al., 2016a, Wolmarans et al., 2013).

Future investigations into a possible causal role of the gut microbiota in the etiology of compulsive phenotypes, are also warranted. Specifically, the relationship between compulsive phenotypes, physiological and psychological stress and immune alterations on the one hand and adaptations in the microbiota of normal and compulsive-like deer mice on the other hand, needs further elucidation. Further, it would be valuable to characterize the behavioral response in LNB[3] deer mice both in the presence and absence of microbiota to establish a clear mechanism for its potent behavioral effect, as reported earlier (Neufeld et al., 2011). By providing a clearer roadmap for future investigation, such studies could possibly contribute to a better understanding of the neurobiology underlying OCD that may ultimately lead to the development of better treatment.

[1] obessive-compulsive
[2] obsessive-compulsive disorder
[3] large nest building

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Addendum A

Letters of permission to submit Chapter 3 for examination purposes

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9th November 2018

RE: Letter of Recommendation to Submit Co-Authored Article of Isma Scheepers for Examination Purposes

Dear Sir/Madam,

We, John F. Cryan, Thomaz Bastiaanssen, Anna Golubeva, Gerard Clarke and Kieran Rea, co-authors of the journal article *'Naturalistic compulsive-like behaviour in the deer mouse (Peromyscus maniculatus bairdii) is associated with alterations in the gut microbiome'* presented in Chapter 3, hereby grant permission for the work to be examined in the dissertation of Isma Scheepers. When the time will come to submit this work for publication, all co-authors involved will first be consulted.

All the best,

Yours faithfully

Gerard Clarke

K Rea

Prof. John F. Cryan

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7 November 2018

Letter of Permission to Submit Co-Authored Article for Examination Purposes

I hereby give permission to submit the following co-authored article for examination purposes:

'Naturalistic compulsive-like behaviour in the deer mouse (Peromyscus maniculatus bairdii) is associated with alterations in the gut microbiome'

Scheepers, IM., Bastiaanssen, T., Harvey BH., Cryan, J., Hemmings, SMJ., Rea, K., Clarke, G., Santana, L., Van der Sluis, R., Malan-Muller, S., Wolmarans, D.

Yours faithfully

Dr R van der Sluis

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To whom it may concern

Letter of Permission to submit co-authored article for MSc examination purposes

This letter serves to confirm that we, Dr Malan-Müller and Prof Hemmings (Department of Psychiatry, Stellenbosch University), grant Ms Scheepers permission to submit our co-authored article, entitled "*Naturalistic compulsive-like behaviour in the deer mouse (Peromyscus maniculatus bairdii) is associated with alterations in the gut microbiome*" as part of her MSc examination. We have been involved, as co-supervisors, in the conceptualisation and planning of the project, Dr Malan-Müller provided bioinformatics training and assistance to Ms Scheepers and we were involved in the manuscript preparation.

Kind regards

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Addendum B

Supplementary data to Chapter 3

QIAamp® PowerFecal® DNA Kit Handbook

August 2017

QIAamp® PowerFecal® DNA Kit Handbook

For the isolation of DNA from stool, gut material and biosolids

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAamp PowerFecal DNA Kit	**(50)**
Catalog no.	**12830-50**
Number of preps	**50**
BeadTubes, Dry Garnet 0.7 mm	50
PowerBead Solution	42 ml
MB Spin Columns	50
Solution C1	6.6 ml
Solution C2	15 ml
Solution C3	15 ml
Solution C4	72 ml
Solution C5	30 ml
Solution C6	9 ml
Collection Tubes (2 ml)	4 x 50
Quick Start Protocol	1

Storage

The QIAamp PowerFecal DNA Kit reagents and components can be stored at room temperature (15–25°C) until the expiration date printed on the box label.

Intended Use

All QIAamp products are intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

WARNING: Solution C5 contains alcohol and is flammable.

WARNING: Do not use bleach to clean the inside of the QIAvac® 24 Plus Manifold.

CAUTION	**DO NOT add bleach or acidic solutions to directly to the sample preparation waste**

PowerBead Solution and Solution C4 contain guanidine salts, which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with a suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAamp PowerFecal DNA Kits is tested against predetermined specifications to ensure consistent product quality.

Introduction

The QIAamp PowerFecal DNA Kit is designed for fast and easy purification of both microbial and host genomic DNA from stool and feces. Based on the DNeasy® PowerSoil® Kit, the QIAamp PowerFecal DNA Kit uses the same Inhibitor Removal Technology® (IRT) for stool that has worked so well for soil. IRT is very effective at removing inhibitory substances commonly found in stool, such as polysaccharides, heme compounds and bile salts. The result is highly pure DNA that is ready to use in demanding downstream applications.

Principle and procedure

We recommend starting with 0.25 grams of stools or biosolids. Each sample is homogenized in a 2 ml bead beating tube containing garnet beads. Cell lysis of host cells, as well as microbial cells, is facilitated by both mechanical collisions between beads and chemical disruption of cell membranes, ensuring efficient extraction from even the toughest microorganisms. The IRT is then used to remove common substances in fecal samples that interfere with PCR. Total genomic DNA is captured on a silica membrane in a spin column format. DNA is then washed and eluted from the membrane. The isolated DNA is ready for PCR analysis and other downstream applications, including qPCR and next generation sequencing analysis.

High-throughput options

We offer a vacuum-based protocol for faster processing without centrifugation for the DNA-binding and column-washing steps using MB Spin Columns. The QIAvac 24 Plus Manifold (cat. no. 19413) allows for processing of up to 24 MB Spin Column preps at a time. For additional high-throughput options, we offer the DNeasy UltraClean 96 Microbial Kit (cat. no. 10196-4) for processing up to 2 x 96 samples using a centrifuge capable of spinning two stacked 96-well blocks (13 cm x 8 cm x 5.5 cm) at 2500 x *g*. For 96-well homogenization of bacteria, we offer the TissueLyser II and Plate Adapter Set (cat. no. 85300 and 11990, respectively).

Automated nucleic acid purification on the QIAcube

Purification of DNA using the QIAamp PowerFecal DNA Kit can be automated on the QIAcube®. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash and elute), enabling you to use the QIAamp PowerFecal DNA Kit for purification of high-quality DNA.

If automating the QIAamp PowerFecal DNA Kit on the QIAcube, the instrument may process fewer than 50 samples due to dead volumes, evaporation and additional reagent consumption by automated pipetting. QIAGEN only guarantees 50 sample preps with manual use of the QIAamp PowerFecal DNA Kit.

For more information about the automated procedure, see the relevant protocol sheet available at **www.qiagen.com/MyQIAcube**. Up-to-date protocol sheets can be downloaded free of charge, or may be obtained by contacting QIAGEN Technical Services at **support.qiagen.com**.

Figure 1. The QIAcube instrument.

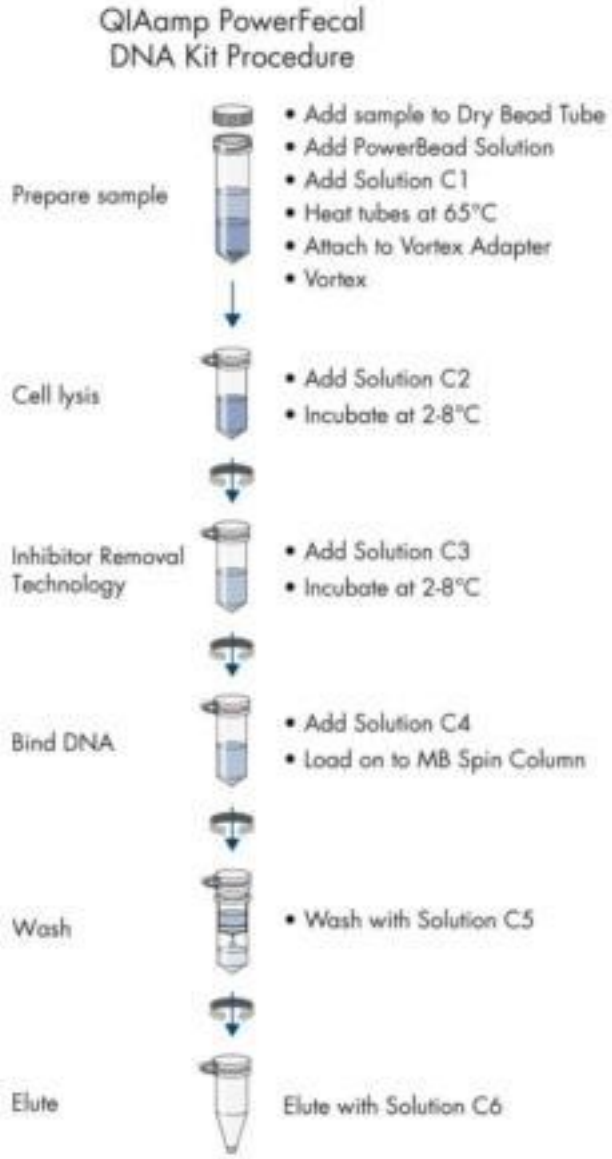


Figure 2. QIAamp PowerFecal DNA Kit procedure.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

- Microcentrifuge (13,000 x g)
- Pipettes (60 µl–750 µl)
- Vortex-Genie® 2 Vortex
- Vortex Adapter for 24 (1.5–2.0 ml) tubes (cat. no. 13000-V1-24)
- 100% ethanol (for the QIAvac 24 Plus Manifold protocol)

Protocol: Experienced User

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- Shake to mix Solution C4 before use.

Procedure

1. Add 0.25 g of stool or biosolid to the Bead Tube provided.
   Note: For fecal samples that are especially high in lipids, polysaccharides and protein (e.g. meconium or some bird feces) smaller amounts of starting material (~0.10 g) may improve DNA yield and purity.
2. Add 750 µl of PowerBead Solution to the Bead Tube.
3. Add 60 µl of Solution C1 and invert several times or vortex briefly.
4. Heat the tubes at 65°C for 10 min.
5. Secure tubes horizontally using a Vortex Adapter (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min.
6. Centrifuge the tubes at 13,000 x *g* for 1 min.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Expect between 400 to 500 µl of supernatant.
8. Add 250 µl of Solution C2 and vortex briefly to mix. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerFecal extractions with the incubation we recommend you retain the step.
9. Centrifuge the tubes at 13,000 x *g* for 1 min.
10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.
11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
    Note: You can skip the 5 min incubation. However, if you have already validated the PowerFecal extractions with the incubation we recommend you retain the step.

12. Centrifuge the tubes at 13,000 x *g* for 1 min.
13. Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube (provided). Do not transfer more than 750 µl at this step.
14. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.
15. Load 650 µl of supernatant onto an MB Spin Column and centrifuge at 13,000 x *g* for 1 min. Discard the flow-through and repeat until all the supernatant has been processed.
    Note: Each sample processed will require a total of three loads.
16. Add 500 µl of Solution C5 and centrifuge for 1 min at 13,000 x *g*.
17. Discard the flow-through and centrifuge again for 1 min at 13,000 x *g*.
18. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided).
    Note: Avoid splashing any Solution C5 onto the MB Spin Column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you may use sterile, DNA-free, PCR-grade water (cat. no. 17000-10) or TE buffer.
    Note: Eluting with 100 µl of Solution C6 will maximize DNA yield. For more concentrated DNA, a **minimum** of 50 µl of Solution C6 can be used.
20. Centrifuge at 13,000 x *g* for 1 min and discard the MB Spin Column. The DNA in the tube is now ready for downstream applications.
    Note: We recommend storing DNA frozen (–20°C to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA, see the Troubleshooting Guide.

Protocol: Detailed

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C)
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves
- Shake to mix Solution C4 before use

Procedure

1. Add up to 0.25 g of stool or biosolid to the Bead Tube provided.
   Note: For fecal samples that are especially high in lipids, polysaccharides and protein (e.g. meconium or some bird feces) smaller amounts of starting material (~0.10 g) may improve DNA yield and purity.
2. Add 750 µl of PowerBead Solution to the Bead Tube.
   Note: Once the sample is loaded into a Dry Bead Tube, the next step is homogenization and lysis. The garnet beads and PowerBead Solution will help disperse the soil particles.
3. If Solution C1 has precipitated, heat at 60°C until precipitate dissolves. Add 60 µl of Solution C1 and invert several times or vortex briefly.
   Note: Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will form a white precipitate in the bottle. Heating to 60°C will dissolve the SDS but will not harm it or the other disruption agents. Solution C1 can be used while it is still warm. Vortexing mixes the components in the Bead Tube and begins to disperse the sample.
4. Heat the tubes at 65°C for 10 min.
   Note: Fecal samples contain a complex array of polysaccharides, lipids, salts and cells. Heating the samples increases the reaction rate between the lysis buffer and these substances and aids cell lysis.
5. Secure tubes horizontally using a Vortex Adapter (cat. no. 13000–V1–24). Vortex at maximum speed for 10 min.

Note: Vortexing is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1–4 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open. Use of the vortex adapter will maximize homogenization, which can lead to higher DNA yields. Avoid using tape, which can become loose and result in reduced homogenization efficiency, inconsistent results and reduced yields.

6. Centrifuge the tubes at 13,000 x *g* for 1 min.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Expect between 400 to 500 µl of supernatant.
   Note: The exact volume will depend on the absorbency of your starting material and is not critical for the procedure to be effective.
8. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step. Solution C2 has Inhibitor Removal Technology (IRT). It contains a reagent that can precipitate non-DNA organic and inorganic material including polysaccharides, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.
9. Centrifuge the tubes at 13,000 x *g* for 1 min.
10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube (provided).
    Note: The pellet at this point contains non-DNA organic and inorganic material including polysaccharides, cell debris and proteins. For best DNA yields and quality, avoid transferring any of the pellet.
11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
    Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step. Solution C3 has Inhibitor Removal Technology (IRT), and is a second reagent to precipitate additional non-DNA organic and inorganic material including

polysaccharides, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

12. Centrifuge the tubes at 13,000 x *g* for 1 min.
13. Avoiding the pellet, transfer up to 750 µl of supernatant into a clean 2 ml Collection Tube (provided).
    Note: The pellet at this point contains non-DNA organic and inorganic material including polysaccharides cell debris and proteins. For best DNA yields and quality, avoid transferring any of the pellet.
14. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.
    Note: Solution C4 is a high-concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the MB Spin Columns.
15. Load 650 µl of supernatant onto an MB Spin Column and centrifuge at 13,000 x *g* for 1 min. Discard the flow-through and repeat until all the supernatant has been processed.
    Note: Each sample processed will require a total of three loads. In the high salt solution, DNA is selectively bound to the MB Spin Column, while contaminants pass through.
16. Add 500 µl of Solution C5 and centrifuge for 30 s at 13,000 x *g*.
    Note: Solution C5 is an ethanol-based wash solution used to further clean the DNA that is bound to the MB Spin Column. This wash solution removes residual salt, and other contaminants while allowing the DNA to stay bound to the MB Spin Column.
17. Discard the flow-through and centrifuge again for 1 min at 13,000 x *g*.
    Note: The flow-through fraction is non-DNA organic and inorganic waste removed from the silica MB Spin Column membrane by the ethanol wash solution. The second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests and gel electrophoresis.
18. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided).
    Note: Avoid splashing any Solution C5 onto the MB Spin Column.

19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you may use sterile DNA-free PCR-grade water (cat. no. 17000-10) or TE buffer.
Note: Eluting with 100 µl of Solution C6 will maximize DNA yield. For more concentrated DNA, a **minimum** of 50 µl of Solution C6 can be used. Placing Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wet. This will result in a more efficient and complete release of the DNA from the MB Spin Column. As Solution C6 passes through the MB Spin Column silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris), which lacks salt.
20. Centrifuge at 13,000 x *g* for 1 min and discard the MB Spin Column. The DNA is now ready for downstream applications.
Note: We recommend storing DNA frozen (–20°C to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA, see the Troubleshooting Guide.

