



US009827276B2

(12) **United States Patent**
Honda et al.(10) **Patent No.:** **US 9,827,276 B2**(45) **Date of Patent:** ***Nov. 28, 2017**(54) **COMPOSITION FOR INDUCING
PROLIFERATION OR ACCUMULATION OF
REGULATORY T CELLS**(71) Applicant: **The University of Tokyo**, Tokyo (JP)(72) Inventors: **Kenya Honda**, Tokyo (JP); **Koji Atarashi**, Tokyo (JP); **Kikuji Itoh**, Tokyo (JP); **Takeshi Tanoue**, Tokyo (JP)(73) Assignee: **The University of Tokyo**, Tokyo (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/472,840**(22) Filed: **Mar. 29, 2017**(65) **Prior Publication Data**

US 2017/0232044 A1 Aug. 17, 2017

Related U.S. Application Data

(63) Continuation of application No. 15/216,015, filed on Jul. 21, 2016, which is a continuation of application No. 14/492,850, filed on Sep. 22, 2014, now Pat. No. 9,433,652, which is a continuation of application No. 13/701,467, filed as application No. PCT/JP2011/063302 on Jun. 3, 2011, now abandoned.

(30) **Foreign Application Priority Data**Jun. 4, 2010 (JP) 2010-129134
Dec. 3, 2010 (WO) PCT/JP2010/071746(51) **Int. Cl.****A61K 35/74** (2015.01)**A61K 35/742** (2015.01)**A61K 9/00** (2006.01)(52) **U.S. Cl.**CPC **A61K 35/742** (2013.01); **A61K 9/0053** (2013.01)(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited****U.S. PATENT DOCUMENTS**4,576,936 A 3/1986 MacDonald
5,599,795 A 2/1997 McCann et al.
5,700,787 A 12/1997 Tzianabos et al.
6,348,452 B1 2/2002 Brown et al.
6,551,632 B2 4/2003 Borody
6,645,530 B1 11/2003 Borody
7,629,330 B2 12/2009 Wang et al.
7,749,494 B2 7/2010 Renaud et al.
8,906,668 B2 12/2014 Henn et al.
9,028,841 B2 5/2015 Henn et al.9,415,079 B2 8/2016 Honda et al.
9,421,230 B2 8/2016 Honda et al.
9,433,652 B2 9/2016 Honda et al.
9,533,014 B2 1/2017 Henn et al.
9,610,307 B2 4/2017 Berry et al.
9,642,881 B2 5/2017 Honda et al.
9,642,882 B2 5/2017 Honda et al.
9,649,345 B2 5/2017 Honda et al.
9,662,381 B2 5/2017 Honda et al.
2003/0113306 A1 6/2003 Collins et al.
2004/0028689 A1 2/2004 Borody
2004/0170617 A1 9/2004 Finegold
2004/0219160 A1 11/2004 Tzianabos et al.
2006/0067924 A1 3/2006 Lee et al.
2006/0240482 A1 10/2006 Kwok et al.
2007/0258953 A1 11/2007 Duncan et al.
2008/0003207 A1 1/2008 Cui
2008/0305089 A1 12/2008 Bufe et al.
2008/0311080 A1 12/2008 Collins et al.
2009/0124573 A1 5/2009 Mazmanian et al.
2009/0317427 A1 12/2009 Kasper et al.
2010/0119488 A1 5/2010 Huber-Haag et al.
2010/0275282 A1 10/2010 Round et al.
2011/0009360 A1 1/2011 Kasper et al.
2012/0027734 A1 2/2012 Van Immerseel et al.
2012/0276149 A1 11/2012 Littman et al.

(Continued)

FOREIGN PATENT DOCUMENTSCA 2850000 A1 4/2013
CN 101310730 A 11/2008

(Continued)

OTHER PUBLICATIONS[No Author Listed] Ruminococcus. Microbe Wiki. Aug. 2010 Last accessed at <https://microbewiki.kenyon.edu/index.php/Ruminococcus> on Apr. 19, 2016.

Abraham et al., Molecular mechanisms of IL-2 gene regulation following costimulation through LFA-1. J Immunol. Nov. 1, 2001;167(9):5193-201.

Abrams, Open-Label, Uncontrolled Trial of Bowel Sterilization and Repopulation With Normal Bowel Flora for Treatment of Inflammatory Bowel Disease. Current Therapeutic Research. Dec. 1997;58(12):1001-1012.

Andoh et al., Faecal microbiota profile of Crohn's disease determined by terminal restriction fragment length polymorphism analysis. Aliment Pharmacol Ther. Jan. 2009;29(1):75-82. doi: 10.1111/j.1365-2036.2008.03860.x. Epub Sep. 26, 2008.

Atarashi et al., ATP drives lamina propria T(H)17 cell differentiation. Nature. Oct. 9, 2008;455(7214):808-12. doi: 10.1038/nature07240. Epub Aug. 20, 2008.

(Continued)

Primary Examiner — Brian Gangle(74) *Attorney, Agent, or Firm* — Wolf, Greenfield & Sacks, P.C.

(57)

ABSTRACTIt was found that bacteria belonging to the genus *Clostridium* induce accumulation of regulatory T cells (Treg cells) in the colon. Moreover, the present inventors found that regulatory T cells (Treg cells) induced by from these bacteria suppressed proliferation of effector T-cells. From these findings, the present inventors found that the use of bacteria belonging to the genus *Clostridium* or a physiologically active substance derived therefrom made it possible to induce proliferation or accumulation of regulatory T cells (Treg cells), and further to suppress immune functions.**30 Claims, 40 Drawing Sheets**

(56)

References Cited

U.S. PATENT DOCUMENTS

2013/0149339	A1	6/2013	Honda et al.
2014/0147425	A1	5/2014	Henn et al.
2014/0171339	A1	6/2014	Keku et al.
2014/0341921	A1	11/2014	Honda et al.
2015/0143557	A1	5/2015	Honda et al.
2015/0224152	A1	8/2015	Littman et al.
2015/0297642	A1	10/2015	Borody
2015/0320805	A9	11/2015	Honda et al.
2016/0143960	A1	5/2016	Honda et al.
2016/0151430	A1	6/2016	Honda et al.
2016/0193256	A1	7/2016	Honda et al.
2016/0193257	A1	7/2016	Honda et al.
2016/0199423	A1	7/2016	McKenzie et al.
2016/0199424	A1	7/2016	Berry et al.
2016/0235792	A1	8/2016	Berry et al.
2016/0243172	A1	8/2016	Cook et al.
2016/0271188	A1	9/2016	Berry et al.
2016/0279177	A1	9/2016	Kelly et al.
2017/0007691	A1	1/2017	Riken et al.
2017/0028061	A1	2/2017	Riken et al.
2017/0087197	A1	3/2017	Honda et al.
2017/0105977	A1	4/2017	Golden et al.
2017/0112915	A1	4/2017	Honda et al.
2017/0209502	A1	7/2017	Honda et al.
2017/0216378	A1	8/2017	Honda et al.
2017/0232045	A1	8/2017	Honda et al.

FOREIGN PATENT DOCUMENTS

DE	102006062250	A1	6/2008
EP	1749538	A1	2/2007
EP	1955706	A1	8/2008
JP	2009-084215	A	4/2009
WO	WO 2009/050486	A2	4/2009
WO	WO 2009/149149	A1	12/2009
WO	WO 2011/005756	A1	1/2011
WO	WO 2011/022542	A2	2/2011
WO	WO 2011/022660	A1	2/2011
WO	WO 2011/027990	A2	3/2011
WO	WO 2011/151941	A1	12/2011
WO	WO 2011/152566	A2	12/2011
WO	WO 2013/080561	A1	6/2013
WO	WO 2015/156419	A1	10/2015
WO	WO 2016/194427	A1	12/2016

OTHER PUBLICATIONS

Atarashi et al., Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*. Jan. 21, 2011;331(6015):337-41. doi: 10.1126/science.1198469. Epub Dec. 23, 2010.

Atarashi et al., Microbiota in autoimmunity and tolerance. *Curr Opin Immunol*. Dec. 2011;23(6):761-8. doi: 10.1016/j.coi.2011.11.002. Epub Nov. 22, 2011.

Atarashi et al., Microbial influence on T cell subset development. *Seminars in Immunology*. Apr. 4, 2011;23(2):146-153.

Atarashi et al., Treg induction by a rationally selected mixture of *Clostridia* strains from the human microbiota. *Nature*. Aug. 8, 2013;500(7461):232-6. doi: 10.1038/nature12331. Epub Jul. 10, 2013.

Barnes et al., Regulatory T cells reinforce intestinal homeostasis. *Immunity*. Sep. 18, 2009;31(3):401-11. doi: 10.1016/j.immuni.2009.08.011.

Bassaganya-Riera et al., Punicic acid modulates mucosal immune responses and prevents gut inflammation through PPAR gamma and delta-dependent mechanisms. *FASEB J*. 2010; 24 (Meeting Abstract Supplement). Abstract.

Bassaganya-Riera et al., Soluble fibers and resistant starch ameliorate disease activity in an experimental model of inflammatory bowel disease. *FASEB J*. 2010; 24 (Meeting Abstract Supplement). Abstract.

Borody et al., Treatment of ulcerative colitis using fecal bacteriotherapy. *J Clin Gastroenterol*. Jul. 2003;37(1):42-7.

Bouskra et al., Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature*. Nov. 27, 2008;456(7221):507-10. doi: 10.1038/nature07450. Epub Nov. 5, 2008.

Browne et al., Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature*. May 4, 2016. doi: 10.1038/nature17645.

Cebra, Influences of microbiota on intestinal immune system development. *Am J Clin Nutr*. May 1999;69(5):1046S-1051S.

Chandrasekaran et al., *Clostridium* difficile Toxin B blocks effector T cells proliferation by inhibiting PLD signaling. *J. Immunol*. Apr. 2010; 184(1): 49.19. Abstract.

Clavel et al., Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating human stomach. *J Appl Microbiol*. 2004;97(1):214-9.

Collins et al., The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol*. Oct. 1994;44(4):812-26.

Curotto De LaFaille et al., Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity*. May 2009;30(5):626-35. doi: 10.1016/j.immuni.2009.05.002.

Di Giacinto et al., Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. *J Immunol*. Mar. 15, 2005;174(6):3237-46.

Eeckhaut et al., The anaerobic butyrate-producing strain *Butyrivibrio pullicaecorum* decreases colonic inflammation and ulceration in a TNBS-induced colitis rat model. In, 5th Probiotics, Prebiotics and New Foods Congress, Rome, Italy (2009).

Ellis et al., Molecular characterization of stool microbiota in HIV-infected subjects by panbacterial and order-level 16S ribosomal DNA (rDNA) quantification and correlations with immune activation. *J Acquir Immune Defic Syndr*. Aug. 15, 2011;57(5):363-70. doi: 10.1097/QAI.0b013e31821a603c.

Favier et al., Development of bacterial and bifidobacterial communities in feces of newborn babies. *Anaerobe*. Oct. 2003;9(5):219-29.

Foditsch et al., Isolation and characterization of *Faecalibacterium prausnitzii* from calves and piglets. *PLoS One*. Dec. 31, 2014;9(12):e116465. doi: 10.1371/journal.pone.0111645. eCollection 2014.

Foligne et al., A key role of dendritic cells in probiotic functionality. *PLoS One*. Mar. 21, 2007;2(3):e313.

Foligne et al., Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World J Gastroenterol*. Jan. 14, 2007;13(2):236-43.

Frank et al., Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A*. Aug. 21, 2007;104(34):13780-5. Epub Aug. 15, 2007.

Gaboriau-Routhiau et al., The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity*. Oct. 16, 2009;31(4):677-89. doi: 10.1016/j.immuni.2009.08.020.

Garrett et al., Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell*. Oct. 5, 2007;131(1):33-45.

Geuking et al., Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity*. May 27, 2011;34(5):794-806. doi: 10.1016/j.immuni.2011.03.021. Epub May 19, 2011.

Grehan et al., Durable alteration of the colonic microbiota by the administration of donor fecal flora. *J Clin Gastroenterol*. Sep. 2010;44(8):551-61. doi: 10.1097/MCG.0b013e3181e5d06b.

Hart et al., Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut*. Nov. 2004;53(11):1602-9.

Hata et al., Blood group B degrading activity of *Ruminococcus gnavus* alpha-galactosidase. *Artif Cells Blood Substit Immobil Biotechnol*. May 2004;32(2):263-74.

Hayashi et al., Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. *Microbiol Immunol*. 2002;46(12):819-31.

Hayashi et al., Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiol Immunol*. 2002;46(8):535-48.

(56)

References Cited

OTHER PUBLICATIONS

- Hazenberget al., Effects of the human intestinal flora on germ-free mice. *J Appl Bacteriol.* Feb. 1981;50(1):95-106.
- Itoh et al., Characterization of clostridia isolated from faeces of limited flora mice and their effect on caecal size when associated with germ-free mice. *Lab Anim.* Apr. 1985;19(2):111-8.
- Itoh et al., Colonization resistance against *Pseudomonas aeruginosa* in gnotobiotic mice. *Lab Anim.* Jul. 1986;20(3):197-201.
- Itoh et al., Intestinal bacteria antagonistic to *Clostridium difficile* in mice. *Lab Anim.* Jan. 1987;21(1):20-5.
- Itoh et al., Production of gnotobiotic mice with normal physiological functions. I. Selection of useful bacteria from feces of conventional mice. *Z Versuchstierkd.* 1980;22(3):173-8.
- Ivanov et al., Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell.* Oct. 30, 2009;139(3):485-98. doi: 10.1016/j.cell.2009.09.033.
- Ivanov et al., Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe.* Oct. 16, 2008;4(4):337-49. doi: 10.1016/j.chom.2008.09.009.
- Jarry et al., Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. *J Clin Invest.* Mar. 2008;118(3):1132-42.
- Kamanaka et al., Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity.* Dec. 2006;25(6):941-52. Epub Nov. 30, 2006.
- Karimi et al., *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med.* Feb. 1, 2009;179(3):186-93. doi: 10.1164/rccm.200806-951OC. Epub Nov. 21, 2008.
- Khoruts et al., Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea. *J Clin Gastroenterol.* May-Jun. 2010;44(5):354-60. doi: 10.1097/MCG.0b013e3181c87e02.
- Kwon et al., Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci U S A.* Feb. 2, 2010;107(5):2159-64. doi: 10.1073/pnas.0904055107. Epub Jan. 13, 2010.
- Latvala et al., Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World J Gastroenterol.* Sep. 28, 2008;14(36):5570-83; discussion 5581-2.
- Lawley et al., Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. *PLoS Pathog.* 2012;8(10):e1002995. doi: 10.1371/journal.ppat.1002995. Epub Oct. 25, 2012.
- Li et al., Effect of oral feeding with *Clostridium leptum* on regulatory T-cell responses and allergic airway inflammation in mice. *Ann Allergy Asthma Immunol.* Sep. 2012;109(3):201-7. doi: 10.1016/j.anai.2012.06.017. Epub Jul. 17, 2012.
- Li et al., Symbiotic gut microbes modulate human metabolic phenotypes. *Proc Natl Acad Sci U S A.* Feb. 12, 2008;105(6):2117-22. doi: 10.1073/pnas.0712038105.
- Liu et al., Reclassification of *Clostridium coccoides*, *Ruminococcus hansenii*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus luti*, *Ruminococcus productus* and *Ruminococcus schinkii* as *Blautia coccoides* gen. nov., comb. nov., *Blautia hansenii* comb. nov., *Blautia hydrogenotrophica* comb. nov., *Blautia luti* comb. nov., *Blautia producta* comb. nov., *Blautia schinkii* comb. nov. and description of *Blautia wexlerae* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol.* Aug. 2008;58(Pt 8):1896-902. doi: 10.1099/ijs.0.65208-0.
- Livingston et al., Gut commensal *Lactobacillus reuteri* 100-23 stimulates an immunoregulatory response. *Immunol Cell Biol.* Jan. 2010;88(1):99-102. doi: 10.1038/icb.2009.71. Epub Sep. 29, 2009.
- Lopetuso et al., Commensal clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog.* Aug. 13, 2013;5(1):23. doi: 10.1186/1757-4749-5-23.
- Louis et al., Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett.* May 2009;294(1):1-8. doi: 10.1111/j.1574-6968.2009.01514.x. Epub Feb. 13, 2009.
- Lu et al., Molecular orchestration of differentiation and function of regulatory T cells. *Genes Dev.* Jun. 1, 2009;23(11):1270-82. doi: 10.1101/gad.1791009.
- MacPherson et al., Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol.* Jun. 2004;4(6):478-85.
- Mandalari et al., In vitro evaluation of the prebiotic properties of almond skins (*Amygdalus communis* L.). *FEMS Microbiol Lett.* Mar. 2010;304(2):116-22. doi: 10.1111/j.1574-6968.2010.01898.x. Epub Jan. 11, 2010.
- Mangin et al., Molecular inventory of faecal microflora in patients with Crohn's disease. *FEMS Microbiol Ecol.* Oct. 1, 2004;50(1):25-36. doi: 10.1016/j.femsec.2004.05.005.
- Maslowski et al., Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature.* Oct. 29, 2009;461(7268):1282-6. doi: 10.1038/nature08530.
- Maynard et al., Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3-precursor cells in the absence of interleukin 10. *Nat Immunol.* Sep. 2007;8(9):931-41. Epub Aug. 12, 2007.
- Mazmanian et al., A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature.* May 29, 2008;453(7195):620-5. doi: 10.1038/nature07008.
- Mazmanian, Gut immune balance is as easy as S-F-B. *Immunity.* Oct. 16, 2009;31(4):536-8. doi: 10.1016/j.immuni.2009.09.005.
- Miquel et al., *Faecalibacterium prausnitzii* and human intestinal health. *Curr Opin Microbiol.* Jun. 2013;16(3):255-61. doi: 10.1016/j.mib.2013.06.003. Epub Jul. 3, 2013. Review.
- Miyake et al., Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to *Clostridia* XIVa and IV Clusters. *PLoS One.* Sep. 14, 2015;10(9):e0137429. doi: 10.1371/journal.pone.0137429. eCollection 2015.
- Narushima et al., Characterization of the 17 strains of regulatory T cell-inducing human-derived *Clostridia*. *Gut Microbes.* May-Jun. 2014;5(3):333-9. doi: 10.4161/gmic.28572. Epub Mar. 18, 2014.
- Okada et al., Effects of fecal microorganisms and their chloroform-resistant variants derived from mice, rats, and humans on immunological and physiological characteristics of the intestines of ex-germfree mice. *Infect Immun.* Dec. 1994;62(12):5442-6.
- O'Mahony et al., Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog.* Aug. 1, 2008;4(8):e1000112. doi: 10.1371/journal.ppat.1000112.
- Paredes-Sabja et al., *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends Microbiol.* Jul. 2014;22(7):406-16. doi: 10.1016/j.tim.2014.04.003. Epub May 7, 2014.
- Qin et al., A human gut microbial gene catalogue established by metagenomic sequencing. *Nature.* Mar. 4, 2010;464(7285):59-65. doi: 10.1038/nature08821.
- Qiu et al., *C. Faecalibacterium prausnitzii* upregulates regulatory T cells and anti-inflammatory cytokines in treating TNBS-induced colitis. *J Crohns Colitis.* Dec. 1, 2013;7(11):e558-68. doi: 10.1016/j.crohns.2013.04.002. Epub May 2, 2013.
- Rehman et al., Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients. *J Med Microbiol.* Sep. 2010;59(Pt 9):1114-22. doi: 10.1099/jmm.0.021170-0. Epub Jun. 3, 2010.
- Rohlke et al., Fecal flora reconstitution for recurrent *Clostridium difficile* infection: results and methodology. *J Clin Gastroenterol.* Sep. 2010;44(8):567-70. doi: 10.1097/MCG.0b013e3181dadab10.
- Rosero et al., Reclassification of *Eubacterium rectale* (Prévot et al., 1967) in a new genus *Agathobacter* gen. nov., as *Agathobacter rectalis* comb. nov., within the family *Lachnospiraceae*, and description of *Agathobacter ruminis* sp. nov., from the rumen. *Int J Syst Evol Microbiol.* Nov. 30, 2015. doi: 10.1099/ijsem.0.000788.

(56)

References Cited

OTHER PUBLICATIONS

- Rossi et al., *Faecalibacterium prausnitzii* A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. *Sci Rep.* Jan. 4, 2016;6:18507. doi:10.1038/srep18507.
- Round et al., Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A.* Jul. 6, 2010;107(27):12204-9. doi: 10.1073/pnas.0909122107. Epub Jun. 21, 2010.
- Round et al., The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol.* May 2009;9(5):313-23. doi:10.1038/nri2515. Review. Erratum in: *Nat Rev Immunol.* Aug. 2009;9(8):600.
- Rubtsov et al., Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity.* Apr. 2008;28(4):546-58. doi: 10.1016/j.immuni.2008.02.017.
- Sakaguchi et al., Regulatory T cells and immune tolerance. *Cell.* May 30, 2008;133(5):775-87. doi: 10.1016/j.cell.2008.05.009.
- Salzman et al., Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol.* Jan. 2010;11(1):76-83. doi:10.1038/ni.1825. Epub Oct. 22, 2009.
- Sanos et al., RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol.* Jan. 2009;10(1):83-91. doi: 10.1038/ni.1684. Epub Nov. 23, 2008.
- Schloss et al., The dynamics of a family's gut microbiota reveal variations on a theme. *Microbiome.* Jul. 21, 2014;2:25. doi:10.1186/2049-2618-2-25. eCollection 2014.
- Schouten et al., Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. *J Nutr.* Apr. 2010;140(4):835-41. doi: 10.3945/jn.109.116061. Epub Feb. 17, 2010.
- Segain et al., Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut.* Sep. 2000;47(3):397-403.
- Shen et al., Molecular profiling of the *Clostridium leptum* subgroup in human fecal microbiota by PCR-denaturing gradient gel electrophoresis and clone library analysis. *Appl Environ Microbiol.* Aug. 2006;72(8):5232-8.
- So et al., *Lactobacillus casei* potentiates induction of oral tolerance in experimental arthritis. *Mol Immunol.* Nov. 2008;46(1):172-80. doi: 10.1016/j.molimm.2008.07.038. Epub Sep. 19, 2008.
- So et al., *Lactobacillus casei* suppresses experimental arthritis by down-regulating T helper 1 effector functions. *Mol Immunol.* May 2008;45(9):2690-9. doi:10.1016/j.molimm.2007.12.010. Epub Feb. 19, 2008.
- Sokol et al., *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A.* Oct. 28, 2008;105(43):16731-6. doi: 10.1073/pnas.0804812105. Epub Oct. 20, 2008.
- Song et al., *Clostridium bartlettii* sp. nov., isolated from human faeces. *Anaerobe.* Jun. 2004;10(3):179-84.
- Sydora et al., CD4+CD25+ regulatory T cells have divergent effects on intestinal inflammation in IL-10 gene-deficient mice. *Dig Dis Sci.* Jun. 2008;53(6):1544-52.
- Tanoue et al., Immune responses to gut microbiota-commensals and pathogens *Gut Microbes.* Jul.-Aug. 2010; 1(4): 224-233.
- Tanoue et al., Regulation of intestinal Th17 and Treg cells by gut microbiota. *Inflammation Regeneration.* May 2015;35(3):99-105.
- Tao et al., Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med.* Nov. 2007;13(11):1299-307. Epub Oct. 7, 2007.
- Thibault et al., Butyrate utilization by the colonic mucosa in inflammatory bowel diseases: a transport deficiency. *Inflamm Bowel Dis.* Apr. 2010;16(4):684-95. doi:10.1002/ibd.21108.
- Third Party Observations filed in European Patent Application No. 11728077.6 on Jan. 29, 2016.
- Umesaki et al., Differential roles of segmented filamentous bacteria and *Clostridia* in development of the intestinal immune system. *Infect Immun.* Jul. 1999;67(7):3504-11.
- Valcheva et al., Prebiotics Prevent Loss of Intestinal Biodiversity and Reduce Colitis in Hla-B27 Transgenic Rats. *Canadian Digestive Diseases Week.* Feb. 2009 Poster Session 2-Immunology and Inflammatory Bowel Disease. Abstract 168.
- Van Immerseel et al., Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. *J Med Microbiol.* Feb. 2010;59(Pt 2):141-3. doi: 10.1099/jmm.0.017541-0. Epub Nov. 26, 2009.
- Van't Land et al., Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides. *Vaccine.* Aug. 9, 2010;28(35):5711-7. doi: 10.1016/j.vaccine.2010.06.046. Epub Jun. 26, 2010.
- Wang et al., Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. *J Appl Microbiol.* Nov. 2011;111(5):1212-23. doi:10.1111/j.1365-2672.2011.05135.x. Epub Sep. 14, 2011.
- Zhang et al., Therapeutic effects of *Clostridium butyricum* on experimental colitis induced by oxazolone in rats. *World J Gastroenterol.* Apr. 21, 2009;15(15):1821-8.
- Opposition against EP Patent 2 575 835 B1 dated Jul. 19, 2017. 27 pages. Müller Fottner Steineke Part mbB.
- Opposition against EP Patent No. 2 575 835 B1 dated Jul. 19, 2017. 37 pages. Nestec S.A.
- Opposition against EP Patent 2 575 835 B1 dated Jul. 19, 2017. 20 pages. Dr. Martin Grund.
- Opposition against EP Patent 2 575 835 B1 dated Jul. 18, 2017. 23 pages. Strawman Limited.
- Opposition against EP Patent 2 575 835 B1 dated Apr. 25, 2017. 35 pages. Seres Therapeutics.
- Opposition against EP Patent 2 575 835 B1 dated Jul. 18, 2017. 4 pages. Mr. Lars Manke.
- AAS et al., Recurrent *Clostridium difficile* colitis: case series involving 18 patients treated with donor stool administered via a nasogastric tube. *Clin Infect Dis.* Mar. 1, 2003;36(5):580-5. Epub Feb. 14, 2003.
- Andoh et al., Terminal restriction fragment polymorphism analyses of fecal microbiota in five siblings including two with ulcerative colitis. *Clin J Gastroenterol.* Oct. 2009;2(5):343-345. doi:10.1007/s12328-009-0106-8. Epub Sep. 19, 2009.
- Atarashi et al., Regulation of colonic regulatory T cells by *Clostridium* species. *WS/PP-064-03 abstract.* 14th International Congress of Immunology. iii132. Aug. 25, 2010.
- Atarashi et al., Induction of Colonic Regulatory T Cells by Indigenous *Clostridium* Species *Science* 331 (2011) 337-341. Supporting Online Material.
- Autoimmune Disease List. American Autoimmune Related Diseases Association. 2017.
- Bakken, Fecal bacteriotherapy for recurrent *Clostridium difficile* infection. *Anaerobe.* Dec. 2009;15(6):285-9. doi: 10.1016/j.anaerobe.2009.09.007. Epub Sep. 22, 2009.
- Belkaid et al., Natural regulatory T cells in infectious disease. *Nat Immunol.* Apr. 2005;6(4):353-60.
- Bueche et al., Quantification of endospore-forming firmicutes by quantitative PCR with the functional gene spo0A. *Appl Environ Microbiol.* Sep. 2013;79(17):5302-12. doi: 10.1128/AEM.01376-13. Epub Jun. 28, 2013.
- Cato et al., *Clostridium oroticum* Comb. Nov. *Int. J. Syst. Bact.* Jan. 1968;17(1):9-13.
- DeWhirst et al., Phylogeny of the defined murine microbiota: altered Schaedler flora. *Appl Environ Microbiol.* Aug. 1999;65(8):3287-92.
- European Office Action for Application No. 11 728 077.6. dated Sep. 18, 2015.
- Honda et al., Regulation of T Cell Responses by Intestinal Commensal Bacteria. 2011;25(2):103-104.
- Janeway et al., Autoimmune responses are directed against self antigens. *Immunobiology: The Immune System in Health and Disease.* 5th edition. 2001. New York: Garland Science.
- Janeway et al., *Immunobiology: The Immune System in Health and Disease.* 6th edition. 2005. Chapter 10. 414. Figure 10.4.

