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Can fecal transplantation treat the physical consequences of chronic stress?

Employing fecal transplantation in a mouse model for chronic psychosocial stress

Dissertation to obtain the doctoral degree of medicine (Dr. med.)
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List of abbreviations

5-HT	5- hydroxytryptamine = serotonin
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
APS	Ammonium persulfate
B. infantis	*Bifidobacterium infantis*
B. longum	*Bifidobacterium longum*
BSA	Bovine serum albumin
C. difficile	*Clostridium difficile*
CD	Crohn's disease
CD11b	Cluster of differentiation molecule 11b
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CORT	Corticosterone
CRAMP	Cathelin-related antimicrobial peptide
CRH	Corticotropin-releasing hormone
CSC	Chronic subordinate colony housing
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleotide triphosphates
DPBS	Dulbecco's Phosphate Buffered Saline
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ENS	Enteric nervous system
FT	Fecal transplantation
GABA	Gamma-aminobutyric acid
GF	Germ-free
HDS	Histological damage score
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid
HPA axis	Hypothalamus-pituitary-adrenal axis
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IDO	Indoleamine 2,3 dioxygenase
IFN-γ	Interferon-gamma
IGEPAL	Octylphenyl-polyethylene glycol
IL-6	Interleukin-6
KC	Keratinocyte chemoattractant
L. rhamnosus	*Lactobacillus rhamnosus*
MCP-1	Monocyte chemotactic protein-1
mRNA	Messenger ribonucleic acid

MWU test	Mann-Whitney *U* test
OF/NO test	Open field/novel object test
PCR	Polymerase chain reaction
PTSD	Post-traumatic stress disorder
RNA	Ribonucleic acid
RPL	Ribosomal proteins
RT	Room temperature
SCFA	Short-chain fatty acids
SDR	Social disruption
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHC	Single-housed controls
SPF	Specific pathogen free
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
TEMED	Tetramethyl-ethylenediamine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
UC	Ulcerative colitis

1 Introduction

1.1 Chronic stress-related disorders

Psychosocial stress, especially chronic psychosocial stress is an acknowledged risk factor for the development and aggravation of affective disorders like depression (McGonagle and Kessler, 1990), anxiety-related disorders (Virtanen et al., 2011) and post-traumatic stress disorder (PTSD) (McLaughlin et al., 2010), as well as somatic disorders like coronary heart disease (Bosma et al., 1998; Orth-Gomér et al., 2000), metabolic syndrome (Chandola et al., 2006), chronic fatigue syndrome (Heim et al., 2009), asthma (Sandberg et al., 2000; Wright et al., 2004), infections (Cohen et al., 1991; Kiecolt-Glaser et al., 1996), cancer (Levav et al., 2000) and especially gastrointestinal disorders like irritable bowel syndrome (IBS) (Bennett et al., 1998), ulcerative colitis (UC) (Bitton et al., 2003; Levenstein et al., 1994) and crohn's disease (CD) (Mardini et al., 2004). Unfortunately, the mechanism how chronic stress exactly influences the development and the pathophysiology of the above-mentioned disorders is not completely understood yet.

However, chronic psychosocial stress has not only shown to be linked to affective and gastrointestinal diseases, also affective and gastrointestinal diseases among themselves seem to be comorbid (Gros et al., 2009; Walker et al., 2008; Gracie et al., 2018). For example, Kurina and colleagues found that on the one hand patients with UC more often suffer from anxiety than healthy controls and that on the other hand the prevalence of anxiety and depression is higher in patients with CD than in the general population (Kurina et al., 2001). These findings suggest that a common origin or pathophysiological pathway could underlie both diseases.

Interestingly, recent human studies indicate that depressed as well as IBS patients show significant differences in their intestinal microbiota composition compared to healthy persons (Rajilić-Stojanović et al., 2011; Jiang et al., 2015). Besides, rodent studies revealed that chronic stress changes the composition of the intestinal microbiota (Bailey et al., 2010; Bailey et al., 2011). Remarkably, it is also known that the latter can influence physiological, behavioural and inflammatory systems of the host (Sudo et al., 2004; Bailey et al., 2011;

Diaz Heijtz et al., 2011). In this context, a novel hypothesis is that the vulnerability to develop chronic stress-related disorders is significantly influenced by chronic stress-induced changes in the composition of the gastrointestinal microbiome.

1.2 The gastrointestinal microbiome and it's functions for the host

In the last few years, a growing body of human and animal research focused on the influence of the gastrointestinal microbiome on the host organism. The microbiome in general is defined as accumulation of microorganisms (bacteria, archaea, eukarya and viruses) and their genome in the host (Gordon et al., 2012. Microorganisms occupy the inner and outer surfaces of the host like the skin, the oral cavity, the gastrointestinal tract, the respiratory system and the genitals (Gordon et al., 2012). The vast majority of those microorganisms reside within the intestinal tract, where they accomplish essential functions for the host organism like the synthesis of vitamin K (Russell et al., 2012), the production of digestive enzymes (Englyst and Macfarlane, 1986; El Kaoutari et al., 2013) as well as the fermentation of glycans, the processing of debilitating H_2 to methane and the participation in the biosynthesis of essential amino acids (Gill et al., 2006). Moreover, it is known that the gastrointestinal microbiota closely interact with the local intestinal immune system (Kamada et al., 2013) and play an important role in the development and maintenance of the intestinal barrier (Farhadi et al., 2003).

Given all these important functions for the health of the host organism, it is not surprising that the gastrointestinal microbiome has also shown to have huge influences on the host beyond the intestinal tract. Accumulating data indicate the existence of the so-called "microbiota-gut-brain axis" through that the gut microbiota and the central nervous system (CNS) communicate in a bidirectional way (Cryan and Dinan, 2012) (see Fig. 1). Various pathways are thought to be involved in the functionality of this axis such as neural, neuro-immune, neuroendocrine and humoral pathways (Cryan and Dinan, 2012).

The vagus nerve that connects the CNS and the enteric nervous system (ENS) by its afferent and efferent nerve fibres displays an important route for the communication between the brain, the gut and the intestinal microbiome. Via vagal efferent nerve fibres, the CNS

regulates various autonomic functions in the gut, including motility as well as secretion and absorption (Vaupel, 2010). Vagal afferent nerve fibres provide information about the state of the gut, such as gastrointestinal tension, absorbed nutrients and presence of hormones and transmitters (Berthoud and Neuhuber, 2000). *Vice versa*, various effects of the gastrointestinal microbiota on brain function have also found to be dependent on the vagus nerve. For example, the administration of the probiotic *Bifidobacterium longum* (*B. longum*) induces anxiolytic behaviour in experimental mice compared to control medium gavaged mice, but this effect is not present when mice are vagotomised (Bercik et al., 2011). Treatment with the probiotic *Lactobacillus rhamnosus* (*L. rhamnosus*) results in anxiolytic behaviour and modulates γ-aminobutyric acid A ($GABA_A$) and $GABA_B$ receptor messenger ribonucleic acid (mRNA) expression in different brain regions of experimental mice compared to broth-fed control mice. Interestingly, both effects seem to be dependent on the vagus nerve as vagotomy prevents the effects of *L. rhamnosus* on anxiolytic behaviour as well as $GABA_A$ receptor expression in the brain (Bravo et al., 2011).

Furthermore, the gastrointestinal microbiota interact closely with the local mucosal as well as the systemic immune system and in this way intercommunicate with the CNS (Maynard et al., 2012). Specific microbial cell membrane components are recognized by toll-like receptors (TLR) expressed in different immune cells (Muzio et al., 2000). This interaction triggers a signalling pathway that results in the production and secretion of inflammatory cytokines (Takeda and Akira, 2004). In turn, pro- and anti-inflammatory cytokines have many different effects in the CNS. For instance, pro-inflammatory cytokines are associated with the development of sickness-behaviour and depression (Raison et al., 2006). Besides, cytokines play an important role in the tryptophan metabolism. In detail, the enzyme indoleamine 2,3 dioxygenase (IDO) that degrades tryptophan through the kynurenine pathway is highly inducible by pro-inflammatory cytokines (Pfefferkorn et al., 1986; Fujigaki et al., 2006). Therefore, kynurenine and its metabolites are seen as inflammatory markers and recent studies suggest an involvement of kynurenine in the pathogenesis of IBS as well as inflammation-associated depressive disorders (Kennedy et al., 2017). Taken together, as intestinal microbiota are involved in the production of cytokines as mentioned before, they are thereby able to influence the activity of IDO and consequently the metabolism of tryptophan along the kynurenine pathway. Moreover, besides influencing the

tryptophan metabolism along the kynurenine pathway, emerging evidence suggest that the intestinal microbiota can modulate circulating levels of tryptophan in general. For instance, experiments on germ-free (GF) mice show that the latter have increased circulating tryptophan levels compared to conventionally colonised mice (Clarke et al., 2013; Wikoff et al., 2009). Interestingly, colonising GF mice post weaning normalises circulating tryptophan levels (Clarke et al., 2013). As tryptophan is the precursor molecule to serotonin (5-HT), the intestinal microbiota thereby influence the availability of 5-HT in the brain and consequently the serotonergic neurotransmission in the CNS (Desbonnet et al., 2008; Clarke et al., 2013).

In addition, the intestinal microbiota communicate with the CNS via short-chain fatty acids (SCFA). The latter are neuroactive bacterial metabolites of dietary fibres that enter the CNS by crossing the blood-brain barrier (Frost et al., 2014). For example, recent studies on rodents show that SCFAs are involved in the central appetite regulation (Frost et al., 2014) and in the alterations of brain phospholipids involved in the pathogenesis of autism spectrum disorder (Thomas et al., 2012). Moreover, the intestinal microbiota produce various neurotransmitters such as GABA (Barrett et al., 2012), free dopamine and norepinephrine (Asano et al., 2012) as well as acetylcholine (Stephenson and Rowatt, 1947) in the gut and thereby intercommunicate closely with the CNS via the humoral pathway.

Vice versa, there are also host-endocrine pathways that are involved in the microbiota-gut-brain communication. Stress-related hormones like corticotrophin-releasing hormone (CRH) and glucocorticoids that are regulated through the hypothalamus-pituitary-adrenal (HPA) axis have various effects on the gut such as altering the intestinal permeability, inducing barrier dysfunction as well as changing the intestinal microbiota composition (Söderholm et al., 2002; Teitelbaum et al., 2008; Smith et al., 2010; Park et al., 2013).

Although many interactions of the intestinal microbiota with the brain and the host organism are well established, the consequences of chronic stress-induced changes in the intestinal microbiota composition for the host organism are still not fully elucidated to date. In the following section, this topic will be elaborated.

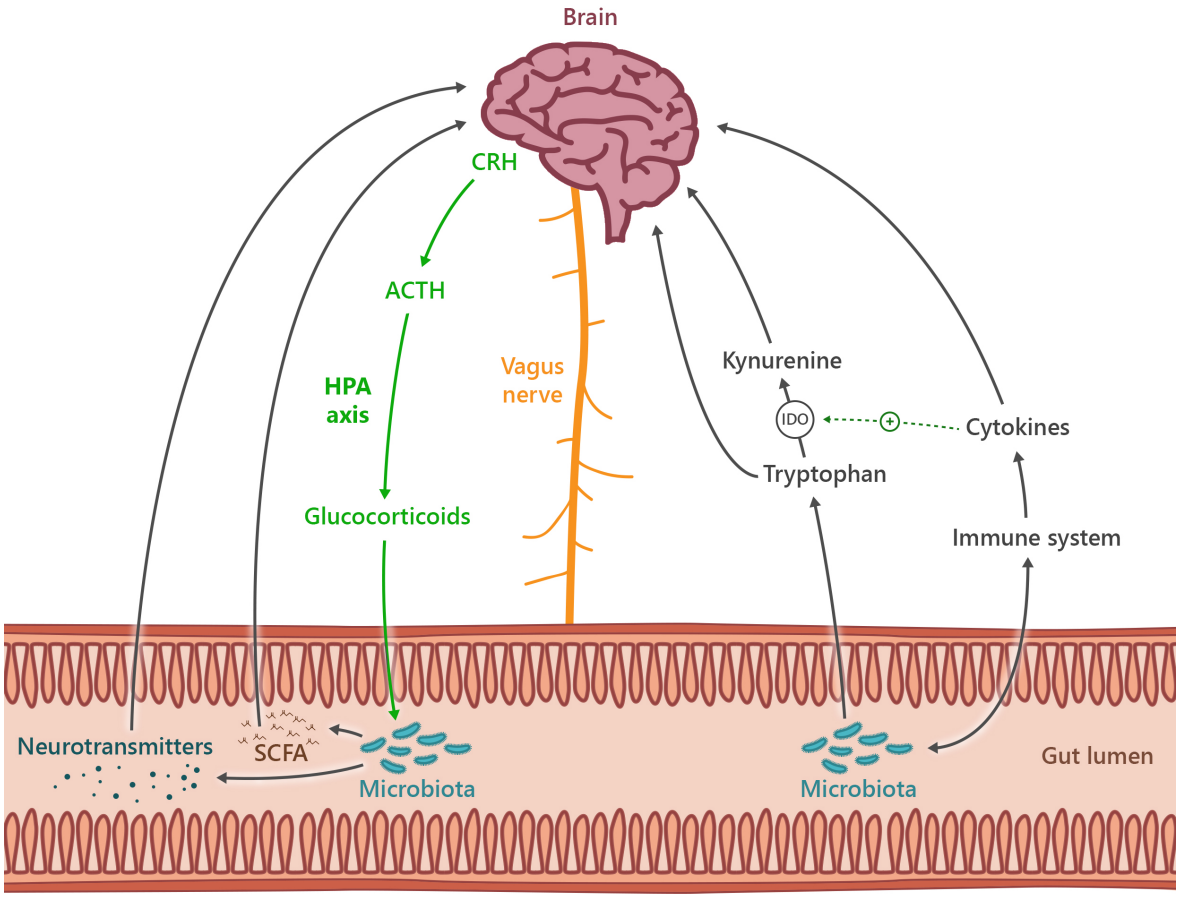

Figure 1: Microbiota-gut-brain axis. Numerous direct and indirect pathways are involved in the bidirectional communication of the brain (purple), the gut (red) and the gut microbiota (cyan). The vagus nerve (yellow) connects the brain and the gut with efferent nerve fibres through which the brain influences the gut motility and absorption and with afferent nerve fibres through which the gut microbiota are able to influence the brain and the behaviour. The gut microbiota produce neurotransmitters (dark blue) as well as short-chain fatty acids (SCFA; brown) that can modulate the brain and its function. In addition, by modifying the level of circulating tryptophan, the gut microbiota have a strong impact on the presence of this neurotransmitter in the brain. Furthermore, the gut microbiota interact closely with the immune system, which triggers the production of cytokines. The latter also modulate brain function and behaviour. Besides, cytokines induce the enzyme indoleamine 2,3 dioxygenase (IDO) that degrades tryptophan through the kynurenine pathway. Kynurenine in turn can have marked effects on brain function. The hypothalamus-pituitary-adrenal (HPA) axis (green) regulates the secretion of corticotropin releasing hormone (CRH), which in turn regulates the secretion of adrenocorticotropic hormone (ACTH) and glucocorticoids hereafter. Glucocorticoids can alter the gut permeability and have the potential to alter the gut microbiota composition.

1.3 Effects of chronic stress-related disorders and chronic stress on the intestinal microbiota composition

As mentioned previously, gastrointestinal and affective disorders have shown to be comorbid in many cases. Interestingly, in recent human studies it has been found that both types of disorders involve alterations in the fecal and intestinal microbiota composition. In detail, sequencing of fecal samples from depressed patients showed that the levels of some bacterial orders and families are increased while others are decreased compared to non-depressed patients (Naseribafrouei et al., 2014; Jiang et al., 2015). Furthermore, the analysis of fecal samples from IBS patients revealed a lower biodiversity and a different formation of the intestinal microbiota compared to a healthy control group (Codling et al., 2010; Carroll et al., 2011; Rajilić-Stojanović et al., 2011).

Essential for this study is that different animal models indicate that chronic stress is able to change the composition of the gastrointestinal microbiota. For instance, the gastrointestinal microbiota composition of mice stressed by water, food and bedding deprivation is measurably distinct from normally fed and kept control animals (Tannock and Savage, 1974). Particularly the level of *Lactobacilli* in the stomach decreases and the level of coliform bacteria in the intestines increases (Tannock and Savage, 1974). Furthermore, stressors early in life have shown to influence the composition of the gut microbiome. In detail, prenatal (acoustic stress for pregnant females) and postnatal stress (maternal separation) reduces the number of intestinal microorganisms in rhesus monkeys, especially the number of *Bifidobacteria* and *Lactobacilli* (Bailey and Coe, 1999; Bailey et al., 2004). Moreover, crowding and heat stress in growing rats and chicks leads to changes in the intestinal microbiota composition such as an increase in aerobic bacteria compared to undisturbed control animals (Suzuki et al., 1983).

