Unlocking the mysterious interconnection between the gut microbiota and catestatin

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Unlocking the Mysterious Interconnection between the Gut Microbiota and Catestatin

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De darm van zoogdieren herbergt een diverse microbiële gemeenschap met een enorme metabolische capaciteit, gezamenlijk aangeduid als het darmmicrobioom. Er vindt een uniek samenspel plaats tussen een subtype van cellen van het darmepitheel, namelijk entero-endocriene cellen en de microbiota. Entero-endocriene cellen scheiden een groot aantal hormonen en bioactieve peptiden af, die een sleutelrol spelen bij het moduleren van mucosale immuniteit, darmmotiliteit en metabolisme. Een van deze bioactieve peptiden is catestatine (CST), dat is afgeleid van het prohormoon chromogranine-A. Van CST is aangetoond dat het de aangeboren mucosale immuniteit, darmpermeabiliteit, hypertensie reguleert en een mogelijke target is voor behandeling van diabetes.

In dit proefschrift hebben we aangetoond dat CST selecteert voor de kolonisatie van darmbacteriële gemeenschappen die in staat zijn om het antimicrobiële effect ervan te weerstaan, wat op zijn beurt de niveaus van butyraat en andere korteketenvetzuren beïnvloedt die optimaal zijn voor de darmfunctie. Verder toonden we, via fecale microbiële transplantatie, aan dat de veranderde darmmicrobiota in muismodellen met depletie van hun CST een oorzakelijke rol spelen in de colondisfunctie waargenomen bij die muizen, gekenmerkt door verhoogde darmpermeabiliteit, fibrose en veranderde immuun- en metabolische -gerelateerde cellulaire paden. Over het algemeen is dit proefschrift een voorbeeld van de cruciale wisselwerking tussen de darmmicrobiota en CST, bij het opzetten en onderhouden van mucosale homeostase en opent het nieuwe wegen voor op microbiota gerichte therapieën bij mensen voor specifieke ziekten die verband houden met veranderde niveaus van CST.

Brief summary

The mammalian gut harbors a diverse microbial community with a vast metabolic capacity, collectively referred to as the gut microbiome. A unique interplay occurs between a subtype of cells of the gut epithelium, namely, enteroendocrine cells, and the microbiota. Enteroendocrine cells secrete an extensive number of hormones and bioactive peptides, which play a key role in modulating mucosal immunity, gut motility, and metabolism. Among these bioactive peptides is catestatin (CST), which is derived from the prohormone chromogranin-A. CST has been shown to regulate innate mucosal immunity, gut permeability, hypertension, as well as a potential diabetes treatment.

In this thesis, we showed that CST selects for the colonization of gut bacterial communities that are capable of resisting its antimicrobial effect, which in turn, impacts the levels of butyrate and other short-chain fatty acids which are optimal for the intestinal function. Furthermore, we showed via fecal microbial transplantation, that the altered gut microbiota in mouse models with depletion in their CST play a causal role in the colonic dysfunction observed in those mice, characterized by increased gut permeability, fibrosis, and altered immune- and metabolic-related cellular pathways. Overall, this thesis exemplifies the pivotal interplay between the gut microbiota and CST, in the establishment and maintenance of mucosal homeostasis and opens new avenues for microbiota-targeted therapeutics in humans for specific diseases associated with altered levels of CST.

Breve resumen

El intestino de los mamíferos alberga una diversa comunidad microbiana con gran capacidad metabólica, denominada colectivamente *microbiota intestinal*. Existen interacciones únicas entre un subtipo de células del epitelio intestinal, llamadas células enteroendocrinas y la microbiota intestinal. Las células enteroendocrinas secretan una gran cantidad de hormonas y péptidos activos biológicamente, que desempeñan un papel clave en la modulación de la inmunidad de la mucosa intestinal, la motilidad del intestino y el metabolismo. Entre estos péptidos se encuentra la catestatina, que se deriva de la hormona cromogranina-A. Se ha demostrado que la catestatina regula la inmunidad innata de la mucosa, la permeabilidad intestinal, la hipertensión y también ha sido propuesta para el tratamiento de la diabetes tipo 2.

En esta tesis, mostramos que la catestatina selecciona comunidades bacterianas intestinales que son capaces de resistir su efecto antimicrobiano, contribuyendo a la colonización del intestino, lo que a su vez afecta los niveles de butirato y otros ácidos grasos de cadena corta, que son esenciales para la buena función intestinal. Además, demostramos a través del trasplante microbiano fecal que la microbiota intestinal alterada en modelos de ratón sin catestatina, desempeña una causa en la disfunción del colon, caracterizada por un aumento de la permeabilidad intestinal, fibrosis y alteraciones en las rutas celulares inmunitarias y metabólicas observada en esos ratones. En general, esta tesis ejemplifica la interacción fundamental entre la microbiota intestinal y la catestatina en el establecimiento y mantenimiento de la homeostasis de la mucosa intestinal y abre nuevas vías para la terapia dirigida a la microbiota en humanos para enfermedades específicas asociadas con niveles alterados de catestatina.

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Chapter 1

General Introduction and Scope of the Thesis

The human gastrointestinal tract (GI) is a complex and dynamic network, where interactions between the host and a vast diversity of microorganisms co-exist in a mutualistic relationship[1,2]. The GI tract harbors around 100 trillion microbial cells consisting of bacteria, fungi, viruses, archaea, and protozoa collectively referred to as *the gut microbiota*[3]. This microbial community encompasses a large repertoire of microbial genes with powerful metabolic capacity that contributes to essential functions in host physiology, metabolism, and immune response[4–6].

The gut microbiota has been shown to modulate several host pathways to maintain host homeostasis since early life[7,8], including the maintenance of the intestinal wall[9], mucus layer integrity[10], epithelial cell function[11], innate and adaptive immunity[12,13], intestinal motility[14], energy metabolism[15], neurotransmission[16] and neuropsychological behaviors[17]. Moreover, the gut microbiota is in a continuous cross-talk with the brain via the microbiota-gut-brain axis (MGB-axis) (**Figure 1**). Several studies using germ-free mice showed the significance of the MGB-axis in brain neurotransmission, brain morphology, and behavior[18–20]. Owning to its crucial function, the imbalance in the gut microbial communities have been strongly linked to the development and progression of chronic diseases such as inflammatory bowel diseases (IBD)[21], autoimmune diseases[22,23], cardiometabolic disorders[24], neuropsychiatric diseases[25], and cancer[26]. IBD, for example, affects up to ~0.5% of the Western population, and the incidence rises in the Western world due to diet and environmental factors such as stress[27]. The pathophysiology of IBD is characterized by intestinal hyperpermeability[28,29], which is associated with the translocation of microbiota into the gut mucosa thereby inducing intestinal inflammation, another hallmark of IBD[30–32]. Similarly, alterations in the gut microbiota have been implicated in mental health, cognitive function, and neurological diseases[33–35]. It is now well established that increased intestinal permeability and intestinal inflammation, seen in IBD, and metabolic disorders, can lead to neuroinflammation through the bloodstream and the MGB-axis[35,36]. Chronic neuroinflammation can induce neuronal cell death implicated in the most common neurodegenerative diseases including Alzheimer's disease, Parkinson's disease (PD), and amyotrophic lateral sclerosis which are leading contributors to worldwide disability[37].

The development of microbial interventions to treat diseases associated with microbial imbalance has been consistently explored. However, this approach is still debatable given that the microbiota of each individual is vastly diverse. One of those microbial interventions is fecal microbial transplantation (FMT)[38,39]. FMT refers to the process of transferring fecal bacteria and other microbes from a healthy individual (referred to as the *donor*) into another individual (referred to as the *recipient*) to introduce beneficial microbes and restore intestinal homeostasis

and improve the immune function[40]. FMT has become the standard of care for the treatment of recurrent *Clostridioides difficile* infection, as antibiotic treatment is the main risk factor for this infection[41,42]. Besides, FMT is potentially effective for patients with ulcerative colitis, a subtype of IBD, however, different features in FMT medical methodology need to be studied further to achieve consistent effects[39,43–45]. In addition, there is increasing pre-clinical and clinical evidence supporting the role of FMT not only in gastrointestinal disorders but also as a potential treatment of other conditions[46] such as cardiometabolic diseases[47,48], neurological disorders[49,50] and its application for the decolonization of multidrug-resistant bacteria[51–54]. Especially, FMT studies in animal models have resulted in discovering unknown mechanisms related to the robust effects of the gut microbiota on the host[55–57].

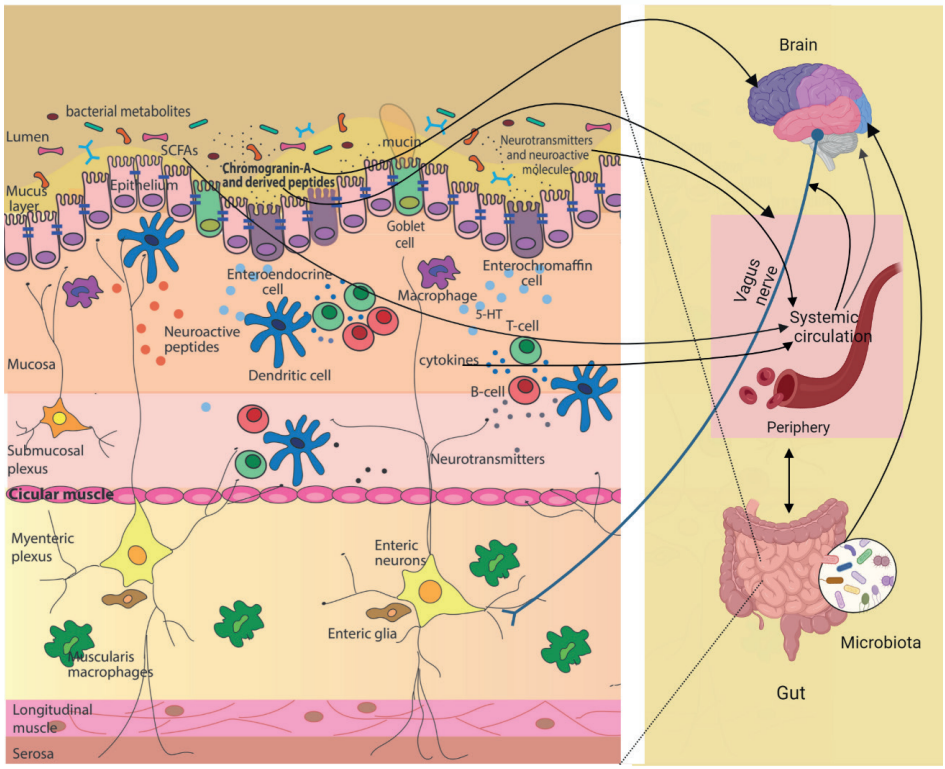

Figure 1. Overview of the communicative routes between the microbiota-gut and the brain. There are several routes by which the microbiota communicates with the gut and beyond. Once stimulated, the epithelial enteroendocrine cells, releases pro-hormones and peptides such as chromogranin-A. Adapted after [207].

Within the context of the host-microbe interactions in the MGB-axis, gut secreted peptides play an important role[58]. For example, the chromogranin/secretogranin (granin) proteins, consist of acidic soluble proteins that are expressed by endocrine, neuroendocrine, and neuronal cells throughout the body, including the gut epithelium[59–61].

The prohormone chromogranin-A

One of the most important proteins from the granin family is the human chromogranin-A (gene, *CHGA*; protein, CgA)[62,63] which is a precursor of several hormones, secreted extensively from enteroendocrine cells (EECs) in the GI tract and may play an important role in the MGB-axis due to its pro-hormone effects (**Figure 1**)[64–66]. CgA is a 48-52 kDa (439 amino acid mature protein), is a highly conserved coiled-coil glycoprotein, especially in its N- and C-terminal regions[67]. CgA was first identified in chromaffin granules of the adrenal medulla[68,69]. Nonetheless, CgA has been identified in other secretory vesicles of endocrine, neuroendocrine, and neuronal tissues[70–73] as well as in human keratinocytes [74], cells from myocardium[75–77], endothelial cells[78,79], and macrophages[78,80]. CgA has been also found in cells of the pancreatic islet[81], secretory granules of glucagon containing α-cells, and insulin-producing β-cells[73,82–85], modulating glucose tolerance and insulin sensitivity through its derived peptides[80,83,86–88]. The broad distribution of CgA has linked it with several disorders including cardiovascular diseases[76,89–93], hypertension[94–98], metabolic disorders[99], IBD[100,101], and neurodegenerative disorders[102–107]. CgA has a substantial influence on human health and disease[108] as its increased levels have been detected in patients afflicted with carcinoid tumors or other neuroendocrine tumors[109–114], heart arrest, kidney failure, hypertension, rheumatoid arthritis, and IBD[64,100,115–118].

Within the MGB-axis, EECs cells are the major source of CgA, where it is colocalized, co-stored, and co-released with several regulatory molecules such as gut hormones and neurotransmitters including catecholamines, serotonin (5HT), and other gut peptides (PYY and GLP-1) essential for the GI function and are known to interact with the gut microbiota[57,72,95,119,120]. In fact, the full-length CgA protein has intra- and extracellular functions. Intracellularly, CgA modulates calcium homeostasis and the biogenesis of dense-core granules[121,122]. CgA activates calcium channels present on the granule membranes, which mobilizes calcium into the cytoplasm[123] this process is important for intestinal smooth muscle contraction[124]. In the brain, CgA was shown to influence the immune system through the activation of microglial cells, which subsequently produce heat-stable diffusible neurotoxic agents, nitric oxide, and tumor necrosis factor-alpha (TNF-α), leading to neuronal apoptosis[125,126]. Moreover,

fecal CgA has been lately proposed as a health biomarker, where low fecal CgA levels correlated with increased microbiota diversity and functional richness, while high fecal CgA concentration was associated with irritable bowel syndrome[127].

Cleavage products of chromogranin-A

CgA exerts its extracellular functions when it is proteolytically processed giving rise to several biologically active peptides (**Figure 2**), which are involved in a wide range of systemic functions acting in an autocrine or paracrine manner but also exert endocrine effects via overflow in the bloodstream[123,128,129]. The basic amino acids are cleaved intracellularly and extracellularly by different proteases including prohormone convertases (PC1 or PC2)[130–132], cathepsin L[133], furin[134], kallikrein[135], thrombin[136], and plasmin[137–139]. Interestingly, CgA processing is cell- and tissue-specific with extensive processing in the gastrointestinal mucosa[123,140,141] from endocrine cells, nerve terminals, and immunocytes[79,130]. Moreover, CgA processing can also occur in stimulated polymorphonuclear neutrophils, thereby affecting a wide range of cells and tissues involved in inflammatory processes[142]. Even when CgA is known to be processed inside the cell by hormone-storage vesicles or after its liberation to the bloodstream, it is still unclear whether any other molecular or enzymatic processes are involved in the cleavage of CgA-derived peptides in a tissue-specific manner[143] In addition, whether CgA peptides are generated in equimolar quantities or produced as a response to physiological demands in certain cells or tissue, is still unknown. CgA concentrations in plasma span from 0.5 to 1 nM[90]. Nonetheless, it has been reported that circulating levels of CgA and peptides change in health and disease[144].

As mentioned earlier, CgA is the precursor of seven biological active peptides, their production depends on the proteolytic cleavage sites, post-translational modifications (glycosylation and phosphorylation), and further proteolytic processing[90,145,146]. The first discovered peptide was the anti-insulin peptide Pancreastatin (PST) ($hCgA_{250-301}$), which regulates glucose internal stability[85,147,148]. The pleiotropic, inhibitor of the catecholamine release and proinsulin peptide catestatin (CST). CST ($hCgA_{352-372}$) has antimicrobial and anti-inflammatory effects[143,149–151]. Moreover, the proteolytic process of CST generates cateslytin ($hCgA_{344-358}$), which is the active domain of CST with antimicrobial properties[146,152]. Vasostatin ($hCgA_{1-76}$) is vasodilator, antiangiogenic, as well as antiadrenergic[153–155]. WE-14 ($hCgA_{324-337}$) acts as an antigen for the highly diabetogenic CD4+ T-cell clones and was detected in midgut carcinoid tumors[156–158]. Chromofungin ($hCgA_{47-66}$) has also antimicrobial effects and it is involved in the regulation of the innate immune response[159,160].

Finally, serpinin ($hCgA_{402-439}$), which is a pro-adrenergic peptide involved in granule biogenesis[161] and cardiac function in rodents[162]. CgA is a relevant protein as its peptides exert antagonistic effects to regulate different biological functions[163,164].

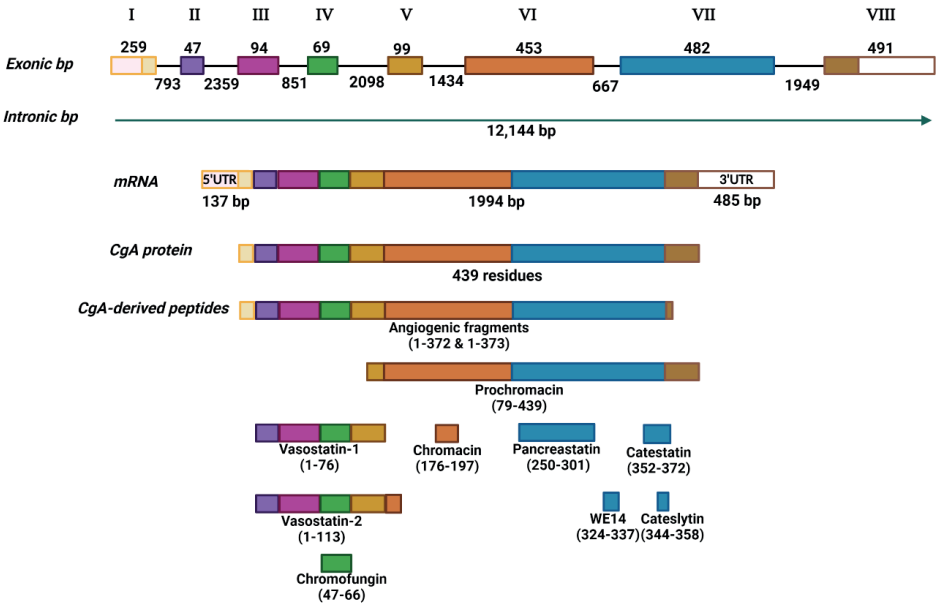

Figure 2. Schematic representation of human chromogranin-A and derived peptides. ChgA gene is localized on chromosome 14, with its transcript, proteins, and its derived-biologically active peptides. The size of the gene is 12,144 bp, it consists of eight exons indicated by Roman numbers, 1994 bp transcript, and 622 bp 'UTR untranslated region. CgA protein consists of 457 amino acids residues, which mature protein consists of 439 residues after removal of the signal peptide. The peptides are colored according to their exon of precedence. CgA mature protein is cleaved by different enzymes resulting in CgA-derived peptides. Amino acid residues positions in the peptides are numbered relative to their positions in the mature protein. Adapted after [208].

Catestatin

The CgA-derived peptide, CST, is generated by intra- and extra-granular proteolytic cleavage of prohormone convertase PC1 and PC2 and the cysteine protease cathepsin-L[133]. CST consists of 21 amino acid residues, its primary sequence (human sequence: S_{352}SMKLSFRARAYGFRGPGPQL$_{372}$) is a highly conserved sequence across species[72,165], and it is flanked by cleavage sites[131,132,139,166]. CST was firstly identified as a potent inhibitor of nicotinic cholinergic-stimulated catecholamine secretion, which may function as an autocrine negative-feedback mechanism modulating

catecholamine production[166–169]. CST has multifunctional properties in health and disease including a major role in regulating innate immunity[74,78,80,142,149,170–174], supporting angiogenesis[175,176], hypertension[98,176–178], cardiovascular functions[179–186], anti-obesity[187], and in metabolic function[80,172,188,189]. The physiological blood levels of CST range from 0.03 to 1.5 nM in healthy individuals, however, altered levels of CST have been found in pathological conditions[90,190,191]. Indeed, plasma levels of CST were reduced in patients suffering from type 2 diabetes, hypertension, and coronary disease[88,190–193], whereas increased CST levels were reported in patients with coronary collateral developments and IBD[163,185]. Moreover, CgA-knockout (CgA-KO) mice suffer from an obese phenotype[85] as well as severe hypertension, which could be rescued by CST treatment[98]. Administration of CST in animal models, resulted in reduced hypertension[98], cardiac damage[80,179], obesity[194], atherosclerosis[78], inflammation[195], decreased the levels of catecholamines[189], and improved glucose tolerance, insulin sensitivity[172,189] and gut permeability[163] These findings indicate that altered CST levels contribute to disease development[143,150].

Catestatin as an antimicrobial peptide

CST is cationic and amphiphilic in nature as its molecular modeling resulted in a β-strand/loop/β-strand structure surrounded by hydrophobic interactions[196]. Cationic residues and hydrophobic domains are the common characteristics of amphiphilic antimicrobial peptides[197]. The cationic charges are crucial in creating electrostatic interactions with negatively charged bacterial membranes[198], while hydrophobic residues, help to penetrate the pathogenic membranes leading to membrane disruption and eventually cell death[199]. Thus, the antimicrobial activity of CST presumably, resides in its N-terminus, also called cateslytin, which is highly cationic[146]. CST has been reported to exert inhibitory effects in *in vitro* studies against several pathogenic gram-positive (*Micrococcus luteus, Bacillus megaterium*), gram-negative bacteria (*Escherichia coli* D22), and filamentous fungi (*Neurospora crassa* and *Aspergillus fumigatus*)[173]. Additionally, human CST showed antimicrobial effects in several skin microbes such as gram-positive (*Staphylococcus aureus* and Group *A Streptococcus*), gram-negative bacteria (*E. coli O29*, and *Pseudomonas aeruginosa*), also (*Candida albicans*), and filamentous fungi (*Aspergillus niger*, *Aspergillus fumigatus*, *Trichophyton rubrum*)[74]. Recently, Rabbi *et al.* showed that *in vivo* treatment of mice with CST significantly modified the fecal gut microbiota composition in mice, particularly in the ratio between the two main phyla; lowering the relative abundance of *Firmicutes* and increasing the abundance of Bacteroidota[200].

Despite the lack of established evidence, it is suggested that cateslytin is the active core of CST[167,173], that interacts with the anionic components of gram-positive and gram-negative bacteria, producing the permeabilization of microbial membranes

and thereafter cell lysis[146,201]. Cateslytin is resistant to degradation by the proteases produced by *Staphylococcus aureus*[201]. Interestingly, both CST and cateslytin showed a wide range of antimicrobial activities against bacteria and yeasts once attached to biomaterials[202,203].

Catestatin and the intestinal epithelial barrier

Recent research has demonstrated extensive processing of CgA to CST in the colon, and CST concentrations in mice colonic tissue are in average 0.37 nmol/mg of protein[163], while the levels in circulating plasma from mice were 0.86 nmol/L[80]. Additionally, mice lacking CST (CST-KO) had alterations in the tight junctions of the colonic tissue, intestinal fibrosis, gut hyperpermeability, and cecal microbiota imbalance. Treatment with CST reduced gut permeability contributing to the integrity of tight junctions, lowering inflammation, and reducing intestinal fibrosis. Another work showed that intrarectal CST treatment reduced the severity of the experimental murine colitis decreasing IL-18 in the colon and maintaining tight junctions integrity[204]. CST administration in experiments with DSS-induced CACO-2 epithelial injury also showed increased migration, proliferation, and viability of tight junction cell proteins as well as reduced pro-inflammatory IL-8 and IL-18 levels, inhibition of STAT3 eliminated these beneficial effects of CST. Finally, CST was suggested to ameliorate disease onset and progression of IBD by reducing the activation of pro-inflammatory macrophages *in vitro* and mice[171,195].

The levels of CST in the feces of healthy volunteers were reported to be ~1 nM, and 3 nM in plasma[205]. This evidence supports that CST diffuses to the lumen, suggesting CST plays a fundamental role in regulating host-microbe interactions. The effects of CST on gut barrier function and immune response could also be a collateral consequence of the CST antimicrobial effect, which would, presumably, alter the intestinal microbiota composition. Nonetheless, further studies are warranted to elucidate the cross-talk between the CST and gut microbiota as well as to demonstrate whether it is the imbalanced gut microbiota or altered levels of CST that underlie a causative role in intestinal inflammatory diseases and beyond.

Scope of the thesis

A growing line of research points towards the role of CST in intestinal homeostasis. However, it is yet unclear whether CST exerts this effect directly or indirectly via its effect on the gut microbiota. It is also unknown what role the gut microbiota has on the levels of intestinal CST, which, in turn, alters the gut microbiota composition and functionality, impacts intestinal permeability, and immune response. This thesis investigates the interplay between CST and the gut microbiota, and its impact on intestinal homeostasis. For this purpose, adult mice with a knockout in CST (CST-KO mice) were compared to wild type C57/BL6 J mice. CST-KO mice have a deletion in the 63 bp CST domain from Exon VII of the CHGA gene[80] in C57BL/6 background. **Chapter 2** illustrates how the endogenous antimicrobial peptide, CST, shapes the microbiota composition in the gut. We postulated that the gut microbiota may have evolved antimicrobial resistance mechanisms to counteract the antimicrobial effect of CST since these microbes are in continuous contact with the gut epithelium, which secretes antimicrobial peptides, including CST. To test our hypothesis, we treated WT and CST-KO with CST and profiled their fecal microbiota. Additionally, we performed *in vitro* inhibitory assays to identify the genes involved in the microbial resistance to CST. Disruption in the levels of CST has been associated with changes in the gut microbiota and mucosal function[163]. However, the extent to which changes in gut microbiota composition and function mediate the dysregulation of these pathways is unknown. Thus, in **Chapter 3** we performed reciprocal fecal microbial transplantation (FMT) in wild-type (WT) mice and CST-KO mice. Combined transcriptome, transmission electron microscopy, and microbiota phylogenetic profiling analyses were employed. This study provides a comprehensive overview of mouse metabolic- and immune-related cellular pathways and processes that were co-mediated by fecal microbiota transplantation. Since the precursor of CST, CgA, is co-localized with the trace-amine associated receptor 1 in EECs[206], we presumed a possible role between the levels of trace amines and the expression levels of their main receptor the trace amine-associated receptor 1 (TAAR1) in the regulation of CST secretion as a gut hormone. Trace amines, such as catecholamines, are host and microbe-derived small molecules that have attracted attention in modulating the MGB-axis. Nonetheless, the experimental work was not continued due to technical difficulties. Instead, **Chapter 4** reviews trace amines and their main receptor TAAR1 in the MGB-axis, as well as their potential implication in inflammatory bowel diseases, and the reported comorbidities of neuropsychiatric and gastrointestinal disorders.

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Chapter 2

Research article

Catestatin selects for colonization of antimicrobial-resistant gut bacterial communities

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Abstract

The gut microbiota is in continuous interaction with the innermost layer of the gut, namely the epithelium. One of the various functions of the gut epithelium, is to keep the microbes at bay to avoid overstimulation of the underlying mucosa immune cells. To do so, the gut epithelia secrete a variety of antimicrobial peptides, such as chromogranin A (CgA) peptide catestatin (CST: $hCgA_{352-372}$). As a defense mechanism, gut microbes have evolved antimicrobial resistance mechanisms to counteract the killing effect of the secreted peptides. To this end, we treated wild-type mice and CST knockout (CST-KO) mice (where only the 63 nucleotides encoding CST have been deleted) with CST for 15 consecutive days. CST treatment was associated with a shift in the diversity and composition of the microbiota in the CST-KO mice. This effect was less prominent in WT mice. Levels of the microbiota-produced short-chain fatty acids, in particular, butyrate and acetate were significantly increased in CST-treated CST-KO mice but not the WT group. Both CST-treated CST-KO and WT mice showed a significant increase in microbiota-harboring phosphoethanolamine transferase-encoding genes, which facilitate their antimicrobial resistance. Finally, we show that CST was degraded by *Escherichia coli* via an *ompT*in-protease and that the abundance of this gene was significantly higher in metagenomic datasets collected from patients with Crohn's disease but not with ulcerative colitis. Overall, this study illustrates how the endogenous antimicrobial peptide, CST, shapes the microbiota composition in the gut and primes further research to uncover the role of bacterial resistance to CST in disease states such as inflammatory bowel disease.

Keywords

Antimicrobial peptide; microbiota; resistance; phosphoethanolamine transferase; *omptin*

Introduction

The complex microbial community inhabiting the mammalian gut, and in particular their metabolic products, has a crucial effect on various health and disease states, such as inflammatory bowel disorders[1]. Gut microbiota, and its products, are in continuous interaction with the innermost layer of the gut, namely the gut epithelium. Part of the gut epithelial function is to keep the microbes at bay to avoid overstimulation of the underlying immune cells in the gut, and the subsequent inflammation[2,3]. To do so, the gut epithelial cells secrete a wide variety of antimicrobial peptides.

CST, a proteolytically processed product of Chromogranin-A (CgA), acts as an antimicrobial peptide[4,5]. The pro-protein CgA is abundantly expressed in endocrine and neuroendocrine cells including the epithelial enteroendocrine cells (EECs)[6–9]. CST consists of 21 amino acid residues and acts on a wide range of regulatory functions including immune, endocrine, metabolic, neurological, and cardiovascular functions[10–14]. For example, mice with a selective deletion of the CST-coding region of the *Chga* gene (CST-KO mice) displayed increased gut permeability, which was restored by CST treatment[15].

To date, a few studies have indicated associations between CST and the gut microbiota. Intrarectal administration of CST for 6 days, in mice, resulted in a shift in the gut microbiota composition, with a decrease of *Firmicutes* and an increase in the *Bacteroidota* phyla[16]. These findings suggest the role of CST as a modulator of the gut microbiota composition. This CST-modulatory effect could be mediated by its antimicrobial activity against a wide range of microorganisms. In fact, CST has been reported to have antimicrobial activity against pathogenic bacteria and filamentous fungi in *in-vitro* studies[5]. In humans, CST showed antimicrobial effects against several skin-microbes[17]. Nevertheless, the underlying mechanisms by which CST exerts its effects *in vivo* on the gut microbiota remain obscure. In this study, we show how CST plays a role in governing the colonization of the gut microbiota.

Results

Catestatin treatment affects the microbiota composition in CST-KO and WT mice.

The composition of the gut microbiota is shaped by, among others, host-produced antimicrobial peptides[18,19]. Here, we treated CST-KO and WT mice (n=12) with 2 µg/g body weight/day CST by intraperitoneal injection for 15 consecutive days. Amplicon

sequencing of the V3-V4 regions of the bacterial 16S rRNA gene was performed on the fecal samples of CST-KO and WT mice with and without CST treatment (n=12). Microbial richness, assessed by the number of observed amplicon sequence variants (ASVs) and ACE index (Abundance-based Coverage Estimator), showed a significant decrease in CST-KO compared to WT mice (**Figure 1A**). In contrast, the richness levels were restored upon treatment of CST-KO mice, but not in WT mice treated with CST. Next, the microbiota diversity was determined by Shannon's H and inverted Simpson's index, both indices are used to measure similar parameters of alpha diversity. Similar to the richness scores, the diversity index was significantly higher in the CST-treated CST-KO mice but not in the CST-treated WT group (**Figure 1B**). The data highlight that the effect of the absence of CST in CST-KO mice on the microbiota diversity and richness could be restored upon treatment with CST, while in WT mice, with normal CST levels in their gut, the microbiota diversity and richness did not change with CST treatment.

As a general exploratory analysis, principal component analysis (PCA) was performed and showed distinct clustering of the CST-KO, WT, and CST-treated groups (**Supplementary Figure 1A**). Particularly, the CST-KO group clustered away from the other 3 groups, while the cluster of CST-treated WT mice showed a great spread and was overlapping with clusters of CST-treated CST-KO mice and WT mice.

To correct for any residual variation in the data, we further employed constrained RDA (redundancy analysis) to determine which bacterial taxa were associated with the different groups of mice at the genus level. The analysis was constrained to both, the mouse genotype and treatment (non-treated and CST-treated). Similar to the cluster separation detected by PCA analysis (**Supplementary Figure 1A**), both constraints had a significant influence on the model ($p < 0.001$, determined by ANOVA-like permutation test), explaining 13.4% (genotype) and 9.8% (treatment) of the variation, respectively (**Figure 1C**). Notably, the CST treated CST-KO group was shifted to the left side along RDA1, indicating a shift in "genotype" upon CST treatment, as opposed to WT, where the treatment group only moved along RDA2, which is associated with the treatment.

To assess the treatment effect exclusively on each genotype, we performed RDA constrained to untreated and CST-treated groups. On the genus level, *Dubosiella* and *Romboutsia* showed the strongest association with CST treatment along RDA1 (explaining ~31.2% of the variation) in the CST-KO mice (**Supplementary Figure 1B**). In WT mice, RDA demonstrated an association of *Faecalibaculum*, *Bifidobacterium*, *Romboutsia*, and *Anaeroplasma* with the baseline along RDA1 (~17.2% explained variance), while *Akkermansia* was more associated with PC1 (~25.1% explained

variance; residual variance). *Alloprevotella* and *Candidatus Saccharimonas* representing the strongest associations with CST treatment in WT (**Supplementary Figure 1B**).