Protocol: QIAvac 24 Plus Vacuum Manifold

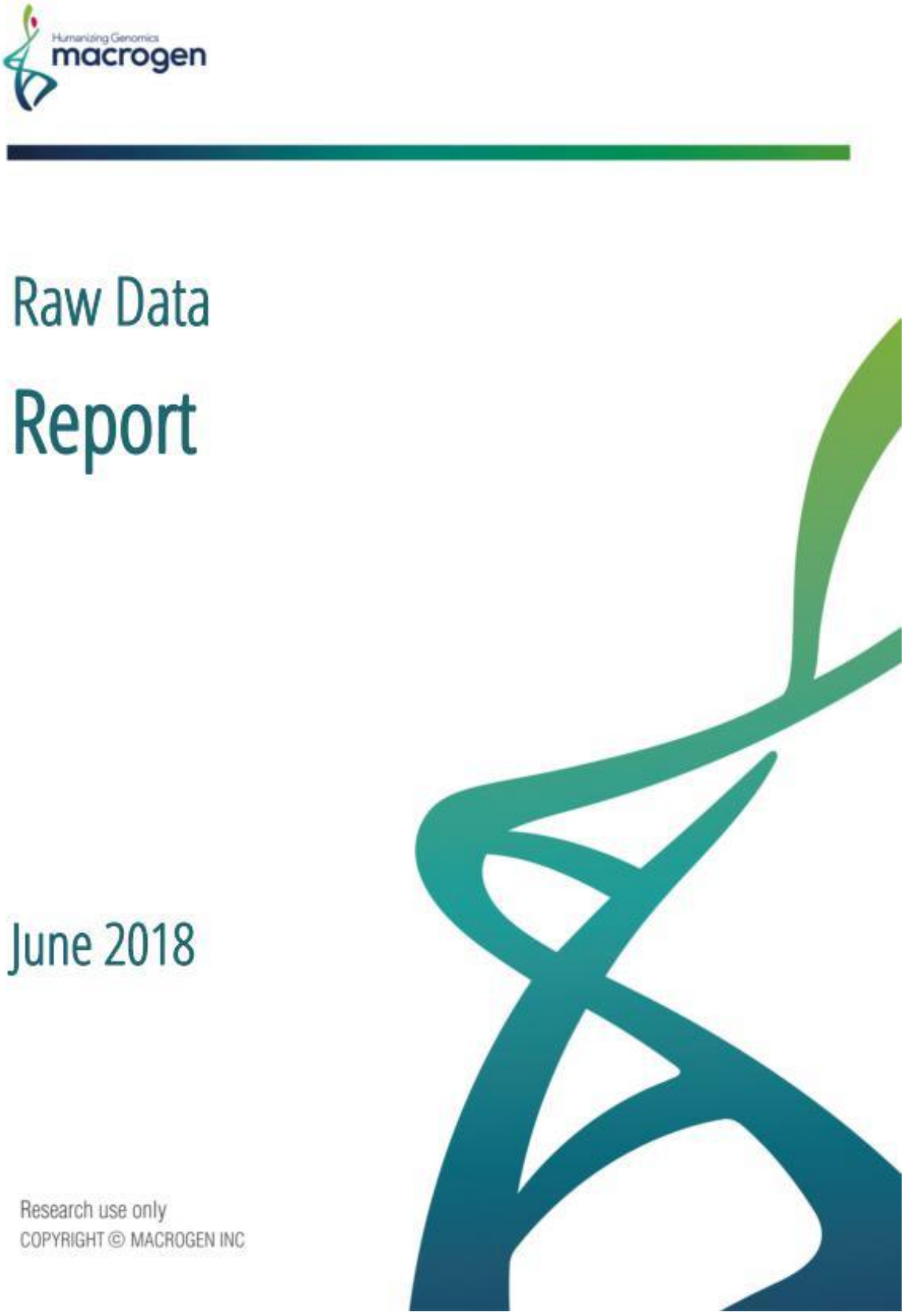
Important point before starting

- Perform all centrifugation steps at room temperature (15–25°C)
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves
- Shake to mix Solution C4 before use
- For each sample lysate, use one MB Spin Column. Keep the MB Spin Column in the attached 2 ml Collection Tube and continue using the Collection Tube as a MB Spin Column holder until needed for the Vacuum Manifold protocol.
- Label each Collection Tube top and MB Spin Column to maintain sample identity. If the MB Spin Column becomes clogged during the vacuum procedure, switch to the centrifugation protocol.
- You will need to provide 100% ethanol for step 8 of this protocol.

Procedure

1. Connect the QIAvac 24 Plus to the vacuum source using the QIAvac Connecting System (for more details, refer to the QIAvac 24 Plus Handbook, Appendix A, page 16).
2. Insert a VacValve into each Luer slot of the QIAvac 24 Plus that is to be used. Close unused Luer slots with Luer plugs or close the inserted VacValve.
3. Insert a VacConnector into each VacValve. Perform this step directly before starting the purification to avoid exposure of VacConnectors to potential contaminants in the air.
4. Place an MB Spin Column into each VacConnector on the manifold.
5. Transfer 650 µl of lysate (after step 14 of centrifugation protocol) to the MB Spin Column.
6. Turn on the vacuum source and open the VacValve of the port. Hold the tube in place when opening the VacValve to keep the spin filter steady. Allow the lysate to pass through the MB Spin Column.

Raw Data Report, Macrogen



Project Information

Client Name	De Wet Wolmarans
Company / Institution	North West University
Order Number	1805KMI-0089
Type of Read	Paired-end
Read Length	301
Number of Samples	35
Library Kit	Herculase II Fusion DNA Plymerase Nextera XT Index Kit V2
Library Protocol	16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B
Type of Sequencer	Illumina platform

Table of Contents

1. Data Download Information

1. 1. Raw Data

This is a zip file of un-trimmed data.
The trimmed data are generated from this data after adapter trimming.

Download link	File size	md5sum
1805KMI-0089_Raw_Data.zip	3.1G	2446b07f020ed70cf292155e19a0f4b8

RawData.zip : Read1/Read2 of All Samples (- One compressed file of all samples)

1. 2. Trimmed Data and Analysis Results

Download link	File size	md5sum
1805KMI-0089.zip	3.0G	0bad657702efa14d9c2219f4795c9f51

*.zip : Read1/Read2 of All Samples

Your data will be retained in our server for 3 months. Should you wish to extend the retention period, please email (ngs@macrogen.com) or contact our sales team.

2. Experimental Methods and Workflow

2. 1. Experiment Overview

Figl. Experiment overview

The Illumina NGS workflow includes 4 basic steps :

1) Sample Prep.(Sample Preparation)

For library construction, DNA/RNA is extracted from a sample. After performing quality control (QC), qualified samples proceed to library construction.

2) Library Construction

The sequencing library is prepared by random fragmentation of the DNA or cDNA sample, followed by 5' and 3' adapter ligation. Alternatively, "tagmentation" combines the fragmentation and ligation reactions into a single step that greatly increases the efficiency of the library preparation process. Adapter-ligated fragments are then PCR amplified and gel purified.

3) Sequencing

For cluster generation, the library is loaded into a flow cell where fragments are captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment is then amplified into distinct, clonal clusters through bridge amplification. When cluster generation is complete, the templates are ready for sequencing.

Illumina SBS technology utilizes a proprietary reversible terminator-based method that detects single bases as they are incorporated into DNA template strands. As all 4 reversible, terminator-bound dNTPs are persent during each sequencing cycle, natural competition minimizes incorporation bias and greatly reduces raw error rates compared to other technologies. The result is highly accurate base-by-base sequencing that virtually eliminates sequence-context-specific errors, even within repetitive sequence regions and homopolymers.

4) Raw data

Sequencing data is converted into raw data for the analysis.

2. 2. Generation of Raw Data

The Illumina seqeuncer generates raw images utilizing sequencing control software for system control and base calling through an integrated primary analysis software called RTA (Real Time Analysis). The BCL (base calls) binary is converted into FASTQ utilizing illumina package bcl2fastq.

3. Summary of Produced Data

3. 1. Raw Data Statistics

The total number of bases, reads, GC (%), Q20 (%), and Q30 (%) are calculated for the 35 samples. For example, in 1, 469,642 reads are produced, and total read bases are 141.4M bp. The GC content (%) is 55.146% and Q30 is 72.088%.

✓ **The following table only shows maximum of 20 samples. If your samples are more than 20, please refer to the attached excel file. View full table** : 1805KMI-0089_Data_Stat.xlsx (Raw_Stat sheet)

Table 1. Raw data Stats (maximun 20 samples)

Sample ID	Total read bases (bp)	Total reads	GC(%)	AT(%)	Q20(%)	Q30(%)
1	141,362,242	469,642	55.146	44.85	84.317	72.088
2	156,690,968	520,568	55.758	44.24	84.943	73.112
3	189,571,004	629,804	55.622	44.38	85.019	73.149
4	180,426,022	599,422	55.854	44.15	84.004	71.791
5	168,307,762	559,162	55.149	44.85	84.657	72.383
6	171,150,406	568,606	55.796	44.2	84.751	72.899
7	177,404,584	589,384	55.287	44.71	85.212	73.292
8	135,521,036	450,236	55.433	44.57	83.266	70.474
9	168,630,434	560,234	55.288	44.71	84.583	72.650
10	174,250,706	578,906	55.861	44.14	84.143	72.146
11	201,661,572	669,972	55.792	44.21	85.042	73.090
12	180,789,630	600,630	55.570	44.43	84.726	72.834
13	145,466,678	483,278	56.073	43.93	84.502	72.595
14	188,211,688	625,288	55.621	44.38	84.933	72.937
15	172,035,948	571,548	55.473	44.53	85.102	73.204
16	179,607,302	596,702	55.245	44.76	84.528	72.423
17	99,420,300	330,300	55.746	44.25	81.811	68.610
18	180,850,432	600,832	55.504	44.5	84.795	72.800
19	195,037,766	647,966	55.642	44.36	84.796	72.678
20	182,750,946	607,146	55.747	44.25	85.021	73.129

Sample ID : Sample name.
Total read bases : Total number of bases sequenced.
Total reads : Total number of reads. For Illumina paired-end sequencing, this value refers to the sum of read 1 and read 2.
GC(%) : GC content.
AT(%) : AT content.
Q20(%) : Ratio of bases that have phred quality score of over 20.
Q30(%) : Ratio of bases that have phred quality score of over 30.

3. 2. Adapter Trimmed Data Statistics

Scythe (v0.994) and Sickle programs are used to remove adapter sequences. After adapter trimming, reads shorter than 36bp are dropped in order to produce clean data.

✓ **The following table only shows maximum of 20 samples. If your samples are more than 20, please refer to the attached excel file. View full table** : 1805KMI-0089_Data_Stat.xlsx (Trimmed_Stat sheet)

Table 2. Adapter trimmed data Stats (maximum 20 samples)

Sample ID	Total read bases (bp)	Total reads	GC(%)	AT(%)	Q20(%)	Q30(%)
1	141,133,604	469,528	55.155	44.84	84.399	72.166
2	156,348,660	520,422	55.776	44.22	85.057	73.222
3	189,349,435	629,580	55.629	44.37	85.083	73.210
4	180,267,215	599,268	55.858	44.14	84.056	71.840
5	168,179,773	559,032	55.152	44.85	84.701	72.424
6	171,020,784	568,470	55.798	44.2	84.797	72.942
7	177,258,365	589,200	55.292	44.71	85.261	73.339
8	135,308,989	450,140	55.442	44.56	83.350	70.553
9	168,404,617	560,060	55.295	44.7	84.655	72.718
10	174,080,637	578,696	55.866	44.13	84.199	72.199
11	201,482,692	669,794	55.797	44.2	85.092	73.138
12	180,588,965	600,464	55.575	44.42	84.789	72.894
13	145,309,136	483,162	56.078	43.92	84.565	72.654
14	187,927,848	625,118	55.633	44.37	85.012	73.014
15	171,868,073	571,428	55.477	44.52	85.158	73.258
16	179,435,812	596,484	55.251	44.75	84.582	72.474
17	99,340,357	330,232	55.748	44.25	81.860	68.655
18	180,666,675	600,658	55.509	44.49	84.851	72.853
19	194,825,930	647,844	55.649	44.35	84.856	72.735
20	182,536,926	606,944	55.754	44.25	85.085	73.191

Sample ID : Sample name.
Total read bases : Total number of bases sequenced.
Total reads : Total number of reads. For Illumina paired-end sequencing, this value refers to the sum of read 1 and read 2.
GC(%) : GC content.
AT(%) : AT content.
Q20(%) : Ratio of bases that have phred quality score of over 20.
Q30(%) : Ratio of bases that have phred quality score of over 30.

3. 3. Total Read Bases

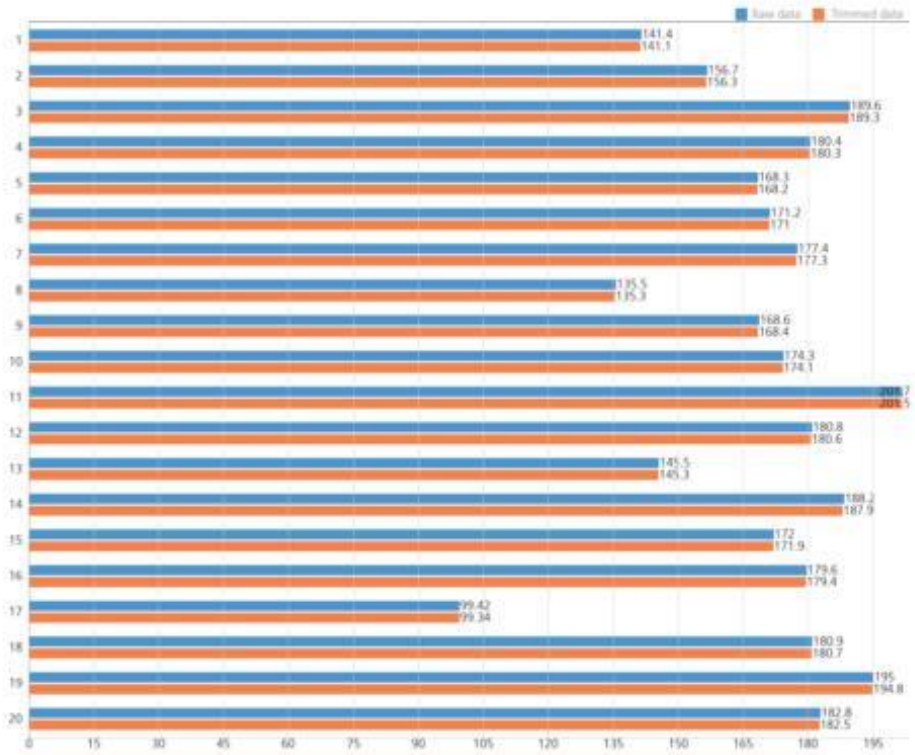


Figure 2. Throughput of Raw data and Adapter trimmed data (Mb)

3. 4. Total Reads

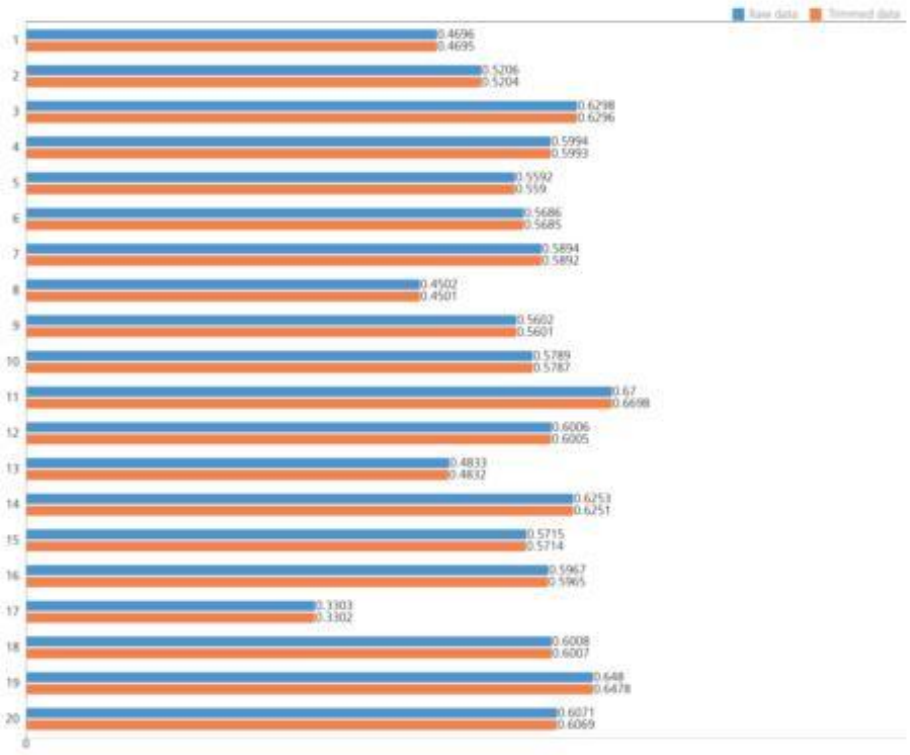


Figure 3. Total read count of Raw data and Adapter trimmed data (M)

3. 5. GC Content

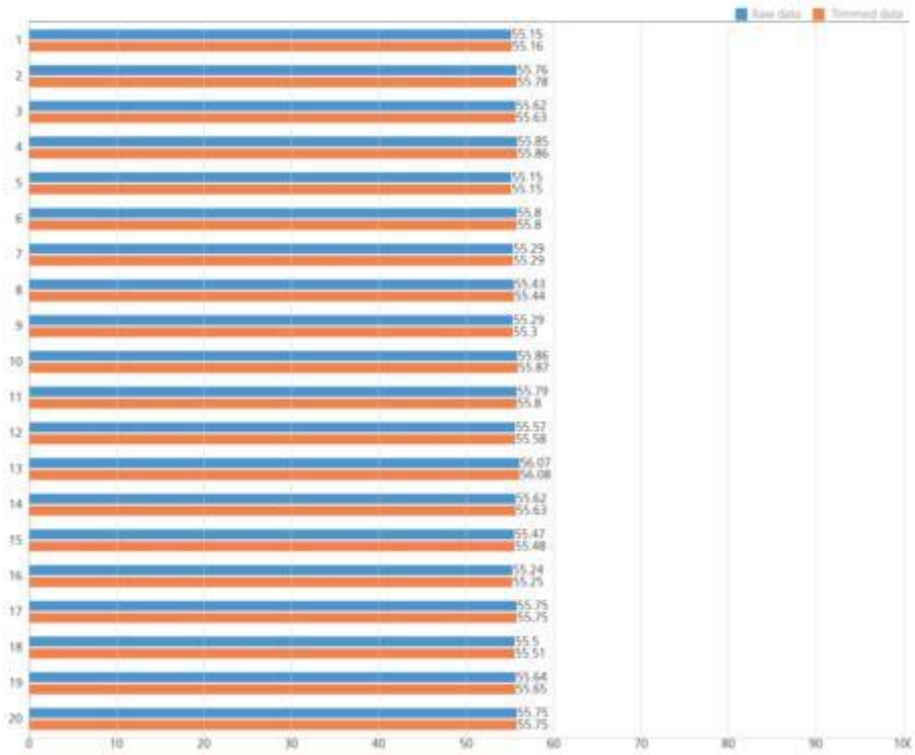


Figure 4. GC Content of Raw data and Adapter trimmed data (%)