Of special interest for this study is that adult chronic psychosocial stress in rodents and humans has been shown to have a strong impact on the richness and diversity of the gastrointestinal microbiome. For example, Bailey and colleagues revealed that social disruption stress as well as prolonged immobilisation stress in mice induces changes in the composition of different bacterial phyla and genera as well as an overall reduction in microbial diversity and richness (Bailey et al., 2010; Bailey et al., 2011). Moreover, in a

human study, it has been shown that during academic stress, students exhibit lower counts of bacteria, particularly of lactic acid bacteria, in fecal samples (Knowles et al., 2008).

Taken together, these points strengthen the relevance of the hypothesis that the vulnerability to develop chronic stress-related disorders might in large parts be mediated by chronic stress-induced alterations in the intestinal microbiota composition (see Fig. 2).

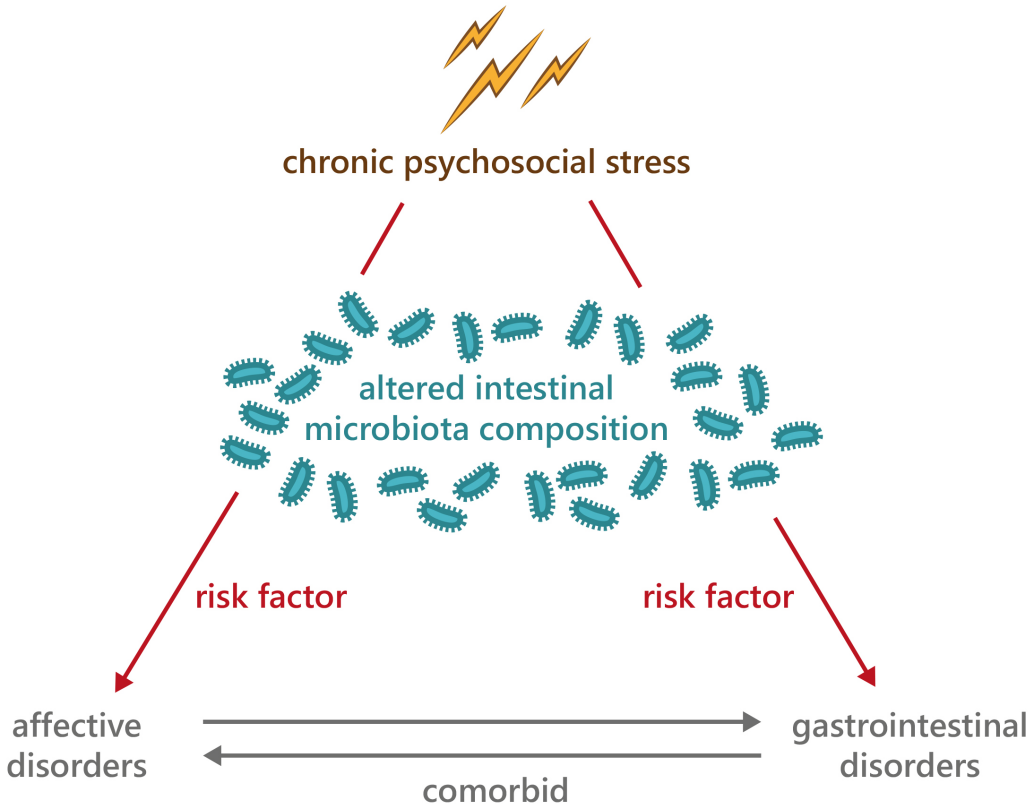

Figure 2: Schematic illustration of the hypothesis that chronic psychosocial stress is a risk factor for chronic stress-related disorders by altering the intestinal microbiota composition.

1.4 Impacts of the intestinal microbiota on stress-related physiological, behavioural and inflammatory parameters

Numerous studies on rodents show that the intestinal microbiome influences stress-related physiological, behavioural and inflammatory parameters. In this context, Sudo and colleagues could show that the postnatal gastrointestinal microbiota composition plays an important role in priming the HPA axis stress response. In detail, restrained stress-exposed GF compared to specific pathogen free (SPF) mice display an exaggerated activation of the HPA axis, visible by elevated levels of plasma adrenocorticotropic hormone (ACTH) and plasma corticosterone (CORT) (Sudo et al., 2004). Interestingly, colonisation of GF mice in early life with the probiotic *Bifidobacterium infantis* (*B. infantis)* or with feces from SPF mice is able to normalise the elevated HPA axis response (Sudo et al., 2004). Likewise, Huo and colleagues showed that the chronic restrained stress-induced activation of stress mediators is higher in stressed vs. unstressed GF compared to SPF animals (Huo et al., 2017).

In line with stress-related physiological parameters, there are also many studies investigating the impact of the intestinal microbiota on host behaviour. Although not fully consistent (Nishino et al., 2013; Crumeyrolle-Arias et al., 2014), most studies could show that GF mice are less anxious than SPF mice (Neufeld et al., 2011; Diaz Heijtz et al., 2011; Clarke et al., 2013). Noteworthy, colonisation of GF animals in early life with conventional microbiota reverses the anxiolytic behaviour (Diaz Heijtz et al., 2011; Clarke et al., 2013).

Moreover, the composition of the intestinal microbiota affects the inflammatory response to stress. For instance, Bailey and colleagues showed that circulating levels of the pro-inflammatory cytokine interleukin-6 (IL-6) are higher in mice that were exposed to the social disruption (SDR) stressor than in non-stressed mice. However, mice having a reduced microbiota density in the gut following antibiotic administration do not show an elevation in IL-6 levels following SDR, indicating that certain microbiota interact closely with the immune system and are needed to generate a proper immune response (Bailey et al., 2011).

Interestingly, studies treating rodents with probiotic bacteria could show that the latter are able to lessen the stress-induced behavioural and physiological consequences for the host. For example, it was just recently shown that the administration of the probiotic *L. rhamnosus*

reduces the acute stress-induced CORT elevation as well as anxiety- and depressive-related behaviour in *L. rhamnosus*-treated mice compared to broth-fed control mice following the exposure to a behavioural testing (Bravo et al., 2011). Furthermore, treatment with the probiotic *B. infantis* reverses depressive-like behaviour following maternal separation in rats (Desbonnet et al., 2008). In line with rodent studies, beneficial effects of a probiotic intake on self-reported everyday life distress and cognitive handling of sadness were recently also found in healthy humans (Messaoudi et al., 2011; Tillisch et al., 2013; Steenbergen et al., 2015).

1.5 The use of fecal transplantation to treat inflammatory and affective disorders

Given that chronic stress changes the intestinal microbiota composition and the latter in turn has an effect on the physiological, behavioural and inflammatory status of an organism, the treatment of stress-induced alterations in the intestinal microbiome might be able to ameliorate the consequences of chronic stress or might decrease the vulnerability to develop chronic stress-related somatic and affective disorders. Therefore, a promising approach to treat or prevent stress-induced alterations in the intestinal microbiota composition and its consequences for the organism is the transplantation of donor feces with a healthy microbiota composition (see Fig. 3).

Introductory, the method called “fecal transplantation” (FT) is an acknowledged therapeutic approach for humans to treat recurrent *Clostridium difficile* (*C. difficile*) infections, especially for patients with a treatment resistance to antibiotics (Kelly et al., 2015). The opportunistic bacterium *C. difficile* thereby proliferates when the normal intestinal flora is diminished and disordered after a long period of antibiotic therapy (Turner et al., 2013). The transplantation of stool via enema, gastroscope or nasojejunal tube from a healthy donor is therefore used to restore the affected intestinal flora and to reduce the niche for pathogenic germs like *C. difficile* (Gough et al., 2011). A lot of studies show that FT is an effective therapy for recurrent *C. difficile* infections (Mattila et al., 2012; Brandt et al., 2012; van Nood et al., 2013). More precisely, a systematic review on 27 reports revealed that 92% of the patients treated with FT are cured of recurrent *C. difficile* infections (Gough et al., 2011).

Since FT achieved high efficiencies in the elimination of *C. difficile* infections, studies employing FT on patients with active inflammatory bowel disease (IBD) were recently performed to test whether FT can be used as a novel strategy to alleviate IBD symptoms or to even reach clinical remission (Gianotti and Moss, 2017). Although some studies were not successful in using FT to treat IBD (Suskind et al., 2015; Rossen et al., 2015), many studies could indeed show beneficial treatment effects of FT such as the improvement in the disease activity in children with UC (Kunde et al., 2013), higher clinical remission rates in UC patients treated with FT vs. placebo (Moayyedi et al., 2015) and an increased bacterial diversity in fecal samples of CD patients following FT (Vaughn et al., 2016). Likewise, a meta-analysis of 18 studies investigating the treatment effects of FT in IBD patients found an overall clinical remission rate of 45% (Colman and Rubin, 2014).

Since lately, FT is also used in preclinical studies to investigate the impact of the intestinal microbiota on the host. Recent studies on rodents revealed that a transplantation of feces from one animal to another is able to affect the behaviour and physiology of the recipient animal. For instance, Bercik and colleagues showed that the oral transplantation of donor feces into GF mice of a different mouse strain is able to transfer the strain-specific behavioural characteristics to the recipient (Bercik et al., 2011). Besides using rodents as FT donors, FT has also recently been performed using human donors and rodent recipients. For instance, rats with an antibiotics-diminished microbiome develop a depressive-like behavioural and physiological phenotype following oral gavage with fecal microbiota obtained from depressed human subjects (Kelly et al., 2016). Moreover, De Palma and colleagues could show that the transplantation of donor feces from IBS-patients into GF mice via oral gavage leads to the development of IBS-specific characteristics, like an increased intestinal barrier dysfunction as well as anxiety-related behaviour in recipient GF mice (De Palma et al., 2017).

Although the method of FT is established very well in animal research, there is less known if FT via rectal infusion instead of oral gavage can also be successfully conduced in rodents. Furthermore, it is still not very clear if FT can be used in recipient animals that were not raised germ-free or pre-treated with antibiotics to reduce their natural microbial richness.

Moreover, it is not known yet if it is possible to use FT to treat chronic stress-related disorders besides using it to treat *C. difficile* infections and IBD.

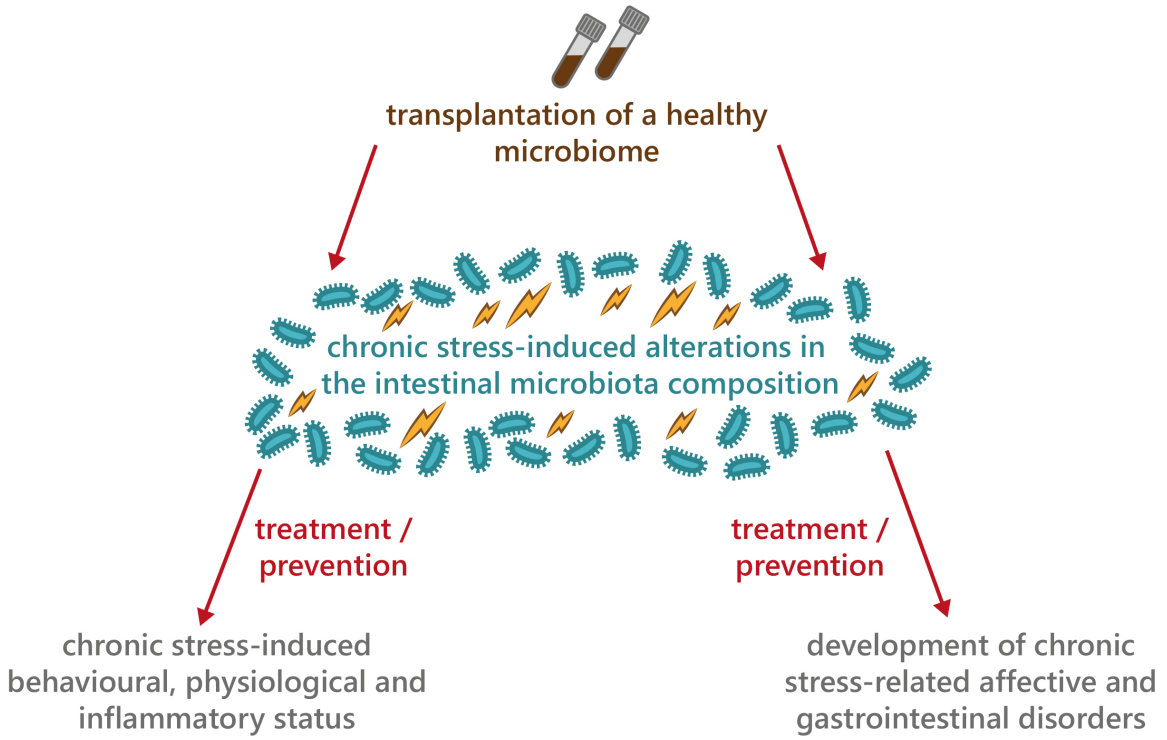

Figure 3: Schematic illustration of the possible use of fecal transplantation as treatment/prevention for chronic stress-induced alterations and chronic stress-related disorders.

1.6 The chronic subordinate colony housing model

In order to investigate the use of FT to treat chronic stress-induced alterations in the intestinal microbiota composition and its consequences for the host organism, an appropriate animal model is required. The latter needs to reliably induce chronic stress as well as somatic and affective consequences and furthermore needs to cause alterations at the level of the gastrointestinal microbiome. There are anyway only a few animal models employing a chronic stress paradigm that causes both somatic and affective disorders at the same time and there are even less that additionally confirm to affect the microbiota composition in the gut. Given that the chronic subordinate colony housing (CSC) paradigm fulfils all the above-mentioned criteria, it represents a promising model to study the interaction between chronic psychosocial stress and the microbiome in experimental mice.

The CSC paradigm induces chronic psychosocial stress in male experimental mice via prolonged subordination and repeated social defeat to a larger dominant male mouse during 19 consecutive days (Reber et al., 2007). In detail, on day 1 of the CSC paradigm, four experimental mice (CSC mice) are placed into the home cage of a dominant mouse (resident). Given that male mice instinctively establish a hierarchy within their colony, the four CSC intruder mice are immediately subordinate to the dominant resident. In order to avoid habituation, all four CSC mice are transferred into the home cage of a novel resident on days 8 and 15. Single housed control (SHC) mice are used as controls. Single housing can be regarded as the most adequate form of housing for control mice because group housing biases behavioural and physiological parameters as unfamiliar male mice of the same size also establish a hierarchy when group housed in the same cage (Singewald et al., 2009).

The CSC paradigm has repeatedly shown to cause stress-induced alterations in behavioural, physiological and inflammatory parameters in experimental mice. More precisely, in behavioural tests conducted on day 19 of the CSC paradigm, CSC mice show increased state as well as social anxiety-related behaviour compared to SHC mice (Reber et al., 2007; Reber and Neumann, 2008; Slattery et al., 2012). Furthermore, after sacrificing the mice in the morning of day 20, various physiological and inflammatory alterations can be observed in the CSC animals. In detail, 19 days of CSC result in stress-induced thymus atrophy and adrenal hypertrophy (Reber et al., 2007). Moreover, CSC mice display an increased susceptibility for systemic as well as local intestinal inflammation. More precisely, CSC results in chronic low-grade inflammation, visible by elevated levels of various cytokines and inflammatory markers in the plasma (Langgartner et al., 2018a). Besides, in the presence of gastrointestinal pathobionts like *Helicobacter* species (Langgartner et al., 2017), CSC causes spontaneous and aggravates chemically-induced colitis as seen in both the increase of the colonic histological damage score (HDS) and the elevation of cytokine secretion from isolated mesenteric lymph node cells (Reber et al., 2007; Reber et al., 2008).

Furthermore, it has been found that the CSC paradigm induces alterations in the intestinal microbiota composition. The latter is indicated by a significant shift in microbial diversity of CSC vs. SHC mice (Reber et al., 2016). Moreover, CSC animals display an increased

bacterial load in the colonic tissue and in fecal samples (Reber et al., 2011). However, so far it is not known if the observed alterations in the gastrointestinal microbiota composition are associated with the above-mentioned behavioural, physiological and inflammatory consequences of CSC.

1.7 Aims of the study

To date it is not known whether transplantations of healthy donor feces can ameliorate the behavioural, physiological and inflammatory consequences of chronic psychosocial stress or even prevent the development of chronic stress-related affective and somatic disorders. In turn, it is also unknown whether transplantations of feces from chronically stressed donors can induce stress-related characteristics in the recipient. Due to the relevance of the CSC paradigm in this context, this model was used to address these issues.