To further identify which bacterial taxa were affected by the CST treatment, pairwise comparisons of bacterial abundances were performed between CST-treated and untreated groups for each genotype. Focusing on the phylum level, *Firmicutes* decreased in relative abundance, while *Bacteroidota*, *Patescibacteria*, *Desulfobacterota*, and *Proteobacteria* increased with CST treatment in both CST-KO and WT groups. *Verrucomicrobiota* showed lower abundance in CST-KO but increased significantly in CST-treated groups (**Figure 1D**). The changes depicted on the phyla level in CST-treated WT mice are consistent with findings from Rabbi *et al,* albeit with different CST administration and dosage regimen[16]. These shifts in the main bacterial phyla upon CST treatment were further confirmed to be consistent in human microbiota by *in vitro* culturing of healthy human fecal samples with 10 µM CST (**Supplementary Figure 1C**). On family level the most prominent changes were observed for *Erysipelotrichaceae*, which was consistently decreased in treated groups, and *Lachnospiraceae*, which was increased in CST-KO, but decreased in WT in the CST treated groups. Further *Lactobacillaceae* was found to be decreased in CST-KO but increased in WT CST treated groups (**Figure 1D**). On the genus level, CST treatment reduced the abundance of *Staphylococcus* and *Turicibacter* in CST-KO and WT mice, while *Alistipes*, *Akkermansia*, and *Roseburia*, were significantly increased only in the CST-KO group (**Supplementary Excel Sheet**). Next, we used LEfSe (Linear discriminant analysis Effect Size;[47]) to complement our differential abundance analysis. The main discriminant feature separating the groups (untreated and CST-treated) in CST-KO mice were species from the genera *Atopostipes*, *Jeotgalicoccus*, *Turicibacter*, *Staphylococcus*, and *Coriobacteriaceae* UCG-002 family (**Figure 1E**). In WT mice, the most discriminating genera were *Lactobacillus*, *Candidatus Saccharimonas*, *Turicibacter*, and *Enterococcus*.

Changes in microbial composition are often accompanied by metabolic changes, in particular, the production of short-chain fatty acids (SCFAs)[20]. Thus, levels of SCFAs; acetate, butyrate, and propionate, were measured in the cecum of untreated and CST-treated CST-KO and WT mice (**Figure 1F**). CST treatment significantly increased the levels of acetate and butyrate in CST-KO mice but not in the WT group. Specifically, butyrate was significantly lower in the CST-KO mice compared to their WT counterparts. Both observations are consistent with the observed significant increase in the SCFA-producing bacteria, *Alistipes*, *Akkermansia*, and *Roseburia* in the CST-KO group.

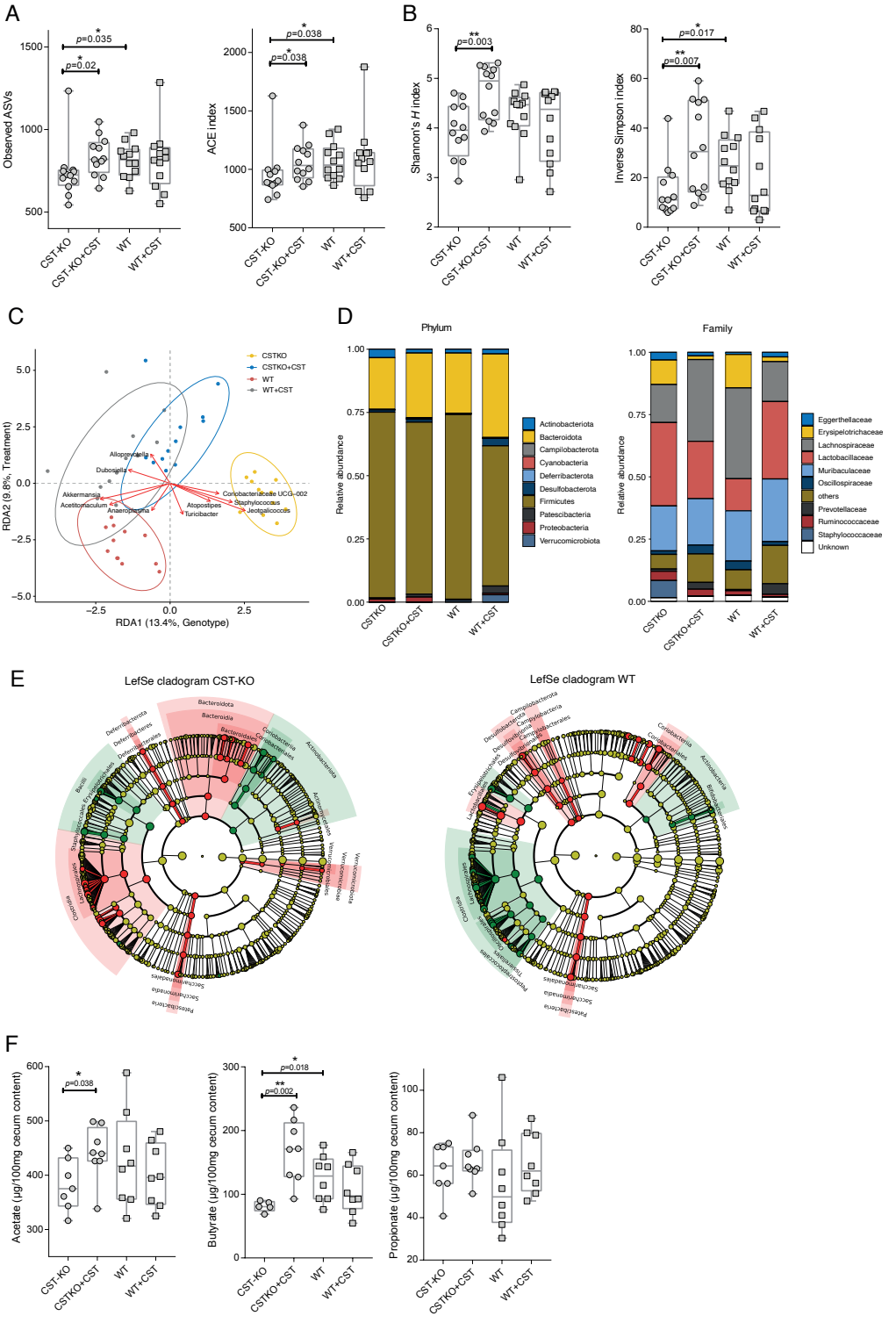
A
Observed ASVs
p=0.035
p=0.02
ACE index
p=0.038
p=0.038
B
Shannon's H index
p=0.003
Inverse Simpson index
p=0.017
p=0.007
CST-KO
CST-KO+CST
WT
WT+CST
C
RDA2 (9.8%, Treatment)
RDA1 (13.4%, Genotype)
CSTKO
CSTKO+CST
WT
WT+CST
Allopreotella
Dubosiella
Akkermansia
Acetitomaculum
Anaeroplasma
Atopobiaceae
Turicibacter
Coriobacteriaceae UCG-002
Staphylococcus
Jeotgalicoccus
D
Phylum
Relative abundance
Actinobacteriota
Bacteroidota
Campilobacterota
Cyanobacteria
Deferribacterota
Desulfobacterota
Firmicutes
Patescibacteria
Proteobacteria
Verrucomicrobiota
Family
Eggerthellaceae
Erysipelotrichaceae
Lachnospiraceae
Lactobacillaceae
Muribaculaceae
Oscillospiraceae
others
Prevotellaceae
Ruminococcaceae
Staphylococcaceae
Unknown
E
LefSe cladogram CST-KO
LefSe cladogram WT
F
Acetate (μg/100mg cecum content)
p=0.038
Butyrate (μg/100mg cecum content)
p=0.018
p=0.002
Propionate (μg/100mg cecum content)

Figure 1. CST treatment affects the murine fecal microbiota. (**A, B**) Alpha diversity was assessed using different metrics and is concordantly significantly increased upon CST treatment. **(A)** represent the species richness (observed ASVs, ACE) and (**B**) species diversity of the CST-KO and WT mice before and after CST treatment. Significance was tested with an unpaired *Mann-Whitney* test. Boxes represent the median with interquartile range, and whiskers represent the maxima and minima. (**C**) Genotype and treatment constrained redundancy analysis (RDA) on genus collapsed abundances. Arrows indicate the association of taxa with samples, with the length being a proxy for the strength of the association. Ellipses represent normal data generated by the R package ggplot2. Significant separation of clusters and contribution of the variables to the variance of the RDA was tested with Permutational Multivariate ANOVA (PERMANOVA) and revealed significant ($p < 0.001$) effects of CST administration, both in CST-KO and WT mice; red - WT, grey – WT+CST, yellow – CST-KO, blue - CST-KO+CST. (**D**) Relative abundance of the present phyla and families. (**E**) Cladograms of a LefSe analysis (Linear discriminant analysis Effect Size) comparing microbiota changes upon CST treatment in CST-KO and WT mice. Significance was tested using a Kruskal-Wallis (KW) test and Linear Discriminant Analysis (LDA). A significant feature was considered when $p < 0.01$ and Log (LDA score) > 3. The green color indicates the microbiota composition before CST treatment and the red color after CST treatment. (**F**) Concentrations of cecal acetate, butyrate, and propionate, of CST-treated (CST-KO, n=7; WT, n=8) and untreated animals (CST-KO, n=8; WT, n=8). Cecal acetate and butyrate were significantly increased in CST-treated CST-KO mice. Boxes represent the median with interquartile range, and whiskers represent the maxima and minima. Data were analyzed using a 2-tailed paired *t*-test, while 2 outliers were removed using ROUT in GraphPad Prism. Error bars are shown as mean ± SEM.

Overall, the results indicated a significant impact of CST treatment on the diversity and composition of the microbiota in the CST-KO mice, where CST is absent. This effect was much less prominent in WT mice, which have normal levels of CST.

CST treatment of CST-KO mice promotes the growth of core taxa present in WT mice.

To investigate whether certain taxa exhibit similar behavior (increase or decrease in abundance) between genotypes when CST is administered, we focused on the core microbiota (common taxa between CST-treated groups), representing microbiota members, which directly (e.g., via resistance genes) or indirectly (e.g., via cross-feeding), resist the antimicrobial effect of CST. To do so, log-fold changes between CST treated and untreated groups for both WT and CST-KO mice were compared (**Figure 2**). Almost all significant changes in core taxa (determined by unpaired Wilcoxon test) were exclusively higher in abundance in CST treated CST-KO mice (quadrants I and II), while, for WT, both significant increases and decreases in the

abundance of core taxa were observed. These results coincide with the RDA analysis (cluster of CST-treated CST-KO group moving closer to WT) and indicate that the effect of CST treatment in CST-KO mice promotes the growth of core taxa associated with WT mice. Taxa present in quadrant II, which represent those common taxa between the untreated WT and treated CST-KO mice, are partially restored in their abundance in the latter group. Of note is, that *Turicibacter* was the only core taxon in both genotypes, exclusively negatively affected by CST treatment, exhibiting a significant decrease in abundance and therefore suggesting higher susceptibility to CST. To confirm this observation, we performed *in vitro* assays, to determine minimum inhibitory concentrations (MIC) for *Turicibacter sanguinis*. MIC was also performed for *Bacteroides thetaiotaomicron*, which increased in abundance upon CST treatment. The screening revealed low MIC for *T. sanguinis* (32 μM CST), while *B. thetaiotaomicron* had a higher MIC (>64 μM CST), thus confirming our *in vivo* observations. Reverse-phase HPLC analysis of spent culture supernatant after 24 h incubation with CST revealed degradation/uptake of CST from the medium, in the case of *B. thetaiotaomicron*, but seemingly less for *T. sanguinis*, hence explaining the higher MIC value in *B. thetaiotaomicron* (**Supplementary Figure 2**). Taken together, the data imply that the changes in the gut microbiota composition associated with the CST treatment (**Figure 1**) may be caused by the antimicrobial resistance of certain taxa to CST. However, the underlying mechanisms remain obscure.

Catestatin treatment promotes the abundance of the gut microbiota harboring antimicrobial resistance phosphoethanolamine transferase-encoding genes

To determine whether bacteria with an increase in abundance in CST-treated groups harbor antimicrobial resistance genes, a metagenomic prediction was performed using PiCRUSt2 on KO (KEGG Orthology) level. From a panel of known AMP resistance genes[21], one KEGG Orthologue (K03760) was found to be significantly different in abundance between CST treated and untreated WT mice and close to significance in CST-KO, when corrected for multiple testing (**Supplementary Excel Sheet**).

Sequences within this KEGG Orthogroup contain orthologs of *EptA* (*E. coli*) phosphoethanolamine transferase. As KEGG Orthologs only contain sequences from reference genomes with no specific gut metagenomic context, we sought to refine our search to investigate the taxonomical distribution of the *EptA*. To do so, the *EptA* protein sequence from *E. coli* BW25113 was used as a query to search the mouse gastrointestinal bacterial catalog (MGBC). Identified protein sequences were further filtered and a phylogenetic tree was constructed, which exhibited a distinct grouping of sequences without great dispersion and most of the sequences of the same

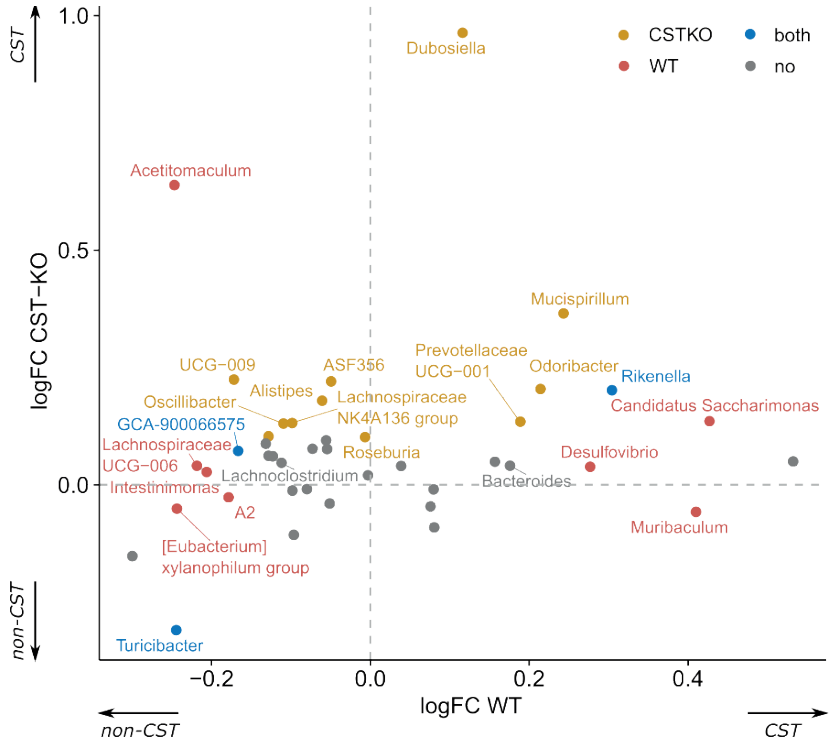

Figure 2 Taxa able to endure CST treatment benefit from its presence in the environment. Scatter plot of log-fold changes, comparing abundance changes for significantly abundant genera between treated and untreated groups per genotype. Colors represent significance when testing for differential abundance between treated and untreated groups for each genotype.

phylum were clustered in separate clades. This showed a major subdivision between the foremost sequences from *Bacteroidota*, *Proteobacteria*, and Campylobacterota (**Figure 3A**).

Using the taxonomical distribution of *eptA*, we reanalyzed the abundance of genera harboring *eptA* upon CST treatment. Here, both CST-treated groups showed a significant increase in microbiota-harboring *eptA*, though the effect in the CST-KO group seemed to be more pronounced (**Figure 3B**). To further support our findings that *eptA*-harboring core taxa increase in abundance in CST treated groups, we looked at individual log-fold changes of single taxa, as described above (**Figure 2**). Indeed, *eptA*-harboring core taxa were higher in abundance in CST treated groups, regardless of the genotype (**Supplement Figure 3A**).

To further confirm the bioinformatics analysis outcome for the involvement of the *eptA* gene in CST resistance, wild-type *E. coli* BW25113 and an *eptA* knockout strain (Δ*eptA*) were employed and were cultured in different concentrations (8-100 μM) of CST (**Figure 3C**). The results indicate that the lack of *eptA* led to a decreased carrying capacity at higher CST concentrations (**Figure 3C**), which is equivalent to the maximum population size of the culture, as well as a concentration-dependent prolonged lag-phase during the growth of the *E. coli* (**Figure 3C**).

Similar to the majority of known antimicrobial peptides, CST is recognized by the *PhoP*Q system, which involves the induction of expression of the gene *pmrD*, followed by activation of *pmrA*, and eventually *eptA* expression[23]. To this end, we tested the expression of the above-mentioned transcription regulators in *E. coli* BW25113 strain. Most notably, *phoP* showed an approximately 3-fold increase at 0.5 h after stimulation of *E. coli* BW25113 strain stimulated with 20 μM CST (sufficient concentration for detection without growth penalty), but only 2-fold and less prolonged in the control, before returning to baseline expression. The *pmrA* showed a later upregulation (after 1h) compared to *phoP* in *E. coli* BW25113 CST-stimulated cultures, while *pmrD* expression spiked in the presence of CST, but was invariant between the different conditions (**Supplementary Figure 3B**). Taken together, the results show a higher abundance of the bacteria-harboring *eptA*-like genes upon CST treatment, potentially providing these CST-resistant bacteria an advantage to colonize the gut and, in turn, indirectly affect the colonization of other bacterial taxa.

Catestatin peptide is degraded by *E. coli* omptin protease

The ability of *E. coli* BW25113$^{\Delta eptA}$ to still resist comparatively high CST concentrations led us to test whether the bacterium harbors an additional resistance mechanism, such as degradation or uptake, similar to what we observed in *B. thetaiotaomicron* (**Figure 3D**). We analyzed the spent culture supernatant by reverse-phase HPLC, which showed absence of CST-corresponding peaks after 24 h of incubation with *E. coli* BW25113$^{\Delta eptA}$ as well as *E. coli* BW25113 (**Supplementary Figure 4A**). To test whether the enzyme involved in the CST degradation is excreted in the supernatant, sterile-filtered supernatants of non-stimulated *E. coli* BW25113 cultures were incubated with CST and subjected to tricine SDS-PAGE to visualize the degradation/uptake of CST. Indeed CST was cleaved by *E. coli* BW25113 after incubation for different time intervals from 30 min up to 24 h (**Figure 4A**). This suggested the involvement of a secreted or membrane-anchored protease, which can also be present in secreted vesicles, in CST degradation. To identify which protease was involved, literature search was performed and revealed that *E. coli* harbors only 2 major outer membrane proteases, predicted to cleave CST [24]. Among those proteases is the *ompT*in, an outer membrane protease, encoded by *ompT*. To confirm that

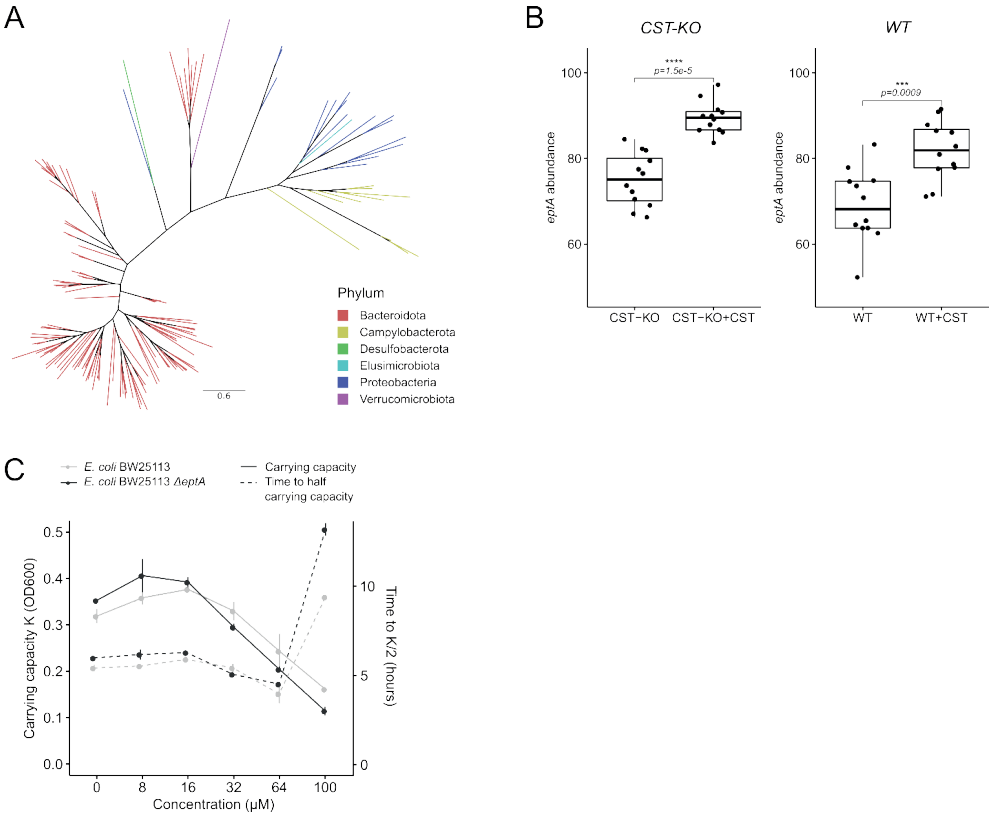

Figure 3. CST treatment promotes the abundance of the antimicrobial resistance gene, *eptA*. (**A**) Unrooted phylogenetic tree depicting the distribution of orthologous *eptA*-like genes throughout bacterial species in the mouse gut. The tree was pruned using Treemmer to aid visualization, while branches were colored by the phylum they belong to. (**B**) Boxplot showing a significant abundance increase of *eptA* harboring bacteria upon CST treatment, both in CST-KO and WT mice. The *eptA* abundance is defined as the sum of abundances from all taxa harboring an *eptA* gene. Boxes represent the median with interquartile range, and whiskers represent the maxima and minima. Significance was assessed by an unpaired *t*-test. (**C**) CST inhibits the growth of wild-type *E. coli* BW25113 and *E. coli* BW25113$^{\Delta eptA}$ strains, visualized by carrying capacity K (a measure of maximum OD600) and time taken to reach half the carrying capacity (inflection point of the growth curve at mid-log-phase) that depends on CST concentrations ranging from 8-100 μM.

*ompT*in was responsible for the CST degradation, an *E. coli ompT* knockout strain (*E. coli* BW25113$^{\Delta ompT}$) was employed and was cultured in different concentrations of CST. Compared to the wild-type and *E. coli* BW25113$^{\Delta eptA}$ strains, *E. coli* BW25113$^{\Delta ompT}$ showed no growth at 100 µM CST and an extensively prolonged lag-phase at 64 µM (**Figure 3D** and **Figure 4B**). Collectively, the results imply that wild-type *E. coli* BW25113 possesses the ability to cleave CST through its outer membrane protease *ompT*in, providing the bacterium with another defense mechanism, together with *EptA*, to resist the antimicrobial action of CST.

To further confirm the complementary function of *EptA* and *ompT*in, we performed qPCR on control (unstimulated) and CST-stimulated cultures of wild-type *E. coli* BW25113 and *E. coli* BW25113$^{\Delta ompT}$. The expression of *eptA* was upregulated about 1.7-fold in *E. coli* BW25113$^{\Delta ompT}$ without CST stimulation compared to the wild-type strain (**Figure 4C**). When stimulated with 20 µM CST, an increase in the expression of *ompT*, but not *eptA*, was detected in wild-type *E. coli* BW25113 without stimulation and at 0.5 h after stimulation, while this effect was reversed at later time points (**Supplementary Figure 3B**).

As *E. coli* BW25113 is considered a lab strain, which might not be representative of naturally occurring *E. coli* strains, we also tested for the presence of *ompT* in two gut isolate strains, namely *E. coli* Nissle 1917 and *E. coli* DSM 11250. Both were found to be *ompT* carrying (**Supplementary Figure 4B**). Taken together, the results indicate the capacity of *E. coli* strains harboring *ompT*in to degrade CST, plausibly altering endogenous levels of the peptide in the gut in cases where the abundance of *E. coli* is significantly increased, such as in inflammatory bowel disease[25] To test this hypothesis, we investigated the abundance of *ompT*-like genes (KO term K01355) in different subtypes of inflammatory bowel diseases (IBD), which are also associated with altered levels of CST[15], using publicly available data from the MetaQuery database[26]. A significant increase in the abundance of *ompT*-like genes was found between non-IBD and Crohn's disease patients. Similarly, there was a significant increase in the abundance of *ompT*-like genes between Crohn's disease patients and patients with ulcerative colitis, but not between ulcerative colitis diseased and non-IBD subjects (**Figure 4D**). This observation was consistent with the abundance of *E. coli* in those patients (**Figure 4D**). Overall, the data indicate that CST degradation by bacteria-harboring *ompT*in-like proteases may play a role in altered levels of the peptide previously detected in IBD patients, which in turn would result in an overgrowth of these bacteria and worsening of the disease situation.

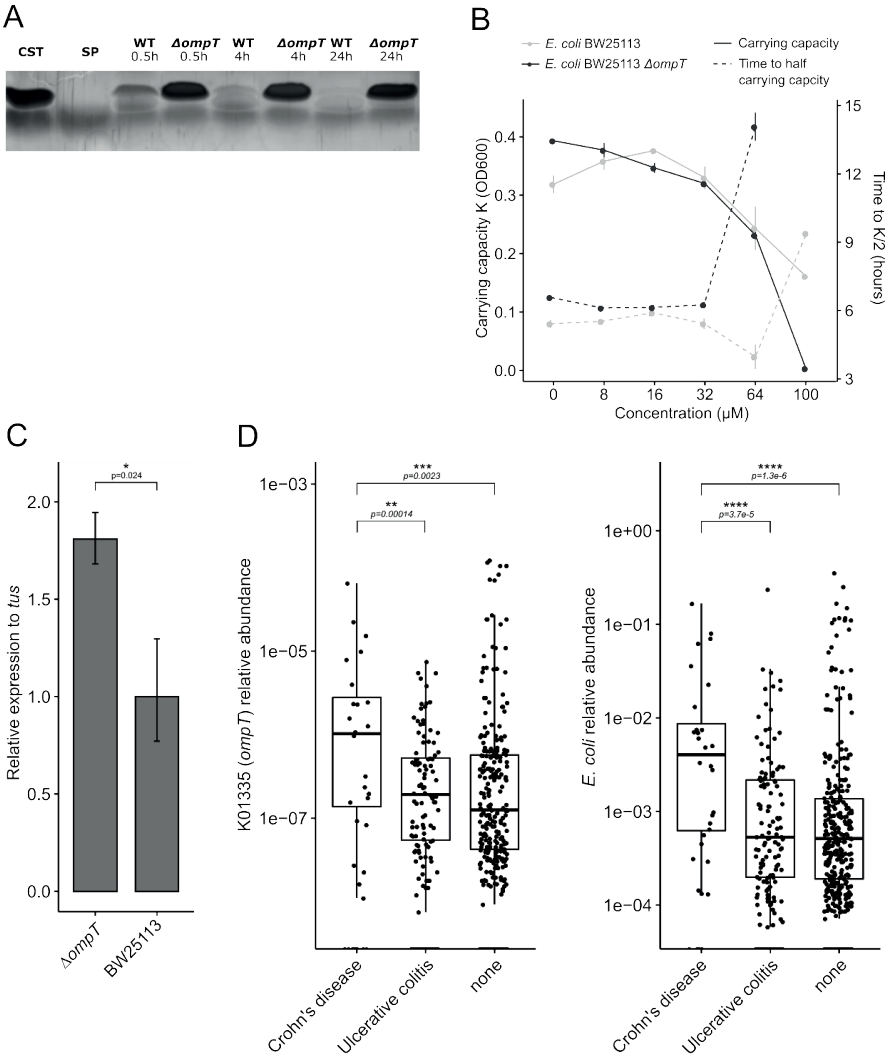

Figure 4. Omptin enzyme is responsible for the degradation of CST in *E coli*. (**A**) Silver-stained tricine SDS-PAGE gels showing the cleavage of CST by wild-type *E. coli* BW25113, but not in *E. coli* BW25113$^{\Delta ompT}$. Sterile non-stimulated culture supernatants were cultured with 100μM CST and samples were collected at different time points; CST: catestatin (2.3kDa), SP: substance P (1.3kDa) (**B**) CST inhibits the growth of *E. coli* BW25113$^{\Delta ompT}$, visualized by carrying capacity K (a measure of maximum OD600) and time taken to reach half the carrying capacity in CST concentrations ranging from 8-100 μM. (**C**) Baseline expression of *eptA* is increased in *E. coli* BW25113$^{\Delta ompT}$ compared and normalized to that of wild-type *E. coli* BW25113. *tus* was used as a housekeeping gene for normalization in both strains and statistical significance was calculated using a Wilcoxon test. (**D**) Relative abundance of *ompT*in like genes or *E. coli* in patients with Crohn's disease, ulcerative colitis, and healthy subjects. Axes are on log10-scale and significance was assessed using Wilcoxon test without stratification.

Discussion

This study unravels the crucial role of CST on the microbiota composition in the fecal samples of WT and CST-KO mice. Specifically, there was a significant increase in the abundance of taxa that harbor the CST peptide-resistance enzyme, phosphoethanolamine transferase in CST-KO and WT mice treated with CST (**Figure 3B**). The shifts observed in the microbial phyla in this study after treating WT mice with CST are in agreement with the findings of Rabbi *et al.*, who also reported an increase in Bacteroidota and a decrease in Firmicutes, albeit a different route for the administration of CST and dosage[19]. Low abundance of Bacteroidota, especially *Bacteroides/Prevotella* have been linked to obesity, and CST-KO mice were described to be insulin resistant and obese besides having elevated blood pressure[13,27]. In contrast, increased Bacteroidota/Firmicutes ratio has been associated with IBD[28,29], which is also characterized by elevated levels of CST[15]. Nevertheless, as CST is a multifunctional peptide, acting directly on the microbiota as well as on the host, any changes related to CST treatment should be viewed as a complex interplay and not direct causation originating from the microbiota[12]. Additionally, the lack of CST in CST-KO mice may have induced the expression of other antimicrobial peptides, which, in turn, may contribute to the changes associated with the CST treatment.

CST treatment could, only to a small extent, restore the microbial composition in a CST-depleted environment, to the composition detected in an environment already primed with CST (WT mice) (**Figure 2**). In general, the effect of CST treatment observed in the CST-KO group was significantly greater than that in the WT group (**Figure 1**). This difference could be attributed to the presence of normal levels of CST in the WT mice; thus, the microbiota is already primed with the peptide and has evolved mechanisms to resist its antimicrobial effect. In contrast, the CST-KO group, in which CST is absent, may harbor bacteria that are susceptible to the CST antimicrobial activity but were eradicated upon CST treatment (**Figure 2**). Especially the core taxa resisting CST treatment seem to benefit from their presence in the gut lumen environment, by exhibiting an increase in abundance. The introduction of CST to a CST-depleted environment did not necessarily promote exclusively health beneficial microbiota, but rather led to a more equilibrated composition, including also pathobiont taxa. For example, in CST-treated CST-KO, *Oscillibacter* and *Mucispirillum* (**Figure 2**), both of which increased in relative abundance, have been found to increase in diet-induced obesity and might be considered as pathobionts[30,31]. Similarly, *Alistipes* was shown to have a detrimental influence on colorectal cancer progression, despite contrasting evidence that it also has protective effects against colitis or cardiovascular diseases[32]. On the other side *Odoribacter*, an SCFA-producer,

which increased in relative abundance in CST-treated WT mice (**Figure 2**) has been linked to immunomodulatory effects and general health benefits presumably also in cooperation with *Akkermansia* [33].

So far, antimicrobial resistance mechanisms have been mostly investigated in pathogens due to their implication for antibiotic resistance. In *in-vitro* studies, CST has been reported to have an inhibitory effect against the pathogenic gram-positive (*Micrococcus luteus, Bacillus megaterium*), gram-negative bacteria (*Escherichia coli* D22), and filamentous fungi (*Neurospora crassa* and *Aspergillus fumigatus*)[5]. Moreover, human CST showed antimicrobial effects against several skin microbes including the gram-positive (*Staphylococcus aureus* and Group *A Streptococcus*), gram-negative bacteria (*E. coli O29*, and *Pseudomonas aeruginosa*), yeast (*Candida albicans*), and filamentous fungi (*Aspergillus niger*, *Aspergillus fumigatus*, *Trichophyton rubrum*)[17].

As a defense mechanism, bacteria have evolved several systems to survive the effect of the antimicrobial peptides[21]. The ability to withstand these peptides provides a potential benefit in colonization over other members of the gut microbiota, especially under pathological conditions[34]. Analogously, antimicrobial peptides are regularly used by the host to select for specific gut microbiota in homeostatic situations. For CST, the antimicrobial activity resides in the N-terminal of the peptide, which is highly cationic[5]. These positive charges in the peptide interact with the anionic components of the bacterial cell membrane, resulting in the permeabilization of microbial membranes, leading to cell lysis[7]. The present study suggests that CST may as well play a role in aiding the host to select which microbes to colonize the gut. The abundance of bacteria encoding the phosphoethanolamine transferase genes increased upon CST treatment (**Figure 3A-D**). This is not surprising since the *E.coli* phosphoethanolamine transferase *eptA*, for example, has been linked to colistin resistance, being a cyclic proteinogenic antibiotic[35]. EptA acts by modifying core lipopolysaccharide components with ethanolamine, increasing membrane charge and thus, repelling the antimicrobial agent. The fact that EptA-like proteins are abundant across a variety of different taxa present in the human gastrointestinal tract (**Figure 3A-C**) implies that antimicrobial resistance is a mutualistic and important component in the selection of a specific microbiota composition by the host, through active modulation of antimicrobial peptide levels, such as CST. Indeed, this paradigm is supported by our findings that CST treatment induced a large-scale alteration in the microbiota composition not only in the CST-KO group but also in the WT group, both of which clustered closer to each other, and further away from the untreated groups (**Figure 1**).

The induced expression of *eptA* gene in *E. coli* BW25113$^{\Delta ompT}$ (**Figure 4C**) implies a complementary mechanism when other defense mechanisms are missing. Indeed, we uncovered the degradation of CST by wild-type *E. coli*, a member of the gut microbiota via *omptin*, an outer membrane protease, known to cleave other cationic antimicrobial peptides, such as protamine or LL-37[36,37]. *Omptin* proteases exhibit a strong requirement for arginine in the P1 position of the cleavage site, while P1' can be either lysine, glycine, or valine[38]. Catestatin harbors one arginine – glycine site, thus *omptin* may potentially cleave at this position, yielding cateslytin and a hexapeptide. As cateslytin has been reported to have an antimicrobial activity[5], this cleavage might not completely ameliorate the effect of CST. Nonetheless, we observed a reduction of the inhibitory concentration in *E. coli* BW25113$^{\Delta ompT}$ (**Figure 4B**). The presence of these fragments in the gut has implications on the microbiota composition, as it may facilitate colonization of other CST-susceptible bacteria[39]. It remains elusive though, whether CST is taken up and accumulating inside the bacteria or is further degraded, and what the fate of the resulting cleavage fragments is, e.g., through uptake by other microbiota or interaction with the host. Analogously it remains unclear how other bacteria, such as *B. thetaiotaomicron* can degrade CST (**Supplementary figure 2**) and how widespread such a phenomenon is among the gut bacteria.