3. 6. Q30 (%)

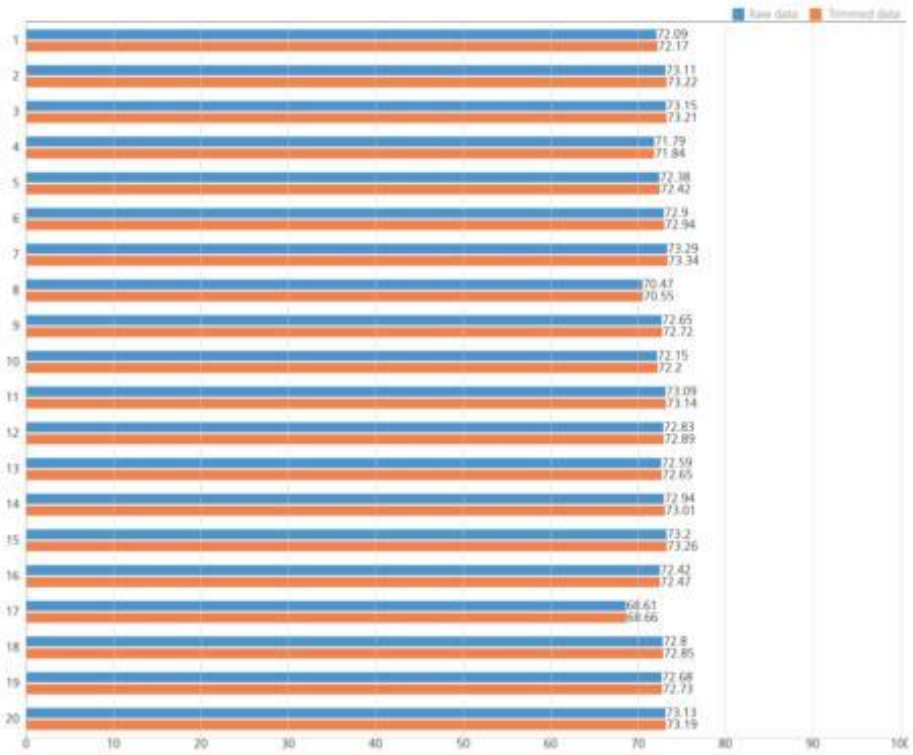


Figure 5. Q30 scores of Raw data and Adapter trimmed data (%)

4. Appendix

4. 1. FAQ

Q: I want to see the produced data. How can I open the files?

A: As the large size zip files provided by our company are hard to process in the Windows environment, we highly recommend using Linux environment for a smoother operation..

4. 2. FASTQ File

Example of FASTQ

```
@HISEQ-MFG:501:HB0TFADXX:1:1101:1247:2183 1:N:0:
CTCAGCTAAATACTTTGACACCNGTANNANNNNNNNNNTNNNNNNNNNNN
+
@@@BDDDDHHHHFHIIIIIIII#3AC##########################
```

FASTQ file is composed of four lines.
Line 1 : ID line includes information such as flow cell lane information.
Line 2 : Sequences line.
Line 3 : Separator line (+ mark).
Line 4 : Quality values line about sequences.

4. 3. Phred Quality Score Chart

Phred quality score numerically expresses the accuracy of each nucleotide. Higher Q number signifies higher accuracy. For example, if Phred assigns a quality score of 30 to a base, the chances of having base call error are 1 in 1000.

Phred Quality Score Q is calculated with $-10\log_{10}P$, where P is probability of erroneous base call.

Quality of phred score	Probability of incorrect base call	Base call accuracy	Characters
10	1 in 10	90%	!"#$%&'()*+
20	1 in 100	99%	,-./012345
30	1 in 1000	99.9%	6789:;h=i?
40	1 in 10000	99.99%	@ABCDEFGHIJ

Encoding : Sanger Quality (ASCII Character Code=Phred Quality Value + 33)

Research use only

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Contact

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Lims : http://dna.macrogen.com

ASV counts per sample, ASV taxonomical assignments, R scripts and metadata

METADATA

Sample	Nestbuilding	Group	Treatment_groups	Gender	Age
sa1	NNB	Baseline_1	NNB_B1	M	8
sa2	NNB	Baseline_1	NNB_B1	M	8
sa3	NNB	Baseline_1	NNB_B1	M	8
sa4	NNB	Baseline_1	NNB_B1	F	8
sa5	NNB	Baseline_1	NNB_B1	F	8
sa6	NNB	Baseline_1	NNB_B1	F	8
sa7	LNB	Baseline_1	LNB_B1	M	8
sa8	LNB	Baseline_1	LNB_B1	M	8
sa9	LNB	Baseline_1	LNB_B1	M	8
sa10	LNB	Baseline_1	LNB_B1	F	8
sa11	LNB	Baseline_1	LNB_B1	F	8
sa12	LNB	Baseline_1	LNB_B1	F	8
sa13	NNB	SO2	Pre_treatment	F	8
sa14	NNB	SO2	Pre_treatment	F	8
sa15	NNB	SO2	Pre_treatment	F	8
sa16	NNB	SO2	Pre_treatment	F	8
sa17	NNB	SO2	Pre_treatment	F	8
sa18	LNB	SO2	Pre_treatment	F	8
sa19	LNB	SO2	Pre_treatment	F	8
sa20	LNB	SO2	Pre_treatment	F	8
sa21	LNB	SO2	Pre_treatment	F	8
sa22	LNB	SO2	Pre_treatment	F	8
sa23	NNB	SO2	Post_treatment	F	12
sa24	NNB	SO2	Post_treatment	F	12
sa25	NNB	SO2	Post_treatment	F	12
sa26	NNB	SO2	Post_treatment	F	12
sa27	NNB	SO2	Post_treatment	F	12
sa28	LNB	SO2	Post_treatment	F	12
sa29	LNB	SO2	Post_treatment	F	12
sa30	LNB	SO2	Post_treatment	F	12
sa31	LNB	SO2	Post_treatment	F	12
sa32	LNB	SO2	Post_treatment	F	12
33-NEG	NA	NA		NA	NA

DADA 2 PIPELINE-SCRIPT

```
source("https://bioconductor.org/biocLite.R")
biocLite("dada2")
biocLite(suppressUpdates = TRUE)

source("http://bioconductor.org/biocLite.R")
biocLite(suppressUpdates = TRUE)
biocLite("ShortRead", suppressUpdates = TRUE)
```

```
library(ShortRead)
library(Biostrings)
library(dada2)
library(ggplot2)
library(Rcpp)
library(RcppParallel)
library(vegan)
library(spatstat)
library(readxl)
getwd()
setwd("C:/Users/Isma Scheepers/Documents/My_Data/filtered")
path <- "C:/Users/Isma Scheepers/Documents/My_Data/filtered"
list.files(path)

memory.limit(size = 8000000000)

fnFs <- sort(list.files(path, pattern="_1.fastq", full.names = TRUE))
fnRs <- sort(list.files(path, pattern="_2.fastq", full.names = TRUE))

sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)

plotQualityProfile(fnFs[1:2])
plotQualityProfile(fnRs[1:2])

##Filering and Trimming
filtFs <- file.path(path, "filtered", paste0(sample.names, "_F_filt.fastq.gz"))
filtRs <- file.path(path, "filtered", paste0(sample.names, "_R_filt.fastq.gz"))

out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(275,250),
trimLeft=c(17,21), maxN=0, maxEE=c(3,5), truncQ=2, rm.phix=TRUE,
compress=TRUE, multithread=FALSE)
##Creating output directory: C:/Users/Isma
Scheepers/Documents/My_Data/filtered/filtered

head(out)

errF <- learnErrors(filtFs, multithread=TRUE)
errR <- learnErrors(filtRs, multithread=TRUE, MAX_CONSIST = 12)
plotErrors(errF, nominalQ=TRUE)

derepFs <- derepFastq(filtFs, verbose=TRUE)
derepRs <- derepFastq(filtRs, verbose=TRUE)

names(derepFs) <- sample.names
names(derepRs) <- sample.names
```

```
dadaFs <- dada(derepFs, err=errF, multithread=TRUE)
dadaRs <- dada(derepRs, err=errR, multithread=TRUE)

dadaFs[[1]]

mergers <- mergePairs(dadaFs, derepFs, dadaRs, derepRs, verbose=TRUE)
head(mergers[[1]])

seqtab <- makeSequenceTable(mergers)

dim(seqtab)

table(nchar(getSequences(seqtab)))


seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus",
multithread=TRUE, verbose=TRUE)
dim(seqtab.nochim)
sum(seqtab.nochim)/sum(seqtab)

getN <- function(x) sum(getUniques(x))
track <- cbind(out, sapply(dadaFs, getN), sapply(dadaRs, getN),
sapply(mergers, getN), rowSums(seqtab.nochim))

colnames(track) <- c("input", "filtered", "denoisedF", "denoisedR",
"merged", "nonchim")
rownames(track) <- sample.names
head(track)

# determining taxonomy
taxa <- assignTaxonomy(seqtab.nochim, "rdp_train_Set_16.fa", multithread=FALSE)

taxa.print <- taxa # Removing sequence rownames for display only
rownames(taxa.print) <- NULL
head(taxa.print)
```

OBJECTIVE 1: PHYLOSEQ SCRIPT

```
## Handoff to phyloseq and installing phyloseq
source("http://bioconductor.org/biocLite.R")
biocLite("phyloseq")
library(phyloseq); packageVersion("phyloseq") ##dit werk nadat rtools
afgelaai is
## vir Citation van bioconducter (from within R, enter citation("phyloseq")):
```

```
theme_set(theme_bw())
metadata <- read_excel ("C:/Users/Isma
Scheepers/Documents/My_Data/filtered/metadata.xlsx", sheet="DNA
ekstraksies data volledig")
metadata_test <- as.data.frame(sample_data(metadata))
taxa_test <- taxa
row.names(seqtab.nochim) <- row.names(metadata_test)
ps_test <- phyloseq(otu_table(seqtab.nochim, taxa_are_rows=FALSE),
sample_data(metadata_test), tax_table(taxa_test))
ps_test
ps_t1 <- prune_samples(sample_names(ps_test) != "sa33", ps_test)
#remove neg ctrl from data set (LET WEL: "sa33" is die NEG ctrl groep)
ps_t1
#Select only baseline samples

ps_B1_test <- subset_samples(ps_t1,
substring(metadata$Treatment_groups,4,6) == "_B1")
ps_B1_test # now we have 12 samples

# To work on Genus level data
ps.genus_B1_test <- tax_glom(ps_B1_test, taxrank="Genus")
ps.genus_B1_test
taxa_names(ps.genus_B1_test) = c(tax_table(ps.genus_B1_test)[,6])
sort(sample_sums(ps.genus_B1_test)) # see distribution of reads/sample
hist(sample_sums(ps.genus_B1_test))

# Rarefy before doing richness and BC distances
sample_sums(ps.genus_B1_test)
set.seed(454)
ps.gen.rar_B1_test <- rarefy_even_depth(ps.genus_B1_test, sample.size
= min(sample_sums(ps.genus_B1_test)),rngseed=FALSE)
sample_sums(ps.gen.rar_B1_test) # all samples now rarefied to 20334 reads/sample

# Visualize alpha diversity
plot_richness(ps.gen.rar_B1_test, x="Nestbuilding",
measures=c("Shannon","Simpson","Chao1", "Observed", "Fisher"),
color="Nestbuilding") + geom_boxplot()

richestimates_B1_test <- estimate_richness(ps.gen.rar_B1_test) # to
show all richness estimates
row.names(richestimates_B1_test) <- row.names(sample_data(ps_B1_test))
richestimates_B1_test
RDEtmp_B1_test <- data.frame(sample_data(ps.gen.rar_B1_test))
RDEtmp2_B1_test <- data.frame(merge(RDEtmp_B1_test,
richestimates_B1_test,by="row.names"), row.names=1)
ps.gen.rar.RDE_B1_test <-
phyloseq(sample_data(RDEtmp2_B1_test),otu_table(ps.gen.rar_B1_test))


# Select out LNB and NNB data from rarefied PhylObject to use later in wilcox
LNB_B1 <- subset_samples(ps.gen.rar.RDE_B1_test,
```

```
metadata$Treatment_groups == "LNB_B1")
LNB.RDE_B1 <- data.frame(sample_data(LNB_B1)[,c("Observed","Fisher","Chao1",
"Shannon", "Simpson")])
NNB_B1 <- subset_samples(ps.gen.rar.RDE_B1_test,
metadata$Treatment_groups == "NNB_B1")
NNB.RDE_B1 <- data.frame(sample_data(NNB_B1)[,c("Observed","Fisher","Chao1",
"Shannon", "Simpson")])

#Test for differences in richness between 2 groups ####
#Perform Wilcox (if wanted to compare between 4 groups, then use Kruskal Wallis)
statistic=c()
wilcox=c()
qvals=c()
medianLNB=c()
medianNNB=c()
pvals = c()
ndata = c()
score= c()
for (j in 1:ncol(LNB.RDE_B1)){
  pvals[j] = wilcox.test(LNB.RDE_B1[,j],NNB.RDE_B1[,j],paired=FALSE)$p.value
  statistic[j] =
wilcox.test(LNB.RDE_B1[,j],NNB.RDE_B1[,j],paired=FALSE)$statistic
  score[j] = colnames(LNB.RDE_B1)[j]
  medianLNB[j] = median(LNB.RDE_B1[,j])
  medianNNB[j] = median(NNB.RDE_B1[,j])
}
qvals = p.adjust(pvals,method ="BH") #multiple testing correction done
for the multiple taxa tested
wilcox_B1 <- rbind(wilcox,cbind(score,statistic,pvals,qvals,medianLNB,medianNNB))
#new object wilcox with rbind combines rows of wilcox test, cbind
combines columns of all those measures
wilcox_B1 <- na.omit(wilcox_B1) #omit NA in data
wilcox2_B1 <- wilcox_B1[as.numeric(wilcox_B1[,4])<=0.05,] #[,4] means
4th column, thus q-value
wilcox3_B1 <- wilcox_B1[as.numeric(wilcox_B1[,4])<=0.1,]

# If you want to write it to a table
write.table(wilcox2_B1,"wilcox-pval-lt-0.05-B1.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
write.table(wilcox3_B1,"wilcox-pval-lt-0.10-B1.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
# No obvious systematic difference in alpha-diversity between patients
and controls

## Ordinate/Bray Curtis
ps.gen.rar.bray_B1 <- ordinate(ps.gen.rar_B1_test, method="NMDS",
distance="bray") # NMDS:nonmetric multidimensional scaling, on the
rarefied genera level phylobject
plot_ordination(ps.gen.rar_B1_test, ps.gen.rar.bray_B1,
color="Nestbuilding", title="Bray NMDS")
ps.gen.rar.bray_B1$stress
```

```
#translate genus composition into interindividual distance
mydist_B1_test <-
vegdist(data.frame(otu_table(ps.gen.rar_B1_test)),"bray") #creating a
distance matrix using vegdist & tell it that otu_table is a data.frame
vars_B1_test <- data.frame(sample_data(ps.gen.rar_B1_test)) #to
include the variables from metadata
cap.gender_B1_test <- capscale(mydist_B1_test ~ vars_B1_test$Gender)
#use mydist distance matrix and test gender variable to see if it had
an effect on BC dist
anova(cap.gender_B1_test) ##cap gender b test not found

## Bar plot_most abundant taxa
# Filter taxa observed more than once in at least 15% of participants
(to not look at all of them and to minimize multiple testing). Note
filter_taxa works on phyloseq objects ONLY. Use filtering ONLY when
comparing genera (not for distance matrices)
ps.rar.mean1_B1_test <- filter_taxa(ps.gen.rar_B1_test, function(x)
mean(x)>=1, TRUE)
ps.rar.mean1.min0.15_B1_test <- filter_taxa(ps.rar.mean1_B1_test,
function(x) sum(x>=1) >= (0.15*length(x)), TRUE)

# Create stacked bar chart for  top 20 genera ##

Top20Gen_B1_test <-names(sort(taxa_sums(ps.rar.mean1.min0.15_B1_test),
TRUE)[1:20]) #sort the sums of the taxa to get 1-20 of the most
abundant genera in PhylObject
Top20Gen.cut_B1_test <-prune_taxa(Top20Gen_B1_test,
ps.rar.mean1.min0.15_B1_test) #now select only those top 20
##Top20Gen.rar <-rarefy_even_depth(Top20Gen_B1_test, set.seed(TRUE),
rngseed = TRUE, Random.seed(454)) werk nie
plot_bar(Top20Gen.cut_B1_test) #plot top 20 taxa
plot_bar(Top20Gen.cut_B1_test,  fill="Family") +
facet_wrap(~Nestbuilding, scales="free_x")
plot_bar(Top20Gen.cut_B1_test, fill="Genus") +
facet_wrap(~Nestbuilding, scales="free_x")

# Select out patients and controls#
LNBtaxa_B1 <- subset_samples(ps.rar.mean1.min0.15_B1_test, Nestbuilding =="LNB")
colnames(otu_table(LNBtaxa_B1)) # NOTE:taxa should be rows! Therefore
need to transpose before loop
rownames(otu_table(LNBtaxa_B1))
LNBtaxa.otu_B1 <- data.frame(otu_table(LNBtaxa_B1))
NNBtaxa_B1 <- subset_samples(ps.rar.mean1.min0.15_B1_test, Nestbuilding =="NNB")
NNBtaxa.otu_B1 <- data.frame(otu_table(NNBtaxa_B1))
colnames(LNBtaxa.otu_B1)
colnames(NNBtaxa.otu_B1)

# Wilcox to see if differences between taxa
pvals <- c()
statistic <- c()
```

```
medianLNB <- c()
medianNNB <- c()
wilcox <- c()
score <- c()
for (i in 1:ncol(LNBtaxa.otu_B1)){
  pvals[i] = wilcox.test(LNBtaxa.otu_B1[,i],NNBtaxa.otu_B1[,i],paired=FALSE)$p.value
  statistic[i] =
wilcox.test(LNBtaxa.otu_B1[,i],NNBtaxa.otu_B1[,i],paired=FALSE)$statistic
  score[i] = colnames(LNBtaxa.otu_B1)[i]
  medianLNB[i] = median(LNBtaxa.otu_B1[,i])
  medianNNB[i] = median(NNBtaxa.otu_B1[,i])
}
qvals = p.adjust(pvals,method ="BH") #multiple testing correction done
for the multiple taxa tested
wilcox <- rbind(wilcox,cbind(score,statistic,pvals,qvals,medianLNB,medianNNB))
#new object wilcox with rbind combines rows of wilcox test, cbind
combines columns of all those measures

wilcoxa_B1 <- na.omit(wilcox) #omit NA in data
wilcoxb_B1 <- wilcoxa_B1[as.numeric(wilcoxa_B1[,4])<=0.05,] #[,4]
means 4th column, thus qvalue - want to compare
wilcoxc_B1 <- wilcoxa_B1[as.numeric(wilcoxa_B1[,4])<=0.1,]

write.table(wilcoxa_B1,"wilcoxa_na.omit_B1.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
write.table(wilcoxb_B1,"wilcoxb-pval-lt-0.05-B1.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
write.table(wilcoxc_B1,"wilcoxc-pval-lt-0.1-B1.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")

write.table(otu_table(ps.rar.mean1.min0.15_B1_test),"Baseline_1.txt",quote=F,col.names=TRUE,ro
w.names=TRUE,
sep="\t")
```