The main aims of my study therefore were to examine i) if transplantations of donor feces from non-stressed SHC mice during the CSC procedure can treat or prevent chronic stress-related physiological, behavioural and inflammatory alterations in CSC mice and ii) if transplantations of donor feces from stressed CSC mice are able to induce chronic stress-related characteristics in unstressed SHC mice. Besides, my study aimed to generally test whether FT via the rectal route and without the use of an antibiotic pre-treatment can affect behavioural, physiological and inflammatory parameters in recipient mice.

Therefore, one set of SHC- and CSC-recipient mice received two rectal infusions of feces from non-stressed SHC-donor mice at days 4 and 11 of the CSC-paradigm. Another set of SHC- and CSC-recipient mice received two rectal infusions of feces from chronically stressed CSC-donor mice at days 4 and 11 of the CSC-paradigm. To see if FT *per se* has an effect on the collected parameters, another set of SHC and CSC mice received two rectal infusions of saline at days 4 and 11 of the CSC-paradigm, respectively.

Notwithstanding the above, a side aim of my thesis was to establish the method of Gram staining in colonic tissue sections in order to correlate the number of positively stained bacteria with the HDS of the colon.

2 Material and methods

2.1 Material

2.1.1 Buffers and solutions

Complete Mini Protease Inhibitor solution	1 Complete Mini Protease Inhibitor cocktail tablet dissolved in 1ml dH_2O
EDTA lysis buffer	1ml 0.02% EDTA 0.01751g sodium chloride 50µl HEPES 5µl IGEPAL 100µl Complete Mini Protease Inhibitor solution
Electrode buffer, 10x	30g Tris 144g Glycine 10g SDS Pellets ad 1l dH_2O
Electrode buffer, 1x	100ml 10x electrode buffer 900ml dH_2O
Loading Dye, 4x	90µl 4x Laemmli Sample Buffer 10µl β-Mercaptoethanol
Resolving gel buffer, 4x	75ml 2M Tris 4ml 10% SDS 21ml dH_2O
Stacking gel buffer, 4x	50ml 1M Tris-Cl 4ml 10% SDS 46ml dH_2O
TBS, 10x (pH 7.4)	61g Tris (500mM) 87.66g sodium chloride ad 1l dH_2O

TBS, 1x	100ml 10x TBS 900ml dH_2O
TBS-T, 1x	100ml 10x TBS 1ml Tween 20 ad 1l dH_2O
Transfer buffer, 10x	30.3g Tris (25mM) 144g Glycine (192mM) ad 1l dH_2O
Transfer buffer, 1x	100ml 10x transfer buffer 700ml dH_2O 200ml Methanol

2.1.2 Chemicals

0.02% EDTA Solution	Sigma Aldrich, St. Louis, MO, USA
10% Ammonium persulfate (APS)	Sigma Aldrich, St. Louis, MO, USA
30% acrylamide/bisacrylamide solution	Carl Roth GmbH&Co KG, Karlsruhe, Germany
4x Laemmli Sample Buffer	Bio-Rad Laboratories, Hercules, CA, USA
Acetone	Sigma Aldrich, St. Louis, MO, USA
Albumin bovine (BSA) Fraction 5	BIOMOL GmbH, Hamburg, Germany
Complete Mini Protease Inhibitor cocktail tablets	Roche Diagnostics GmbH, Rotkreuz, Switzerland
DNase I	Qiagen, Venlo, Netherlands
Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (1x) (DMEM/F-12)	Gibco®, Life technologies™, Thermo Fisher Scientific, Waltham, MA, USA
Dulbecco's Phosphate Buffered Saline (1x) (DPBS)	Gibco®, Life technologies™, Thermo Fisher Scientific, Waltham, MA, USA

Eosine Y Solution	Waldeck GmbH&Co KG, Muenster, Germany
Ethanol 99.8%	Sigma Aldrich, St. Louis, MO, USA
Formaldehyde 3.5 - 3.7%	Otto Fischar GmbH&Co KG, Saarbruecken, Germany
Glycine	BioFroxx GmbH, Einhausen, Germany
2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (HEPES)	Biochrom GmbH, Berlin, Germany
Octylphenyl-polyethylene glycol (IGEPAL)	Alfa Aesar, Thermo Fisher Scientific, Heysham, UK
Isotonic saline solution	Fresenius Kabi GmbH, Bad Homburg, Germany
Mayer's hemalum solution	Merck Chemicals GmbH, Darmstadt, Germany
N, N, N', N',- Tetramethyl-ethylenediamine (TEMED)	Carl Roth GmbH&Co KG, Karlsruhe, Germany
PageRuler™ Plus Prestained Protein Ladder	Thermo Fisher Scientific, Waltham, MA, USA
Paraplast Plus embedding medium	McCormick Scientific, St Louis, MO, USA
Platinum ® SYBR ® Green	Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA
Re-Blot Plus Strong Solution, 10x	Merck Chemicals GmbH, Darmstadt, Germany
Roti®-Histokitt	Carl Roth GmbH&Co KG, Karlsruhe, Germany
Roti®-Histol (Xylene)	Carl Roth GmbH&Co KG, Karlsruhe, Germany
Skim Milk Powder	Sigma Aldrich, St. Louis, MO, USA
Sodiumdodecylsulfate Pellets (SDS)	Carl Roth GmbH&Co KG, Karlsruhe, Germany
Sodium chloride	Sigma Aldrich, St. Louis, MO, USA

Tris	AppliChem GmbH, Darmstadt, Germany
Tween ® 20	VWR International, Radnor, PA, USA
β-Mercaptoethanol	AppliChem GmbH, Darmstadt, Germany

2.1.3 Kits

Clarity ™ Western ECL Substrate	Bio-Rad Laboratories, Hercules, CA, USA
High Capacity cDNA Reverse Transcriptase Kit	Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
RNeasy Mini kit	Qiagen, Venlo, Netherlands

2.1.4 Consumable materials

96-well plates	Sarstedt, Nuernbrecht, Germany
384-well plates	Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA
Cell Strainer 70 µm	Corning Incorporated, Durham, NC, USA
Cover glasses (24 x 40 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
EDTA micro tube (1.3 ml)	Sarstedt, Nuernbrecht, Germany
Eppendorf tubes (0.5/1.5/2 ml)	Eppendorf, Hamburg, Germany
Falcon® (15/ 50 ml)	Corning Incorporated, Durham, NC, USA
Mini Trans-Blot® Filter paper Nitrocellulose Blotting Membrane, Amersham™ Protran™ (Premium 0.45 µm NC)	Bio-Rad Laboratories, Hercules, CA, USA GE Healthcare Life Science, Chalfont St Giles, UK

PCR SingleCap tubes	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Precellys Lysing Kit	Bertin Technologies, Tarnos, France
Rotilabo®-embedding cassettes, POM	Carl Roth GmbH & Co KG, Karlsruhe, Germany
SuperFrost Ultra Plus® microscope slides	Thermo Fisher Scientific, Waltham, MA, USA
Syringe (2 ml)	B. Braun, Melsungen, Germany

2.1.5 Appliances and labware

ChemiDoc™ MP Imaging System	Bio-Rad Laboratories, Hercules, CA, USA
CO2 Incubator BB15	Thermo Fisher Scientific, Waltham, MA, USA
Drying-oven VENTI-Line	VWR International, Radnor, PA, USA
Freezer (-20°C)	Liebherr, Bulle, Switzerland
Freezer (-80°C)	Thermo Fisher Scientific, Waltham, MA, USA
Fridge (4°C) Medline	Liebherr, Bulle, Switzerland
Gavage needle	Heidelberg, Germany
Ice Maker, Manitowoc® FR 0266 A	Manitowoc Company, Manitowoc, WI, USA
Micro Star 17R centrifuge	VWR International, Radnor, PA, USA
Microm HM 430	Thermo Fisher Scientific, Waltham, MA, USA
Microscope Microscope Leica DMI 6000B	Carl Zeiss AG, Oberkochen, Germany Leica Microsystems GmbH, Wetzlar, Germany
Mini PROTEAN® System short and spacer plates (0.75 mm)	Bio-Rad Laboratories, Hercules, CA, USA

Mini PROTEAN® Tetra Handcast Systems	Bio-Rad Laboratories, Hercules, CA, USA
Mini Star centrifuge	VWR International, Radnor, PA, USA
Multipipette® stream	Eppendorf, Hamburg, Germany
NanoDrop 2000	Thermo Fisher Scientific, Waltham, MA, USA
Olympus DP73 digital microscope camera	Olympus, Shinjuku, Tokio, Japan
Pipettes (2.5 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf, Hamburg, Germany
Plate Reader FLUOstar OPTIMA	BMG LABTECH GmbH, Ortenberg, Germany
Power Pac ™ HC basic power suppy	Bio-Rad Laboratories, Hercules, CA, USA
Real-Time PCR System ViiA7	Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA
Scale CPA 10001	Sartorius Weighing Technology GmbH, Goettingen, Germany
Semi-dry transfer cell, Trans-Blot ® SD	Bio-Rad Laboratories, Hercules, CA, USA
Shaker ROCKER 3D digital	IKA® GmbH & Co KG, Staufen, Germany
Thermocycler Peqstar	VWR International, Radnor, PA, USA
Thermomixer comfort	Eppendorf, Hamburg, Germany
Vortex Genie 2	Scientific Industries Inc., Bohemia, NY, USA
Water bath W20	VWR International, Radnor, PA, USA

2.1.6 Software

CellSens Entry	Olympus, Shinjuku, Tokio, Japan
EthoVision XT (version 9)	Noldus Information Technology, Wageningen, The Netherlands
Image Lab™	Bio-Rad Laboratories, Hercules, CA, USA
SigmaPlot (version 11.0)	Systat Software Inc., San José, CA, USA
SPSS statistics (version 23.0)	IBM, Armonk, NY, USA

2.1.7 Antibodies

Anti-CD11b Antibody (ERP 1244)	Abcam®, Cambridge, UK
Anti-rabbit IgG HRP-linked Antibody	Cell Signaling Technology, Danvers, MA, USA
F4/80 (M-300)	Santa Cruz Biotechnology, Dallas, TX, USA
β-Tubulin Antibody	Cell Signaling Technology, Danvers, MA, USA

2.1.8 Primers

CRAMP forward	5’CAGCCCTTTCGGTTCAAGAA3’	Sigma Aldrich, St. Louis, MO, USA
CRAMP reverse	5’CCCACCTTTGCGGAGAAGT3’	Sigma Aldrich, St. Louis, MO, USA
IFN-γ forward	5’TGCTGATGGGAGGAGATGTCT3’	Sigma Aldrich, St. Louis, MO, USA
IFN-γ reverse	5’TGCTGTCTGGCCTGCTGTTA3’	Sigma Aldrich, St. Louis, MO, USA

RPL forward	5’CCTGCTGCTCTCAAGGTT3’	Sigma Aldrich, St. Louis, MO, USA
RPL reverse	5’TGGCTGTCACTGCCTGGTACTT3’	Sigma Aldrich, St. Louis, MO, USA
TNF-α forward	5’AGGGGCCACCACGCTCTTCT3’	Sigma Aldrich, St. Louis, MO, USA
TNF-α reverse	5’TGAGTGTGAGGGTCTGGGCCAT3’	Sigma Aldrich, St. Louis, MO, USA

2.2 Methods

2.2.1 Animals

Male C57BL/6N mice (Charles River, Sulzfeld, Germany) at the age of 36 - 45 days were used as both fecal donor and fecal recipient animals. Male CD1 mice (Charles River, Sulzfeld, Germany) that were older, larger and heavier were used as dominant residents. After arrival, donor as well as recipient mice were kept in a SPF animal facility and stayed separately in standard polycarbonate mouse cages (16 x 22 x 14 cm) for at least seven days before the CSC paradigm started. All mice were kept under standard laboratory conditions (12h light/12h dark cycle, lights on at 06.00 a.m., 22°C, 60% humidity) and had free access to tap water and standard mouse diet.

All experimental protocols were approved by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals (Regierungspraesidium Tuebingen, Germany; reference number 35/9185.81-3, experimental number 1195). All efforts were made to minimise the number and suffering of the used animals.

2.2.2 Experimental design

One to two weeks after arrival, mice were either exposed to the CSC paradigm (day 1-20) or kept as SHC mice (day 1-20). In this study, three different sets of SHC and CSC recipient mice were used. In detail, on days 4 and 11, animal set 1 (SHC: n = 20, CSC: n = 14) was rectally infused with saline, animal set 2 (SHC: n = 12, CSC: n = 12) with SHC-donor feces and animal set 3 (SHC: n = 12, CSC: n = 12) with CSC-donor feces. SHC or CSC exposure for donor mice started on the same day (day 1) as SHC or CSC exposure for recipient mice. Therefore, feces infused into recipient mice on days 4 and 11 were obtained from donor mice exposed to SHC or CSC for either 4 or 11 days, respectively. Altogether n = 28 SHC and n = 24 CSC mice were used as donor animals.

All recipient mice were tested for anxiety-related behaviour in the open field/novel object (OF/NO) test on day 19 between 07.00 a.m. and 10.00 a.m. After undergoing brief CO_2 anaesthesia, all mice were killed by rapid decapitation in the morning of day 20 between 07.00 a.m. and 10.00 a.m. to analyse the stress-related physiological parameters. Thymus and adrenal glands of all recipient mice were removed, pruned of fat and weighted separately. The colon of all recipient mice was removed and the anal part was taken to assess the HDS. Another distal piece of the colon was used in a subset of animals (animal set 1: SHC: n = 8, CSC: n = 4; animal set 2: SHC: n = 12, CSC: n = 12; animal set 3: SHC: n = 12, CSC: n = 12) to assess the colonic protein levels of F4/80 and cluster of differentiation molecule 11b (CD11b) as well as the colonic mRNA levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and cathelin-related antimicrobial peptide (CRAMP).

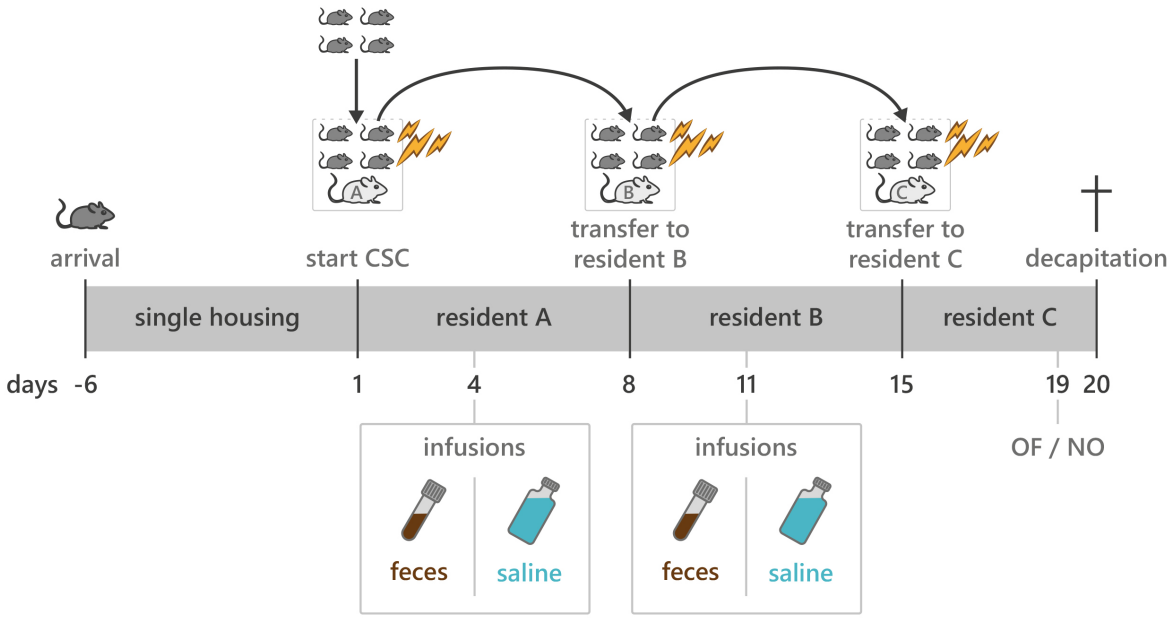

Figure 4: Timeline of the experimental design. After arrival, all mice were housed individually for one week. On day 1, the chronic subordinate colony housing (CSC) paradigm started. To induce chronic psychosocial stress, four CSC mice were put into the home cage of a larger dominant resident mouse. To avoid habituation, the CSC mice were transferred into the home cage of a novel resident on days 8 and 15. On days 4 and 11, experimental mice received infusions of either saline (animal set 1), feces from single-housed control (SHC)-donor mice (animal set 2) or feces from CSC-donor mice (animal set 3). Behavioural tests (open field/novel object (OF/NO) test) were carried out on day 19. Mice were decapitated in the morning of day 20.