Levels of CST are increased in IBD patients, while the presence of *E. coli* was associated with the development and progression of different IBD subtypes such as Crohn's disease or ulcerative colitis[25,40]. The higher abundance of *ompT*-like genes in samples from Crohn's disease patients, which coincided with a higher abundance of *E.coli* (**Figure 4D**), suggests that bacteria harboring *ompT*-like genes, such as *E. coli*, may have a benefit to colonize the gut due to its ability to resist CST. In cases where the overall microbial composition is compromised, and the endogenous levels of CST are altered, like in IBD, such bacterial resistance may cause particular bacterial strains to be involved in worsening the gut inflammation. Overgrowth of gut bacteria with the capacity to resist CST may lead the host to secrete higher levels of CST as a defense mechanism. The levels of CST were reported to be higher in IBD patients compared to healthy subjects[15]. It remains unclear why only samples from Crohn's disease patients but not ulcerative colitis showed a higher abundance of *ompT* as well as *E. coli*. One plausible explanation could be due to the different *E. coli* strains predominantly present in either Crohn's disease or ulcerative colitis[25] (**Figure 4D**).

Overall, the present study highlights the significant role of the CST peptide in determining the microbiota composition. The fact that gut bacteria with a capacity to resist the antimicrobial effect of CST become more abundant upon CST treatment,

suggests that these bacteria may have gained an advantage over other microbiota members to survive the gut environment, which, indirectly shapes the colonization of the total community. Further studies should also explore the changes elicited by CST administration on the host side to gain a clearer picture of the direct and indirect effects of CST on the gut microbiota.

Materials and Methods

Animals

All studies with mice were approved by the University of California San Diego (UCSD) and Veteran Affairs San Diego Healthcare System (VASDHS) Institutional Animal Care and Use Committees for the Mahata laboratory (UCSD: #S00048M; VA: #A13-002) and were performed in San Diego in adherence to the NIH Guide for the Care and Use of Laboratory Animals. Our experiment was based on a single cohort of mice which consisted of twelve mice per group each (control and CST-treatment, per genotype); out of those, twenty-four male adult C57BL/6 J mice (age 20 weeks) were purchased from Jackson Laboratory (Bar Harbor, ME) and twenty-four CST-KO mice (age 20-28 weeks) generated in the Mahata laboratory, were used. CST-KO mice have a deletion in the 63 bp CST domain from Exon VII of the *Chga* gene[27] in C57BL/6 background. Mice from WT and CST-KO groups were injected intraperitoneally once daily with CST (2 µg/g body weight; Genscript Biotech Corporation, Piscataway, NJ) for 15 days based on previous experimentation [15]. Mice were housed in 4 to 5 animals per cage and had free access to water and food (Normal Chow Diet, LabDiet 5001) in temperature and humidity-controlled rooms with a 12-hr light/dark cycle.

Determination of short-chain fatty acids (SCFAs) in cecal samples

Cecal samples (100 mg) were suspended in 1 mL of saturated NaCl (36%) solution. An internal standard (50 µL of 10.7 µM 2-methyl butyric acid in MQ water) was added and the samples were homogenized using glass beads. After the addition of 150 µL H_2SO_4 96%, SCFAs were extracted with 3 ml of ether. The ether layer was collected and dried with Na_2SO_4 (150 mg). The supernatant (0.5 µL) was analyzed using gas chromatography with flame ionization detection (Agilent, Santa Clara, California, USA). The system was equipped with a DB FFAP analytical column (30m x 0.53 mm ID, 1.0 µm; Agilent) and helium GC grade (5.6) was used as carrier gas with a constant flow of 4.2 ml/min. The initial oven temperature was held at 100 °C for 3 min, ramped with 4 °C/min to 140 °C (isothermal for 5 min) and further with 40 °C/min to 235 °C (isothermal for 15 min). The resulting chromatograms were processed using ChemStation (Agilent Technologies).

DNA isolation and 16S rRNA gene sequencing

DNA isolation was performed on fecal samples that were collected from untreated and CST-treated WT and CST-KO mice. A phenol-chloroform-isoamyl alcohol procedure was used for DNA extraction[41]. Briefly, the pellet was resuspended in 1 ml of lysis buffer (940 µl TE buffer, 50 µl SDS 10%, and 10 µl proteinase K 20 mg/ml) in a 2 ml screw cap micro-tube containing a mix of zirconium and glass beads. Then, samples were incubated at 58 °C for 1 hour and 150 µl of buffered phenol (Invitrogen, 15513-047) was added. To support lysis, samples were homogenized 3 × 30 sec with 1-min intervals on ice in a mini bead-beater (Biospec, Bartlesville, USA). This was followed by the addition of 150 µl chloroform/isoamyl alcohol (24:1) and centrifugation at 16,000x g for 10 min and 4 °C. The upper layer was carefully transferred to a clean tube, 300 µl of phenol-chloroform-isoamyl alcohol [25:24:1] was added, centrifuged and the process was repeated by adding 300 µl of chloroform-isoamyl alcohol [24:1]. To precipitate the DNA, the upper layer was transferred to a new tube and 1 volume of absolute isopropanol and 1/10 volume of 3 M sodium acetate was added. Samples were incubated overnight at -20 °C. Subsequently, samples were centrifuged at 16,000x g for 20 min at 4 °C, the supernatant was discarded and the pellet was washed with 700 µl of 70% ethanol. After ethanol aspiration, the DNA pellet was air-dried for 30 min and resuspended in 100 µl TE buffer. In each step, samples were vigorously mixed using a vortex.

Sequencing of the V3-V4 region of the bacterial 16S rRNA gene was carried out by Novogene Co. Ltd. Briefly, for sequencing library preparation, raw DNA extracts were diluted to 1 ng/µl in sterile water, and amplicons were generated by PCR (primers 341F and 806R) using a Phusion High-Fidelity PCR Master Mix (New England Biolabs). Amplification product quality was assessed by gel electrophoreses and samples were pooled in equimolar ratios. Libraries were generated with a NEBNext Ultra DNA Library Prep Kit for Illumina and sequencing was carried out on an MiSeq (Illumina) 250 bp paired-end platform. Initial processing of reads involved trimming of adapters and primers using a Novogene in-house pipeline (Novogene Co. Ltd, Cambridge, UK).

Microbiota analysis

Paired-end sequencing reads were filtered, denoised, merged, and classified with the dada2 package in the statistical programming language R while processing forward and reverse reads separately until merge[42,43]. Briefly, reads were truncated to 220 bases and low-quality reads were filtered followed by dereplication. Error models were learned, while manually enforcing the monotonicity of the error function. Reads were denoised and merged with a minimal overlap of 12 bp, while

non-merging reads were concatenated. Singletons have been removed before performing bimera removal, followed by read classification using SILVA (V138) as a taxonomical reference database.

For downstream analysis, the phyloseq and microbiome packages were used, samples were rarefied to even depth, and richness was determined by the number of observed amplicon sequence variants (ASVs), as well as ACE index and alpha diversity was assessed via Shannon's H and inverted Simpson indexes[44,45]. All ASVs were collapsed on genus level and cumulative sum scaling was applied using metagenomeSeq[46]. The resulting genus abundance table served as input for different types of ordinations such as principal component analysis (PCA) or redundancy analysis (RDA). The significance of constraints in constrained RDA was determined by permutation-like ANOVA. Differential abundance was assessed by unpaired Wilcoxon Rank Sum test using $p < 0.05$ as a significance threshold followed by FDR correction.

To generate the double log-fold change plot, first, the core microbiota were estimated for each group by filtering raw ASV counts to contain only more than 10 reads and be present in more than 10% of the samples. Next, only taxa intersecting between CST treated groups were selected and analyzed further. Differential abundance was assessed by a Wilcoxon Rank Sum test between treated and untreated groups per genotype and FDR corrected. Only taxa with an adjusted $p < 0.05$ were considered further. Their mean log-fold changes between groups were calculated and plotted against each other.

Further LefSe analysis was carried out, adjusting the alpha value for factorial Kruskal-Wallis test and the alpha value for pairwise Wilcoxon tests to 0.01. Additionally, the LDA score threshold was set to 3.0[47].

For the functional analysis, the metagenomic prediction from 16S rRNA gene sequencing data was done using PiCRUSt2, using raw ASVs and unnormalized ASV abundances[48]. For this, ASVs were first filtered to have a minimal abundance of 10 reads and to be present in at least 1/10 of the samples. PiCRUSt2 was run with standard parameters and stratification enabled. The metagenomic data was further analyzed by using STAMP v2.1.3[49]. For statistical analysis, White's non-parametric t-test, two-sided, and Benjamini-Hochberg multiple test correction were used to reveal differences between groups.

Phylogenetic analysis

The amino acid sequence of the *E. coli eptA* gene (UniProt P30845) was searched with phmmer from the hmmer suite (v3.3.1; hmmer.org) against a non-redundant dataset of the mouse gastrointestinal bacterial catalog (MGBC)[50]. Hits were filtered on full sequence E-value, excluding all sequences with an E-value greater than 0.5x the median absolute deviation from the median. Additionally, only sequences originating from genomes with >90% completeness and <1 contamination were taken into account. Sequences were dereplicated, aligned with MAFFT (v7.453) and a phylogenetic tree was constructed using FastTree (v2.1.11)[51,52]. The produced gene tree was visualized with FigTree (v1.4.4), to aid visibility the tree was pruned using Treemmer (v0.3; with parameter -RTL 0.95)[53].

Bacteria and growth inhibition assay

E. coli BW25113, BW25113$^{\Delta ompT}$ (JW0554) and BW25113$^{\Delta eptA}$ (JW5730) (**Supplementary Table 1**) were routinely grown aerobically in Difco Antibiotic Medium 3 (BD, Franklin Lakes, USA) at 37 °C degrees without agitation. Bacteria were inoculated from −80 °C stocks and grown overnight. Before the experiment, cultures were diluted at 1:100 in fresh medium from overnight cultures. Five different CST concentrations were tested in triplicates in a 96-well plate. For this, 10 µl of a 10x concentrated stock of CST were added to 90µl of diluted culture to obtain final concentrations of 8, 16, 32, 64, and 100 µM. Bacterial growth was monitored for 24 h at 600 nm (OD600) with shaking for 5s every 15 min using a BioTek ELx808 microplate reader (BioTek, Winooski, USA). Growth curves were fit using the growthcurver R package (v0.3.0).

Tricine SDS-PAGE

Overnight cultures of *E. coli* strains BW25113 and BW25113$^{\Delta ompT}$ (**Supplementary Table 1**) were centrifuged at 10,000 g for 10 min at 4°C and the supernatant was collected. The supernatant was further sterile filtered (0.2 µm filters) and to 90 µl of supernatant 10 µl of a 10x CST stock was added to reach a final concentration of 100 µM. Aliquots of 20 µl were taken at time points 0.5, 4, and 24 h.

To visualize CST degradation, culture supernatants were run on Tricine SDS-PAGE following a modified protocol[54]. Briefly, gels consisted out of a 4% stacking gel and a 16% resolving gel both with a cross-linker ratio of 19:1, and were prepared to standard procedure. For sample prep, 10 µl of each sample were mixed with 10 ml of reducing sample buffer (100 mM Tris-HCl, 1% SDS, 4% 2-mercaptoethanol, 0.02% Coomassie Brilliant Blue, 24% glycerol) and incubated at 70°C for 5 min. Subsequently, 10 µl of the sample was loaded on the gel, first, run for ~30 min at 30 V, and then at 170 V for 90 min. Silver staining was applied to visualize proteins using various modifications in fixation to avoid loss of small proteins[55]. For this, gels were

fixed (30% ethanol, 15% formalin, 5% acetic acid) for 60 min and quickly washed once with 50% ethanol and twice with MilliQ-filtered water. Gels were incubated for 40 min in 0.005% sodium thiosulfate, followed by incubation for 40 min in 0.1% silver nitrate solution containing 0.07% formalin. Washing with MilliQ-filtered water was performed and a 2% sodium carbonate solution containing 0.1% formalin was added. Gels were incubated for 1-2 min until bands became visible and development was stopped by removing the developer and adding 50 mM EDTA solution. Gels were then imaged in a ChemiDoc MP Imaging System (BioRad, Hercules, USA).

Expression analysis

Overnight cultures of *E. coli* BW25113, *E. coli* BW25113$^{\Delta eptA}$, and *E. coli* BW25113$^{\Delta ompT}$ were resuspended 1:1000 in 6 ml of fresh Difco Antibiotic Medium 3 and grown until OD600 0.5 was reached. Aliquots of 1 ml were harvested, centrifuged at 10,000 g for 10 min, and immediately stored at -80 °C. This and all subsequent centrifugation steps were done at 4 °C.

For culture stimulation with CST *E. coli* BW25113 or *E. coli* BW25113$^{\Delta ompT}$ was resuspended 1:1000 in 6 ml fresh Difco Antibiotic Medium 3 and grown until OD600 0.5. An aliquot of 5 ml was harvested and centrifuged at 3,000 g for 10 min. The pellet was resuspended in a fresh medium containing 20 µM CST and gently mixed. Samples of 1 ml were taken at time points 0h (before resuspension), 0.5h, 1h, 2h, and 4h, subsequently centrifuged at 10,000 g for 10 min and immediately stored at -80 °C.

RNA was isolated using a phenol:chloroform:isoamyl alcohol procedure as previously described [56]. The harvested bacterial pellet was resuspended in 200 µl TE buffer and transferred to a tube containing 0.5 g of 0.1 mm zirconia/silica beads, 25 µl 10% SDS, and 200 µl phenol:chloroform:isoamyl alcohol solution. The subsequent procedure was performed as described and isolates were stored at -80°C until further use.

qPCR was carried out according to standard procedures[57]. Briefly, 2 µg of RNA were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (Applied Biosystems, Vilnius, Lithuania). Subsequently, 10 ng of cDNA were used for qPCR employing a PowerUp SYBR Green Master Mix (Applied Biosystems, Vilnius, Lithuania) and primers for genes *eptA*, *ompT*, *phoP*, *pmrD*, *pmrA* and tus. Reactions were performed in a clear 96-well plate using a CFX96 Real-Time PCR System (BioRad, Hercules, USA). Gene expression was assessed by normalizing to housekeeping gene tus using the R package qpcR (v1.4) for curve fitting and the pcr (v1.2.2) package for differential expression calculation employing the $2^{-\Delta\Delta Ct}$ method as described in[58].

Statistical analysis

All statistical tests were performed using GraphPad Prism 7 or the statistical programming language R. Specific statistical tests are indicated in the text or figure legends. Normality was assessed by either D'Augustino-Pearson omnibus normality test or Shapiro-Wilk normality test. If normality was met a t-test was chosen, otherwise non-parametric Mann-Whitney was used. Outliers were assessed with GraphPads ROUT method (Q=1).

Data availability statement

All data generated or analyzed during this study are included in this manuscript and its supplementary information files. The 16S rRNA gene amplicon sequence data were deposited under BioProject number PRJNA741992.

Authors' Contributions

P.G-D., M.S, S.E.A and S.K.M conceived and designed the study. P.G-D., M.S., B.D, and S.K.M. performed the experiments, and P.G-D., M.S, B.D., and S.E.A analyzed the data. P.G-D, M.S, S.E.A. wrote the original manuscript that was reviewed by A.D, B.D., K.V., and S.K.M. Funding for these studies was acquired by S.E.A. and S.K.M. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no competing interests

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Supplementary Methods

Culturing of fecal content

A pre-culture was prepared by pooling the fecal content of 20 healthy human subjects, which was previously collected and stored in (1:1) liquid amies at -80 °C. For this, approximately 1.1 g of fecal content from each subject were used and suspended with a total of 71 ml of Gifu Anaerobic Broth (HiMedia Laboratories GmbH, Einhausen, Germany). The culture was gently homogenized by vortexing and transferred into a 100 ml Erlenmeyer flask with a cotton stopper and incubated anaerobically (2% H_2, 5% CO_2, balanced with N_2) in a Coy Laboratory Anaerobic Chamber (neo-Lab Migge GmbH, Heidelberg, Germany) at 37 °C overnight. A 10x stock solution of CST was prepared by dilution with MilliQ-filtered water. For each culture, 2.7 ml of pre-culture was added to a tube (5ml tubes; Geiner Bio-One, Frickenhausen, Germany), incubated for 30 min before the addition of 300µl of 10x peptide stock solution, to reach a final volume of 3ml and final concentration of 10 µM CST. Samples of 200 µl were taken after 24 and 48 hours, respectively, centrifuged at 16000 x g for 15 min, and supernatant and pellet were stored separately at -80 °C until further use.

DNA isolation followed a previously proposed protocol by Yu and Morrison (2004). Briefly, the pellet was resuspended in 750 µl of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, 50 mM EDTA, 4 % SDS) and transferred to a screw cap tube containing ~ 500mg of 0.1mm zirconium bead and four 3mm glass beads. Samples were homogenized 3 × 1 min with 1-minute intervals on ice in a mini bead-beater (Biospec, Bartlesville, USA). Subsequently, samples were further incubated at 95°C for 15min and centrifuged at 16000 x g for 15 min at 4 °C. The supernatant was transferred to a clean tube and 20µl of 10M ammonium acetate was added and incubated on ice for 10 min before centrifuging at 16000 x g for 15 min at 4 °C. The supernatant was again transferred to a clean tube and one volume of 100% ice-cold isopropanol was added. Samples were incubated on ice for 1.5-2 h and centrifuged at 16000 x g for 15 min at 4 °C. The supernatant was aspirated and the pellet was washed with 200 µl of 70% ethanol with the following centrifugation to collect the pellet. Ethanol was removed and tubes were left to air-dry for ~1 h before resuspension of the pellet in 200 µl of TE buffer. Sequencing and microbiome analysis was carried out as described in the main text.

Inhibition assay

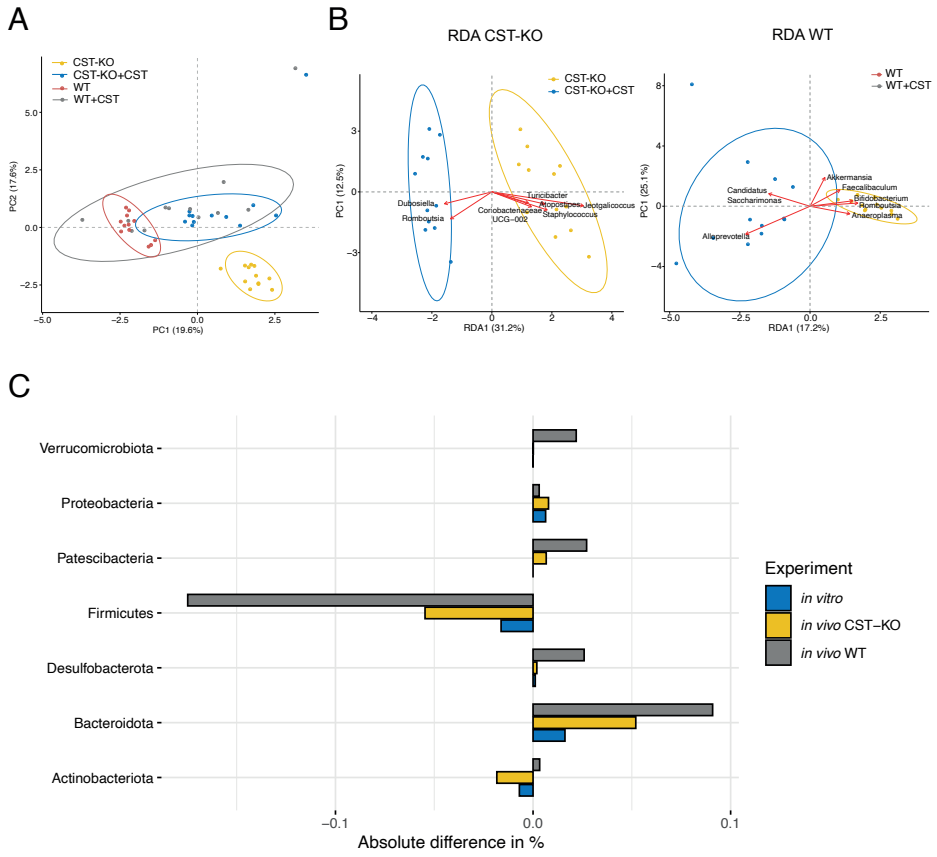
We determined minimal inhibiting concentrations for several gut-related bacteria, which showed up as differentially abundant taxa in previous unpublished analyses and were thought to be related to a CST environment. Bacteria were grown overnight in 1ml of modified DSMZ medium 110 to stationary phase and subsequently diluted

1/100 in medium. Five different CST concentrations were tested in triplicates in a 96-well plate. For this 10 µl of a 10x concentrated stock of CST were added to 90µl of diluted 6 culture to obtain final concentrations of 4, 16, 32, and 64 µM. Growth was monitored for 24h at 600nm (OD600) with shaking for 5 s every 15 min using an Infinity 200 Pro microplate reader (Tecan, Grödig, Austria). Growth curves were fit using the growthcurver R package and minimum inhibiting concentrations (MIC) were determined upon visual inspection of the growth curves.

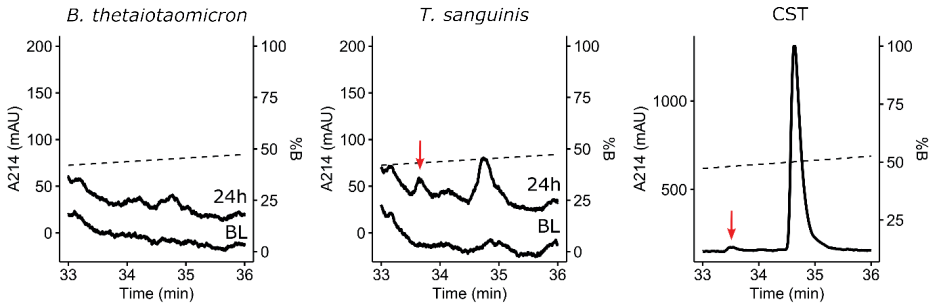
HPLC

Bacterial cultures were centrifuged at 16000 x g for 10 min at 4 °C and 100 µl of supernatant were transferred to a clean tube. 200 µl of denaturation solution (7 M urea, 2 M thiourea, 20 mM dithiothreitol) were added, briefly vortexed and 1ml of ice-cold 100% acetone was added. Samples were incubated on ice for 1.5 h before centrifugation at 16000 x g for 15 min at 4°C. The supernatant was discarded and 200 µl of resolubilization solution (70% acetonitrile, 12 mM HCl) was added followed by incubation on ice for 1.5 h. Samples were centrifuged again at 16000 x g for 15 min at 4 °C, the supernatant was transferred to a clean tube and dried in a Savant speed-vacuum dryer (SPD131, Fisher Scientific, Landsmeer, The Netherlands) at 45 °C for ~1 h. The dry pellet was reconstituted in 120 µl of 0.1% trifluoroacetic acid (TFA) and filtered through a 0.2 µm filter (adapted from[2]). HPLC analysis was carried out on a Dionex UltiMate3000 system (Thermo Fisher Scientific, The Netherlands) with a Kinetex C18 reverse-phase column (5 µm, 100 Å, 250x4.6 mm, Phenomenex, Utrecht, The Netherlands) and gradient elution. The mobile phase system consisted of 0.1% TFA/water (solvent A) and 70% ACN/water + 0.1% TFA (solvent B), while the gradient was as follows: 0-10 min 0%B, 10-50 min to 70%B, 50-55 min 70%B. Detection was performed via a Waters 474 fluorescent detector (Waters Chromatography B.V, Etten-Leur, The Netherlands) with excitation wavelength set to 274 nm and emission set to 303nm (slit width 18 nm).

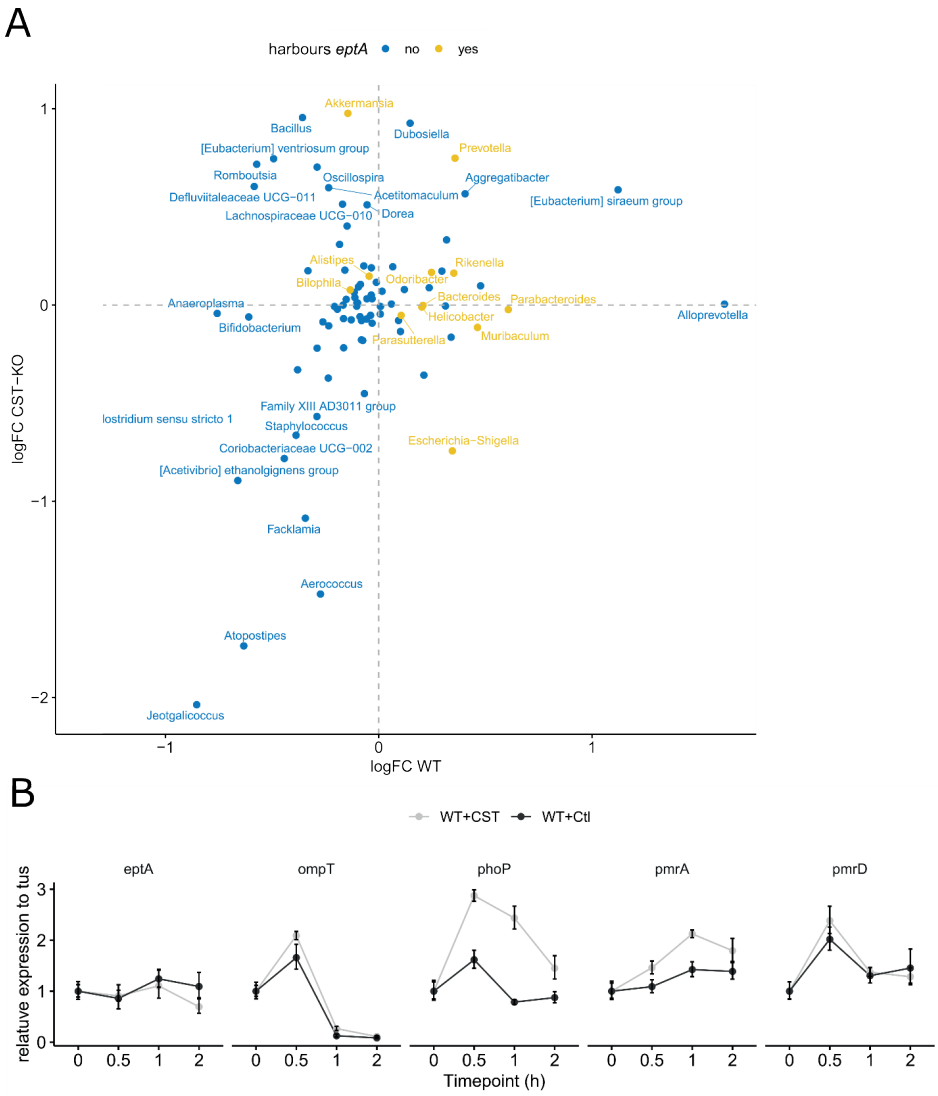
Supplementary Results



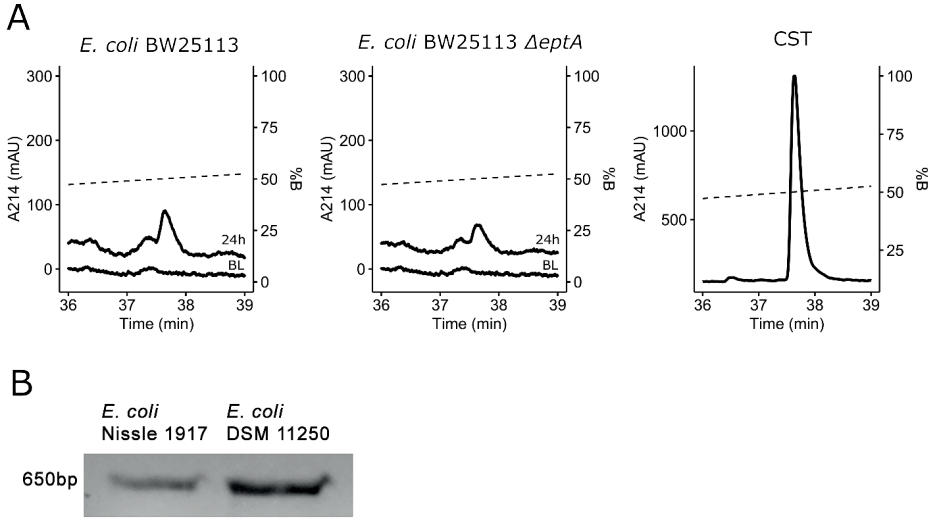
Supplementary Figure 1. (A) Principal component analysis (PCA) at the genus level using CSS scaled abundances determined by 16S genotyping. The plot reveals a distinct clustering of treatment groups, with the treated wild-type cluster being greatly spread. Ellipses represent normal data ellipses produced by methods from the R package ggplot2. (B) Treatment-constrained RDA of either CST-KO or WT. Arrows indicate taxa associated with either CST-treated (blue) or non-treated (yellow) groups, while the length of the arrow indicates the strength of the association. Ellipses represent normal data ellipses produced by methods from the R package ggplot2. (C) Differences in relative microbial compositions on phylum level between CST treated and untreated for the *in vivo* experiment (WT and CST-KO) and non-peptide control and CST for the *in vitro* experiment. Values are expressed as absolute difference, such that 0.1 translates to a relative compositional change of 10%, while only phyla with a mean abundance >1% are displayed for convenience.



Supplementary Figure 2. Reverse-phase HPLC chromatograms of culture for *B. thetaiotaomicron* and *T. sanguinis* stimulated with 32μM CST. Red arrows indicate the presence of an additional CST-related peak, which also appeared in the CST standard, and remained in *T. sanguinis* culture, suggestive of a lack of CST degradation. The mobile phase gradient (%B, percentage of mobile phase B) is indicated at the dashed line. CST –catestatin standard, 24h – culture supernatant after 24h of incubation, BL – baseline of culture supernatant without CST.



Supplementary Figure 3. (A) Scatter plot of log-fold changes, comparing abundance changes for significantly abundant genera between treated and untreated groups per genotype. The coloring indicates the presence of an *eptA*-like gene. (B) Expression analysis of wild-type *E. coli* BW25113 stimulated with CST or medium as control (Ctl). *phoP* and *pmrA* are substantially upregulated compared to WT control and knockout, while the increase in *ompT* expression is only minor compared to WT control. *eptA* expression remains invariant upon stimulation. Expression is assessed by the -ΔΔCt method with normalization to housekeeping gene *tus* and timepoint 0 preceding stimulation. Bars indicate point ranges.



Supplementary Figure 4. (A) Reverse-phase HPLC chromatograms of culture supernatant from *E. coli* BW25113 and *E. coli* BW25113$^{\Delta eptA}$ stimulated with 32μM CST. The mobile phase gradient (%B, percentage of mobile phase B) is indicated at the dashed line. CST – catestatin standard, 24h – culture supernatant after 24h of incubation, BL – baseline of culture supernatant without CST. (B) Polymerase chain reaction (PCR) of a 650bp fragment of the *E. coli ompT* gene, resolved on 1% agarose for two gut isolates.

Supplementary Tables

Supplementary Table 1. Resources

Strain or Primer		Relevant feature	Reference
Strains			
	E. coli BW25113	Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514	[3]
	E. coli JW5730	*E. coli* BW25113 with Δ*eptA*740::kan	[3]
	E. coli JW0554	*E. coli* BW25113 with Δ*ompT*774::kan	[3]
	Bacteroides thetaiotaomicron ATCC29148		ATCC
	Turicibacter sanguinis DSM14220		DSMZ
Primers	*phoP*_fwd	GCAGACGTCCCATCAGTACAT	This study
	*phoP*_rev	GTCGATGACGCAGAAGATGC	This study
	*eptA*_fwd	CTCTTCAAGCCCGTGGAACA	This study
	*eptA*_rev	CGCTGATGACACCGCAAATG	This study
	*ompT*_fwd	CGATTCCATGCGCCTTCAAC	This study
	*ompT*_rev	GGAGGCCGAAAAGTCAGTCA	This study
	*pmrD*_fwd	TAACTGCTTGCCGAGAGGAC	This study
	*pmrD*_rev	CTTATGCTCTGCGATGCTGG	This study
	*pmrA*_fwd	ATGCACTTCCAGGGTGTTGG	This study
	*pmrA*_rev	CGTGCGATAGCGTGACAACC	This study
	*tus*_fwd	AACAGTCCGAAAATCGCAGC	This study
	*tus*_rev	GCAATCAGTGGTGTAGGGCA	This study

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Chapter 3

Research article

Gut microbiota transplantation drives the adoptive transfer of colonic genotype-phenotype characteristics between mice lacking catestatin and their wild-type counterparts

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Abstract

The gut microbiota is in continuous interaction with the intestinal mucosa via metabolic, neuro-immunological, and neuroendocrine pathways. Disruption in levels of antimicrobial peptides produced by the enteroendocrine cells, such as catestatin, has been associated with changes in the gut microbiota and an imbalance in intestinal homeostasis. However, whether the changes in the gut microbiota have a causational role in intestinal dyshomeostasis has remained elusive. To this end, we performed reciprocal fecal microbial transplantation in wild-type mice and mice with a knockout in the catestatin coding region of the chromogranin-A gene (CST-KO mice). Combined microbiota phylogenetic profiling, RNA sequencing, and transmission electron microscopy were employed. Fecal microbiota transplantation from mice deficient in catestatin (CST-KO) to microbiota-depleted wild-type mice induced transcriptional and physiological features characteristic of a distorted colon in the recipient animals, including impairment in tight junctions, as well as an increased collagen area fraction indicating colonic fibrosis. In contrast, fecal microbiota transplantation from wild-type mice to microbiota-depleted CST-KO mice reduced collagen fibrotic area, restored disrupted tight junction morphology, and altered fatty acid metabolism in recipient CST-KO mice. This study provides a comprehensive overview of the murine metabolic- and immune-related cellular pathways and processes that are co-mediated by the fecal microbiota transplantation and supports a prominent role for the gut microbiota in the colonic distortion associated with the lack of catestatin in mice. Overall, the data show that the gut microbiota may play a causal role in the development of features of intestinal inflammation and metabolic disorders, known to be associated with altered levels of catestatin and may, thus, provide a tractable target in the treatment and prevention of these disorders.