OBJECTIVE 2: PHYLOSEQ-SCRIPT

```
## Handoff to phyloseq and installing phyloseq
source("http://bioconductor.org/biocLite.R")
biocLite("phyloseq")
library(phyloseq); packageVersion("phyloseq")
## vir Citation van bioconducter (from within R, enter citation("phyloseq")):

theme_set(theme_bw())
metadata <- read_excel ("C:/Users/Isma
Scheepers/Documents/My_Data/filtered/Metadata.xlsx", sheet="DNA
ekstraksies data volledig")
meta_test <- as.data.frame(sample_data(metadata))
taxa_test <- taxa
row.names(seqtab.nochim) <- row.names(meta_test)
ps_test <- phyloseq(otu_table(seqtab.nochim, taxa_are_rows=FALSE),
sample_data(meta_test), tax_table(taxa_test))
ps_test
```

```
ps_t1 <- prune_samples(sample_names(ps_test) != "sa33" &
sample_names(ps_test) != "sa27", ps_test) #remove neg ctrl from data
set (LET WEL: "sa33" is die NEG ctrl groep)
ps_t1 <- prune_samples(sample_names(ps_test) != "sa33"  &
sample_names(ps_test) != "sa27" & sample_names(ps_test) != "sa32",
ps_test) #remove neg ctrl from data set (LET WEL: "sa33" is die NEG
ctrl groep)
ps_t1
#Select
#Select only baseline samples
ps_T_test <- subset_samples(ps_t1, ps_t1@sam_data$Group == "SO2")
ps_T_test#

# To work on Genus level data
ps.genus_T_test <- tax_glom(ps_T_test, taxrank="Genus")
ps.genus_T_test
taxa_names(ps.genus_T_test) = c(tax_table(ps.genus_T_test)[,6])
sort(sample_sums(ps.genus_T_test)) # see distribution of reads/sample
hist(sample_sums(ps.genus_T_test))

# Rarefy before doing richness and BC distances
sample_sums(ps.genus_T_test)
set.seed(454)
ps.gen.rar_T_test <- rarefy_even_depth(ps.genus_T_test, sample.size =
min(sample_sums(ps.genus_T_test)),rngseed=FALSE)
sample_sums(ps.gen.rar_T_test) # all samples now rarefied to 21824 reads/sample

# Visualize alpha diversity
plot_richness(ps.gen.rar_T_test, x="Treatment_groups",
measures=c("Shannon","Simpson","Chao1", "Observed", "Fisher"),
color="Treatment_groups") + geom_boxplot()

richestimates_T_test <- estimate_richness(ps.gen.rar_T_test) # to show
all richness estimates
row.names(richestimates_T_test) <- row.names(sample_data(ps_T_test))
richestimates_T_test
RDEtmp_T_test <- data.frame(sample_data(ps.gen.rar_T_test))
RDEtmp2_T_test <- data.frame(merge(RDEtmp_T_test,
richestimates_T_test,by="row.names"), row.names=1)
ps.gen.rar.RDE_T_test <-
phyloseq(sample_data(RDEtmp2_T_test),otu_table(ps.gen.rar_T_test))

# Select out Post_treatment and Pre_treatment data from rarefied
PhylObject to use later in wilcox
Pre_treatment_T <- subset_samples(ps.gen.rar.RDE_T_test,
Treatment_groups == "Pre_treatment")
Pre_treatment.RDE_T<-
data.frame(sample_data(Pre_treatment_T)[,c("Observed","Fisher","Chao1",
"Shannon", "Simpson")])
Post_treatment_T<- subset_samples(ps.gen.rar.RDE_T_test,
Treatment_groups == "Post_treatment")
```

```
Post_treatment.RDE_T <-
data.frame(sample_data(Post_treatment_T)[,c("Observed","Fisher","Chao1",
"Shannon", "Simpson")])

#Test for differences in richness between 2 groups ####
#Perform Wilcox (if wanted to compare between 4 groups, then use Kruskal Wallis)
statistic=c()
wilcox=c()
qvals=c()
median_Post_treatment=c()
median_Pre_treatment=c()
pvals = c()
ndata = c()
score= c()
for (j in 1:ncol(Post_treatment.RDE_T)){
  pvals[j] = wilcox.test(Post_treatment.RDE_T[,j],Pre_treatment.RDE_T[,j],paired=FALSE)$p.value
  statistic[j] =
wilcox.test(Post_treatment.RDE_T[,j],Pre_treatment.RDE_T[,j],paired=FALSE)$statistic
  score[j] = colnames(Post_treatment.RDE_T)[j]
  median_Post_treatment[j] = median(Post_treatment.RDE_T[,j])
  median_Pre_treatment[j] = median(Pre_treatment.RDE_T[,j])
}
qvals = p.adjust(pvals,method ="BH") #multiple testing correction done
for the multiple taxa tested
wilcox_T <-
rbind(wilcox,cbind(score,statistic,pvals,qvals,median_Pre_treatment,median_Post_treatment))
#new object wilcox with rbind combines rows of wilcox test, cbind
combines columns of all those measures
wilcox_T <- na.omit(wilcox_T) #omit NA in data
wilcox2_T <- wilcox_T[as.numeric(wilcox_T[,4])<=0.05,] #[,4] means 4th
column, thus q-value
wilcox3_T <- wilcox_T[as.numeric(wilcox_T[,4])<=0.1,]

# If you want to write it to a table
write.table(wilcox2_T,"wilcox-pval-lt-0.05-T.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
write.table(wilcox3_T,"wilcox-pval-lt-0.10-T.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
# No obvious systematic difference in alpha-diversity between patients
and controls

## Ordinate/Bray Curtis
ps.gen.rar.bray_T <- ordinate(ps.gen.rar_T_test, method="NMDS",
distance="bray") # NMDS:nonmetric multidimensional scaling, on the
rarefied genera level phylobject
plot_ordination(ps.gen.rar_T_test, ps.gen.rar.bray_T,
color="Treatment_groups", title="Bray NMDS")
ps.gen.rar.bray_T$stress

#translate genus composition into interindividual distance
mydist_T_test <-
```

```
vegdist(data.frame(otu_table(ps.gen.rar_T_test)),"bray") #creating a
distance matrix using vegdist & tell it that otu_table is a data.frame
vars_T_test <- data.frame(sample_data(ps.gen.rar_T_test)) #to include
the variables from metadata
cap.gender_T_test <- capscale(mydist_T_test ~ vars_T_test$Gender)
#(wil nie doen nie)#use mydist distance matrix and test gender
variable to see if it had an effect on BC dist
anova(cap.gender_T_test) ##cap gender b test not found

## Bar plot_most abundant taxa
# Filter taxa observed more than once in at least 15% of participants
(to not look at all of them and to minimize multiple testing). Note
filter_taxa works on phyloseq objects ONLY. Use filtering ONLY when
comparing genera (not for distance matrices)
ps.rar.mean1_T_test <- filter_taxa(ps.gen.rar_T_test, function(x)
mean(x)>=1, TRUE)
ps.rar.mean1.min0.15_T_test <- filter_taxa(ps.rar.mean1_T_test,
function(x) sum(x>=1) >= (0.15*length(x)), TRUE)
# Create stacked bar chart for  top 20 genera ####
Top20Gen_T_test <-names(sort(taxa_sums(ps.rar.mean1.min0.15_T_test),
TRUE)[1:20]) #sort the sums of the taxa to get 1-20 of the most
abundant genera in PhylObject
Top20Gen.cut_T_test <-prune_taxa(Top20Gen_T_test,
ps.rar.mean1.min0.15_T_test) #now select only those top 20
plot_bar(Top20Gen.cut_T_test) #plot top 20 taxa
plot_bar(Top20Gen.cut_T_test,  fill="Family") +
facet_wrap(~Treatment_groups, scales="free_x")
plot_bar(Top20Gen.cut_T_test, fill="Genus") +
facet_wrap(~Treatment_groups, scales="free_x")

# Select out patients and controls#
Post_treatment_taxa_T <- subset_samples(ps.rar.mean1.min0.15_T_test,
Treatment_groups =="Post_treatment")
colnames(otu_table(Post_treatment_taxa_T)) # NOTE:taxa should be rows!
Therefore need to transpose before loop
rownames(otu_table(Post_treatment_taxa_T))
Post_treatment_taxa.otu_T <- data.frame(otu_table(Post_treatment_taxa_T))
Pre_treatment_taxa_T <- subset_samples(ps.rar.mean1.min0.15_T_test,
Treatment_groups =="Pre_treatment")
Pre_treatment_taxa.otu_T <- data.frame(otu_table(Pre_treatment_taxa_T))
colnames(Post_treatment_taxa.otu_T)
colnames(Pre_treatment_taxa.otu_T)

# Wilcox to see if differences between taxa
pvals <- c()
statistic <- c()
median_Post_treatment <- c()
median_Pre_treatment <- c()
wilcox <- c()
score <- c()
for (i in 1:ncol(Post_treatment_taxa.otu_T)){
```

```
  pvals[i] =
wilcox.test(Post_treatment_taxa.otu_T[,i],Pre_treatment_taxa.otu_T[,i],paired=FALSE)$p.value
  statistic[i] =
wilcox.test(Post_treatment_taxa.otu_T[,i],Pre_treatment_taxa.otu_T[,i],paired=FALSE)$statistic
  score[i] = colnames(Post_treatment_taxa.otu_T)[i]
  median_Post_treatment[i] = median(Post_treatment_taxa.otu_T[,i])
  median_Pre_treatment[i] = median(Pre_treatment_taxa.otu_T[,i])
}
qvals = p.adjust(pvals,method ="BH") #multiple testing correction done
for the multiple taxa tested
wilcox <-
rbind(wilcox,cbind(score,statistic,pvals,qvals,median_Post_treatment,median_Pre_treatment))
#new object wilcox with rbind combines rows of wilcox test, cbind
combines columns of all those measures

wilcoxa_T <- na.omit(wilcox) #omit NA in data
wilcoxb_T <- wilcoxa_T[as.numeric(wilcoxa_T[,4])<=0.05,] #[,4] means
4th column, thus qvalue - want to compare
wilcoxc_T <- wilcoxa_T[as.numeric(wilcoxa_T[,4])<=0.1,]

write.table(wilcoxa_T,"wilcoxa_na.omit_T.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
write.table(wilcoxb_T,"wilcoxb-pval-lt-0.05-T.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")
write.table(wilcoxc_T,"wilcoxc-pval-lt-0.1-T.txt",quote=F,col.names=TRUE,row.names=TRUE,
sep="\t")

otu_table(ps.gen.rar_T_test)
tax_table(ps.gen.rar_T_test)

write.table(otu_table(ps.rar.mean1.min0.15_T_test),"ps_T_test.txt",quote=F,col.names=TRUE,row.n
ames=TRUE,
sep="\t")
```