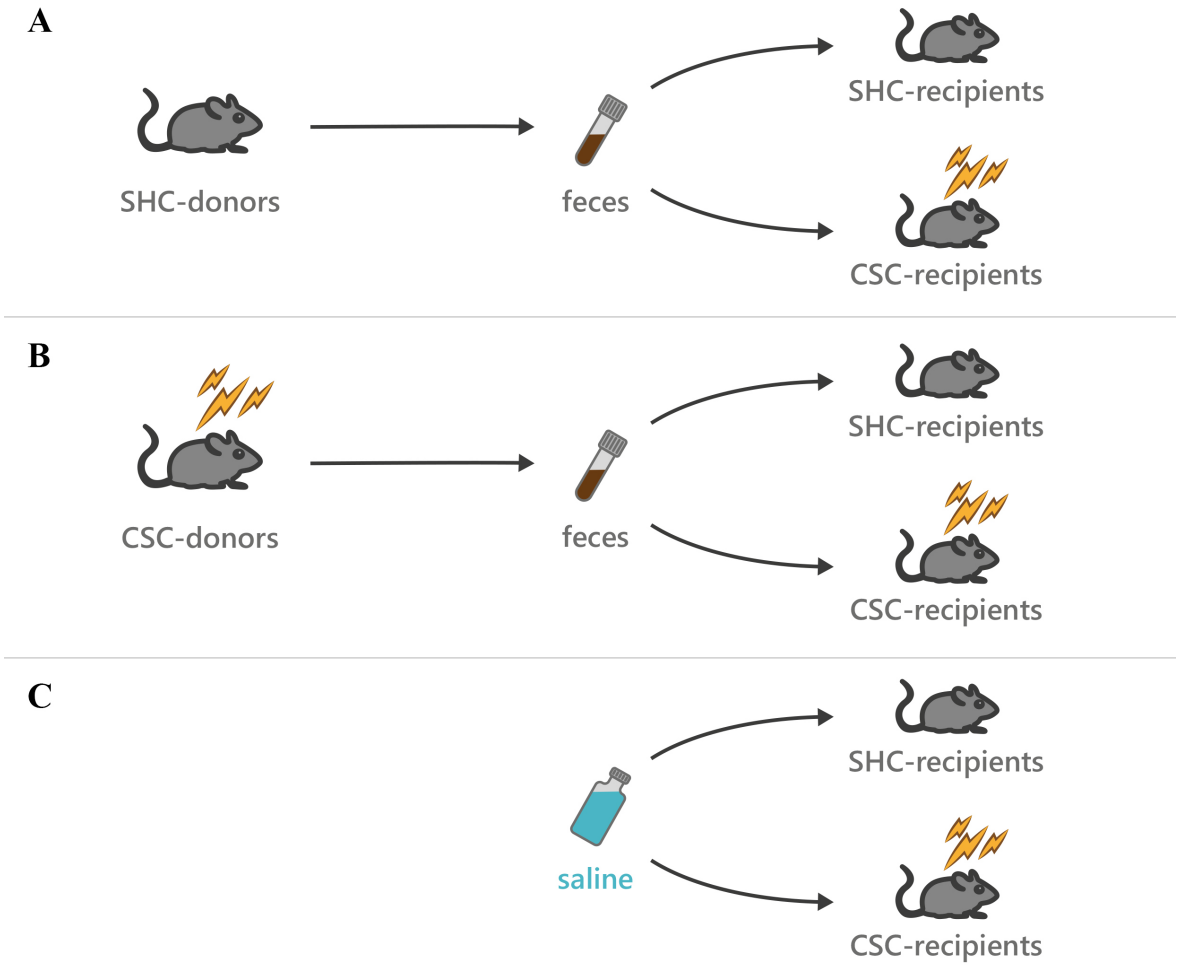

Figure 5: Schematic illustration of the experimental setup. A) Feces from non-stressed single-housed control (SHC)-donor mice were transplanted into a set of SHC- and chronic subordinate colony housing (CSC)-recipient mice. B) Feces from stressed CSC-donor mice were transplanted into another set of SHC- and CSC-recipient mice. C) As controls, saline was infused into another set of SHC and CSC mice.

2.2.3 Chronic subordinate colony housing paradigm

The CSC paradigm is a validated rodent model to induce chronic psychosocial stress in male mice (Reber et al., 2007). The CSC paradigm was performed as described in previous publications (Reber et al., 2007; Langgartner et al., 2015). Briefly, after one to two weeks of single housing, experimental mice were divided into either the SHC or the CSC group. To achieve comparability, all mice were weighted on day 1 and distributed to the respective groups according to their body weights Consequently, the average weight in both groups was approximately the same. On day 1 of CSC, four CSC mice were placed into the cage (plexiglass, 38 x 22 x 35 cm) of a dominant CD-1 mouse (see Fig. 6). Before using the dominant mice as future residents in the CSC paradigm, they were tested for dominance and aggressiveness. Resident mice that were either easily defeated by their opponent or seriously injured their opponent were excluded from following experiments. On days 8 and 15, CSC mice were placed to a novel resident in order to avoid habituation. SHC animals were kept separately and undisturbed except of changing the bedding once a week.

Figure 6: The chronic subordinate colony housing paradigm. The four black experimental mice show submissive behaviour to the white dominant resident mouse, indicated by an upright position.

2.2.4 Preparation of fecal suspensions and fecal transplantation procedure

In the morning of days 4 and 11, fecal suspensions were prepared shortly before transplantation. In order to collect their feces, SHC- and CSC-donor animals were placed separately in cages without bedding (approximately 15-20 min). After collecting a sufficient amount of stool from the respective group of donors (2-3 fecal pellets per mouse; see Fig. 7A), mice were replaced into their home cages and fecal pellets were stored on ice (4°C). Subsequently, fecal pellets were pooled (by group) and homogenised 1:4 in isotonic saline solution (1 part feces + 4 parts saline; Fresenius Kabi, Bad Homburg, Germany; see Fig. 7B) using vortex genie 2 (Scientific Industries Inc., Bohemia, NY, USA). Afterwards, the homogenate was filtered several times through a cell strainer (70 µm, Corning Incorporated, Durham, NC, USA; see Fig. 7C) to avoid congestion of the gavage needle (Heidelberg, Germany). For the FT procedure, the gavage needle was inserted into the anus of the recipient mouse (see Fig. 7E) and 100 µl of the respective solution (saline for Animal set 1, SHC-donor feces for Animal set 2 and CSC-donor feces for Animal set 3; see Fig. 7D) were carefully infused.

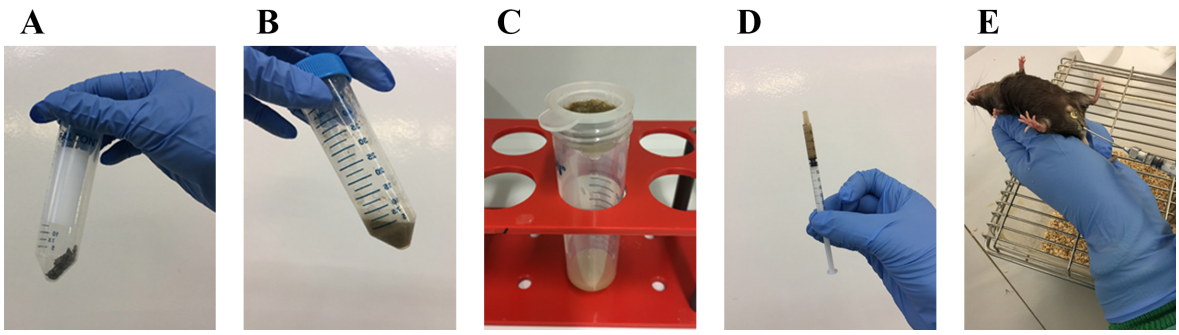

Figure 7: Preparation of fecal suspensions and fecal transplantation procedure. A) Fecal pellets from donor mice were pooled per group and collected, **B)** homogenised **C)** and filtered through a cell strainer. **D)** 100 µl of the respective solution **E)** were rectally infused into the recipient mice.

2.2.5 Open field/ novel object test

To assess CSC and/or FT effects on anxiety-like behaviour, recipient mice were exposed to the OF/NO test on day 19 between 07.00 a.m. and 10.00 a.m. as described before (Langgartner et al., 2017). Briefly, the arena (45 cm length x 27 cm width x 27 cm height) consisted of an inner (27 cm length x 9 cm width) and an outer zone and was illuminated by white light (350 lux). The arena was cleaned entirely before each trial. Within each trial, the mouse was placed into the inner zone (= open field) and was allowed to explore its new surroundings for 5 min. After that, an unfamiliar object (= novel object; plastic lid of a falcon, diameter: 3.5 cm, height: 1.5 cm) was placed into the centre of the inner zone and the mouse had to spend another 5 min in the arena before putting it back into its home cage.

During the OF test, various parameters were analysed using EthoVision XT (Version 9, Noldus Information Technology, Wageningen, The Netherlands), specifically the number of inner zone entries, the time spent in the corners and the total distance moved. During the NO test, the number of object explorations, the time spent in the corners and the distance moved were measured.

2.2.6 Assessment of thymus and adrenal weight

Thymi were removed and stored in ice-cold Dulbecco's Phosphate Buffered Saline (DPBS; Gibco®, Life technologies™, Thermo Fisher Scientific, Waltham, MA, USA) until all of them were collected. Subsequently, thymi were pruned of fat and weighted separately.

Adrenal glands were collected and stored in ice-cold Dulbecco's Modified Eagle Medium (DMEM/F-12, Gibco®, Life technologies™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.1% bovine serum albumin (BSA; BIOMOL GmbH, Hamburg, Germany). Afterwards, adrenals were pruned of fat and weighted separately.

2.2.7 Semi-dry western blot

In order to assess treatment (CSC and/or FT) effects on the level of inflammatory proteins in the colon, semi-dry western blot analysis of the cell surface proteins F4/80 and CD11b was performed.

For protein extraction, frozen (-80°C) pieces (0.5 cm long) of the anal part of the colon were homogenised in ethylenediaminetetraacetic acid (EDTA) lysis buffer (for detail see 2.1.1 buffers and solutions) and incubated 1h on ice at 4°C, followed by centrifugation (13.000 rpm, 15 min, 4°C). Supernatants were stored at -20°C until further analysis. Total protein concentration was measured using a commercial kit (Bicinchoninic Acid Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA).

For the sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), 30 µg of the extracted protein samples were loaded onto sodiumdodecylsulphate polyacrylamide gels (see table 1). Electrophoresis was performed at 60 V for 20 min, then at 110 V for 1 h 55 min using Power Pac ™ HC (Bio-Rad Laboratories GmbH, Hercules, CA, USA). Transfer to nitrocellulose membranes (GE Healthcare Life Science, Chalfont St Giles, UK) was conducted at 0.15 A, using the semi-dry transfer cell Trans-Blot ® SD (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then blocked at room temperature (RT) for 1h with 5% milk powder (Carl Roth GmbH & Co KG, Karlsruhe, Germany) diluted in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T; VWR International, Radnor, PA, USA)). Next, membranes were incubated over night at 4° C with the primary antibody F4/80 (Santa Cruz Biotechnology, Dallas, TX, USA), diluted 1:1000 in TBS-T (5% milk powder). After washing three times for 10 min in TBS-T, membranes were incubated with horseradish peroxidase (HRP)- conjugated goat anti-rabbit antibody (diluted 1:2000 in TBS-T, 1 h at RT; Cell Signaling Technology, Danvers, MA, USA) and then again washed three times for 10 min in TBS-T and once for 5 min in TBS. For visualisation, membranes were incubated with Western ECL Substrate (2 min; Bio-Rad Laboratories, Hercules, CA, USA) and the chemiluminescence was digitalised and analysed by Molecular Imager® ChemiDoc™ XRS+ system and Image Lab™ (Bio-Rad Laboratories, Hercules, CA, USA).

In the next step, membranes were stripped with Re-Blot Plus Strong Antibody Stripping Solution (8 min; Merck Chemicals GmbH, Darmstadt, Germany), blocked twice in 5% milk powder (5 min, diluted in TBS-T) and then incubated with the housekeeper β-Tubulin (diluted 1:1000 in TBS-T with 5% milk powder, 1 h at RT; Cell Signaling Technology, Danvers, MA, USA). After washing three times in TBS-T (each for 10 min) HRP-conjugated anti-rabbit antibody (1:1000 in TBS-T) was used as secondary antibody (30 min at RT). Again, after three steps of washing in TBS-T, membranes were incubated with ECL (4 min), then digitalised and analysed as described above.

After that, membranes were again stripped as mentioned above and incubated over night at 4°C with CD11b antibody (1:2000 in TBS-T with 5% milk powder; Abcam®, Cambridge, UK) and afterwards 1 h at RT with HRP-conjugated anti-rabbit antibody (1:2000 in TBS-T). Visualisation, digitalisation and analysis were performed as described above.

Bands were analysed using Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA). Protein expressions of F4/80 (160 kDa) and CD11b (170 kDa) were normalised to the respective protein expression of the housekeeper β-Tubulin (55 kDa).

Table 1: Preparation of sodiumdodecylsulfate polyacrylamide gels

Resolving gel (10%)	**2x**	**Stacking gel (5%)**	**2x**
Distilled H_2O	3.75 ml	Distilled H_2O	1.725 ml
4x resolving gel buffer	2.25 ml	4x stacking gel buffer	0.75 ml
30% acrylamide/bisacrylamide solution	3 ml	30% acrylamide/bisacrylamide solution	502 µl
10% Ammonium persulfate (APS)	45 µl	10% Ammonium persulfate (APS)	22.5 µl
Tetramethyl-ethylenediamine (TEMED)	4.5 µl	Tetramethyl-ethylenediamine (TEMED)	3.75 µl

2.2.8 mRNA isolation and Real-Time polymerase chain reaction (PCR)

To determine treatment (CSC and/or FT) effects on colonic mRNA levels of pro-inflammatory markers, one piece of 0.5 cm length from the distal part of the colon was frozen at -80°C until mRNA extraction.

The RNeasy Mini kit (Qiagen, Venlo, Netherlands) was used for mRNA isolation according to the manufacturer's instructions. DNA was digested by DNase I (Qiagen, Venlo, Netherlands) and nucleic acid was collected in 30 µl RNase-free water. RNA concentration was measured using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). To generate complementary deoxyribonucleic acid (cDNA), 1 µg of each RNA sample was added to 10 µl of the master mix (see table 2) and then processed according to the cDNA synthesis program (25°C for 10 min, 37°C for 2 h, 85°C for 5 min; storage at -20°C) using polymerase chain reaction (PCR)-System Thermocycler Peqstar (VWR International, Radnor, PA, USA).

Table 2: Preparation of complementary deoxyribonucleic acid (cDNA) master mix

Master Mix Component	**1x**
10x Reverse Transcriptase Buffer	2 µl
25x 100mM Deoxynucleotide Triphosphates (dNTPs)	0.8 µl
10x Reverse Transcriptase Random Primers	2 µl
MultiSubscribe Reverse Transcriptase	0.6 µl
RNase Inhibitor	0.3 µl
RNAse-free water	4.3 µl
Total volume	**10 µl**

For Real-Time PCR, cDNA samples were diluted 1:20 in RNAse-free water. 2.5 µl of the diluted cDNA was mixed with the PCR master mix (see table 3). Real Time PCR was conducted using a ViiA7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with detection of Platinum SYBR Green (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) using the following cycling conditions:

50 °C for 2 min, 95 °C for 10 min, 40 cycles each consisting of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Then, melting-point acquisition was performed (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s). The used primers (Sigma Aldrich, St. Louis, MO, USA) were the housekeeper Ribosomal Proteins (RPL; forward: 5'CCTGCTGCTCTCAAGGTT3', reverse: 5'TGGCTGTCACTGCCTGGTACTT3'), TNF-α (forward: 5'AGGGGCCACC ACGCTCTTCT3', reverse: 5'TGAGTGTGAGGGTCTGGGCCAT3'), CRAMP (forward: 5'CAGCCCTTTCGGTTCAAGAA3', reverse: 5'CCCACCTTTGCGGAGAAGT3') and IFN-γ (forward: 5'TGCTGATGGGAGGAGATGTCT3', reverse 5'TGCTGTCTGGCCTG CTGTTA3'). For quantification of the results, the corresponding Applied Biosystems Software was used and ΔΔ Ct analysis was performed.