Introduction

It is well-established that the gut microbiota has an essential role in the development and maintenance of human physiology by sustaining homeostatic processes such as gut barrier function[1], host immunity[2], energy metabolism[3], and neuropsychological behaviors[4]. Disruptions in the intimate interactions between the gut microbiota and the host are positively correlated with pathologies such as inflammatory gastrointestinal diseases, metabolic diseases, and neuropsychiatry disorders[5–7].

The pro-hormone chromogranin-A (CgA) is proteolytically processed into several biologically active peptides including Catestatin (CST: $hCgA_{352-372}$)[8,9]. CST consists of 21 amino acids and acts as an inhibitor of the catecholamine secretion through activation of nicotinic cholinergic receptors in cultured cells and mice adrenal glands[8,10,11]. Several metabolic and inflammatory disorders have been linked to altered levels of CST[12–15]. For example, the administration of human CST in mouse models of chronic inflammation, such as colitis ameliorated the intestinal pro-inflammatory parameters including macrophage function, reduction of pro-inflammatory cytokines, and pathways such as interleukin 6 (IL-6), interleukin 1β, tumor necrosis factor α (TNF-α) and signal transducer and activator of transcription 3 (STAT3) dependent pathway[16–18]. Moreover, the phenotype of mice with selective deletion of the CST-coding region of the ChgA gene (CST-KO mice) was shown to display obesity, insulin resistance, hypertension, macrophage infiltration, hyperadrenergic state, as well as high levels of pro-inflammatory cytokines[12,19], and more recently an IBD-like phenotype, including intestinal permeability[20].

Recently, we and others have shown that CST-KO mice display altered gut microbiota composition compared to their wild-type (WT) counterparts[21,22]. In particular, CST treatment reduced the abundance of *Staphylococcus* and *Turicibacter* in CST-KO and WT mice, while *Alistipes*, *Akkermansia*, and *Roseburia* were significantly increased in the CST-KO group[22].

Additionally, levels of the short-chain fatty acids (SCFAs), butyrate, and acetate were significantly increased in CST-KO mice treated with CST[22]. Analogously, supplementation of CST-KO mice with CST restored paracellular intestinal epithelial permeability, reversed inflammation, and fibrosis, all of which are characteristic of CST-KO mice[20]. This led us to hypothesize that the altered gut microbiota may play a key role in developing the disrupted CST-KO-associated phenotypes. One of the most frequently used experimental approaches to study the causal role of the gut microbiota in gut dysbiosis-related diseases is fecal microbiota transplantation (FMT)[23]. Thus, in this study, we performed transplantation of the perturbed

microbiota from CST-KO mice to microbiota-depleted WT mice and vice versa in an attempt to study the contribution and the mechanisms by which the gut microbiota may contribute to the phenotype observed in the CST-KO mice.

Results

Reciprocal fecal microbiota transfer between CST-KO and wild-type mice harboring distinct microbial populations

Recently, we and others have shown that mice with a CST knock-out (CST-KO) have a significantly altered gut microbiota composition compared to their WT counterparts[22]. Additionally, previous studies reported significant differences in gastrointestinal morphology, mucosal immune function, and gut permeability in CST-KO mice[20]. Since CST-KO mice displayed altered gut microbiota composition, we hypothesized that the microbiota may play a substantial role in causing these differences. To unravel this causality, we performed reciprocal fecal microbiota transplantation (FMT), where C57BL/6 WT mice (n=12) were orally gavaged with a fecal microbiota suspension of CST-KO mice and vice versa (**Figure 1A**; methods section; **Supplementary Figure 1A**). The transplanted microbiota was allowed to recolonize the gut of the recipient mice for 14 consecutive days. Fecal pellets collected from CST-KO$^{FMT\text{-}WT}$ and WT$^{FMT\text{-}CST\text{-}KO}$ recipient mice and their controls (n=12, per group) were used for amplicon sequencing of the V3-V4 regions of the bacterial 16S gene.

As a general exploratory analysis, principal component analysis (PCA) was performed on genus level collapsed data and showed significantly distinct clustering of the CST-KO and WT before and after FMT. Particularly, the CST-KO and WT donors clustered separately, while the recipients, CST-KO$^{FMT\text{-}WT}$ and WT$^{FMT\text{-}CST\text{-}KO}$ mice, were overlapping into a middle point, indicating that the transplant induced changes in the microbiota composition in both genotypes (**Supplementary Figure 1B**).

Next, microbial richness was assessed by the observed number of amplicon sequence variants (ASVs) and Chao1 index. Interestingly, the richness levels were restored in CST-KO$^{FMT\text{-}WT}$, but not in WT$^{FMT\text{-}CST\text{-}KO}$ mice (**Figure 1B**). Contrary to the richness scores, the diversity indexes determined by Shannon's H and inverted Simpson's index, showed no significant changes between the recipient groups (**Figure 1B**). Analogous to our previous findings[22], the CST-KO mice consistently showed decreased richness and diversity compared to their WT counterparts. Taken together, the data infers

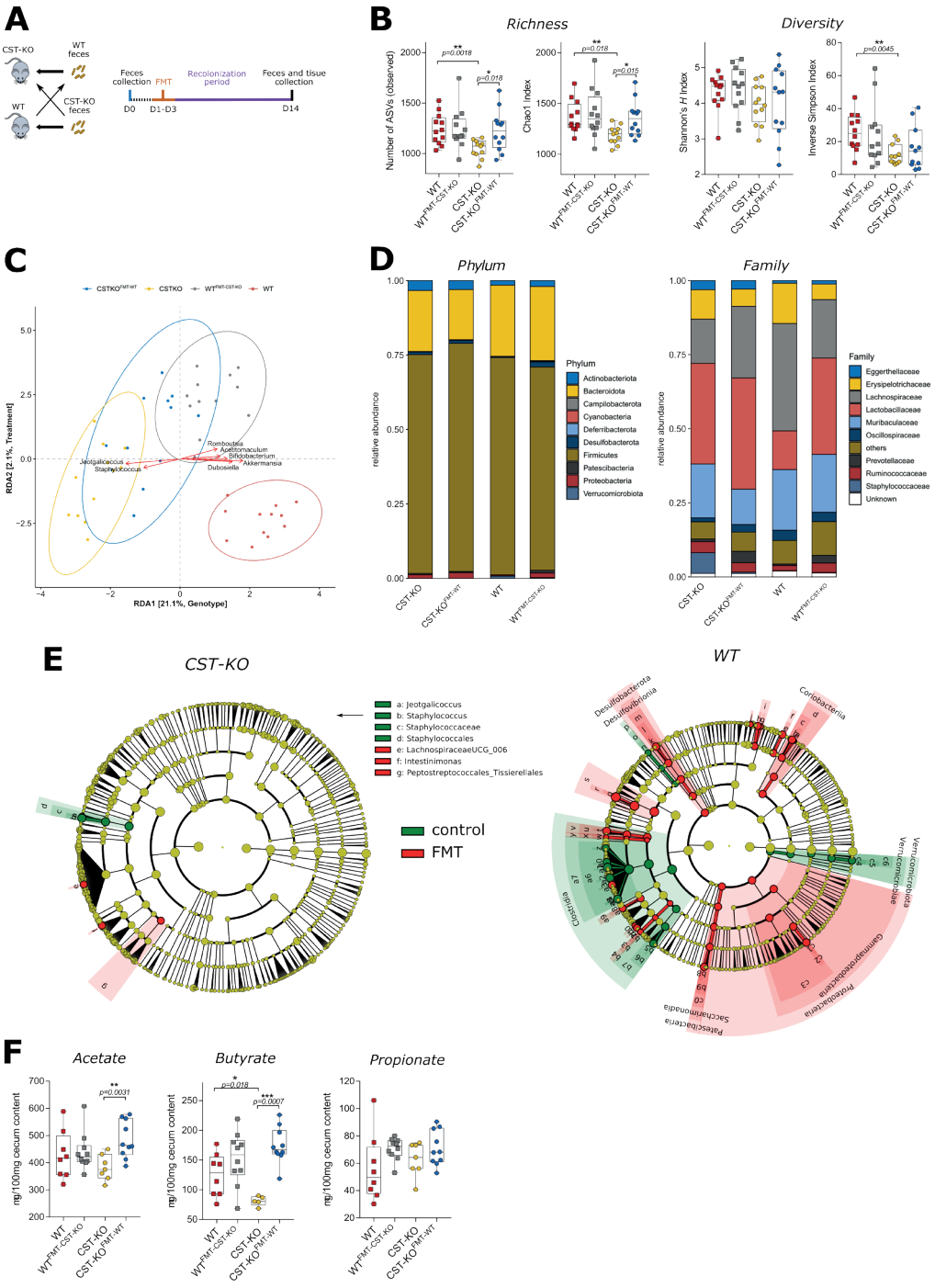
that FMT induced changes in the microbiota composition in both genotypes, with a significant effect in restoring the richness of the microbiota community in CST-KO mice to the WT level.

To correct for any residual variation in the data, genotype and treatment-constrained (FMT) redundancy analysis (RDA) was performed. In agreement with the PCA (**Supplementary Figure 1**), distinct clustering was visible and both constraints also had a significant influence on the model ($p<0.001$, determined by ANOVA-like permutation test), explaining 21.1% (genotype) and 2.1% (FMT treatment) of the variation (**Figure 1C**). RDA determined which bacterial taxa were associated with each group of mice. Focusing on the strongest associations, the largest differences were determined by the genotype. In agreement with our previous findings[22], among others, *Akkermansia*, *Dubosiella*, and *Bifidobacterium* showed the strongest association with WT along RDA1 while *Jeotgalicoccus* and *Staphylococcus* were associated with CST-KO mice. More general we observed a more pronounced change in $WT^{FMT\text{-}CST\text{-}KO}$ compared to $CST\text{-}KO^{FMT\text{-}WT}$, as WT and $WT^{FMT\text{-}CST\text{-}KO}$ clusters show greater distance than CST-KO and $CST\text{-}KO^{FMT\text{-}WT}$. This was also supported by analysis using SourceTracker, which estimated that 65±19% of all taxa were transferred from CST-KO to $WT^{FMT\text{-}CST\text{-}KO}$, while only 27±25% were transferred from WT to $CST\text{-}KO^{FMT\text{-}WT}$. Source samples (controls) contained 86±13% of taxa in unique composition for CST-KO and 84±23% for WT.

To further identify which bacterial taxa were transferred by the FMT, pairwise comparisons of bacterial abundances were performed between the donor and recipient groups for each genotype.

Focusing on the phylum level, *Bacteroidota* decreased, while *Firmicutes* increased in relative abundance, although not significant, in $CST\text{-}KO^{FMT\text{-}WT}$ mice, similar to the microbiota of WT mice (**Figure 1D**). In contrast, in $WT^{FMT\text{-}CST\text{-}KO}$ mice, the opposite effects were observed, where *Firmicutes* and *Verrucomicrobiota* significantly decreased in relative abundance, while *Bacteroidota*, *Actinobacteriota*, and *Patescibacteria* were significantly increased, again similar to the microbiota of CST-KO mice (**Supplement Excel Sheet 1**). On the family level, the most significant changes were observed for the increased abundance of *Peptostreptococcaceae*, *Staphylococcaceae*, *Defluviitaleaceaea*, as well as *UCG-010* in $CST\text{-}KO^{FMT\text{-}WT}$ mice, all of which decreased in abundance in $WT^{FMT\text{-}CST\text{-}KO}$ mice (**Supplement Excel Sheet 1**).

To complement our analysis and to search for predictors at all taxonomic levels including genera, LEfSe was employed (Linear discriminant analysis Effect Size,[24]) (**Figure 1E**). Consistent with the RDA, the main discriminant feature separating


A
CST-KO
WT feces
WT
CST-KO feces
Feces collection
FMT
Recolonization period
Feces and tisue collection
D0
D1-D3
D14
B
Richness
Diversity
Number of ASVs (observed)
Chao1 Index
Shannon's H Index
Inverse Simpson Index
p=0.0018
p=0.018
p=0.015
p=0.0045
WT
WT^FMT-CST-KO
CST-KO
CST-KO^FMT-WT
C
CSTKO^FMT-WT
CSTKO
WT^FMT-CST-KO
WT
RDA2 [2.1%, Treatment]
RDA1 [21.1%, Genotype]
Romboutsia
Adlercreutzia
Adlercreutzia
Bifidobacterium
Akkermansia
Dubosiella
Jeotgalicoccus
Staphylococcus
D
Phylum
Family
relative abundance
Phylum
Actinobacteriota
Bacteroidota
Campilobacterota
Cyanobacteria
Deferribacterota
Desulfobacterota
Firmicutes
Patescibacteria
Proteobacteria
Verrucomicrobiota
Family
Eggerthellaceae
Erysipelotrichaceae
Lachnospiraceae
Lactobacillaceae
Muribaculaceae
Oscillospiraceae
others
Prevotellaceae
Ruminococcaceae
Staphylococcaceae
Unknown
E
CST-KO
WT
a: Jeotgalicoccus
b: Staphylococcus
c: Staphylococcaceae
d: Staphylococcales
e: LachnospiraceaeUCG_006
f: Intestinimonas
g: Peptostreptococcales_Tissierellales
control
FMT
Desulfobacterota
Desulfovibrionia
Coriobacteriia
Verrucomicrobiae
Verrucomicrobiota
Gammaproteobacteria
Proteobacteria
Saccharimonadia
Patescibacteria
Clostridia
F
Acetate
Butyrate
Propionate
mg/100mg cecum content
p=0.0031
p=0.018
p=0.0007

< Figure 1: Altered microbiota composition following cross-FMT in CST-KO and WT mice. (**A**) Experimental strategy with a timeline. (**B**) Alpha diversity was assessed using different metrics. In CST-KO mice, FMT increased the bacterial richness (observed ASVs and Chao1 index) and diversity (inverted Simpson's index). Significance was tested with an unpaired *Mann-Whitney* test. Boxes represent the median with interquartile range, and whiskers represent the maxima and minima. (**C**) Genotype and FMT treatment constrained redundancy analysis (RDA) on genus collapsed abundances. Arrows indicate the association of taxa with samples, with the length being a proxy for the strength of the association. Significant separation of clusters and contribution of the variables to the variance of the RDA was tested with Permutational Multivariate ANOVA (PERMANOVA) and revealed significant ($p<0.001$) effects of FMT, in both CST-KO and WT mice; yellow – CST-KO, blue – CST-KO FMT, grey – WT FMT and red – WT. (**D**) Relative abundance of the present phyla and families (**E**) Cladograms of a LEfSe analysis (Linear discriminant analysis Effect Size) comparing microbiota changes upon FMT in CST-KO and WT mice. The cladogram indicates the microbiota composition represented by rings with phyla in the outermost ring and genera in the innermost ring. The green color represents taxonomic levels enriched in control animals, while the red color represents enrichment in the FMT groups. Each circle is a member within that level. (**F**) Concentrations of cecal acetate, butyrate and propionate of untreated mice (CST-KO, n=7; WT, n=8) and mice after FMT (CST-KO FMT, n=10; WT FMT, n=10). Data were analyzed using a 2-tailed paired *t*-test.

the groups in CST-KO mice were species from the genera *Jeotgalicoccus* and *Staphylococcus*, while CST-KO^{FMT-WT} mice were represented with *Lachnospiraceae* UCG_006, *Intestinimonas*, and *Peptostreptococcaceae*. Notably, compared to the CST-KO groups, alterations in the microbiota composition were more pronounced in the WT mice, where these animals were enriched in species from the class Clostridia and Verrucomicrobiota, while the WT$^{FMT-CST-KO}$ microbiota was constituted with species from Proteobacteria, Patescibacteria, Desulfobacterota, and Coriobacteriia. The increased abundance in those taxa in the WT$^{FMT-CST-KO}$ mice coincides with their higher abundance in the donor CST-KO, which showed a dominance of Proteobacteria, Patescibacteria, Bacilli, Desulfobacterota, and Actinobacteriota, further confirming the success of the FMT procedure.

Changes in microbial composition are often accompanied by metabolic changes, in particular, the production of short-chain fatty acids (SCFAs)[25]. Thus, levels of acetate, butyrate, and propionate, were measured in the cecum of donor and recipient mice of each genotype. Specifically, butyrate was significantly lower in the donor CST-KO mice compared to their WT counterparts as reported previously (**Figure 1F**)[22]. Levels of acetate and butyrate increased significantly in CST-KO^{FMT-WT} compared to CST-KO

(**Figure 1F**). In WT$^{FMT\text{-}CST\text{-}KO}$ mice, no changes were visible compared to the WT control group. These observations are consistent with the observed significant increase in the butyrate and acetate-producing genus *Intestimonas* in the CST-KO$^{FMT\text{-}WT}$ mice[26]. As it is the only well-characterized and significantly changing taxon, this suggests that this specific genus is responsible for the strong increase in SCFA levels. Overall, the results imply that donor-specific taxa reliably colonized the recipients. Despite

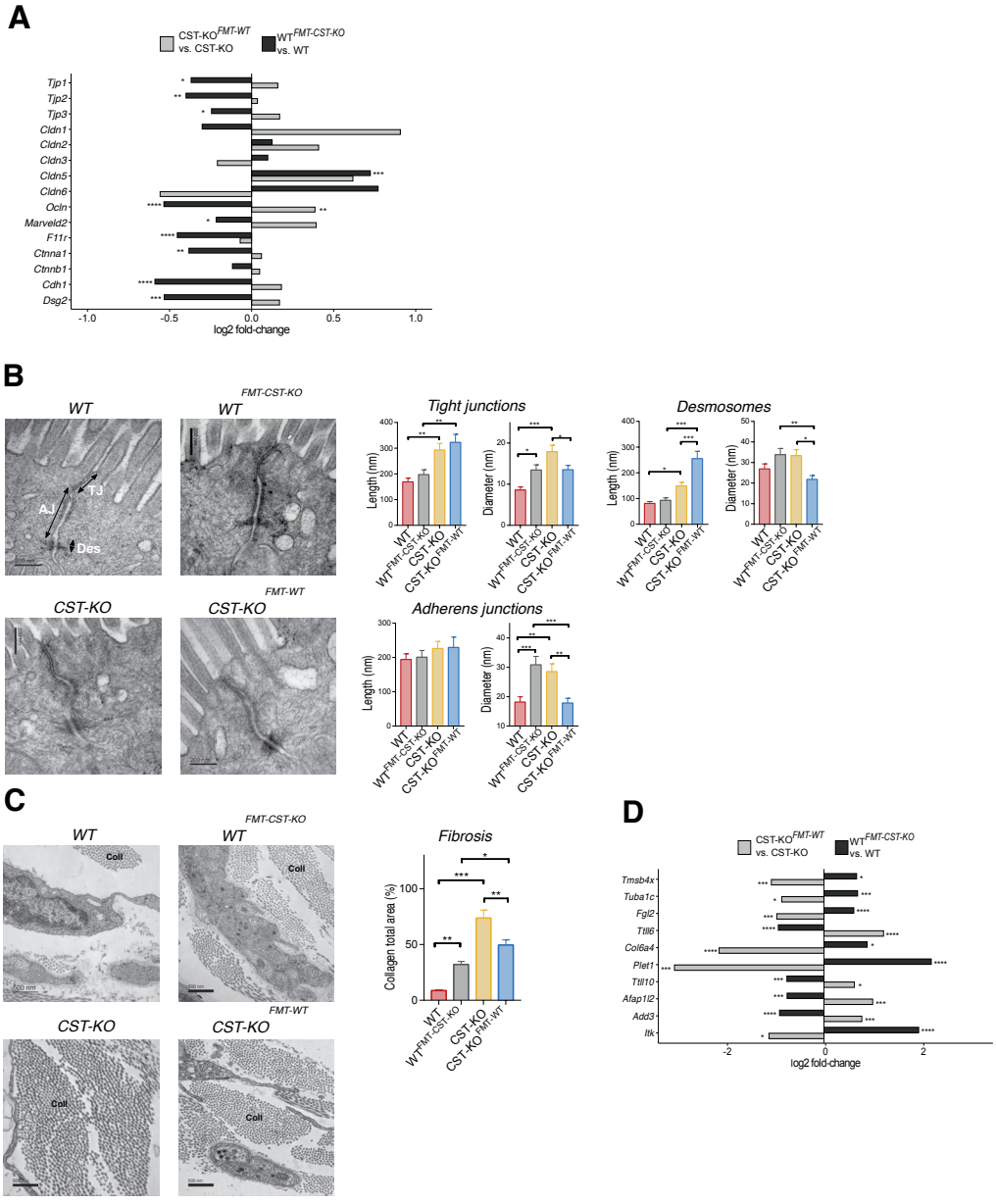


Figure 2: Adoptive transfer of colonic distortions from CST-KO to WT mice after fecal transplant. (A) Bar plot of genes involved in epithelial barrier regulation, showing log2 fold-changes for either CST-KO vs CST-KO^{FMT-WT} or WT vs WT$^{FMT-CST-KO}$. Log2 fold-changes and significance ($p_{adjusted} < 0.05$) of that change were determined by general differential expression analysis using *DESeq2* (see **Supplementary Excel Sheet 2**) **(B)** Representative transmission electron microscopy (TEM) micrographs showing epithelial barrier morphology for each group of mice. Arrows indicate the different parts: TJ – tight junction, AJ – adherens junction, Des – desmosome. The right side of the panel presents morphometric analyses results for TJ, AJ and Des, assessing length and diameter respectively; Bars show mean ± SEM **(C)** TEM micrographs showing the collagen fibers (cross-sectional view) for each group of mice. The images are representative of each group. Coll – collagen. The right side of the panel shows results of morphometric analysis showing fiber density as an assessment of fibrosis; Bars show mean ± SEM **(D)** Bar plot of genes related to the development of fibrosis, showing log2 fold-changes for either CSTKO-KO vs CST-KO^{FMT-WT} or WT vs WT$^{FMT-CST-KO}$.

more prominent specific taxonomical changes in the WT$^{FMT-CST-KO}$ mice, FMT altered microbial richness and SCFAs production in CST-KO^{FMT-WT} but not in the WT$^{FMT-CST-KO}$ recipient mice.

Adoptive transfer of dysfunctional epithelial barrier and colonic fibrosis from CST-KO to WT mice

The gut microbiota has been strongly linked to intestinal homeostasis[27]. Especially, in patients with IBD or colorectal cancer, altered gut microbiota has been associated with a dysfunctional epithelial barrier and tissue inflammation, which, in turn, leads to sub-mucosal fibrosis[28,29]. Importantly, CST-KO mice have recently been shown to exhibit these disrupted intestinal mucosal processes, e.g. increased length and diameter of tight junctions, adherens junctions, and desmosomes, all coinciding with increased gut permeability[20]. Given the altered microbiota composition in CST-KO mice (**Figure 1**;[22]), we hypothesized that the gut microbiota may play a causal role in the development of these features. To test our hypothesis, we employed whole-genome transcriptomic analysis on colonic tissue, as well as transmission electron microscopy (TEM) to examine the morphology of the intestinal colonic epithelium as well as the sub-mucosa of CST-KO and WT controls and the WT$^{FMT-CST-KO}$ and CST-KO^{FMT-WT} recipient mice (**see methods and supplementary information for details**).

The intestinal epithelium is regulated by a series of intercellular junctions between polarized cells: an apical tight junction (TJ) which guards paracellular permeability, the subjacent adherens junction (AJ), and desmosomes, both provide essential adhesive and mechanical properties that contribute to paracellular barrier functions[30]. On the transcriptome level, several genes involved in cell and TJ regulation and multiple TJ-markers such as occludin (*Ocln*), MARVEL domain-containing protein 2 (*Marveld2*), tight junction proteins (*Tjp1-3*)[31], as well as the desmosomal protein desmoglein-2

(*Dsg2*), which is required for the maintenance of intestinal barrier function[32], and AJ genes coding for alpha-E-catenin (*Ctnna1*), all showed substantial downregulation in WT$^{FMT\text{-}CST\text{-}KO}$. In contrast, claudins (*Cldn1-6*) showed inconclusive expression profiles, as some were upregulated in WT$^{FMT\text{-}CST\text{-}KO}$ but also CST-KO$^{FMT\text{-}WT}$ (**Figure 2A**). The transcriptome data was further confirmed by ultrastructural examination of the gut epithelium using TEM, which revealed that the TJ, AJ, and desmosome morphology was different in CST-KO compared to WT, mostly exhibiting increased diameter for all three components, in line with previous findings[20] (**Figure 2B**). The fecal transplantations affected the morphology of the epithelial junctions of the recipient mice. TJs, AJs, and desmosomes diameter appeared to be decreased in CST-KO$^{FMT\text{-}WT}$, whereas in WT$^{FMT\text{-}CST\text{-}KO}$ the diameter increased significantly compared to their donors. The TJs and AJs length was not affected in mice receiving FMT. However, desmosomes were significantly elongated in CST-KO$^{FMT\text{-}WT}$ compared to CST-KO, while in WT$^{FMT\text{-}CST\text{-}KO}$ there were no significant changes compared to WT. Collectively, the findings observed from the epithelial junction morphology indicated a phenotype transfer from their donor's microbiota.

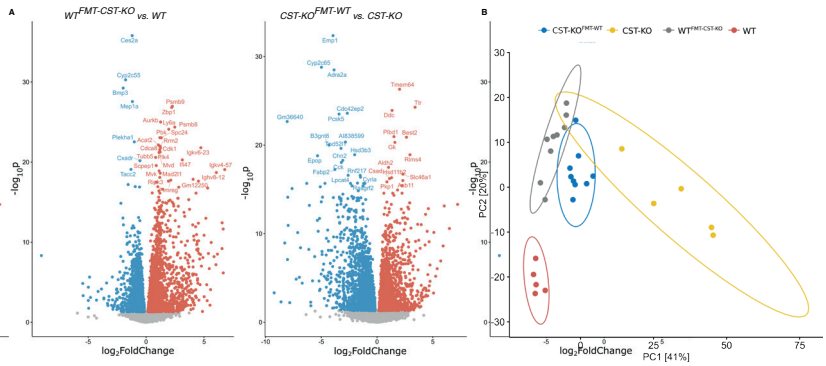
Moreover, the ultrastructural findings revealed by TEM demonstrated an excessive accumulation of the extracellular matrix component, collagen in the colonic tissue of WT$^{FMT\text{-}CST\text{-}KO}$ and CST-KO mice compared to the CST-KO$^{FMT\text{-}WT}$ and WT mice (**Figure 2C**). Excessive accumulation of collagen is indicative of intestinal fibrosis, which is a characteristic of CST-KO mice[20]. Fibrosis results from tissue inflammation and has been associated with the upregulation of several immune-related genes, which are linked to the prognosis of IBD[33]. Indeed, several genes involved in innate and adaptive immune response, cell adhesion, migration, proliferation, angiogenesis, skeletal development, and tissue wound repair were differentially regulated in the recipient groups compared to the donors, including thymosin beta-4 X-linked (*Tmsb4x*), tubulin alpha-1c (*Tuba1c*), fibrinogen-like 2 (*Fgl2*), tubulin tyrosine ligase-like 6 and 10 (*Ttll6* & *Ttll10*), collagen alpha-4 type VI (*Col6a4*), placenta expressed transcript 1 (*Plet1*), actin filament associated protein 1 like-2 (*Afap1l2*), gamma aducin (*Add3*), IL2 inducible T-cell kinase (*Itk*) (**Figure 2D**). Altogether, the results infer that the CST-KO-associated microbiota may play a key role in the development of the altered tight junction regulation and fibrosis in CST-KO mice as inferred from the phenotype transfer after fecal microbiota transplantation in WT$^{FMT\text{-}CST\text{-}KO}$ and that FMT from WT donors reversed, to a great extent, this distortion as shown in CST-KO$^{FMT\text{-}WT}$.

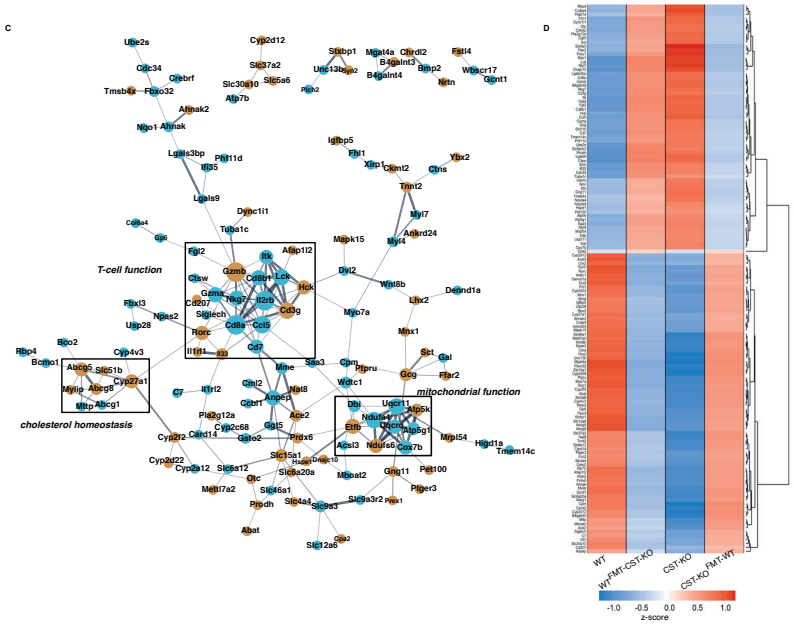
Core regulatory network that governs the transcriptional changes and their association with the transplanted microbiota

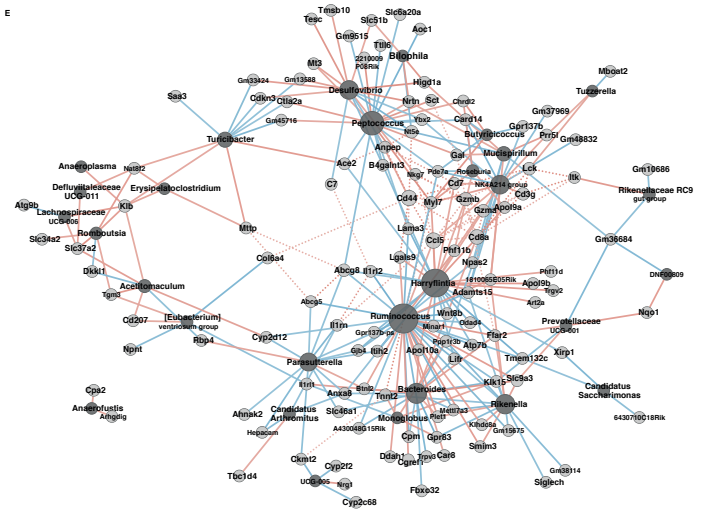
In order to gain a more mechanistic understanding of the transferred microbiota interfaces with the host and which pathways are involved to express a specific phenotype, we performed a comprehensive analysis of the obtained transcriptomic data. A total of 5233 genes were differentially expressed ($p_{adjusted} < 0.05$) in the colonic tissue of $WT^{FMT\text{-}CST\text{-}KO}$ compared to the WT mice, with 2712 (log2FC >2: 765) of these genes being upregulated and 2521 (log2FC >2: 295) downregulated (**Figure 3A**). In $CST\text{-}KO^{FMT\text{-}WT}$, a total of 4273 genes were found to be differentially expressed ($p_{adjusted} < 0.05$) in the colonic tissue compared to the CST-KO mice, with 1987 (log2FC >2: 629) of these genes being upregulated and 2286 (log2FC >2: 916) downregulated (**Figure 3A**). Principal component analysis (PCA) performed on normalized gene expression showed a similar convergent clustering of the recipient mice compared to the donors (**Figure 3B**), in line with the microbiota clustering (**Figure 1C**, **Supplementary Figure 1**).

A comprehensive functional analysis of altered biological processes was carried out between the recipient and donor mice and revealed alterations in immunological processes, including immunoglobulin production, B-cell activation and adaptive immunity regulation to be upregulated in $WT^{FMT\text{-}CST\text{-}KO}$ compared to the WT control. In contrast, changes in $CST\text{-}KO^{FMT\text{-}WT}$ were characterized by downregulation of lipid metabolism and ion transport compared to the CST-KO mice (Detailed analysis can be found in (**Supplementary Figures 2 and 3**). Overall, the transcriptome data revealed a significant impact of the FMT on the transcriptome level in both CST-KO and WT mice.

