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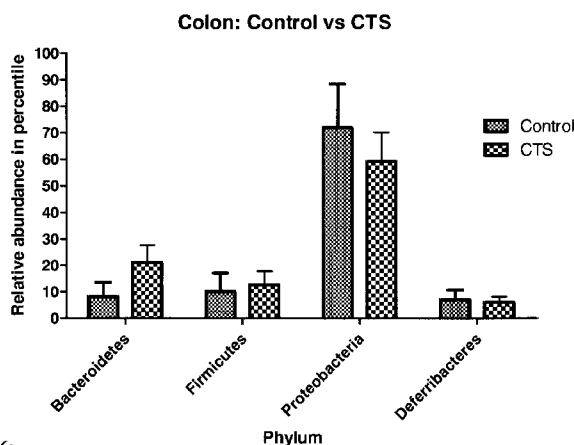


Figure 6:

(57) Abstract: Treatment with catestatin changes the proportion of two major phyla Bacteroidetes and Firmicutes in the gut microbiota in an opposite manner observed in intestinal disorders like IBD, IBS or non-intestinal disorders like obesity. Specifically, administration of an effective amount of catestatin increased the relative percentage of Bacteroidetes and decreased the relative percentage of Firmicutes in the gut microbiota.



HUMAN CATESTATIN INDUCES GUT MICROBIOTA DYSBIOSIS

PRIOR APPLICATION INFORMATION

The instant application claims the benefit of US Provisional Patent Application
5 62/155,572, filed May 1, 2015.

BACKGROUND OF THE INVENTION

Over the last 15 years, bacterial multi-drug resistance (MDR) has emerged and it has several socio-economical causes, from the use of surface antibacterial agents that are now available in many household products¹ to antibiotic over-prescription or
10 failing to complete a course of antibiotics¹. Although due to MDR new lines of antibiotics are required, the development of new antibiotics has been reduced by pharmaceutical companies because of the cost and complexity of clinical trials². Currently, there are relatively few new antimicrobials in development.

The gastrointestinal tract is heavily colonized with an average of 10^{14} microbes
15 that represent thousands of species, which is 10 times more than the total number of cells in the human body³. More than 90% of this bacterial population falls under two major phyla: Bacteroidetes (a gram-negative phylum) and Firmicutes (a gram-positive phylum)^{4, 5}, while the remaining belong to phyla such as Proteobacteria, and Actinobacteria^{4, 5}. In healthy individuals, microbial diversity in the intestine is stable
20 over time and demonstrates a symbiotic relationship with the host³, but a shift in microbial composition, named dysbiosis, targeting mainly Firmicutes and Bacteroidetes, has been described in several pathologies, including related and non-related gastrointestinal pathologies⁶⁻⁸. For example, microbial dysbiosis in gut is observed in intestinal disorders like intestinal bowel syndrome (IBS), intestinal bowel
25 disease (IBD) and also non intestinal disorders like obesity and type 1 and type-2 diabetes. Specifically, gut microbiota helps to digest food items and various metabolites and chemicals are produced by the resident microbiota, which plays a significant role in host health or disease state. For example, *Bacteriodes thetaiotaomicron* can activate the toll-like receptors (TLRs) in the gut epithelium,
30 which in turn can affect the expression of antimicrobial peptides, such as

angiogenins^{9, 10}. In addition to the innate immune system, gut microbiota can also control the host's adaptive immune system through T cell receptor $\alpha\beta$ -positive intraepithelial lymphocytes, regulatory T cells and T helper 17 cells⁵. Overall, gut homeostasis is largely dependent on the normal gut microbiome¹¹.

5 At the mucosal level the epithelium plays a major role in limiting the passage of bacteria to the sub-mucosa and restricts the presence of bacteria to the gut lumen; cell division is an important factor when the epithelial cells are altered and the epithelium needs to be regenerated^{12,13}. Antimicrobial peptides (AMPs) secreted by epithelial cells have a broad spectrum effect against bacteria and they are part of an
10 ancient defense mechanism that is present in virtually all mammals¹⁴. In the gastrointestinal tract, specialized intestinal epithelial cells or circulating inflammatory cells are a major source of these AMPs¹⁴. Within the epithelium, Paneth cells are the main producer of AMPs but new data indicate that enterochromaffin (EC) cells can hypothetically also produce certain types of AMPs¹⁵.

15 The EC cells are the major source of chromogranin A (CgA)¹⁶, a family of highly acidic proteins. The CgA gene is localized at 14q32 in the human genome, consisting of 8 exons and 7 introns, and its 2-Kb transcript is translated into the 457-residue CgA protein. The overall homology for CgA in different vertebrates is approximately 40%, but the most highly conserved regions occur at the N- and C-
20 termini, which show up to 88% sequence homology. Cell- and tissue-specific CgA processing has been described in the rat, mouse and human GI tract¹⁷⁻¹⁹. The CgA primary structure from its cDNA sequence shows the presence of numerous pairs of basic amino acids. These are potential sites for cleavage by prohormone convertases (PC) 1/3 or 2, and carboxypeptidase E/H²⁰, which is consistent with evidence that
25 CgA may serve as a prohormone for shorter bioactive fragments²¹; this is also suggested by the high sequence conservation of CgA-derived peptides. But in the gut, peptides can be highly sensitive to enzymes present in the luminal environment. Proteolytic fragments of CgA-derived peptides exert a broad spectrum of regulatory activities on the cardiovascular, endocrine and immune systems. Among its highly
30 conserved C-terminal regions, CgA gives rise to a peptide of biological importance:

the antihypertensive peptide catestatin (human CTS; CgA₃₅₂₋₃₇₂)²²⁻²⁴, which has restricted antimicrobial activity against *Staphylococcus aureus in vitro*²⁵. Similar to other AMPs, CTS can interact with anionic components of fungi and viruses. As a result, the microbial membrane is permeabilized, leading to cell lysis²⁶. *In vitro* studies have demonstrated that CTS is effective against gram-positive bacteria such as *Staphylococcus aureus* and group A *Streptococcus*, gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, yeasts such as *Candida albicans* and filamentous fungi such as *Aspergillus niger*, *A. fumigates* and *Trichophyton rubrum*^{26, 27}. However, to date, there has been no indication that the *in vitro* data can be reproduced using an *in vivo* model, as due to the presence of several enzymes located in the gut lumen, CTS peptide can be rapidly inactivated. Moreover, there is no indication about the type of microbiota affected, as the colonic mucosa associated population differs completely for the population present in the feces.

Despite the close association between CTS and *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa in vitro*, the effects of *in vivo* CTS treatment on the different type of gut microbiota are unknown. Our aim was to assess the composition of fecal and colonic mucosa associated microbiota and functional alterations in mice that were exposed to CTS for 6 days.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method for increasing levels of Bacteroidetes relative to levels of other bacteria in the gut of an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

According to a further aspect of the invention, there is provided a method for decreasing relative levels of Firmicutes relative to other bacteria in the gut of an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

According to yet another aspect of the invention, there is provided use of catestatin (CTS) for increasing levels of Bacteroidetes relative to levels of other

bacteria in the gut of an individual in need of such treatment.

According to still another aspect of the invention, there is provided use of catestatin (CTS) for decreasing relative levels of Firmicutes relative to other bacteria in the gut of an individual in need of such treatment.

5 According to a yet further aspect of the invention, there is provided a method of modulating gut microbiota composition comprising administering to an individual in need of such treatment an effective amount of catestatin (CTS).

According to a still further aspect of the invention, there is provided use of catestatin (CTS) for modulating gut microbiota composition.

10 According to another aspect of the invention, there is provided a method of treating or preventing or prophylactically treating type 1 diabetes, type 2 diabetes, obesity, IBS or IBD in an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

15 According to a further aspect of the invention, there is provided use of catestatin (CTS) for treating or preventing or prophylactically treating type 1 diabetes, type 2 diabetes, obesity, IBS or IBD in an individual in need of such treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure1: Rarefaction analysis on Chao 1, a measure of species richness based on operational taxonomic unit (OTU), for the fecal samples; control and CTS. Control and CTS treated mice fecal samples are more diverse compared to DSS and DSS+CTS mice treated samples, which have a low diversity. Diversity is not significantly affected by CTS treatment.

25 Figure 2: Rarefaction analysis on Chao 1, a measure of species richness based on operational taxonomic unit (OTU), for the colonic mucosa associated samples; Control and CTS. Diversity is not significantly affected by CTS treatment.

30 Figure 3: Principal coordinate analysis (PCoA) based on the unweighted UniFrac distance metric. Each colored point represents a fecal sample obtained from one mice and it is shaded according to different treatment (CTS or Control). P values were calculated using PERMANOVA. Samples clustered according to treatment

status of the mice ($P < 0.05$), suggesting that CTS and Control mice fecal samples are composed of distinct bacterial communities.

Figure 4: Principal coordinate analysis (PCoA) based on the weighted and unweighted UniFrac distance metric. Each colored point represents a colonic mucosa associated sample obtained from one mice and it is shaded according to different treatment (CTS or Control). P values were calculated using PERMANOVA. Samples did not cluster according to treatment status of the mice ($P > 0.05$), suggesting that CTS treatment did not significantly change bacterial communities.

Figure 5: Effect of CTS treatment on the abundant phyla ($\geq 1\%$) present in the fecal samples. After quality filtering steps 10 phyla was identified in fecal samples. Among these 4 phyla were considered abundant within the population ($\geq 1\%$), including Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. CTS treated mice had a significantly higher level of Bacteroidetes population compared to Control ($P < 0.05$, t-test). On the other hand Firmicutes population were lowered significantly in the feces of CTS treated animals ($P < 0.01$, t-test)

Figure 6: Effect of CTS treatment on the abundant phyla ($\geq 1\%$) present in the colonic mucosa associated samples. After quality filtering steps 19 phyla was identified in colonic mucosa associated samples. Among these 4 phyla were considered abundant within the population ($\geq 1\%$), including Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. CTS treatment did not have a significant impact on these abundant phyla present in the colonic mucosa associated samples.

Figure 7: Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the Control or CTS treatments in the mice fecal samples. All taxa are colored based on the phyla to which they belong. Some sequences could only be affiliated to phylum (P), order (O), family (F) or class (C) levels. Specific taxa were significantly associated with each treatment group, which may be an indicator of shifts in the physiological or metabolic processes that the taxa may influence.

Figure 8: Partial least square discriminant analysis (PLS-DA) of bacterial

communities comparing taxa that were associated with the Control or CTS treatments in the mice colonic mucosa associated samples. All taxa are colored based on the phyla to which they belong. Some sequences could only be affiliated to phylum (P), order (O), family (F) or class (C) levels. Specific taxa were significantly associated with each treatment group, which may be an indicator of shifts in the physiological or metabolic processes that the taxa may influence.