(56)

References Cited

OTHER PUBLICATIONS

- Jawetz et al., Spore-forming gram-positive bacilli: *Bacillus* and *Clostridium* species. Medical Microbiology. Chapter 11. Nov. 2011.
- Kakihana et al., Fecal microbiota transplantation for patients with steroid-resistant acute graft-versus-host disease of the gut. Blood. Oct. 20, 2016;128(16):2083-2088. doi: 10.1182/blood-2016-05-717652. Epub Jul. 26, 2016.
- Kelly et al., Commensal gut bacteria: mechanisms of immune modulation. Trends Immunol. Jun. 2005;26(6):326-33.
- Keynan et al., The role of regulatory T cells in chronic and acute viral infections. Clin Infect Dis. Apr. 1, 2008;46(7):1046-52. doi: 10.1086/529379.
- Krogus-Kurikka et al., Sequence analysis of percent G+C fraction libraries of human faecal bacterial DNA reveals a high number of Actinobacteria. BMC Microbiol. Apr. 8, 2009;9:68. doi: 10.1186/1471-2180-9-68.
- Lau et al., Bacteraemia caused by *Anaerotruncus colihominis* and emended description of the species. J Clin Pathol. Jul. 2006;59(7):748-52. Epub Feb. 7, 2006.
- Lawson et al., *Anaerotruncus colihominis* gen. nov., sp. nov., from human faeces. Int J Syst Evol Microbiol. Mar. 2004;54(Pt 2):413-7.
- Lawson, *Anaerotruncus*. Bergey's Manual of Systematics of Archaea and Bacteria. Sep. 14, 2015;1-4.
- Maizels et al., Regulatory T cells in infection. Adv Immunol. 2011;112:73-136. doi: 10.1016/B978-0-12-387827-4.00003-6.
- Momose et al., 16S rRNA gene sequence-based analysis of *Clostridia* related to conversion of germfree mice to the normal state. J Appl Microbiol. Dec. 1, 2009;107(6):2088-97. doi:10.1111/j.1365-2672.2009.04431.x. Epub Jul. 15, 2009.
- O'Garra et al., IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. J Clin Invest. Nov. 2004;114(10):1372-8.
- Response to European Office Action for Application No. 11 728 077.6. dated Nov. 18, 2015.
- Response to European Office Action for Application No. 11 728 077.6. dated Feb. 25, 2014.
- Response to European Office Action for Application No. 11 728 077.6. dated Jan. 28, 2015.
- Roberts, CSO Vedanta Biosciences Presentation. 1st Microbiome Drug Development Summit. Jun. 28, 2016.
- Sanchez et al., The role of natural regulatory T cells in infection. Immunol Res. Apr. 2011;49(1-3):124-34. doi: 10.1007/s12026-010-8176-8.
- Sartor, Therapeutic correction of bacterial dysbiosis discovered by molecular techniques. Proc Natl Acad Sci U S A. Oct. 28, 2008;105(43):16413-4. doi:10.1073/pnas.0809363105. Epub Oct. 23, 2008.
- Screenshot of https://academic.oup.com/intimm/issue/22/Suppl_1_Pt3 showing the publication of Warren et al. at the Workshop abstract WS64 of XIVth International congress of Immunology held in Kobe in Japan on Aug. 2010.
- Sequence listing of WO 2011/152566.
- Sghir et al., Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol. May 2000;66(5):2263-6.
- Sokol et al., Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. Inflamm Bowel Dis. Aug. 2009;15(8):1183-9. doi:10.1002/ibd.20903.
- Takaishi et al., Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. Int J Med Microbiol. Jul. 2008;298(5-6):463-72. Epub Sep. 25, 2007.
- Wachsman et al., Characterization of an orotic acid fermenting bacterium, *Zymobacterium oroticum*, nov. gen., nov. spec. J Bacteriol. Oct. 1954;68(4):400-4.
- Warren et al. *Clostridium aldenense* sp. nov. and *Clostridium citroniae* sp. nov. isolated from human clinical infections. J Clin Microbiol. Jul. 2006;44(7):2416-22.
- Wells et al., *Clostridia*: Sporeforming Anaerobic Bacilli. Medical Microbiology, 4th edition. Ed:Baron. 1996. University of Texas Medical Branch at Galveston. Mar. 7, 2017.
- Zhou et al., Total fecal microbiota transplantation alleviates high-fat diet-induced steatohepatitis in mice via beneficial regulation of gut microbiota. Sci Rep. May 8, 2017;7(1):1529. doi: 10.1038/s41598-017-01751-y.
- Atarashi et al., Regulation of colonic regulatory T cells by *Clostridium* species. Poster 064-03. Department of Immunology. Aug. 25, 2010.

FIG.1

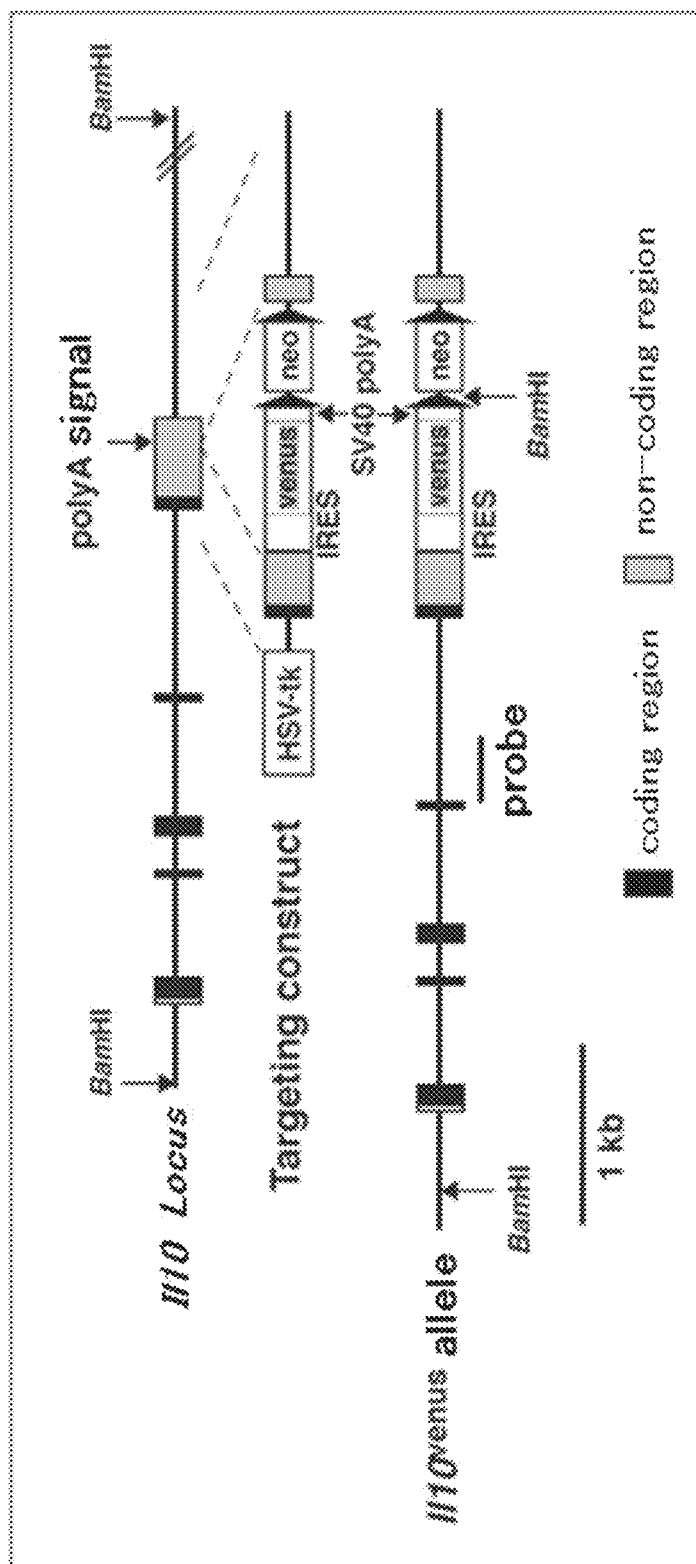


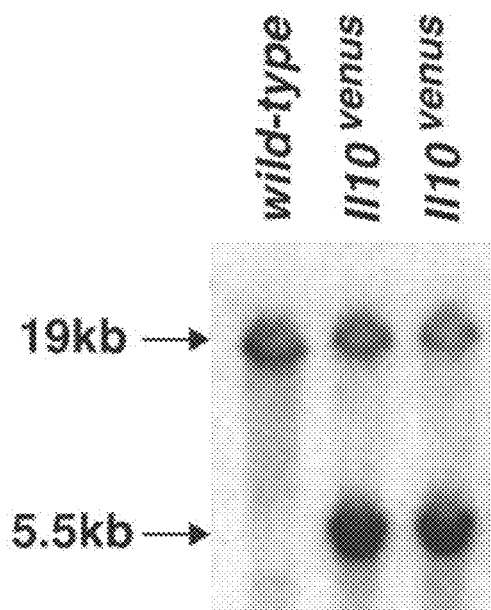
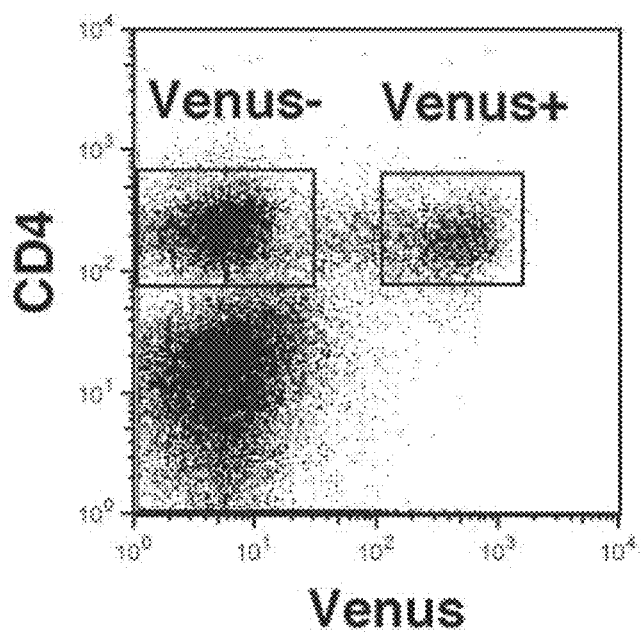
FIG. 2**FIG. 3**

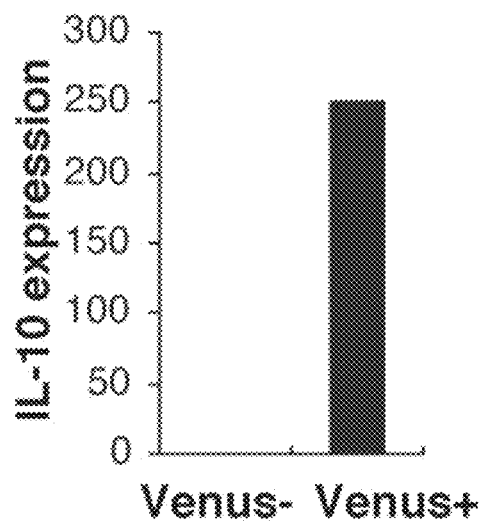
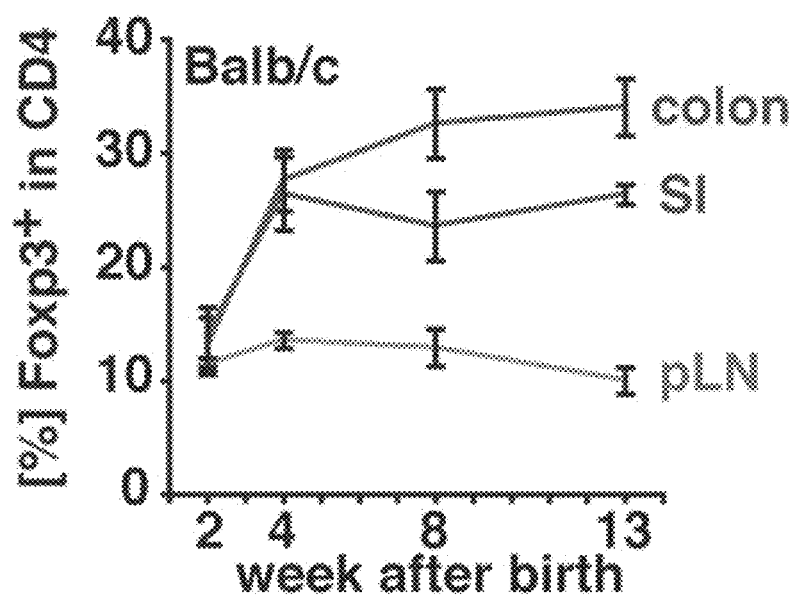
FIG. 4**FIG. 5**

FIG. 6

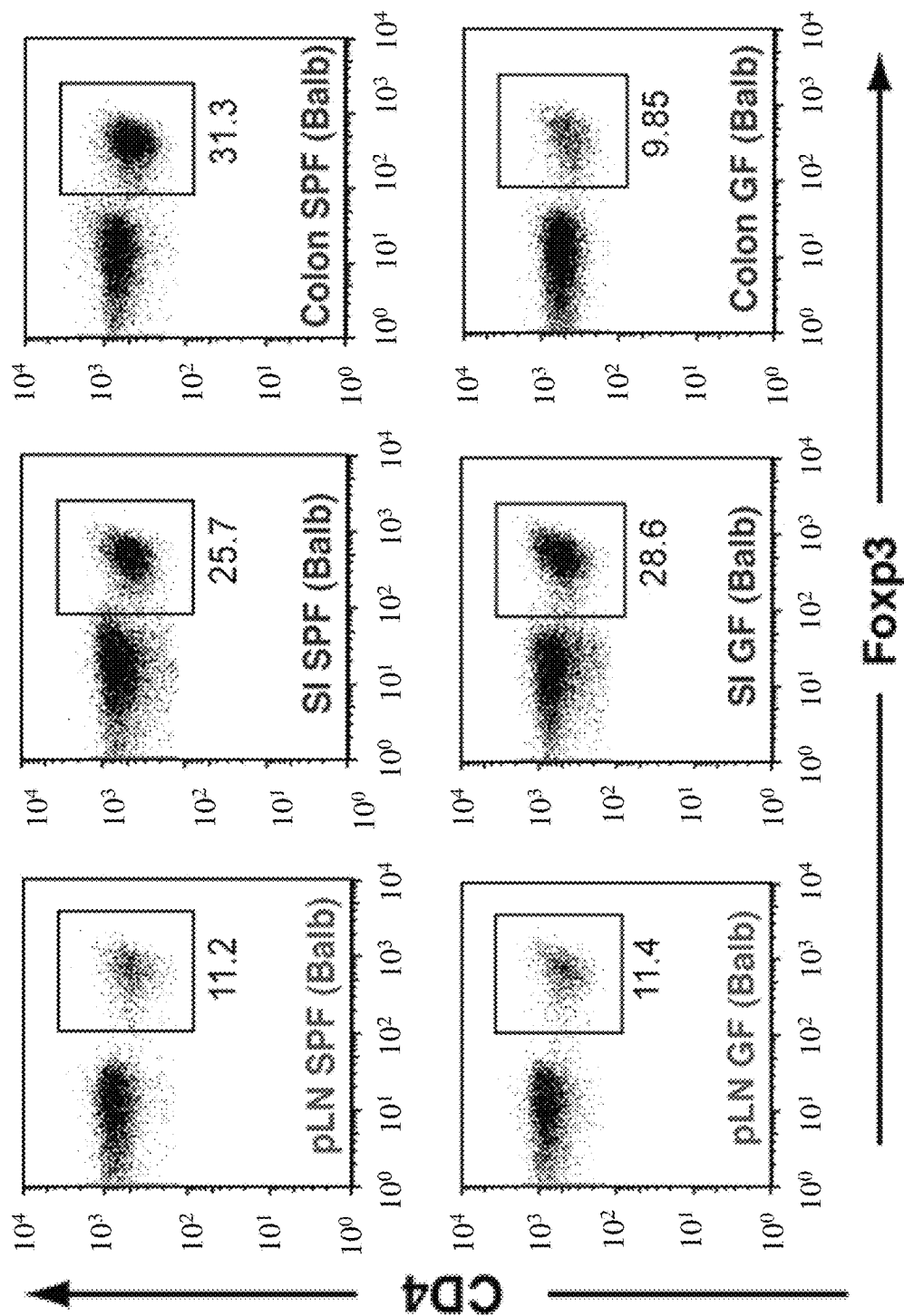


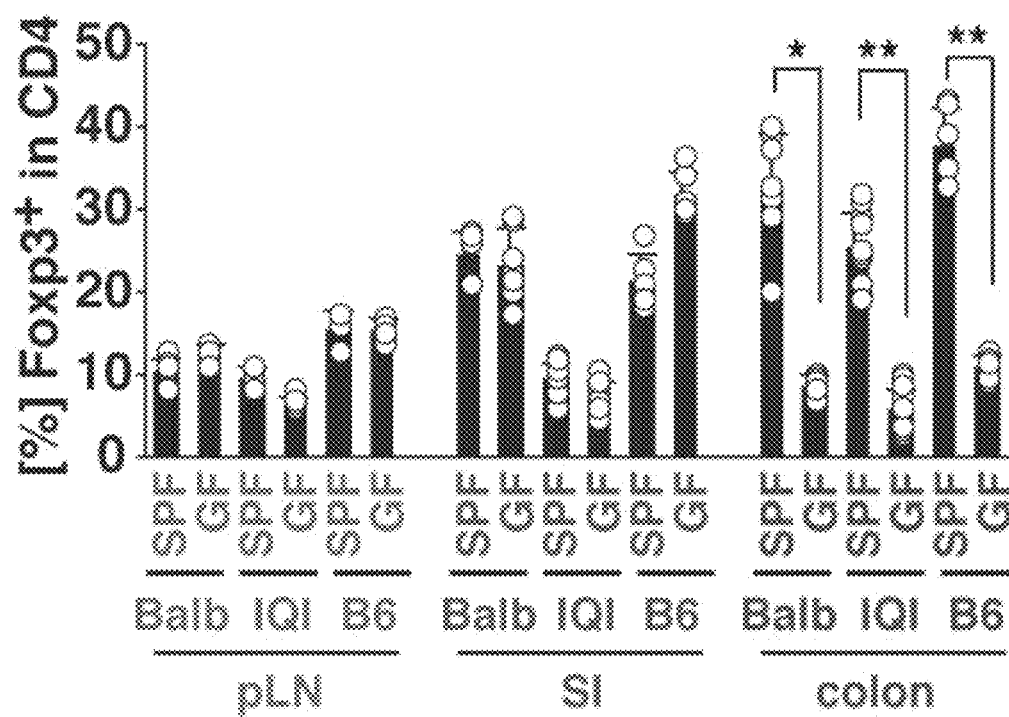
FIG. 7

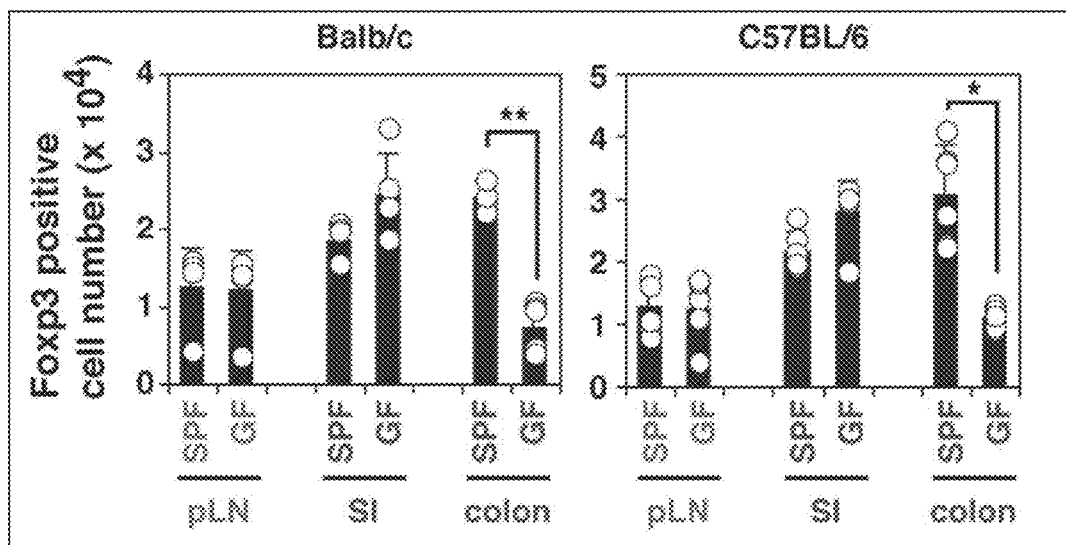
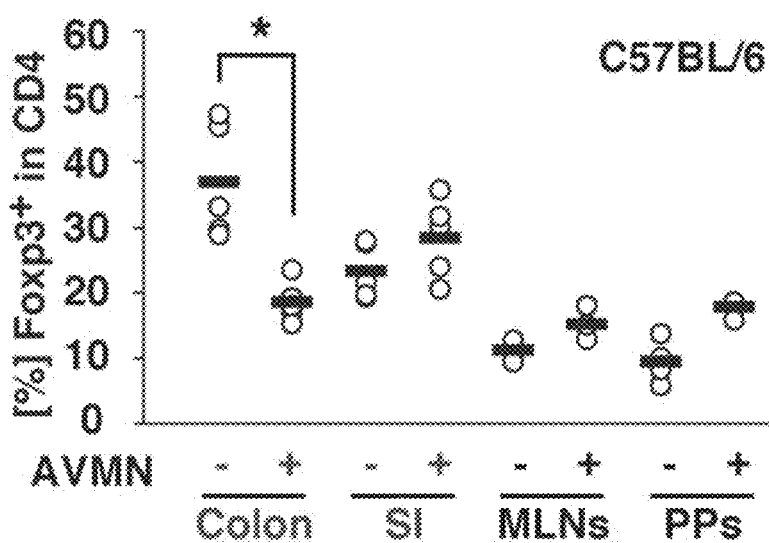
FIG. 8**FIG. 9**