From the differential expression analysis, we hypothesized that there may be a core set of regulatory genes that could serve as transcriptional signatures for the altered mucosal homeostasis upon adoptive microbiota transfer between CST-KO and WT mice in the colon. Therefore, all DEGs with an opposite differential regulation in the recipient groups ($WT^{FMT\text{-}CST\text{-}KO}$ and $CST\text{-}KO^{FMT\text{-}WT}$ compared to their donors: i.e., the same gene is upregulated in one recipient group but downregulated in the other recipient group; (log2-fold change >0.58, p adjusted <0.05; (**Supplementary Excel Sheet 2**), were mined to search for potential transcriptional signatures (see **Methods** for detailed description). This approach allowed us to distinguish between genes that are potentially differentially expressed solely due to the FMT treatment (same log2-fold change) and genes which govern biological meaning, being associated with the specific genotypes microbiota (opposite log2-fold change). A total of 292 genes were found to be commonly expressed in both the $CST\text{-}KO^{FMT\text{-}WT}$ and $WT^{FMT\text{-}CST\text{-}KO}$ but in opposite directions. The associations among the proteins translated


A
WT^FMT-CST-KO vs. WT
CST-KO^FMT-WT vs. CST-KO
-log10p
log2FoldChange
B
CST-KO^FMT-WT
CST-KO
WT^FMT-CST-KO
WT
PC2 [20%]
PC1 [41%]


C
T-cell function
cholesterol homeostasis
mitochondrial function
D
WT
WT^FMT-CST-KO
CST-KO
CST-KO^FMT-WT
z-score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< Figure 3: Core-regulatory network that governs the transcriptional changes and their association with the transplanted microbiota. (A) Volcano plots of differentially expressed genes comparing either WT vs $WT^{FMT\text{-}CST\text{-}KO}$ or CST-KO vs $CST\text{-}KO^{FMT\text{-}WT}$. Log2 fold-changes and significance ($p_{adjusted} < 0.05$) of that change were determined by general differential expression analysis using *DESeq2* (see **Supplementary Excel Sheet 2**). Genes significantly upregulated in the FMT groups are highlighted in red, while significantly downregulated genes are colored in blue. **(B)** Principal component analysis (PCA) of normalized gene expression data reveals distinct clustering between all of the groups. Ellipses represent normal data ellipses produced by methods from the R package ggplot2. **(C)** Network representation of genes regulated in opposite direction in the recipient groups ($WT^{FMT\text{-}CST\text{-}KO}$ and $CST\text{-}KO^{FMT\text{-}WT}$ compared to their donors: i.e., the same gene is upregulated in one recipient group but downregulated in the other recipient group; log2 fold-change >0.58, $p_{adjusted} < 0.05$). Connections are inferred from the STRING database (combined score >0.4), while edge width corresponds to interaction strength. Node size is determined by its degree. Brown color indicates involvement in IBD determined by mining from the TaMMA meta-transcriptome catalogue [34] **(D)** Heatmap of all core genes (including the ones shown in (C)) clustered by their group/gene z-score normalized expression, illustrating the dualistic effect of FMT for those genes in each genotype. **(E)** Network representation of correlation analysis (Pearson, $p_{adjusted} < 0.05$) between the abundance of microbial taxa and the expression of certain genes presented in (D). The network is only representative of WT. Light grey – Gene, Dark grey – Microbial taxon, Blue – negative correlation, Red – positive correlation; Solid lines refer to direct correlation, while dotted lines depict interactions inferred from the STRING database.

by the commonly identified DEGs in the recipient groups in comparison to their controls were assessed and interactions among the query proteins were further visualized. This resulted in a network that comprised 143 nodes and 233 edges of protein-protein interactions after excluding unconnected nodes or clusters of only two genes (**Figure 3C**). The resulting network exemplified the strong impact of adoptive microbiota transfer between CST-KO and WT mice in the colon on both immune and metabolic gene expression throughout the colonic mucosa and encompassed several core regulatory genes that are known to control the induction of metabolic and immune responses. Cluster analysis was used to identify closely interlinked regions from the network of proteins (**Figure 3C**). The top 3 clusters were found to be highly significant and included the most nodes out of the ones reported (**Supplementary Table 1**). Using functional enrichment of the clustered proteins, potential biological processes were assigned to each cluster. Each cluster was related to a separate process, including genes annotated to belong to T-cell function, energy metabolism (mitochondrial functions), and cholesterol homeostasis.

Genes from the cluster involved in T-cell function exclusively showed higher expression in WT$^{FMT\text{-}CST\text{-}KO}$ and CST-KO donors, indicating potential activation of T-cell-related immunological pathways by CST-KO transplanted microbiota. A similar expression pattern was also found for the genes in the energy metabolism cluster. Intriguingly, the genes present in the cholesterol homeostasis cluster were found to be enriched (except *Slc51b*) in CST-KO$^{FMT\text{-}WT}$ and WT donors, indicating a simulative effect of the WT-transferred microbiota (**Figure 3D**). To further assess whether FMT was able to restore the expression levels of the genes identified in the core regulatory network to their original expression levels in the recipient groups, i.e., showing no significant differential expression anymore, we performed a comparative analysis between the CST-KO$^{FMT\text{-}WT}$ vs WT and WT$^{FMT\text{-}CST\text{-}KO}$ vs CST-KO groups. Among all genes in the identified transcriptome signatures, 234 were found to be restored, while 134 were also present in the core network (**Supplementary Excel Sheet 2**). Considering the high fractions of the identified genes, the data highlight the powerful efficacy of the FMT in restoring the transcriptional patterns.

Next, possible associations between the colonic microbiota, which was transferred from the donor to the recipient genotypes (**Figure 1**), and the core regulatory genes (**Figure 3D**) were explored using pairwise Pearson correlations. The correlation analysis revealed that 41% of the core differentially expressed genes were associated with at least one microbial taxon, which was detected to be significantly differentially abundant, only in the WT$^{FMT\text{-}CST\text{-}KO}$ recipient group (**Supplementary Excel Sheet 2**). The associations between core network gene expression and taxon abundance, as well as interaction among the query proteins, were further visualized (**Figure 3E**). Five main clusters were identified, among them, one was found to be less connected to the others, mostly containing low-degree taxon nodes. *Ruminococcus*, *Harryflintia*, and *Peptococcus* had the most correlations among the taxa (average of 29 correlations), while *Klb*, *Klk15*, and *Myl7* were the most connected genes (6, 5, and 6 edges, respectively). Genes from the core T-cell cluster and generally immune-related genes connected to *Peptococcus*, *Harryflintia*, *NK4214* group, *Desulfovibrio*, and *Ruminococcus*. Genes from the energy metabolism cluster did not show any apparent significant correlations to any of the taxa, while genes involved in cholesterol homeostasis (including core gene cluster 3), such as *Mttp*, *Acbg5/8*, and *Slc51b* were correlated with *Turicibacter*, *Peptococcus*, *Parasutterella*, *Desulfovibrio*, and *Bilophila*.

As CST is proposed to play a crucial role in IBD, we investigated which genes from our core regulatory network are involved in this disease. Intriguingly, among the identified tissue transcriptome signatures, the human counterparts of 108 genes (62 genes in the identified core network) have a known role in IBD determined by

matching them to the IBD Transcriptome and Metatranscriptome Meta-Analysis (IBD TaMMA) transcriptomics catalog[34], and 36 of which are linked to bacteria. These findings further support the biological relevance of the identified transcriptional signatures for mucosal control of homeostasis along the gut and the role of altered microbiota composition in CST-KO mice as a cause of differential expression of genes involved in mucosal immune and metabolic processes.

Discussion

The present findings demonstrate that CST-KO-associated alterations in the gut microbiota are sufficient to disrupt colonic homeostasis in healthy mice. Similarly, the transfer of the gut microbiota of healthy mice restored the distorted colonic function in CST-KO mice. Specifically, transplantation of the perturbed microbiota signature from CST-KO mice to microbiota-depleted WT mice induced the development of several colonic dysfunctional features of the CST-KO phenotype on the gene and tissue levels. CST-KO mice are associated with an altered gut microbiota composition, and richness (**Figure 1** and González-Dávila, et al. 2022). A major parallel between the CST-KO microbiota profiles and the WT mice that received the FMT from the CST-KO ($WT^{FMT\text{-}CST\text{-}KO}$) encompassed a reduction of Clostridia and *Akkermansia*, which has been previously linked to metabolic disorders and insulin resistance[35,36], a CST-KO related phenotype[37] as well as a prominent increase in the Proteobacteria population, all of which have been found previously to be indicative for active IBD states[38,39]. In contrast, CST-KO that received the FMT from the WT mice ($CST\text{-}KO^{FMT\text{-}WT}$) encompassed an increase in richness and a notable reduction of *Staphylococcus*, as well as an increase in the butyrate-producing *Intestinimonas* (**Figure 1**). In fact, reduced levels of butyrate have been strongly linked to IBD as well as metabolic disorders[40,41].

Detailed transcriptome analysis allowed the identification of transcriptional signatures of genes commonly differentially regulated in recipient mice in opposite directions (**Figure 3C, D**). These transcriptional signatures included the correlative expression of metabolism-related genes and immune-related genes. Most of these genes showed full restoration of their transcriptional levels when compared to their opposite controls. Among the identified transcriptional signatures, were several genes known to be involved in fibrosis; which was indeed confirmed by TEM, where an excessive accumulation of the extracellular matrix component, collagen was observed in the colonic tissue of $WT^{FMT\text{-}CST\text{-}KO}$ and CST-KO mice compared to the $CST\text{-}KO^{FMT\text{-}WT}$ and WT mice (**Figure 2C**). Furthermore, the identified signatures

comprised genes of which the human orthologues are dysregulated in IBD. Based on genes identified in large-scale transcriptomic meta-analysis (TaMMA), at least 37% of the found genes were associated with IBD.

Our transcriptome and transmission electron microscopy data demonstrated that transferring the gut microbiota from CST-KO mice to WT mice with a depleted gut microbiota could induce the development of some of the features of the dysfunctional colon, such as distorted barrier integrity and fibrosis resulting in a physiological profile similar to CST-KO (**Figure 2**). Further, GO enrichment and pathway analysis consistently identified "immune response" as the main umbrella category affected in response to FMT from CST-KO mice. CST-KO mice exhibited an increased expression in pro-inflammatory genes, namely interferon-gamma (*Infg*), integrin subunit alpha-M (*Itgam*), integrin subunit alpha-X (*Itgax*), CXC motif chemokine ligand-1 (*Cxcl1*), interleukin 12b (*Il12b*), and nitric oxide synthase 2 (*Nos2*) which, were also increased in $WT^{FMT\text{-}CST\text{-}KO}$ (**Supplementary Figure 3** and **Supplementary Excel Sheet 2**). The results are in line with the recent findings showing that CST-KO mice have infiltration of macrophages and $CD4^+$ T-cells, and higher gene and protein expression levels of pro-inflammatory molecules in the gut, indicative for IBD-like states [20]. The same study[20] reported that CST-KO mice have increased gut permeability, altered tight junctions morphometry, which is in agreement with the decreased gene expression of several TJ-markers such as *Clnd5*, *Dsg2*, *Ctnna1*, *Tjp2*, *Tpj3*, *Ocln*, *Marveld2* in CST-KO (**Figure 2A**) as well as their distorted TJ, AJ, and desmosome morphology (**Figure 2B**), compared to WT, all of which (except for *Cldn5*) were restored in $WT^{FMT\text{-}CST\text{-}KO}$ mice decreased the gene expression in the same TJ-markers.

In contrast to the induced immune response in WT mice that received FMT from CST-KO, $CST\text{-}KO^{FMT\text{-}WT}$ mice displayed a downregulation in several cell processes involved in lipid metabolism, suggesting that metabolic reorientation of colonic tissue from an oxidative energy supply had occurred. Notably, CST is known to be involved in body weight regulation through its effects on lipolysis and fatty acid oxidation, as well as its beneficial effects in mice with diet-induced obesity[12,42], and is correlated with plasma HDL-cholesterol levels[43]. However, such changes are usually systemic nature, affecting the expression of genes in adipose or liver tissue. In the intestinal milieu, microsomal triglyceride transfer protein (*Mttp*), the ABCG membrane-associated transporters (*Abcg5*), and (*Abcg8*), which have been attributed to cholesterol/triglyceride uptake are elements of the core regulatory network that regulate the transcriptional changes and their association with the transplanted microbiota (**Figure 3C-E**), altogether indicating strong cross-correlation in their expression[44]. Intestine-specific ablation mutants of *Mttp* showed a decrease in cholesterol transport from the intestine to the plasma and a subsequent reduction in the plasma cholesterol and triglyceride levels,

but an accumulation of triglycerides in the intestine[45]. Alterations in lipid metabolism and elevated immune responses are interrelated, and the gut microbiota was shown to play a key role in this connection [46]. Patients suffering from IBD were also found to have major alterations in lipid metabolic processes both in intestinal tissue as well as systemically[47,48]. More generally immune responses due to pathogen infection have been linked to abnormal energy metabolism in chicken[49], further confirming a strong interplay between metabolic reorientations in the intestine and the restoration of inflammatory responses. Gut microbiota metabolizes lipids in the intestinal lumen, consequently affecting the host (lipid) metabolic profile[50]. Specifically, *Turicibacter*, which, in our data, was decreased in $WT^{FMT\text{-}CSTKO}$ compared to WT, and showed a strong positive correlation with the core gene network, in particular with *Mttp* from the cholesterol homeostasis gene cluster (**Figure 3E**), has been associated with the modulation of lipid metabolism in mice[51]. This, in conjunction with the significant increase in butyrate in the CST-KO mice that received the WT FMT (**Figure 1B**), suggests that FMT caused a change in the microbiota metabolic output, with a consequent shift in host metabolism and epithelial barrier restoration.

Overall, our data suggest that the gut microbiota may play a causal role in the complex mechanisms underlying the development of diseases related to increasing levels of CST, such as IBD and metabolic diseases. The profile of physiological and gene alterations in the colon following FMT may represent a novel paradigm in intestinal pharmacology to investigate potential microbiota-associated disorders. The identified transcriptome signatures included several genes of which the human orthologues are IBD and metabolic disease-associated genes that have also been discovered in large-scale transcriptome studies, suggesting their relevance for the mucosal control of homeostasis, and supporting their importance in the dysregulation of immune- and metabolic-associated pathways in patients with altered levels of CST. Findings from this study may advance the concept that targeting the gut microbiota could be a viable therapeutic strategy for a novel development of diseases associated with altered levels of CST, therefore may augment prevention strategies in these diseases.

Materials and Methods

Animals

All studies with mice were approved by the University of California San Diego (UCSD) and Veteran Affairs San Diego Healthcare System (VASDHS) Institutional Animal Care and Use Committees for the Mahata laboratory (UCSD: #S00048M; VA: #A13-002) and were performed in San Diego, California in adherence to the NIH Guide for the Care and Use of Laboratory Animals. For FMT experimental set-up twelve male adult C57BL/6 J mice (age 20 weeks) were purchased from Jackson Laboratory (Bar Harbor, ME) and were acclimatized for 3 weeks before experimentation. Additionally, twelve CST-KO mice generated in the Mahata laboratory were used for this experiment. CST-KO mice have a deletion in the 63 bp CST domain from Exon VII of the *Chga* gene[27] in C57BL/6 background. For transcriptome analysis, an additional 5 and 7 age and sex-matched mice from WT and CST-KO respectively, served as controls. Mice were housed in 4 to 5 animals per cage and had free access to water and food (Normal Chow Diet, LabDiet 5001) in temperature and humidity-controlled rooms with a 12-hr light/dark cycle.

Fecal microbiota transplantation

After 3 weeks of acclimatization period, FMT was performed reciprocally: CST-KO animals received a fecal transplant from WT donors and WT animals received a fecal transplant from CST-KO donors. To avoid the adverse effects of antibiotic treatment on the intestinal transcriptome and physiology[52], bowel cleansing was performed with polyethylene glycol (PEG), an osmotic laxative agent, as previously described[53]. In brief, CST-KO and WT mice (*n= 12*) were fasted 4 hr before bowel cleansing, with free access to water. Mice received four consecutive bowel cleansings of 425 g/L PEG4000 (Sigma Aldrich) by oral-gastric gavage. Each round of bowel cleansing was performed at 20 min intervals. Due to the laxative effect of PEG, the animals were moved constantly to sterile clean cages without bedding to avoid coprophagy and re-inoculation of the former microbiota. To verify the success of the cleansing procedure, sample mice (n= 2) were chosen randomly and euthanized and the total gut was checked to detect luminal content and it was compared to the intestinal content of a control mouse treated with 0.9% saline (**Supplementary Figure 4**).

Bowel cleansing was only performed on day 1 Five hours later, mice received the first fecal transplant (200 µl fecal suspension) by oral-gastric gavage. In total, mice received 3 fecal suspensions, one per day. Fecal suspension for FMT was prepared as follows one day before FMT, a pool of feces from 10 mice/group was collected freshly, frozen immediately, and stored at -80°C. The fecal suspension (1:10 w/v) was prepared each day of FMT using the frozen pool of feces and sterile PBS. The solution was vortexed

gently for 10 min and centrifuged at 800 rpm for 3 minutes. Only the supernatant was used for FMT oral gavage. After the first FMT, mice had normal access to food, water, and housing conditions. Mice were sacrificed 14 days after recolonization.

DNA isolation and 16S sequencing

DNA extraction was performed on fecal samples that were collected before and post-FMT, using a phenol-chloroform-isoamyl alcohol procedure[54]. Briefly, the pellet was resuspended in 1 ml of lysis buffer (940µl TE buffer, 50 µl SDS 10%, and 10 µl Proteinase K 20 mg/ml) in a 2 ml screw cap microtube containing a mix of zirconium and glass beads. Then, samples were incubated at 58°C for 1 hour, and 150 µl buffered phenol (Invitrogen, 15513-047) was added. In each step, samples were vigorously mixed using a vortex. To support lysis, samples were homogenized 3 × 30 sec with 1-min intervals on ice in a mini bead-beater (Biospec, Bartlesville, USA). This was followed by the addition of 150 µl chloroform/isoamyl alcohol (24:1) and centrifuged at 16,000x g for 10 min at 4°C. The upper layer, which contains the DNA, was carefully transferred to a clean tube and 300 µl of phenol/chloroform/isoamyl alcohol [25:24:1] was added. Again, the upper layer was transferred to a fresh tube and 300 µl of chloroform/isoamyl alcohol [24:1] was added. To precipitate the DNA, the upper layer was transferred to a new tube and 1 volume of absolute isopropanol and 1/10 volume of 3M sodium acetate were added. Samples were incubated overnight at -20°C. To get the DNA pellet, the samples were centrifuged at 16,000x g for 20 min at 4°C. The supernatant was removed and 700 µl of 70% ethanol was added to remove the remaining salts from the pellet. Ethanol was removed and the DNA pellet was air-dried for 30 min before resuspension of the pellet in 100 µl TE buffer.

Sequencing of the V3-V4 region of the bacterial 16S gene was carried out by Novogene Co. Ltd. Briefly, for sequencing library preparation, raw DNA extracts were diluted to 1ng/µl in sterile water, and amplicons were generated by PCR (primers 341F and 806R) using a Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Amplification product quality was assessed by gel electrophoreses and samples were pooled in equimolar ratios. Libraries were generated with a NEBNext® UltraTM DNA Library Prep Kit for Illumina and sequencing was carried out on an Illumina 250bp paired-end platform. Initial processing of reads involved trimming of adapters and primers using a Novogene in-house pipeline (Novogene Co. Ltd, Cambridge, UK).

Microbiota analysis

Paired-end sequencing reads were filtered, denoised, merged, and classified with the dada2 package in the statistical programming language R while processing forward and reverse reads separately until merging[55,56]. Briefly, reads were truncated to 220bp and low-quality reads were filtered followed by dereplication. Error models

were learned while manually enforcing the monotonicity of the error function. Reads were denoised and merged with minimal overlap of 12bp, while non-merging reads were concatenated. Singletons were removed before performing bimera removal, followed by a read classification using SILVA (V138) as a taxonomical reference database.

For downstream analysis, the phyloseq and microbiome packages to determine richness and alpha diversity were assessed via observed amplicon sequence variants (ASVs), chao1, Shannon's H, and inverted Simpson's index[74, 75]. All ASVs were collapsed on the genus level and cumulative sum scaling was applied using metagenomeSeq[57]. The resulting genus abundance table served as input for different types of ordinations such as principal component analysis (PCA) or redundancy analysis (RDA). To test for differences between all clusters, PERMANOVA was applied using the adonis function from the vegan package[58]. Differential abundance between before and post-FMT groups was assessed by paired Wilcoxon Rank Sum test followed by FDR correction using $p < 0.05$ as a significance threshold. SourceTracker was used in default settings, with alpha values set to 0.001 and rarefaction to 1000, to lower computational cost[56]. Control samples (WT and CST-KO) were used as source, while $WT^{FMT\text{-}CST\text{-}KO}$ and $CST\text{-}KO^{FMT\text{-}WT}$ were used as sink. Additionally, LefSe analysis (Linear discriminant analysis Effect Size[59]) was carried out, using standard parameters.

Determination of short-chain fatty acids in cecal samples

Cecal samples (100 mg) were suspended in 1 mL of saturated NaCl (36%) solution. An internal standard (50 µL of 10.7 µM 2-ethyl butyric acid in MQ water) was added and the samples were homogenized using glass beads. After the addition of 150 µL H_2SO_4 96%, SCFAs were extracted with 3 ml of ether. The ether layer was collected and dried with Na_2SO_4 (150 mg). The supernatant (0.5 µL) was analyzed using gas chromatography with flame ionization detection (Agilent, Santa Clara, California, USA). The system was equipped with a DB FFAP analytical column (30m x 0.53 mm ID, 1.0 µm; Agilent) and helium GC grade (5.6) was used as carrier gas with a constant flow of 4.2 ml/min. The initial oven temperature was held at 100 °C for 3 min, ramped with 4 °C/min to 140 °C (isothermal for 5 min) and further with 40 °C/min to 235 °C (isothermal for 15 min). Graphs and statistical analysis were performed with GraphPad Prism, using unpaired t-tests. Significance is indicated in the figure legend.

Transmission Electron Microscopy (TEM) and morphometric analysis in mice colon

To displace blood and wash tissues before fixation, mice were deeply anesthetized and were cannulated through the apex of the heart and perfused with a pre-warmed (37 C) calcium and magnesium buffer with 10 mM KCl for 3 min followed by perfusion with freshly prepared pre-warmed (37 C) fixative containing 2.5% glutaraldehyde, 2% paraformaldehyde in 0.15 M cacodylate buffer for 3 min as described previously[60]. The mouse colon was dissected. The fixation, embedding, sectioning, and staining of the mouse colon were performed as described also by[20]. Grids were viewed using a JEOL JEM1400-plus TEM (JEOL, Peabody, MA) and photographed using a Gatan OneView digital camera with 4×4k resolution (Gatan, Pleasanton, CA). Micrographs were randomly taken from 3 mice per group (8 to 10 photographs per mouse with a total of 25 to 28 photographs) and the morphometry of tight junctions and fibrosis were performed as described previously[20,60]. Briefly, the line segment tool in NIH ImageJ was used to measure the lengths and perpendicular widths (diameter) of TJ, AJ, and desmosomes. The free-hand tool in NIH ImageJ was used to manually trace around the area occupied by the collagen fibers. For the determination of the collagen area, the sum of the collagen area in a randomly-chosen photograph was divided by the total area of that photograph and multiplied by 100.

RNA extraction from colonic samples

Samples were collected and submerged in RNA later (Qiagen) to avoid RNA degradation and stored at -80 °C. A hybrid protocol using TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen) was used to obtain high-quality RNA. Briefly, 10-20 mg of tissue was submerged in 1 ml ice-cold TRIzol in a 2 ml screw cap microtube containing 3 mm glass beads. To perform lysis, samples were homogenized 3 × 30 sec with 1-min intervals on ice in a mini bead-beater (Biospec, Bartlesville, USA). The sample was centrifuged at 12,500x g for 15 min at 4 °C. Then, it was transferred to a clean tube where 200 µl of chloroform was added and mixed with a vortexer. After centrifugation at 12,500x g for 15 min at 4 °C, the upper clear layer was collected in a fresh tube and two volumes of 100% ethanol were added and mixed gently. Immediately, the mixture was transferred to an RNAeasy mini kit column and centrifuged for 30 sec at room temperature. To wash the RNA, two times 500 µl RPE solution was added, and RNA was eluted in a tube with RNAse-free water. RNA quality was assessed by gel electrophoresis.

RNA sequencing in colon samples

RNA library was assembled using NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490) and NEBNext Ultra II RNA Library Prep Kit for Illumina (E7770, New England Biolabs) as per manufacturer instructions. Single-end sequencing

was performed using a NextSeq 500 machine (Illumina; up to 75 cycles). The generated data were subsequently demultiplexed using sample-specific barcodes and changed into fastq files using bcl2fastq (Illumina; version 1.8.4). The quality of the data was assessed using FastQC (v0.11.8)[61]. Low quality bases and (parts of) adapter sequences were removed with Cutadapt (v1.12; settings: q=15, O=5, e=0.1, m=36)[62]. Sequenced poly-A tails were removed as well, by using a poly-A and a poly-T sequence as adapter sequences (A{100} and T{100}). Reads shorter than 36 bases were discarded. The trimmed fragment sequences were subsequently aligned to the mouse reference genome (GRCm39; From Ensembl; release 103) and the number of reads per gene were determined with the use of STAR ([63]; v2.7.8a; settings: --outSAMstrandField=intronMotif, --quantMode=GeneCounts, --outFilterMultimapNmax=1). Duplicate reads were marked with samtools markdup (v1.9; using htslib 1.9) and the extent of PCR amplification (PCR artifacts) was assessed with the use of the R package dupRadar (v.16.0) [64,65]. There was no indication of PCR artifacts for any of the samples (Intercept: 0.006 – 0.013; Slope: 4.50 – 5.69; webpage of dupRadar was used as a guideline; https://bioconductor.org/packages/release/bioc/vignettes/dupRadar/inst/doc/dupRadar.html).

Differential gene expression and gene ontology analysis

The principal component analysis was performed in R (v3.6.3) using the R package DESeq2 (v1.26.0)[56,66]. To visualize the overall effect of experimental covariates as well as batch effects (function: plotPCA). Differential gene expression analyses were performed with the same R package using default settings (Negative Binomial GLM fitting and Wald statistics), following standard normalization procedures.

The function enrichGO of the Bioconductor R package clusterProfiler (v3.14.3) was used to test whether certain gene ontology (GO) categories were enriched among the detected genes (settings: OrgDb=org.Mm.eg.db [v3.10.0], keyType=ENSEMBL, universe=[all genes with an adjusted p-value; padj is not NA], qvalueCutoff=0.05, minGSSize=1, maxGSSize=100,000), while only considering the bioprocess aspect of GO[67,68].

The expected number of genes for each category (see bar plots) were calculated with the gene ratio (GeneRatio) and background ratio (bgRatio) information from the output of the enrichGO function (background ratio times the total number of genes with GO annotation that were differentially expressed).

Differential gene expression analysis with GSEA

Normalized expression levels were subjected to Gene Set Enrichment Analysis (GSEA) using default parameters [69]. GO bioprocess was used as gene set category and enrichment results were visualized using the EnrichmentMap plugin (v3.3.3) of Cytoscape (v3.9), by considering all enriched categories below a nominal p-value of 0.05 [70]. Clusters were grouped and annotated using the Autoannotate plugin (v1.2) with default parameters.

Construction of a core-regulatory network

DEGs ($p_{adjusted}$<0.05) were subset to contain only genes that exhibited an opposite foldchange upon FMT, being greater than +-0.58 log2FC. Associations among the proteins translated by the commonly identified DEGs were assessed using the StringApp plugin (v1.7) from Cytoscape (v3.9) [71,72]. The minimum required interaction score was set to 0.4 and interactions among the query proteins were further visualized. Single nodes and two-node clusters were removed and clusters were identified with Cytocluster ClusterONE (v.1.0)[73] with default settings. Clusters were considered significant when p-value < 0.05 was met. Subsequently, functional enrichment using StringApp was performed on the resulting clusters. Visualization was carried out in Cytoscape.

Correlation analysis of gene expression levels and microbial taxon abundance

Genes were subset to only contain genes that change in a significant manner upon FMT. Pairwise Pearson correlations were computed between normalized expression levels (DSeq2) for each gene against normalized abundance values (CSS) of each microbial taxon using the psych R package. This was done separately per genotype only on FMT timepoints. Only significant correlations were kept for further analysis. To overcome the fact of unpaired samples for the control groups, gene and taxon log2 fold-changes (log2FC) were used to discard any false positive correlations. When the sign of the correlation was positive the sign of the gene and taxon log2FC were required to be equal, contrary if the correlation sign was negative the signs of the log2FCs had to be different.

To further refine the analysis correlations were subset to contain only genes that change oppositely, eg. positive log2FC in WT and negative log2FC in CSTKO, as well as a log2FC greater than 0.58. We performed graph analysis and visualization in Cytoscape v3.9 also employing the StringApp plugin.

Statistical analysis

All statistical tests were performed using GraphPad Prism 7 or the statistical programming language R. Specific statistical tests are indicated in the text or figure legends. Normality was assessed by either D'Augustino-Pearson omnibus normality test or Shapiro-Wilk normality test. If normality was met a t-test was chosen, otherwise non-parametric Mann-Whitney was used. Outliers were assessed with GraphPad ROUT method (Q=1).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Whole transcriptome and 16S rRNA gene amplicon sequence data were deposited under BioProject numbers PRJNA800626 and PRJNA741992.

Disclosure of interest

The authors declare no competing interests.

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Authors' Contributions

P.G-D., M.S, S.E.A, and S.K.M conceived and designed the study. P.G-D., A.D., M.S., B.D, and S.K.M. performed the experiments, and P.G-D., M.S, R.W, B.D., S.K.M., and S.E.A analyzed the data. P.G-D, M.S, S.E.A. wrote the original manuscript that was reviewed by A.D, R.W., B.D., K.V., and S.K.M. Funding for these studies was acquired by S.E.A. and S.K.M. All authors read and approved the final manuscript.

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Supplementary results

CST-KO animals show distinct transcriptional patterns compared to their WT counterparts

Transcriptome analysis was performed using DeSeq2, we detected a total of 3690 differentially expressed genes (DEGs) in CST-KO mice, 2195 were upregulated and 1765 were downregulated ($p_{adj} < 0.05$) compared to WT counterparts. To complement our analysis, we further employed the overrepresentation analysis (OA) with ClusterProfiler tool on the previously determined DEGs as an alternate method to determine significantly changing GO biological processes in CST-KO mice compared to WT (**Supplementary Figure 2, A-C**). This analysis identified differentially expressed genes related to the regulation of hormone metabolic processes, lipid metabolism, molecular transport, and peptide secretion.

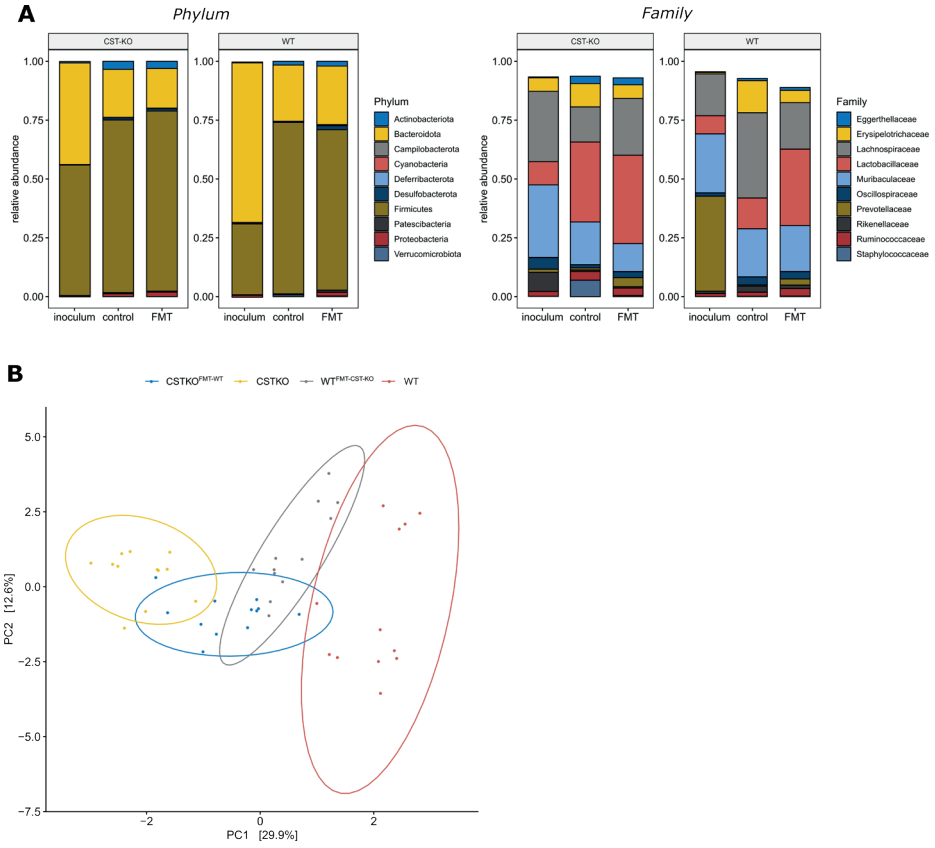
In parallel, we employed Gene Set Enrichment Analysis (GSEA) to search for groups of genes that are significantly enriched in either genotype [1]. For the GSEA, we used again the Gene Ontology biological processes database consisting of 4055 gene sets out of which 26, and 35 gene sets were significantly enriched in CST-KO and WT respectively (nominal p-value < 0.05). GSEA identified enriched GO biological processes involved in catecholamine metabolic processes, especially for norepinephrine uptake and secretion, maintenance of the gastrointestinal epithelium, nervous system development, T-cell mediated immunity, and regulation in leukocyte chemotaxis (**Supplementary Figure 2, D-G**). Collectively, the transcriptome data is in agreement with the known role of catestatin as a nicotinic cholinergic antagonist that affects the catecholamine release[2], a regulator of the gut epithelium barrier[3–5], a regulator of metabolic and immune homeostasis[6,7], anti-obesogenic, and regulator of lipid metabolism[8].