Figure 9. Subsystems and pathways enriched or decreased within the CTS or (Control) mice fecal samples. Corrected P-values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented in the CTS or Control mice fecal samples have a positive or (negative) difference between mean proportions and are indicated by purple or (orange) coloring respectively.

Figure 10. Subsystems and pathways enriched or decreased within the CTS or (Control) mice colonic mucosa associated samples. Subsystems or pathways overrepresented in the CTS or Control mice colonic mucosa associated samples have a positive or (negative) difference between mean proportions and are indicated by Purple or (orange) coloring respectively.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

The mammalian intestinal tract is heavily colonized with a dense, complex and diversified microbial population. In healthy individuals, host and gut microbiota enjoy a symbiotic relationship by maintaining intestinal homeostasis and an array of epithelial antimicrobial agents is secreted into the gut to promote intestinal homeostasis. Enterochromaffin cells in the intestinal epithelium are a major source of chromogranin A (CgA), which is a pro-hormone and can be cleaved into a shorter bioactive peptide

called catestatin (CTS). This study was carried out to evaluate the possible impact of CTS on gut microbiota *in vivo* using a mouse model. The CTS treatment did not modify the richness of the bacterial species in the fecal and colonic mucosa associated samples; however, the treatment significantly modified the bacterial community composition between the groups. The PLS-DA analysis revealed an association between specific taxa and the CTS-treated group at lower taxonomic levels. The CTS-treated mice had a significantly lower relative abundance of Firmicutes and higher abundance of Bacteroidetes. No significant change at the phylum level was observed in CTS-treated mice colonic mucosa associated samples. However, at lower phylogenetic levels, some bacterial taxa were significantly associated with CTS-treated mice in both fecal and colonic mucosa associated samples. Differences in microbial functional pathways in both fecal and colonic mucosa associated samples were detected. These results support the hypothesis that CTS treatment modulates gut microbiota composition under physiological conditions. Accordingly, these data indicate that CTS can be used to induce gut homeostasis which in turn can prevent, treat or prophylactically treat diseases such as obesity, type 1 diabetes, type 2 diabetes, inflammatory bowel disease (IBD), inflammatory bowel syndrome (IBS) or other health conditions.

As used herein, "CTS" refers to the antihypertensive peptide catestatin (human CTS). The CTS is derived from amino acids 352-372 chromogranin A (CgA), a family of highly acidic proteins. In some embodiments, CTS has the amino acid sequence SSMKLSFRARAYGFRGPGPQL (SEQ ID NO:1) although variants of this sequence, both naturally occurring and recombinant, may be used within the invention.

Rabbi et al. (2014, Biochem. Pharma. 89: 386-398) teaches that CTS is increased during colitis and that CTS modulates intestinal inflammation via the macrophage population and through a STAT-3 dependent pathway. Specifically, treatment with full length CTS (amino acids 352-372), proximal CTS fragment (amino acids 352-366) and distal CTS fragment (amino acids 360-372) resulted in less severe induced colitis, which indicates that CTS and the fragments thereof can be used to treat intestinal inflammation.

As will be appreciated by one of skill in the art, this indicates that treatment with CTS or either the proximal or distal fragment thereof will reduce inflammation in an individual suffering from colitis. Specifically, it is believed that CTS influences macrophage production of cytokines, which in turn reduces inflammation. Antigen presenting cell like macrophages, are one of the main producer of proinflammatory cytokines. CTS blocks the intracellular pathway implicated in the regulation of proinflammatory cytokines (i.e. IL-1b, IL-6).

Consequently, Rabbi et al. (2014) teaches that CTS can be used to reduce intestinal inflammation in an individual, which in turn would be expected to reduce the severity of symptoms associated with a colitis attack and/or reduce the frequency of colitis episodes.

However, this anti-inflammatory activity is separate and distinct from the known antimicrobial activity of CTS, which had previously only been demonstrated *in vitro*.

Rabbi et al. (2014) further speculates that intrarectal infusion of CTS peptides might induce a beneficial gut microbiota dysbiosis, which subsequently can affect the development of colitis.

As discussed above, *in vitro* studies have demonstrated that CTS is effective against gram-positive bacteria such as *Staphylococcus aureus* and group A *Streptococcus*, gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, yeasts such as *Candida albicans* and filamentous fungi such as *Aspergillus niger*, *A. fumigates* and *Trichophyton rubrum*^{26, 27}. However, previously, there had been no indication that the *in vitro* data can be reproduced using an *in vivo* model, as due to the presence of several enzymes located in the gut lumen CTS peptide can be rapidly inactivated. Specifically, there are a number of proteases which can act on serine, cysteine and aspartic amino acids²⁸. As the human CTS sequence used in this study has serine and aspartic sequences (SSMKLSFRARAYGFRGPGPQL), it was anticipated that CTS could be inactivated in the gut.

Specifically, CTS is a cationic peptide and as such the inventors hypothesized that if CTS avoided degradation in the gastrointestinal environment, this peptide might

exerts its effect by electrostatic interactions with the negatively charged phospholipid microbial cell walls. As a result of these interactions, any suitable microbial membrane that CTS could interact with would be disrupted. It was further anticipated that CTS could have a general effect, for example, reducing levels of all bacteria.

5 However, as discussed herein, the gastrointestinal environment *in vivo* is incredibly complex and it is impossible to predict the exact effect that a general antimicrobial such as CTS would have in the gut, assuming that it was not degraded by the serine and aspartic acid proteases. Furthermore, it is simply not possible to create a complex gastrointestinal environment *in vitro* that has the enormous
10 microbial diversity to sufficiently mimic the *in vitro* gut.

 Furthermore, as discussed below, in fact, *S. aureus* and *E. coli* levels in the gut were not affected by CTS administration *in vivo*, demonstrating the difference that can be found between *in vitro* studies and *in vivo* experiments. As such, it is clear that not all *in vitro* results can be extrapolated to *in vivo* analysis, especially in the context that
15 *in vitro*, many bacteria are non-cultivable due to the lack of anaerobic condition and are present when the whole microbiome is studied.

 Furthermore, as discussed herein, CTS treatment did not modify the richness of the bacterial species in fecal and colonic mucosa associated samples. Specifically, as shown in Figure 1 and as discussed herein, control and CTS-treated mice treated
20 samples were more diverse than DSS and DSS+CTS mice treated samples, which have a low diversity. These data demonstrated that bacterial diversity or richness was not significantly affected by CTS treatment.

 Based on these results, one conclusion would have been that the *in vitro* antimicrobial activity could not function *in vivo*, possibly due to protease degradation.

25 The inventors theorized that coating CTS might prevent or delay degradation; however, there were concerns that the coating would interfere with the antibiotic activity of CTS which, as discussed above, interacts with anionic components of the microbial membrane and permeabilizes the membrane, leading to cell lysis. As will be appreciated by one of skill in the art, there was considerable concern that a suitably
30 protective coating that would protect CTS from the proteases would also prevent CTS

from interacting with the negatively charged components of the bacterial membranes.

Another possibility would have been that CTS was having a general or broad spectrum antimicrobial affect.

As shown in Figure 4, CTS treatment also did not significantly change the
5 bacterial communities in the colonic mucosa associated samples, as discussed in greater detail below.

However, surprisingly, as shown in Figure 3, it was determined that CTS and control mice fecal samples were composed of distinct bacterial communities. More significantly, as shown in Figure 5, CTS treated mice had a significantly higher level of
10 Bacteroidetes compared to the control mice. The CTS treated mice also had a significantly lower level of Firmicutes compared to the control mice. Thus, in the fecal samples, CTS treatment significantly modified the bacterial community composition between the CTS treatment group and the control.

While CTS was not having a specific effect on diversity of the microbiota or in
15 the colonic mucosa associated samples, CTS treatment was having a significant effect on distinct bacterial communities within fecal samples, indicating that coating was not necessary for rectal administration.

This modification or modulation of the gut microbiota for example to increase relative levels of Bacteroidetes is significant as lower levels of Bacteroidetes may
20 result in adverse health effects as these bacteria have important starch degrading enzymes and also help to establish normal gut immunity. For example, *B. thetaiotaomicron* prevents activation of the proinflammatory transcription factor NF κ B⁴¹. Bacteroidetes also help to prevent production of virulent factors from pathogenic *E. coli*⁴². Moreover, there is data showing that fecal transplantation from a
25 healthy person to IBD patients helps to reduce the symptoms^{43, 44} and that administration of probiotics which modify the microbiota can improve IBS and IBD.

Described herein is a method for increasing levels of Bacteroidetes relative to levels of other bacteria in the gut of an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

30 Described herein is a method for decreasing relative levels of Firmicutes

relative to other bacteria in the gut of an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

Also described herein is the use of catestatin (CTS) for increasing levels of Bacteroidetes relative to levels of other bacteria in the gut of an individual in need of
5 such treatment.

Also described herein is the use of catestatin (CTS) for decreasing relative levels of Firmicutes relative to other bacteria in the gut of an individual in need of such treatment.

Also contemplated is a method of modulating gut microbiota composition comprising administering to an individual in need of such treatment an effective
10 amount of catestatin (CTS).

The use of catestatin (CTS) for modulating gut microbiota composition is also contemplated.

As will be appreciated by one of skill in the art and as discussed herein, an
15 "effective amount" is an amount which is sufficient to achieve the desired result, that is, modulating the gut microbiota composition of an individual, for example, increasing the relative percentage or proportion of Bacteroidetes relative to other bacteria within the gut microbiota and/or decreasing the relative percentage or proportion of Firmicutes relative to other bacteria within the gut microbiota. Such an effective
20 amount will of course depend on the severity of the dysbiosis within the gut microbiota of the individual, as well as other factors, such as the age, weight and general condition of the individual. Determination of such an effective amount is considered to be within the routine skill of one knowledgeable in the art.

The effective amount may be administered by a variety of means, as discussed
25 below and may be administered on a schedule, for example, daily, every other day or multiple times a day, again depending on the state of the individual being treated.

As used herein, an "individual in need of such treatment" is an individual whose gut microbiota is in a dysbiotic state or who is thought to have developed dysbiosis or is at risk of developing dysbiosis.

30 For example, the individual may have developed dysbiosis as a result of

another treatment or as a result of being subjected to a particular diet or environmental conditions. Alternatively, the individual may be an individual who is at risk of developing type 1 diabetes, type 2 diabetes, obesity, IBS or IBD either as a result of heredity, genetic pre-disposition and/or lifestyle.

5 Described herein is a method of treating or preventing or prophylactically treating type 1 diabetes, obesity, IBS or IBD in an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

Also described herein is the use of catestatin (CTS) for treating or preventing or prophylactically treating type 1 diabetes, type 2 diabetes, obesity, IBS or IBD in an
10 individual in need of such treatment.