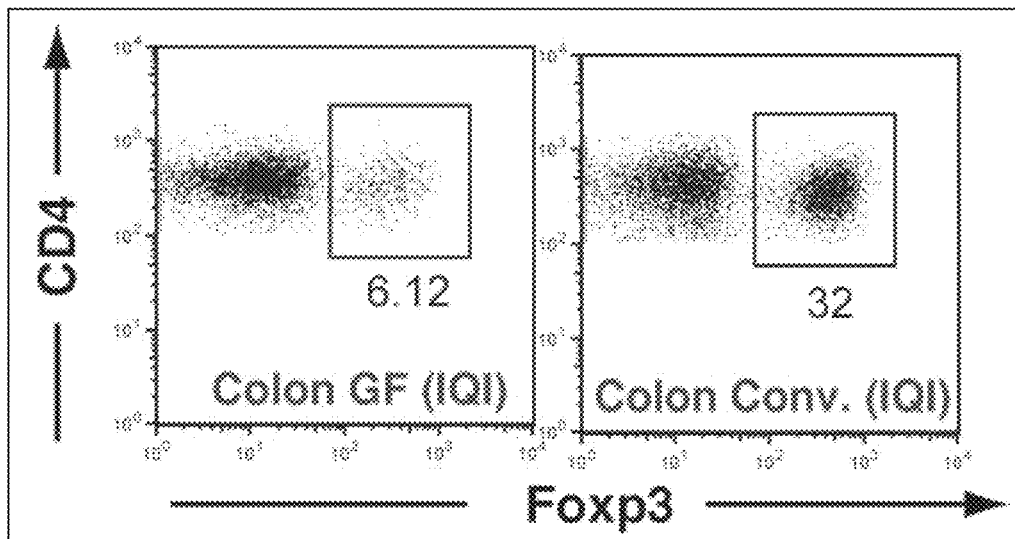
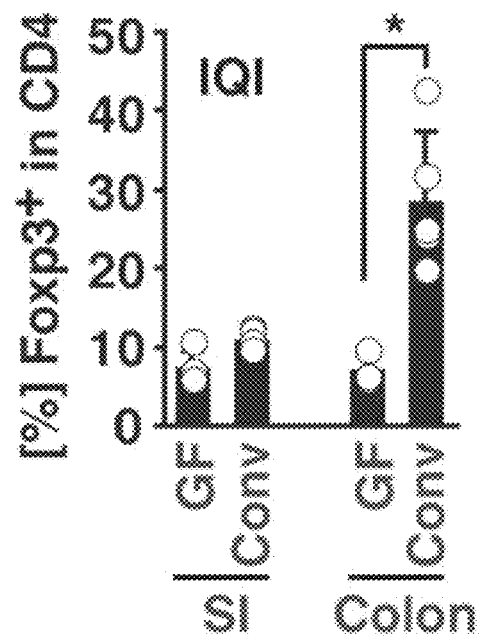
FIG. 10**FIG. 11**

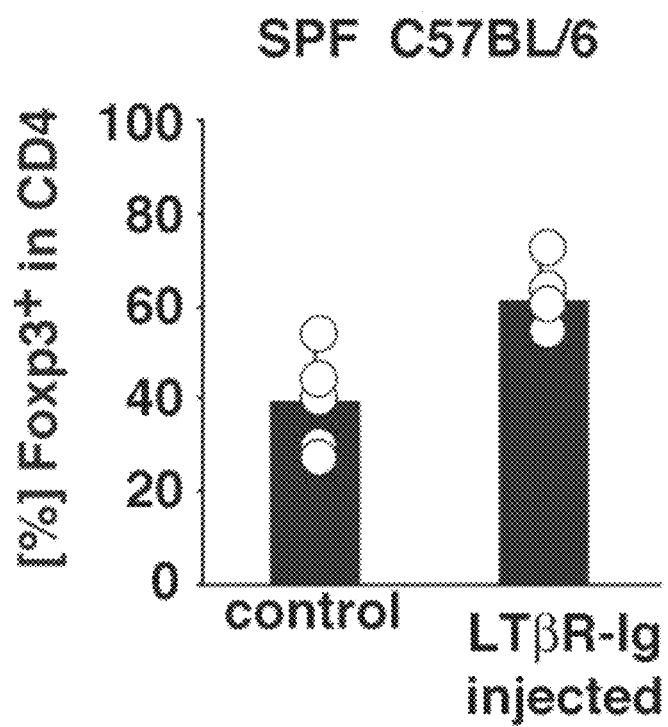
FIG. 12

FIG. 13

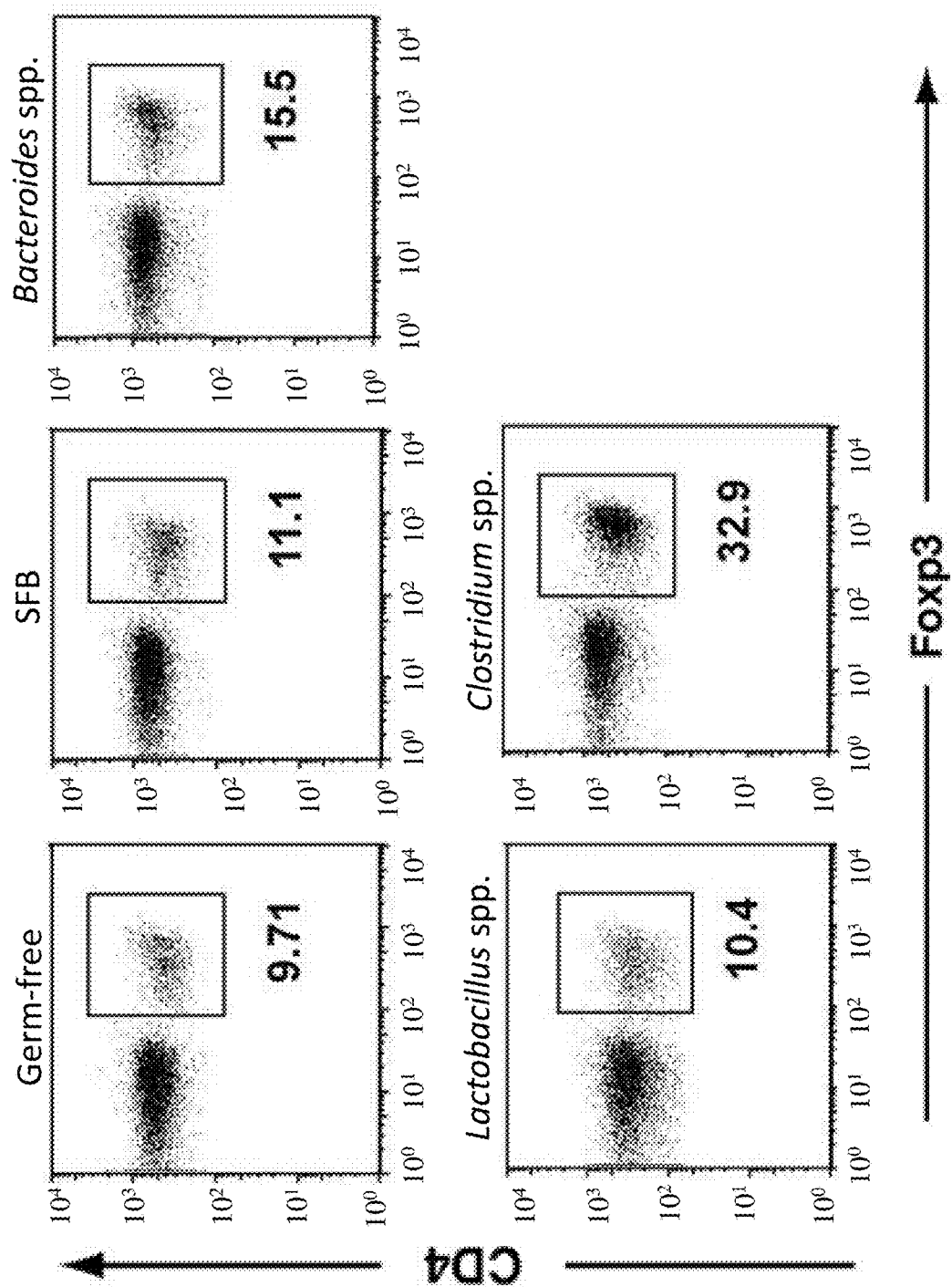


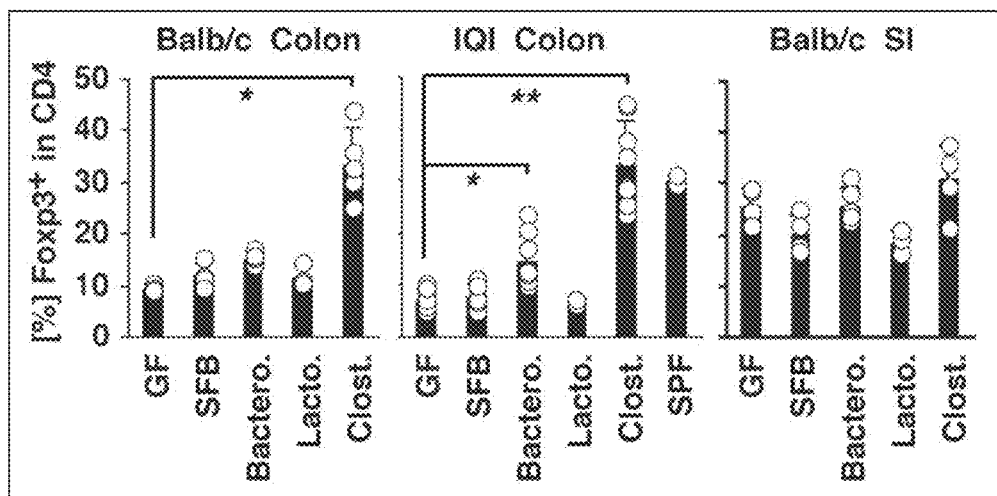
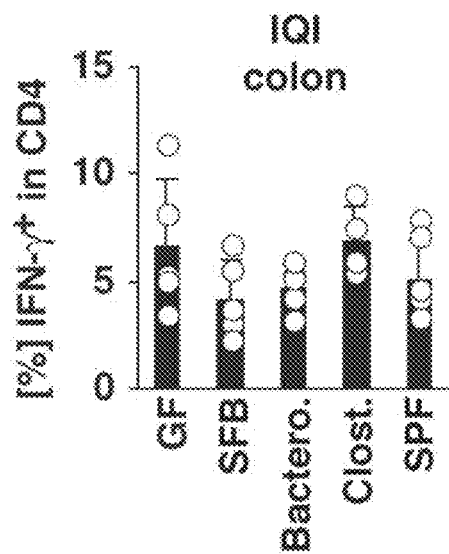
FIG. 14**FIG. 15**

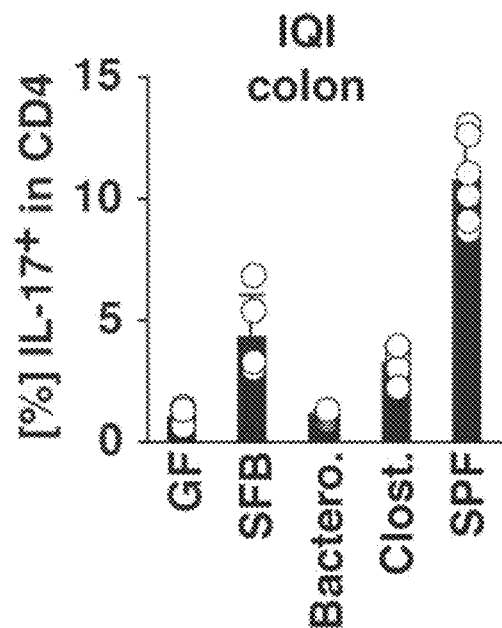
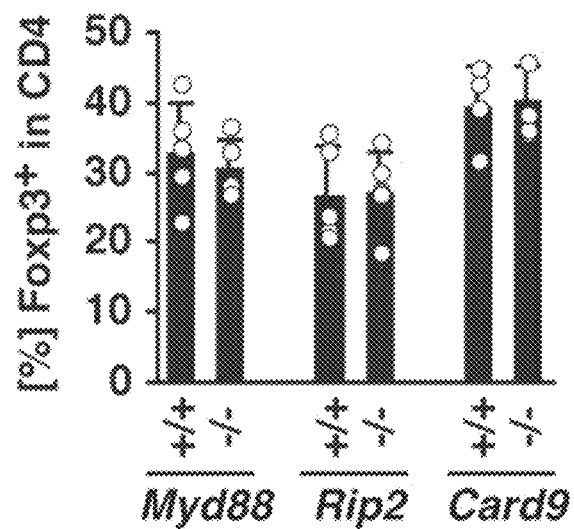
FIG. 16**FIG. 17**

FIG. 18

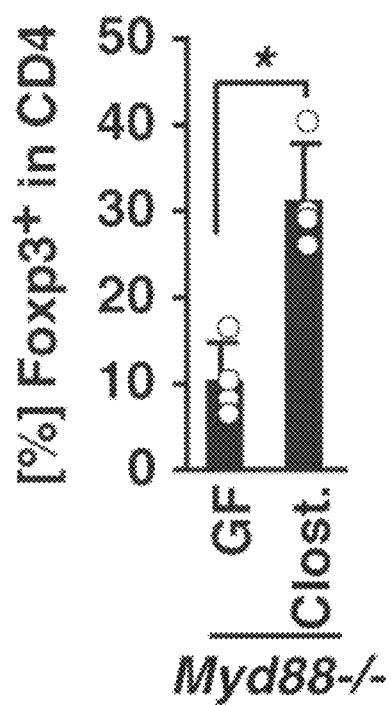


FIG. 19

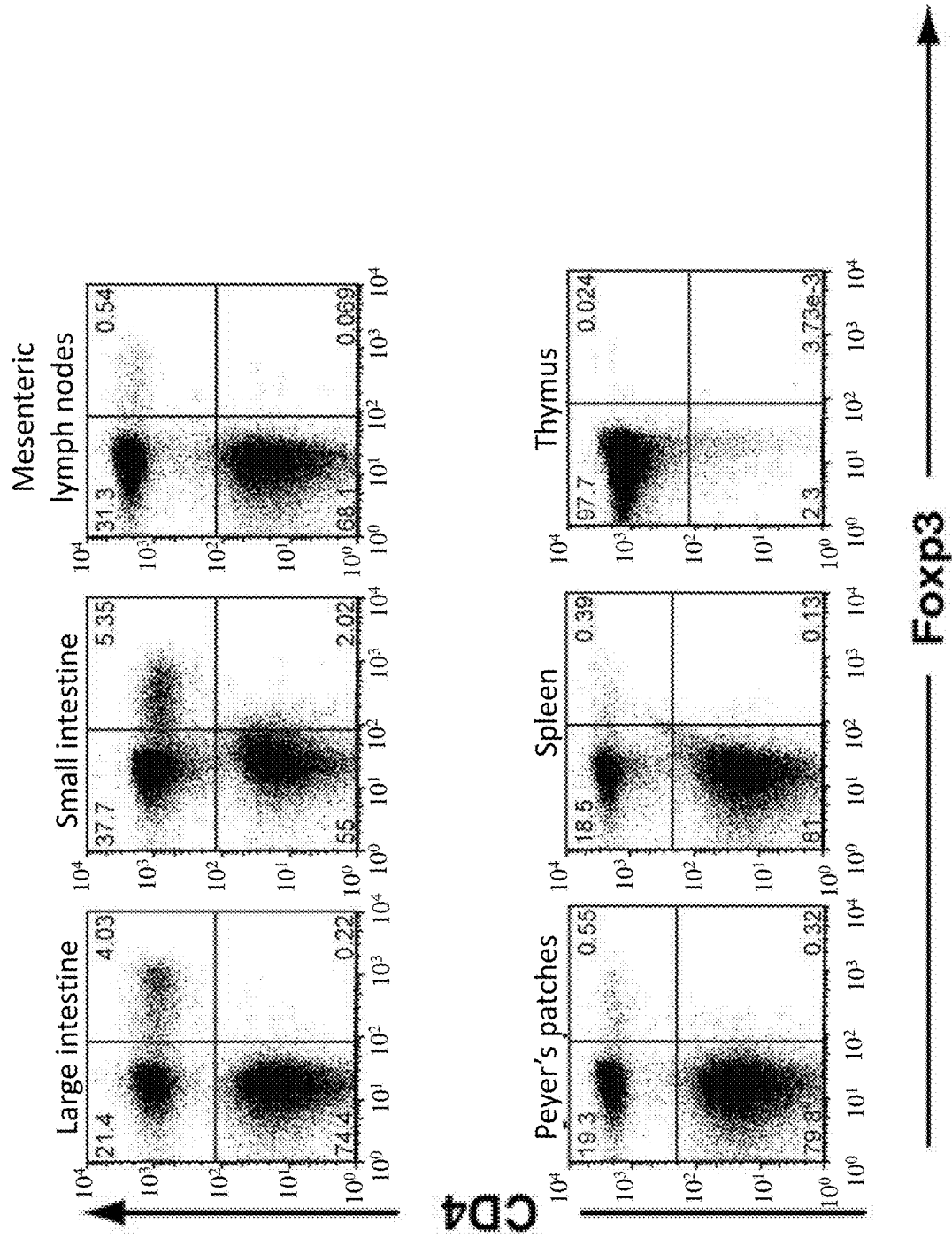


FIG. 19 cont.