PCA SCRIPT

```
options(stringsAsFactors = FALSE)
#source("http://bioconductor.org/biocLite.R")
library(vegan)
library(ggplot2)
library(grid)
library(ggrepel)
library(fossil)
library(reshape2)
## try http:// if https:// URLs are not supported
source("https://bioconductor.org/biocLite.R")
biocLite("metagenomeSeq")
library(metagenomeSeq)
library(coda.base)
library(zCompositions)
## try http:// if https:// URLs are not supported
source("https://bioconductor.org/biocLite.R")
```

```
biocLite("ALDEx2")
library(ALDEx2)
library(Tjazi)

setwd("C:/Users/Isma
Scheepers/Documents/My_Data/filtered/NWU_CORK/DeerMouse/DeerMouse")

doc1 = t(read.delim("Doc 1 Baseline NNB vs LNB male and female OTU -
Metadata 1.csv", sep = "\t", row.names = 1))
doc3 = t(read.delim("Doc 3 Treatment group_OTU.csv", sep = "\t", row.names = 1))

metadata = (read.delim("Metadata file complete.csv", sep = "\t", row.names = 1))

metadata_doc1 = metadata[colnames(doc1),]
metadata_doc3 = metadata[colnames(doc3),]
metadata_doc3$Animal_ID = rep(1:10, 2)[c(1:14,16:19)]

species   <- doc1
species   <- apply(species,c(1,2),function(x) as.numeric(as.character(x)))

conds      <- c(rep("B", ncol(species)-10 ), rep("A", 10)) #If you
have less than 12 animals, adjust!
species.clr <- aldex.clr(species, conds, mc.samples = 1000,
denom="all", verbose=TRUE, useMC=TRUE)
species.eff <- aldex.effect(species.clr, conds, verbose = TRUE,
include.sample.summary = TRUE)

species.exp <- (species.eff[,c(4:(ncol(species.eff)-4))]) #remove the
useless t-test-like results

#### The following was done for boxplot ####
library(ggplot2)
library(ggpubr)
species.rob <- t(species.exp[19,])
group.rob <- as.data.frame(c(rep("NNB", ncol(species)-6 ), rep("LNB", 6)))
rob.df  <- as.data.frame(cbind(species.rob, group.rob))
names(rob.df)[2]<-"Group"
View(rob.df)

ggboxplot(rob.df,x="Group",y="Robinsoniella", add = "dotplot",
color="Group",palette=c("#00AFBB","#E7A800"),order=c("NNB","LNB"),ylab="Robinsoniella
(clr-transformed )",xlab="Baseline Nestbuilding status")

data.a.pca  <- prcomp(t(species.exp))

pc1 <- round(data.a.pca$sdev[1]^2/sum(data.a.pca$sdev^2),4) *100
pc2 <- round(data.a.pca$sdev[2]^2/sum(data.a.pca$sdev^2),4) *100
pc3 <- round(data.a.pca$sdev[3]^2/sum(data.a.pca$sdev^2),4) *100
pc4 <- round(data.a.pca$sdev[4]^2/sum(data.a.pca$sdev^2),4) *100

pca  = data.frame(PC1 = data.a.pca$x[,1],
```

```
            PC2 = data.a.pca$x[,2],
            PC3 = data.a.pca$x[,3],
            PC4 = data.a.pca$x[,4])

#metadata        <- metadata[metadata$Mapping_file == "Adolescence_002",]

pca$ID               = rownames(metadata_doc1)
pca$Group              = metadata_doc1$Group
pca$Nestbuilder         = metadata_doc1$Nestbuilding
pca$Gender          = metadata_doc1$Gender

ggdoc1 = ggplot(pca, aes(x=PC1, y=PC2, fill = Nestbuilder, label = ID,
colour  = Gender)) +
 geom_point(size=6, stroke = 2, shape = 21) +
 xlab(paste("PC1: ", pc1,  "%", sep="")) +
 ylab(paste("PC2: ", pc2,  "%", sep="")) +
 theme_dark() +
 scale_colour_manual(values = c("F" = "black", "M" = "white"))+
 scale_fill_manual(values=c("LNB" = "#ca0020",
                    "NNB" = "#0571b0"))+
 guides(fill  = guide_legend(override.aes = list(shape = 21)),
      shape = guide_legend(override.aes = list(fill = "black")))

ggdoc1

adonis(as.data.frame(data.a.pca$x)~metadata_doc1$Nestbuilding, method
= "euclidean")

########

species   <- doc3
species   <- apply(species,c(1,2),function(x) as.numeric(as.character(x)))

conds      <- c(rep("B", ncol(species)-10 ), rep("A", 10)) #If you
have less than 12 animals, adjust!
species.clr <- aldex.clr(species, conds, mc.samples = 1000,
denom="all", verbose=TRUE, useMC=TRUE)
species.eff <- aldex.effect(species.clr, conds, verbose = TRUE,
include.sample.summary = TRUE)

species.exp <- (species.eff[,c(4:(ncol(species.eff)-4))]) #remove the
useless t-test-like results


data.a.pca  <- prcomp(t(species.exp))

pc1 <- round(data.a.pca$sdev[1]^2/sum(data.a.pca$sdev^2),4) *100
pc2 <- round(data.a.pca$sdev[2]^2/sum(data.a.pca$sdev^2),4) *100
pc3 <- round(data.a.pca$sdev[3]^2/sum(data.a.pca$sdev^2),4) *100
pc4 <- round(data.a.pca$sdev[4]^2/sum(data.a.pca$sdev^2),4) *100
```

```
pca  = data.frame(PC1 = data.a.pca$x[,1],
                  PC2 = data.a.pca$x[,2],
                  PC3 = data.a.pca$x[,3],
                  PC4 = data.a.pca$x[,4])

pca$ID                = rownames(metadata_doc3)
pca$Group             = metadata_doc3$Group
pca$Nestbuilder       = metadata_doc3$Nestbuilding
pca$Gender            = metadata_doc3$Gender
pca$Timepoint         = metadata_doc3$Treatment_groups
pca$Aminal_ID         = metadata_doc3$Animal_ID

ggdoc3 = ggplot(pca, aes(x=PC1,
                         y=PC2,
                         fill = Nestbuilder,
                         label = ID,
                         shape  = Timepoint,
                         colour = Gender )) +
 geom_point(size=6, stroke = 2) +
 xlab(paste("PC1: ", pc1,  "%", sep="")) +
 ylab(paste("PC2: ", pc2,  "%", sep="")) +
 theme_dark() +
 geom_line(    data = pca[pca$Nestbuilder=="LNB",],
               aes(group = pca[pca$Nestbuilder=="LNB",]$Aminal_ID),
               size = 2,
               show.legend = FALSE,
               color = "#ca0020") +
 geom_line(    data = pca[pca$Nestbuilder=="NNB",],
               aes(group = pca[pca$Nestbuilder=="NNB",]$Aminal_ID),
               size = 2,
               show.legend = FALSE,
               color = "#0571b0") +
 scale_colour_manual(values = c("F" = "black", "M" = "white"))+
 scale_shape_manual(values = c("Pre_treatment" = 21,
                               "Post_treatment"= 22)) +
 scale_fill_manual(values=c("LNB" = "#ca0020",
                            "NNB" = "#0571b0"))+
 guides(fill  = guide_legend(override.aes = list(shape = 21)),
        shape = guide_legend(override.aes = list(fill = "black")))

ggdoc3

adonis(as.data.frame(data.a.pca$x)~metadata_doc3$Treatment_groups+metadata_doc3$Nestbuildi
ng,
method = "euclidean")

ta.pca <- (t(data.a.pca$x ))* ((data.a.pca$sdev^2)/sum(data.a.pca$sdev^2))
a.pca  <- t(ta.pca)

ndim = 1:18
pre_ordered  = rownames(a.pca[c(1, 2, 3, 4, 6, 7, 8, 9),])
```

```
post_ordered = rownames(a.pca[c(1, 2, 3, 4, 5, 6, 7, 8)+10,])
disait = sqrt(rowSums( (a.pca[pre_ordered,ndim] -
a.pca[post_ordered,ndim])^2) ) #save for future comparison with BC

dfdist <- data.frame(Measurement = disait, Group =
metadata_doc3$Nestbuilding[c(1, 2, 3, 4, 6, 7, 8, 9)], ID =
rownames(metadata_doc3)[c(1, 2, 3, 4, 6, 7, 8, 9)])

ggait <- ggplot(data = dfdist, aes(x = Group,
                         y = Measurement,
                         label = ID,
                         fill = Group)) +
  geom_point(size = 6, shape = 21, stroke =2) +
  scale_fill_manual(values=c("LNB" = "#ca0020",
                      "NNB" = "#0571b0"), name = "Legend")+
  ylab("Aitchison Distance") +
  xlab("Treatment") +
  theme_dark()

ggait

wilcox.test(dfdist$Measurement~dfdist$Group, paired = F)

comps_doc1 = t(combn(unique(metadata_doc1$Nestbuilding), 2))
comps_doc1

out_df <- pairwise_DA_wrapper(reads = doc1,
                      groups = metadata_doc1$Nestbuilding,
                      comparisons = comps_doc1, parametric =
T, mc.samples = 128)

out_df[out_df$`NNB vs LNB BH.adjusted.p.value`<0.2,]

####
metadata_doc3$indiv_group = paste(metadata_doc3$Nestbuilding,
metadata_doc3$Treatment_groups)
comps_doc3 = t(combn(unique(metadata_doc3$indiv_group), 2))
comps_doc3 = comps_doc3[-c(3, 4),]


out_df <- pairwise_DA_wrapper(reads = doc3,
                      groups = metadata_doc3$indiv_group,
                      comparisons = comps_doc3, parametric = T)
```

VOLCANO PLOT: *ROBINSONIELLA*

```
options(stringsAsFactors = FALSE)
#source("http://bioconductor.org/biocLite.R")
library(vegan)
library(ggplot2)
library(grid)
library(ggrepel)
```

```
library(fossil)
library(reshape2)
library(metagenomeSeq)
library(coda.base)
library(zCompositions)
source("https://bioconductor.org/biocLite.R")
biocLite("ALDEx2")
library(ALDEx2)
source("https://bioconductor.org/biocLite.R")
biocLite("Tjazi")
library(Tjazi)

setwd("C:/Users/Isma
Scheepers/Documents/My_Data/filtered/NWU_CORK/DeerMouse/DeerMouse")

doc1 = t(read.delim("Doc 1 Baseline NNB vs LNB male and female OTU -
Metadata 1.csv", sep = "\t", row.names = 1))

metadata = (read.delim("Metadata file complete.csv", sep = "\t", row.names = 1))

metadata_doc1 = metadata[colnames(doc1),]

species   <- doc1
species   <- apply(species,c(1,2),function(x) as.numeric(as.character(x)))

conds      <- c(rep("A", ncol(species)-10 ), rep("B", 10)) #If you
have less than 12 animals, adjust!
species.clr <- aldex.clr(species, conds, mc.samples = 1000,
denom="all", verbose=TRUE, useMC=TRUE)
species.eff <- aldex.effect(species.clr, conds, verbose = TRUE,
include.sample.summary = TRUE)

species.exp <- (species.eff[,c(4:(ncol(species.eff)-4))]) #remove the
useless t-test-like results

data.a.pca  <- prcomp(t(species.exp))

pc1 <- round(data.a.pca$sdev[1]^2/sum(data.a.pca$sdev^2),4) *100
pc2 <- round(data.a.pca$sdev[2]^2/sum(data.a.pca$sdev^2),4) *100
pc3 <- round(data.a.pca$sdev[3]^2/sum(data.a.pca$sdev^2),4) *100
pc4 <- round(data.a.pca$sdev[4]^2/sum(data.a.pca$sdev^2),4) *100

pca  = data.frame(PC1 = data.a.pca$x[,1],
              PC2 = data.a.pca$x[,2],
              PC3 = data.a.pca$x[,3],
              PC4 = data.a.pca$x[,4])

#metadata        <- metadata[metadata$Mapping_file == "Adolescence_002",]

pca$ID               = rownames(metadata_doc1)
pca$Group              = metadata_doc1$Group
```

```
pca$Nestbuilder         = metadata_doc1$Nestbuilding
pca$Gender          = metadata_doc1$Gender

ggdoc1 = ggplot(pca, aes(x=PC1, y=PC2, fill = Nestbuilder, label = ID,
colour  = Gender)) +
  geom_point(size=6, stroke = 2, shape = 21) +
  xlab(paste("PC1: ", pc1,  "%", sep="")) +
  ylab(paste("PC2: ", pc2,  "%", sep="")) +
  theme_dark() +
  scale_colour_manual(values = c("F" = "black", "M" = "white"))+
  scale_fill_manual(values=c("LNB" = "#ca0020",
                             "NNB" = "#0571b0"))+
  guides(fill  = guide_legend(override.aes = list(shape = 21)),
         shape = guide_legend(override.aes = list(fill = "black")))

ggdoc1

adonis(as.data.frame(data.a.pca$x)~metadata_doc1$Nestbuilding, method
= "euclidean")

comps_doc1 = t(combn(unique(metadata_doc1$Nestbuilding), 2))
comps_doc1

out_df <- pairwise_DA_wrapper(reads = doc1,
                  groups = metadata_doc1$Nestbuilding,
                  comparisons = comps_doc1, parametric = T)

out_df[out_df$`NNB vs LNB BH.adjusted.p.value`<0.2,]

gg_volcano_wrapper(DA_df = out_df,
                   p.vals = 0.2,
                   e.vals = c(-1, 1),
                   e.name.threshold = 1,
                   p.name.threshold = 0.2,
                   labelsize = 4,
                   xlim = c(-2, 2))
```

Supplementary data

STUDY OBJECTIVE 1

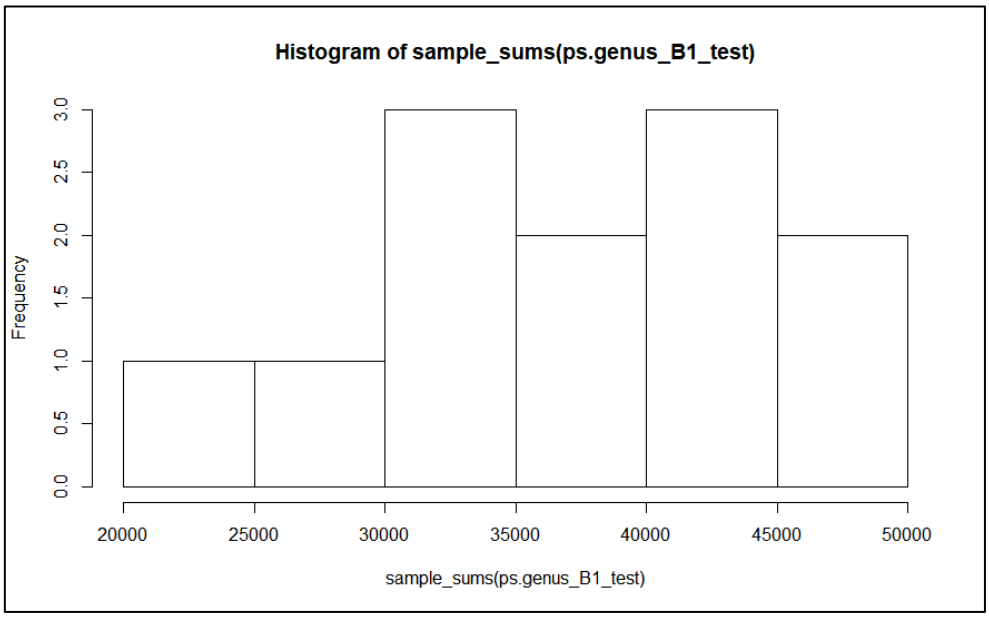

Figure B-1: Histogram of the read distribution of the microbial profiles of NNB and LNB following DADA 2 analysis.

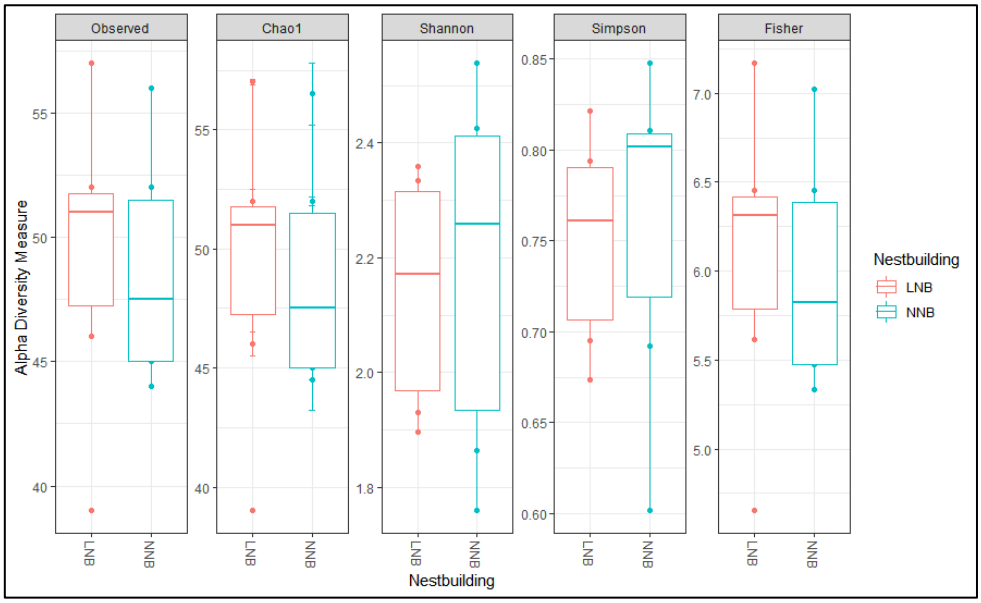

Figure B-2: α-diversity of NNB and LNB animals using Observed, Fisher, Chao1, Shannon and Simpson indices (p > 0.05). Median, 25th and 75th percentile displayed. Whiskers are indicative of the minimum and maximum α-diversity measure.

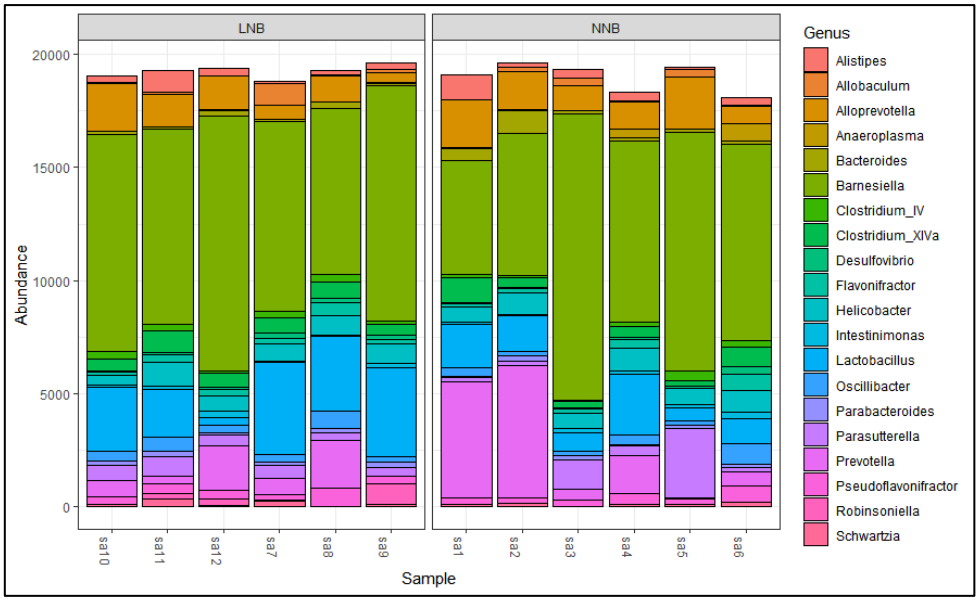

Figure B-3: Top 20 genus present in both NNB and LNB animals; visual representation of total abundance of each genus within each respective sample.