Preferably, the CTS is human CTS.

In some embodiments, the CTS comprises the amino acid sequence as set forth in SEQ ID NO:1 (SSMKLSFRARAYGFRGPGPQL) or a variant thereof which retains anti-microbial activity.

15 As used herein in regards IBS or IBD, an "effective amount" of CTS is an amount of CTS that is sufficient to reduce one or more symptoms associated with IBD (inflammatory bowel disease) or IBS (irritable bowel syndrome). Specifically, this "effective amount" will be sufficient to reduce the severity of one or more symptoms associated with IBD or IBS, for example, abdominal cramps, abdominal pain,
20 diarrhea, fever, weight loss, loss of appetite, bloating and vomiting. The "effective amount" may also reduce the frequency of attacks of IBD and/or IBS and the severity of these symptoms and may also increase the length of time between symptoms, as will be apparent to one of skill in the art.

It can be postulated that there is a bilateral communication between the gut
25 microbiota and mucosal cells present in the inflamed mucosa. However, to date although numbers of studies have been performed using all the anti-inflammatory drug presents on the market, none- have demonstrated a direct or indirect effect on the gut microbiota. Therefore, we can speculate that treating inflammation in gastrointestinal related disorders will not inherently treat dysbiosis.

30 Conversely, it has been demonstrated that pre- or probiotics and the use of

some antibiotic can modify the gut microbiota and modify symptoms associated with IBD and IBS. This demonstrates that treating the underlying disease, gut dysbiosis, can subsequently modify the symptoms associated to mucosal inflammation. In our context, the treatment with CTS will modify gut dysbiosis and ultimately lead to a
5 correction of mucosal inflammatory-associated symptoms.

As will be appreciated by one of skill in the art, the prior teaching of CTS as effective at reducing inflammation during colitis indicates that CTS could be administered during a colitis attack to reduce the severity of symptoms caused by the inflammation and more specifically by immune cell represented by the macrophages
10 population. However, this does not teach or suggest that CTS could be used to correct or treat dysbiosis or prophylactically treat IBD or IBS. Furthermore, it does not teach or suggest treatment or prophylactic treatment or prevention of obesity, type 1 diabetes or type 2 diabetes.

Accordingly, in another aspect of the invention, the use of CTS to treat or
15 prevent IBD or IBS is contemplated with the proviso that symptoms other than inflammation are being treated.

In other embodiments, CTS is administered to an individual suffering from or at risk of developing IBD or IBS to treat dysbiosis associated with IBD or IBS by increasing levels of Bacteroidetes relative to levels of Firmicutes in the gut of said
20 individual.

Preferably, the CTS is formulated for rectal administration to the individual in need of such treatment.

Typically, active agents are delivered rectally by suppository, enema, ointment, cream, foam or injection.

25 Suppositories are generally fatty in nature but water-soluble or water-miscible bases can also be used.

Typically, enemas comprise the active agent dispersed in a suitable flowable carrier vehicle, such as water, alcohol or an aqueous-alcohol fluid. The carrier may be thickened with natural and/or synthetic thickeners such as gums, acrylates or modified
30 celluloses. Unit dosages of enema formulations can be administered from prefilled

bags or syringes.

Foams may include a foaming agent such as n-butane, propane or i-butane. Such foam formulations may be delivered from a pressurized container.

Suitable excipients for rectal administration include but are by no means limited
5 to preservatives, surfactants, emulsifiers, mineral oils, propellants, thickening agents, lubricants, preservatives, pH adjusting agents, chelating agents, emollients and/or humectants, permeation enhancers, suspension-forming agents or mucoadhesive agents.

In some embodiments, the effective amount of CTS if formulated as a
10 suppository or enema.

The mammalian intestine continuously encounters more microorganisms than any other tissue, and survival of the mammalian population largely depends on their unique adaption in the world of microorganisms. Specific intestinal epithelial cells release several antimicrobial peptides, which are critical for maintaining a stable
15 ecological environment that favors commensal and targeting pathological microorganisms³⁷. Moreover, these are also important for inhibiting ongoing inflammatory responses. CTS, a highly conserved CgA peptide that is present in intestinal EC cells, was shown to be an peptide with some immunomodulatory activities and restricted *in vitro* antibacterial activities³⁸, but also antifungal and
20 antiviral activity^{26, 27}. However, to date, and due to the presence of several enzymes present in the gut lumen, there are no documented studies that demonstrate the effect of this peptide on gut microbiota using *in vivo* models. Here, it is shown that i.r. administration of CTS modulates gut microbiota composition under physiological conditions.

25 Based on the α -diversity, it was observed that richness and the bacterial population diversity in both fecal and colonic mucosa associated samples did not change after CTS administration. However, β -diversity analysis using the unweighted Unifrac distances through QIIME revealed that CTS-treated mice had a fecal microbial composition that was significantly separated from the control group, suggesting that a
30 short-term exposure of this peptide in the gut might change the microbial composition

profile. This is in accordance with studies demonstrating the effect of other antibacterial peptide released by Paneth cells (*i.e.* defensin) on gut microbiota³⁹.

Recent investigation shows that intestinal inflammatory conditions such as inflammatory bowel disease (IBD) or inflammatory bowel syndrome (IBS) are associated with altered intestinal homeostasis^{7, 40}. Although microbial dysbiosis has been suggested to be a cause of intestinal pathophysiological conditions, this is still controversial. However, it has been observed that microbial diversity is significantly altered in human and animal models of IBS^{7, 40}. In the context of IBS, although an exact causal microbe has not yet been identified, a reduction in the microbial diversity has been documented and this temporal gut microbiota instability can result in altered host physiology, resulting in heterogeneous symptoms such as those observed in IBS patients^{7, 40}. At the phylum level, IBS patients have a higher relative abundance of Firmicutes and lower abundance of Bacteroidetes compared to controls.⁷

In this study, it was observed that CTS treatment is significantly associated with a relative reduction of Firmicutes in the feces compared with saline-treated mice. Conversely, CTS treatment was associated with a significant higher relative abundance of Bacteroidetes in the feces compared with saline-treated mice. Beside IBS, in colitic conditions, studies demonstrated a reduction in relative abundances of Bacteroidetes⁴⁵ and Verrucomicrobia⁴⁵, a group of bacteria that is present in low numbers in the mammalian intestine, and which are able to degrade mucin⁴⁶, suggesting for a shift in the microbial community. In this study, it was observed that CTS treatment lowered Verrucomicrobia in both fecal and colonic mucosa associated samples. In this study, CTS treatment did not significantly change the relative abundance of Proteobacteria in mice, but a reduction in members of this phylum in colonic mucosa associated samples was observed. In addition to colonic mucosa associated pathologies, studies showed that Firmicutes is significantly more abundant relative to Bacteroidetes in obese mice compared to lean mice⁸; these results were also observed in humans⁸. In this study, CTS treatment was associated with a significant abundance of Bacteroidetes relative to Firmicutes in fecal samples, which was opposite to results from obese animals and humans. However, this change in

Bacteroidetes and Firmicutes abundance were not observed in the colonic mucosa associated samples, suggesting that prolonged administration of this peptide might be required to observe a possible change in the colon wall. At the lower taxonomical level, CTS administration was associated with significant increase in *Prevotella*,
5 *Bacteroides*, and *Parabacteroides* populations in the feces. A recent meta-analysis (Walters et al, FEBS Letters 588 (2014) 4223–4233) of gut microbiome in patients with ulcerative colitis, colonic Crohn's disease or ileal crohn's disease indicated that these three populations were significantly depleted in patients with ulcerative colitis and Crohn's disease and that the these genera are among the signature taxa that can be
10 used to predict if subject likely has IBD or not. It is, therefore, very interesting that CTS supplementation could enrich the population of *Prevotella*, *Bacteroides*, and *Parabacteroides*, which are significantly depleted in IBD patients.

As discussed above, as CTS is cationic in nature, it was expected that there would be a broad spectrum antimicrobial activity, or, if the peptide was not able to
15 remain active in gastrointestinal environment, that there would be no effect.

As will be appreciated by one of skill in the art, it is impossible to create a complex gastrointestinal environment *in vitro* with enormous microbial diversity. But the illumina sequencing technique targeting bacterial 16s rRNA provided the overall impact of CTS on gut microbiota in the actual gastrointestinal environment. Although
20 the overall microbial richness was not modified, it has been shown for the first time that CTS treatment changed the proportion of two major phyla Bacteroidetes and Firmicutes in the feces in an opposite manner observed in intestinal disorders like IBD, IBS or non-intestinal disorders like obesity and type 1 and type 2 diabetes.

The CTS treatment also caused microbial changes in lower taxonomic levels.
25 Using our current approach, we classified microbes to the genus level. It was observed that certain bacterial taxa were positively associated with CTS treatment in both the fecal and colonic mucosa associated samples. Among these taxa, *Bacteroides* (genus) and *Parabacteroides* (genus) showed a positive association with CTS treatment in the fecal samples. Both of these belong to the Bacteroidales order,
30 which also showed a positive association with CTS treatment in the colonic mucosa

associated samples. *Bacteroides* and *Parabacteroides* species represent ~25% of the colonic mucosa associated microbiota and are commensal to the host when present in the gut⁴⁸. These anaerobic rods can transform simple and complex sugars into volatile fatty acids, which can be absorbed by the large intestine as a nutrient.

- 5 *Bacteroides thetaiotaomicron* has several starch-binding genes and can produce significant amount of glycosylhydrolases, which can be crucial to prevent obesity⁴⁹. This might explain why *Bacteroidetes* are more abundant in obese mice compared to lean mice. Beside the enormous starch-utilizing capacity, *Bacteroides* species are important for developing gut immunity. For example, *B. thetaiotaomicron* can
- 10 stimulate Paneth cells to produce Paneth cell protein (Ang4), which is lethal to certain pathogenic microorganisms (e.g. *Listeria monocytogenes*)³⁴. In addition, *B. fragilis* produces zwitterionic polysaccharide (ZPS), which is important for developing CD4 T cells. ZPS-activated CD4 T cells produce interleukin-10 (IL-10), which is essential to prevent abscess formation and other unchecked inflammatory responses⁴⁹⁻⁵¹.
- 15 Increased *Bacteroides* population in mice in response to CTS exposure might be beneficial to control obesity and inflammatory conditions such as IBD or IBS. These results might also explain the underlying mechanisms for improving gut inflammation that we observed previously in colitic mice exposed to CTS³⁰.