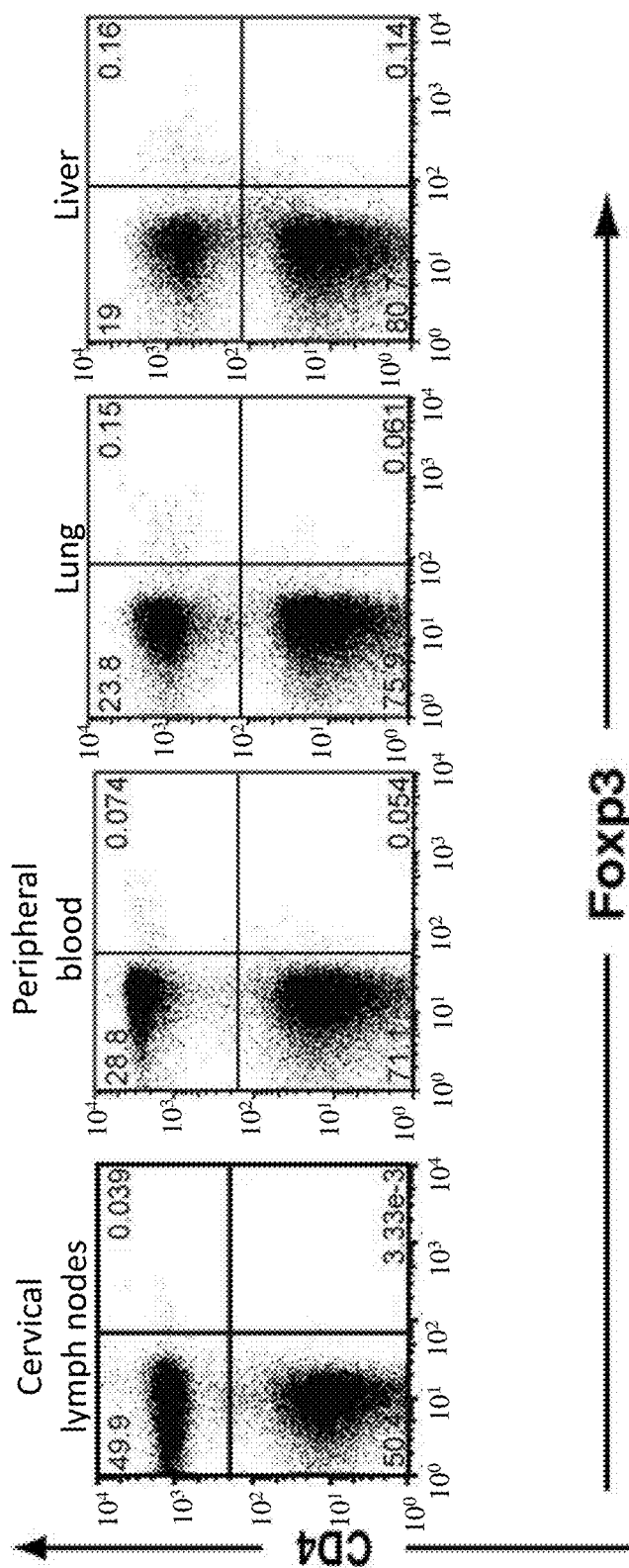


FIG. 20

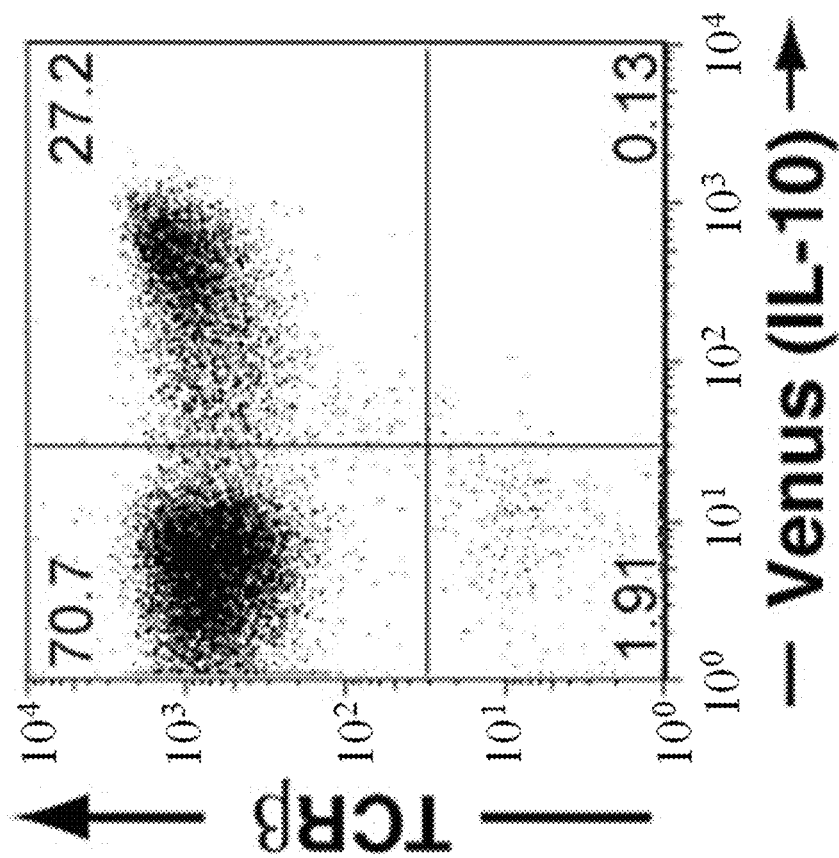


FIG. 21

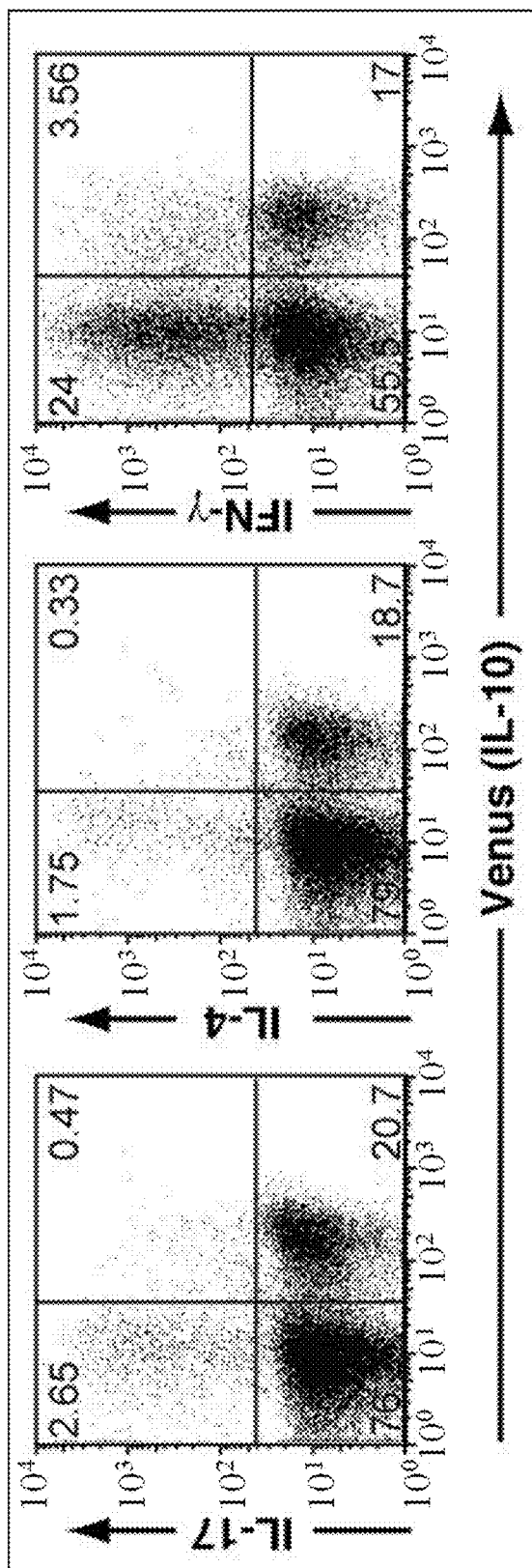


FIG. 22

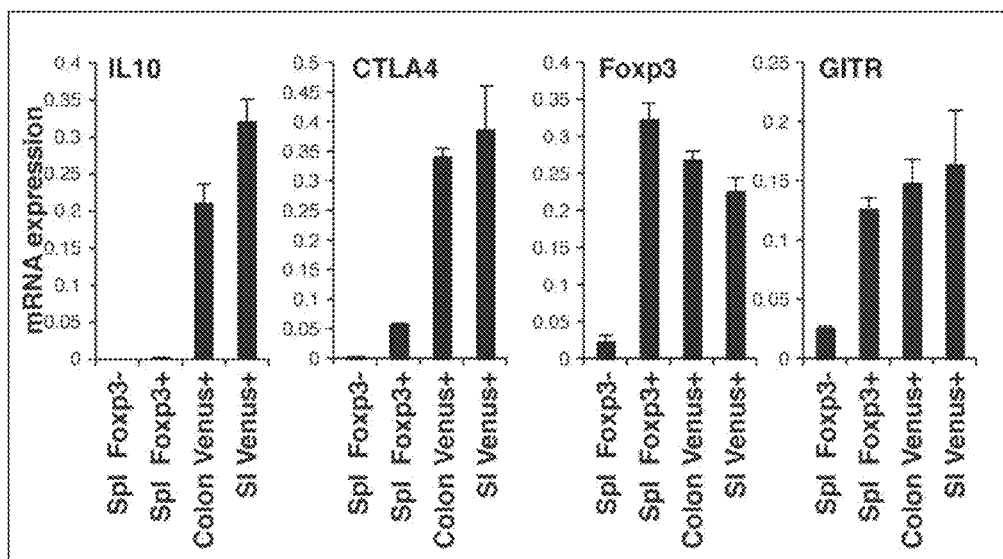


FIG. 23

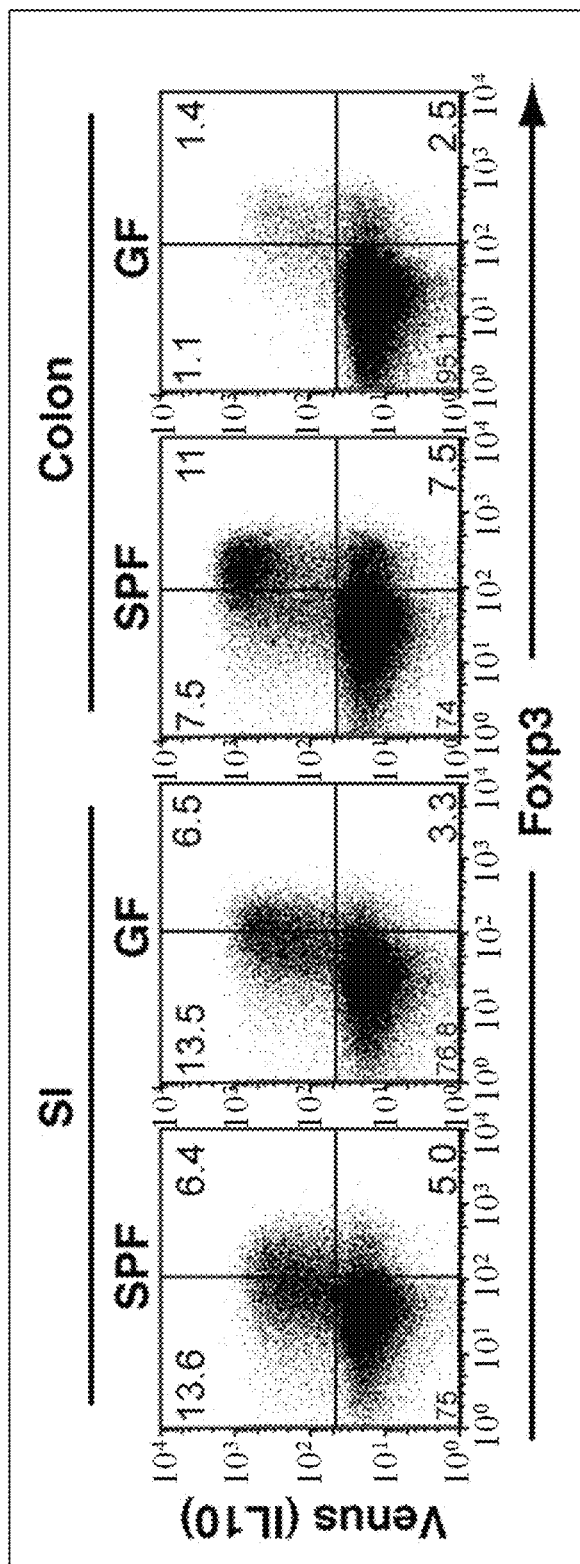
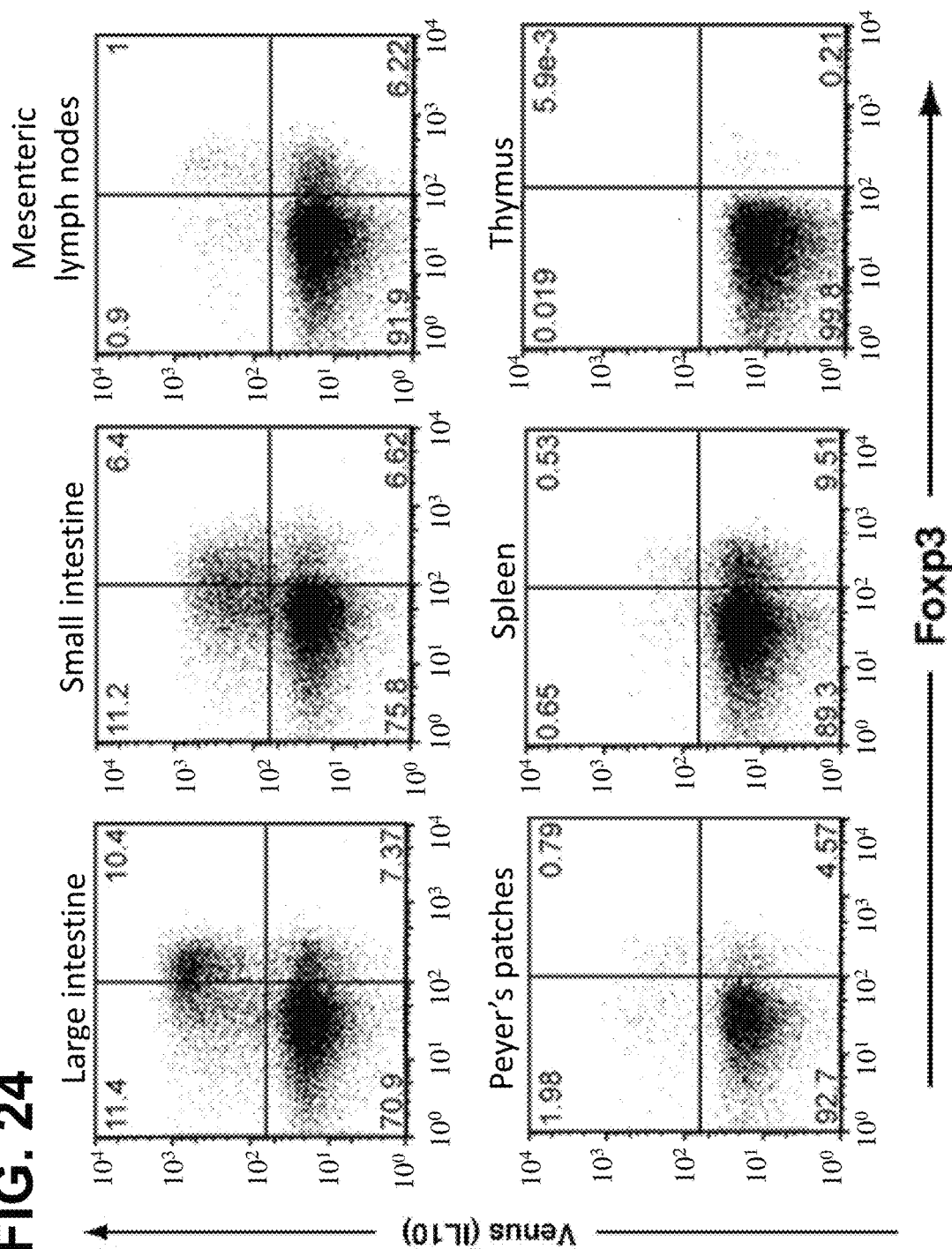


FIG. 24



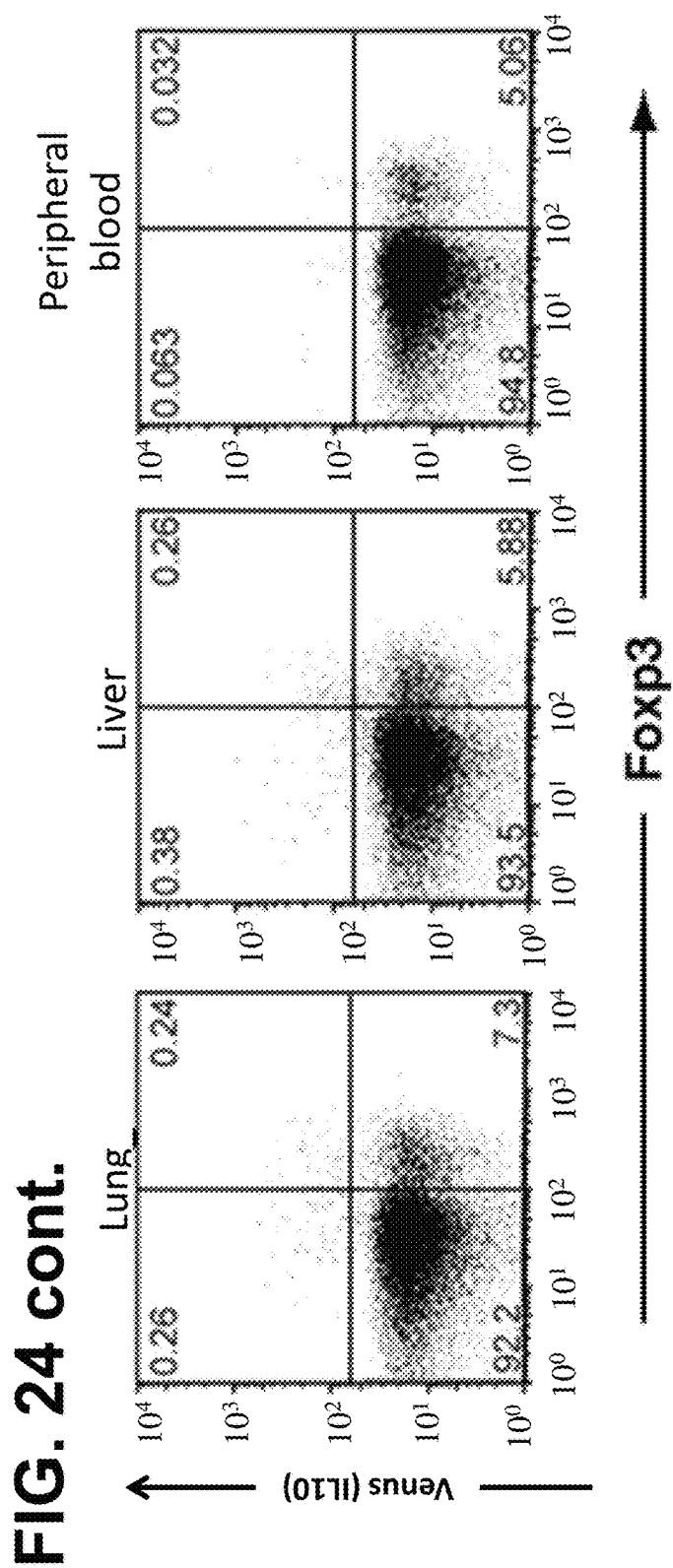


FIG. 25

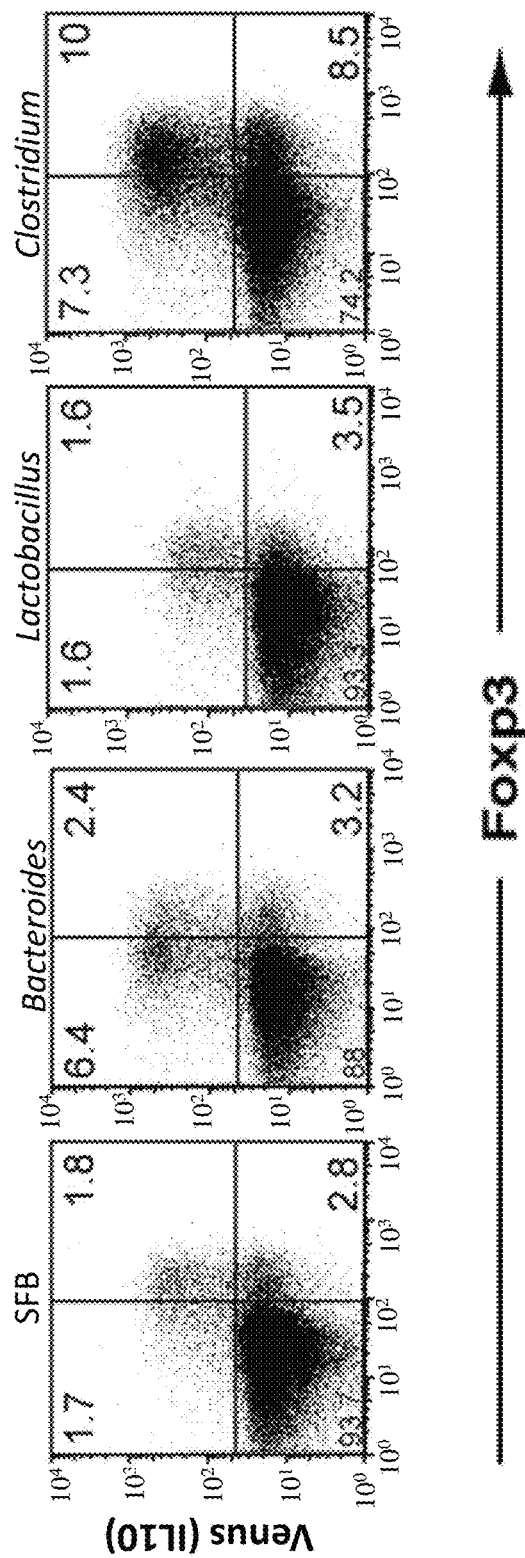


FIG. 26

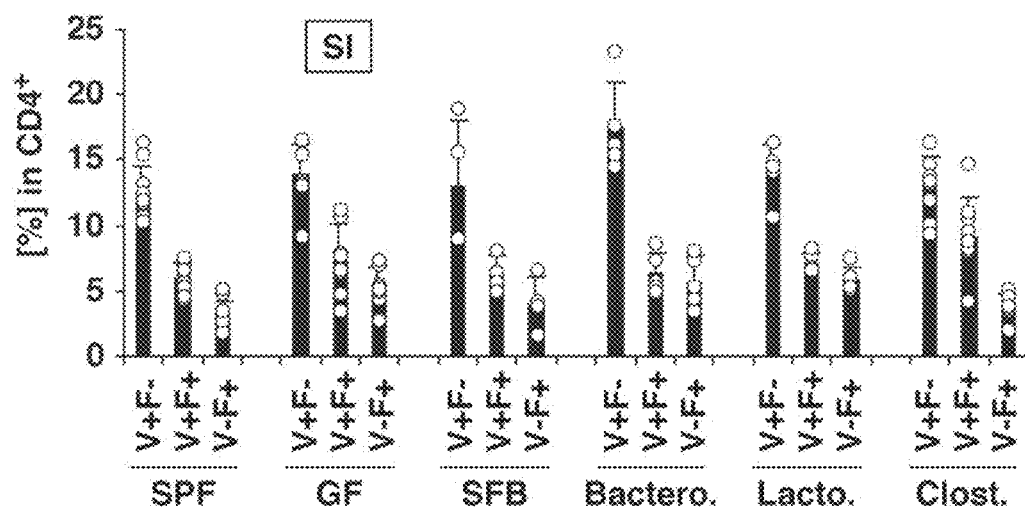


FIG. 27

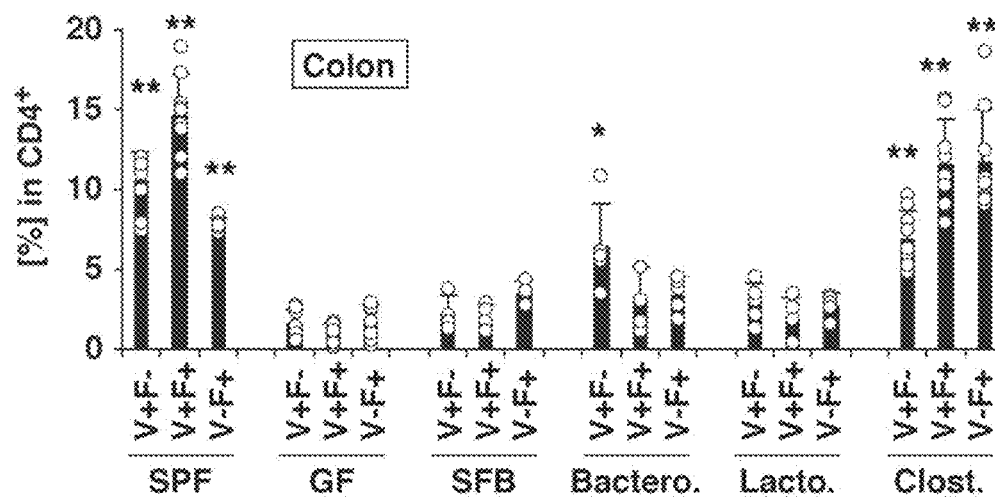


FIG. 28

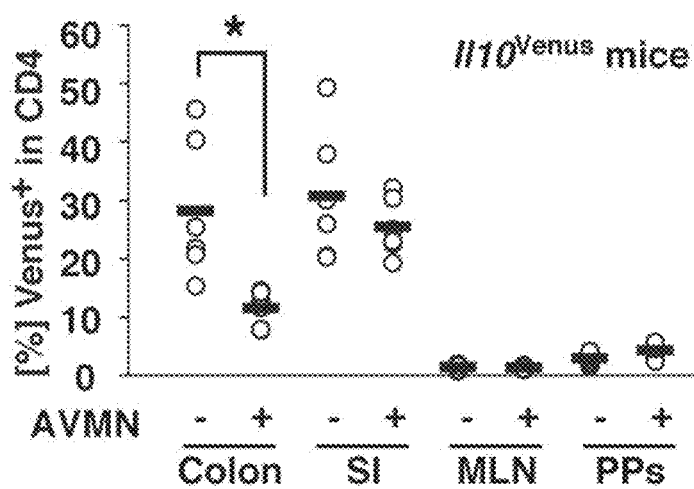


FIG. 29

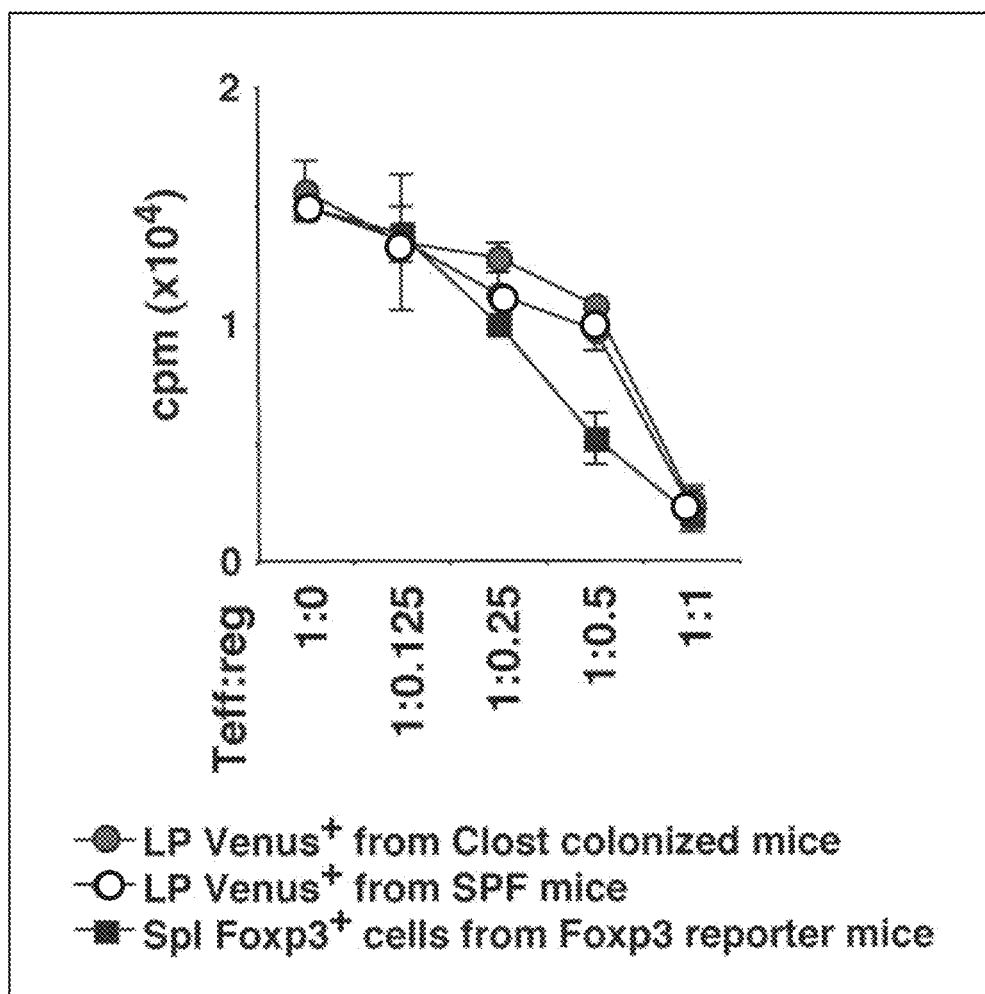


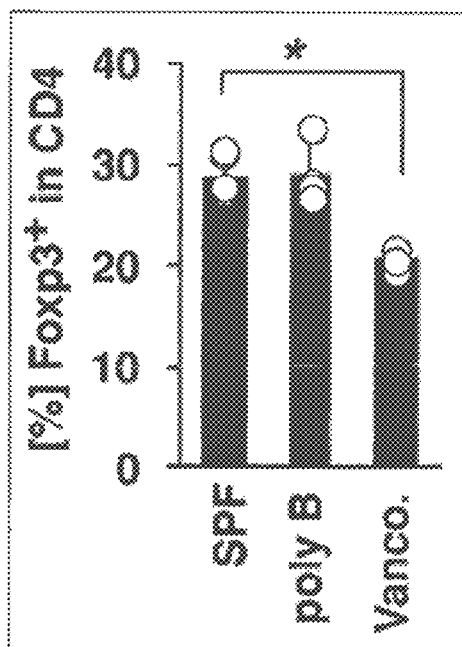
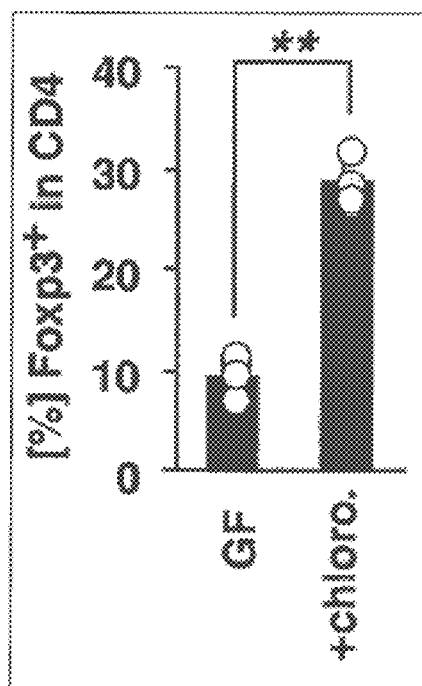
FIG. 30**FIG. 31**