Table 3: Preparation of Real-Time polymerase chain reaction (PCR) master mix

Master Mix Component	**1x**
Platinum SYBR green	5 µl
Forward Primer (1:10)	0.4 µl
Reverse Primer (1:10)	0.4 µl
RNase-free water	1.7 µl
Total volume	**7.5 µl**

2.2.9 Determination of the histological damage score in the colon

To investigate treatment effects (CSC and/or FT) on the histological structure of the anal part of the intestinal tract, the colon tissue of all recipient mice was removed after decapitation and cleaned carefully. The HDS was assessed as described previously (Reber et al., 2007) with some modifications. One centimetre of the distal part of the colon was cut longitudinally, fixed in 5% formaldehyde (Otto Fischar GmbH & Co KG, Saarbruecken, Germany) for approximately 48 h and then embedded in paraffin (McCormick Scientific, St Louis, MO, USA). Two 4 µm tissue sections taken at 100 µm distance were cut longitudinally, put on a microscope slide (SuperFrost Ultra Plus®, Thermo Fisher Scientific, Waltham, MA, USA) and stained with haematoxylin-eosin (Hemalun Solution, Merck Chemicals GmbH, Darmstadt, Germany; Eosine Solution, Waldeck GmbH & Co KG,

Muenster, Germany) to assess the histological damage score. For evaluation, three sections of the stained colonic tissue next to the anal part were analysed by histological scoring. Each individual score represents the mean appearance of the sections.

The HDS ranges from 0 to 8 and consists of the sum of two parameters, the epithelium score and the infiltration score (classifications shown in table 4). An investigator blind to treatment performed all evaluations.

Table 4: Classifications of the histological damage score

Epithelium score		**Infiltration score**	
0	Normal morphology	0	No infiltration
1	Loss of goblet cells	1	Infiltrate around crypt bases
2	Loss of goblet cells in large areas	2	Infiltrate reaching to lamina muscularis mucosae
3	Loss of crypts	3	Extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema
4	Loss of crypts in large areas	4	Infiltration of the lamina submucosa

2.2.10 Establishment of Gram staining in colonic tissue

The method of Gram staining in colonic tissue samples was established to analyse whether the number of bacteria stained in the colonic tissue correlates with the HDS of the colon in general and to investigate treatment effects (CSC and/or FT) of the current study on this correlation.

The colonic tissue samples were prepared as follows: one centimetre of the distal part of the colon was cut longitudinally, fixed in 5% formaldehyde (Otto Fischar GmbH & Co KG, Saarbruecken, Germany) for approximately 48 h and then embedded in paraffin (McCormick Scientific, St Louis, MO, USA). 5 µm tissue sections were cut longitudinally, individually transferred to a microscope slide (SuperFrost Ultra Plus®, Thermo Fisher

Scientific, Waltham, MA, USA) and then dried in 37°C over night. To remove the paraffin, slides were heated in a 60°C hot drying-oven (VENTI-Line, VWR International, Radnor, PA, USA) and afterwards put twice into xylene (Roti®-Histol, Carl Roth GmbH & Co KG, Karlsruhe, Germany) for 10 min. To rehydrate the tissue, slides were put twice into 99.8% ethanol (Sigma Aldrich, St. Louis, MO, USA), once into 90% and once into 70% ethanol (each for 2 min; Sigma Aldrich, St. Louis, MO, USA). Gram staining was performed according to the manufacturer's instructions with modifications concerning the differentiation in 99.8% ethanol and/or acetone (see table 5).

Table 5: Procedure of Gram staining

Solution	Time
Crystal Violet solution	1 min
Gram's Iodine solution	5 min
Differentiate in 99.8% ethanol and/or acetone	Depending on mixing ratio: seconds to minutes
Safranin O solution	45 s
Tartrazine solution	10 s
Rinse twice in 99.8% ethanol	2 x 3 s

In general, Gram staining consists of three main steps, the Crystal Violet staining, the differentiation procedure and the Safranin O counterstaining (see Fig. 8). In the first step, the Crystal Violet solution is used to stain the whole colonic tissue cells and the embedded bacteria. By adding Gram's Iodine solution, Crystal Violet-Iodine complexes are formed. In the following differentiation step, ethanol and/or acetone is used to wash out the Crystal Violet solution from tissue cells and from gram-negative bacteria. The latter own a very thin peptidoglycan cell wall layer with low affinity to Crystal Violet-Iodine complexes. Due to the thick layer of peptidoglycans in the cell wall of gram-positive bacteria, Crystal Violet-Iodine complexes cannot be washed-out easily, so these bacteria stay purple. Subsequent counterstaining with the red Safranin O solution only colours colonic tissue cells and gram-negative bacteria, as the cell wall of gram-positive bacteria is already saturated with Crystal Violet-Iodine complexes. In the following step, two rinses in ethanol remove the Safranin O

solution from the tissue cells but not from gram-negative bacteria, which consequently stay red. Finally, yellow Tartrazine solution is used to colour the colonic tissue cells.

For visualisation, images were taken with the Olympus DP73 digital microscope camera (Olympus, Shinjuku, Tokio, Japan) connected to the Leica microscope DMI 6000B (Leica Microsystems GmbH, Wetzlar, Germany).

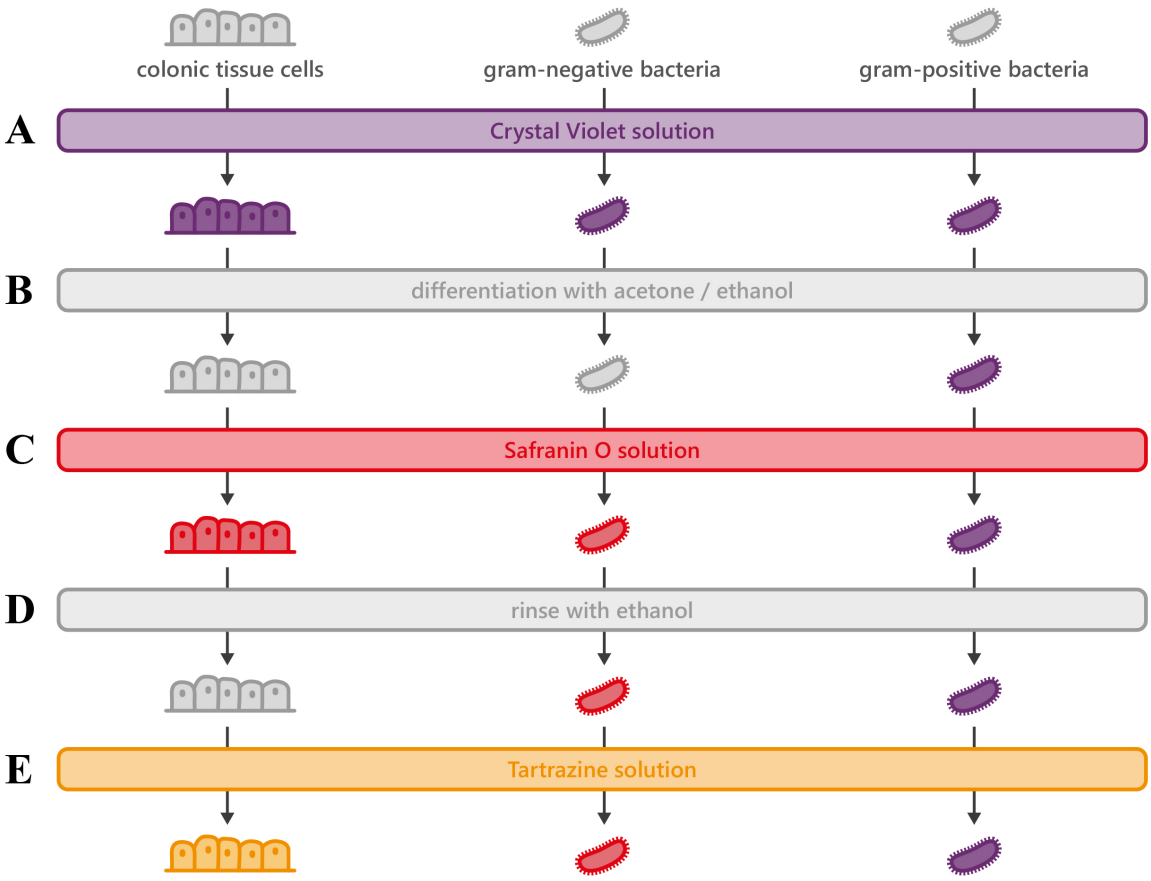

Figure 8: Schematic illustration of Gram Staining. A) The Crystal Violet solution (purple) colours colonic tissue cells, gram-negative and gram-positive bacteria purple. **B)** Differentiation with acetone and/or ethanol (light grey) decolourises colonic tissue cells and gram-negative bacteria; gram-positive bacteria stay purple. **C)** The Safranin O solution (red) colours colonic tissue cells and gram-negative bacteria red. **D)** Rinses in ethanol (light grey) decolourise colonic tissue cells; gram-negative bacteria stay red. **E)** The Tartrazine solution (yellow) colours colonic tissue cells yellow.

2.2.10.1 Establishment of the Gram staining procedure in general

First of all, a test animal (used from remaining tissue samples of previous CSC experiments) possessing a high HDS was used to establish the procedure of Gram staining in general. During the establishment, differentiation with 99.8% ethanol and/or acetone emerged as critical step in the staining procedure (shown in table 5).

At the first attempt, differentiation with 99.8% ethanol for a couple of minutes did not sufficiently wash out the Crystal Violet solution and the tissue stayed purple (see Fig. 9). Consequently, gram-positive bacteria could not be distinguished from the tissue cells.

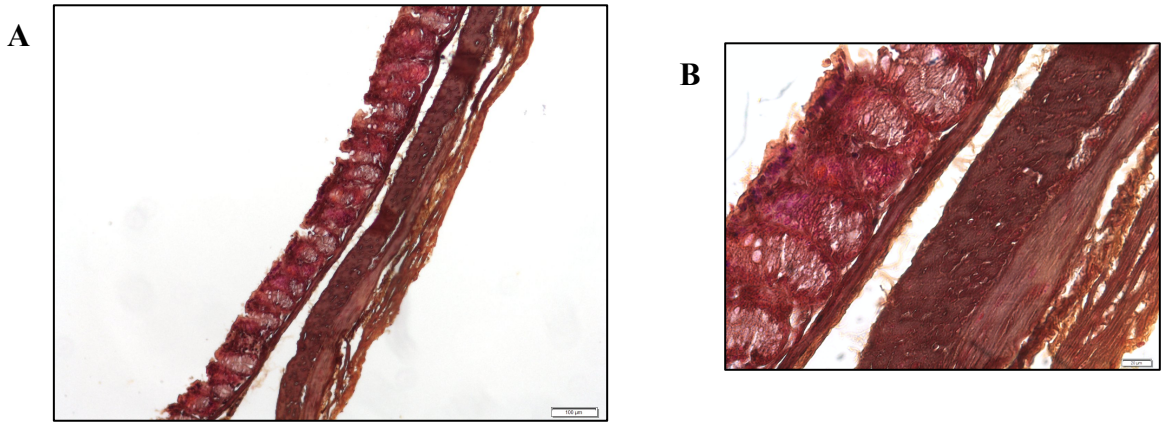

Figure 9: Image of a Gram-stained colonic tissue section with 99.8% ethanol differentiation.
Image **A** (10x magnification) and **B** (40x magnification) show that differentiation with 99.8% ethanol for some minutes during the Gram staining procedure in colonic tissue samples did not remove the Crystal Violet solution.

At the second try, differentiation with acetone for a few seconds completely removed the Crystal Violet staining, leading to complete decolourisation of the gram-positive bacteria (see Fig. 10).

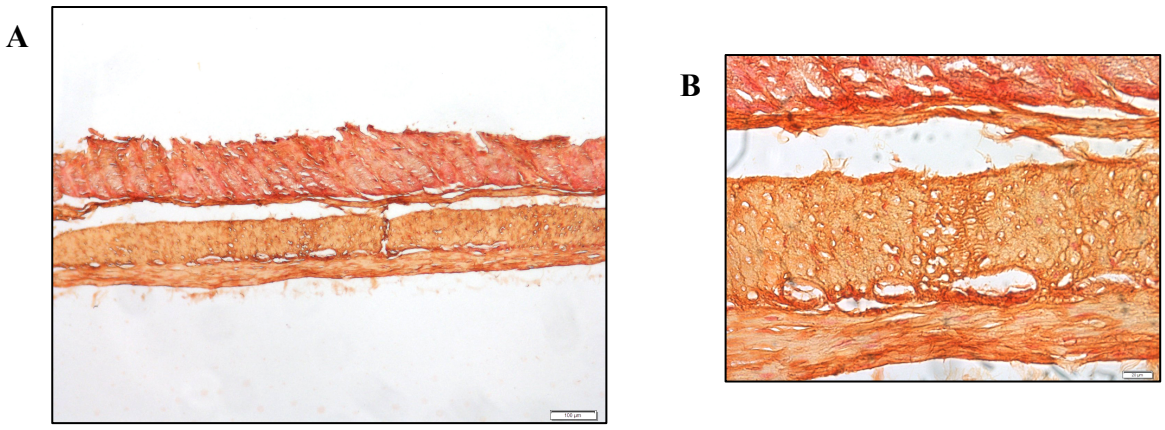

Figure 10: Image of a Gram-stained colonic tissue section with acetone differentiation.
Image **A** (10x magnification) and **B** (40x magnification) show that differentiation with acetone for some seconds during the Gram staining procedure in colonic tissue samples completely washed out the Crystal Violet solution.

The next step was to mix 99.8% ethanol with acetone in different concentrations. The more acetone was added, the more and the faster the tissue and the embedded bacteria decolourised (50:50 (acetone / 99.8% ethanol): 3 s; 40:60 (acetone / 99.8% ethanol): 5 s; 10:90 (acetone / 99.8% ethanol): 1:30 min; 1:100 (acetone / 99.8% ethanol): 20 min). The best outcome was achieved with the concentration 1:100 (acetone / 99.8% ethanol) for 20 min (see Fig. 11). This concentration was used in the following staining procedures.

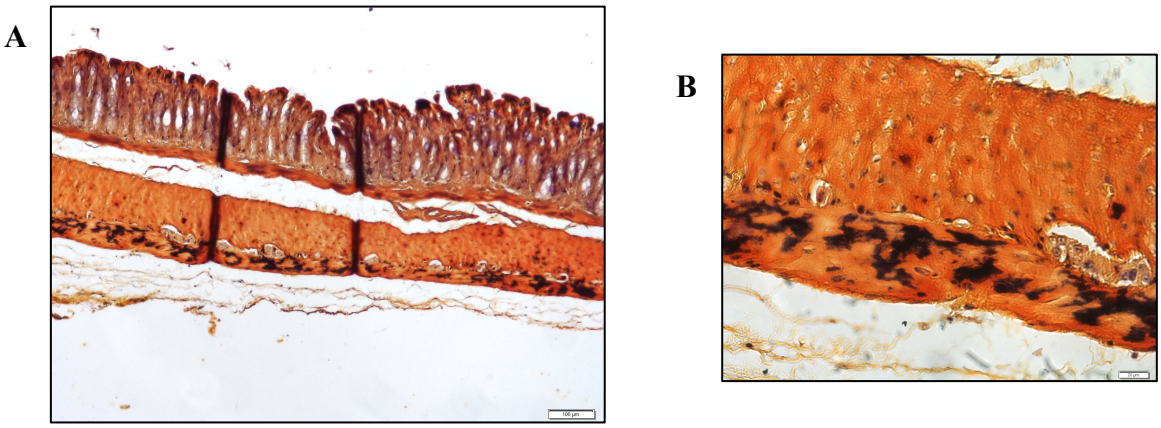

Figure 11: Image of a Gram-stained colonic tissue section with acetone/ 99.8% ethanol (1:100) differentiation.

Image **A** (10x magnification) and **B** (40x magnification) show that differentiation with acetone/ 99.8% ethanol (mixing ratio 1:100) for 20 minutes during the Gram staining procedure in colonic tissue samples led to a clear visualisation of purple-stained gram-positive bacteria.

2.2.10.2 Correlation of the number of stained bacteria with the histological damage score

To correlate the number of bacteria embedded in the colonic tissue with the HDS, colonic tissue sections of nine animals (used from remaining tissue samples of previous CSC experiments) with different HDS (animal 1: 0.7; animal 2: 1.3; animal 3: 2.0; animal 4: 3.0; animal 5: 4.3; animal 6: 5.0; animal 7: 6.0; animal 8: 7.3; animal 9: 7.7) were stained simultaneously in a staining chamber with Gram staining (Sigma-Aldrich, St. Louis, MO, USA) according to the established procedure acquired in the previous step (as described in chapter 2.2.10.1). Differentiation was done with acetone and 99.8% ethanol (1:100).