- In this study, metagenomic functional prediction analysis was performed. This
- 20 approach helps to connect the microbiota as a whole to specific functions in the environment⁵². Using this approach, it was observed that certain subsystems or pathways are enriched after CTS treatment in both the fecal and colonic mucosa samples, surprisingly suggesting that although we were not be able to see any effect for some markers studied after 6 days treatment with CTS, CTS treatment can modify
- 25 specific functions in the environment. One of the most important observations was increased cell division in the CTS-treated mice. In physiological and pathophysiological conditions, the mammalian intestine needs to constantly replenish its epithelial tissues, which are at risk of continuous environmental attacks such as oxidative stress, microbial antigens or inflammatory mediator. Increased cell division
- 30 stimulated by CTS treatment might help to reverse the damage to the intestine

caused by increased inflammation⁵³.

Surprisingly, beside the fact that the purpose of the study was to define the effect of CTS on gut bacteria and its potential implication in gut-related diseases, the prediction analysis also demonstrated that CTS treatment induced functional shifts in the murine intestinal microbiota, with different metabolic pathways that are enriched in the mucosal microbiota of fecal and colonic mucosa associated samples compared to the control mice. CTS treatment showed a positive association with amyotrophic lateral sclerosis (ALS), a condition where the CgA peptide levels are lower in affected patients⁵⁴. However, the exact role of CgA peptides in ALS remains unknown and further data is needed to decipher their exact role in the context of stimulating microglial immune response. Type 1 diabetes was also positively associated with CTS treatment in our study and this observation is in agreement with other studies that demonstrated the abundance of *Bacteroides* in this pathological condition⁵⁵. It was also shown that WE14, a CgA-derived peptide from the distal part of CgA can activate CD4 T cell clones, which can induce diabetes *in vivo*⁵⁶.

These findings provide new insight into the mechanisms of gut microbiota regulation by CTS. We observed a not predicted shift in the microbial profile in response to CTS treatment, which was more prominent in the feces than in colonic mucosa. These results establish the utility of new anti-microbiota peptides and their use as therapeutic agents to treat several conditions of the gastrointestinal tract, such as IBD, IBS, obesity but also other health conditions, such as ALS, obesity and T1 diabetes.

Results

Descriptive analysis

Mice were divided into two groups: one group (control) was injected i.r. with normal saline while the other group received CTS (1.5 mg/kg/day, i.r. for 6 days). Weight loss, stool consistency and blood in the feces were determined every day for each animal. The peptide itself had no direct effect on these parameters, which is consistent with our previous findings³⁰.

Sample assessment by Illumina sequencing

After sacrifice, we collected fecal and colonic mucosa associated samples from a total of 12 mice. Among these, the control group received normal saline intrarectally and the others received CTS intrarectally (1.5 mg per kg body weight for 6 days). During the DNA extraction process, one colonic mucosa associated sample from the saline-treated group was discarded because of poor quality or purity, resulting in a total of 12 useable fecal samples and 11 useable colonic mucosa associated samples for Illumina sequencing. For fecal samples, a total of 328,085 sequences were generated. After quality-filtering steps, an average of 27,340.42 high quality sequences per sample was determined. For colonic mucosa associated samples, a total of 207,123 sequences were generated. After quality-filtering steps, an average of 18,829.36 high-quality sequences per sample was determined.

CTS exposure did not significantly decrease α -diversity in fecal and colonic mucosa associated samples in mice

Bacterial richness and diversity from both fecal and colonic mucosa associated samples between control and CTS-treated groups were calculated. CTS treatment had no significant impact on Chao1, ACE, Shannon and Simpson indices of α -diversity in fecal or colonic mucosa associated bacterial community composition (Figures 1 and 2).

CTS treatment significantly influenced β -diversity in fecal samples but did not significantly influence colonic mucosa associated samples in mice

Bacterial community composition in the fecal and colonic associated microbiota of control and CTS-treated animals (β -diversity) was determined using PERMANOVA analyses of Bray-Curtis distances (Figures 3 and 4). Using unweighted β -diversity, we observed that bacterial communities from fecal samples of CTS-treated mice were clustered separately ($P < 0.05$) from controls, suggesting that the treatment modified the fecal bacterial profile (Figure 3). However, there was no significant change in the

bacterial community in colonic mucosa associated samples in CTS-treated mice compared to controls (**Figure 4**).

CTS treatment influenced colonic but not fecal bacterial microbiota composition at the phylum level in mice

In the fecal samples, a total of 10 phyla were identified, of which 4 phyla were considered to be abundant within the population ($\geq 1\%$); these included Firmicutes, Bacteroidetes, Proteobacteria and Deferribacteres. The other six phyla were considered less abundant within the population ($< 1\%$), and included Actinobacteria, Cyanobacteria, Fibrobacteres, TM7, Tenericutes and Verrucomicrobia (**Table 1**). Among the 4 abundant populations, CTS treatment significantly increased the relative abundance of Bacteroidetes ($P < 0.05$) and significantly decreased the Firmicutes population ($P < 0.001$) in the feces (**Figure 5**).

In the colonic mucosa associated samples, a total of 19 phyla were identified, of which 4 phyla were considered to be abundant within the population; these included Firmicutes, Bacteroidetes, Proteobacteria and Deferribacteres. The other 15 phyla were considered less abundant within the population, and included Acidobacteria, Actinobacteria, Armatimonadetes, Chlamydiae, Chlorobi, Cyanobacteria, Fibrobacteres, Lentisphaerae, OD1, OP3, Planctomycetes, Spirochaetes, TM7, Tenericutes and Verrucomicrobia (**Table 2**). CTS treatment had no significant impact on the relative abundance of bacterial phyla within the population (**Figure 6**).

CTS treatment influences fecal bacterial microbiota composition at lower phylogenetic levels in mice

A total of 328,085 sequences were generated after quality-filtering steps, with an average of 27,340 high-quality sequences per sample. This resulted in identification of 10 phyla, as shown above, and 86 taxa, but some taxa were only classified up to the Phylum (P), Class (C), Order (O), Family (F), Genus or species level. Of the 86 taxa, 54 taxa were considered abundant within the population, while

32 taxa were considered less abundant within the population. Results of the relative abundance of various genera with percentages of sequences $\geq 0.01\%$ were analyzed using PLS-DA to identify bacteria that were most characteristic of CTS or Control treatments. The PLS-DA analysis showed that the genera *Prevotella*, *Bacteroides*
5 *Ovatus*, *Bacteroides*, *Parabacteroides distarosis*, *Parabacteroides* and *Dorea* were positively associated with the CTS treatment in the fecal samples ($R^2 = 0.94$, $Q^2 = 0.57$) (**Figure 7**). In addition, members of Alpharotobacteria (Class), Bacteroidales (Order), RF32 (Order), mitochondria (Family) and YS2 (Order) also showed a positive association with CTS treatment in the fecal samples ($R^2 = 0.94$, $Q^2 = 0.57$). A negative
10 association with the members of *Adlercreutzia*, *Allobaculum*, Bacteroidaceae (Family), Clostridia (Class) and Ruminococcaceae (Family) were evident in the fecal samples collected from CTS-treated mice ($R^2 = 0.94$, $Q^2 = 0.57$).

CTS treatment influences colonic mucosa associated bacterial microbiota composition at lower phylogenetic levels in mice
15

A total of 207,123 sequences were generated after quality-filtering steps, with an average of 18,829 high-quality sequences per sample. This resulted in identification of 19 phyla, as shown above, and 179 taxa, but some taxa were only classified up to the Phylum (P), Class (C), Order (O), Family (F), Genus or species
20 level. Of the 179 taxa, 84 taxa were considered abundant within the population, while 95 taxa were considered less abundant within the population. Relative abundance of various genera/taxa of colonic mucosa associated samples with sequence percentages $\geq 0.01\%$ were analyzed using PLS-DA to identify bacteria that were most characteristic of the CTS or control treatments. The PLS-DA analysis of the colonic
25 mucosa associated samples showed that genera *Bifidobacterium* and *Stenotrophomonas* had a positive association with the CTS treatment ($R^2 = 0.32$, $Q^2 = 0.242$) (**Figure 8**). Members of Bacteroidales (Order), Chitinophagaceae (Family), Clostridiaceae (Family), Clostridiales (Order), Coriobacteriaceae (Family), Pseudomonadaceae (Family), Rikenellaceae (Family), Ruminococcaceae (Family)
30 and YS2 (Order) also showed a positive association with the CTS treatment in the

colonic mucosa associated samples ($R^2 = 0.32$, $Q^2 = 0.242$) (**Figure 8**).

CTS treatment significantly influences fecal and colonic mucosa associated microbiota functional and metabolic pathways in mice

To determine the functional KEGG pathways that could be associated with the observed microbial changes, we compared the functional pathways for the microbiota in fecal and colonic mucosa associated samples from the CTS-treated group with those of the control mice. Several metabolic pathways were determined. Subsystems or pathways that have a significant positive or negative correlation with CTS treatment are shown in **Figures 9 and 10**. In the fecal samples from CTS-treated mice, chlorocyclohexane and chlorobenzene degradation were significantly downregulated ($P=0.015$) (**Figure 9**). However, nitrogen metabolism was enriched in the fecal samples from CTS-treated mice ($P=0.033$; **Figure 9**). Pathways associated with amyotrophic lateral sclerosis (ALS) were significantly enriched in the fecal samples of this group of mice ($P=2.82 \times 10^{-3}$). In the colonic mucosa associated samples from CTS treated mice, nicotinate and nicotinamide metabolism, cell division and ribosome biogenesis were significantly enriched compared to the controls ($P<0.05$; **Figure 10**). However, analysis of colonic mucosa associated samples from CTS-treated mice also had a significant positive correlation with type 1 diabetes mellitus ($P=0.043$; **Figure 10**).

Materials and Methods

Animals

Male C57BL/6 mice (7–9 weeks old) were purchased from Charles River (Canada) and maintained in the animal care facility at the University of Manitoba. The experimental protocol was approved by the University of Manitoba Animal Ethics Committee (10-073) and the research was conducted according to the Canadian Guidelines for Animal Research. Two groups of 4 and 6 mice were studied, one receiving the vehicle solution and one receiving intra-rectal infusion of CTS for 6 days.

Peptide

CTS (Human CgA₃₅₂₋₃₇₂: SSMKLSFRARAYGFRGPGPQL, SEQ ID NO:1)²³ was used (Biopeptide Co., Inc, San Diego, CA), and the peptide was injected intrarectally at 1.5 mg/per kg body weight per day for 6 days. Saline (0.9%) was injected in the control group. Mice were anaesthetized using Isoflurane (Abbott, Toronto, Canada). PE-90 tubing (10 cm long; ClayAdam, Parisppany, NJ), which was attached to a tuberculin syringe (BD, Mississauga, Canada), was inserted 3.5 cm into the colon.