STUDY OBJECTIVE 2

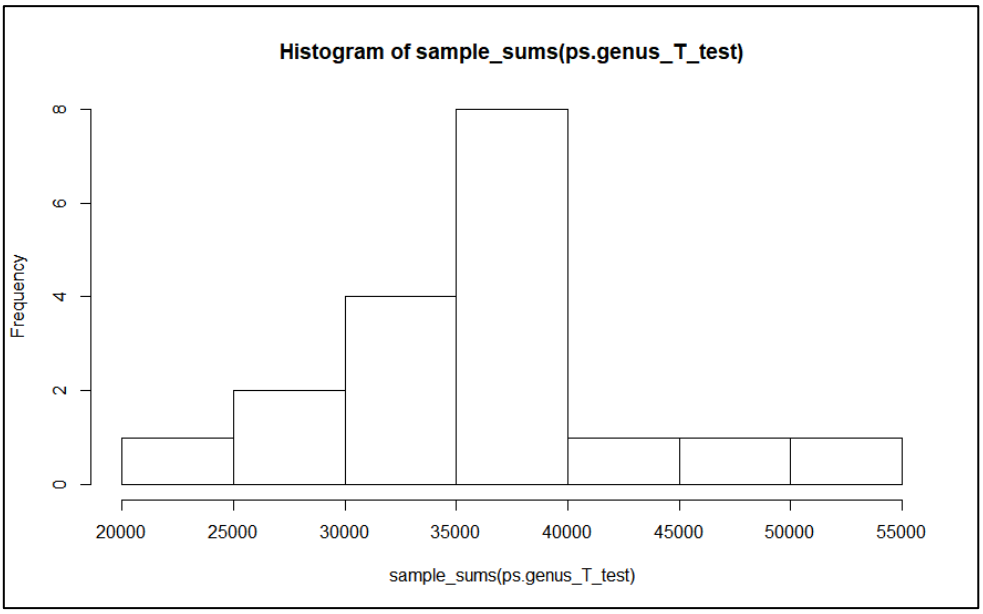

Figure B-4: Histogram of the read distribution of the microbial profiles in pre-treated and treated NNB and LNB animals following DADA 2 analysis

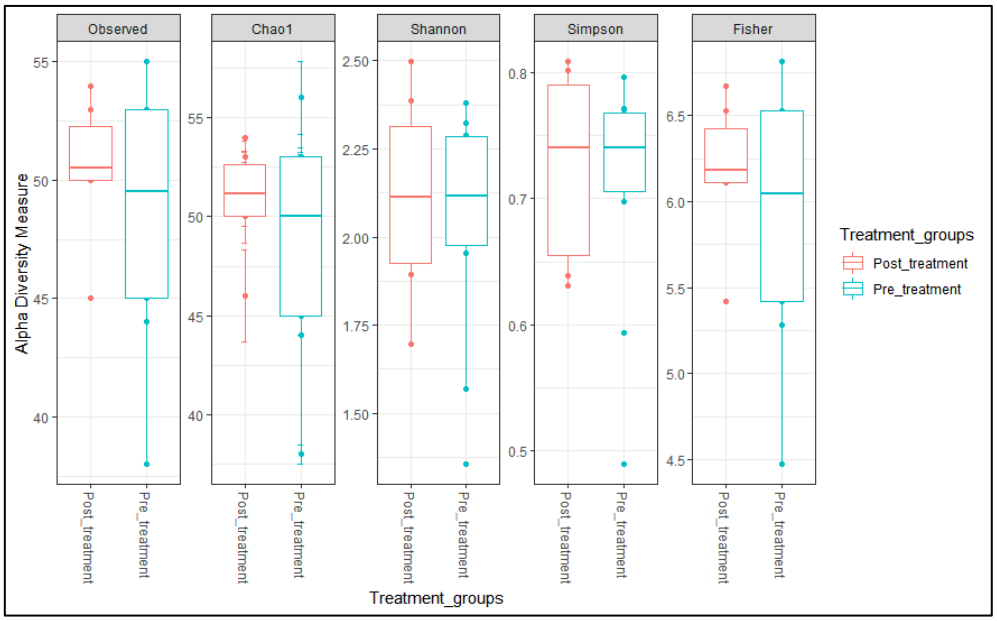

Figure B-5: α-diversities in pre-treated and treated NNB and LNB animals using Observed, Fisher, Chao1, Shannon and Simpson indices (p > 0.05). Median, 25th and 75th percentile is displayed. Whiskers are indicative of the minimum and maximum α-diversity measure.

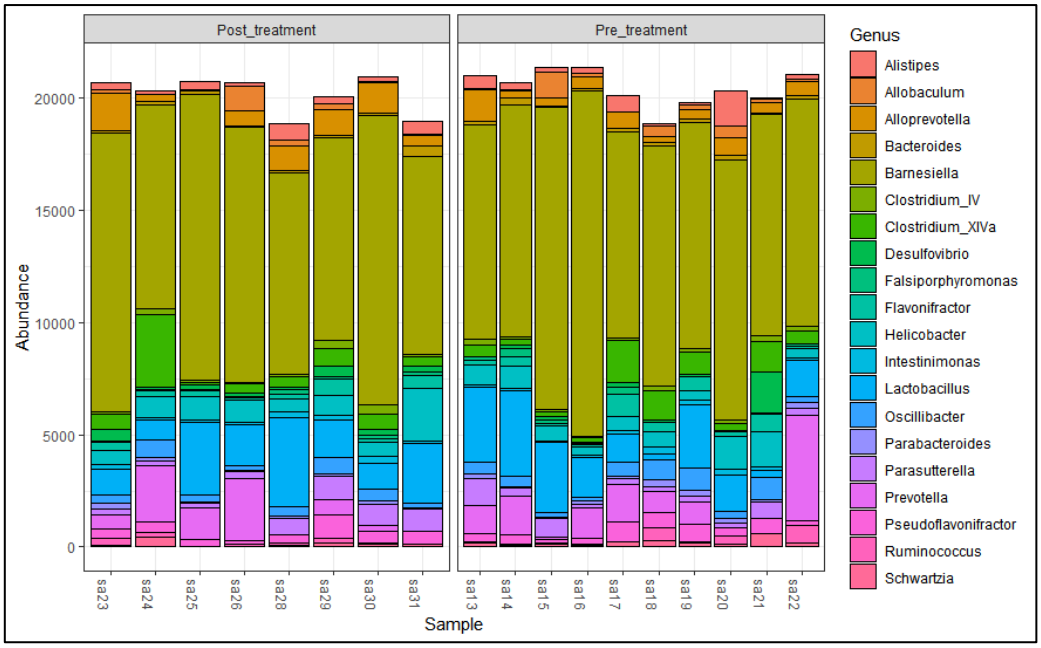

Figure B-6: Top 20 genus present in pre-treated and treated NNB and LNB animals; visual representation of total abundance of each genus within each respective sample.

4.2.1.1 Wilcoxon's signed rank test results

STUDY OBJECTIVE 1

α-diversity results

	Baseline group: 6 NNB vs 6 LNB:					
	score	statistic	pvals	qvals	medianLNB	medianNNB
[1,]	"Observed"	21.5	0.629149731081013	0.699134199134199	51	47.5
[2,]	"Fisher"	21.6	0.629149731081013	0.699134199134199	6.31373071316235	5.82347593538567
[3,]	"Chao1"	21.7	0.629149731081013	0.699134199134199	51	47.5
[4,]	"Shannon"	15	0.699134199134199	0.699134199134199	2.19	2.25898992371963
[5,]	"Simpson"	14	0.588744588744589	0.699134199134199	0.760884760837091	0.801631829784199

β-diversity results

STUDY OBJECTIVE 2

α-diversity results

	Treatment group: Pre-treatment vs. Post-treatment					
	score	statistic	pvals	qvals	median_Pre_treatment	median_Post_treatment
[1,]	"Observed"	45	0.686349461649764	0.828557063851181	49.5	50.5
[2,]	"Fisher"	45	0.686349461649764	0.828557063851181	6.04367885160861	6.18159846069906
[3,]	"Chao1"	46,5	0.591650357484209	0.828557063851181	50	51.1666666666667
[4,]	"Shannon"	43	0.828557063851181	0.828557063851181	2.11698845045694	2.11251214309934
[5,]	"Simpson"	46	0.633438456967869	0.828557063851181	0.740622754295091	0.740605661658907

Addendum C

Supplementary files pertaining to DNA extraction

Spectrophotometric measurements

Sample Nr	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280	Nucleic Acid Factor	Baseline Correction (nm)	Baseline Absorbance
1	102,095	1,804	1,784	2,042	1,132	50	340	0,08
2	169,673	1,822	1,93	3,393	1,863	50	340	0,057
3	138,374	1,816	1,958	2,767	1,524	50	340	0,053
4	131,497	1,806	1,832	2,63	1,456	50	340	0,06
5	179,969	1,823	1,967	3,599	1,975	50	340	0,112
6	191,069	1,832	2,095	3,821	2,086	50	340	0,058
7	105,11	1,803	1,797	2,102	1,166	50	340	0,055
8	67,313	1,778	1,657	1,346	0,757	50	340	0,073
9	132,869	1,819	1,922	2,657	1,461	50	340	0,064
10	154,215	1,813	1,816	3,084	1,701	50	340	0,123
11	147,869	1,82	1,92	2,957	1,625	50	340	0,122
12	161,211	1,827	1,989	3,224	1,764	50	340	0,063
13	113,328	1,831	1,895	2,267	1,238	50	340	0,093
14	104,496	1,807	1,978	2,09	1,156	50	340	0
15	108,559	1,792	1,804	2,171	1,211	50	340	0,053
16	184,758	1,799	1,804	3,695	2,055	50	340	0,16
17	129,508	1,814	1,951	2,59	1,428	50	340	0,042
18	144,381	1,804	1,905	2,888	1,601	50	340	0,049
19	179,098	1,778	1,737	3,582	2,014	50	340	0,189
20	189,18	1,833	2,072	3,784	2,064	50	340	-0,047
21	124,198	1,774	1,651	2,484	1,4	50	340	0,151
22	142,716	1,811	1,707	2,854	1,576	50	340	0,165
23	163,723	1,815	1,878	3,274	1,804	50	340	0,129
24	190,657	1,825	1,956	3,813	2,089	50	340	0,145
25	183,423	1,835	2,041	3,668	1,999	50	340	0,078
26	168,487	1,833	1,973	3,37	1,838	50	340	0,156
27	93,474	1,817	1,709	1,869	1,029	50	340	0,069
28	167,269	1,829	2,066	3,345	1,829	50	340	0,06
29	166,197	1,83	1,916	3,324	1,816	50	340	0,144
30	127,387	1,834	1,934	2,548	1,389	50	340	0,105
31	130,347	1,84	1,963	2,607	1,417	50	340	0,081
32	93,021	1,836	1,817	1,86	1,013	50	340	0,124
33(Blank)	0,366	-3,251	0,116	0,007	-0,002	50	340	0,022

Addendum D

Supplementary files pertaining to the microbiome clarification

Supplementary figures

Quality plot

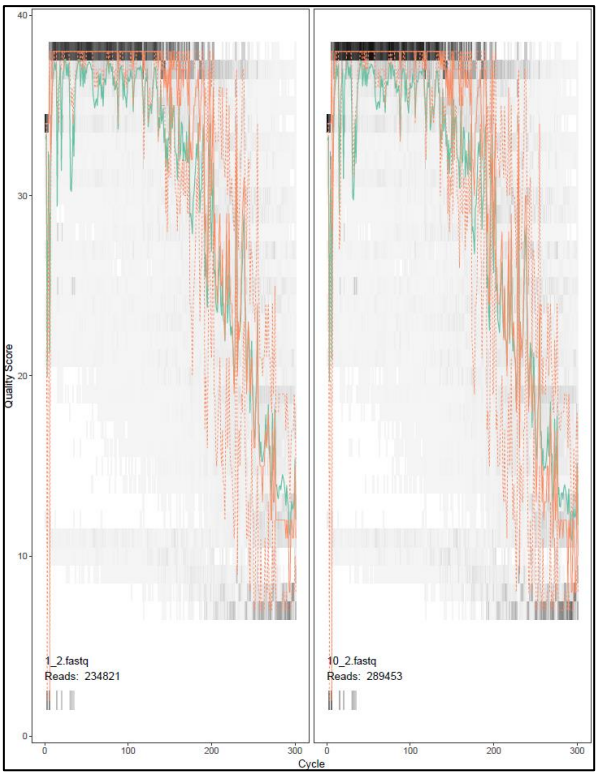

Figure D-1: Quality plot used for initial determination of filtering and trimming values

Supplementary R data

Total reads following each step in the DADA 2 pipeline

Sample nr.	Input	Filtered	DenoisedF	DenoisedR	Merged	Nonchim
1	234764	143438	143438	143438	96102	59848
2	260211	156833	156833	156833	95419	59506
3	314790	190564	190564	190564	117165	74292
4	299634	176695	176695	176695	117898	71956
5	279516	170098	170098	170098	109293	72913
6	284235	174166	174166	174166	108580	70451
7	294600	182206	182206	182206	121331	66500
8	225070	138371	138371	138371	88181	61644
9	280030	170543	170543	170543	103103	61819
10	289348	174292	174292	174292	114048	77919
11	334897	205515	205515	205515	126590	74832
12	300232	184305	184305	184305	118280	75139
13	241581	149672	149672	149672	87791	53460
14	312559	188816	188816	188816	121132	77908

15	285714	175912	175912	175912	111605	67334
16	298242	180697	180697	180697	115329	82532
17	165116	99722	99722	99722	64973	45152
18	300329	181634	181634	181634	115121	76849
19	323922	197005	197005	197005	123843	84419
20	303472	185775	185775	185775	109049	75829
21	269216	166760	166760	166760	107938	67076
22	264231	164089	164089	164089	103321	61454
23	306654	181849	181849	181849	108300	70967
24	253467	157869	157869	157869	100207	69215
25	257364	155608	155608	155608	97205	65670
26	238917	144397	144397	144397	87099	60444
27	321112	192419	192419	192419	115370	73467
28	313199	182386	182386	182386	116607	72906
29	288890	176812	176812	176812	111369	76475
30	297150	179897	179897	179897	114601	79301
31	353614	219757	219757	219757	141255	81568
32	268050	165388	165388	165388	99995	68295
33-NEG	268037	153931	153931	153931	130700	130044

Addendum E

Additional work by the candidate relating to the deer mouse model of OCD

Metabolic Brain Disease (2018) 33:443–455
https://doi.org/10.1007/s11011-017-0161-7

ORIGINAL ARTICLE

Peromyscus maniculatus bairdii as a naturalistic mammalian model of obsessive-compulsive disorder: current status and future challenges

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Abstract
Obsessive-compulsive disorder (OCD) is a prevalent and debilitating condition, characterized by intrusive thoughts and repetitive behavior. Animal models of OCD arguably have the potential to contribute to our understanding of the condition. Deer mice *(Peromyscus maniculatus bairdii)* are characterized by stereotypic behavior which is reminiscent of OCD symptomology, and which may serve as a naturalistic animal model of this disorder. Moreover, a range of deer mouse repetitive behaviors may be representative of different compulsive-like phenotypes. This paper will review work on deer mouse behavior, and evaluate the extent to which this serves as a valid and useful model of OCD. We argue that findings over the past decade indicate that the deer mouse model has face, construct and predictive validity.

Keywords Deer mouse · Obsessive-compulsive · Stereotypy · Nest building · Marble burying · Social · Animal model

Introduction

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric disorder, with lifetime prevalence estimates of 2.6% (Ruscio et al. 2010). Obsessions are intrusive thoughts or ideas that are often associated with compulsive, or repetitive and rigid, behavior (American Psychiatric Association 2013). On factor analyses of OCD symptoms, several symptom dimensions emerge, with varying content of obsessions and/or compulsions (O/C) (Table 1). The symptoms of OCD are time-consuming, distressing, and result in significant interference in occupational and social function (American Psychiatric Association 2013). Since the publication of the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), OCD is included with body dysmorphic disorder, hoarding disorder, trichotillomania, and excoriation disorder in a single diagnostic chapter on 'Obsessive-Compulsive and Related Disorders' (OCRDs; American Psychiatric Association 2013). OCD is not only often co-morbid with conditions classified in the OCRD cluster, but also with mood and anxiety disorders (Torres et al. 2016).

Animal models may be useful for investigating the underlying etiopathology of psychiatric illness. Not only can they contribute to understanding underlying neurobiology, but they may also allow investigation of novel treatments (Fineberg et al. 2011). In the past, a number of animal models of OCD have been proposed. Given that obsessions are difficult to demonstrate in animals, models of OCD have focused on repetitive and rigid behavior that is reminiscent of the symptoms of OCRDs. Such behavior includes excessive lever-pressing (Joel 2006) and nest building behavior (Hoffman and Rueda Morales 2009; Li et al. 2006; Wolmarans et al. 2016a; Greene-Schloesser et al. 2011), rigid locomotive patterns (Szechtman et al. 1998; Wolmarans et al. 2013; Yadin et al. 1991), aberrant grooming and hair pulling behavior (Greer and Capecchi 2002; Kinnear et al. 2000; Welch et al. 2007), compulsive-like chewing (Chou-Green et al. 2003a, b), compulsive-like marble burying (Greene-Schloesser et al. 2011) and hoarding (Andersen et al. 2010). Together, these models conceivably represent different OC-like phenotypes that may contribute to our understanding of the mechanisms underlying different

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Table 1 Common OC symptom dimensions. *Adapted from* (Abramovitch and Cooperman 2015; Markarian et al. 2010)

Obsessions	Compulsions
Concerns about contamination	Washing
Concerns about harming oneself or others	Checking
Concerns about symmetry and order	Ordering, arranging and counting
Obsessions about sexual, religious or aggressive nature	Mental rituals aimed to avoid or neutralize obsessive thoughts e.g. praying
Concerns about saving	Collecting and hoarding (hoarding can also be diagnosed as a separate condition; American Psychiatric Association 2013)

symptoms. Animal models of OCD can be based on naturalistic or conditioned behavior, pharmacological challenges, or genetic manipulation (Alonso et al. 2015). Whereas naturalistic and conditioned models may provide more insight into the behavioral triggers and course of OC-like behavior, pharmacological and genetic models may provide targeted frameworks for studying specific neurobiological and genetic mechanisms underlying O/Cs (Alonso et al. 2015).

The deer mouse *(Peromyscus maniculatus bairdii)* model of OCD (Korff et al. 2008) can be regarded as a naturalistic model characterized by spontaneous OC-like behavior (Hoffman 2011; Wolmarans et al. 2013). Over the past decade, our group has published a number of investigations of this model. The current paper will review progress to date.