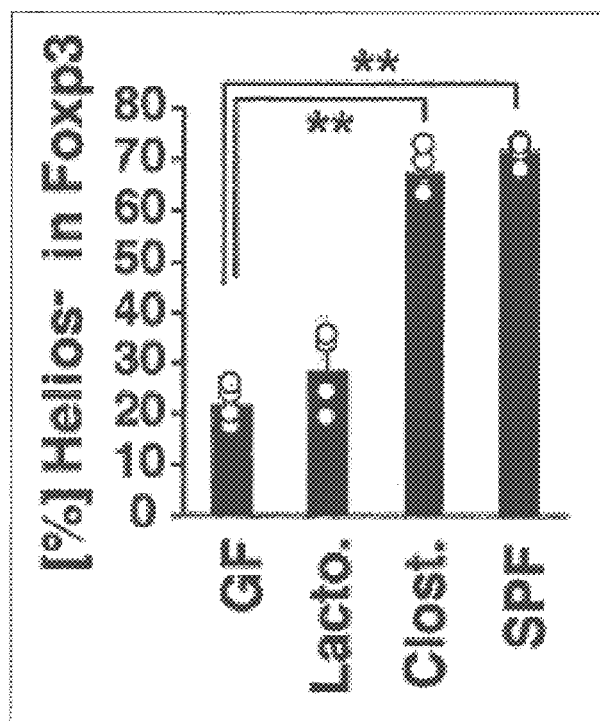
FIG. 32

FIG. 33

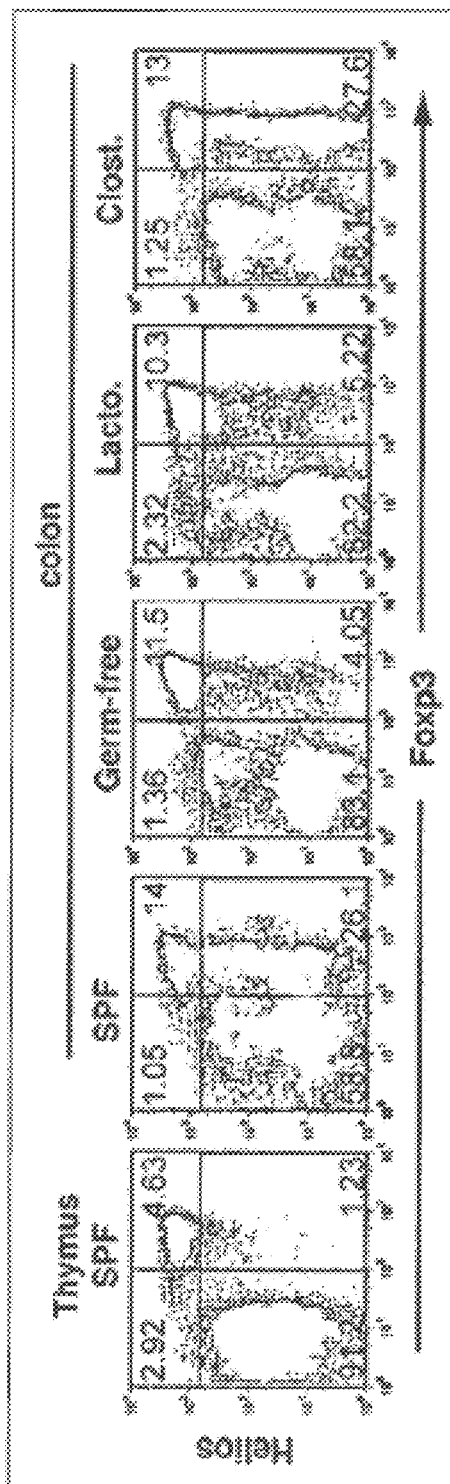


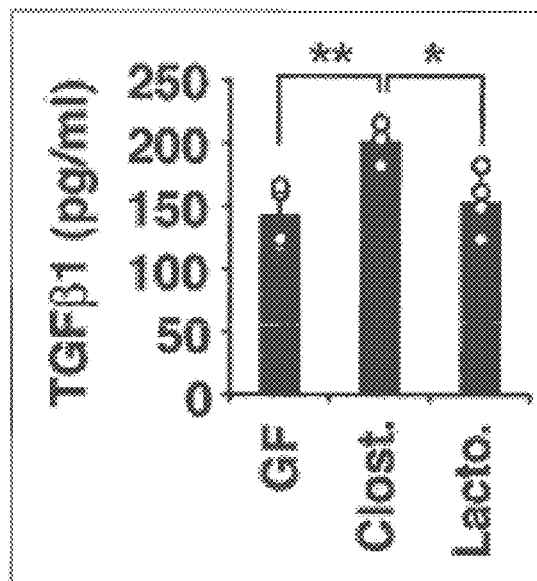
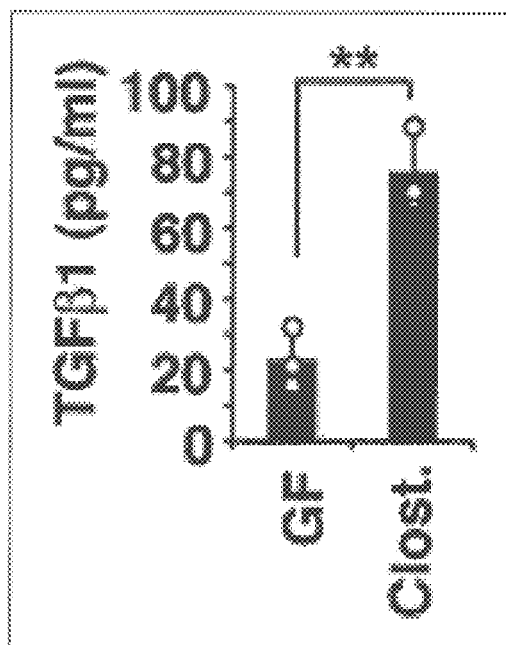
FIG. 34**FIG. 35**

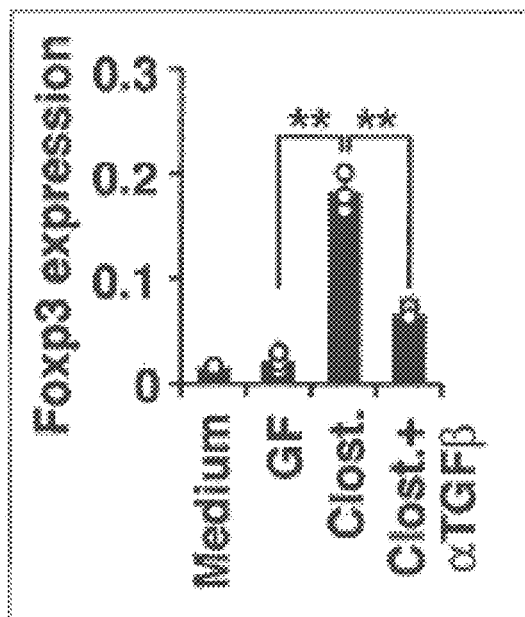
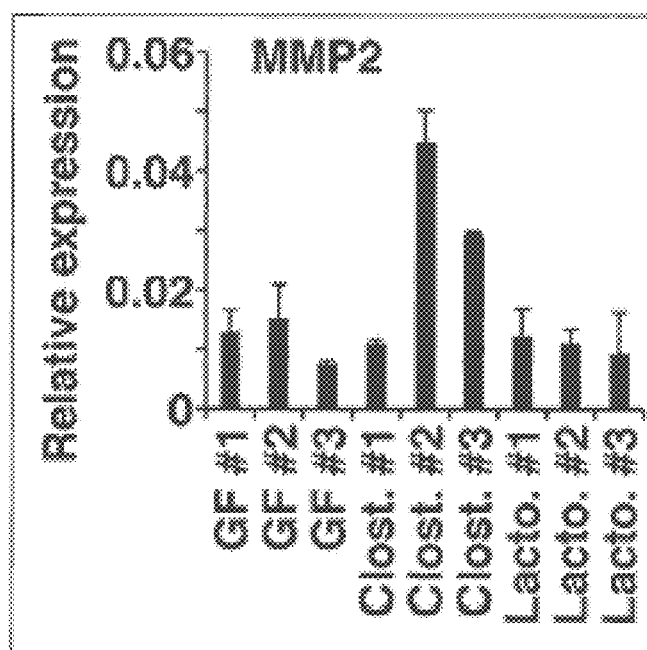
FIG. 36**FIG. 37**

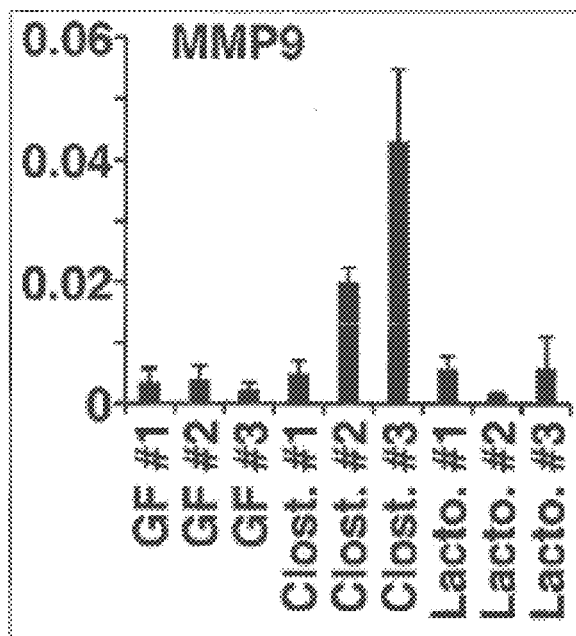
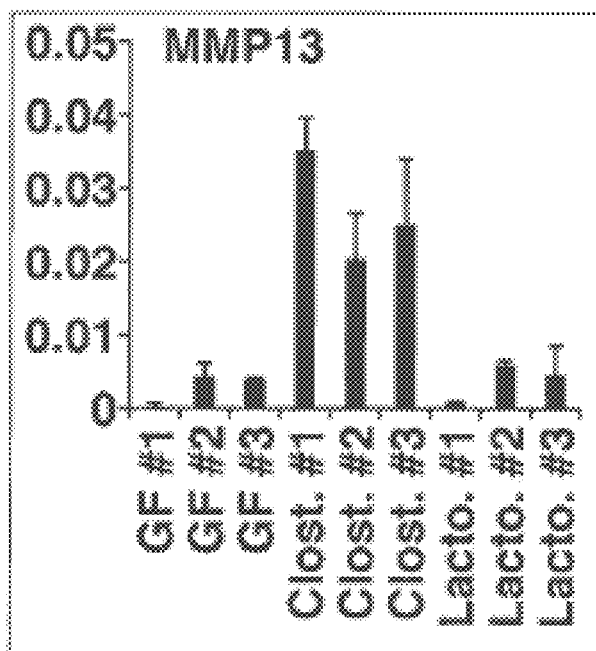
FIG. 38**FIG. 39**

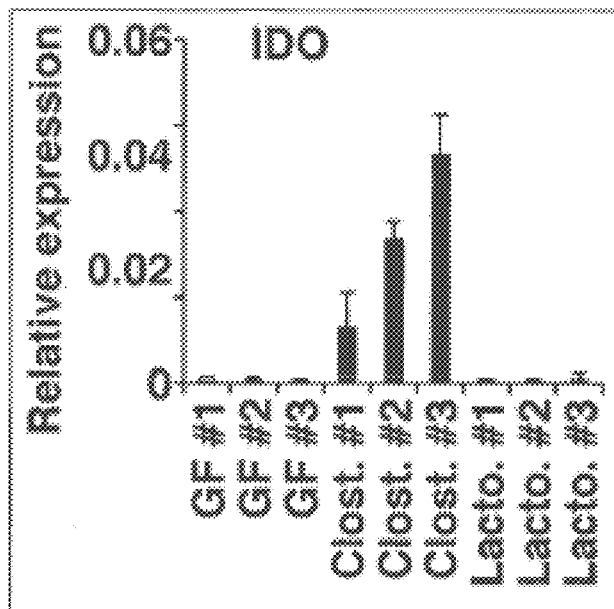
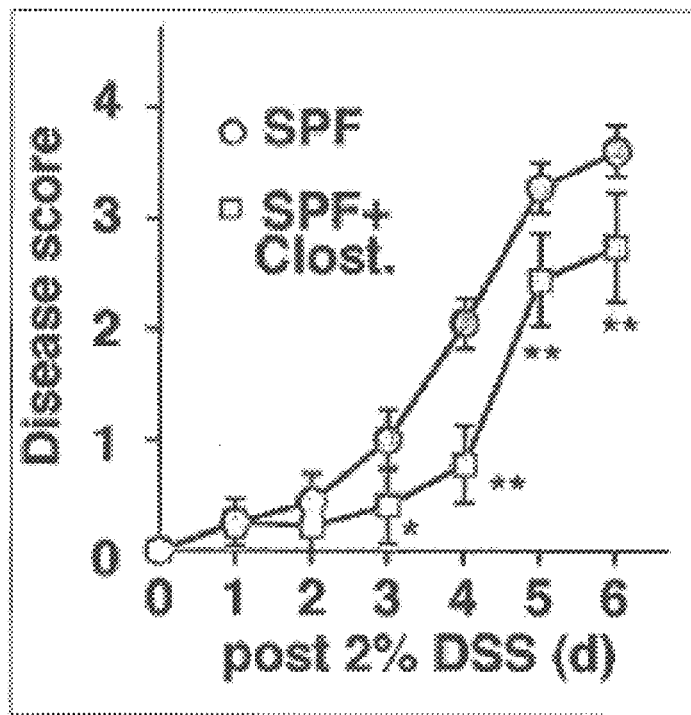
FIG. 40**FIG. 41**