As a result, it was not possible to compare the colonic tissue sections of the nine animals to establish a correlation between the HDS and the bacterial load in the colonic tissue. Although all nine microscope slides with the colonic tissue sections were treated equally and stained simultaneously in the very same staining chamber, the stained tissue looked immensely different. Some tissue sections were light-coloured and the Crystal Violet solution was completely removed (see Fig. 12A and B). Some other tissue sections showed blurry violet artefacts with the result that defined gram-positive bacteria could not be distinguished from the tissue cells (see Fig 12C and D). Nevertheless, there were also tissue sections with clearly visible gram-positive bacteria embedded in the lamina muscularis mucosae and the muscle layer (see Fig. 12E - G). Besides, it was never possible to identify gram-negative bacteria.

An explanation for the incomparability of the colonic tissue sections might be the execution of the differentiation step with ethanol and acetone in the staining chamber. As the tissue sections were not exactly equidistant within the staining chamber, it could be possible that the solution of ethanol and acetone did not reach all the tissue sections to the same extent and therefore washed out the Crystal violet solution a little less in slightly closer tissue sections and a little more in slightly distant tissue sections. Due to the time limitation of my experiments in the laboratory, it was not possible to test further approaches such as another setup for the differentiation step. Consequently, the non-completed establishment of the method of Gram staining could not be employed on the experimental animals of this study.

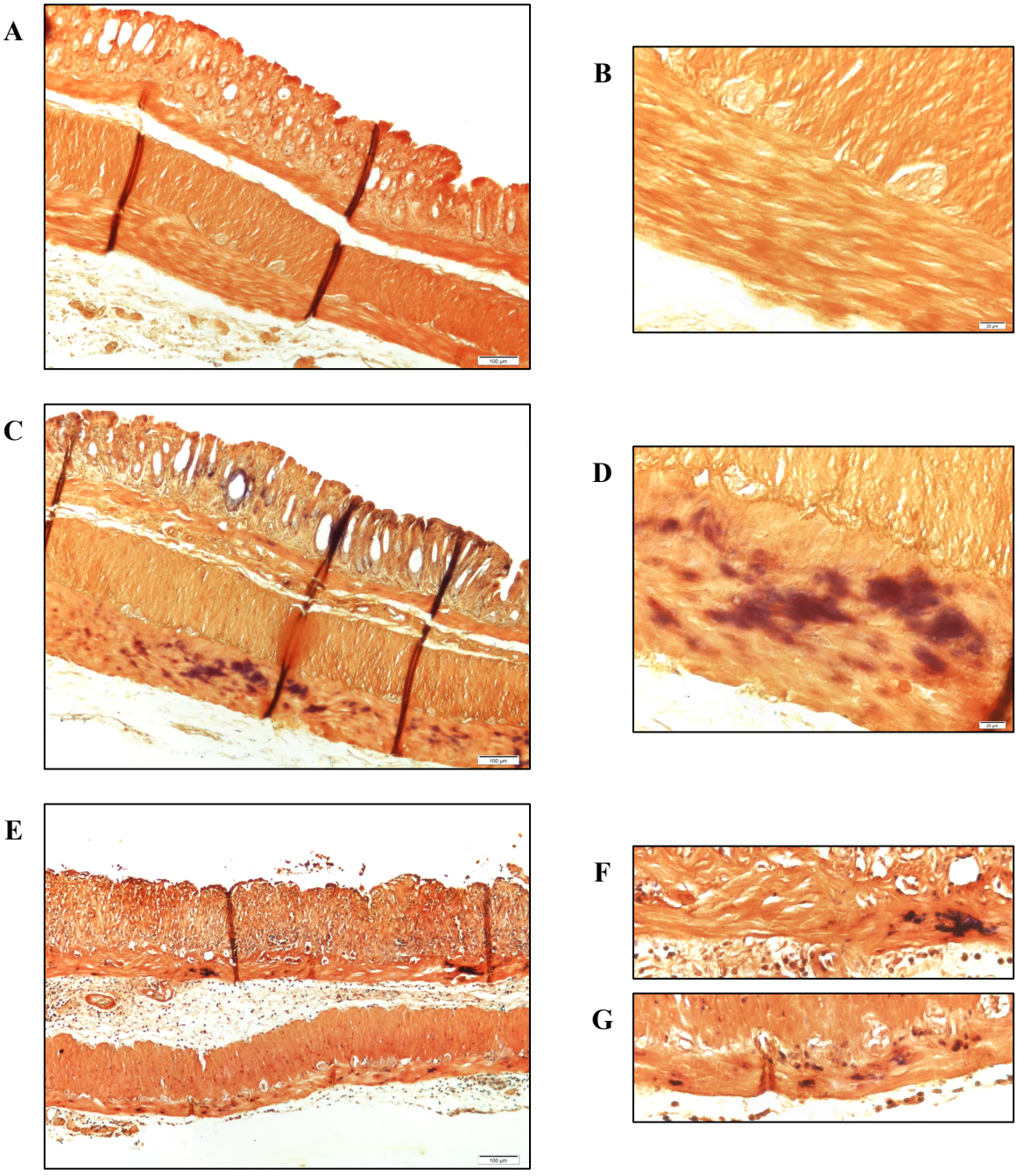

Figure 12: Representative images of colonic tissue sections stained with Gram staining in the same staining chamber. Differentiation with acetone / 99.8% ethanol (mixing ratio: 1:100) completely washed out the Crystal Violet solution and no gram-positive bacteria can be seen in image **A** (10x magnification) or **B** (40x magnification). Image **C** (10x magnification) and **D** (40x magnification) show blurry violet artefacts of the Crystal Violet staining. Gram-positive bacteria can be seen in image **E** (10x magnification). Image **F** (40x magnification) shows gram-positive bacteria in the lamina muscularis mucosae. Image **G** (40x magnification) shows gram-positive bacteria in the muscle layer.

2.2.11 Statistics

For statistical comparisons, the software package IBM SPSS statistics (version 23.0; IBM Corporation, Armonk, NY, USA) was used. Kolmogorov-Smirnov test using Lilliefors` significance was employed to test normal distribution of all acquired data sets. Outliers in normally distributed data sets were identified using Grubb's test and excluded from further analysis. Normally distributed data sets were subsequently analysed using parametric statistics, i.e. two-way ANOVA (two factors, two or more independent samples; readouts: time in corners OF, entries to OF, distance moved OF, distance moved NO, absolute adrenal weight, absolute thymus weight) and parametric Student's *t*-test (one factor, two independent samples; readouts: absolute thymus weight). Non-normally distributed data sets were analysed using non-parametric statistics, i.e. Mann-Whitney *U* (MWU) test (one factor, two independent samples; readouts: time in corners NO, NO explorations, relative TNF-α mRNA, relative CRAMP mRNA, relative IFN-γ mRNA, F4/80 protein expression, CD11b protein expression, histological damage score) and Kruskal-Wallis ANOVA (one factor, more than two independent samples; readouts: time in corners NO, NO explorations, relative TNF-α mRNA, relative CRAMP mRNA, relative IFN-γ mRNA, F4/80 protein expression, CD11b protein expression, histological damage score). All tests comparing more than two samples were followed, when a significant main effect was found, by *post-hoc* analysis using Bonferroni pairwise comparison. For graphical illustration, the software package SigmaPlot (version 11.0; Systat Software Inc., San José, CA, USA) was used. Normally distributed data are presented as bars (mean + standard error of the mean (SEM)). Non-normally distributed data are presented as box plots. Solid line represents the median, dashed line represents the mean for each data set. Lower box indicates 25th, upper box indicates 75th percentile; 10th (lower error bar) and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. The level of significance was set at $p \leq 0.05$, $p \leq 0.09$ was considered as a trend.

3 Results

3.1 Effects of CSC and FT on anxiety-like behaviour in the OF/NO-test

Exposure to 19 days of CSC increased some parameters of anxiety-related behaviour in the OF/NO test in all experimental groups compared to the respective SHC group as indicated by a significant main effect of the factor stress on the time spent in the corners during the OF test (two-way ANOVA: $F_{1,76} = 18.207$; $P < 0.001$). In detail, saline-treated animals ($P = 0.03$), recipients of SHC-donor feces ($P = 0.021$) and recipients of CSC-donor feces ($P = 0.006$) significantly spent more time in the corners than the respective SHC control group (see Fig. 13A). Correspondingly, saline-treated (MWU-test: $P = 0.001$), SHC-recipient (MWU-test: $P = 0.039$) as well as CSC-recipient (MWU-test: $P = 0.001$) CSC versus (vs.) SHC mice significantly spent more time in the corners during the NO test (see Fig. 13D).

Furthermore, there was a significant main effect in the distance moved during the NO test (two-way ANOVA: $F_{1,75} = 28.876$; $P < 0.001$). In detail, saline-treated CSC ($P = 0.016$), SHC-recipient CSC ($P = 0.018$) and CSC-recipient CSC ($P < 0.001$) showed a decrease in locomotor activity during the NO test compared to SHC control mice (see Fig. 13F). However, the number of entries to the OF (two-way ANOVA: $F_{1,76} = 11.169$; $P = 0.001$) was only significantly decreased in saline-treated CSC ($P = 0.018$) as well as CSC-recipient CSC vs. SHC animals ($P = 0.045$; see Fig. 13B), but not in SHC-recipient mice. Similarly, only saline-treated CSC (MWU-test: $P = 0.027$) as well as CSC-recipient CSC mice (MWU-test: $P = 0.012$) exhibited a decreased number of object explorations during the NO test compared to unstressed SHC mice (see Fig. 13E).

Moreover, there was a significant main effect of the factor stress in the distance moved during the OF test (two-way ANOVA: $F_{1,76} = 5.011$; $P = 0.028$). In detail, SHC-recipient CSC ($P = 0.04$) but not saline-treated CSC and CSC-recipient CSC animals displayed a decrease in locomotor activity during the OF test compared to the respective SHC control group (see Fig. 13C).

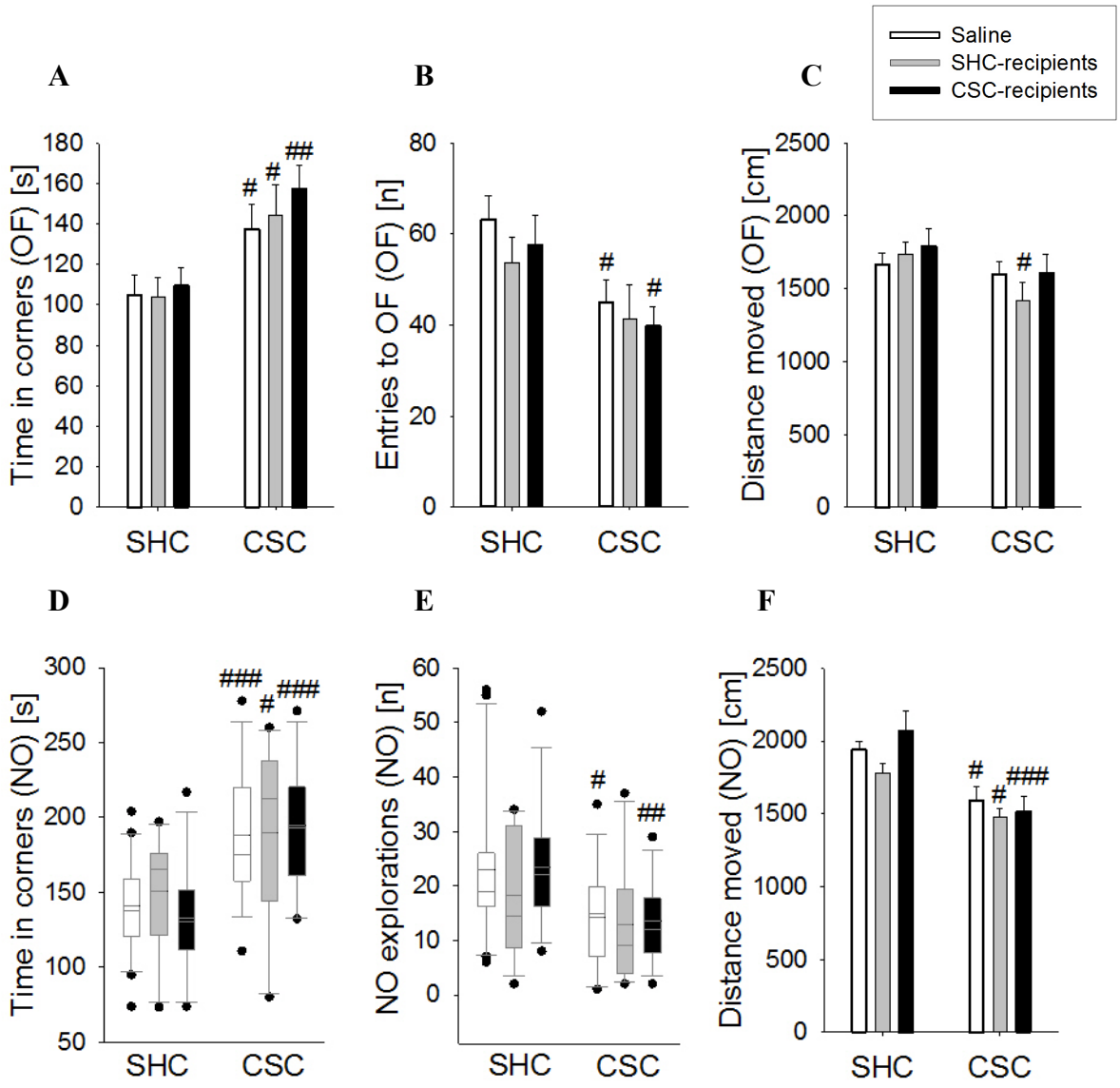

Figure 13: Behavioural parameters of the open field/novel object (OF/NO) test on day 19 of the chronic subordinate colony housing (CSC) paradigm. Exposure to 19 days of CSC **A)** increased the time spent in corners during the OF test of saline-treated animals, single-housed control (SHC)-recipients and CSC-recipients, **B)** decreased the number of entries to the OF of saline-treated and CSC-recipient animals during the OF test, **C)** decreased the distance moved during the OF test of SHC-recipients, **D)** increased the time spent in corners during the NO test of saline-treated animals, SHC-recipients and CSC-recipients, **E)** decreased the number of object explorations of saline-treated animals and CSC-recipients during the NO test and **F)** decreased the distance moved during the NO test of saline-treated animals, SHC-recipients and CSC-recipients compared to the respective SHC group. s = seconds, n = numbers, cm = centimetre. Saline-treated animals (SHC: n = 20, CSC: n = 14), SHC-recipients (SHC: n = 12, CSC: n = 12), CSC-recipients (SHC: n = 12, CSC: n = 12). Normally distributed data are presented as bars (mean + standard error of the mean). Non-normally distributed data are presented as box plots. Solid line represents the median, dashed line represents the mean for each data. Lower box indicates 25th, upper box indicates 75th percentile. 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are shown. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ versus respective SHC. Modified from (Langgartner et al., 2018b), CC BY 4.0, http://creativecommons.org/licenses/by/4.0/

3.2 Effects of CSC and FT on thymus and adrenal weight

Analysis of the absolute thymus weight showed a significant main effect in the factor stress (two-way ANOVA: $F_{1,73} = 6.283$; $P = 0.014$). In detail, CSC-recipient mice ($P = 0.05$) showed a significant decrease in the absolute thymus weight compared to the respective SHC group. Besides, independent t-test analysis revealed a trend towards a loss in thymus weight in saline-treated CSC vs. SHC mice ($P = 0.082$). However, this effect was not visible in SHC-recipient mice (see Fig. 14A).

Independent of the factor FT, chronic exposure to the CSC paradigm significantly increased the absolute adrenal weight in all groups compared to the respective SHC group (two-way ANOVA: $F_{1,69} = 56.139$; P = < 0.001; saline-treated animals: $P < 0.001$; SHC-recipients: $P < 0.001$; CSC-recipients: $P = 0.003$; see Fig. 14B).