Enriched biological functions and pathways modulated in response to the adoptive microbiota transfer in CST-KO$^{FMT\text{-}WT}$ and WT$^{FMT\text{-}CST\text{-}KO}$

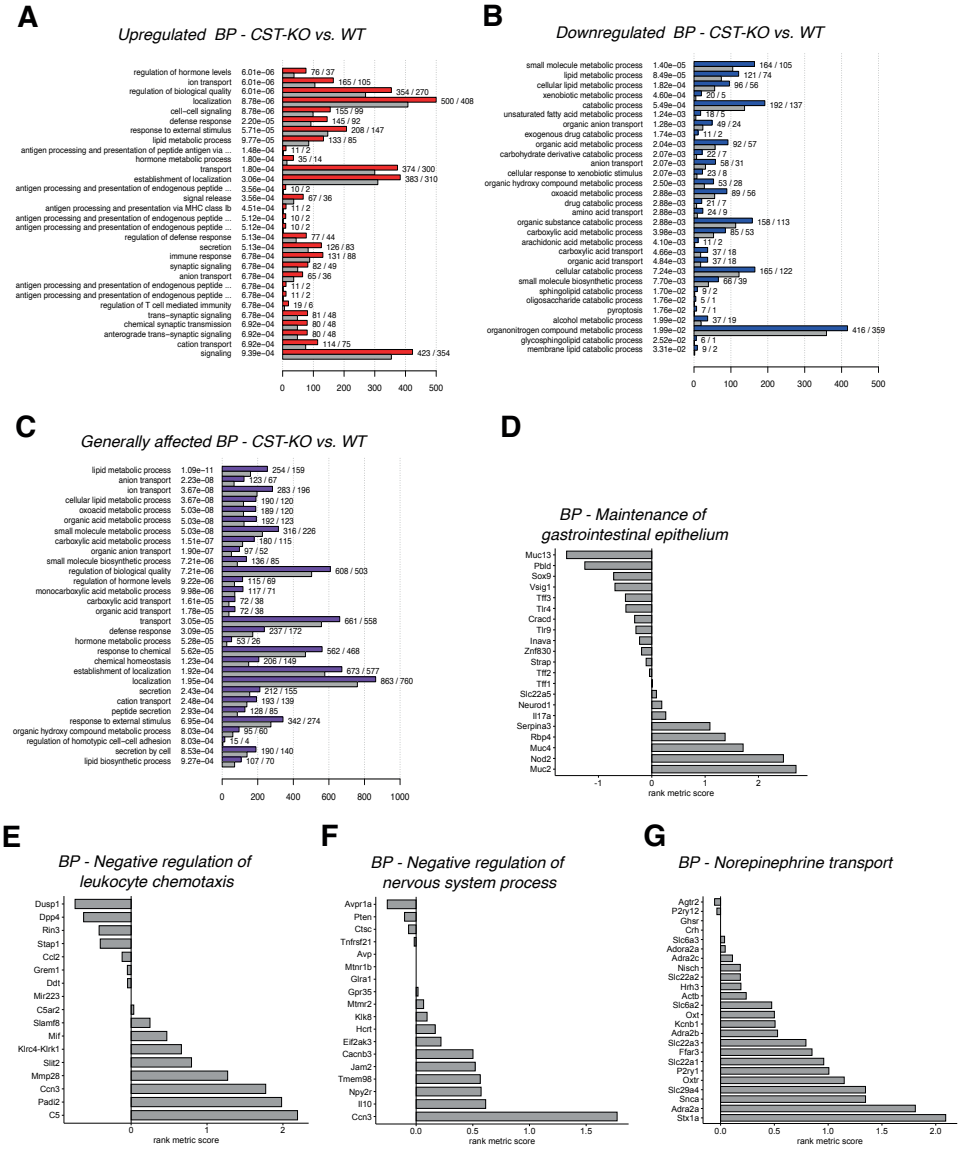
To comprehensively describe which biological processes are altered in CST-KO$^{FMT\text{-}WT}$ and WT$^{FMT\text{-}CST\text{-}KO}$ compared to their controls, we employed GSEA on the biological process gene ontology (GO) level. A total of 120 gene sets were enriched in CST-KO$^{FMT\text{-}WT}$ compared to the CST-KO mice (nominal p-value < 0.05), while 275 gene sets were enriched in the WT $^{FMT\text{-}CST\text{-}KO}$ compared to WT mice (**Supplementary Excel Sheet 2**). To complement our analysis, we further used overrepresentation analysis (OA) with the ClusterProfiler tool on the previously determined DEGs as an alternate method to determine significantly changing biological pathways (**Supplementary Figure 3**). Collectively, both analyses identified several clusters of gene sets involved in

immunological processes, including immunoglobulin production, B-cell activation, and adaptive immunity regulation; regulation of innate immunity, immune system activation, and MHC antigen processing, to be upregulated in $WT^{FMT\text{-}CST\text{-}KO}$ compared to the WT control. Additionally, GSEA identified several clusters of gene sets involved in the upregulation of chromosome separation, chromatin remodeling, DNA replication, and cell cycle-related gene sets in WT $^{FMT\text{-}CST\text{-}KO}$ compared to the WT control. OA additionally identified a large downregulated cluster related to cell regulatory functions in WT $^{FMT\text{-}CST\text{-}KO}$. Remarkably, changes in CST-$KO^{FMT\text{-}WT}$ were less prominent compared to the $WT^{FMT\text{-}CST\text{-}KO}$. Moreover, the CST-$KO^{FMT\text{-}WT}$ group displayed fewer upregulated genes but a greater number of downregulated genes compared to the $WT^{FMT\text{-}CST\text{-}KO}$. GSEA identified a few clusters of gene sets involved in DNA repair damage, pyrimidine metabolic processes, glycolipid metabolism, blood pressure regulation, and vitamin (folate) metabolism, to be upregulated in CST-$KO^{FMT\text{-}WT}$ compared to the CST-KO group. These results were supported by OA, which additionally showed upregulation in gene sets involved in lipid metabolism.

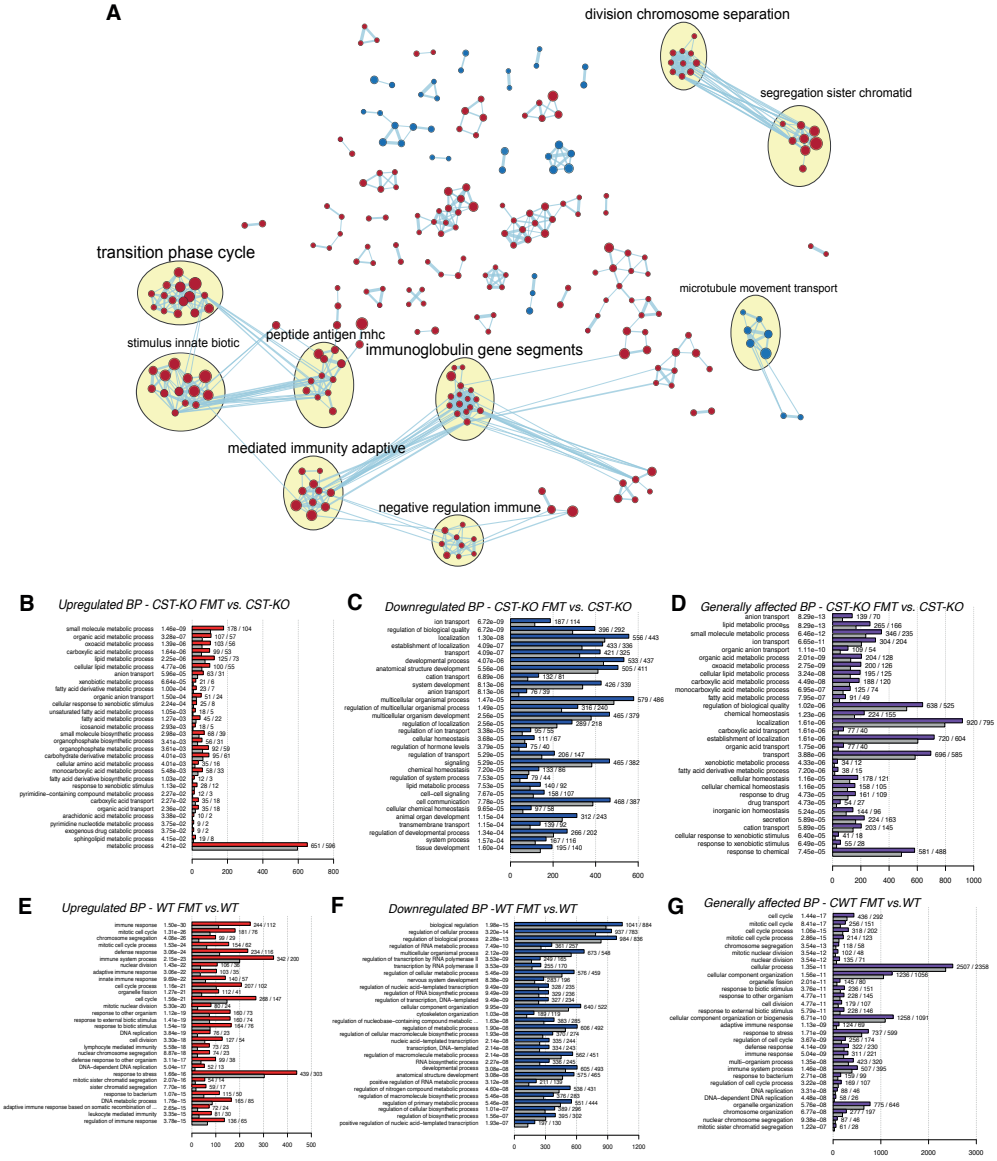
Supplementary Figures



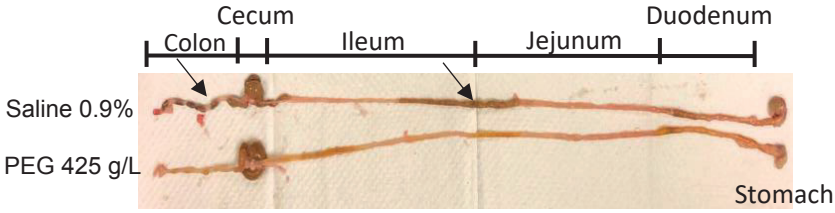
Supplementary Figure 1. (**A**) Relative abundance of the present phyla and families from the inoculum, and before and after FMT in CST-KO and WT (**B**) Principal component analysis (PCA) of genus-level collapsed abundance data, shows distinct clustering of the different groups. Especially the control groups are separated, while the FMT treated groups converge to a middle point. Ellipses represent normal data ellipses produced by methods from the R package ggplot2.



Supplementary Figure 2. Bar plots of the 30 most significant GO bioprocesses identified by ClusterProfiler for the comparison between WT and CST-KO control groups. As input the list of DEGs ($p_{adjusted} < 0.01$) was either subset to only contain upregulated (**A**) or downregulated (**B**) genes or not filtered at all (**C**). Colored bars indicate the number of found DEGs in a specific category, while grey bars indicate the amount of DEGs to be expected by chance in each category. The q-values are indicated after each process (in the middle) and numbers at the end of the bars indicate the number of observed and expected genes. (D-G) Selection of bioprocesses, which were identified by GSEA to be significantly enriched in either genotype. Values indicate the ranking metric (signal2noise ratio) for each gene contained in the bioprocess. Positive values designate enrichment in CST-KO and negative values in WT mice respectively.



Supplementary Figure 3. (**A**) Enrichment map of significantly expressed gene sets (GO terms; nominal p-value <0.05) identified by GSEA between WT and $WT^{FMT\text{-}CST\text{-}KO}$. Blue indicates enrichment in WT, while red indicates enrichment in $WT^{FMT\text{-}CST\text{-}KO}$. Clusters were annotated with Cytoskape AutoAnnotate plugin. Connections between nodes indicate more than 25% of shared DEGs, while edge thickness correlates with the shared fraction. (**B-G**) Bar plots of the 30 most significant GO bioprocesses identified by ClusterProfiler for the comparison between CST-KO and $CST\text{-}KO^{FMT\text{-}WT}$ and between WT and $WT^{FMT\text{-}CST\text{-}KO}$. As input the list of DEGs ($p_{adjusted} < 0.01$) was either subset to only contain upregulated (**B, E**) or downregulated (**C, F**) genes or not filtered at all (**D, G**). Colored bars indicate the number of found DEGs in a specific category, while grey bars indicate the amount of DEGs to be expected by chance in each category. The q-values are indicated after each process (in the middle) and numbers at the end of the bars indicate the number of observed and expected genes.



Supplementary Figure 4 . Representative photograph showing the gastrointestinal tract of a mouse after four bowel cleansings using 0.9% saline (up) and polyethylene glycol PEG (down). Arrows depict the luminal content in the bowel still present after using a saline solution but not in the PEG treated group.

Supplementary Tables

Supplementary Table 1: Clustering results from core network analysis

Custer	No. of Nodes	Node Density	Cluster Quality	p-value	Included Nodes	Functional enrichment
1	13	0.628	0.742	1.84E-05	*Nkg7, Cd7, Cd8b1, Gzma, Ctsw, Cd8a, Cd3g, Itk, Ccl5, Hck, Lck, Il2rb*	Adaptive Immune Response, Leucocyte activation, T-cell differentiation
2	9	0.778	0.778	2.11E-04	*Uqcr11, Etfb, Atp5k, Ndufs6, Cox7b, Atp5g1, Uqcrq, Ndufa4, Dbi*	Generation of precursor metabolites and energy, Electron transport chain, ATP metabolic process
3	7	0.619	0.722	0.0016	*Mttp, Abcg8, Cyp27a1, Abcg5, Mylip, Slc51b*	Cholesterol homeostasis, Organic hydroxy compound transport, Cholesterol efflux

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Chapter 4

Review article

Actions of trace amines in the brain-gut-microbiome axis via trace amine-associated receptor 1 (TAAR1)

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Abstract

Trace amines and their primary receptor, Trace Amine-Associated Receptor-1 (TAAR1) are widely studied for their involvement in the pathogenesis of neuropsychiatric disorders despite being found in the gastrointestinal tract at physiological levels. With the emergence of the "brain-gut-microbiome axis," we take the opportunity to review what is known about trace amines in the brain, the defined sources of trace amines in the gut, and emerging understandings on the levels of trace amines in various gastrointestinal disorders. Similarly, we discuss localization of TAAR1 expression in the gut, novel findings that TAAR1 may be implicated in inflammatory bowel diseases, and the reported comorbidities of neuropsychiatric disorders and gastrointestinal disorders. With the emergence of TAAR1 specific compounds as next-generation therapeutics for schizophrenia (Roche) and Parkinson's related psychoses (Sunovion), we hypothesize a therapeutic benefit of these compounds in clinical trials in the brain-gut-microbiome axis, as well as a potential for thoughtful manipulation of the brain-gut-microbiome axis to modulate symptoms of neuropsychiatric disease.

Introduction

The brain-gut-microbiome axis is an emerging area of research highlighting the involvement of gastrointestinal microbes with the comorbidities of several neuropsychiatric disorders. The intestinal tract harbors the most abundant ecosystem of bacteria with concentrations ranging from 103 to 1014 bacteria depending on tissue localization (Hillman et al 2017). The idea of microbes having a beneficial impact on human health predates our current understanding of the microbiome by 100 years, as E. Metchnikoff associated fermented food products with longevity in a rural population, and suggested that lacto*Bacilli* could counteract the effects of illness and aging (Metchnikoff 1908). In 2007, the United States National Institutes of Health established the "Human Microbiome Project" to improve the understanding of the microbial flora in human health. The collective genome of the microbial species living on our body, termed metagenome, outnumbers the human genome by a factor of 200 (Qin et al. 2010). Thus, it is not surprising that the metagenome and its encoded metabolic activities play a crucial role in all aspects of human health and disease (Marcobal et al. 2013). As such, there has been a focus on the role of bacterial metabolic byproducts in human health (Jacobs et al. 2016; Santoru et al. 2017; Smith and Macfarlane 1997; Vandenberg et al. 2003; Kisuse et al. 2018). Meanwhile, advances have been made in animal studies using germ-free mice, suggesting that disturbances in the intestinal microbial flora can alter brain chemistry and behavior (Park et al. 2013). About 60% of anxiety and depression patients are described to have intestinal function disturbance, such as in irritable bowel syndrome (IBS). Recently, IBS has also been related to changes in intestinal microbiota, including disruption of the intestinal microflora. While there has been a focus on the role of complex carbohydrates and neuroactive short-chain fatty acids (e.g. butyrate, acetate and propionate) in the brain-gut-microbiome axis, some of these same studies provide evidence that trace amine (TA) levels are elevated in gastrointestinal disorders and neuropsychiatric disorders. Here, we propose the novel hypothesis, that the putative trace amine receptor, Trace Amine-Associated Receptor 1 (TAAR1) can augment gastrointestinal illness and neuropsychiatric disorders as a result of a dysregulated intestinal microbial flora. This review discusses several elements of the brain-gut-microbiome axis as it relates to trace amines, TAAR1, and the role they may play in both neuropsychiatric and comorbid gastrointestinal disorders.

TAAR1 is a G protein-coupled receptor that was deorphanized in 2001 (Borowsky et al. 2001; Bunzow et al. 2001) and has been widely studied as a major regulator of dopamine in neuropsychiatric disorders and in acute and neuroadaptive responses to drugs of abuse; and extensively reviewed (Berry et. Al 2017; Christian and Berry 2018; Grandy et al. 2016; Schwartz et al. 2018). Currently, specific TAAR1 compounds

are nearing the completion of clinical trials for the treatment of schizophrenia and Parkinson's related psychoses (Roche, Sunovion). The predominant endogenous ligands for TAAR1 are classified as 'trace amines' and include p-tyramine, β-phenylethylamine, tryptamine, 3-iodothyronamine, and octopamine as well as 'classical' monoamine neurotransmitters including histamine, serotonin, and dopamine (Borowsky et al. 2001; Chiellini et al. 2012; Hoefig et al. 2015; Pugin et al. 2017; Sotnikova et al. 2010; van Kessel et al. 2019). TAs activate TAAR1 at nanomolar affinities, whereas classical monoamine neurotransmitters activate the receptor at or near micromolar concentrations (Panas et al. 2012; Xie et al. 2007).

The term TA was adopted by a study group at the 1975 meeting of the American College of Neuropsychopharmacology (Usdin and Sandler 1976), and it is now often mentioned that the levels of TAs are <10ng/g (Berry 2004; Gainetdinov et al. 2018). TAs are classically defined as any monoamine with physiological levels less than 1-100ng/g of tissue weight (Boulton 1974) though oftentimes higher levels are subsequently identified in new tissue assessments of particular amines. Historical studies of TAs in the body have correlated imbalances in TA levels to neuropsychiatric disorders including schizophrenia, substance abuse, depression, ADHD, and Parkinson's. The role of TAs in the gut has not been systematically studied, likely because the identification of TAs and their hypothesized role in neuropsychiatric disorders was decades before the understanding of the microbiome and metabolome. Perhaps because of the focus of TAs in psychiatric illness over prior decades, the discovery of TAAR1 in 2001 led to a body of research studying the effects of TAAR1 in modulating monoaminergic signaling in the brain. The proposed and known functions of TAAR1 in neuropsychiatric disorders has been extensively reviewed and as such will only be briefly described here when relevant.

Recent research has increasingly drawn connections between perturbations to the gut microbiota and both gastrointestinal and psychiatric conditions (Felice and O'mahony 2017). The gastrointestinal tract is a heterogeneous layer of tissue comprised of smooth muscle, neuronal cells, immune cells, and epithelial cells. Maintainance of gastrointestinal homeostasis is dynamic and involves the regulation of the epithelial cell monolayer to protect the underlying immune cells and neurons to prevent excessive inflammation (Rao and Wang 2010). There are billions of neurons interconnected via trillions of synapses in the gut and brain, all of which are primarily governed by communication mediated by neuromodulators. One way these modulators are hypothesized to link the gut and brain is by the production of aminergic compounds from the gut microbiota – a diverse collection of microbial communities that are thought to influence a wide array of biological processes. Within the realm of neuromodulators originating from the microbiota exists the

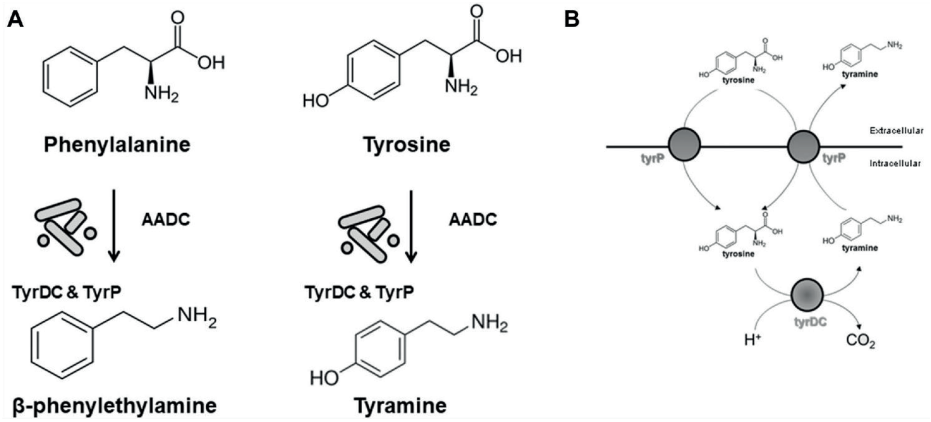

Figure 1. (**A**) Two Metabolic byproducts of dietary amino acid metabolism by mammalian and microbial enzymatic systems. Phenylalanine and tyrosine are decarboxylated by the microbial TyrDC & TyrP system and mammalian aromatic amino acid decarboxylases (AADC) to phenylethylamine and tyramine respectively. (**B**) Microbial transport and decarboxylation of tyrosine. Tyrosine is taken in by the tyrosine transporter (tyrP) into microbes where the enzymatic decarboxylation of tyrosine to tyramine occurs by the tyrosine decarboxylase (TyrDC) enzyme. Tyramine is then transported to the extracellular space of the microbe by tyrP (Bargossi et al. 2017; Bargossi et al. 2015; Pessione et al. 2009; Wolken et al. 2006). A similar mechanism occurs for decarboxylation of phenylalanine to β-phenylethylamine by tyrosine decarboxylase.

TAs (Pugin et al. 2017). Several enteric and food-borne microorganisms are known to produce tyramine and β-phenylethylamine, as listed in **Table 1**. In fact, around 3 g of un-degraded proteins and peptides enter the human intestine every day from the diet, as well as from endogenous sources such as host tissues, pancreatic enzymes, and other secretions (Chacko and Cummings 1988). Such large amounts of organic nitrogen-containing compounds are available for catabolism to amino acids, providing essential amino acids to the host (e.g. phenylalanine) and further metabolic degradation by intestinal microorganisms (**Fig 1A**) (Rasnik et al. 2017). In response to an acidification of the environment, microorganisms, such as those listed in **Table 1**, upregulate several transporters including the tyrosine transporter and a tyrosine decarboxylase. Once transported intracellularly, tyrosine is rapidly decarboxylated to tyramine by the bacterial tyrosine decarboxylase (TyrDC), where it is then exported from the microorganism by TyrP, mechanistically described in **Figure 1B** (Wolken et al. 2006). When produced in adequate amounts, gut bacterial-produced TAs have been shown to have differing effects for the host. For example, β-phenylethylamine is reported as an antimicrobial against pathogenic *E. coli* (Lynnes et al. 2014), and tyramine has been shown to stimulate fast ileal contractions

and neuropeptide Y release (Broadley et al. 2009), as well as increasing synthesis and secretion into circulation of monoamine neurotransmitters (Yano et al. 2015). Modulation of intestinal motility and secretion can have profound effects on luminal pH, mucosal immune response, and delivery of important nutrients to the host cells and enteric microbiota.

Table 1. Enteric and/or food-borne bacterial species able to synthesize trace amines and subsequent levels in the gastrointestinal tract

Trace Amine	Trace amine concentrations in or transiting human gut	Trace amine-producing enteric/food-borne microbes	References
Tyramine	400-700 mg/L, Pugin et al. (2017)	*A.haemolyticus, A. hydrophila, A.faecalis, B. cereus, B. subtilis C. braaki, C. freundii, C.gallinarum, C. piscicola, C. maltaromaticum, C. divergens, C. freundii, E. faecalis, E. faecium, Enterococcus. sp., E. coli, E.durans, E. hirae, E. casseliflavus, E. mundtii, E. aerogenes, E. cloacae, K. pneumoniae, L. brevis, Lactobacillus sp., L. curvatus, L. plantarum, L. buchneri, L.casei, L. paracasei, L. reuteri, L. hilgardii, L. homohiochii, L. delbruecki, L. lactis, L. alimentarius, L.curvatus, L. mesenteroides, M. morganii, P. mirabilis, P. vulgaris, P. aerugina, R. ornithinolytica, S. thermophilus, S. faecalis, S. macedonicus, Sporolactobacillus sp., T. halophilus, W. cibaria, W. confusa, W. paramesenteroides, W. viridiscens.*	Barbieri et al. (2019), Bonnin-Jusserand et al. (2012), Borresen et al. (1989), Buňková et al. (2009), Coton and Coton (2009), Coton et al. (2004), Coton et al. (2011), La Gioia et al. (2011), Ladero et al. (2012), Ladero et al. (2013), Leisner et al. (2007), Linares et al. (2011), Maifreni et al. (2013), Marcobal et al. (2012), Marcobal et al. (2006), Min et al. (2004), Moreno-Arribas et al. (2001), Pessione et al. (2005), Pessione et al. (2009), Pircher et al. (2007), Pugin et al. (2017), van Kessel et al. (2019) and Zhu et al. (2016)
β-Phenylethylamine	10 nmol/g, Turroni et al (2016)	*B. cereus, C. divergens, C. carnosus, E. faecium, E. faecalis, E. casseliflavus, E. durans, E. mundtii, E. hirae, L. lactis, L. brevis, L. mesenteroides, Staphylococcus sp., P. aerugina.*	Bargossi et al. (2015), de Las Rivas et al. (2008), Landete et al. (2005), Linares et al. (2011), Min et al. (2004), Perin et al. (2017) and Pessione et al. (2009)

TAAR1 localization has been identified in both the stomach and the intestine in mouse and human (Chiellini et al. 2012; Adriaenssens et al. 2015; Ito et al. 2009; Kidd et al. 2008; Ohta et al. 2017; Raab et al. 2015; Revel et al. 2013), but the exact function in the polarized gastrointestinal epithelium remains largely unexplored. Here, it is worth noting that despite no definitive function being identified in the polarized epithelium of the stomach and intestine, TAAR1 functionality has been

described in the beta-cells of the pancreas as activation of the receptor resulted in hormone (PYY and GLP-1) secretion (Raab et al. 2015), though these studies are not relevant to our discussion the brain-gut- microbiome axis. Functional TAAR1 was found in almost all peripheral immune cells (Babusyte et al. 2013; Panas et al. 2012; Sriram et al. 2016; Wasik et al. 2012), with evidence that TAAR1 can modulate not only intracellular signaling (Panas et al. 2012), but also immune cell functions such as chemotaxis (Babusyte et al. 2013), phagocytosis (unpublished abstract, Miller Lab, (Gwilt et al. 2018)) and altered expression of cytokines (Bugda Gwilt et al. 2019a; Babusyte et al. 2013). Immune cells are known to infiltrate the gastrointestinal tract with epithelial damage and inflammation, and given the reported chemotactic capacity of TAAR1 positive cells towards TAs, TAAR1 positive immune cells should be noted in the gastrointestinal microenvironment given the propensity of patients with neuropsychiatric disorders to also present with peripheral inflammation.

Our lab has identified TAAR1 expression in the gastrointestinal tract of C57BL/6 mice, summarized in **Fig 2** (Bugda Gwilt et al. 2019b; Ito et al. 2009), in human intestinal epithelial cell lines (unpublished data), as well as enteric glia (unpublished data). Interestingly, TAAR1 localization is primarily intracellularly in the colonic epithelium (**Fig2A**) whereas in the small intestine it is predominantly found on epithelial membranes (**Fig 2B**). The dynamic expression and localization of TAAR1 have been previously described (Bugda Gwilt et al. 2019a; Sriram et al. 2016; Stavrou et al. 2018) and the production of TAs by several species in the microbiome (e.g. Bargossi et al. 2015; Pessione et al. 2009; van Kessel et al 2019) may account for the predominant membranous localization of TAAR1 compared to colonic tissue. In the brain-gut-microbiome axis, gut microbes are able to signal by the vagal nerve, mediating behavioral effects in animals (Forsythe et al. 2014). While vagal nerve signaling is demonstrated to have roles in these effects, studies are lacking that investigate the role of non- neuronal-like tissues in the brain-gut-microbiome axis. Given evidence for the localization of TAAR1 in the gastrointestinal epithelium, enteric glia (**Fig 2A**), and all peripheral immune cells, it is important to understand the role that these tissues have in mediating gut health and modulation of inflammation by the varied sources of TAs in the gut. Perhaps, TAAR1 activation in these tissues can be damaging, exposing the sensitive underlying tissue to pathogenic microbes, providing a mechanism of exposure of the vagal nerve pathways to microorganisms, or food byproducts.

In addition to bacterial origins, another prominent source of TAs in the human body is through consumption of fermented food like cheese, pickles, and wine, where the lactic acid bacteria are responsible for the production of TAs in these food products (Marcobal et al. 2012). While the entire *Lactobacillus* species are considered producers of tyramine (Pessione et al. 2009), only specific *Lactobacillus* species are found in food

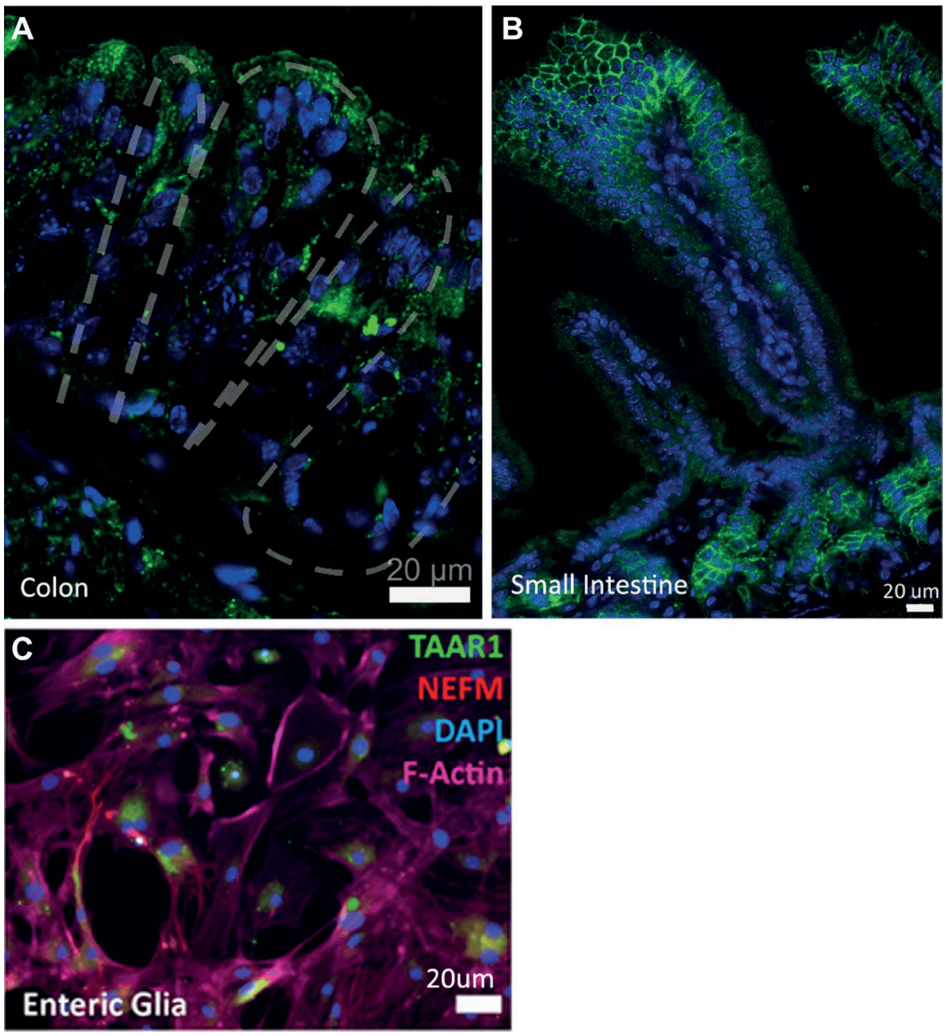

Figure 2. Summary of TAAR1 expression in mouse gastrointestinal tissue formalin-fixed paraffin-embedded mouse tissue (**A, B**) was stained with specific TAAR1 antibody, "D274" designed and published by the Miller lab (Bugda Gwilt et al. 2019a). (**A**) TAAR1 localization is seen in apical epithelial cells and is both intracellularly and membrane, localized in colon tissue. Colon tissue morphology is as expected, and crypts shapes are depicted by dashed lines for clarity. (**B**) TAAR1 localization is seen primarily on membranes of the villus (finger-like projections) and in the base of the crypts. (**C**) Ex vivo isolated enteric glial cultures were stained with TAAR1 (green) neurofilament medium (NEFM Red), Actin (pink), and DAPI (blue), demonstrating profound intracellular localization of TAAR1 in ex vivo enteric glial cultures.

products, and some have been found to survive transit through the gastrointestinal tract (Pugin et al. 2017). Bacterial jejunal contents were found to coincide with the production of TYR in the presence of tyrosine decarboxylase ex vivo (Fernandez De Palencia et al. 2011; Van Kessel et al. 2019). When fermented food products are ingested, levels of TAs in the gut can be raised to undesirable levels (Pugin et al. 2017). As previously discussed, acidification of an environment enriches tyramine production through the TyrP and TyrDC enzymatic systems. Accordingly, tyramine production by *Enterococcus* species in food is enhanced by lowered pH in the small intestine that can not only simulate rapid passage through the gastrointestinal tract (Fernandez de Palencia et al. 2011), but is common in patients with inflammatory bowel disease (Press et al. 1998).

Dietary TAs were first described as having a physiological relevance in the 1980's with the advent of a new class of antidepressants: the monoamine oxidase inhibitors. This phenomenon –known as "The Cheese Effect" – has been attributed to the accumulation of very high levels of tyramine and β- phenylethylamine in patients being treated with monoamine oxidase inhibitors (Anderson et al. 1993; Price and Smith 1971; Shalaby 1996; Stratton et al. 1991). Patients suffer from severe vasoconstriction as a result of the accumulation of very high levels of consumed and bacterial-produced tyramine and phenylethylamine (Anderson et al. 1993; Price and Smith 1971; Shalaby 1996; Stratton et al. 1991). Though the mechanism of the so-called "cheese effect" is not mediated by TAAR1, a similar decrease in monoamine oxidase activity may be present in patients with gastrointestinal illness. A hallmark of several gastrointestinal diseases leads to an ablation of the polarized epithelium, commonly seen in inflammatory bowel diseases, or an altered microbial composition. In either case, it is reasonable to predict that monoamine oxidase enzymatic activity can be affected by an overabundance of aminergic compounds, or an ablation of the cells harboring the enzyme.