The deer mouse model of OCD

Peromyscus maniculatus (deer mouse) and congeneric species are the most common mammals native to the North-American continent (Shorter et al. 2012). As opposed to rats, dogs, cows, sheep, and laboratory mice *(Mus)* where selective breeding has led to genomic alterations (Vrana 2007), deer mice represent a true wild-type mammalian model system, although bred in captivity at the *Peromyscus* genetic stock center of the University of South Carolina (Joyner et al. 1998). Wild-type strains differ from inbred strains in being genetically more diverse (Yang et al. 2011), and deer mice have proven to be useful in the study of genetic variability and epi-genetic influences underlying different behavioral phenotypes. One subspecies, *viz.* the tall grass prairie *P. maniculatus bairdii* strain (hereafter only referred to as deer mice), of which stock animals have been derived from 40 wild-type ancestors caught in Washtenaw County, Michigan, has been used in studies of spontaneous repetitive behavior (Hadley et al. 2006; Presti et al. 2004; Shorter et al. 2014).

Notably, the repetitive and stereotypic behavior observed in these animals, i.e. jumping, backward somersaulting and pattern running, is expressed in varying frequencies across the population in laboratory settings, suggesting that these behaviors are influenced by a combination of genetic and environmental factors (Shorter et al. 2014). While repetitive motor patterns are not necessarily indicative of pathology (Eilam et al. 2006; Langen et al. 2011), the seemingly purposeless and time-consuming stereotypies of varying forms and intensity in deer mice, have been proposed to resemble the repetitive and rigid symptomology of OCD (Korff et al. 2008) and autism (Lewis et al. 2007). That said, given that stereotypic behavior is seen in deer mice in naturalistic settings also (Shorter et al. 2012, 2014), it is likely that this may be an adaptive response.

Spontaneous stereotypy in deer mice as a phenotype of persistent, but compulsive behavior

Jumping, backward somersaulting, and pattern running in deer mice were originally studied based on their resemblance to stereotypical movement disorders, in particular the motor manifestations of autism (Powell et al. 1999). Compared to animals housed in standard cages, individuals maintained in enriched cages express lower levels of stereotypy (most notably pattern running), characterized by a delayed onset and a lower incidence (Bechard et al. 2016; Hadley et al. 2006; Powell et al. 1999). However, it has been suggested that up to 62% of deer mice housed in standard laboratory cages developed stereotypy.

In early work, behavioral categorization was performed by means of visual observation (Powell et al. 1999), with each animal observed twice weekly for 5 min. Subsequently, the same group introduced automated screening (Presti et al. 2004), and animals were classified into low- (L) and high- (H) stereotypical groups based on the mean stereotypy score, i.e. the number of distinct stereotypical movements, generated over an eighteen-hour-long session (Presti et al. 2004). Further, to obtain accurate results from neurochemical investigations, only animals expressing the lowest and highest levels of stereotypy were included in follow-up studies, excluding a grey margin of animals and that yielded a more distinct separation of the two behavioural phenotypes. This

approach has since been adopted by others (Wolmarans et al. 2013, 2016a, b, 2017a).

Korff et al. (2008) classified deer mice into non- (N), low- (L), and high- (H) stereotypic groups on the basis of a *mean* stereotypy score obtained during three individual *one-hour long* behavioral screening sessions, each one week apart. Deer mouse stereotypy was variable within the population with 45% of animals classified as H, 41% as L, and 14% as N, irrespective of sex. As deer mice are nocturnal animals, follow-up investigations measured the time spent executing stereotypy over 12-h during the dark cycle (Wolmarans et al. 2013); this allowed us to demonstrate that the stereotypy frequency and intensity vary across subjects and between different assessments, and that H deer mice express time-consuming stereotypy during specific bouts of the dark cycle only (Fig. 1, two of the normal 5 baseline stereotypy trials shown). This is arguably reminiscent of the waxing and waning nature of OC symptomology (American Psychiatric Association 2013; Wolmarans et al. 2013).

A central question to modeling OCD in animals is whether it is possible to characterize stereotypy not simply as a motoric phenomenon, but rather as representing an underlying cognitive-affective alteration (Tanimura et al. 2009, 2008; Wolmarans et al. 2017a). In this regard, previous findings must be considered. Repeated administration of psychostimulants induces stereotypy and facilitates the transition of goal-directed to habitual responses (Burguière et al. 2015; Graybiel 2008). However, since deer mouse stereotypy is not subject to amphetamine-induced behavioral sensitization (Tanimura et al. 2009), it is likely that the stereotypical phenotype described in deer mice differs from a purely habitual phenomenon.

In addition, we have noted differences in sociability between H and N deer mice, both within- and between cohorts (Wolmarans et al. 2017a), with changes in sociability after administration of escitalopram. This again suggests that stereotypic behavior in deer mice is not merely a motoric phenomenon, but reflects more broad-spread mechanisms. By investigating group interactions between three animals in different social paradigms, *viz.* HHH, NNN, HHN and NNH, we demonstrated that H animals group together in the presence of an N conspecific (HHN paradigm), while being marginalized by animals of the N cohort (NNH paradigm). Our data relating to the sociability of deer mice has key implications. Notably, *P. maniculatus bairdii* engage in fewer social interactions compared to *P. polionotus subgriseus*, another species within the genus (Shorter et al. 2014). However, stereotypical behavior in *P. polionotus subgriseus* is negligible. Taken together, results from the Shorter et al. (2014) and Wolmarans et al. (2017a) investigations may indicate that the sociability of *P. maniculatus bairdii* is modified by the level of stereotypy displayed by conspecifics, although it is premature to draw strong conclusions about a causal relationship between stereotypy and altered social competence. This is consistent with clinical evidence regarding the social behavior of OCD patients and their social experiences in the presence of healthy peers (Berrocal et al. 2006; Kim et al. 2012; Rosa et al. 2012; Storch et al. 2006).

Taken together, deer mouse stereotypy resembles OC behavior in that 1) it is repetitive, persistent and time consuming, 2) it manifests as a narrow range of phenotypes, i.e. jumping, pattern running and backward somersaulting that can possibly be differentiated at a neurobiological level, 3) it demonstrates within- and between-subject variance in frequency and intensity, 4) it is expressed in a waxing and waning pattern, 5) it is resistant to behavioral sensitization and so may represent a form of abnormally regulated goal-directed behavior, rather than a habit, 6) it is characterized by social deficits, and 7) it is influenced by both environmental and genetic factors.

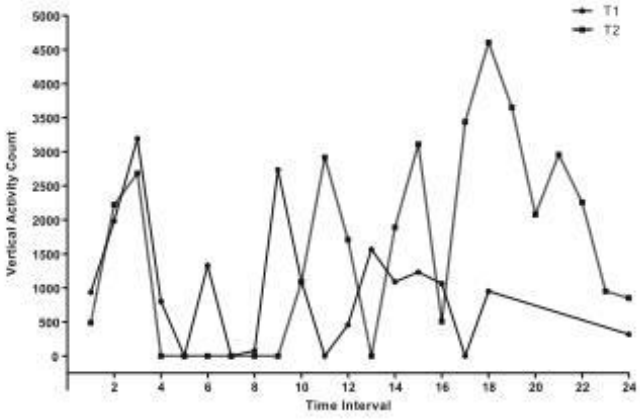

Fig. 1 Varying intra- and inter-trial expression of stereotypy. *Data represents vertical activity counts of one animal generated across 2 respective 12-h dark cycles, each divided into 24 30-min time-intervals.* ***T1 – T2****: Trial 1–2, spaced one week apart*

Parallels between the treatment response of deer mouse stereotypy and OCD

Response to chronic, but not sub-chronic selective serotonin reuptake inhibitors (SSRIs)

During the initial stages of investigating deer mouse stereotypy as a putative animal model of OCD, Korff et al. (2008) demonstrated that chronic (21-day) intraperitoneal treatment with a high dose SSRI, i.e. fluoxetine 20 mg/kg/day, but not the noradrenaline reuptake inhibitor (NRI), desipramine, attenuated stereotypy intensity in H and L animals without affecting normal locomotion. As chronic and high dose SSRI treatment is the first-line pharmacological treatment for OCD, while noradrenergic compounds are ineffective (Fineberg and Craig 2007), these findings accredit deer mouse stereotypy with valuable predictive validity as an animal model of OCD. Subsequent work extended these initial findings. To account for individual fluctuations in stereotypy over the course of a single dark cycle and considering that the distinct forms of stereotypy do not demonstrate any significant association with one another, a focus was established on time *and* stereotypy intensity (Fig. 2) (Wolmarans et al. 2013). This classification system also allows for genetic predisposition and epigenetic influence in the stock colony as it appraises deer mouse behavior on a continuum from normal to severe manifestations of stereotypy. As opposed to the use of cut-off criteria, the balance may shift in any direction without changing the fundamental goal of the investigation, *viz.* comparing normal and stereotypic animals.

To exclude the effect of injection stress on the manifestation of stereotypy, oral dosing with high dose escitalopram (50 mg/kg/day), a highly potent selective serotonin reuptake inhibitor (Owens et al. 2001), was introduced, together with a comparison between chronic (4 weeks) and sub-chronic (1 week) treatment. Subsequently, *while adjusting H bouts to periods of normal rodent activity*, we demonstrated that chronic *but not* sub-chronic escitalopram treatment reduced the time spent executing stereotypy (Wolmarans et al. 2013). Notably however, such animals still engaged in bouts of spontaneous H behavior (Wolmarans et al. 2013), arguably not unlike that observed in OCD (Overduin and Furnham 2012). These observations are important as we have previously suggested that by increasing the number of bouts of normal rodent activity, escitalopram engenders control over the urge to engage in H behavior (Wolmarans et al. 2013). Instead of persistently expressing less severe compulsive-like behavior, H animals can engage in normal rodent activities for a greater part of their wake cycle, although not being able to abstain entirely from OC bouts.

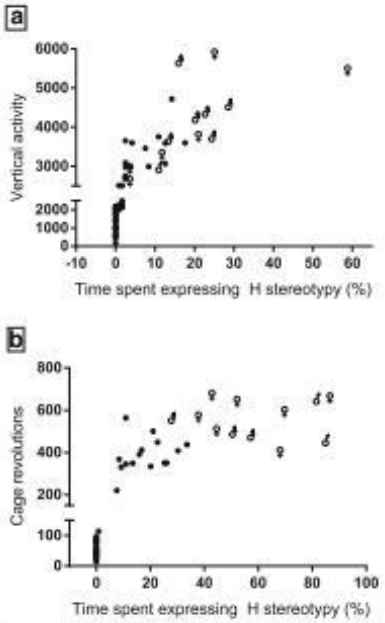

Fig. 2 Stereotypy intensity versus time spent engaging in high stereotypical activity over 12 h.*(a) Mean of highest individual daily vertical activity scores across the first five baseline behavioral trials versus time spent engaging in H activity. (b) Mean of highest individual daily cage revolution scores over the first five baseline behavioral trials versus time spent engaging in H activity. Sex symbols indicate selected male and female H animals*

Overlaps between deer mouse behavior and treatment resistance in OCD

Treatment resistance remains a clinical obstacle in approximately 30% - 50% of OCD patients who remain unresponsive to SSRI monotherapy (Fineberg et al. 2006). The treatment of refractory OCD may include an increase in the dose of the SSRI and a longer duration of treatment (Bejerot and Bodlund 1998), or switching treatment to another SSRI (Fineberg et al. 2006). A third strategy is to augment SSRI therapy with a low dose D_2 blocker (Erzegovesi et al. 2005; Hollander et al. 2003; Ipser et al. 2006; McDougle et al. 2000). Recently, we investigated marble burying (MB) behavior in deer mice as a measure of anxiety- and/or compulsive-like behavior (Dixit et al. 2014; Kedia and Chattarji 2014). We identified persistent burying behavior in 11% of deer mice; such behavior was found independent of stereotypy levels or sex (Wolmarans et al. 2016b). Moreover, in contrast with previous work in different species (Ichimaru et al. 1995; Li et al. 2006), chronic high dose oral escitalopram (50 mg/kg/day) failed to attenuate this behavior. We therefore speculate that

MB in deer mice may be useful in modeling treatment resistant OCD; however, this remains to be proven.

Together with findings that backward somersaulting, but not jumping and pattern running, may reflect a behavior that is resistant to change (Tanimura et al. 2008), it is possible that some phenotypes of deer mouse behavior may be representative of different underlying psychobiological processes that may respond differentially to OCD treatment strategies. Although backward somersaulting does respond to escitalopram treatment, it may be useful to establish whether it demonstrates differential response to augmentation treatment strategies, compared to vertical jumping and pattern running. There is clinical evidence to suggest that different psychobiological mechanisms are variably affected in different patients with OCD, perhaps influencing treatment outcomes (Mataix-Cols et al. 1999; Rufer et al. 2006). To date, little work on animal models of OCD has focused on the issue of treatment-resistance, and this may be a useful focus for future investigation.

Biobehavioral overlaps between deer mouse stereotypy and OCD

Cortico-striatal-thalamic-cortical (CSTC) circuitry in OCD

OCD may reflect underlying disruption in cortico-striatal-thalamic-cortical (CSTC) signaling (Ahmari 2016; Tanimura et al. 2008; Wolmarans et al. 2013). Abnormal regulation of goal-directed behavior may be central to OCD symptomology (Gillan et al. 2011). Brain areas implicated in OCD mediate goal-directed behavior. These include the prefrontal cortex, striatum and thalamic nuclei which communicate with each other via different pathways (Evans et al. 2004; Nambu 2008). The CSTC circuit (Fig. 3) is organized in such a manner that the anterior cingulate cortex (ACC), via innervation of the ventral striatum, exerts feedback through the thalamus to the orbitofrontal cortex (OFC) (Haber and Knutson 2010). Consisting of a direct *(behaviorally activating)* and an indirect *(behaviorally inactivating)* pathway, the CSTC circuit is important for planning, executing and terminating complex motor responses and to facilitate reward learning (Morein-Zamir et al. 2014; Stocco et al. 2010). A relative bias in favor of the direct over the indirect pathway may underlie OC symptomology (Saxena and Rauch 2000; van den Heuvel et al. 2005). This not only results in an overactive OFC, resulting in dysfunctional reward processing (Haber and Knutson 2010), but also increases the activity in the CSTC circuit as a whole (Bartz and Hollander 2006; Saxena and Rauch 2000).

The striatum mainly consists of GABAergic projections that divide into two subgroups, i.e. striato-nigral (SN) neurons of the direct pathway (projecting to and inhibiting the GPi/SNr), and striato-pallidal (SP) neurons constituting the indirect pathway (projecting to and inhibiting the GPe) (Rymar et al. 2004; Yelnik et al. 1991). Further, both pathways are tonically inhibited under resting conditions (Wilson and Groves 1981). However, upon initiating a behavioral action,

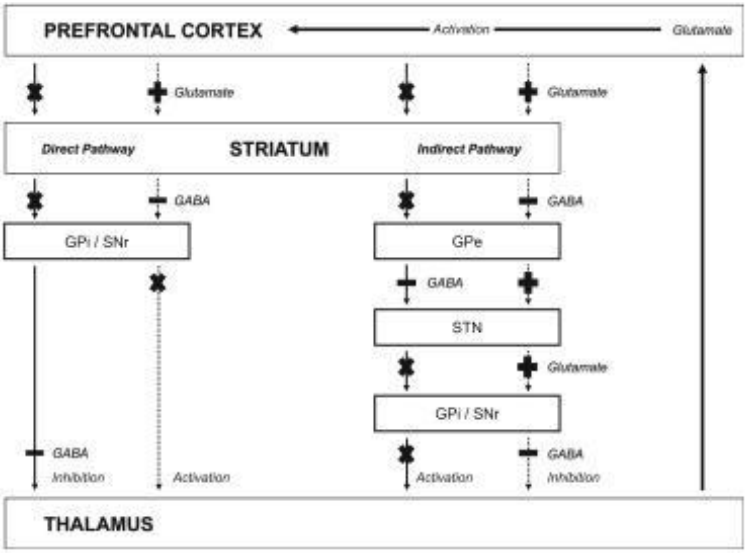

Fig. 3 The cortico-striatal-thalamic-cortical (CSTC) circuit. ***Solid lines***, *no cortical activation of pathways;* ***dotted lines***, *cortically activated pathways;* ***crosses***, *no considerable neurotransmitter release;* ***minus signs***, *GABAergic inhibition;* ***plus signs***, *disinhibition of target / glutamatergic activation;* ***GPi / SNr***, *globus pallidus interna/ substantia nigra pars reticulata;* ***GPe***, *globus pallidus externa;* ***STN***, *subthalamic nucleus;* ***GABA***, *gamma-amino butyric acid*

signaling in both the *activating* (direct) and *inactivating* (indirect) pathways are triggered. This functional antagonism is resolved by the substantia nigra pars compacta (SNc) that modulates both pathways via dopaminergic signaling. Whereas the SN neurons of the direct pathway express G_s associated dopamine-1 (D_1) receptors, the SP neurons of the indirect pathway express G_i associated D_2 receptors (Tanimura et al. 2010). As such, stimulation of D_1 elevates cAMP and increases GABA release, resulting in the activation of the direct pathway (Fig. 3). Conversely, activation of the SP D_2 receptors decreases cAMP concentrations and inhibits the release of GABA, thereby inhibiting the indirect pathway (Gerfen et al. 1990; Tepper and Bolam 2004). Striatal dopamine release will therefore initiate motor behavior by shifting the executive balance to the direct pathway (Beiser et al. 1997; Chesselet and Delfs 1996).