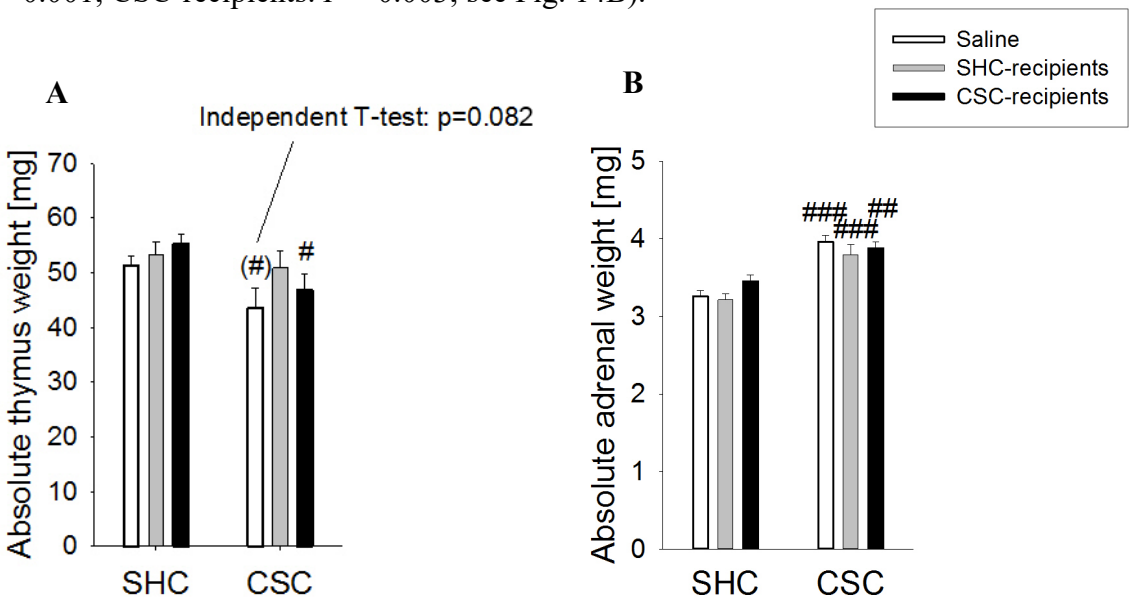

Figure 14: Absolute thymus and adrenal weight after decapitation in the morning of day 20 of the chronic subordinate colony housing (CSC) paradigm. Exposure to 19 days of CSC **A)** decreased the absolute thymus weight in saline-treated and CSC-recipient animals and **B)** increased the absolute adrenal weight in saline-treated animals, single-housed control (SHC)-recipients and CSC-recipients compared to the respective SHC group. mg = milligram. Assessment of thymus weight: saline-treated animals (SHC: n = 20, CSC: n = 13), SHC-recipients (SHC: n = 12, CSC: n = 12), CSC-recipients (SHC: n = 12, CSC: n = 12). Assessment of adrenal weight: saline-treated animals (SHC: n = 18, CSC: n = 11), SHC-recipients (SHC: n = 12, CSC: n = 12), CSC-recipients (SHC: n = 12, CSC: n = 12). Normally distributed data are presented as bars (mean + standard error of the mean). $^{(\#)}P$ = strong trend, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ versus respective SHC. Modified from (Langgartner et al., 2018b), CC BY 4.0, http://creativecommons.org/licenses/by/4.0/

3.3 Effects of CSC and FT on colonic inflammation

Statistical analysis revealed no effect of the factors stress and/or FT on colonic protein levels of F4/80 (see Fig. 15A) and CD11b (see Fig. 15B), colonic mRNA levels of TNF-α (see Fig. 15C), CRAMP (see Fig. 15D) and IFN-γ (see Fig. 15E) as well as the HDS (see Fig. 15F).

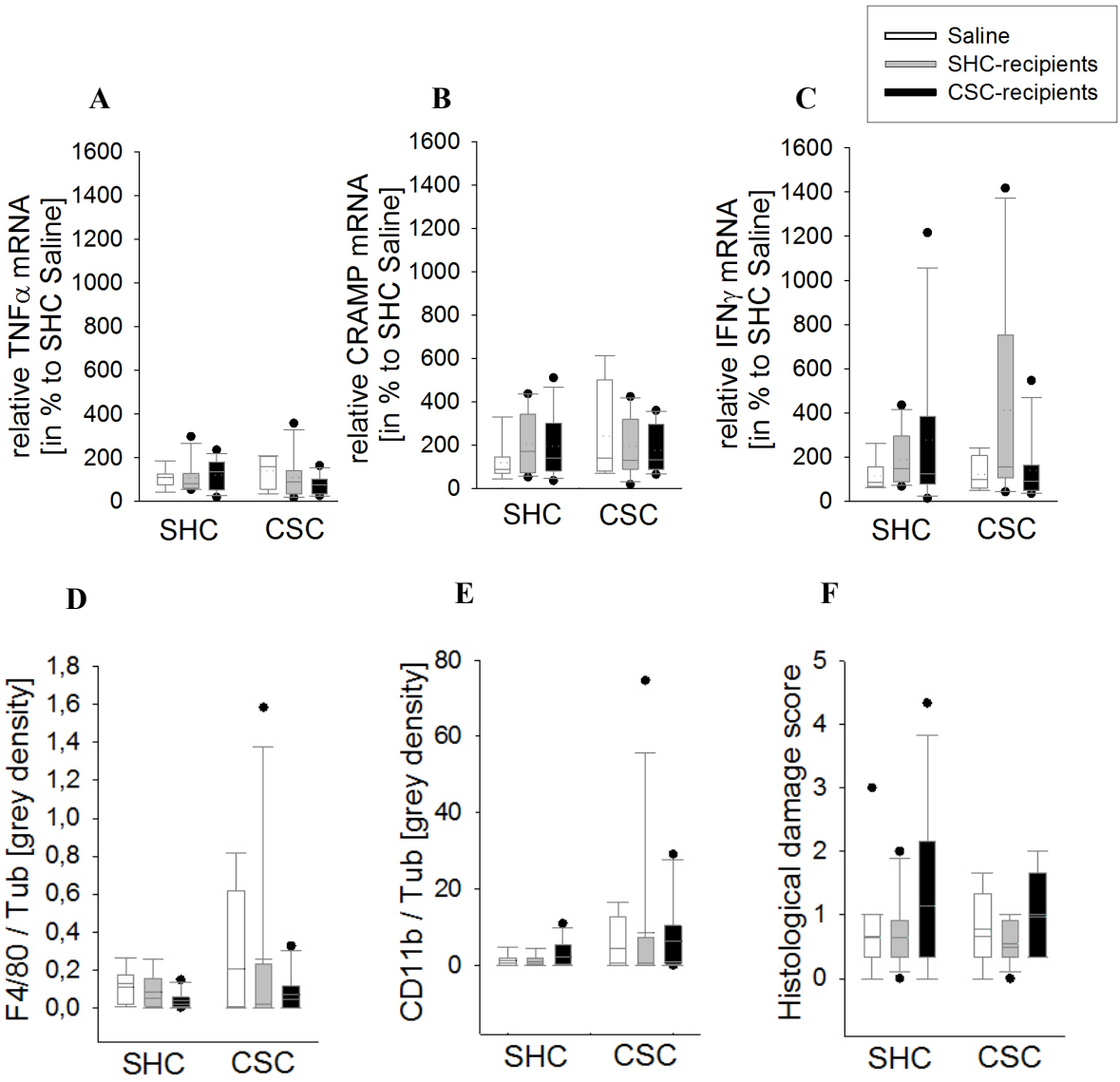

Figure 15: Parameters of colonic inflammation after decapitation in the morning of day 20 of the chronic subordinate colony housing (CSC) paradigm. Exposure to 19 days of CSC **A)** did not influence the colonic messenger ribonucleic acid (mRNA) levels of tumor necrosis factor-alpha (TNF-α), **B)** cathelin-related antimicrobial peptide (CRAMP) or **C)** interferon-gamma (IFN-γ) and **D)** did not change the colonic protein levels of F4/80 or **E)** cluster of differentiation molecule 11b (CD11b) and **F)** did not alter the histological damage score compared to the respective single-housed control (SHC) group. β-Tubulin (Tub) represents the loading control for F4/80 and CD11b protein expression. Assessment of colonic protein and mRNA levels: saline-treated animals (SHC: n = 8, CSC: n = 4), SHC-recipients (SHC: n = 11, CSC: n = 11), CSC-recipients (SHC: n = 12, CSC: n = 12). Assessment of histological damage score: saline-treated animals (SHC: n = 19, CSC: n = 13), SHC-recipients (SHC: n = 12, CSC: n = 12), CSC-recipients (SHC: n = 12, CSC: n = 11). Normally distributed data are presented as bars (mean + standard error of the mean). Non-normally distributed data are presented as box plots. Solid line represents the median, dashed line represents the mean for each data. Lower box indicates 25th, upper box indicates 75th percentile. 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are shown. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ versus respective SHC. Modified from (Langgartner et al., 2018b), CC BY 4.0, http://creativecommons.org/licenses/by/4.0/

4 Discussion

The results of my thesis provide first evidence that rectal transplantations of feces from non-stressed SHC-donor mice into chronically stressed CSC-recipient mice are able to ameliorate stress-induced anxiety-related behaviour in the OF/NO test as well as stress-induced alterations in the physiology as indicated by the prevention of thymus atrophy. Though, transplantations of SHC-donor feces did not prevent adrenal hypertrophy of the recipients following chronic stress exposure.

In turn, rectal transplantations of feces obtained from chronically stressed CSC-donor mice into non-stressed SHC-recipient mice did neither lead to increased anxiety-like behaviour in the OF/NO test nor to stress-related alterations in physiological parameters, indicating that FT did not result in the transmission of stress-related characteristics from donors to recipients, at least not in the parameters that I assessed in my study.

Besides, the results of my experiments indicate that FT via the rectal route and without the use of an antibiotic pre-treatment is able to influence certain behavioural and physiological parameters in the recipients. Furthermore, FT had no effects on the expression of colonic inflammatory markers and the level of histological damage in the colon of the recipients, indicating that the rectal route represents a safe type of application for FT.

The side aim to establish the method of Gram staining in colonic tissue sections and a correlation of the positively stained bacteria with the HDS could not be achieved without exceeding the time limit of my experiments in the laboratory. To finalise the establishment, the suggested approaches in section 2.2.10.2 should be tested in further study.

4.1 Effects of FT on the behaviour of recipient mice

Numerous studies show that chronic stress affects the anxiety-related behaviour of experimental animals (Sterlemann et al., 2008). Besides, it has been shown that the intestinal microbiota composition is able to influence anxiety-related behaviour (Clarke et al., 2013; De Palma et al., 2017). Given that the CSC paradigm has an effect on both the anxiety-related behaviour (Reber et al., 2007; Reber and Neumann, 2008) and the intestinal microbiota composition (Reber et al., 2011; Reber et al., 2016), one aim of this study was to investigate if transplantations of feces from unstressed SHC-donor mice into chronically stressed CSC mice would lead to a reduction of stress-induced anxiety-related behaviour in the recipients and *vice versa* if fecal microbiota obtained from chronically stressed CSC-donor mice transplanted into non-stressed SHC mice would induce anxiety-like behaviour in the recipients. Therefore, mice underwent behavioural testing in the OF/NO test at the end (day 19) of the paradigm.

In accordance with previous findings (Reber et al., 2007; Langgartner et al., 2017), the CSC procedure increased anxiety-related behaviour in the saline-treated group, indicating that the rectal transplantation procedure itself did not have an influence on the outcome. More precisely, saline-treated CSC animals displayed a decreased number of entries to the centre of the arena during the OF test, spent significantly more time in the shaded corners during both the OF and the subsequent NO test and moved a shorter distance during the NO test compared to saline-treated SHC mice. Since mice naturally live in dark and narrow subways and have an aversion to wide spaces, this behaviour is generally interpreted as an expression of anxiety whereas increased time spent in the brighter inner zone of an arena can be seen as anxiolytic-like behaviour (Prut and Belzung, 2003). Besides, saline-treated CSC animals showed a decreased number of NO explorations during the NO test compared to saline-treated SHC mice, indicating that their exploratory behaviour was reduced due to increased fear (Tang and Sanford, 2005). Different from the other parameters, the total distance moved during the OF test did not differ between saline-treated SHC and CSC animals, consistent with the view that the total distance moved in the OF test rather displays a parameter for the general locomotor activity and exploratory behaviour than a parameter for anxiety-like behaviour (Mathis et al., 1994; Bailey et al., 2009). Consequently, the CSC-induced elevation of anxiety-related behaviour seen in the above-mentioned parameters of the

OF/NO test were not influenced by differences in general locomotion (Langgartner et al., 2017; Foertsch et al., 2017).

Though, looking at the results of the OF/NO test of SHC-recipient animals, SHC-donor feces seemed to have an effect on stress-induced anxiety-related behaviour. In detail, SHC-recipient CSC mice did neither show the typical CSC-induced decrease in OF entries nor in NO explorations. This could mean that the infusions of SHC-donor feces slightly ameliorated CSC-induced anxiety, at least in these two parameters. However, it is important to note that SHC-recipient CSC mice displayed a reduction in the total distance moved during the OF test compared to the respective SHC group. On the one hand this could mean that the assessed parameters for anxiety-related behaviour in the OF/NO test are confounded by the decrease in general locomotion as mentioned above. On the other hand, however, in some literature, the total distance moved during the OF test is indeed taken as a parameter for anxiety-related behaviour (Denenberg, 1969; Prut and Belzung, 2003). Interpreting the results in this way, SHC-recipient CSC mice could show increased anxiety-like behaviour, referring to the total distance moved during the OF test, whereas saline-treated and CSC-recipient CSC animals do not. This could be due to a stronger resident in the SHC-recipient CSC colony who intensified the submission and increased anxiety-like behaviour. However, this hypothesis does not fit to the decrease of anxiety-like behaviour of SHC-recipient CSC mice seen in the other parameters assessed in the OF/NO test.

Recipients of CSC-donor feces showed similar outcomes of the assessed parameters to saline-treated animals, suggesting that CSC-donor feces did neither induce nor worsen anxiety-related behaviour in SHC or CSC recipients, respectively.

Comparing my study to similar studies using FT to assess the impact of the transplanted microbiome on the behaviour of the recipient, others also worked with the OF test but additionally chose further behavioural tests to investigate the anxiety-like behaviour. For example, the step-down test, the light/dark preference test and the elevated plus-maze test were additionally employed in these studies (Bruce-Keller et al., 2015; Kelly et al., 2016; De Palma et al., 2017). By the use of at least two behavioural tests, these studies were able to confirm the transmission of the behavioural phenotype of the FT donors to the recipients

in different test settings. Therefore, in future CSC-studies, further behavioural tests that cover various aspects of anxiety-related behaviour and general locomotion are needed to receive a detailed insight into the potential of FT to influence anxiety-related behaviour of experimental CSC animals. In this context, in addition to the OF/NO test, the 24h locomotion tracking could be used to investigate the general locomotion in an individual experimental set-up.

4.2 Effects of FT on the physiology of recipient mice

It is well known that chronic stress leads to several physiological alterations in the organism like exaggerated HPA axis activation, enlargement of the adrenal glands and thymus involution (Selye, 1946; Klein et al., 1992; Engler and Stefanski, 2003). Moreover, several studies show that the intestinal microbiota composition plays an important role in the physiology of the host (Sudo et al., 2004; Huo et al., 2017). Therefore, another aim of my study was to examine if transplantations of feces from SHC-donor mice into CSC-recipient mice could prevent chronic stress-induced effects on physiological parameters and if infusions of CSC-donor feces into SHC-recipients could induce chronic stress-induced effects in control animals, respectively.

In line with previous findings (Reber et al., 2007; Langgartner et al., 2018a), the CSC paradigm in my study induced adrenal hypertrophy in saline-treated CSC vs. SHC mice. Furthermore, the CSC paradigm also induced adrenal hypertrophy in SHC-recipient CSC and CSC-recipient CSC mice vs. respective SHC control mice, indicating that the transplanted microbiota had no effect on the adrenal weight.