Interestingly, fecal metabolomic studies have identified a higher relative abundance of the *TyrDC* gene and its harboring bacteria *Enterococcus* have been recently detected in Parkinson's disease patients who require higher frequency of the levodopa daily dosage regime compared to other Parkinson's disease patients (van Kessel et al. 2019). TyrDC has the capacity to decarboxylate levodopa into dopamine, which coincides with the conversion of tyrosine into tyramine (van Kessel et al. 2019). Tyramine has been recently been suggested as an early stage biomarker for Parkinson's due to increased urine tyramine compared to healthy controls (D'andrea et al. 2019). Thus, higher availability of tyrosine or TyrDC in the intestinal tract of those patients may result in accumulation of tyramine, causing detrimental side effects. For example: many patients experience dyskinesias, which have been previously

correlated with modulation of the β-arrestin 2 signaling pathway, a pathway that has been previously linked to TAAR1 signaling (Espinoza et al. 2015; Harmeier et al. 2015; Urs et al. 2015).

Tyramine has additional known functions in human intestinal epithelial cell lines (Del Rio et al. 2017), though there are currently no published functional links to a receptor-mediated mechanism by TAAR1 in these epithelial cell models. Briefly – tyramine transiting the gut, presumably from consumption of tyramine rich food – can promote the adherence of microbes to the intestinal epithelial cells (Fernandez De Palencia et al. 2011; Luqman et al. 2018) and can modulate inflammatory cytokine signaling in intestinal epithelial cells (Fernandez de Palencia et al 2011). Tyramine can also increase the synthesis of serotonin by enteroendocrine cells in the gut, elevating its release into circulation (Yano et al 2015). Additionally, work from our lab has demonstrated that tyramine activation of bone marrow derived macrophages from C57BL/6 mice augments secretion of inflammatory cytokine gene expression, an effect that is attenuated by the specific TAAR1 antagonist EPPTB (Bugda Gwilt et al. 2019a). Based on reports that dietary TAs can activate human TAAR1 in primary epithelial cells (Ohta et al. 2017), these specific effects of tyramine in *in vitro* human epithelial cell models may be attributed to a specific receptor-mediated mechanism by TAAR1 activation. Our ongoing work is currently seeking to delineate this effect.

Both mouse studies and human patients present with elevated tyramine levels compared to healthy controls in gastrointestinal diseases with comorbid neuropsychiatric disorders including: celiac disease (De Angelis et al. 2016; Di Cagno et al. 2011), colorectal cancer (Goedert et al. 2014; Sinha et al. 2016) and inflammatory bowel disease (Santoru et al. 2017; Nagao-Kitamoto et al. 2016). Metabolomic studies have also identified a role of β-phenylethylamine in the fecal metabolome, and altered phenylalanine metabolism in inflammatory bowel disease (Kolho et al. 2017; Paley 2019; Santoru et al. 2017; Yuan et al. 2018). In a human cohort, the fat composition of the diet can mediate the levels of β- phenylethylamine in the fecal metabolome (Kisuse et al. 2018). No receptor-mediated mechanism has been confirmed for either tyramine or β-phenylethylamine to act on the polarized epithelia of the gut, though a recent review by Christian et al suggests that TAAR1 may mediate some effects in inflammatory bowel diseases (Christian and Berry 2018).

TAs from dietary or microbial synthetic pathways have many potential fates in the gut, some of which may be context-specific. In the brain, TAs are rapidly degraded by tissue monoamine oxidases of neurons and supportive cells, although production and circulation in the brain may provide a more limited source of amines than

the plentiful sources in the gut. On a cellular level, TAs can be absorbed by simple diffusion (Berry et al. 2013; Blakeley and Nicol 1978; Tchercansky et al. 1994), facilitated diffusion (Blakeley and Nicol 1978), or by specific monoamine transporters (Xie and Miller 2008). *In vitro* studies investigating the small intestine epithelial cell line report β-phenylethylamine absorption to be pH-dependent, and showing minimal degradation of luminal β-phenylethylamine by intestinal bacteria (Fischer et al. 2010). Similarly, Tchercansky et al. (1994) showed tyramine is absorbed by rat small intestine epithelium by simple diffusion (Tchercansky et al. 1994), and tyramine plasma levels are reported to reach levels of 0.2μM after ingestion of 200mg of tyramine in healthy individuals (Vandenberg et al. 2003). Reports of absorption of TAs *in vitro* and in ex vivo systems suggest that TAs in the gut may escape the degradative effects of monoamine oxidase enzymes, even in a healthy epithelium.

TA signaling has historically been studied in a wide spectrum of neuropsychiatric disorders, including attention deficit hyperactive disorder (ADHD), major depressive disorder, and schizophrenia. TAAR1 is strongly implicated in schizophrenia diagnoses and progression. Several studies have found that patients with schizophrenia have increased levels of tyramine or β-phenylethylamine in the urine (Potkin et al. 1979) and plasma (O'reilly et al. 1991; Shirkande et al. 1995), as well as an increase in comorbid IBD or IBS diagnoses (Gupta et al. 1997; Hemmings 2004). Perturbations to the microbiome are reported in both inflammatory bowel disease (e.g. Santoru et al. 2017) and schizophrenia (Severance, Emily G. et al. 2017; Severance, E. G. et al. 2015), a phenomenon that is reversed with the successful administration of antipsychotics.

Attention deficit hyperactive disorder (ADHD) is commonly associated with a dysregulation of the trace amine β-phenylethylamine. Extensive studies on the comorbidities of ADHD and gastrointestinal diagnoses are lacking, though current studies are suggestive that disruption to the gut-brain axis may play a role in ADHD. Children diagnosed with ADHD exhibit changes to their microbiome compared to healthy controls, and administration of certain strains of bacteria within the first six months of life has been shown to have protective effects against ADHD (Felice and O'Mahony 2017). Additionally, preliminary studies indicate an increased level of pro-inflammatory cytokines and decreased levels of both tyramine and β-phenylethylamine in patients with ADHD (Baker et al. 1991; Sandgren and Brummer 2018), indicating a potential connection between the psychological condition and TA levels in the body.

Major depressive symptoms are also correlated with decreased urinary levels of β- phenylethylamine, (Wolf and Mosnaim 1983) and therapeutics seeking to increase β-phenylethylamine levels naturally with exercise (Szabo et al. 2001) or

replacement therapy with β-phenylethylamine (Sabelli and Javaid 1995) both appear to provide relief of major depressive disorder symptoms. Conversely, elevated urine β-phenylethylamine levels are correlated with manic disorders including bipolar affective disorder (Karoum et al. 1982; O'reilly et al. 1991). Interestingly, there is a correlation of either a diagnosis of irritable bowel syndrome or inflammatory bowel disease within one year of diagnoses of depression (Kurina et al. 2001).

Discussion

The recognition of TAAR1 as a mediator for TAs to act as chemical modulators of the brain-gut- microbiome axis opens up a new avenue for investigation on psychiatric and gastrointestinal disorder comorbidity as well as new treatment avenues for these common disorders. The prevailing hypotheses in neuropsychiatric and gastrointestinal disorders suggest an interplay of genetic and environmental factors in the onset and propagation of disease. TAs and their primary receptor, TAAR1, have been widely studied for their involvement in the pathogenesis of neuropsychiatric disorders, which have high comorbidity with gastrointestinal disorders. With the emergence of greater understanding of the brain- gut-microbiome axis, it is now clear that both brain and gut share common communication molecules which can originate endogenously in the host or resident microbiome, or exogenously from ingested food. Here, we take the opportunity to review what is known about TAs in the brain, the defined sources of TAs in the gut, and our emerging understanding the levels of TAs in various gastrointestinal disorders. We summarize evidence that TAs are ingested as well as produced by the microbiome, and that their receptor, TAAR1, is present in the gastrointestinal tract. Accordingly, novel TAAR1-targetted therapeutic compounds being advanced in clinical trials as new treatments for neuropsychiatric disorders could potentially have a therapeutic benefit through manipulation of the brain-gut- microbiome axis to modulate symptoms of neuropsychiatric disease. The localization of TAAR1 expression in the gut implicates a mechanism by which TAs, as well as other endogenous or exogenous TAAR1 ligands, are implicated in inflammatory bowel diseases and the reported comorbidities of neuropsychiatric disorders and gastrointestinal disorders.

Although we focused on reviewing tyramine and β-phenylethylamine, it is important to acknowledge that there are additional TAs, e.g. tryptamine, which are known in the human metabolome (Jeffery et al. 2012), which have similar effects to tyramine and β-phenylethylamine on gut motility and neurons (Williams et al. 2014), promoting adherence of bacteria to epithelial cells (Luqman et al. 2018), with identified accumulation in both colon cancer and irritable bowel syndrome (Ahmed et al. 2016;

Bearcroft et al. 1998; Hong et al. 2011; Ponnusamy et al. 2011). It is also important to recognize that the levels of TAs and the expression patterns of TAAR1 are highly dynamic and can be affected by diet, drugs, disease and psychological state. Likewise, the variable levels of TAs may augment the secretion of neuromodulators into circulation, thereby modulating the levels of neurotransmitters in the brain (Yano et al 2015). Both direct actions of TAs on TAAR1 in the cells of the intestine and brain, as well as the secondary effects on neurotransmitters (e.g. serotonin, norepinephrine) remains to be further explored.

Localization in the intestine and luminal apical localization (**Fig 2**) of epithelial cells in the gut and other polarized epithelia (thyroid) (Szumska et al. 2015) demonstrate a potential yet unexplored significance of TAAR1 in the gut as commensal microbe microbiome niches on luminal apical membranes of the intestine. Additional studies are needed to understand if the effects of tyramine, β-phenylethylamine, and other TAs that are seen in *in vitro* epithelial cell lines are mediated by TAAR1. Similarly, TAAR1 may have an unappreciated role in the regulation of homeostasis in the gut, as TAAR1 may serve as a microbial sensor in the GIT mediating differentiation of the lumen or polarization of epithelial cells. To understand the role of the microbiota in the regulation of TAAR1 expression and activation, it would be prudent to study TAAR1 expression in germ-free mice or specific-pathogen-free mice, as some datasets in NCBI GeoData suggest a low level of TAAR1 expression in germ-free and SPF mice, though the conflicting detection of TAAR1 in RNA-seq data may be confounding these effects.

There is an underappreciated function and role of the so-called 'elusive trace amines' and their role in normal human physiology. The emergence of fecal metabolomic studies has classified TA levels at micromolar concentrations in the body for the first time, suggesting TAs may be physiologically active in the gut (Jacobs et al. 2016; Santoru et al. 2017; Smith and Macfarlane 1997; Vandenberg et al. 2003; Kisuse et al. 2018). With the identification of TAAR1 expression in myriad cells in the intestine, there presents a great opportunity to further study complex mechanisms of the brain-gut-microbiome axis as it relates to intestine development, immune cell maturation as it relates to the 'hygiene hypothesis' for allergies and immunological disorders. Further, TAAR1 may serve as a novel therapeutic drug target to be further investigated for the treatment of comorbid gut and neuropsychiatric disorders.

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Chapter 5

General discussion, future perspectives, and concluding remarks

General discussion

Our knowledge of the gut microbiota and its metabolic capacity has been extensively enhanced over the past years[1–3]. The intimate interplay between the intestinal mucosa and its epithelial enteroendocrine cells (EECs) is crucial in the secretion of bioactive compounds and hormones [4,5], which are involved in host nutrition, appetite, immunity, blood secretion, and gut motility[6,7] including members of the chromogranin family [8,9]. Chromogranin-A (CgA) is one of the major proteins stored and secreted by the secretory granules of EECs in the gut[10]. CgA is proteolytically processed to generate several biologically active peptides, including catestatin (CST), whose effects are essential for the regulation of intestinal homeostasis by modulating innate immunity in the gut epithelium as an antimicrobial peptide[11–14] as well as maintenance of gut barrier function[15]. Experiments in mice lacking CST (CST-KO) indicated that these mice have abnormal colonic physiology and function[15]. Moreover, the administration of human CST to mice induced changes in fecal microbiota composition[16]. The exact molecular mechanisms in which CST modulates the gut microbiota have remained elusive. The work in this thesis strived to narrow the gap in our mechanistic understanding of the interrelation between CST and the gut microbiota. This thesis was based on experiments performed in CST-KO mice, where these mice were treated either with CST or with a standard mouse microbiota (Fecal Microbiota Transfer, FMT) and were compared with their healthy counterparts. The major findings reported in the experimental chapter of this thesis, and their future perspectives will be discussed in the following chapter.

Catestatin antimicrobial activity shapes the colonic microbiota composition

Antimicrobial peptides (AMPs) play important roles in protecting against infection while maintaining intestinal homeostasis by supporting mutualistic interactions with the gut microbiota, they possess an extensive antimicrobial activity with broad mechanisms of action and also regulate the gut microbiota composition[17,18]. In turn, the gut microbiota produces metabolites that directly regulate AMP production to orchestrate immune responses[19]. **Chapter 2** unraveled the crucial role of CST antimicrobial peptide on the microbiota composition. The colonic microbiota profiling indicated that the absence of CST is associated with a lower abundance of short-chain fatty acid (SCFA-producing bacteria such as *Roseburia* and *Intestinimonas* and, in turn, lower levels of SCFAs, in particular, butyrate and acetate (**Chapters 2, 3**). However, the supplementation of CST in a CST-depleted environment did not promote exclusively health beneficial microbiota, but rather led to a more equilibrated composition, including also pathobiont taxa (**Chapter 2**). Although changes in the microbiota composition have been observed in healthy wild-type

(WT) mice treated with CST (**Chapter 2**), the changes were much less prominent and were dominated by the colonization of gut bacteria with a capacity to resist the antimicrobial effect of CST that became more abundant upon CST treatment. A significant increase in the abundance of taxa that harbor the CST peptide-resistance enzyme, phosphoethanolamine transferase (*eptA*) was observed in CST-KO and WT mice treated with CST. The latter finding implies that CST may regulate the microbiota composition via its antimicrobial properties (**Chapter 2**) and that during its depletion, the microbiota was not primed with CST, thus do not evolve mechanisms to tolerate its antimicrobial effect.

Moreover, the work performed in **chapter 2** uncovered members of the gut microbiota to harbor proteases with the capacity to degrade CST peptide. For example, we showed that the gut bacteria, *E. coli*, could degrade CST via the action of its outer membrane protease *omptin*, which is known to cleave other cationic AMPs, such as protamine or LL-37[20,21]. Although the resulting peptides were not further tested, we speculate that *omptin* may cleave at arginine–glycine residues in CST, yielding cateslytin and a hexapeptide. As cateslytin has been reported to have antimicrobial activity[13], this cleavage might not completely ameliorate the antimicrobial effect of CST and or cateslytin, the antimicrobial specificity while testing cateslytin might be different from the outcomes present in this thesis with the use of complete CST. Furthermore, other different peptides can emerge due to the proteolytic process of CST in the intestinal lumen from different members of the gut microbiota in specific homeostatic or pathogenic conditions. Findings from this thesis on the effect of CST on shaping the microbiota composition through its antimicrobial properties are of significant importance to diseases associated with altered levels of intestinal AMPs, such as inflammatory bowel disease (IBD). Indeed, altered levels of endogenous AMPs have been associated with IBD-like animal models, and the administration of these peptides has ameliorated intestinal inflammation in murine models of IBD[22–24].

Gut microbiota plays a key role in developing the disrupted colonic homeostasis associated with altered levels of CST

The establishment of microbiota-accommodating homeostasis following the transfer of fecal material from healthy WT mice to CST-KO mice was marked by the restoration of the distorted colonic epithelial function. Transfer of WT-microbiota restored the dysfunctional epithelial junctions in mice with CST depletion as well as intestinal fibrosis (**Chapter 3**, **figure 2**). Indeed, an increase in the expression of (innate) immune cell gene-markers was observed in CST-KO mice and WT mice after receiving the fecal material of the CST-KO mice. CST-KO mice were reported to have intestinal complications involving infiltration of macrophages and CD4+ T-cells, colonic

hyperpermeability due to impaired function of tight junctions, increased fibrosis, and higher gene and protein expression levels of pro-inflammatory molecules in the gut, all of these are indicative for IBD-like states[15]. In parallel, the initial low diversity microbial populations in CST-KO mice shifted to high diversity, WT-like community (**Chapter 3**, **Figure 1**). The newly established microbiota comprised members that could produce SCFAs that can, in turn, be efficiently used by the host intestinal cells. This scenario was inferred from the high concentrations of the SCFAs butyrate, and acetate in the large intestinal lumen of CST-KO mice after receiving the WT-microbiota.

Remarkably, several *Muc* genes and genes involved in mucin glycosylation were significantly differentially expressed in CST-KO mice after receiving the WT-microbiota transplant (**Figure 1**), consistently with the study from Muntjewerff, *et.al* [25], which showed significantly increased expression levels of *Muc2*, as well as an increased number of goblet cells in CST-KO mice, and upon their treatment with CST, the expression of *Muc2* was downregulated to WT levels. The fact that the expression of *Muc2* was reversed in CST-KO mice upon transfer of fecal material from WT mice suggests that the effect of depletion of CST on overexpression of *Muc2* in CST-KO mice[25] might be caused by the altered microbiota, in particular, the mucin-degrading bacteria, in this mouse model. Indeed, the abundance of *Akkermansia muciniphila* was lower in CST-KO mice compared to WT mice (**Chapter 2**), this gram-negative anaerobic bacterium uses the mucus layer as an energy source by breaking down mucins with mucus-degrading enzymes, and in turn, synthesize SCFAs such as butyrate and propionate, or produce monosaccharides which can be used by other mucus-residing bacteria such as *Lachnospiraceae*[26].

Altered mucosal homeostasis is governed by a gene regulatory network

The reconstructed gene regulatory networks in **chapter 3** appeared to be involved in the regulation of the immune and metabolic adaptation of the intestinal mucosa upon adoptive microbiota transfer between CST-KO and WT mice in the colon. The central regulatory network identified in this study in the colon may therefore serve as a genetic signature for control of colonic homeostasis in healthy mice and could be used as a hypothesis-generating model for further studies of certain diseases with a clear genetic basis that includes the network genes. Such studies should then investigate the modulations and dysregulation of these networks in patients. Moreover, the intriguing link between the genes in the regulatory network, microbiota, and the human orthologue genes with an inferred role on IBD, identified for the colon in **chapter 3** suggests that re-establishment of the imbalanced microbiota associated with altered levels of CST affects the regulation of immune-

and metabolic-associated pathways in patients with altered levels of CST. It is thus likely that the microbiota-induced changes in the colonic immune profile may underlie the health-promoting effects associated with CST treatment.

Future perspectives

Combining fecal material transfer with the use of a mouse model with deletion of CST-peptide has brought new insight into the role of CST in shaping the gut microbiota, which in turn, affects the gut homeostasis and thereby complements other studies that studied the role of CST in gastrointestinal inflammation and other disorders. These studies did not take the gut microbiota into account or only analyzed the microbiota profile to evaluate the host responses to altered levels of CST.

The role of CST in shaping the gut microbial community most likely via its antimicrobial effect makes it attractive to precisely determine the levels of CST in the colonic lumen versus the mucosal layer. Data on the physiological concentration of CST in the gut epithelium is sparse, except a study reporting CST concentration in mice colonic tissue is on average 0.37 nmol/mg of protein[15], while in circulating plasma was 0.86 nmol/L[27]. These data indicate that the CST concentrations are significantly higher in the colon compared to the plasma. Additionally, mice treated with CST for 15 consecutive days, intraperitoneally, showed an increase in plasma CST from 0.86 to 1.29 nmol/L in WT mice, while CST in CST-KO mice levels changed from 0 to 0.65 nmol/L[27]. The use of oral administration instead of intraperitoneal injection, which was employed in this thesis, would help a more accurate delivery of the peptide to the intestine. Published and unpublished data from the Mahata lab show that the pharmacokinetic features of CST in WT mice after oral administration of ~17.19 nmol/L CST; bioavailability: 15 min; C_{max} (maximal plasma concentration): ~11.24 nmol/L; T_{max} (time to reach maximal plasma concentration): 1 h; $T_{1/2}$: ~6.7 h; plasma concentration after 24 h of oral administration: ~4.36 nmol/L as compared to ~1.17 nmol/L after 24 h of intraperitoneal administration; degradation of CST after 24 h of oral administration: ~74.6%, suggesting the peptide to be very stable[28]. The degradation of CST by the gut microbiota identified in this thesis requires additional studies to identify the degradation products, and their antimicrobial activity as well as their effect on the host. In particular, whether one of the degradation products is cateslytin, the potential active antimicrobial segment of CST bCGA($_{344-358}$)[13,29], it would be necessary to perform a comparative study using cateslytin for the treatment of CST-KO and WT mice as its antimicrobial specificity might be different than CST

whole peptide. CST is present along the gastrointestinal tract[12,30]. Data from this thesis focused only on the colon and did not investigate the interplay between small intestinal microbiota and CST.

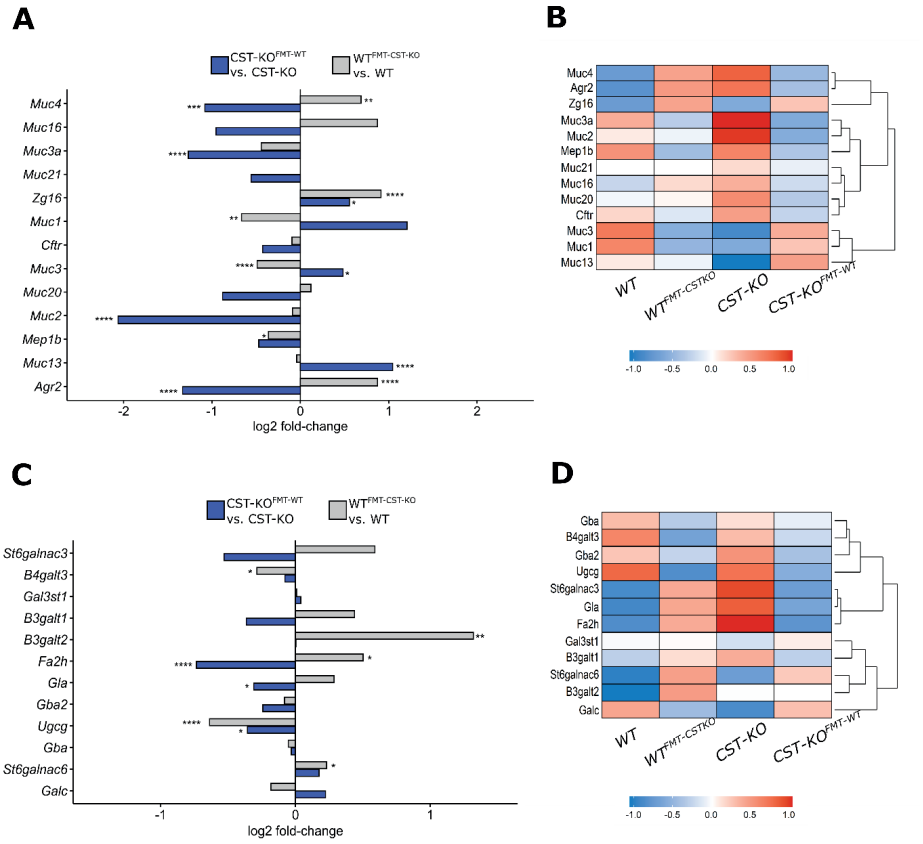

Figure 1. Adoptive transfer of intestinal mucus layer genotype in CST-KO and WT mice after fecal transplant. (A, C) Bar plot of genes involved in mucus layer regulation, mucin glycosylation, and composition showing log2 fold-changes for either CST-KO vs CST-KO^{FMT-WT} or WT vs WT$^{FMT-CST-KO}$. Log2 fold-changes and significance ($p_{adjusted} < 0.05$) of that change were determined by general differential expression analysis using *DESeq2* (see **chapter 3**, **Supplementary Excel Sheet 2**). **(B, D)** Heatmap of genes involved in mucin synthesis, glycosylation, and function. Values are expressed as mean z-scores, normalized by genotype and gene, to show the strength and direction of the expression change.

Role of Catestatin in the regulation of gut motility?

Gut motility is a key element in the regulation of intestinal physiology, but also of the microbiota composition as well as the cross-talk between the gut and brain[31,32]. Gut motility is tightly controlled by a multitude of regulatory components such as enteric neurons, interstitial cells of Cajal, smooth muscle cells, hormones, bacterial metabolites, and neurotransmitters[31,33,34], including acetylcholine (ACh), whose excitatory effect is depolarizing intestinal smooth muscle through the muscarinic ACh receptors[35,36]. CST has been shown to activate the type-2 muscarinic (M2) acetylcholine receptor[37]. The transmission electron microscopic analysis performed in this thesis highlighted some intriguing changes in the ultrastructure of the submucosal plexus, where cholinergic vesicles and peptidergic vesicles are stored and released. CST-KO mice had a significantly lower number of cholinergic vesicles per μm^2 of colonic tissue compared to the WT group, which was significantly increased upon CST treatment. In contrast, the number of inhibitory peptidergic vesicles per μm^2 of colonic tissue was higher in CST-KO compared to the WT group, and this number was significantly reduced upon CST injection solely in the CST-KO group. Intriguingly, CST-KO, which demonstrated a remarkable tendency of a delayed gut transit, as measured by total gut transit time (TGTT) with carmine red, compared to WT had significantly decreased TGTT as well as higher defecation frequency compared to the baseline measurements after two weeks of CST treatment (**Figure 2, C**). Notably, these changes were not observed in WT mice treated with CST. The findings coincide with the increase in the levels of butyrate and acetate in CST-KO but not in WT mice after treatment with CST (**Chapter 2**, **Figure 1**). Butyrate has been shown to increase intestinal motility and augment the contractions in colonic segments in rats[38,39]. Moreover, butyrate has been also shown to modulate the activity of the enteric nervous system by increasing the proportion of cholinergic neurons[39]. Together with the modulatory role of CST on the catecholamine secretion through cholinergic receptors[37,40,41], the data show that CST treatment may stimulate gut motility by enhancing the ascending contraction via increasing the number of cholinergic vesicles and reducing the descending relaxation by lowering the number of peptidergic vesicles in CST-KO mice.

Besides the regulatory role in gut motility, ACh and the vasoactive intestinal peptide (VIP) have been also shown to regulate the production of mucus[42–45]. Goblet cells are responsive to ACh[46] while VIP enhances mucus secretion promoting *Muc-2* expression levels[46], which was significantly downregulated in CST-KO mice that received WT-microbiota transplant (**Figure 2 A, B**).

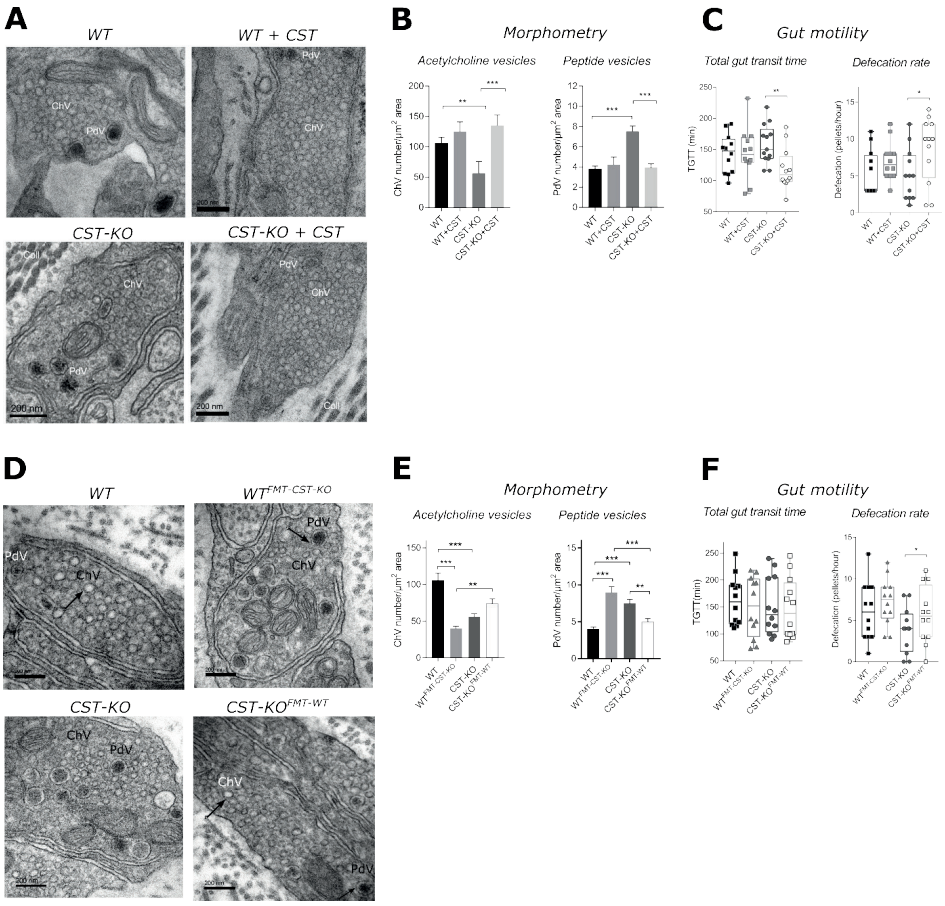

Figure 2. Catestatin treatment reduces total gut transit time.

TGTT was performed administering 6 % (w/v) carmine red by oral gavage to mice that were housed individually in metabolic cages during this experimentation. TGTT baseline measurements were carried out in WT and CST-KO mice before CST treatment or FMT (n=12 per group). Subsequently, these mice were treated with either CST (i.p. 2 µg/g body weight for 15 days) or FMT (methods described in **chapter 3)** and TGTT was again measured in all mice. **(A, C)** Effects of CST or FMT on TGTT and defecation frequency in CST-KO and WT mice. Significance was tested with an unpaired *Mann-Whitney* test. Boxes represent the median with interquartile range, and whiskers represent the maxima and minima. (*$p < 0.05$). **(B, D)** TEM micrographs show the submucosal plexus where cholinergic (light vesicles) and peptidergic (dark) vesicles can be seen. The images are representatives of each group. ChV - acetylcholine vesicles, PdV – peptidergic vesicles. Morphometric analysis using TEM showing density which corresponds to the number of cholinergic and peptidergic vesicles/area from mice colon (n=3). Significance was tested using (1-way ANOVA); Bars show mean ± SEM. TGTT: total gut transit time. i.p. intraperitoneal injection. TEM: transmission electron microscopy.

Comparable to CST treatment, the transfer of fecal material from WT to CST-KO mice resulted in a significantly lower number of peptidergic vesicles in colonic tissue, but not for acetylcholine vesicles which FMT effect was inconsistent among groups (**Figure 2 D, E**). Butyrate was also significantly increased in the FMT-recipient CST-KO mice (Chapter 3, **Figure 1**). While defecation frequency was increased in these mice, no significant changes in the TGTT were observed, in contrast to CST treatment (**Figure 2, F**). Plausibly, increasing the number of mice in this experiment may render significant changes owing to the high inter-individual variation.

Interrelation between the hyperadrenergic state in CST-KO mice and gut microbiota?

CST-KO mice are known to be hypertensive[27]; consistently, CST treatment in hypertensive rodents exerted anti-hypertensive effects, reduced blood pressure and catecholamine levels. These results are further supported by the TEM analysis of the colonic EECs, where the dense core vesicles (DCVs), are responsible for the storage and release of neurotransmitters like catecholamines[47]. In line with previous reports[15], our TEM analysis showed that the numbers of DCVs were significantly reduced in CST-KO compared to WT counterparts and CST-KO-associated microbiota could transfer this phenotype to the WT mice, which showed a significant reduction in their DCVs area upon receiving the CST-KO associated microbiota (**Figure 3 A, B**). In contrast, the transfer of WT-associated microbiota to CST-KO mice restored the reduced levels of DCVs area in the latter. The results infer a role of the gut microbiota associated with CST depletion in the elevated levels of these catecholamines in CST-KO mice and their potential role in the development of a state of hypertension. How the microbiota and CST play these interactions needs further attention.

Relevance of the identified gene-regulatory networks as gene signatures for human

This study identified a gene regulatory network that appeared to be involved in the regulation of the immune and metabolic adaptation of the intestinal mucosa upon adoptive microbiota transfer between CST-KO and WT mice in the colon. However, the assessment of the applicability of the identified gene regulatory networks used as molecular signatures for the evaluation of disease risk in humans is yet a considerable challenge[48]. This approach can be performed by integrative analysis of multi-omics in mouse models and humans, by comparing the core regulatory networks that will yield novel models of biological interest[49]. There, essential core or non-essential peripheral genes can be identified and useful information about diseases or disease development can be extracted, and ultimately, new disease models will emerge from this approach. Even though the existence of completely reliable human regulatory networks and their role in disease is debated[50], the identification of shared regulators

in the mouse model used in this study and of their human orthologues in IBD would support the validity of the animal models for human studies. The success of this endeavor will also depend on the development and selection of the most appropriate computational methods[51]. This, in turn, may inspire molecular hypotheses for human gastrointestinal pathologies and may complement clinical trials by identifying novel gene targets and exploring their functional interdependency with other genes, as diseases are normally a consequence of abnormalities in many genes.