Hyperactivity of the CSTC circuitry in OCD is hypothesized to be related to deficits in reward processing (Ferreira et al. 2017; Figee et al. 2011; Palminteri et al. 2012; Pinto et al. 2014), a process that is closely correlated with cortico-striatal dopaminergic signaling (Ljungberg et al. 1991; Mirenowicz and Schultz 1994; Schultz et al. 1993). Briefly, *initial* anticipation of a possible reward activates nearly 75% of the dopaminergic neurons in the basal ganglia (Ljungberg et al. 1991). However, repetitive exposure to the same stimulus facilitates a process of reward conditioning that enables the brain to evaluate future confrontations with the same set of factors it was conditioned to (Romo and Schultz 1990). It has been shown that the basal ganglia code differences between predicted and actual rewards with 'reward prediction errors' (Schultz et al. 1997; Schultz 2002). These are important for reward- and punishment based learning, as it is applied to implement sensorimotor changes to either keep experiencing the same reward in the case of a positive error, or achieving a better outcome in the case of a negative error. Therefore, the relative lack of a significant dopaminergic response after the manifestation of a fully predicted reward may account in part for inadequate closure after task completion in patients with OCD (Figee et al. 2011). It can thus be hypothesized that a dysfunctional reward system and altered dopaminergic signaling plays a role in OCD (Denys et al. 2004; Gillan et al. 2011; Husted et al. 2006).

Although dopamine plays a prominent role to facilitate and maintain motor behavior, it is drugs that target serotonergic and not dopaminergic signaling that have proved most useful in the first-line treatment of OCD (Fineberg and Craig 2007). The fact that behavioral inhibition has been associated with serotonergic neurotransmission (Cools et al. 2008; Daw et al. 2002) may be relevant. In a review of the opponent interactions between serotonin and dopamine (Daw et al. 2002), the term 'opponency' describes a paradigm in which more than one system codes for different affective events. While it is known that the dopaminergic system codes rewarding stimuli, serotonin is activated during the experience of aversive stimuli (Fletcher 1995; Fletcher and Korth 1999; Fletcher et al. 1999; Kapur and Remington 1996). Indeed, by enhancing serotonergic signaling, both conditioned behaviors (such as lever pressing for food) and unconditioned behaviors (such as feeding) normally associated with dopaminergic signaling, are inhibited. Consequently, the opposite effect is achieved when antagonizing serotonin or stimulating dopamine. This corresponds with data demonstrating that serotonin antagonizes the effects of dopamine in the SN neurons of the direct pathway (Daw et al. 2002; Kapur and Remington 1996). Therefore, with respect to OCD, it is possible that the balance between reward seeking behavior and aversive reactions is related to the balance between dopaminergic and serotonergic signaling (Ferreira et al. 2017).

Deer mouse stereotypy and aberrant CSTC signaling

A strong body of evidence confirms cortico-striatal involvement in deer mouse stereotypy. First, a bias in favor of the direct SN pathway has been demonstrated by findings that the phenotypic expression of deer mouse stereotypy, but not normal patterns of motor behavior, can be inhibited via selective blockade of striatal D_1 and N-methyl-D aspartate (NMDA) receptors (Presti et al. 2003). Moreover, this is supported by a significantly higher SN-dynorphin / SP-enkephalin ratio (Presti and Lewis 2005) as well as reduced activity in the subthalamic nuclei (STN) of H, compared to N animals (Tanimura et al. 2010). There is also evidence that such striatal dysfunction underlies an association between deer mouse stereotypy and deficits in cognitive ability (Bechard et al. 2016; Tanimura et al. 2008). As alluded to earlier, rearing deer mice in EE cages improves procedural learning ability in jumpers and pattern runners, but not in backward somersaulters, implicating a possible role for different psychobiological mechanisms underlying unique forms of stereotypy. Importantly, these cognitive changes were linked to *striatal* rather than *hippocampal* mechanisms, the former normally associated with age-related deficits in learning ability (Frick and Fernandez 2003; Frick et al. 2003). In addition, in line with findings demonstrating increased EE-induced neuronal firing in the indirect SP pathway (Bechard et al. 2016), a positive correlation was found between the rate of stereotypy and cognitive rigidity in animals that benefited from EE. Thus, improvements in procedural learning ability occurred in parallel with striatally-mediated adaptations in expression of stereotypy. Although CSTC-associated deficits in learning ability have been demonstrated in OCD patients (Eng et al. 2015; Olley et al. 2007), these are not specific to OCD (Colomer et al. 2017; Lewis et al. 2007; Palminteri et al. 2009). While behavioral rigidity in jumpers and pattern runners responds to intervention, backward somersaulting may represent a behavior that is resistant to change (Tanimura et al. 2008).

Dopamine in deer mouse stereotypy

Although dopamine is known to be a pivotal role player in the CSTC circuitry, its role in the pathogenesis of deer mouse stereotypy remains to be clarified. Most parameters of dopaminergic signaling remain unaltered when comparing H vs. N mice, including striatal D_1 and D_2 receptor density (Powell et al. 1999) and regional brain levels of dopamine (Güldenpfennig et al. 2011; Powell et al. 1999; Presti et al. 2004), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) (Güldenpfennig et al. 2011; Powell et al. 1999). Also, while systemic and intrastriatal administration of the D_1/D_2 receptor agonist, apomorphine, elicits typical rodent stereotypies, e.g. hyperlocomotion, gnawing and excessive grooming, it fails to exacerbate the characteristic deer mouse stereotypies, i.e. jumping, pattern running and backward somersaulting (Presti et al. 2002).

Further, deer mouse stereotypy seems to be unrelated to selective interference by D_1 or D_2 receptor modulators, which neither trigger nor exacerbate its expression (Korff et al. 2008; Presti et al. 2004). Korff et al. (2008) demonstrated attenuation of stereotypical behavior following administration of the selective D_2 agonist, quinpirole (5 mg/kg/day × 4 days), while Presti et al. (2004) demonstrated no significant behavioral alteration. While Korff et al. (2008) used a 4-day intraperitoneal dosing and administration schedule, Presti et al. (2004) administered quinpirole intra-striatally at a dose of 5 μg/side over 60 s, which may account for the disparate results. However, findings from both these investigations seem inconsistent with the quinpirole compulsive-like checking model of OCD (Szechtman et al. 2001, 1998). Still, given important phenotypical differences between pharmacologically induced and spontaneous stereotypy in deer mice (Presti et al. 2002) and considering that neither the Korff et al. (2008), nor the Presti et al. (2004) investigations administered quinpirole for a duration comparable to that of the Szcechtman group (1998, 2001), both these models may be useful in understand the range of mechanisms that may underlie OC-like behavior (Szechtman et al. 2017). The dopaminergic system may play a role in processing context, salience, or reward which differ across these models. Indeed, recent work related to different OC phenotypes and its association with context related deficits in reward and punishment processing (Ferreira et al. 2017; Figee et al. 2011; Palminteri et al. 2012; Pinto et al. 2014), have to be considered.

Altered serotonergic signaling and deer mouse behavior

Deer mouse stereotypy is associated with significant evidence for serotonergic involvement, namely selective response to an SSRI but not an NRI, and significantly reduced striatal SERT density in H-animals (Korff et al. 2008; Wolmarans et al. 2013). Regional brain analysis of the cyclic adenosine monophosphate (cAMP) - phosphodiesterase type 4 (PDE_4) cascade in deer mice may assist in learning more on receptor signaling in the CSTC in these animals, and possibly in OCD. The intensity of stereotypy expressed in deer mice is positively correlated to frontal-cortical cAMP concentrations, while being inversely related to PDE_4 activity (Korff et al. 2009). Furthermore, chronic SSRI treatment attenuated this response (Korff et al. 2009). This not only supports frontal-cortical dysfunction in deer mouse stereotypy at a neurobiological level (Evans et al. 2004), but suggests involvement of the adenylate cyclase-cAMP-PDE_4 cascade. The inverse correlation between PDE_4 activity and the intensity of stereotypy may indicate that H-associated increased cAMP is related to increased post-synaptic $5HT_{1A}$ adenylate cyclase (AS)-cAMP activity (Korff et al. 2009). This hypothesis is strengthened by the demonstration that stimulation of pre-synaptic $5HT_{1A/1B/1D}$ auto-receptors induce perseverative locomotor paths (Shanahan et al. 2011; Yadin et al. 1991), while their desensitization is thought to mediate some of the ameliorative effects of the SSRIs (Blier et al. 1996). Desensitization of frontal-cortical $5HT_{1A/1B/D}$ auto-receptors results in increased release of serotonin which in turn is associated with anti-compulsive effects (El Mansari and Blier 2006; Goddard et al. 2008). This may have relevance to earlier studies describing the attenuation of deer mouse stereotypy to meta-chlorophenylpiperazine (mCPP), a non-selective serotonergic agonist (Korff et al. 2008). Although some of the human literature on mCPP indicates an association with exacerbation of OC-symptoms (Aouizerate et al. 2005), mCPP attenuates quinpirole-induced compulsive checking (Tucci et al. 2013; Tucci et al. 2015) providing congruence across at least two animal models of OCD. However, that selective PDE_4 inhibition with rolipram decreases methamphetamine-induced stereotypy (Iyo et al. 1995) hints at a possible causal role for disordered PDE_4 activity in deer mouse stereotypy.

Disturbances in SERT are well-described in OCD (Hesse et al. 2005; Reimold et al. 2007; Zitterl et al. 2008), while this protein represents an important biological target for the SSRI group of drugs (El Mansari and Blier 2006; Fineberg and Craig 2007). To test the hypothesis that hyposerotonergic signaling underlies OC behavior, we determined frontal-cortical and striatal serotonin transporter (SERT) densities in H and N deer mice (Wolmarans et al. 2013). In line with the theory of behavioral opponency between dopamine and serotonin (Daw et al. 2002) and consistent with findings that deer mouse stereotypy involves a relative bias in favor of the direct SN pathway, we found a significant reduction in striatal but not frontal-cortical SERT density in H, compared to N animals (Wolmarans et al. 2013). This is consistent with clinical (Hesse et al. 2005) and pre-clinical (Vermeire et al. 2012) literature and supports the hypothesis that the biobehavioral effects of a relative increase in SN dopaminergic signaling in

Table 2 Summary of key findings from the deer mouse model of OCD

Face Validity	• Genetic and environmental predispositions to H behavior	(Powell et al. 1999; Shorter et al. 2014)
	• Stereotypies are time-consuming, of varying frequency and intensity; not sensitive to behavioral sensitization, suggesting goal-directed behavior	(Tanimura et al. 2009; Wolmarans et al. 2013)
	• Heterogenous behavioral phenotypes: • jumping, pattern running and backward somersaulting stereotypies in 45% of the population; • aberrant nest building in 30% of the population not associated with H behavior; • persistent marble burying in 11% of the population not associated with H behavior	(Powell et al. 1999; Wolmarans et al. 2013, 2016a, b)
	• Deficits in striatum mediated learning ability in H, but not N animals	(Tanimura et al. 2008)
	• Behavioral rigidity is correlated with stereotypy severity and differs between the various phenotypes, implicating distinct neurobiological mechanisms underlying different phenotypes	(Bechard et al. 2016; Tanimura et al. 2008)
	• Altered social interaction between H-N and N-H, compared to H-H or N-N	(Wolmarans et al. 2017b)
Construct Validity	• CSTC circuit involvement: relative bias in favor of the direct SN pathway; reduced activity in the STN	(Presti and Lewis 2005; Presti et al. 2003; Tanimura et al. 2010)
	• Stereotypy is dependent on such bias and not to global dopaminergic dysfunction, as opposed to other rodent stereotypies, e.g. gnawing and grooming	(Presti et al. 2003, 2002)
	• Frontal-cortical serotonergic involvement: increased cAMP and reduced PDE_4 activity	(Korff et al. 2009)
	• Striatal serotonergic involvement: reduced striatal SERT density	(Wolmarans et al. 2013)
	• Reversal of deer mouse stereotypy with mCPP and quinpirole; indicating serotonergic and dopaminergic involvement in treatment response	(Korff et al. 2008)
	• Oxidative stress: disturbed frontal-cortical redox balance, i.e. deficient frontal-cortical glutathione availability, decreased frontal-cortical GSH and GSSG	(Güldenpfennig et al. 2011; Wu et al. 2009)
Predictive Validity	• Demonstrates response to chronic, but not sub-chronic high dose escitalopram treatment: • Treatment response is demonstrated for H stereotypy and large nest building, but not for persistent marble-burying. • Treatment response is demonstrated with respect to sociability in H-H interactions	(Wolmarans et al. 2013, 2016a, b, 2017b)
	• Selective response to an SSRI (fluoxetine) but non-responsive to treatment with an NRI, i.e. desipramine	(Korff et al. 2008)

deer mice (Presti and Lewis 2005; Presti et al. 2003) are not sufficiently countered by serotonin (Daw et al. 2002).

Deer mouse stereotypy and oxidative stress

Recent clinical studies have indicated oxidative stress in OCD (Behl et al. 2010; Chakraborty et al. 2009a, b; Selek et al. 2008), as well as effective augmentation of standard SSRI treatment with the glutathione precursor, N-acetyl cysteine (NAC) (Camfield et al. 2011; Lafleur et al. 2006; Sayyah et al. 2010). Consistent with these findings, we have demonstrated that H deer mice present with a disturbed frontal-cortical redox balance, i.e. reduced activity of the glutathione system as evinced by diminished concentrations of reduced (GSH) and oxidized glutathione (GSSG) in frontal cortical circuits with these deficits correlated with stereotypy severity (Güldenpfennig et al. 2011). A positive correlation was found between the intensity of stereotypy and the glutathione redox balance (Güldenpfennig et al. 2011), possibly suggesting a *relative* protective upregulation of glutathione synthesis as a function of stereotypy. Similar findings were demonstrated in deer mice exposed to low levels of environmental toxins (Wu et al. 2009) perhaps suggesting that *P. maniculatus bairdii* is able to counter the effects of low to moderate degrees of oxidative stress.

Taken together, these findings possibly indicate that H behavior is associated with levels of oxidative stress akin to that of mild pathology. However, caution must be applied when drawing causal relationships between deer mouse stereotypy and OCD, and it is notable that oxidative stress has been found in a number of psychiatric disorders (Berk et al. 2011; Chauhan and Chauhan 2006; Sarandol et al. 2007; Wang et al. 2009). In addition, the response of OC symptoms to NAC augmentation may be related to its modulation of NMDA receptor signaling, rather than specific effects on oxidative stress (Lafleur et al. 2006). Further study of the association between deer mouse stereotypy and oxidative stress, and of the response of such stereotypy to anti-oxidants is needed.

Peromyscus maniculatus bairdii as a model of heterogeneous OC behavior

OCD is characterized by a narrow range of different symptoms. The most prevalent obsessions are concerns about contamination (55%), inappropriate aggressive and

sexual thoughts (50% and 32% respectively), and concerns about symmetry and order (36%). The most common compulsions are ritualistic checking (80%), cleaning and decontamination rituals (46%) and counting (21%) (Abramowitz et al. 2010). Recently, we began to investigate whether *P. maniculatus bairdii* may express different OC-related phenotypes in addition to spontaneous stereotypy. We studied MB, previously proposed as a measure of compulsive activity (Albelda and Joel 2012) and nest building (NB) (Greene-Schloesser et al. 2011), which represents normal rodent activity but with between- and within-species variance (Jirkof 2014; Smithers 1983). As referred to earlier, high MB behavior was observed in 11% of our deer mouse cohort, independent of stereotypy or sex (Wolmarans et al. 2016a), while 30% of animals, again independent of stereotypy or sex, displayed persistent large NB behavior. Escitalopram had no effect on high MB, but reduced high NB (Wolmarans et al. 2016a, b). Further work is needed to determine whether such observations are analogous to clinical findings (including differential response of different symptom dimensions to stressors and to SSRIs). However, that large NB but not high MB responded to escitalopram, and that neither behavior was associated with a specific stereotypical cohort, suggests that NB and MB in the deer mouse reflect different underlying neurobiological mechanisms. Further, it is possible that such neurobiological differences may be species specific (Greene-Schloesser et al. 2011). While large NB also occurs naturally, mainly in response to environmental change (Jirkof 2014), the persistent and severe nature of large NB in some laboratory houseddeer mice only, suggests that in this sub-group, large NB may not be goal-directed or adaptive. This tentatively establishes a degree of face and predictive validity for large NB as reflecting a different, but also naturalistic OC-like phenotype in deer mice.

Conclusion

The deer mouse *(Peromyscus maniculatus bairdii)* offers an opportunity to study the neurobiology of OC-like behavior within a naturalistic framework. The species presents with stereotypies that are reminiscent of OCRD symptoms, while such stereotypies appear to share, at least in part, underlying psychobiological mechanisms and treatment response typical of OCD (Table 2). Given that deer mice display a narrow range of stereotypical behaviors, it is possible that some of these may be useful for studying specific symptom dimensions found in OCRDs. Further research on the genetic and epigenetic associations of stereotypies in the deer mouse model may be useful.

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