In addition to the adrenal weight, further physiological parameters were assessed in the recently published paper about this study (Langgartner et al., 2018b). In detail, basal plasma CORT concentrations, measured in trunk blood after sacrificing the mice in the morning of day 20, were increased in all of the three CSC groups compared to the respective SHC group (Langgartner et al., 2018b). However, SHC-recipient CSC mice showed a significantly lower increase of the basal plasma CORT level compared to saline-treated and CSC-recipient CSC mice (Langgartner et al., 2018b). Moreover, adrenal *in vitro* ACTH

stimulation revealed that the adrenal glands of all SHC and CSC groups secreted higher levels of CORT after incubation with ACTH compared to respective basal CORT levels after saline incubation (Langgartner et al., 2018b), indicating that adrenals of CSC animals do not suffer from ACTH insufficiency (Uschold-Schmidt et al., 2012). Importantly, both the elevated basal morning plasma CORT concentrations and the maintained sensitivity of the adrenal glands to *in vitro* ACTH stimulation in CSC vs. SHC mice in my study do not correspond to previous CSC-studies. It has been consistently shown that the adrenal glands of CSC vs. SHC mice fail to produce higher amounts of CORT after *in vitro* stimulation with increasing ACTH doses, indicating that the adrenals become insensitive to an ACTH stimulation after prolonged stressor exposure (Reber et al., 2007; Uschold-Schmidt et al., 2012). Moreover, previous CSC-studies have repeatedly shown that basal plasma CORT levels in the morning of day 20 are not elevated in CSC vs. SHC mice when CSC mice were not acutely stressed before killing (Reber et al., 2007; Uschold-Schmidt et al., 2012). Due to the finding that basal plasma CORT concentrations are indeed higher in CSC vs. SHC mice exposed to an acute heterotypic stressor (elevated platform exposure) 5 min before killing (Uschold-Schmidt et al., 2012), CSC colonies are constantly videotaped during the hour before killing to see whether or not CSC mice are acutely stressed by attacks of the resident. In my study, the videos show that the resident did not attack the CSC mice in the hour before killing, indicating that the elevated CORT levels cannot be explained in this way (Langgartner et al., 2018b).

Taken together, in my study, the CSC paradigm in combination with the FT procedure must have influenced the HPA axis in a different way than the CSC paradigm alone. It is possible that, compared to CSC alone, both the exposure to CSC and the infusions of saline or feces on day 4 and again on day 11 display a more severe stressor for the animals. Consequently, the basal morning CORT levels stay elevated in the recipient CSC mice. However, given that the increase of the basal morning plasma CORT concentration is less pronounced in SHC-recipient CSC mice compared to saline-treated and CSC-recipient CSC mice, it is possible that the transplanted microbiota obtained from SHC-donor mice alleviated the effect of CSC and FT on the plasma morning CORT levels.

This hypothesis would also be in line with the findings of my study concerning the thymus weight. In detail, in line with previous studies (Reber et al., 2007), the CSC paradigm induced thymus atrophy in saline-treated CSC vs. SHC mice. CSC-recipient CSC mice also showed thymus atrophy compared to respective SHC mice. However, infusions of SHC-donor feces prevented thymus atrophy in recipient CSC mice.

In the following, a few studies will be presented to have a broader overview about this topic. In particular, the study of Engler and Stefanski shows that subordinate male rats display a significant loss of thymus weight after a 24-hour confrontation with a dominant male resident compared to undisturbed control rats (Engler and Stefanski, 2003). Given that the thymus possesses a high density of glucocorticoid type-II receptors that make the thymocytes highly sensitive to glucocorticoids (Lowy, 1989), thymus atrophy can be seen as the consequence of elevated serum CORT levels in the experimental rats following 24h of social stress. Adrenalectomy four weeks prior to the social stress exposure prevents thymus atrophy, verifying that glucocorticoids play a key role in the regulation of thymic function (Engler and Stefanski, 2003). Interestingly, the loss of thymus weight was not reversible within seven days upon completion of the social stress exposure, assuming that the effect of CORT on thymus weight can be long lasting, at least more than seven days (Engler and Stefanski, 2003). In line with this, a previous study of our laboratory employing the CSC procedure for 19 days in mice measured plasma CORT concentrations and the thymus weight at several time points (days 2, 7, 14, 20) of the CSC procedure (Reber et al., 2007). Interestingly, plasma CORT levels are only increased on day 2 of stressor exposure and afterwards return to baseline levels whereas thymus atrophy can be found at all time points (Reber et al., 2007).

In my study, in contrast to the other experimental CSC groups, SHC-recipient CSC mice do not show a loss of thymus weight. Interestingly, this experimental group also exhibits a significantly less pronounced elevation of plasma CORT levels in the morning of day 20. In line with the above-mentioned study of Engler and Stefanski (Engler and Stefanski, 2003), it is plausible that the thymus weight in SHC-recipients did not decrease because SHC-donor feces prevented highly increased plasma CORT levels. At this point, it is important to note that the first transplantation of SHC-donor feces took place on day 4 of the CSC procedure,

while the above-quoted CSC-study (Reber et al., 2007) describes that the effect of plasma CORT on thymus atrophy occurs on day 2 of stressor exposure. Nevertheless, in this case, it is quite likely that transplantations of SHC-donor feces prevented thymic involution by preventing highly increased plasma CORT levels already in the early phase (around day 2) of stressor exposure.

Concluding this part, together with the possible amelioration of stress-induced anxiety, transplantations of SHC-donor feces seem to have stress-protective effects on behavioural as well as specific physiological parameters.

4.3 Effects of FT on colonic inflammation

My study is among the first ones that performed FT via the rectal route. Before, studies employing FT mostly used oral gavage to infuse donor feces into the recipient animal (Bercik et al., 2011; Bruce-Keller et al., 2015; Kelly et al., 2016; De Palma et al., 2017). In my study, fecal suspensions were infused through a gavage needle that was carefully inserted into the rectum of the recipient mice. The difficulty of this procedure is that the gavage needle is stiff and could possibly injure the sensitive rectal mucosa and consequently cause inflammation. Furthermore, as the rectal infusions are extraneous substances in the colon of the host, they could probably activate the local intestinal immune system and could hereby induce inflammation. Given that the method of rectal infusion is new and untested, local colonic inflammatory parameters were analysed to exclude that the rectal infusion procedure itself causes colonic injury and inflammation. Additionally, colonic inflammatory parameters were assessed to test whether transplantations of SHC-donor feces into CSC-recipients and *vice versa* CSC-donor feces into SHC-recipients lead to interactions with the local intestinal immune system.

In line with a recent study of our laboratory that found that CSC under SPF conditions does not induce local colonic inflammation (Langgartner et al., 2017), CSC in my study under SPF conditions and in combination with rectal infusions of saline did also not cause local colonic inflammation. In detail, saline-treated CSC vs. SHC mice did not show any differences in the histological damage score of the colon, showing that neither the CSC

exposure nor the rectal infusion procedure caused macroscopic damages of the mucosal layer of the colon. Furthermore, saline-treated CSC vs. SHC mice did not differ in the colonic F4/80 and CD11b protein expression, showing that colonic macrophages, monocytes and granulocytes were not significantly attracted or activated. Moreover, the colonic mRNA levels of the pro-inflammatory cytokines TNF-α and IFN-γ and the colonic mRNA levels of CRAMP that represents the gene for an antimicrobial peptide were comparable between saline-treated SHC and CSC mice, revealing that the local colonic immune system was not activated. In line with the absent local colonic inflammation of saline-treated CSC mice, SHC- and CSC- recipient CSC vs. SHC mice did also not show increased colonic inflammatory parameters, respectively.

Taken together, these results show that the CSC paradigm under SPF conditions and in combination with FT does not have a different influence on colonic inflammatory parameters than the CSC paradigm alone, a finding that ensures that the CSC paradigm is an appropriate model to employ FT. Besides, the investigation of the local colonic inflammation parameters in saline- as well as feces-infused mice shows that neither the inserted gavage needle nor the rectal infusions cause any inflammation in the colon of the recipient mice, suggesting that the transplantation procedure via the rectal route represents an appropriate type of application for FT.

Like mentioned above, it is important to note that in contrast to SPF conditions, CSC has different effects on intestinal inflammatory parameters when performed under non-SPF conditions (Langgartner et al., 2017). Specifically, under non-SPF conditions, CSC exposure indeed causes colonic inflammation, seen in an elevated HDS and an increased secretion of pro-inflammatory cytokines by *in vitro* stimulated mesenteric lymph node cells (Reber et al., 2007). In a recent study of our laboratory, it was found that the development of colonic inflammation following CSC exposure is dependent on certain pathobionts, namely *Helicobacter* species, in the intestinal flora of the stressed mice (Langgartner et al., 2017). Consequently, the lack of these pathobionts under SPF conditions prevents the development of colonic inflammation. In this context, it would be very interesting to repeat my study under non-SPF conditions in order to investigate if a transplantation of feces from SPF donor mice would have any consequences on the presence of *Helicobacter* species in the intestinal

microbiota composition and consequently on the development of colonic inflammation in the recipient animals.

In contrast to the absence of effects in local colonic inflammatory parameters, the published paper about this study shows that CSC under SPF condition is indeed able to increase systemic inflammation, seen by elevated levels of cytokines and inflammatory markers in the plasma of saline-treated CSC vs. SHC mice, specifically of keratinocyte chemoattractant (KC), monocyte chemotactic protein-1 (MCP-1) and IL-6 (Langgartner et al., 2018b). Interestingly, FT was able to influence the latter (Langgartner et al., 2018b). More precisely, the results of the paper reveal that transplantations of SHC-donor feces prevented CSC-induced increases of plasma KC and plasma IL-6 in the recipients, indicating that the transplanted microbiota modified the interaction with the immune system and hereby prevented systemic inflammation (Langgartner et al., 2018b).

4.4 Limitations of the study

When interpreting the results of my study, it has to be taken into account that there is no proof yet that the donor microbiota were able to colonise the recipient's intestines sufficiently and thus, are directly responsible for the effects on the behaviour and physiology detected in my experiments. Regarding other studies on this subject, the recipient's natural microbiome was mostly either depleted by an orally given antibiotic cocktail for at least 14 days prior to FT (Bruce-Keller et al., 2015; Kelly et al., 2016) or the recipients were born and raised in a germ-free environment and consequently did not possess an endogenous microbiome (Bercik et al., 2011; De Palma et al., 2017). Furthermore, these studies confirmed that the recipients successfully adopted the donor microbiota. In detail, Bercik and colleagues verified via DGGE (denaturing gradient gel electrophoresis) analysis of fecal samples that almost 100% of the bacterial strains were transferred to the recipient (Bercik et al., 2011). Moreover, Bruce-Keller and colleagues used 16S ribosomal RNA sequencing to confirm the establishment of a diverse microbiota composition (Bruce-Keller et al., 2015). Given that the FT procedure in my study has been modified on the one hand by taking the rectal instead of the oral route and on the other hand by refraining from antibiotic pre-

treatment, it cannot be concluded from other FT-studies whether or not the recipients adopted the donor microbiota.

Interestingly however, a recent study found that rats without an antibiotic pre-treatment prior to FT adopt a higher amount of donor microbiota phylotypes than rats with an antibiotic pre-treatment (Manichanh et al., 2010). On the contrary, another study on mice detected the opposite. More precisely, they found that FT is more efficient when mice are pre-treated with antibiotics compared to a pre-treatment with bowel cleansing or no pre-treatment, suggesting that antibiotics open niches in the intestinal microbiota composition for exogenous microbiota to colonise the gut (Ji et al., 2017).

In order to clarify if the FT-recipients adopted the donor microbiota, in future studies, it needs to be confirmed if the modified FT procedure used in my study has the potential to alter the intestinal microbiota composition of the recipient and if so, it has to be analysed which specific bacterial phyla and species are colonising the recipients.

4.5 Conclusions and outlook

In conclusion, the results of my thesis show that transplantations of an "unstressed" microbiome might be a promising method to treat or to even prevent behavioural and physiological alterations of the recipient following chronic psychosocial stress. Additionally, as described in the published paper about this experiment, also inflammatory alterations following chronic psychosocial stress exposure can be treated/prevented by FT (Langgartner et al., 2018b). Besides, the results of the published paper show that transplantations of SHC-donor feces are able to treat/prevent CSC-induced alterations in the bone homeostasis of the recipients (Langgartner et al., 2018b). In this context, in a prospective clinical setting, transplantations of a "healthy" microbiome might be used as an effective therapy for chronic stress-related diseases in human beings.

Importantly, in the published paper about this experiment it was also shown that transplantations of CSC-donor feces into SHC-recipients result in the transfer of stress-induces changes in bone homeostasis, a finding that for the first time demonstrates that FT

also entails risks for the recipient (Langgartner et al., 2018b). As a consequence, therapeutic approaches that use FT to treat recurrent *C. difficile* infections, IBD symptoms or prospective applications of FT need to intensely screen fecal donors for chronic stress exposure in the presence or the past to protect the recipient from acquiring a "stressed" microbiome and its consequences for the organism.

Moreover, the results of my study indicate that FT via the rectal route and without an antibiotic pre-treatment can affect certain behavioural, physiological and inflammatory parameters of the recipient. Given that local colonic inflammatory parameters were not increased in recipient animals, fecal infusions via the rectal route can be regarded as an appropriate type of application for FT.

Besides, the findings of my study corroborate the hypothesis that chronic stress-induced changes in the intestinal microbiota composition are markedly involved in chronic stress-induced behavioural, physiological and inflammatory alterations of the host and therefore emphasise the relevance of the intestinal microbiome in the entire organism.

In the future, further studies are needed to test if the effects of FT on the behavioural, physiological and inflammatory status of recipient CSC mice can be intensified and expanded, for example by varying the frequency of the transplantations or by starting FT already prior to CSC exposure. Moreover, it needs to be identified, which specific microbiota are involved in the beneficial effects of FT.

5 Summary

Chronic psychosocial stress is known to affect the behavioural, physiological and inflammatory status of the organism and represents an accepted risk factor for the development and aggravation of affective and somatic diseases. Besides, chronic psychosocial stress is able to change the intestinal microbiota composition. Given that stress-related affective or gastrointestinal diseases are often accompanied by an altered intestinal microbiota composition and that the intestinal microbiome is able to influence the behaviour and physiology of an organism, it is very likely that chronic psychosocial stress mediates the vulnerability to develop chronic stress-induced diseases by changing the intestinal microbiota composition. If this hypothesis is true, a promising therapeutical approach to treat or to prevent chronic stress-induced changes in the intestinal microbiota composition and the associated consequences for the organism might be the transplantation of a healthy intestinal microbiota composition.

An ideal model to address this hypothesis is the so called “chronic subordinate colony housing” (CSC) paradigm, as it induces chronic psychosocial stress in male mice and has repeatedly shown to alter the anxiety-related behaviour, physiological and inflammatory parameters and the intestinal microbiota composition.

Therefore, to address the above mentioned hypothesis, it was the main aim of my thesis to examine i) if transplantations of feces from non-stressed single-housed control (SHC) mice into chronically stressed CSC mice can prevent CSC-induced alterations and ii) if transplantations of a “stressed” microbiome from CSC-donor mice can transfer CSC-induced effects into SHC mice. Besides, the study aimed to generally test if fecal transplantation (FT) via the rectal route instead of the commonly used oral route and without an antibiotic pre-treatment can affect stress-induced parameters in recipient mice.

To do so, SHC- and CSC-recipient mice received repeated rectal infusions of SHC-donor feces on days 4 and 11 of the CSC paradigm. Anxiety-related behaviour was tested on day 19 and physiological and local colonic inflammatory parameters were assessed after sacrificing the mice in the morning of day 20. The same procedure was done with another

set of SHC- and CSC-recipient mice that received CSC-donor feces. To see if rectal infusions *per se* have any effects on the assessed parameters, a set of SHC and CSC control mice received saline infusions at the respective days.

The results of my thesis reveal that transplantations of SHC-donor feces had slightly stress-protective effects, indicated by the reduction of CSC-induced anxiety-related behaviour and by the prevention of CSC-induced thymus atrophy. However, transplantations of SHC-donor feces had no effects on CSC-induced adrenal enlargement. Moreover, the results of my study show that transplantations of CSC-donor feces did not transfer CSC-induced anxiety-related behaviour and physiological alterations to SHC-recipients. Moreover, the results of my study indicate that FT via the rectal route and without an antibiotic pre-treatment can affect certain chronic stress-induced parameters of the recipients as shown in the above-mentioned effects of SHC-donor feces on the behaviour and physiology of recipient CSC mice. Besides, my results show that local colonic inflammatory parameters were neither increased in saline nor in SHC- and CSC-recipient mice, indicating that the procedure of rectal infusions does not cause colonic inflammation in the recipients. Therefore, the rectal route can be regarded as an appropriate type of application for FT.

In conclusion, the results of my study in combination with the recently published paper about this study (with me as second author) show that rectal transplantations of an “unstressed” microbiome can treat and/or prevent certain consequences of chronic psychosocial stress in the recipients. Therefore, in the future, transplantations of a “healthy” microbiome might be used as a therapeutical application for humans to treat and/or prevent negative consequences of chronic stress and to reduce the vulnerability to develop chronic stress-related diseases. Besides, the results of the published paper about this experiment reveal that some chronic stress-induced effects can be transferred to the recipient via FT, indicating that FT can also have undesirable side effects. Therefore, therapeutic approaches that use FT need to intensely screen fecal donors for present or previous chronic stress exposure.

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Appendix

List of figures

List of tables

Acknowledgements

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Curriculum vitae

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