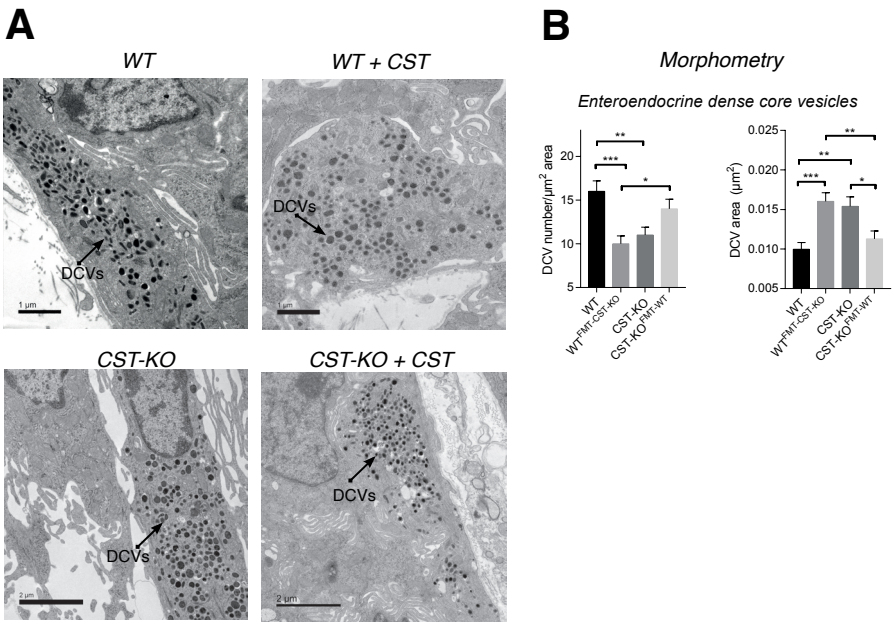

Figure 3. Fecal microbiota transplantation affects enteroendocrine dense core vesicles. **(A)** TEM micrographs show the DCVs from enteroendocrine cells. The images are representatives of mice in each group (n=3). **(B)** Morphometric analysis using TEM showing measurements DCV density, and vesicle area in mouse colon. Significance was tested using (1-way ANOVA); Bars show mean ± SEM. DCVs: dense-core vesicles. TEM: transmission electron microscopy.

Concluding remarks

The altered levels of the antimicrobial CST affect the gut microbiota composition and their metabolism disrupting the mutualistic and symbiotic interactions between bacteria and the host epithelial cells (enteroendocrine cells, goblet cells, and nerve vesicles) affecting intestinal balance and causing disease. This study provides a comprehensive overview of the murine metabolic- and immune-related cellular pathways and processes that are co-mediated by the fecal microbiota transplantation and supports a prominent role for the gut microbiota in the colonic distortion associated with the lack of CST in mice.

The combination of the mouse model with the deletion in CST together with the fecal material transfer applied in this thesis provides detailed resources on the processes involved in the microbiota-governed CST effects in the host. This approach appears to have been very successful in increasing our understanding of the CST-microbiota interplay, thus it complements previous studies that have targeted the intestinal CST in the same or similar mouse models. The correlations identified in this thesis between the multi-variate datasets, including the linkages identified between microbial groups and specific host functions, support the prominent role of the microbiota in the modulation of CST role in host physiology. The interplay that was most extensively explored in this thesis focused on the intestinal mucosa, but some impact of the microbiota on other aspects of host physiology may also be of great relevance to explore for host health and well-being.

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Appendices

Nederlandse samenvatting

De mysterieuze interacties tussen de darmmicrobiota en menselijke catestatine

Het menselijke maagdarmkanaal (GI) is een complex en dynamisch netwerk, waar interacties tussen de gastheer en een enorme diversiteit aan micro-organismen naast elkaar bestaan in een mutualistische relatie. Het maagdarmkanaal herbergt ongeveer 100 biljoen microbiële cellen bestaande uit bacteriën, schimmels, virussen, archaea en protozoa die gezamenlijk de darmmicrobiota worden genoemd. De bacteriële gemeenschappen in onze darm hebben een groot repertoire van genen met een krachtige metabolische capaciteit door de productie van microbiële metabolieten die bijdragen aan vitale functies van de menselijke fysiologie en metabolisme, inclusief de regulatie van de immuunrespons in alle stadia van ons leven, optimale functie van de darmbarrière die microben op afstand houdt om bacteriële translocatie naar de darm, darmmotiliteit, opname van voedingsstoffen door de fermentatieprocessen te voorkomen en ook bij te dragen aan neuropsychologische reacties. Eventuele verstoringen in de nauwe interacties tussen de darmmicrobiota en de menselijke gastheer zijn sterk in verband gebracht met de ontwikkeling en progressie van pathologieën, waaronder chronische ontstekingsaandoeningen en metabole stoornissen zoals inflammatoire darmaandoeningen (IBD), type 2 diabetes, obesitas, reumatoïde artritis, multiple sclerose; psychologische stoornissen, waaronder depressie en angst, en zelfs neurodegeneratieve ziekten zoals de ziekte van Parkinson en autisme. Groeiend bewijs heeft aangetoond dat microbiële producten verschillende routes kunnen beïnvloeden die de hersenen beïnvloeden. Deze bidirectionele communicatie tussen de hersenen en de darmflora wordt de microbiota-darm-brein (MGB) as genoemd.

Binnen de context van interacties tussen gastheer en microben zijn er actieve moleculen die de belangrijkste functies van de MGB-as moduleren, ook wel 'darmpeptiden' genoemd, die worden uitgescheiden door de epitheliale entero-endocriene cellen (EEC's) uit de darmcelbarrière. Bepaalde darmpeptiden hebben antimicrobiële eigenschappen die de bacteriële samenstelling in onze darmen reguleren. Een van die uitgescheiden bioactieve peptiden is catestatine, waarvan de voorloper het pro-hormoon chromogranine-A is. Studies uitgevoerd op de darm geven aan dat catestatine bijdraagt aan de optimale functie van de darm, omdat het een ontstekingsremmend peptide is en de juiste functie ondersteunt van de 'tight junctions', dit zijn eiwitstructuren gelokaliseerd tussen de darmcellen die zorgen voor een integrale vorm en functie voor de darmbarrière. Bovendien helpt catestatine het functionele evenwicht van het maagdarmkanaal door zijn antimicrobiële effect

uit te oefenen. *In-vitro*- onderzoeken hebben aangetoond dat catestatine een antimicrobieel peptide is voor bepaalde soorten bacteriën, dat het membraan van de micro-organismen permeabiliseert en bacteriedood induceert. Bovendien is catestatine gevonden in het lumen van de darm waar de darmmicrobiota leeft, wat wijst op mogelijke interacties daartussen. Toch was dit samenspel nog onduidelijk. Om die reden was het onderzoek in dit proefschrift gericht op het meer in detail uitleggen van het samenspel tussen catestatine en de darmmicrobiota en de impact ervan op de darmbalans. Om dit te bereiken, werden muizenexperimenten uitgevoerd en toonden aan dat de antimicrobiële werking van catestatine een belangrijke rol speelde bij het vormgeven van de microbiota-samenstelling en de productie van vetzuren met een korte keten **(Hoofdstuk 2)** . We hebben waargenomen dat CST-KO-muizen een onevenwichtige microbiële gemeenschap hadden en lagere niveaus van essentiële bacteriële metabolieten zoals butyraat en acetaat, die van vitaal belang zijn voor de menselijke gezondheid en die de optimale functie van de darmepitheelbarrière ondersteunen. Behandeling met catestatine verhoogde significant de niveaus van butyraat en acetaat die gunstig zijn voor de gastheer. Bovendien waren de antimicrobiële effecten van catestatine gunstig voor de kolonisatie van bepaalde bacteriestammen, in het bijzonder die stammen die antimicrobiële resistentiemechanismen hebben ontwikkeld om het dodende effect van catestatine tegen te gaan. Een van deze resistentiemechanismen is gebaseerd op bacteriële membranen door middel van fosfo-ethanolaminetransferasen (*EptA*) die tot expressie worden gebracht in verschillende bacteriesoorten in de menselijke darm. Dit impliceert dat microbiële resistentie een mutualistische en belangrijke component is bij de selectie van specifieke microbiota-samenstelling door de gastheer, via de modulatie van antimicrobiële peptiden, zoals catestatine.

Een ander van deze bacteriële resistentiemechanismen tegen catestatine vond plaats via *ompT*in, een buitenste enzym dat catestatine afbreekt. We toonden aan dat de genexpressie van *ompT*in-protease significant hoger is bij de ziekte van Crohn (een subtype van inflammatoire darmziekte), waarbij de niveaus van catestatine verhoogd zijn in vergelijking met gezonde mensen. Over het algemeen benadrukt deze studie de belangrijke rol van catestatine-peptide bij het bepalen van de samenstelling van de microbiota die essentieel is voor een gezonde darmfunctie.

CST-KO-muizen lijden aan darmproblemen die verband houden met de darmbarrière, waaronder het slecht functioneren van de tight junctions, een veranderde immuunrespons met ontstekingen en een onevenwichtige samenstelling van de darmmicrobiota als gevolg van het gebrek aan catestatine (zoals we eerder beschreven in hoofdstuk 2). Om de effecten en routes te onderzoeken waarin de darmmicrobiota betrokken is bij de darm- en gezondheidsproblemen van de CST-

KO muizen, hebben we een wederzijdse fecale microbiële transplantatie (FMT) uitgevoerd **(Hoofdstuk 3)** . FMT is een procedure waarbij sommige uitwerpselen van een donor worden getransplanteerd naar een ontvanger, de uitwerpselen bevatten de darmmicrobiota die metabolische effecten in de gastheer genereert. We transplanteerden 1) de ontlasting van de normale gezonde muizen (WT) naar de muizen met darmaandoeningen (CST-KO) en 2) de ontlasting van de muizen met darmaandoeningen (CST-KO) naar gezonde normale muizen (WT). De microbiota overgebracht van CST-KO-muizen verstoorde de normale transcriptionele en fysiologische kenmerken van een muizencolon, waaronder schade aan de tight junctions, intestinale fibrose en verhoogde ontstekingsparameters. Daarentegen herstelde de transplantatie van normale muizen naar CST-KO-muizen hun disfunctionele dikke darm en verhoogde de niveaus van vetzuren met een korte keten, die de darmbarrière ten goede komen. Bovendien hebben we een kernregulerend netwerk gereconstrueerd waar we specifieke leden van de darmmicrobiota konden associëren met immuun- en metabole gastheerreacties. Over het algemeen biedt deze studie een uitgebreid overzicht van metabole en immuungerelateerde processen van muizen die worden gemedieerd door fecale microbiële transplantatie en ondersteunt het een prominente rol van de darmmicrobiota bij darmdisfuncties die verband houden met onevenwichtige catestatinespiegels, zoals bij patiënten met inflammatoire darmaandoeningen.

Slotopmerkingen

De experimenten uitgevoerd in dit proefschrift beschreven de effecten van catestatine als een antimicrobieel peptide in de darm, het reguleren van de samenstelling en het metabolisme van de microbiota, en benadrukten de processen waarmee de darmmicrobiota en catestatine darmmodulatie uitoefenen. Onze aanpak is succesvol geweest in het vergroten van ons begrip tussen catestatine en de onderlinge verbinding van de darmmicrobiota in de MGB-as, en vormt dus een aanvulling op eerdere onderzoeken die zich in dezelfde of vergelijkbare muismodellen op de intestinale catestatine hebben gericht. De correlaties die in dit proefschrift worden geïdentificeerd tussen de microbiota en specifieke gastheerfuncties, benadrukken de prominente rol van de microbiota in de modulatie van de gezondheid van de gastheer. Bevindingen uit onze onderzoeken kunnen het concept bevorderen dat het richten op de darmmicrobiota een levensvatbare therapeutische strategie zou kunnen zijn voor een nieuwe ontwikkeling van nieuwe ziektebehandelingen die verband houden met veranderde niveaus van catestatine, en daarom de preventiestrategieën bij deze ziekten kunnen versterken.

Layman's summary

The mysterious interactions between the gut microbiota and human catestatin

The human gastrointestinal (GI) tract is a complex and dynamic network, where interactions between the host and a vast diversity of microorganisms co-exist in a mutualistic relationship, benefiting each other. The GI tract harbors around 100 trillion microbial cells consisting of bacteria, fungi, viruses, archaea, and protozoa collectively referred to as the gut microbiota. The bacterial communities in our gut have a large repertoire of genes with powerful metabolic capacity through the production of microbial metabolites that contribute to vital functions of human physiology and metabolism including the regulation of the immune response from all stages of our life, optimal function of the intestinal barrier keeping microbes at bay to avoid bacterial translocation to the intestine, gut motility, absorption of nutrients by the fermentation processes, and also contributing to neuropsychological responses. Any disturbances in the close interactions between the gut microbiota and the human host have been strongly linked to the development and progression of pathologies including chronic inflammatory conditions and metabolic disorders such as inflammatory bowel diseases (IBD), type 2 diabetes, obesity, rheumatoid arthritis, multiple sclerosis; psychological disorders including depression, and anxiety, and even neurodegenerative diseases like Parkinson's disease and autism. Growing evidence has shown that microbial-derived products can influence several routes that affect the brain, this bidirectional communication between the brain and the gut microbiota is called the microbiota-gut-brain (MGB) axis.

Within the context of host-microbe interactions, there are bioactive molecules that modulate key functions of the MGB-axis, also known as 'gut peptides', which are secreted by the epithelial enteroendocrine cells (EECs) from the intestinal cell barrier. Certain gut peptides have antimicrobial properties regulating the bacterial composition in our intestines. One of those secreted bioactive peptides is catestatin, which precursor is the pro-hormone chromogranin-A. Studies performed on the gut indicate that catestatin contributes to the optimal function of the intestine, being an anti-inflammatory peptide, and supporting the correct function of the 'tight junctions', which are protein structures localized between the intestinal cells that provide integral shape and function to the intestinal barrier. Moreover, catestatin assists the functional balance of the GI tract by exerting its antimicrobial effect. *In-vitro* studies demonstrated catestatin is an antimicrobial peptide for certain types of bacteria, permeabilizing the membrane of the microorganisms and inducing bacterial death. Moreover, catestatin has been found in the lumen of the intestine

where the gut microbiota inhabits, indicating potential interactions among them. Nevertheless, this interplay was still unclear. For that reason, the research in this thesis aimed to explain more in detail the interplay between catestatin and the gut microbiota and its impact on intestinal balance. To achieve this, mice experiments were performed and showed that the antimicrobial action of catestatin played an important role in shaping the microbiota composition and its production of short chain fatty acids **(Chapter 2)**. We observed that CST-KO mice had imbalanced microbial communities, and lower levels of essential bacterial metabolites such as butyrate and acetate, which are vital for human health assisting the optimal function of the intestinal epithelial barrier. Treatment with catestatin increased significantly the levels of butyrate and acetate which are beneficial for the host. Moreover, the antimicrobial effects of catestatin benefited the colonization of certain strains of bacteria, in particular those that have developed antimicrobial resistance mechanisms to counteract the killing effect of catestatin. One of these resistance mechanisms relies on bacterial membranes through phosphoethanolamine transferases (EptA) expressed throughout several bacterial species in the human gut, this implies that microbial resistance is a mutualistic and important component in the selection of specific microbiota composition by the host, through the modulation of antimicrobial peptides, such as catestatin.

Another of these bacterial resistance mechanism against catestatin occurred via *omptin*, which is an outer enzyme that breaks down catestatin. We showed that *omptin* protease gene expression is significantly higher in Crohn's disease (a subtype of inflammatory bowel disease), in which the levels of catestatin are increased compared to healthy people. Overall, this study highlights the significant role of catestatin peptide in determining the microbiota composition which is essential for a healthy gut function.

CST-KO mice suffer from intestinal problems related to the intestinal barrier including tight junctions malfunction, an altered immune response involving inflammation, and an imbalanced gut microbiota composition due to the lack of catestatin (as we described before in **chapter 2**). In **chapter 3** we aimed to investigate the effects and routes in which the gut microbiota is involved in the intestinal and health problems of the CST-KO mice, we performed a reciprocal fecal microbial transplantation (FMT). FMT is a procedure where some feces of a donor are transplanted to a recipient, the feces contain the gut microbiota generating metabolic effects in the host. We transplanted 1) The feces of the normal healthy mice (WT) to the mice with intestinal conditions (CST-KO) and 2) the feces of the mice with intestinal conditions (CST-KO) to healthy normal mice (WT). The microbiota transferred from CST-KO mice disrupted the normal transcriptional and physiological characteristics

of a mouse colon including damage to the tight junctions, intestinal fibrosis, and increased inflammatory parameters. In contrast, the transplant from normal mice to CST-KO mice restored their dysfunctional colon by increasing the levels of short-chain fatty acids that benefit the intestinal barrier and improving the integrity of their mucus layer. Moreover, we reconstructed a core-regulatory network where we could associate specific members of the gut microbiota with immune and metabolic host responses. Overall this study provides a comprehensive overview of murine metabolic- and immune-related processes that are mediated by fecal microbial transplantation and supports a prominent role of the gut microbiota in gut dysfunctions associated with imbalanced catestatin levels such as in patients with inflammatory bowel disease.

Concluding remarks

The experiments performed in this thesis described the effects of catestatin as an antimicrobial peptide in the gut, regulating the microbiota composition and metabolism, and highlighted the processes by which the gut microbiota and catestatin exert intestinal modulation including the gut motility. Our approach has been successful in increasing our understanding between catestatin and the gut microbiota interconnection in the MGB-axis, thus it complements previous studies that have targeted the intestinal catestatin in the same or similar mouse models. The correlations identified in this thesis between the microbiota and specific host functions, emphasize the prominent role of the microbiota in the modulation of host health. Findings from our studies may advance the concept that targeting the gut microbiota could be a viable therapeutic strategy for a novel treatment development of diseases associated with altered levels of catestatin, therefore may augment prevention strategies in these diseases.

Resumen de la tesis

Las interacciones misteriosas entre la microbiota intestinal y la catestatina humana

El tracto gastrointestinal humano es una red compleja y dinámica, donde las interacciones entre el huésped (humano) y nuestra gran diversidad de microorganismos coexisten en una relación mutualista. El tracto gastrointestinal alberga alrededor de 100 billones de células microbianas que consisten en bacterias, hongos, virus, arqueas y protozoos, denominados colectivamente *microbiota intestinal*. Las comunidades bacterianas en nuestro intestino tienen un gran repertorio de genes con una poderosa capacidad metabólica a través de la producción de metabolitos microbianos que contribuyen a funciones vitales de la fisiología y el metabolismo humanos, incluida la regulación de la respuesta inmune en todas las etapas de nuestra vida, función óptima de la barrera intestinal que protege al humano de los microbios para evitar la inclusión bacteriana al intestino, la motilidad intestinal, la absorción de nutrientes por los procesos de fermentación bacteriana y también contribuye a las respuestas neuropsicológicas del huésped. Cualquier alteración en las estrechas interacciones entre la microbiota intestinal y el ser humano se ha relacionado fuertemente con el desarrollo y la progresión de patologías que incluyen afecciones inflamatorias crónicas y trastornos metabólicos como la enfermedad inflamatoria intestinal, diabetes tipo 2, obesidad, artritis reumatoide, esclerosis múltiple; también trastornos psicológicos que incluyen depresión y ansiedad, e incluso enfermedades neurodegenerativas como la enfermedad de Parkinson y el autismo. El incremento en la evidencia ha demostrado que los productos derivados de la microbiota intestinal pueden influir en varias rutas que afectan el cerebro, esta comunicación bidireccional entre el cerebro y la microbiota intestinal se denomina eje microbiota-intestino-cerebro (MIC).

En el contexto de las interacciones huésped-microbio, existen moléculas activas que modulan funciones clave del eje MIC, también conocidas como "péptidos intestinales", que son secretados por las células epiteliales enteroendocrinas (EEC) de la barrera celular intestinal. Ciertos péptidos intestinales tienen propiedades antimicrobianas que regulan la composición bacteriana en nuestros intestinos. Uno de esos péptidos secretados es la catestatina, cuyo precursor es la hormona cromogranina-A, la división de la cromogranina-A por enzimas da lugar a varios péptidos, entre ellos la catestatina. Estudios en el intestino indican que la catestatina es un péptido esencial para el funcionamiento óptimo del intestino, siendo un péptido antiinflamatorio que apoya la función correcta de las 'uniones estrechas', que son estructuras protéicas localizadas entre las células intestinales que proporcionan

forma y función integrales a la barrera intestinal. Además, la catestatina ayuda al equilibrio funcional del tracto gastrointestinal al ejercer su efecto antimicrobiano. Los estudios *in vitro* demostraron que la catestatina es un péptido antimicrobiano para ciertos tipos de bacterias, que permeabiliza la membrana de los microorganismos e induce la muerte bacteriana. Además, se ha encontrado catestatina en el lumen del intestino donde habita la microbiota intestinal, lo que indica posibles interacciones entre ellos. Sin embargo, esta interacción aún no se ha esclarecido. Por ello, la investigación de esta tesis pretende explicar con más detalle la interrelación entre la catestatina y la microbiota intestinal y su impacto en el equilibrio intestinal. Para lograr esto, se realizaron estudios comparativos entre ratones que carecían el gen de catestatina (ratones CST-KO) y ratones normales y sanos.

El capítulo 2 describe cómo los efectos antimicrobianos de la catestatina dan forma a la composición de la microbiota y su metabolismo, descubrimos que los ratones CST-KO tienen comunidades microbianas desequilibradas y niveles más bajos de metabolitos bacterianos como el butirato y el acetato, que son vitales para la salud humana y ayudan a la función óptima de la barrera epitelial intestinal. Sorprendentemente, el tratamiento con catestatina aumentó significativamente los niveles de butirato y acetato que son beneficiosos para el huésped. Además, los efectos antimicrobianos de la catestatina beneficiaron la colonización de ciertas cepas de bacterias, en particular aquellas que han desarrollado mecanismos de resistencia antimicrobiana para contrarrestar el efecto letal de la catestatina. Uno de estos mecanismos de resistencia se basa en las membranas bacterianas a través de las fosfoetanolamina transferasas (*EptA*) expresadas en varias especies bacterianas del intestino humano, lo que implica que la resistencia microbiana es un componente mutualista e importante para la selección de la composición específica de la microbiota por parte del huésped, a través de la modulación de péptidos antimicrobianos, como la catestatina.

Descubrimos un segundo mecanismo de resistencia bacteriana contra la catestatina a través de la *ompT*ina, que es una enzima externa que rompe la molécula de la catestatina. Finalmente, mostramos que la expresión del gen de la proteasa *ompT*in es significativamente mayor en la enfermedad de Crohn (un subtipo de enfermedad inflamatoria intestinal), en la que los niveles de catestatina están elevados en comparación con las personas sanas. En general, este estudio destaca el papel significativo del péptido de catestatina en la determinación de la composición de la microbiota, que es esencial para una función intestinal saludable.

Los ratones CST-KO sufren problemas intestinales relacionados con deficiencias en la barrera intestinal, incluido el mal funcionamiento de las uniones celulares estrechas, respuesta inmunitaria alterada que implica inflamación y una composición de la microbiota intestinal desequilibrada debido a la falta de catestatina (como describimos anteriormente en el capítulo 2). En el **capítulo 3** realizamos un transplante microbiano fecal para descubrir los efectos y rutas en las que la microbiota intestinal está involucrada en los problemas intestinales y de salud de los ratones CST-KO. El transplante fecal es un procedimiento en el que se trasfieren heces fecales de un donante a un receptor, las heces contienen la microbiota intestinal que genera efectos metabólicos en el huésped.

Trasplantamos 1) las heces de los ratones sanos normales (WT) a los ratones con condiciones intestinales (CST-KO) y 2) las heces de los ratones con condiciones intestinales (CST-KO) a ratones normales sanos (WT). La microbiota de los ratones CST-KO alteró las características normales transcripcionales y fisiológicas del colon de los ratones sanos, incluyendo daño a las uniones estrechas, incremento de fibrosis intestinal y aumento de los parámetros inflamatorios. Por el contrario, el trasplante de la microbiota de ratones normales a ratones CST-KO restauró su colon disfuncional, aumentando los niveles de ácidos grasos de cadena corta que benefician a la barrera intestinal y mejorando la integridad de la mucosa intestinal. Además, reconstruimos una red reguladora central en la que pudimos asociar miembros específicos de la microbiota intestinal con respuestas inmunitarias y metabólicas del huésped. En general, este estudio proporciona una descripción completa de los procesos relacionados con el sistema inmunológico y metabólico murino que están mediados por el trasplante microbiano fecal que respalda un papel destacado de la microbiota intestinal en las disfunciones intestinales asociadas con niveles de catestatina desequilibrados, como es el caso de los pacientes con enfermedad inflamatoria intestinal.

Conclusiones

Los experimentos realizados en esta tesis describieron los efectos de la catestatina como péptido antimicrobiano en el intestino, regulando la composición y el metabolismo de la microbiota, y destacando los procesos en los que la microbiota intestinal y la catestatina ejercen modulación intestinal incluyendo también la motilidad intestinal. Nuestro enfoque ha tenido éxito en incrementar nuestra comprensión entre la catestatina y su interconexión con la microbiota intestinal en el eje MIC, por lo que complementa estudios previos que se han centrado en la catestatina intestinal en dichos modelos de ratón. Además, las correlaciones identificadas en esta tesis entre la microbiota y las funciones específicas del huésped enfatizan el papel destacado de la microbiota en la modulación de la salud del

huésped. Los hallazgos de nuestros estudios pueden promover el concepto de que dirigirse a la microbiota intestinal podría ser una estrategia terapéutica viable para el desarrollo novedoso de tratamientos para enfermedades asociadas con niveles alterados de catestatina, por lo tanto, puede aumentar las estrategias para tratar y prevenir estas enfermedades en humanos.

About the author

Dulce Pamela González Dávila was born on December 29th, 1990 in Mexico City. Pamela obtained her Bachelor's degree in Biotechnology engineering in 2013 at Instituto Tecnológico de Estudios Superiores de México (ITESM-CEM). She worked for one year at the chemical industry in Diversey, Mexico but she wanted to continue a research career. Pamela applied for a national scholarship from CONACYT Mexico which supported her Master's and PhD studies at the University of Groningen. She graduated in 2017 with a Master in Science in Molecular Biology and Biotechnology being part of the Top Programme in Biomolecular Sciences. Her first master's internship was at the Host-Microbe Interactions part of Microbial Physiology Department where Pamela conducted a screening for gut bacterial proteases and metabolites targeting mammalian neuroactive compounds at Dr. Sahar El Aidy's lab in 2016. Her last master's research internship was at the Institute National de la Recherche Agronomique (INRAE), in Jouy en Josas, Paris where she conducted research employing animal models, epithelial cell lines, and commensal bacterium with Dr. Luis Bermúdez Humarán in 2017.

In March 2018, Pamela started her PhD at the Graduate School of Science and Engineering (GSSE), Groningen Biomolecular Sciences and Biotechnology Institute (GBB), again at Host-Microbe Interactions Department led by her promoter Prof. Sahar El Aidy in collaboration with her co-promoter Dr. Sushil Mahata from the University of California, San Diego (UCSD). She performed her research presented in this thesis about the interconnections between the gut microbiota and the antimicrobial peptide catestatin. During her PhD, Pamela also supervised master's and bachelor's projects and presented her research work at scientific conferences.

Moreover, Pamela participated for almost two years at the PhD council of the Faculty of Science and Engineering of the University of Groningen, where she could organize activities for the training and well-being of the PhD community. She also participated for one year as an active member of the GSSE Sounding Board providing input on the PhD policy and student management. Pamela participated in 2020 as PhD member of the external committee for the assessment of VLAG Graduate School at the University of Wageningen according to the Dutch Strategy Evaluation Protocol.

Currently, Pamela is the chair of the organizing committee of the first interdisciplinary Summer School on Psychedelic Research at the University Medical Center Groningen (UMCG), which will be held in July 2022 where the latest developments on psychedelic research will be presented by international experts. Pamela recognizes the great value of psychedelic substances not only for the treatment of chronic mental illnesses but also to explore different states of consciousness. She is interested to develop a holistic approach to psychedelic-assisted therapy in the Western world by integrating ancient wisdom, and traditional indigenous practices into our modern scientific medical system.

Acerca del Autor

Dulce Pamela González Dávila nació el 29 de diciembre de 1990 en la Ciudad de México. Pamela obtuvo su título de Ingeniero en Biotecnología en 2013 en el Instituto Tecnológico de Estudios Superiores de México (ITESM-CEM). Trabajó durante un año en la industria química en Diversey, México, ahí se dio cuenta que quería continuar una carrera de investigación. Pamela solicitó una beca nacional de CONACYT México que apoyó sus estudios de maestría y doctorado en la Universidad de Groningen, Países Bajos. Se graduó en 2017 con una Maestría en Ciencias en Biología Molecular y Biotecnología siendo parte del Programa Superior en Ciencias Biomoleculares. Su primera pasantía de investigación de maestría fue en el grupo de Interacciones Huésped-Microbio, donde Pamela realizó una detección de proteasas y metabolitos bacterianos intestinales enfocados a activar compuestos neuroactivos del huésped en el laboratorio de Dr. Sahar El Aidy en 2016. Su última pasantía de investigación de maestría fue en el Instituto Nacional de Investigación Agronómica de Francia (INRAE), en Jouy es Josas, París donde realizó investigaciones empleando modelos animales, líneas celulares epiteliales y bacterias comensales con el Dr. Luis Bermúdez Humarán en 2017.

En marzo de 2018, Pamela comenzó su doctorado en la Escuela de Graduados en Ciencias e Ingeniería en el Instituto de Biotecnología y Ciencias Biomoleculares de Groningen (GBB), Departamento de Interacciones Huésped-Microbio dirigido por su promotora Prof. Sahar El Aidy en colaboración con su co-promotor Dr. Sushil Mahata de la Universidad de California, San Diego (UCSD). Pamela realizó su investigación presentada en esta tesis sobre las interconexiones entre la microbiota intestinal y el péptido antimicrobiano catestatina. Durante su doctorado, Pamela también supervisó proyectos de maestría y licenciatura y presentó su trabajo de investigación en conferencias científicas.

Además, Pamela participó durante casi dos años en el consejo de doctorado (PhD Council) de la Facultad de Ciencias e Ingeniería de la Universidad de Groningen, donde pudo organizar actividades para la formación y el bienestar de la comunidad de estudiantes de doctorado. Pamela participó en 2020 como miembro del comité externo para la evaluación de la escuela de graduados VLAG en la Universidad de Wageningen de acuerdo con el Protocolo de Evaluación de la Estrategia nacional de Países Bajos.

Actualmente, Pamela es la cabeza del comité organizador de la primera escuela de verano interdisciplinaria sobre Investigación Científica Psicodélica en el Centro Médico Universitario de Groningen (UMCG) que se llevará a cabo en julio de 2022, donde expertos internacionales presentarán los últimos avances en investigación psicodélica. Pamela reconoce el gran valor de las sustancias psicodélicas no solo para el tratamiento de enfermedades mentales crónicas sino también para explorar estados expandidos de conciencia. Está interesada en implementar conscientemente un enfoque holístico de la terapia asistida por psicodélicos en el mundo occidental mediante la integración de la sabiduría ancestral y las prácticas indígenas tradicionales en nuestro sistema científico moderno.

Publications in this thesis

González-Dávila, P., Schwalbe,M., Danewalia, A., Wardenaar, R., Dalile, B., Verbeke, K., Mahata, S.K. & El Aidy, S. (**2022**). Gut microbiota transplantation drives the adoptive transfer of colonic genotype-phenotype characteristics between mice lacking catestatin and their wild-type counterparts. *Gut Microbes.* 14:1, 2081476. https://doi.org/10.1080/19490976.2022.2081476

González-Dávila, P., Schwalbe, M., Danewalia, A., Dalile, B., Verbeke, K., Mahata, S. K., & El Aidy, S. (**2022**). Catestatin selects for colonization of antimicrobial-resistant gut bacterial communities. *The ISME Journal*. https://doi.org/10.1038/s41396-022-01240-9

Bugda Gwilt, K., **González, D. P.**, Olliffe, N., Oller, H., Hoffing, R., Puzan, M., El Aidy, S., & Miller, G. M. (**2020**). Actions of Trace Amines in the Brain-Gut-Microbiome Axis via Trace Amine-Associated Receptor-1 (TAAR1). *Cellular and Molecular Neurobiology*. 40, 191–201. https://doi.org/10.1007/s10571-019-00772-7

Other publications

Lenoir, M., Martín, R., Torres-Maravilla, E., Chadi, S., **González-Dávila, P.,** Sokol, H., Langella, P., Sánchez-Pardo M., & Bermúdez-Humarán, L. G. (**2020**). Butyrate mediates anti-inflammatory effects of *Faecalibacterium prausnitzii* in intestinal epithelial cells through Dact3. *Gut Microbes*. 12:1, 1826748. https://doi.org/10.1080/19490976.2020.1826748

PhD Training DP González Dávila

Sum of registered credits (EC): 46

Training Activities	Year	EC
Chairperson of the Summer School on Psychedelic Research, University Medical Center Groningen	2022	2
PhD Council Member, Graduate School of Science and Engineering, University of Groningen	2020-2022	2
How to deal intercultural differences workshop	2022	0,2
Member of the review committee to assess VLAG Graduate School, Wageningen University & Research	2021	2
Entrepreneurship course for scientists	2021	0,3
Dutch language course A2/B1	2021	2
Groningen Biomolecular Sciences and Biotechnology Institute symposium (research pitch)	2021	0,2
Write a scientific article course	2020	4
PhD Council's support workshops	2020	0,3
Member of GSSE Sounding Board, University of Groningen	2020-2021	1
Supervision writing assignment (Laurie Kerkhof)	2020	0,5
Master's project supervision (Markus Schwalbe)	2020-2021	1
Time management yourself and your PhD - course	2020	0,5
English language support 3: Analytical storytelling on paper	2020	0,5
Member of the PhD Scholarship Programme Committee FSE, University of Groningen	2020-2021	0,5
International research experience, University of California, San Diego (UCSD)	2019	3
PhD Day - Volunteer	2019	2
Groningen Biomolecular Sciences and Biotechnology Institute symposium	2019	1
Bachelor's project supervision (Stephanie Roethig)	2019	1
Dutch language course A1-A2	2019	2
Article 9 License - Laboratory Animal Science	2018	4
PhD day - Volunteer	2018	1
Groningen Biomolecular Sciences and Biotechnology Institute symposium (2nd best poster award)	2018	1
Scientific Integrity Course	2018	2
Introductory event	2018	1
Master's project supervision (Aldert de Jong)	2018	2
Introductory essay "Role of trace amines on gut trace aminergic system"	2018	6
Mastering your PhD	2018-2022	2

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