Assessment of physical condition

Weight loss, stool consistency and bleeding were assessed daily to determine any possible physical changes in the mice as a result of CTS treatment.⁵⁷ Scores were defined as follows: weight: 0, no loss; 1, 5–10%; 2, 10–15%; 3, 15–20%; and 4, 20% weight loss; stool: 0, normal; 2, loose stool; and 4, diarrhea; and bleeding: 0, no blood; 2, presence of blood; and 4, gross blood. Blood was assessed using the Hemocult II test (Beckman Coulter, Oakville, Canada).

Fecal and tissue sample collection

Samples were collected 6 days post-treatment induction, after euthanasia under isoflurane (Abbot) anaesthesia. The macroscopic score was determined on the sacrifice day based on stool consistency, rectal prolapse and rectal and colonic bleeding. On the day of sacrifice, a 250 mg fecal sample and a portion of the colon were collected in individual collector tubes from each animal. All samples were preserved at -80°C before use.

DNA extraction and quality check

Colons were opened and 50 mg of scrapings were taken from the inside. Samples were homogenized at room temperature, and genomic DNA was extracted from 250 mg fecal mass using a ZR fecal DNA Kit (Zymo Research Corp., Orange, CA). DNA extraction was performed using a Qiagen DNeasy blood & tissue kit

(Qiagen corp, Valencia, USA). Both DNA extraction kits have a bead-beating step to lyse microbial cells. To match the concentration requirement for pyrosequencing, DNA quantity was determined using a Beckman DU/800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Genomic DNA was normalized to achieve a concentration of 20 ng/μL, and quality-checked by 16S rRNA gene using PCR amplification of 27F (5'-GAAGAGTTTGATCATGGCTCAG-3', SEQ ID NO:2) and 342R (5'-CTGCTGCCTCCCGTAG-3', SEQ ID NO:3)^{58, 59}. Amplicons were verified by agarose gel electrophoresis.

10 ***Library construction and Illumina sequencing***

Library construction and Illumina sequencing were performed as described by Derakhshani et al.⁶⁰. Briefly, the V3-V4 region of the 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers⁶¹. A reverse PCR primer was indexed with 12-base Golay barcodes to allow for sample multiplexing. The PCR reaction for each sample was performed in duplicate and contained 1.0 μl of pre-normalized DNA, 1.0 μl each of forward and reverse primers (10 μM), 12 μl HPLC grade water (Fisher Scientific, ON, Canada) and 10 μl 5 Prime Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec and 72°C for 90 sec; this was followed by an extension step at 72°C for 10 min in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany). PCR products were then purified using a ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) to remove primers, dNTPs and reaction components. The V4 library was then generated by pooling 200 ng of each sample, and quantified using Picogreen dsDNA (Invitrogen, NY, USA). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, and the concentration was measured through optical density using a Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). Finally, 15% of the PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized

sequencing primers for read1 (5'-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3', SEQ ID NO:4), read2 (5'-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3', SEQ ID NO:5) and index read (5'-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3', SEQ ID NO:6) were synthesized and purified using polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

Bioinformatics analyses

Bioinformatics analyses were performed as described by Derakhshani et al.⁶⁰. Briefly, the PANDAseq assembler⁶² was used to merge overlapping paired-end Illumina fastq files. All the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed using downstream computational pipelines in the open source software package QIIME⁶³. Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality filters so that reads with more than 3 consecutive bases that had quality scores below $1e^{-5}$ were truncated, and those with a read length shorter than 75 bases were removed from the downstream analysis. Chimeric reads were filtered using UCHIME⁶⁴ and sequences were assigned to operational taxonomic units (OTU) using the QIIME implementation of UCLUST⁶⁵ at the 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using an RDP classifier⁶⁶ and aligned with the Greengenes core reference database⁶⁷ using PyNAST algorithms⁶⁸. The phylogenetic tree was built with FastTree 2.1.3⁶⁹ for additional comparisons between microbial communities.

Alpha (α)- and beta (β)-diversity analyses

Within-community diversity (α -diversity) was calculated using QIIME. An α rarefaction curve was generated using a Chao 1 estimator of species richness⁷⁰ with

10 sampling repetitions at each sampling depth. An even depth of approximately 15,700 sequences per sample was used for calculation of richness and diversity indices. To compare microbial composition between samples, β -diversity was measured by calculating the weighted and unweighted Unifrac distances⁷¹ using 5 QIIME default scripts. Principal coordinate analysis (PCoA) was applied on the resulting distance matrices to generate two-dimensional plots using PRIMER v6 software⁷². Permutational multivariate analysis of variance of Bray-Curtis distance (PERMANOVA)⁷³ was used to calculate *P*-values and test for significant differences in β -diversity among treatment groups. α -diversity differences between control and CTS 10 groups were determined using SAS (SAS 9.3, 2012).

Partial Least Square Discriminant Analysis

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on the genus data to identify the effects of 15 treatments^{74, 60}. The PLS-DA is a particular case of partial least square regression analysis in which *Y* is a set of variables describing the categories of a categorical variable on *X*. In this case, *X* variables were the bacterial genera and the *Y* variables were observations on different days. For this analysis, data were scaled using Unit Variance in SIMCA. Cross-validation was then performed to determine the number of 20 significant PLS components and a permutation testing was conducted to validate the model. To avoid over-parameterization of the model, the variable influence on the projection (VIP) value was estimated for each genus, and genera with $VIP < 0.50$ were removed from the final model^{75, 76}. An r^2 estimate then was used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the 25 model. The PLS-regression coefficients were used to identify genera that were most characteristic of each treatment group and the results were visualized by PLS-DA loading scatter plots.

Metagenomic Functional Prediction

30 Phylogenetic Investigation of Communities by Reconstruction of Unobserved

States (PICRUST), a computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes, was used on the Greengenes-selected OTUs to generate metagenomic data, and to derive relative Kyoto Encyclopedia of Genes and Genome (KEGG) pathway abundance. KEGG data were analyzed using Statistical Analysis of Metagenomic Profiles (STAMP).

Statistical analysis

The SAS UNIVARIATE procedure (SAS 9.3, 2012) was used to test the normality of residuals for α biodiversity data. Non-normally distributed data were log transformed and then used to assess the effect of sampling using the SAS MIXED procedure. Phylum percentage data was also used to evaluate statistical differences among different days. The SAS MIXED procedure was used, as described above, to test for significant changes in the proportions of different phyla among the groups of interest. All the phyla were divided into two groups of abundant (above 1% of the population) and low-abundance (below 1% of the population). The differences between groups were considered significant at $P < 0.05$ while trends were observed at $P < 0.1$.

The scope of the claims should not be limited by the preferred embodiments set forth in the examples but should be given the broadest interpretation with the description as a whole.

5 **Table 1:** Relative abundance of bacterial phyla from pyrosequenced 16s rRNA sequences in fecal samples. A total of 328, 085 sequences were generated after quality-filtering steps, with an average of 27,340.42 high-quality sequences per sample. This resulted in identification of 10 phyla. Of the 10 phyla, 4 phyla were considered as abundant within the population ($\geq 1\%$), including Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. The other six phyla were considered less abundant within the population ($< 1\%$), including Actinobacteria, Cyanobacteria, Fibrobacteres, TM7, Tenericutes, and Verrucomicrobia.

Phylum	Groups	Mean percentage of sequence in total bacterial population	Std. Error Mean
Unclassified	Control	0.496921	0.092571
	CTS	0.414249	0.04264
Actinobacteria	Control	0.511464	0.202226
	CTS	0.187706	0.09371
Bacteroidetes	Control	59.59139	4.253899
	CTS	73.99889	2.680188
Cyanobacteria	Control	0.017379	0.006038
	CTS	0.152001	0.041196
Deferribacteres	Control	1.709693	1.021632
	CTS	1.143747	0.782717
Fibrobacteres	Control	0.001941	0.00115
	CTS	0.000541	0.000541
Firmicutes	Control	33.80289	3.56062
	CTS	20.55645	1.827242
Proteobacteria	Control	2.218421	0.409325
	CTS	2.872719	0.488761

TM7	Control	0.003883	0.002301
	CTS	0.003098	0.001035
Tenericutes	Control	0.314479	0.089961
	CTS	0.404554	0.187125
Verrucomicrobia	Control	1.331539	0.730169
	CTS	0.266048	0.129101

Table 2: Relative abundance of bacterial phyla from pyrosequenced 16s rRNA sequences in colonic mucosa associated samples. A total of 207, 123 sequences were generated after quality-filtering steps, with an average of 18, 829.36 high-quality sequences per sample. This resulted in identification of 19 phyla. Of the 19 phyla, 4 phyla were considered as abundant within the population ($\geq 1\%$), including Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. The other 15 phyla were considered less abundant within the population ($< 1\%$), including Acidobacteria, Actinobacteria, Armatimonadetes, Chlamydiae, Chlorobi, Cyanobacteria, Fibrobacteres, Lentisphaerae, OD1, OP3, Planctomycetes, Spirochaetes, TM7, Tenericutes, and Verrucomicrobia.

Phylum	Groups	Mean percentage of sequence in total bacterial population	Std. Error Mean
Unclassified	Control	0.122989	0.050167
	CTS	0.291527	0.19307
Acidobacteria	Control	0.025735	0.010402
	CTS	0.033388	0.00685
Actinobacteria	Control	0.095085	0.095085
	CTS	0.072405	0.031928
Armatimonadetes	Control	0	0
	CTS	0.000879	0.000879
Bacteroidetes	Control	8.06612	5.42359
	CTS	21.0435	6.556034
Chlamydiae	Control	0	0
	CTS	0.00306	0.002007
Chlorobi	Control	0	0
	CTS	0.002098	0.002098
Cyanobacteria	Control	0.039247	0.022309

	CTS	0.175241	0.063441
Deferribacteres	Control	6.923494	3.656936
	CTS	5.949701	2.168418
Fibrobacteres	Control	0	0
	CTS	0.013779	0.012973
Firmicutes	Control	10.13424	6.855156
	CTS	12.51866	5.161134
Lentisphaerae	Control	0	0
	CTS	0.00457	0.00457
OD1	Control	0	0
	CTS	0.004988	0.00332
OP3	Control	0	0
	CTS	0.001604	0.001604
Planctomycetes	Control	0.002067	0.002067
	CTS	0.004664	0.001662
Proteobacteria	Control	72.02106	16.32041
	CTS	59.16764	11.01085
Spirochaetes	Control	0	0
	CTS	0.082258	0.082258
TM7	Control	0	0
	CTS	0.007409	0.00482
Tenericutes	Control	0.037096	0.019253
	CTS	0.371448	0.170574

Verrucomicrobia	Control	2.532872	2.52412
	CTS	0.142801	0.117295

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CLAIMS

1. A method for increasing levels of Bacteroidetes relative to levels of other bacteria in the gut of an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

5 2. A method for decreasing relative levels of Firmicutes relative to other bacteria in the gut of an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

3. Use of catestatin (CTS) for increasing levels of Bacteroidetes relative to levels of other bacteria in the gut of an individual in need of such treatment.