FIG. 42

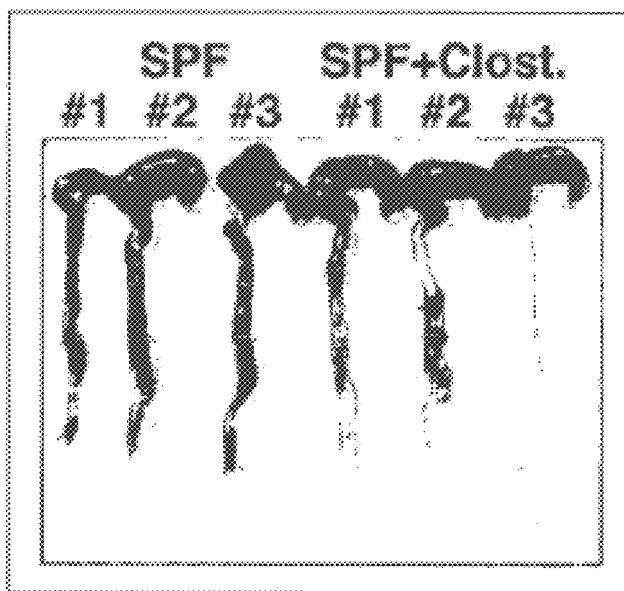


FIG. 43

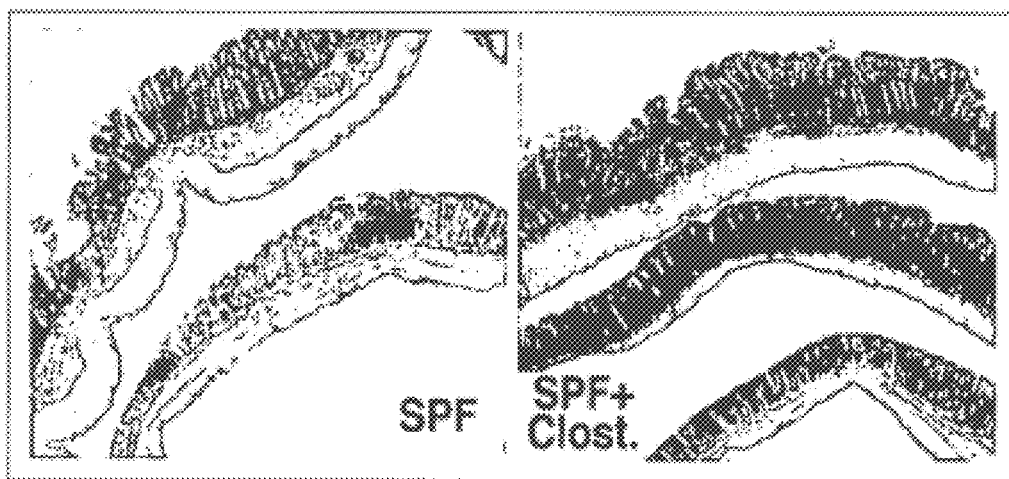


FIG. 44

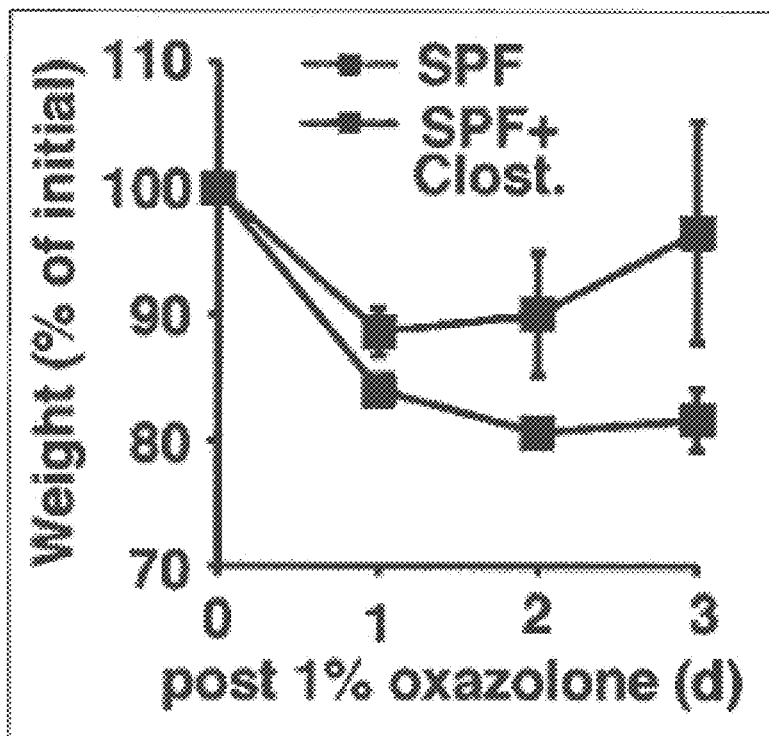


FIG. 45

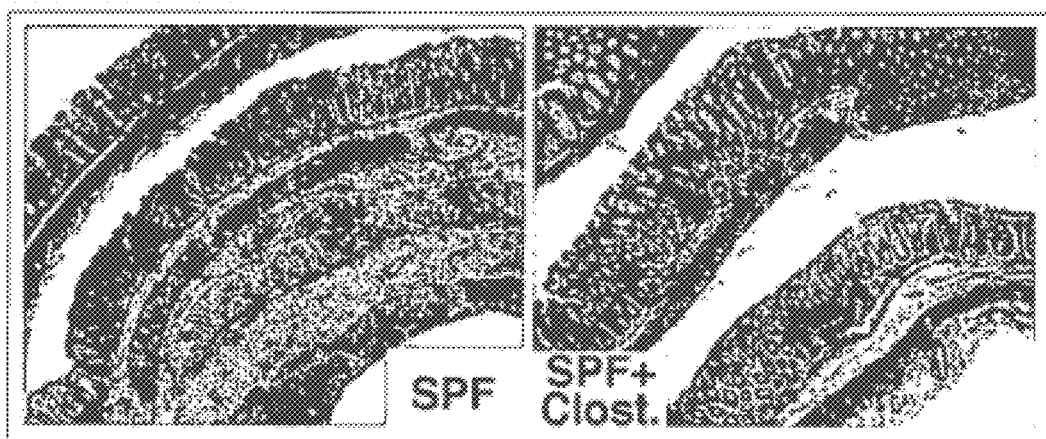


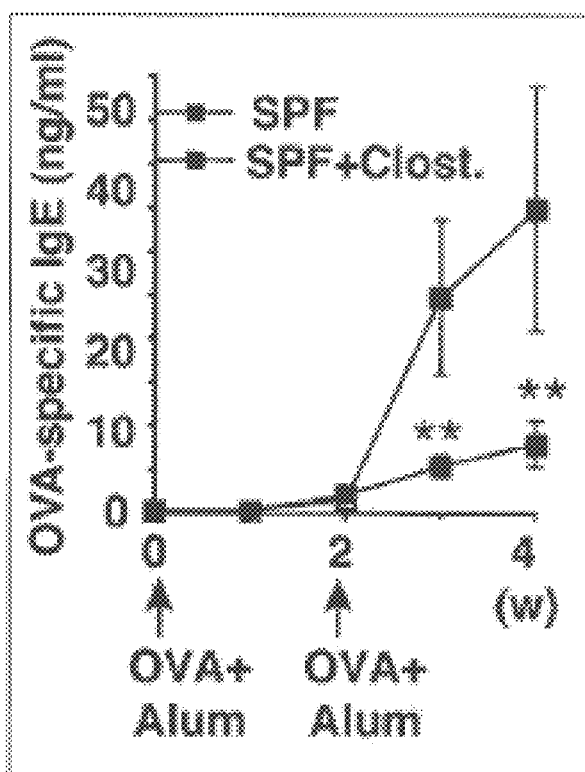
FIG. 46

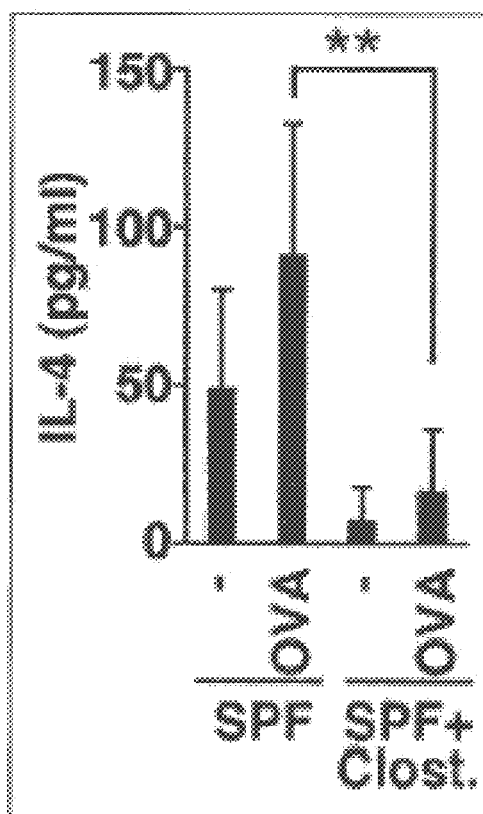
FIG. 47

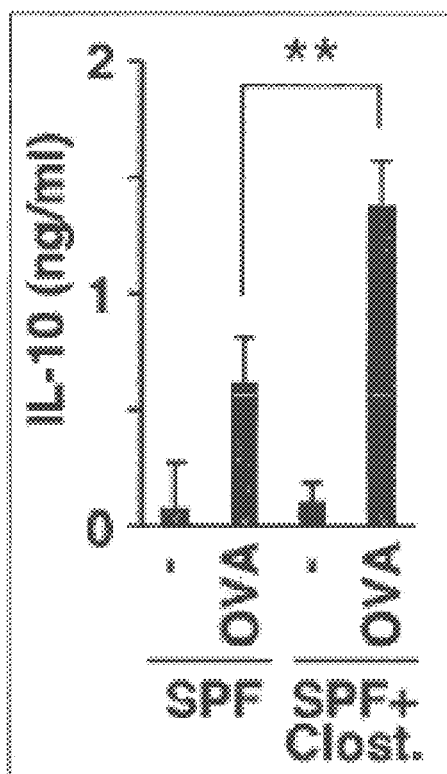
FIG. 48

FIG. 49

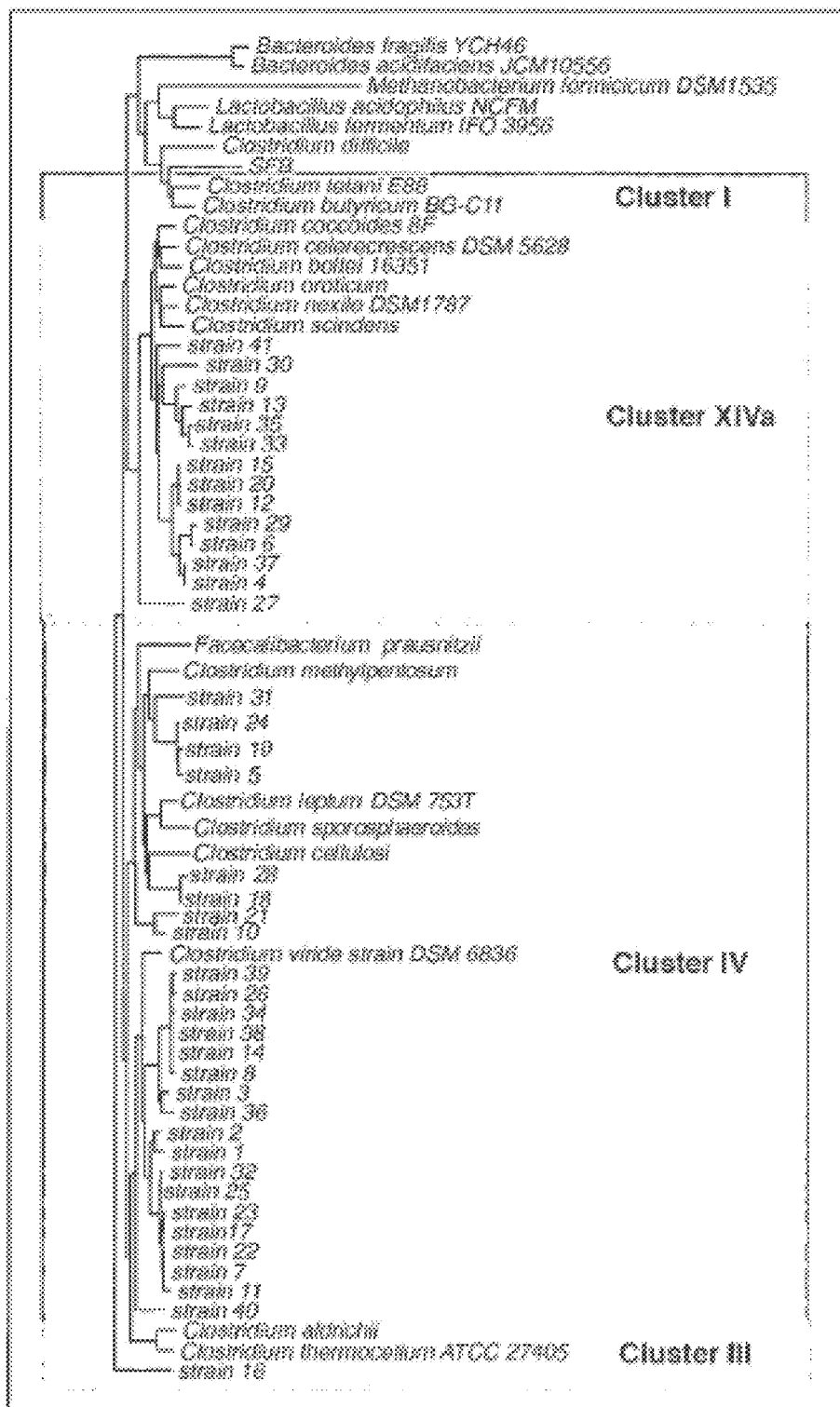


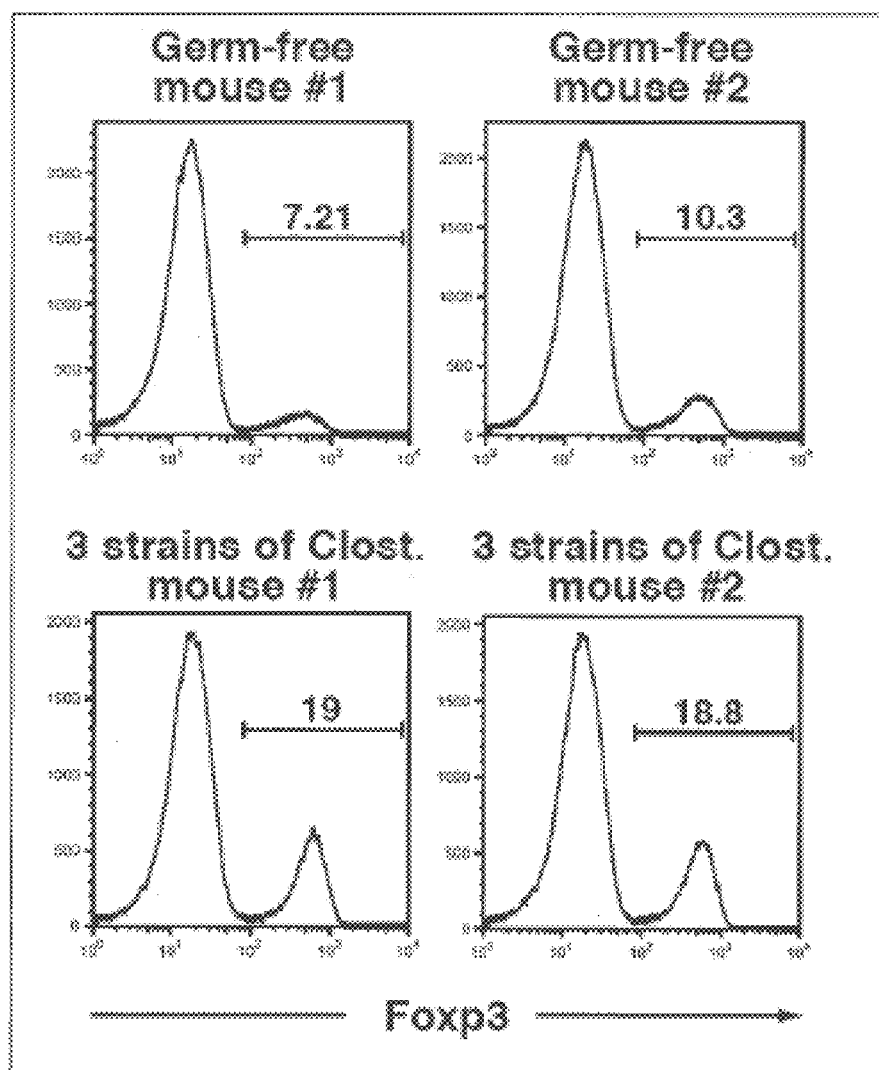
FIG. 50

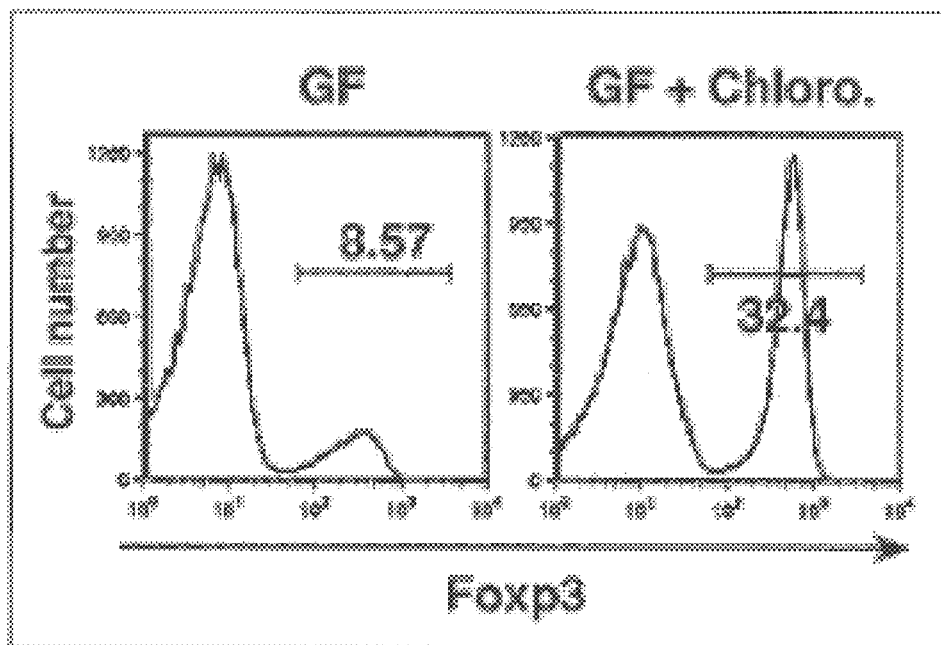
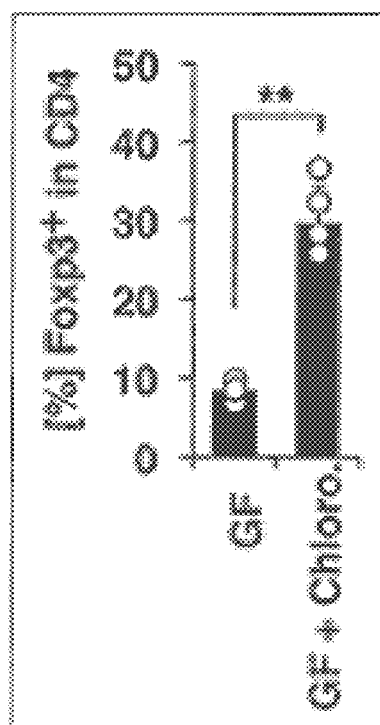
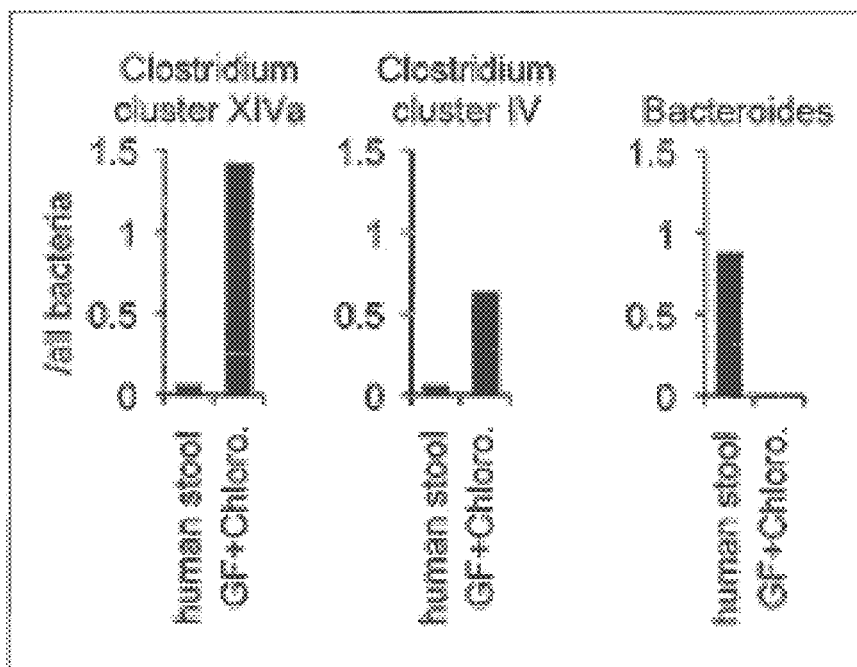
FIG. 51**FIG. 52**

FIG. 53

1

COMPOSITION FOR INDUCING PROLIFERATION OR ACCUMULATION OF REGULATORY T CELLS

RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 15/216,015, filed Jul. 21, 2016, which is a continuation of U.S. application Ser. No. 14/492,850, filed Sep. 22, 2014, now issued as U.S. Pat. No. 9,433,652, which is a continuation of U.S. application Ser. No. 13/701,467, filed Feb. 11, 2013, which is a national stage filing under 35 U.S.C. 371 of International Application PCT/JP2011/063302, filed Jun. 3, 2011, which claims the benefit of and priority to JP 2010-129134, filed Jun. 4, 2010 and PCT/JP2010/071746, filed Dec. 3, 2010. The entire teachings of the referenced applications are incorporated by reference herein.

TECHNICAL FIELD

The present invention relates to a composition which has an effect of inducing proliferation or accumulation of regulatory T cells, and which comprises, as an active ingredient, bacteria belonging to the genus *Clostridium*, a physiologically active substance derived from the bacteria, bacterial spores, or the like. The present invention also relates to a method for inducing proliferation or accumulation of regulatory T cells, as well as a method for inhibiting such proliferation or accumulation. Moreover, the present invention relates to a vaccine composition containing at least one strain of bacteria belonging to the genus *Clostridium* or a spore of bacteria, as well as a method for treating or preventing at least one disease or condition selected from infectious diseases and autoimmune diseases by administering the vaccine composition to an individual in need thereof. The present invention also relates to a method for screening for a compound that promotes proliferation or accumulation of regulatory T cells, as well as a non-human mammal which is used in this method, and in which a reporter gene is expressed under control of IL-10 gene expression.

BACKGROUND ART

Hundreds of species of commensal microorganisms are harbored in gastrointestinal tracts of mammals, and intimately interact with the host immune systems. Results of researches using germ-free (GF) animals have shown that the commensal microorganisms exert great influences on the development of mucosal immune systems such as histogenesis of Peyer's patches (PPs) and isolated lymphoid follicles (ILFs), secretion of antimicrobial peptides from epithelium, and accumulation of unique lymphocytes in mucosal tissues, the unique lymphocytes including immunoglobulin A-producing plasma cells, intraepithelial lymphocytes, IL-17-producing CD4-positive T cells (Th 17), and IL-22-producing NK-like cells (Non-Patent Documents 1 to 7). Consequently, the presence of intestinal bacteria enhances protective functions of the mucous membranes, providing the hosts with robust immune responses against pathogenic microbes invading the bodies. On the other hand, the mucosal immune systems maintain unresponsiveness to dietary antigens and harmless microbes (Non-Patent Document 3). For this reason, abnormality in the regulation of cross-talk between commensal bacteria and an immune system (intestinal dysbiosis) may lead to overly robust

2

immune response to environmental antigens, so that inflammatory bowel disease (IBD) is caused (Non-Patent Documents 8 to 10).

Results of Recent studies have shown that individual commensal bacteria control differentiation of their specific immune cells in the mucosal immune system. For example, *Bacteroides fragilis*, which is a commensal bacterium in humans, specifically induces a systemic Th1 cell response and a mucosal IL-10-producing T cell response in mice, and plays a role in protecting the host from colitis, which would otherwise be caused by a pathogen (Non-Patent Document 3). Segmented filamentous bacteria, which are intestinal commensal bacteria in mice, are shown to induce mucosal Th17 cell response and thereby to enhance resistance against infection of gastrointestinal tracts of the host with a pathogen (Non-Patent Documents 11 to 13). In addition, short-chain fatty acids derived from several commensal bacteria are known to suppress intestinal inflammation (Non-Patent Document 14). Moreover, it is presumed that the presence of some species of intestinal microbiota exerts a great influence on the differentiation of regulatory T cells (hereafter referred to as "Treg cells") which maintain homeostasis of the immune system.

Meanwhile, regulatory T cells which have been identified as a subset suppressing immunity are CD4⁺ T cells in which a transcription factor Foxp3 is expressed, and are known to play an important role in maintaining immunological homeostasis (Non-Patent Documents 8, 9, 15, and 16). Moreover, it has been known that the Foxp3-expressing cells are present in a large number especially in the colon, and only Treg cells present locally in the colon constantly expresses IL-10, which is an immunosuppressive cytokine, at a high level (Non-Patent Document 17). It is also known that animals having CD4⁺ Foxp3⁺ cells from which IL-10 is specifically removed develop inflammatory bowel disease (Non-Patent Document 18).

Accordingly, if the mechanism of the induction of Treg cells which produce IL-10 in the colon at a high level is elucidated, immunosuppression can be enhanced, which in turn can be applied to treatment of autoimmune diseases such as inflammatory bowel disease, as well as to organ transplantation.

However, mechanisms of how a large number of Treg cells come to be present in the colon and how the Treg cells produce IL-10 in the colon at a high level are still unclear. Moreover, it is also still unclear what species of bacteria constituting the intestinal commensal bacterial flora exerts the influence on the induction of regulatory T cells.

CITATION LIST

Non Patent Literature

- [NPL 1] J. J. Cebra, "Am J Clin Nutr", May, 1999, 69, 1046S
- [NPL 2] A. J. Macpherson, N. L. Harris, "Nat Rev Immunol", June 2004, 4, 478
- [NPL 3] J. L. Round, S. K. Mazmanian, "Nat Rev Immunol", May 2009, 9, 313
- [NPL 4] D. Bouskra et al., "Nature", Nov. 27, 2008, 456, 507
- [NPL 5] K. Atarashi et al., "Nature", Oct. 9, 2008, 455, 808
- [NPL 6] Ivanov, II et al., "Cell Host Microbe", Oct. 16, 2008, 4, 337
- [NPL 7] S. L. Sanos et al., "Nat Immunol", January 2009, 10, 83

[NPL 8] M. A. Curotto de Lafaille, J. J. Lafaille, "Immunity", May 2009, 30, 626

[NPL 9] M. J. Barnes, F. Powrie, "Immunity", Sep. 18, 2009, 31, 401

[NPL 10] W. S. Garrett et al., "Cell", Oct. 5, 2007, 131, 33

[NPL 11] Ivanov, II et al., "Cell", Oct. 30, 2009, 139, 485.

[NPL 12] V. Gaboriau-Routhiau et al., "Immunity", Oct. 16, 2009, 31, 677

[NPL 13] N. H. Salzman et al., "Nat Immunol", 11, 76.

[NPL 14] K. M. Maslowski et al., "Nature", Oct. 29, 2009, 461, 1282

[NPL 15] L. F. Lu, A. Rudensky, "Genes Dev", Jun. 1, 2009, 23, 1270

[NPL 16] S. Sakaguchi, T. Yamaguchi, T. Nomura, M. Ono, "Cell", May 30, 2008, 133, 775

[NPL 17] C. L. Maynard et al., "Nat Immunol", September 2007, 8, 931

[NPL 18] Y. P. Rubtsov et al., "Immunity", April 2008, 28, 546

SUMMARY OF INVENTION

Technical Problem

The present invention has been made in view of the above-described problems of the conventional techniques. Accordingly, an object of the present invention is to identify intestinal commensal bacteria which induce the proliferation or accumulation of regulatory T cells. Another object of the present invention is to provide compositions or the like which comprise the identified intestinal commensal bacteria or a physiologically active substance derived therefrom, and which thus have induce the proliferation or accumulation of regulatory T cells (Treg cells).

Solution to Problem

The present inventors have made earnest studies to solve the above-described problems. As a result, the present inventors have found that a chloroform-treated fraction and a spore-forming fraction of a fecal sample obtained from a mammal induces accumulation of regulatory T cells (Treg cells) in the colon. Moreover, the present inventors have found that bacteria belonging to the genus *Clostridium* induce proliferation or accumulation of regulatory T cells in the colon. The present inventors have also found that the regulatory T cells induced by these bacteria suppress proliferation of effector T cells. Furthermore, the present inventors have also found that colonization of bacteria belonging to the genus *Clostridium* and resultant proliferation or accumulation of Treg cells regulate local and systemic immune responses.

From these findings, the present inventors have found that the use of bacteria belonging to the genus *Clostridium*, spores thereof, or a physiologically active substance derived therefrom makes it possible to induce the proliferation or accumulation of regulatory T cells (Treg cells), and further to suppress immune functions.

More specifically, the present invention has the following aspects:

(1) A composition that induces proliferation or accumulation of regulatory T cells, the composition comprising, as an active ingredient, at least one substance selected from the group consisting of the following (a) to (c):

(a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;

(b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and

(c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

(2) Any of the compositions described herein, wherein the regulatory T cells are transcription factor Foxp3-positive regulatory T cells or IL-10-producing regulatory T cells.

(3) The composition according to any one of (1) and (2), wherein the composition has an immunosuppressive effect.

(4) The composition according to any one of (1) to (3), wherein the composition is a pharmaceutical composition.

(5) A method for inducing proliferation or accumulation of regulatory T cells in an individual (e.g., an individual in need thereof, such as an individual in need of induction of proliferation or accumulation of regulatory T cells), the method comprising a step of administering, to the individual, at least one substance selected from the group consisting of the following (a) to (c):

(a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;

(b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and

(c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

(6) A method for inducing proliferation or accumulation of regulatory T cells in an individual (e.g., an individual in need thereof, such as an individual in need of induction of proliferation or accumulation of regulatory T cells), the method comprising a step of administering an antibiotic against Gram-negative bacteria to the individual. And the antibiotic can be administered alone or in combination with at least one substance selected from the group consisting of the following (a) to (c):

(a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;

(b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and

(c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

(7) A method for inducing proliferation or accumulation of regulatory T cells in an individual, the method comprising a step of administering, to the individual, at least one substance selected from the group consisting of almond skin, inulin, oligofructose, raffinose, lactulose, pectin, hemicellulose, amylopectin, acetyl-Co A, biotin, beet molasses, yeast extracts, and resistant starch.

(8) The method according to any one of (5) to (7), wherein a therapeutic composition is further administered to the individual.

Note that, the "therapeutic composition" here is meant to be something other than (a)-(c) described in (5) and (6), the antibiotic against Gram-negative bacteria described in (6), or the substances described in (7).

(9) The method according to (8), wherein

the therapeutic composition is at least one composition selected from the group consisting of corticosteroids, mesalazine, mesalamine, sulfasalazine, sulfasalazine derivatives, immunosuppressive drugs, cyclosporin A, mercaptopurine, azathiopurine, prednisone, methotrexate, antihistamines, glucocorticoids, epinephrine, theophylline, cromolyn sodium, anti-leukotrienes, anti-cholinergic drugs

for rhinitis, anti-cholinergic decongestants, mast-cell stabilizers, monoclonal anti-IgE antibodies, vaccines, and combinations thereof.

(10) The method according to any one of (5) to (9), wherein one measurement selected from the group consisting of promotion of IL-10 expression, promotion of CTLA4 expression, promotion of IDO expression, and suppression of IL-4 expression is used as an index of the induction of proliferation or accumulation of regulatory T cells in the individual.

(11) A method for inhibiting proliferation or accumulation of regulatory T cells in an individual (e.g., an individual thereof), the method comprising a step of administering an antibiotic against Gram-positive bacteria to the individual.