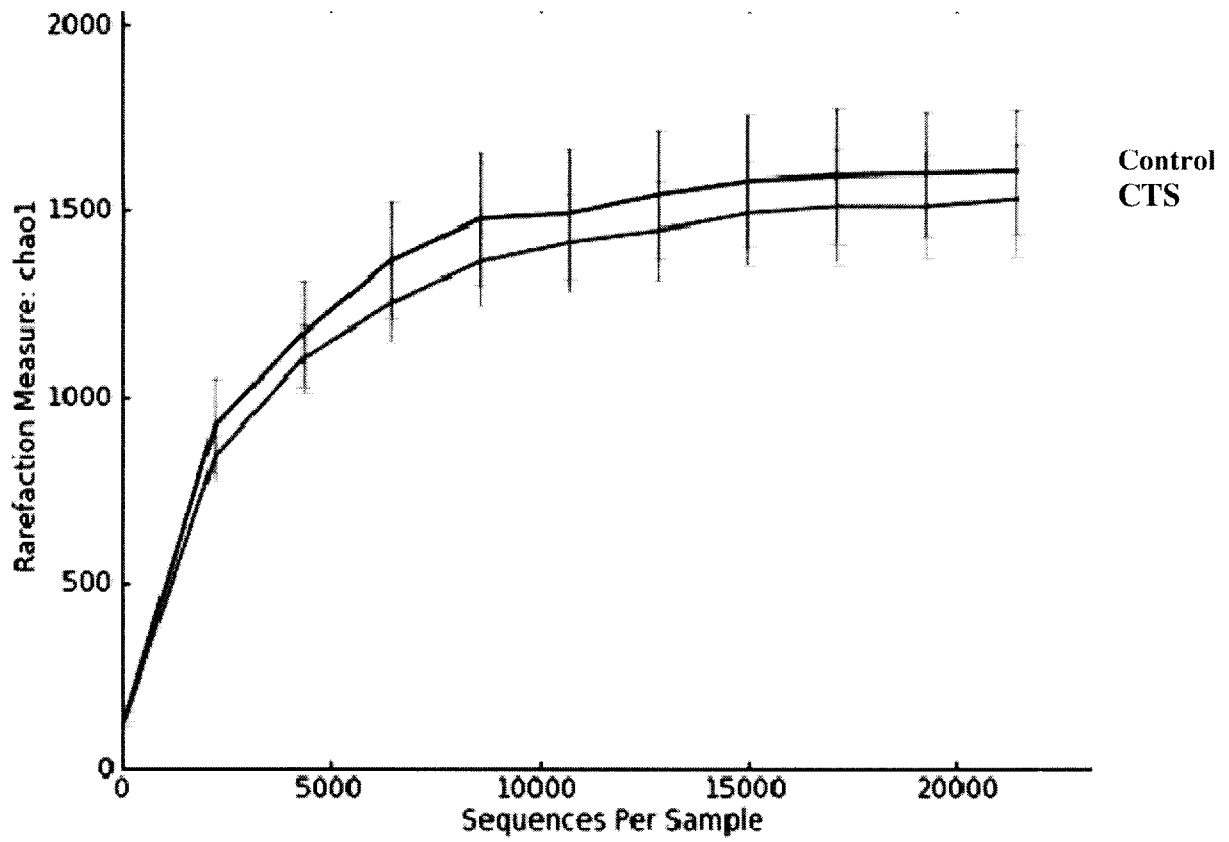
10 4. Use of catestatin (CTS) for decreasing relative levels of Firmicutes relative to other bacteria in the gut of an individual in need of such treatment.

5. A method of modulating gut microbiota composition comprising administering to an individual in need of such treatment an effective amount of catestatin (CTS).

15 6. Use of catestatin (CTS) for modulating gut microbiota composition.

7. A method of treating or preventing or prophylactically treating type 1 diabetes, obesity, IBS or IBD in an individual in need of such treatment comprising administering to said individual an effective amount of catestatin (CTS).

20 8. Use of catestatin (CTS) for treating or preventing or prophylactically treating type 1 diabetes, obesity, IBS or IBD in an individual in need of such treatment.

**Figure1:**

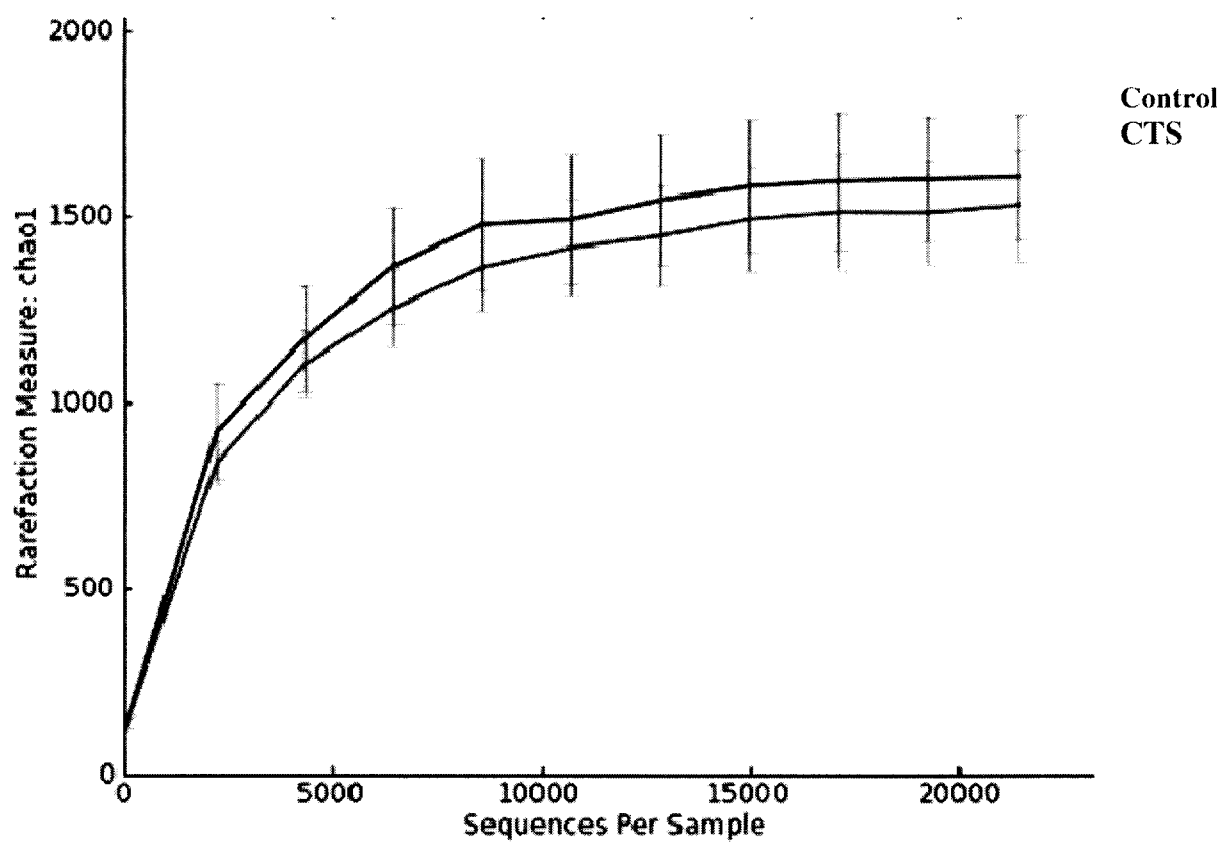


Figure 2:

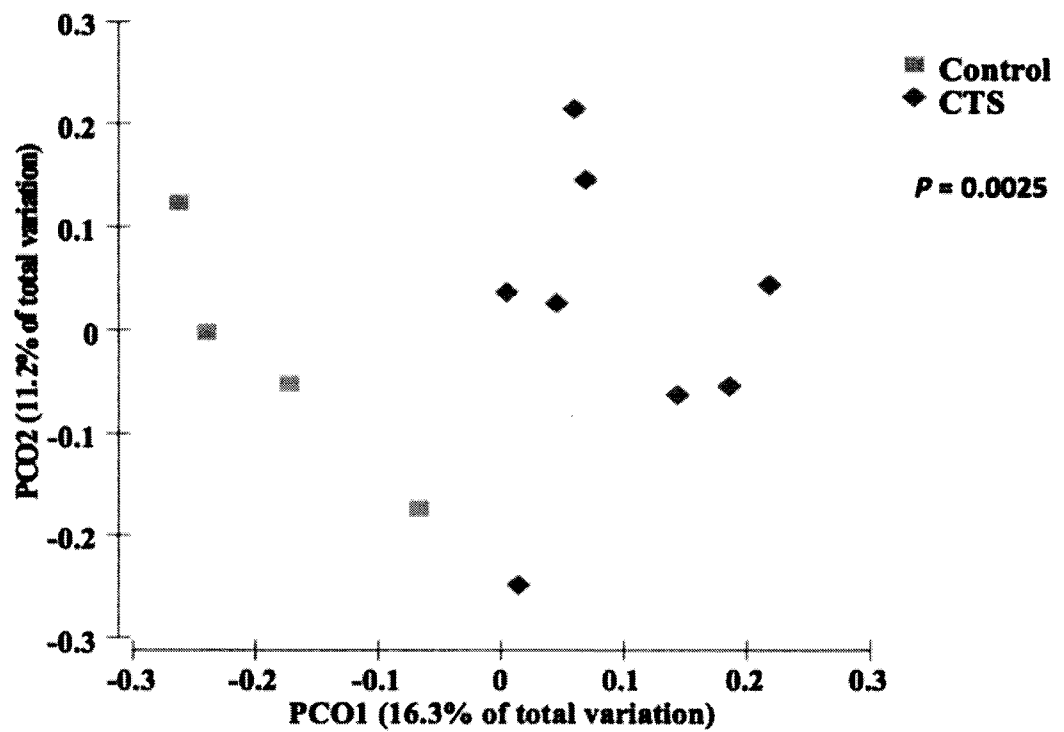


Figure 3:

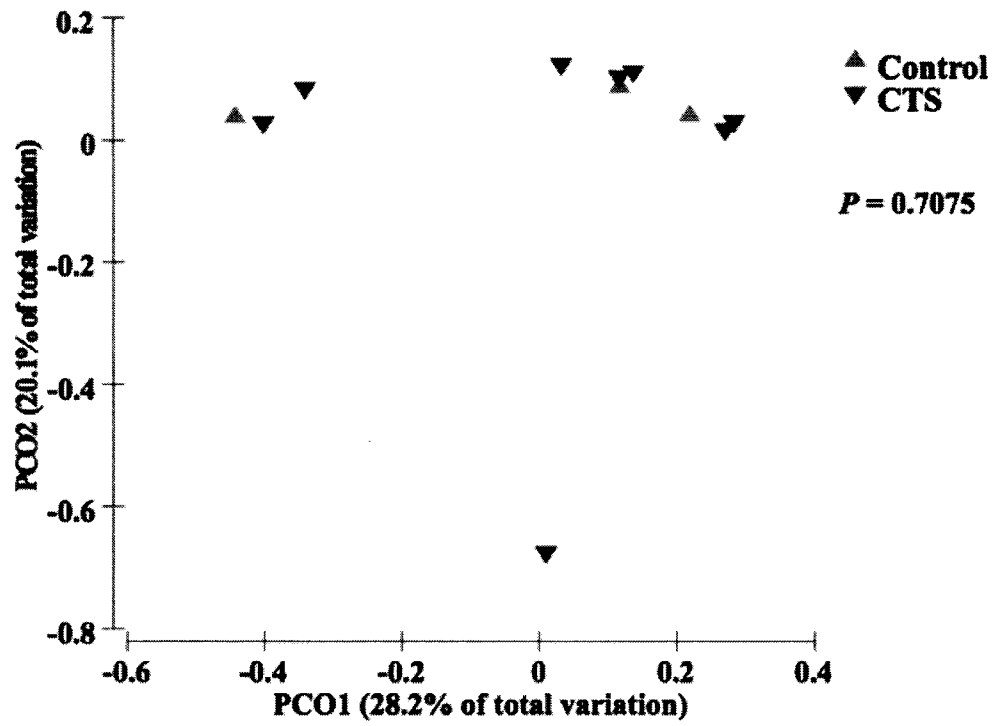
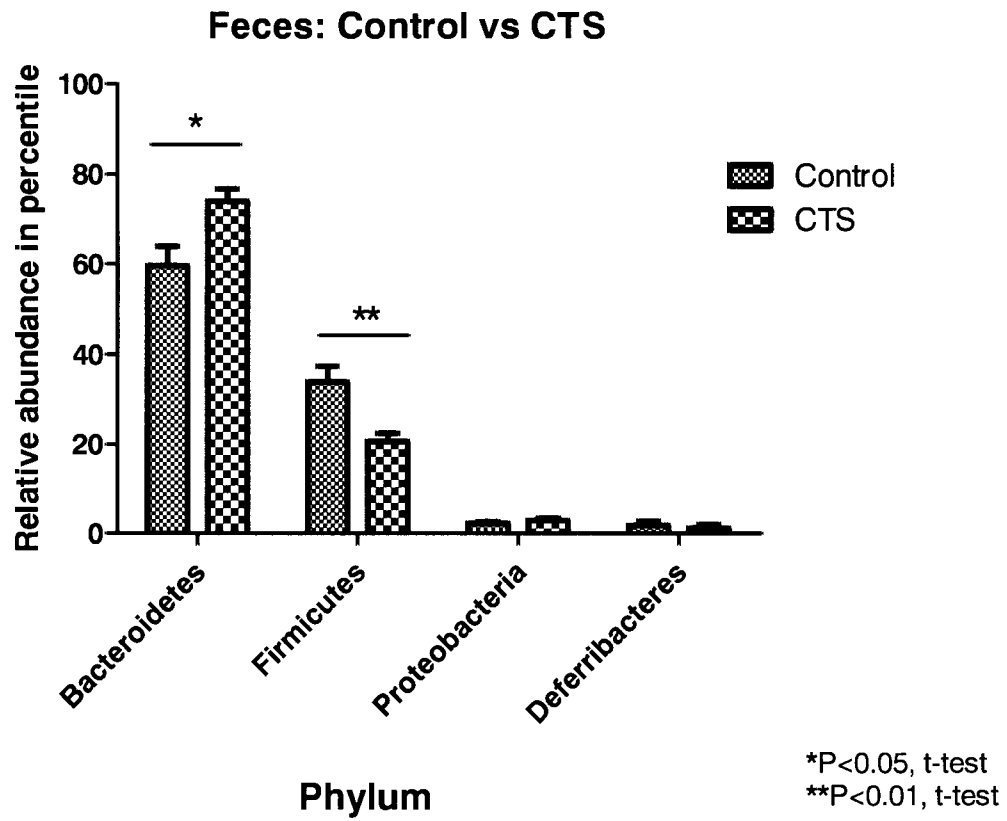


Figure 4:

**Figure 5:**

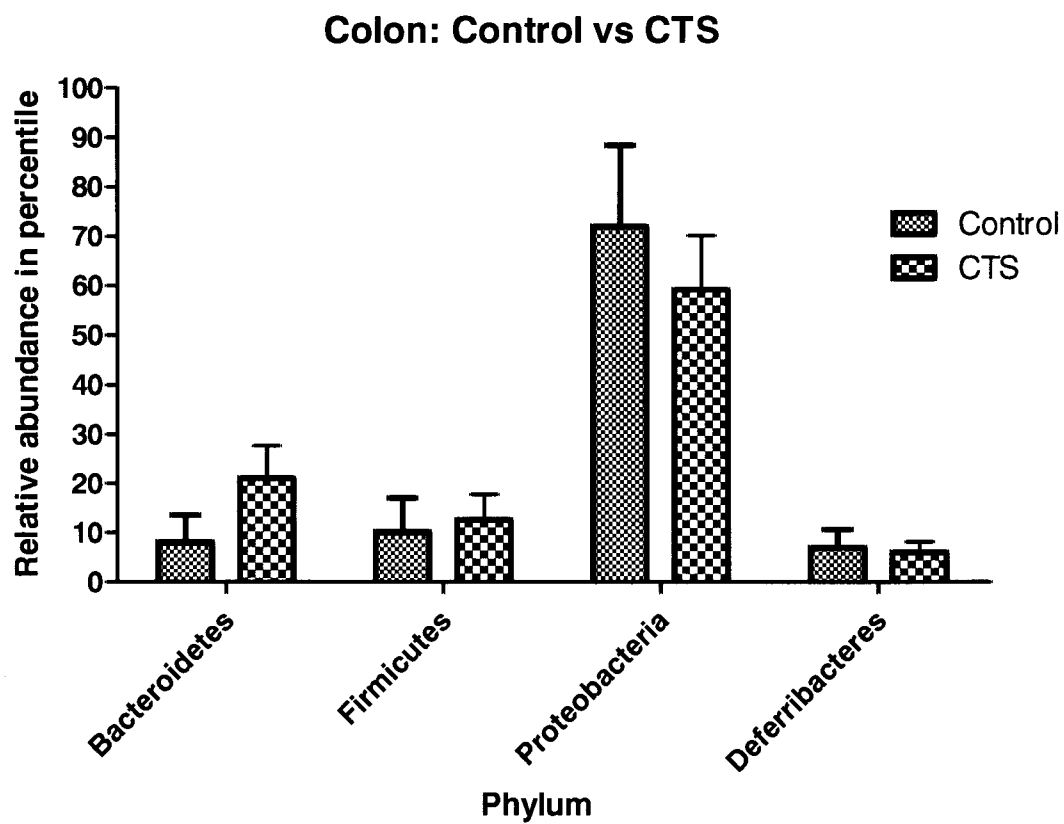
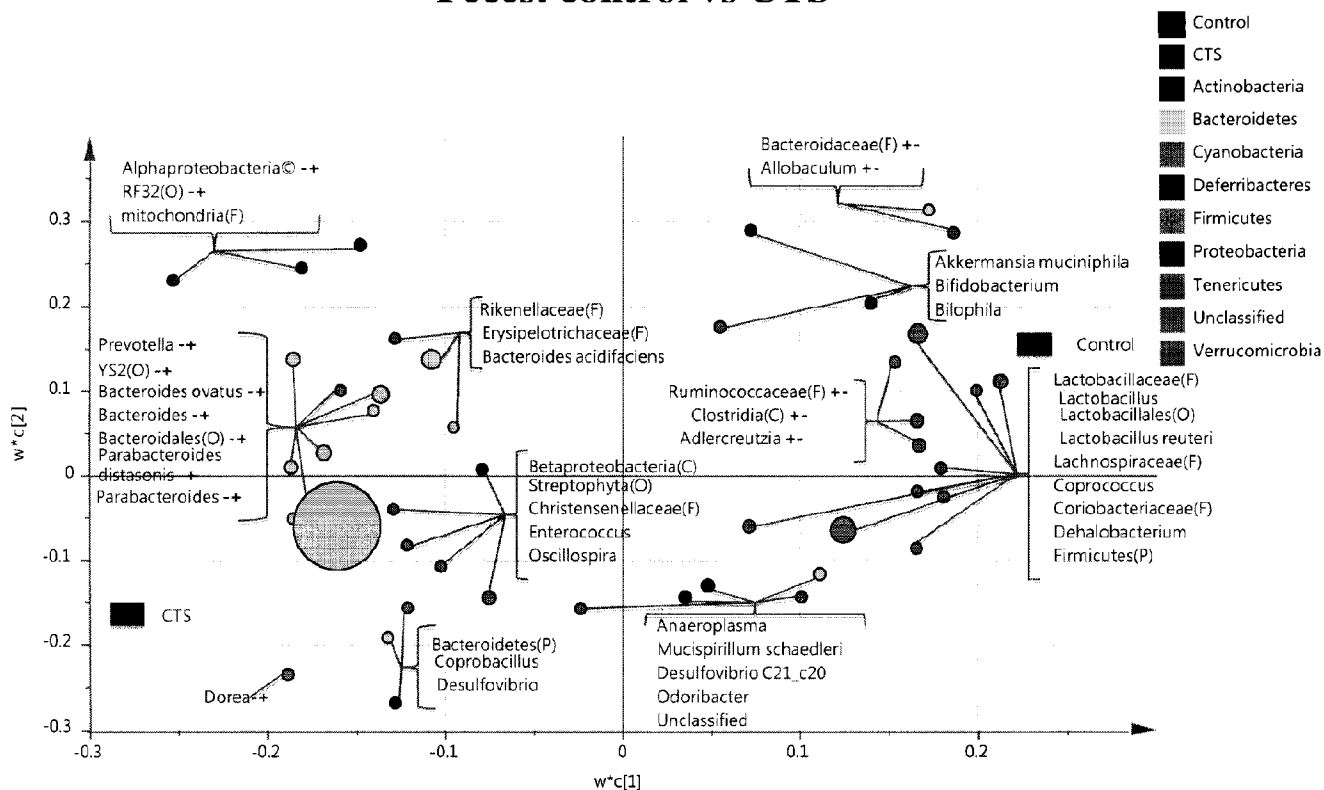