(12) The composition according to any one of (5) to (11), wherein the regulatory T cells are transcription factor Foxp3-positive regulatory T cells or IL-10-producing regulatory T cells.

(13) A vaccine composition comprising at least one substance selected from the group consisting of the following (a) to (c):

- (a) bacteria belonging to the genus *Clostridium*;
- (b) a spore of bacteria in a spore-forming fraction of a fecal sample obtained from a mammal; and
- (c) bacteria in a chloroform-treated fraction of a fecal sample obtained from a mammal.

(14) A method for treating aiding in treating, reducing the severity of, or preventing at least one disease selected from infectious diseases and autoimmune diseases in an individual (e.g., an individual in need thereof, such as an individual in need of treatment, reduction in the severity of or prevention of at least one such disease), the method comprising administering the vaccine composition according to (13) to the individual.

(15) A method for screening for a compound having an activity to promote proliferation or accumulation of regulatory T cells, the method comprising:

(I) preparing a test substance from at least one substance selected from the group consisting of the following (a) to (c):

- (a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;
- (b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and
- (c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

(II) preparing non-human mammals in which a reporter gene is to be expressed under control of IL-10 gene expression;

(III) bringing the test substance into contact with the non-human mammal;

(IV) after the contact with the test substance, detecting cells expressing the reporter gene in a CD4⁺ Foxp3⁺ cell group of the non-human mammal, and determining the number of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene or a ratio of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene to cells in the

CD4⁺ Foxp3⁺ cell group not expressing the reporter gene;

(V) detecting cells expressing the reporter gene in a CD4⁺ Foxp3⁺ cell group of the non-human mammal which has not been in contact with the test substance, and determining the number of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene or a ratio of cells in the CD4⁺ Foxp3⁺ cell

group expressing the reporter gene to cells in the CD4⁺ Foxp3⁺ cell group not expressing the reporter gene; and

(VI) comparing the number or the ratio determined in (IV) with the number or the ratio determined in (V), and determining, when the number or the ratio determined in (IV) is greater than that determined in (V), that the test substance is a compound that promotes proliferation or accumulation of Treg cells.

(16) A non-human mammal which is used for the method according to (15), and in which the reporter gene is expressed under the control of the IL-10 gene expression.

(17) A method for isolating, from a sample of bacteria belonging to the genus *Clostridium*, a compound having an activity to promote proliferation or accumulation of regulatory T cells, the method comprising (I) to (III):

(I) preparing a genomic DNA from the sample of bacteria belonging to the genus *Clostridium*;

(II) inserting the genomic DNA into a cloning system, and preparing a gene library derived from the sample of bacteria belonging to the genus *Clostridium*; and

(III) isolating a compound having an activity to promote proliferation or accumulation of regulatory T cells, by use of the gene library obtained in step (II).

(18) A method of treatment comprising (I) to (III):

(I) measuring the percentage and/or absolute amounts of *Clostridium* Clusters IV and XIV in the microbiota of a subject,

(II) comparing them to the same measurements in a healthy individual; and

(III) administering a substance to the subject, if a statistically significant decrease in the number/amounts of *Clostridium* cluster IV, XIV in the subject compared to the healthy individual is detected, wherein the substance is at least one substance selected from the group consisting of the following (a) to (c):

- (a) any of the substances described herein
- (b) an antibiotic against Gram-negative bacteria; and
- (c) the substance selected from the group consisting of almond skin, inulin, oligofructose, raffinose, lactulose, pectin, hemicellulose, amylopectin, acetyl-Co-A, biotin, beet molasses, yeast extracts, and resistant starch.

(19) A method of monitoring, comprising (I) to (II):

(I) measuring level of *Clostridium* cluster IV, XIV in a subject after administering at least one substance selected from the group consisting of the following (a) to (c):

- (a) any of the substances described herein;
- (b) an antibiotic against Gram-negative bacteria; and
- (c) the substance selected from the group consisting of almond skin, inulin, oligofructose, raffinose, lactulose, pectin, hemicellulose, amylopectin, acetyl-Co A, biotin, beet molasses, yeast extracts, and resistant starch; and

(II) if the level increases, it is judged to be a sign that the subject is responding favorably.

Advantageous Effects of Invention

The compositions of the present invention containing as an active ingredient bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria serves as an excellent composition for inducing the proliferation or accumulation of regulatory T cells (Treg cells). Immunity in a living organism can be suppressed through administration of the composition of the present invention as a pharmaceutical product or ingestion of the composition as a food or beverage. Accordingly, the composition of the present invention can be used, for

example, to prevent or treat autoimmune diseases or allergic diseases, as well as to suppress immunological rejection in organ transplantation or the like. In addition, if a food or beverage such as a health food comprises the composition of the present invention, healthy individuals can ingest the composition easily and routinely. As a result, it is possible to induce the proliferation or accumulation of regulatory T cells and thereby to improve immune functions.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic diagram showing a method of producing $\text{Il10}^{\text{venus}}$ mouse.

FIG. 2 is a diagram showing results of Southern blotting performed for analysis as to whether or not the $\text{Il10}^{\text{venus}}$ mice have an $\text{Il10}^{\text{venus}}$ allele.

FIG. 3 is a FACS dot-plot diagram showing results obtained when Venus-positive cells and Venus-negative cells from the $\text{Il10}^{\text{venus}}$ mice were sorted.

FIG. 4 is a graph showing the results obtained when the amounts of IL-10 mRNA expressed in Venus positive-cells and Venus-negative cells of the $\text{Il10}^{\text{venus}}$ mice were analyzed by real-time RT-PCR.

FIG. 5 is a graph showing change in the ratio of Foxp3⁺ cells in CD4⁺ lymphocytes of SPF mice.

FIG. 6 shows FACS dot-plot diagrams showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the small intestine, the colon, and the peripheral lymph nodes of GF mice and SPF mice.

FIG. 7 is a graph showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the small intestine, the colon, and the peripheral lymph nodes of GF mice and SPF mice.

FIG. 8 shows graphs showing analysis results of the numbers of CD4⁺ Foxp3⁺ cells isolated from the small intestine, the colon, and the peripheral lymph nodes of GF mice and SPF mice.

FIG. 9 is a plot diagram showing analysis results of the ratios of Venus⁺ cells in CD4⁺ cells in various tissues of SPF mice treated with antibiotics.

FIG. 10 shows FACS dot-plot diagrams showing analysis results of the ratio of Foxp3⁺ cell in CD4⁺ lymphocytes isolated from the colonic lamina propria of GF mice to which a fecal suspension of SPF mice was administered.

FIG. 11 is a graph showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the lamina propria of the colon and the lamina propria of the small intestine of GF mice to which a fecal suspension of SPF mice was administered.

FIG. 12 is a graph showing analysis results of the ratio of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the lamina propria of mice deficient in ILFs, PPs, and colonic-patches.

FIG. 13 shows FACS dot-plot diagrams showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the colonic lamina propria of GF mice to which specific commensal bacteria were administered.

FIG. 14 shows graphs showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the colonic lamina propria of GF mice to which specific commensal bacteria were administered.

FIG. 15 is a graph showing analysis results of the ratios of IFN- γ ⁺ cells in CD4⁺ lymphocytes isolated from the colonic lamina propria of mice in which specific commensal bacteria were colonized.

FIG. 16 is a graph showing analysis results of the ratios of IL-17⁺ cells in CD4⁺ lymphocytes isolated from the colonic lamina propria of mice in which specific commensal bacteria were colonized.

FIG. 17 is a graph showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the colon of kinds of SPF mice each being deficient in a pathogen-associated molecular pattern recognition receptor-associated factor.

FIG. 18 is a graph showing analysis results of the ratios of Foxp3⁺ cells in CD4⁺ lymphocytes isolated from the colonic lamina propria of Myd88^{-/-} mice in which the *Clostridium* was colonized.

FIG. 19 shows FACS dot-plot diagrams showing analysis results of the ratios of Venus⁺ cells in lymphocytes isolated from various tissues of $\text{Il10}^{\text{venus}}$ mice.

FIG. 20 is a FACS dot-plot diagram showing analysis results of the expression of a T cell receptor β chain on cell surfaces of lymphocytes isolated from the colonic lamina propria of $\text{Il10}^{\text{venus}}$ mice.

FIG. 21 shows FACS dot-plot diagrams showing analysis results of the expression of IL-17, IL-4, and IFN- γ in lymphocytes isolated from the colonic lamina propria of $\text{Il10}^{\text{venus}}$ mice.

FIG. 22 shows graphs showing analysis results of the amounts of mRNAs of IL-10, CTLA4, Foxp3, and GITR expressed in spleen Foxp3⁺CD4⁺ cells, spleen Foxp3⁺CD4⁺ cells, colonic lamina propria Venus⁺ cells, and small intestinal lamina propria Venus⁺ cells.

FIG. 23 shows FACS dot-plot diagrams showing analysis results of the expression of CD4, Foxp3, and Venus in the lamina propria of the small intestine and the lamina propria of the colon of GF $\text{Il10}^{\text{venus}}$ mice and SPF $\text{Il10}^{\text{venus}}$ mice.

FIG. 24 shows FACS dot-plot diagrams showing analysis results of the expression of Venus and Foxp3 of CD4 cells in various tissues of SPF $\text{Il10}^{\text{venus}}$ mice.

FIG. 25 shows FACS dot-plot diagrams showing analysis results of the expression of Foxp3 and Venus in $\text{Il10}^{\text{venus}}$ mice in which specific commensal bacteria were colonized.

FIG. 26 is a graph showing analysis results of the expression of Foxp3 and/or Venus of CD4⁺ cells in the small intestine of $\text{Il10}^{\text{venus}}$ mice in which specific commensal bacteria were colonized.

FIG. 27 is a graph showing analysis results of the expression of Foxp3 and/or Venus of CD4⁺ cells in the colon of $\text{Il10}^{\text{venus}}$ mice in which specific commensal bacteria were colonized.

FIG. 28 is a plot diagram showing analysis results of the ratios of Venus⁺ cells in CD4⁺ cells isolated from various tissues of $\text{Il10}^{\text{venus}}$ mice treated with antibiotics.

FIG. 29 is a graph showing analysis results of immunoregulatory functions of CD4⁺ Venus⁺ cells from the colonic lamina propria of GF $\text{Il10}^{\text{venus}}$ mice in which the genus *Clostridium* was colonized, CD4⁺ Venus⁺ cells from the colonic lamina propria of SPF $\text{Il10}^{\text{venus}}$ mice, and CD4⁺ GFP⁺ cells from the spleen of Foxp3^{eGFP} reporter mice.

FIG. 30 is a graph showing the results obtained when SPF B6 mice were treated with polymyxin B or vancomycin for 4 weeks, and then analyzed for the ratio of Foxp3⁺ cells in the CD4⁺ cell group.

FIG. 31 is a graph showing the results obtained when SPF mice-derived chloroform-treated feces were orally administered to GF mice, and then the ratio of Foxp3⁺ cells in the CD4⁺ cell group was analyzed.

FIG. 32 is a graph showing the general results of flow cytometry analysis on Helios expression in LP lymphocytes

in the thymuses or the colons of SPF mice, GF mice, *Lactobacillus*-colonized mice, or *Clostridium*-colonized mice.

FIG. 33 shows plot diagrams showing representative results of flow cytometry analysis on CD4 expression, Foxp3 expression, and Helios expression in the LP lymphocytes in the thymuses or the colons of the SPF mice, the GF mice, the *Lactobacillus*-colonized mice, or the *Clostridium*-colonized mice.

FIG. 34 is a graph showing the results obtained when the whole colons derived from GF mice, *Lactobacillus*-colonized mice, or *Clostridium*-colonized mice were cultured, and the culture supernatants thereof were analyzed for the TGF- β 1 concentration by ELISA.

FIG. 35 is a graph showing the results obtained when intestinal epithelial cells (IECs) derived from GF mice or *Clostridium*-colonized mice were cultured, and the culture supernatants thereof were analyzed for the TGF- β 1 concentration by ELISA.

FIG. 36 is a graph showing the results obtained when splenic CD4⁺ T cells were cultured together with an anti-CD3 antibody and with a culture supernatant of IECs isolated from GF mice or mice colonized with 46 bacterial strains of the genus *Clostridium* (Clost.) in the presence or absence of an anti-TGF- β antibody, and the T cells were collected on day 5 of the culture and analyzed for Foxp3 expression by real-time RT-PCR.

FIG. 37 is a graph showing the results obtained when C57BL/6 GF mice were orally inoculated with 46 bacterial strains of the genus *Clostridium* (Clost.) or three bacterial strains of the genus *Lactobacillus* (Lacto.), and IECs were collected three weeks after the inoculation and analyzed for the relative mRNA expression level of the MMP2 gene by real-time RT-PCR.

FIG. 38 is a graph showing the results obtained when C57BL/6 GF mice were orally inoculated with 46 bacterial strains of the genus *Clostridium* (Clost.) or three bacterial strains of the genus *Lactobacillus* (Lacto.), and IECs were collected three weeks after the inoculation and analyzed for the relative mRNA expression level of the MMP9 gene by real-time RT-PCR.

FIG. 39 is a graph showing the results obtained when C57BL/6 GF mice were orally inoculated with 46 bacterial strains of the genus *Clostridium* (Clost.) or three bacterial strains of the genus *Lactobacillus* (Lacto.), and IECs were collected three weeks after the inoculation and analyzed for the relative mRNA expression level of the MMP13 gene by real-time RT-PCR.

FIG. 40 is a graph showing the results obtained when C57BL/6 GF mice were orally inoculated with 46 bacterial strains of the genus *Clostridium* (Clost.) or three bacterial strains of the genus *Lactobacillus* (Lacto.), and IECs were collected three weeks after the inoculation and analyzed for the relative mRNA expression level of the IDO gene by real-time RT-PCR.

FIG. 41 is a graph showing the results obtained when control mice (SPF) and *Clostridium*-administered mice (SPF+Clost.) were treated with 2% DSS, observed and measured for the body weight loss, the hardness of stool, and bleeding for six days, and then evaluated numerically.

FIG. 42 is a photograph showing the state of the colons collected on day 6 after the control mice (SPF) and the *Clostridium*-administered mice (SPF+Clost.) were treated with 2% DSS.

FIG. 43 shows photomicrographs showing the results obtained when the control mice (SPF) and the *Clostridium*-administered mice (SPF+Clost.) were treated with 2% DSS,

and the colons thereof were collected on day 6 and analyzed histologically by HE staining.

FIG. 44 is a graph showing the results obtained when control mice (SPF) and *Clostridium*-administered mice (SPF+Clost.) were sensitized with oxazolone, and subsequently the inside of each rectum was treated with a 1% oxazolone/50% ethanol solution, and the body weight loss was measured.

FIG. 45 shows photomicrographs showing the results obtained when the control mice (SPF) and the *Clostridium*-administered mice (SPF+Clost.) were sensitized with oxazolone, and subsequently the inside of each rectum was treated with a 1% oxazolone/50% ethanol solution, and the colons obtained by the treatment were analyzed histologically by HE staining.

FIG. 46 is a graph showing the results obtained when control mice (SPF) and *Clostridium*-administered mice (SPF+Clost.) were immunized by administering alum-absorbed ovalbumin (OVA) twice at a 2-week interval, and the sera were collected therefrom and analyzed for the concentration of OVA-specific IgE in these sera by ELISA.

FIG. 47 is a graph showing the results obtained when the control mice (SPF) and the *Clostridium*-administered mice (SPF+Clost.) were immunized by administering the alum-absorbed OVA twice at a 2-week interval, and splenic cells were collected and analyzed for IL-4 production of these splenic cells by in-vitro OVA restimulation.

FIG. 48 is a graph showing the results obtained when the control mice (SPF) and the *Clostridium*-administered mice (SPF+Clost.) were immunized by administering the alum-absorbed OVA twice at a 2-week interval, and the splenic cells were collected and analyzed for IL-10 production of these splenic cells by the in-vitro OVA restimulation.

FIG. 49 is Phylogenetic tree constructed by the neighbor-joining method with the resulting sequences of the 41 strains of *Clostridium* and those of known bacteria obtained from Genbank database using Mega software.

FIG. 50 is histograms showing Foxp3 expression gated CD4 cells from GF mice (Germ-free mouse #1 and #2) or GF mice colonized with three strains of *Clostridium* belonging to cluster IV (3 strains of Clost. mouse #1 and #2).

FIG. 51 is histograms showing Foxp3 expression by CD4 positive lymphocytes from GF mice (GF) or GF mice gavaged with chloroform-treated human stool (GF+Chloro).

FIG. 52 is a graph showing Foxp3 expression by CD4 positive lymphocytes from GF mice (GF) or GF mice gavaged with chloroform-treated human stool (GF+Chloro).

FIG. 53 is a graph showing amounts of *Clostridium* and Bacteroides in feces of mice gavaged with chloroform-treated human stool

DESCRIPTION OF EMBODIMENTS

<Composition Having Effect of Inducing Proliferation or Accumulation of Regulatory T Cells>

The present invention provides a composition that induces proliferation or accumulation of regulatory T cells, the composition comprising, as an active ingredient, at least one substance selected from the group consisting of the following (a) to (c):

- (a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;
- (b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and
- (c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

In the present invention “regulatory T cells” mean T cells which have a function of suppressing an abnormal or excessive immune response, and which play a role in immune tolerance. The regulatory T cells are typically transcription factor Foxp3-positive CD4-positive T cells. However, the regulatory T cells of the present invention also include transcription factor Foxp3-negative regulatory T cells, as long as the regulatory T cells are IL-10-producing CD4-positive T cells.

The meaning of the “induces proliferation or accumulation of regulatory T cells” in the present invention includes an effect of inducing the differentiation of immature T cells into regulatory T cells, which differentiation leads to the proliferation or the accumulation of regulatory T cells. In addition, the meaning of the “induces proliferation or accumulation of regulatory T cells” in the present invention includes in-vivo effects, in vitro effects, and ex vivo effects. Accordingly, all of the following effects are included: an effect of inducing in vivo proliferation or accumulation of regulatory T cells through administration or ingestion of the bacteria belonging to the genus *Clostridium* or the physiologically active substance or the like derived from the bacteria; an effect of inducing proliferation or accumulation of cultured regulatory T cells by causing the bacteria belonging to the genus *Clostridium* or the physiologically active substance or the like derived from the bacteria to act on the cultured regulatory T cells; and an effect of inducing proliferation or accumulation of regulatory T cells which are collected from a living organism and which are intended to be subsequently introduced into a living organism, such as the organism from which they were obtained or another organism, by causing the bacteria belonging to the genus *Clostridium* or the physiologically active substance or the like derived from the bacteria to act on the regulatory T cells. The effect of inducing proliferation or accumulation of regulatory T cells can be evaluated, for example, as follows. Specifically, the bacteria belonging to the genus *Clostridium* or the physiologically active substance or the like derived from the bacteria is orally administered to an experimental animal such as a germ-free mouse, then CD4-positive cells in the colon are isolated, and the ratio of regulatory T cells contained in the CD4-positive cells is measured by flow cytometry (refer to Example 7).

The regulatory T cells of which proliferation or accumulation is induced by the composition of the present invention are preferably transcription factor Foxp3-positive regulatory T cells or IL-10-producing regulatory T cells.

The “bacteria belonging to the genus *Clostridium*,” which are the active ingredient in the composition of the present invention, is not particularly limited as long as the bacteria have the effect of inducing proliferation or accumulation of regulatory T cells. The bacteria preferably belong to the cluster XIVa or the cluster IV. One strain of the bacteria alone can be used for the composition of the present invention, but two or more strains of the bacteria can be used together for the composition of the present invention. The use of multiple strains of bacteria belonging to the cluster XIVa or the cluster IV in combination can bring about an excellent effect on regulatory T cells. In addition to the bacteria belonging to these clusters, bacteria belonging to other clusters (for example, bacteria belonging to the cluster III) can also be used in combination. If more than one strain of bacteria is used (e.g., one or more strain belonging to cluster XIVa, one or more strain belonging to cluster IV, one or more strain belonging to a cluster other than cluster XIVa or cluster IV, such as one or more strain belonging to cluster III), the type and number of strains used can vary widely.

The type and number to be used can be determined based on a variety of factors (e.g., the desired effect, such as induction or inhibition of proliferation or accumulation of regulatory T cells; the disease or condition to be treated, prevented or reduced in severity; the age or gender of the recipient) The strains can be present in a single composition, in which case they will be consumed or ingested together, or can be present in more than one composition (e.g., each can be in a separate composition), in which case they can be consumed individually or the compositions can be combined and the resulting combination (combined compositions) consumed or ingested. Any number or combination of strains that proves effective (e.g., any number from one to 200, such as 1 to 100, 1 to 50, 1 to 40, 1 to 30, 1 to 20, 1 to 10, 1 to 5 and any number therebetween) can be administered. In certain embodiments of the present invention, a combination of some or all of the 46 strains described in Document (Itoh, K., and Mitsuoka, T. Characterization of clostridia isolated from faeces of limited flora mice and their effect on caecal size when associated with germ-free mice. Lab. Animals 19: 111-118 (1985)) is used. For example, at least one, two or more, three, three or more, four, four or more, five, five or more, six, six or more or any other number of the 46 described strains, including 46 strains, can be used. They can be used in combination with one another and in combination with strains not described in the cited reference (e.g., in combination with one or more strains belonging to cluster III). Note that, the cluster of “bacteria belonging to the genus *Clostridium*” can be identified, for example, as follows. Specifically, the bacteria belonging to the genus *Clostridium* are classified by PCR using a primer set consisting of SEQ ID NOs 64 and 65 (for *Clostridium* spp. belonging to the cluster XIVa) or a primer set consisting of SEQ ID NOs 66 and 67 (for *Clostridium* spp. belonging to the cluster IV) (refer to Example 18). In addition, the bacteria belonging to the genus *Clostridium* are classified by sequencing of 16S rRNA gene amplified using a primer set consisting of SEQ ID NOs 19 and 20 (refer to Example 7).

Viable cells of the bacteria belonging to the genus *Clostridium* can be used for the composition of the present invention, and killed cells thereof may also be used for the composition. In addition, from the viewpoint of stability to heat, resistance to antibiotics and the like, and long storage period, the bacteria belonging to the genus *Clostridium* are preferably in the form of spore.

The meaning of the “physiologically active substance derived from bacteria belonging to the genus *Clostridium*” of the present invention includes substances contained in the bacteria, secretion products of the bacteria, and metabolites of the bacteria. Such a physiologically active substance can be identified by purifying an active component from the bacteria, a culture supernatant thereof, or intestinal tract contents in the intestinal tract of a mouse in which only bacteria belonging to the genus *Clostridium* are colonized by an already known purification method.

The active ingredient “spore-forming fraction of a fecal sample obtained from a mammal” in the composition of the present invention is not particularly limited, as long as the fraction includes spore-forming bacteria present in feces of a mammal, and has the effect of inducing proliferation or accumulation of regulatory T cells.

The active ingredient “chloroform-treated fraction of a fecal sample obtained from a mammal” in the composition of the present invention is not particularly limited, as long as the fraction is obtained by treating feces of a mammal with

chloroform (for example, 3% chloroform), and has the effect of inducing proliferation or accumulation of regulatory T cells.

Note that the “mammal” in the present invention is not particularly limited, and examples thereof include humans, mice, rats, cattle, horses, pigs, sheep, monkeys, dogs, and cats.

Meanwhile, when the “spore-forming fraction of a fecal sample obtained from a mammal” or the “chloroform-treated fraction of a fecal sample obtained from a mammal” is cultured in a medium, substances contained in the bacteria, secretion products of the bacteria, metabolites of the bacteria are released from the bacteria and the like contained in the fraction. The meaning of the active ingredient “culture supernatant of the fraction” in the composition of the present invention includes such substances, secretion products, and metabolites. The culture supernatant is not particularly limited, as long as the culture supernatant has the effect of inducing proliferation or accumulation of regulatory T cells. Examples of the culture supernatant include a protein fraction of the culture supernatant, a polysaccharide fraction of the culture supernatant, a lipid fraction of the culture supernatant, and a low-molecular weight metabolite fraction of the culture supernatant.