Figure 6:

7/9

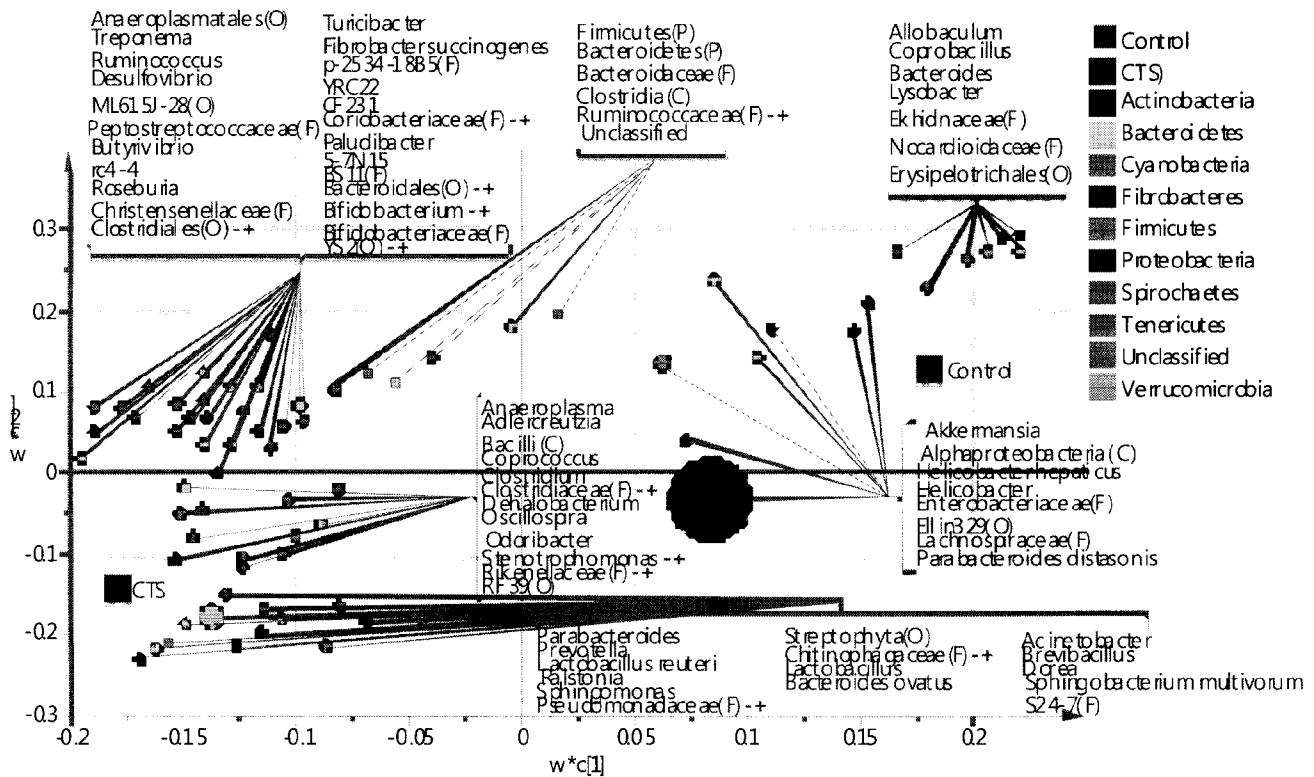
Feces: control vs CTS

(+,-) a positive or negative association with Control
 (+,-) a positive or negative association with CTS

R² = 0.94 Q² = 0.57, based on 2 components

Figure 7

Colon: Control vs CTS



(+, -) a positive or negative association with Control

(+, -) a positive or negative association with CTS

$R^2 = 0.94$ $Q^2 = 0.57$, based on 2 components

Figure 8:

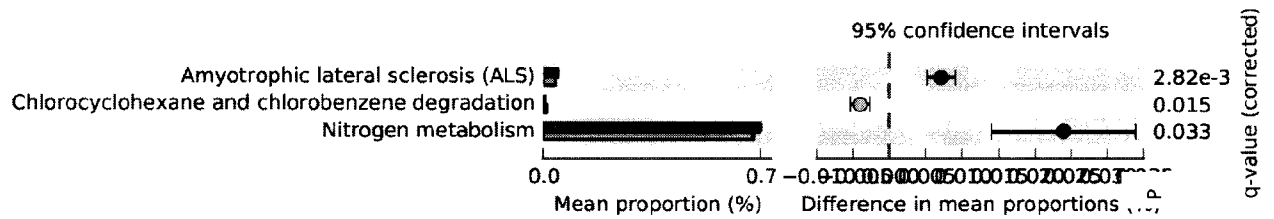


Figure 9. Subsystems and pathways enriched or decreased within the CTS or (Control) mice fecal samples. Corrected P-values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented in the CTS or Control mice fecal samples have a positive or (negative) difference between mean proportions and are indicated by purple or (orange) coloring respectively.

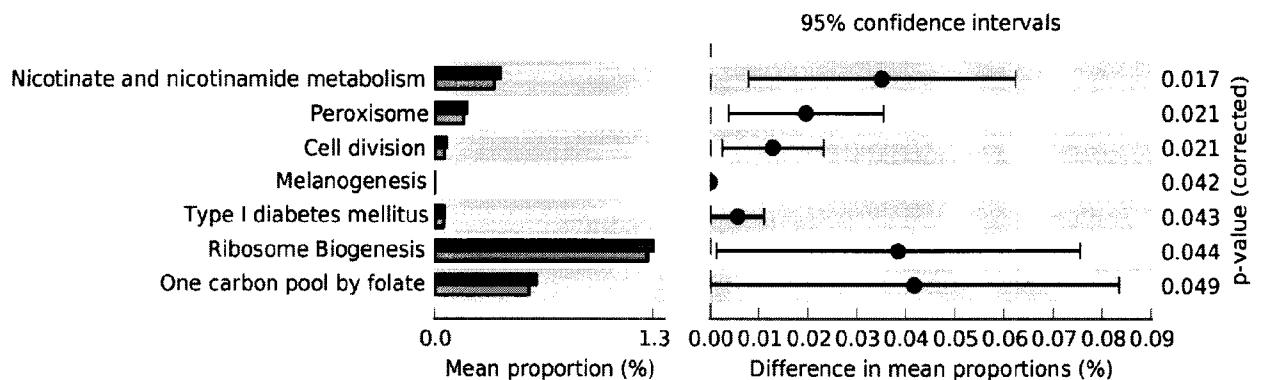


Figure 10. Subsystems and pathways enriched or decreased within the CTS or (Control) mice colon samples. Subsystems or pathways overrepresented in the CTS or Control mice colon samples have a positive or (negative) difference between mean proportions and are indicated by Purple or (orange) coloring respectively.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2016/050477

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 38/17** (2006.01), **A61P 3/00** (2006.01), **C07K 14/575** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: **A61K 38/17** (2006.01), **A61P 3/00** (2006.01), **C07K 14/575** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: STN (Medline CaPlus, BIOSIS), Questel-Orbit, PubMed

Keywords: Catestatin, CTS, gut, intestine*, mucos*, micro*, flora, fec*, faec*, antimicrob*, antibacter*, *bacter*, *microb*, treat*, prevent*, Ghia, Rabbi, Khafipour, Munyaka

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2014188373A1 (GHIA, J.-E. et al.) 27 November 2014 (27-11-2014) see page 44, last paragraph	1-6
X	Rabbi, M.F. et al. Catestatin decreases macrophage function in two mouse models of experimental colitis. 2014. Biochem. Pharmacol. 89(3):386-398, ISSN 1873-2968 see page 397, third full paragraph	1-6
A	SHOOSHTARIZADEH, P et al. The antimicrobial peptides derived from chromogranin/secretogranin family, new actors of innate immunity. 2010. Regul Pept. 165(1):102-110, ISSN 1873-1686 See whole document	1-6

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search

28 July 2016 (28-07-2016)

Date of mailing of the international search report

04 August 2016 (04-08-2016)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2016/050477

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRIOLAT, J. New antimicrobial activity for the catecholamine release-inhibitory peptide from chromogranin A. Cell Mol Life Sci. 2005. 62(3):377-385, ISSN 1420-682X See whole document	1-6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2016/050477**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 1, 2 and 5
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1, 2 and 5 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. The product recited in these claims, which may have therapeutic benefits, is administered to a subject. Regardless, this Authority has carried out a search based on the alleged effects or purposes/uses of the product(s) referred to in claims 1, 2 and 5.
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

1-6

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III

This international Searching Authority found multiple inventions (groups) in this international application as follows:

Group 1: Claims 1-6

A method of modulating gut microbiota composition comprising administering to an individual an effective amount of catestatin.

Group 2: Claims 7-8 (partially)

A method of treating or preventing type 1 diabetes in an individual comprising administering to an individual an effective amount of catestatin.

Group 3: Claims 7-8 (partially)

A method of treating or preventing obesity in an individual comprising administering to an individual an effective amount of catestatin.

Group 4: Claims 7-8 (partially)

A method of treating or preventing irritable bowel syndrome (IBS) in an individual comprising administering to an individual an effective amount of catestatin.

Group 5: Claims 7-8 (partially)

A method of treating or preventing inflammatory bowel disease (IBD) in an individual comprising administering to an individual an effective amount of catestatin.

The requirements of unity of invention are not fulfilled in that there is no technical relationship among the inventions as they do not involve one or more of the same or corresponding technical features. The expression "special technical features" means those features which define a contribution which each of the claimed inventions considered as a whole makes over the prior art.

The technical relationship among the inventions appears to be catestatin. Catestatin is known as is its effectiveness to treat diseases. See for example, WO2013149259 (2013-10-03; MAHATA et al.) and WO2009137091 (2009-11-12; MAHATA et al.) which teach a method of treating obesity and diabetes respectively. Treatable diseases also include disorders of the gut such as colitis (see RABBI et al. (Biochem Pharmacol.; 2014, 89:386-98). As well, it was known that catestatin has anti-microbial activity (see BOMAN et al., Infect Immun; 1993, 61:377-85; DORSCHNER et al., J Invest Dermatol., 2001; 117:91-97) which would be directly related to Group 1 and anti-inflammatory activity (see RABBI, supra). For these reasons, the listed inventions cannot be united by a single general inventive concept.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2016/050477

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2014188373A1	27 November 2014 (27-11-2014)	None	