The composition of the present invention may be in the form of a pharmaceutical composition, a food or beverage (which may also be an animal feed), or a reagent used for an animal model experiment, the pharmaceutical composition, the food or beverage, and the reagent having the effect of inducing proliferation or accumulation of regulatory T cells. An example of the present invention revealed that regulatory T cells (Treg cells) induced by bacteria or the like belonging to the genus *Clostridium* suppressed the proliferation of effector T-cells. Accordingly, the composition of the present invention can be used suitably as a composition having an immunosuppressive effect. The immunosuppressive effect can be evaluated, for example, as follows. Specifically, regulatory T cells isolated from an experimental animal, such as a mouse, to which the composition of the present invention is orally administered are caused to act on effector T-cells (CD4⁺ CD25⁻ cells) isolated from the spleen, and then proliferation ability thereof is measured by using the intake amount of [³H]-thymidine as an index (refer to Example 14).

The composition of the present invention can be used, for example, as a pharmaceutical composition for preventing or treating an autoimmune disease such as chronic inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, or Hashimoto's disease, or an allergic disease such as pollenosis or asthma; a pharmaceutical composition for suppressing rejection in organ transplantation or the like; a food or beverage for improving immune functions; or a reagent for suppressing the proliferation or function of effector T-cells.

More specific examples of target diseases of the composition of the present invention include autoimmune diseases, allergic diseases, and rejection in organ transplantations and the like, such as inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, sprue, autoimmune arthritis, rheumatoid arthritis, Type I diabetes, multiple sclerosis, graft vs. host disease following bone marrow transplantation, osteoarthritis, juvenile chronic arthritis, Lyme arthritis, psoriatic arthritis, reactive arthritis, spondyloarthritis, systemic lupus erythematosus, insulin dependent diabetes mellitus, thyroiditis, asthma, psoriasis, dermatitis scleroderma, atopic dermatitis, graft versus host disease, acute or chronic immune disease associated with organ transplan-

tion, sarcoidosis, atherosclerosis, disseminated intravascular coagulation, Kawasaki's disease, Grave's disease, nephrotic syndrome, chronic fatigue syndrome, Wegener's granulomatosis, Henoch-Schoenlein purpura, microscopic vasculitis of the kidneys, chronic active hepatitis, uveitis, septic shock, toxic shock syndrome, sepsis syndrome, cachexia, acquired immunodeficiency syndrome, acute transverse myelitis, Huntington's chorea, Parkinson's disease, Alzheimer's disease, stroke, primary biliary cirrhosis, hemolytic anemia, polyglandular deficiency type I syndrome and polyglandular deficiency type II syndrome, Schmidt's syndrome, adult (acute) respiratory distress syndrome, alopecia, alopecia areata, seronegative arthropathy, arthropathy, Reiter's disease, psoriatic arthropathy, chlamydia, yersinia and salmonella associated arthropathy spondyloarthritis, atheromatous disease/arteriosclerosis, atopic allergy, food allergies, autoimmune bullous disease, pemphigus vulgaris, pemphigus foliaceus, pemphigoid, linear IgA disease, autoimmune haemolytic anaemia, Coombs positive haemolytic anaemia, acquired pernicious anaemia, juvenile pernicious anaemia, myalgic encephalitis/Royal Free Disease, chronic mucocutaneous candidiasis, giant cell arteritis, primary sclerosing hepatitis, cryptogenic autoimmune hepatitis, Acquired Immunodeficiency Disease Syndrome, Acquired Immunodeficiency Related Diseases, Hepatitis C, common varied immunodeficiency (common variable hypogammaglobulinaemia), dilated cardiomyopathy, fibrotic lung disease, cryptogenic fibrosing alveolitis, postinflammatory interstitial lung disease, interstitial pneumonitis, connective tissue disease associated interstitial lung disease, mixed connective tissue disease associated lung disease, systemic sclerosis associated interstitial lung disease, rheumatoid arthritis associated interstitial lung disease, systemic lupus erythematosus associated lung disease, dermatomyositis/polymyositis associated lung disease, Sjogren's disease associated lung disease, ankylosing spondylitis associated lung disease, vasculitic diffuse lung disease, haemosiderosis associated lung disease, drug-induced interstitial lung disease, radiation fibrosis, bronchiolitis obliterans, chronic eosinophilic pneumonia, lymphocytic infiltrative lung disease, postinfectious interstitial lung disease, gouty arthritis, autoimmune hepatitis, type-1 autoimmune hepatitis (classical autoimmune or lupoid hepatitis), type-2 autoimmune hepatitis (anti-LKM antibody hepatitis), autoimmune mediated hypoglycemia, type B insulin resistance with acanthosis nigricans, hypoparathyroidism, acute immune disease associated with organ transplantation, chronic immune disease associated with organ transplantation, osteoarthritis, primary sclerosing cholangitis, idiopathic leucopenia, autoimmune neutropenia, renal disease NOS, glomerulonephritis, microscopic vasculitis of the kidneys, discoid lupus, erythematosus, male infertility idiopathic or NOS, sperm autoimmunity, multiple sclerosis (all subtypes), insulin-dependent diabetes mellitus, sympathetic ophthalmia, pulmonary hypertension secondary to connective tissue disease, Goodpasture's syndrome, pulmonary manifestation of polyarteritis nodosa, acute rheumatoid fever, rheumatoid spondylitis, Still's disease, systemic sclerosis, Takayasu's disease/arteritis, autoimmune thrombocytopenia, idiopathic thrombocytopenia, autoimmune thyroid disease, hyperthyroidism, goitrous autoimmune hypothyroidism (Hashimoto's disease), atrophic autoimmune hypothyroidism, primary myxoedema, phacogenic uveitis, primary vasculitis, vitiligo, allergic rhinitis (pollen allergies), anaphylaxis, pet allergies, latex allergies, drug allergies, allergic rhinoconjunctivitis, eosinophilic esophagitis, hypereosinophilic syndrome,

eosinophilic gastroenteritis cutaneous lupus erythematosus, eosinophilic esophagitis, hypereosinophilic syndrome, and eosinophilic gastroenteritis.

The composition of the present invention can also be used as a pharmaceutical composition for preventing or treating infectious diseases in an individual whose resistance to the infectious diseases is impaired because of damage due to excessive inflammation caused by the immunity.

Example of infectious pathogens which impair maintenance or recovery of homeostasis of a host, and which eventually bring about such immunopathological tissue damage include *Salmonella*, *Shigella*, *Clostridium difficile*, *Mycobacterium* (which cause the disease tuberculosis), protozoa (which cause the disease malaria), filarial nematodes (which cause the disease filariasis), *Schistosoma* (which cause the disease schistosomiasis), *Toxoplasma* (which cause the disease toxoplasmosis), *Leishmania* (which cause the disease leishmaniasis), HCV and HBV (which cause the disease hepatitis C and hepatitis B), and herpes simplex viruses (which cause the disease herpes).

Pharmaceutical preparations can be formulated from the composition of the present invention by already known drug formulation methods. For example, the composition of the present invention can be used orally or parenterally in the forms of capsules, tablets, pills, liquids, powders, granules, fine granules, film-coated preparations, pellets, troches, sublingual preparations, chewables, buccal preparations, pastes, syrups, suspensions, elixirs, emulsions, liniments, ointments, plasters, cataplasms, transdermal absorption systems, lotions, inhalations, aerosols, injections, suppositories, and the like.

For formulating these preparations, the composition of the present invention can be used in appropriate combination with carriers acceptable pharmacologically or acceptable for a food or beverage, specifically, with sterile water, physiological saline, vegetable oil, solvent, a base material, an emulsifier, a suspending agent, a surfactant, a stabilizer, a flavoring agent, an aromatic, an excipient, a vehicle, a preservative, a binder, a diluent, a tonicity adjusting agent, a soothing agent, a bulking agent, a disintegrating agent, a buffer agent, a coating agent, a lubricant, a colorant, a sweetener, a thickening agent, a flavor corrigent, a solubilizer, other additives, or the like.

Meanwhile, for formulating a pharmaceutical preparation thereof, and particularly for formulating a pharmaceutical preparation for oral administration, it is preferable to use in combination a composition which enables an efficient delivery of the composition of the present invention to the colon, from the viewpoint of more efficiently inducing the proliferation or accumulation of regulatory T cells in the colon.

Such a composition or method which enables the delivery to the colon is not particularly limited, and known compositions or methods can be employed as appropriate. Examples thereof include pH sensitive compositions, more specifically, enteric polymers which release their contents when the pH becomes alkaline after the enteric polymers pass through the stomach. When a pH sensitive composition is used for formulating the pharmaceutical preparation, the pH sensitive composition is preferably a polymer whose pH threshold of the decomposition of the composition is 6.8 to 7.5. Such a numeric value range is a range where the pH shifts toward the alkaline side at a distal portion of the stomach, and hence is a suitable range for use in the delivery to the colon.

Moreover, another example of the composition enabling the delivery to the colon is a composition which ensures the delivery to the colon by delaying the release of the contents

by approximately 3 to 5 hours, which corresponds to the small intestinal transit time. In an example of formulating a pharmaceutical preparation using the composition for delaying the release, a hydrogel is used as a shell. The hydrogel is hydrated and swells upon contact with gastrointestinal fluid, so that the contents are effectively released. Furthermore the delayed release dosage units include drug-containing compositions having a material which coats or selectively coats a drug. Examples of such a selective coating material include in vivo degradable polymers, gradually hydrolyzable polymers, gradually water-soluble polymers, and/or enzyme degradable polymers. A preferred coating material for efficiently delaying the release is not particularly limited, and examples thereof include cellulose-based polymers such as hydroxypropyl cellulose, acrylic acid polymers and copolymers such as methacrylic acid polymers and copolymers, and vinyl polymers and copolymers such as polyvinylpyrrolidone.

Examples of the composition enabling the delivery to the colon further include bioadhesive compositions which specifically adhere to the colonic mucosal membrane (for example, a polymer described in the specification of U.S. Pat. No. 6,368,586), and compositions into which a protease inhibitor is incorporated for protecting particularly a biopharmaceutical preparation in the gastrointestinal tracts from decomposition due to an activity of a protease.

An example of a system enabling the delivery to the colon is a system of delivering a composition to the colon by pressure change in such a way that the contents are released by utilizing pressure change caused by generation of gas in bacterial fermentation at a distal portion of the stomach. Such a system is not particularly limited, and a more specific example thereof is a capsule which has contents dispersed in a suppository base and which is coated with a hydrophobic polymer (for example, ethyl cellulose).

Another example of the system enabling the delivery to the colon is a system of delivering a composition to the colon, the system being specifically decomposed by an enzyme (for example, a carbohydrate hydrolase or a carbohydrate reductase) present in the colon. Such a system is not particularly limited, and more specific examples thereof include systems which use food components such as non-starch polysaccharides, amylose, xanthan gum, and azopolymers.

When used as a pharmaceutical composition, the composition of the present invention may be used in combination with an already known pharmaceutical composition for use in immunosuppression. Such a known pharmaceutical composition is not particularly limited, and may be at least one therapeutic composition selected from the group consisting of corticosteroids, mesalazine, mesalamine, sulfasalazine, sulfasalazine derivatives, immunosuppressive drugs, cyclosporin A, mercaptopurine, azathiopurine, prednisone, methotrexate, antihistamines, glucocorticoids, epinephrine, theophylline, cromolyn sodium, anti-leukotrienes, anti-cholinergic drugs for rhinitis, anti-cholinergic decongestants, mast-cell stabilizers, monoclonal anti-IgE antibodies, vaccines (preferably vaccines used for vaccination where the amount of an allergen is gradually increased), and combinations thereof. It is preferable to use these therapeutic compositions in combination with the composition of the present invention.

When the composition of the present invention is used as a food or beverage, the food or beverage can be, for example, a health food, a functional food, a food for specified health use, a dietary supplement, a food for patients, or an animal feed. The food or beverage of the

present invention can be ingested in the forms of the compositions as described above, and also can be ingested in the forms of various foods and beverages. Specific examples of the foods and beverages include various beverages such as juices, refreshing beverages, tea beverages, drink preparations, jelly beverages, and functional beverages; alcoholic beverages such as beers; carbohydrate-containing foods such as rice food products, noodles, breads, and pastas; paste products such as fish hams, sausages, paste products of seafood; retort pouch products such as curries, food dressed with a thick starchy sauces, and Chinese soups; soups; dairy products such as milk, dairy beverages, ice creams, cheeses, and yogurts; fermented products such as fermented soybean pastes, yogurts, fermented beverages, and pickles; bean products; various confectionery products such as Western confectionery products including biscuits, cookies, and the like, Japanese confectionery products including steamed bean-jam buns, soft adzuki-bean jellies, and the like, candies, chewing gums, gummies, cold desserts including jellies, crème caramels, and frozen desserts; instant foods such as instant soups and instant soy-bean soups; microwavable foods; and the like. Further, the examples also include health foods and beverages prepared in the forms of powders, granules, tablets, capsules, liquids, pastes, and jellies. The composition of the present invention can be used for animals including humans. The animals, other than humans, are not particularly limited, and the composition can be used for various livestock, poultry, pets, experimental animals, and the like. Specific examples of the animals include pigs, cattle, horses, sheep, goats, chickens, wild ducks, ostriches, domestic ducks, dogs, cats, rabbits, hamsters, mice, rats, monkeys, and the like, but the animals are not limited thereto.

Without wishing to be bound by theory, in the present invention, individuals in which the relative abundance of bacteria belonging to the group Firmicutes (the group to which the *Clostridium* clusters IV and XIVa belong) is large gain more body weight than individuals in which the relative abundance of bacteria belonging to the group Bacteroidetes is large. Accordingly, the composition of the present invention is capable of conditioning absorption of nutrients and improving feed efficiency. From such a viewpoint, the composition of the present invention can be used for promoting body weight gain, or for an animal feed good in feed efficiency.

Moreover, the addition of the composition of the present invention to an antibiotic-free animal feed makes it possible to increase the body weight of a subject that ingests the animal feed to a level equal to or higher than those achieved by antibiotic-containing animal feeds, and also makes it possible to reduce pathogenic bacteria in the stomach to a level equal to those achieved by typical antibiotic-containing animal feeds. Accordingly, the composition of the present invention can be used for an animal feed which does not need the addition of antibiotics.

In addition, unlike conventional bacteria (*Lactobacillus* and *Bifidobacteria*) in commercial use which are not easy to incorporate into the livestock production, the composition of the present invention in the spore form can be pelletized, sprayed, or easily mixed with an animal feed, and also can be added to drinking water.

The feeding of such an animal feed using the composition of the present invention is not particularly limited, and the animal feed may be fed to a subject at regular intervals in a selective manner, or may be fed for a certain period (for example, at its birth, during weaning, or when the subject to be fed is relocated or shipped).

Moreover, from the above-described viewpoint, the composition of the present invention can be preferably used for malnourished humans. In other words, also when the subject who ingests the composition is a human, the composition of the present invention can preferably be used for promoting the body weight gain, and enhancing the energy absorption from foods.

The food or beverage of the present invention can be manufactured by a manufacturing technique which is well known in the technical field. To the food or beverage, one or more components (for example, a nutrient) which are effective for the improvement of an immune function by the immunosuppressive effect may be added. In addition, the food or beverage may be combined with another component or another functional food exhibiting a function other than the function of the improvement of an immune function to thereby serve as a multi-functional food or beverage.

Moreover, the composition of the present invention can be preferably incorporated into foods requiring a processing step which may destroy ordinary probiotic strains. Specifically, most commercially usable probiotic strains cannot be incorporated into foods which need to be processed by any one of a heat treatment, long term storage, a freezing treatment, a mechanical stress treatment, and a high-pressure treatment (for example, extrusion forming or roll forming). On the other hand, because of an advantageous nature of forming spores, the composition of the present invention can be easily incorporated into such processed foods.

For example, the composition of the present invention in the form of spore can survive even in a dried food, and can remain living even after being ingested. Likewise, the composition of the present invention can withstand low-temperature sterilization processes, typically processes at a temperature in a range from 70° C. to the boiling point, both inclusive. Thus, the composition of the present invention can be incorporated into all kinds of dairy products. Furthermore, the composition of the present invention can withstand long-term storage of many years; high-temperature processing such as baking and boiling; low-temperature processing such as freezing and cold storage; and high-pressure treatments such as extrusion forming and roll forming.

The foods which need to be processed under such harsh conditions are not particularly limited, and examples thereof include foods which need to be processed in a microwave oven to be edible (for example, oatmeal), foods which need to be baked to be edible (for example, muffin), foods which need to be subjected to a sterilization high-temperature treatment for a short period of time to be edible (for example, milk), and foods which need to be heated to be drinkable (for example, hot tea).

When the composition of the present invention is administered or ingested, the amount thereof for the administration or ingestion is selected as appropriate depending on the age, body weight, symptoms, health conditions, of a subject, the kind of the composition (a pharmaceutical product, a food or beverage, or the like), and the like. For example, the amount per administration or ingestion is generally 0.01 mg/kg body weight to 100 mg/kg body weight, and preferably 1 mg/kg body weight to 10 mg/kg body weight. The present invention also provides a method for suppressing the immunity of a subject, the method being characterized in that the bacteria belonging to the genus *Clostridium* or the physiologically active substance derived from the bacteria is administered into or ingested by the subject as described above.

A product of the composition of the present invention (a pharmaceutical product, a food or beverage, or a reagent) or

a manual thereof may be provided with a note stating that the product can be used to suppress the immunity (including a note stating that the product has an immunosuppressive effect, and a note stating that the product has an effect of suppressing the proliferation or function of effector T-cells). Here, the “provision to the product or the manual thereof with the note” means that the note is provided to a main body, a container, a package, or the like of the product, or the note is provided to a manual, a package insert, a leaflet, or other printed matters, which disclose information on the product.

<Method for Inducing Proliferation or Accumulation of Regulatory T Cells>

As described above, and as will be shown in Examples, the administration of the composition of the present invention to an individual makes it possible to induce proliferation or accumulation of regulatory T cells in the individual. Thus, the present invention can provide a method for inducing proliferation or accumulation of regulatory T cells in an individual, the method comprising a step of administering, to the individual, at least one substance selected from the group consisting of the following (a) to (c):

- (a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;
- (b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and
- (c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

Note that, the “individual” in the present invention is not particularly limited, and examples thereof include humans, various kinds of livestock, poultry, pets, experimental animals, and the like. The “individual” may be in a healthy state or a diseased state.

Moreover, as will be shown in Example 5 to be described later, Gram-positive commensal bacteria play principal roles in the proliferation or accumulation of regulatory T cells. Accordingly, the present invention can also provide a method for inducing proliferation or accumulation of regulatory T cells in an individual, the method comprising a step of administering an antibiotic against Gram-negative bacteria to the individual.

In the present invention, the “antibiotic against Gram-negative bacteria” is not particularly limited, and examples thereof include aminoglycoside antibiotics (amikacin, gentamicin, kanamycin, neomycin, netilmicin, tobramycin, and paromomycin), cephalosporin antibiotics (cefactor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefixime, cefdinir, cefditoren, cefoperazone, cefotaxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, and cefoxitin), sulfonamides, ampicillin, and streptomycin. Without wishing to be bound by theory, the “antibiotic against Gram-negative bacteria” according to the present invention is preferably one which reduces Gram-negative bacteria, and contributes to the colonization of Gram-positive bacteria.

Moreover, a prebiotic composition such as almond skin, inulin, oligofructose, raffinose, lactulose, pectin, hemicellulose (such as xyloglucan and alpha-glucans), amylopectin, and resistant starch which are not decomposed in the upper gastrointestinal tract and promote the growth of intestinal microbes in the intestinal tract, as well as growth factors such as acetyl-Co A, biotin, beet molasses, and yeast extracts, contribute to the proliferation of bacteria belonging to the genus *Clostridium*. Accordingly, the present invention can also provide a method for inducing proliferation or accumulation of regulatory T cells in an individual, the method comprising a step of administering, to the indi-

vidual, at least one substance selected from the group consisting of these substances.

Meanwhile, in the “method for inducing proliferation or accumulation of regulatory T cells” of the present invention, the composition of the present invention, the above-described “antibiotic against Gram-negative bacteria,” and the above-described “prebiotic composition or growth factor” may be used in combination. Such combined use is not particularly limited, and examples of the combined use are as follows: the “antibiotic against Gram-negative bacteria” is administered to an individual in advance, and then the composition of the present invention is administered; the “antibiotic against Gram-negative bacteria” and the composition of the present invention are simultaneously administered to an individual; the “prebiotic composition or growth factor” is administered to an individual in advance, and then the composition of the present invention is administered; the “prebiotic composition or growth factor” and the composition of the present invention are simultaneously administered to an individual; the composition of the present invention, the “antibiotic against Gram-negative bacteria,” and the “prebiotic composition or growth factor” are administered to an individual simultaneously or individually at any appropriate time.

Moreover, a therapeutic composition may be administered to an individual together with at least one substance selected from the group consisting of the composition of the present invention, the “antibiotic against Gram-negative bacteria,” and the “prebiotic composition or growth factor.”

Such a therapeutic composition is not particularly limited, and may be at least one therapeutic composition selected from the group consisting of corticosteroids, mesalazine, mesalamine, sulfasalazine, sulfasalazine derivatives, immunosuppressive drugs, cyclosporin A, mercaptopurine, azathiopurine, prednisone, methotrexate, antihistamines, glucocorticoids, epinephrine, theophylline, cromolyn sodium, anti-leukotrienes, anti-cholinergic drugs for rhinitis, anti-cholinergic decongestants, mast-cell stabilizers, monoclonal anti-IgE antibodies, vaccines (preferably, vaccines used for vaccination where the amount of an allergen is gradually increased), and combinations thereof. It is preferable to use these therapeutic compositions in combination with the above-described substance.

Moreover, there is no particular limitation imposed on the combined use of the therapeutic composition with at least one substance selected from the group consisting of the composition of the present invention, the “antibiotic against Gram-negative bacteria,” and the “prebiotic composition or growth factor”. For example, the “one substance” and the therapeutic composition are administered orally or parenterally to an individual simultaneously or individually at any appropriate time. Moreover, in the above-described “method for inducing proliferation or accumulation of regulatory T cells,” whether or not the administration of the composition of the present invention or the like actually induces the proliferation or accumulation of regulatory T cells can be determined by using, as an index, increase or reinforcement of at least one selected from the group consisting of the number of regulatory T cells, the ratio of regulatory T cells in the T cell group of the colon, a function of regulatory T cells, and expression of a marker of regulatory T cells. It is preferable to use one measurement selected from the group consisting of promotion of IL-10 expression, promotion of CTLA4 expression, promotion of IDO expression, and suppression of IL-4 expression, as the index of the induction of proliferation or accumulation of regulatory T cells.

Note that examples of a method for detecting such expression include the northern blotting, the RT-PCR, and the dot blotting for detection of gene expression at the transcription level; and the ELISA, the radioimmunoassay, the immunoblotting, the immunoprecipitation, and the flow cytometry for detection of gene expression at the translation level.

Meanwhile, a sample used for measuring such an index is not particularly limited, and examples thereof include blood sampled from an individual and tissue pieces obtained in a biopsy.

<Method for Predicting Response of Individual to Composition of Present Invention and/or Prognosis of Individual>

The present invention can provide a method in which the absolute amount or the ratio of bacteria belonging to the genus *Clostridium* in a microbiota of an individual is determined, and, when the ratio or the absolute value of the bacteria belonging to the genus *Clostridium* is reduced in comparison with a base line value obtained by performing a similar determination on an individual in a typical health state, it is determined that the individual is possibly responsive to the composition of the present invention.

In one embodiment, a method to predict a subject's response to a substance and/or the subject's prognosis is provided. The method comprises measuring the percentage or absolute amounts of *Clostridium* clusters IV and XIV in the microbiota of the subject and comparing them to a baseline value of the same measurements in a prototypical healthy subject, wherein a decreased absolute amount or percentage level of *Clostridium* clusters IV and/or XIV indicates that the subject may respond favorably to the compositions of the invention.

In one embodiment, the method further comprises measuring the composition of the microbiota of the subject after administration of the substance, wherein an increase in the percentage or absolute number of *Clostridium* spp. belonging to clusters IV, XIV after administration of the compositions of the present invention relative to prior to the administering is a positive indicator of enhanced immunosuppression (or immunoregulation). The measurement of the composition of the subject's microbiota can be made with techniques known in the art, such as 16srRNA sequencing.

Note that, in these embodiments, the substance is at least one substance selected from the group consisting of the following (a) to (e):

(a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;

(b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction;

(c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction;

(d) an antibiotic against Gram-negative bacteria according to the present invention; and

(e) at least one substance selected from the group consisting of almond skin, inulin, oligofructose, raffinose, lactulose, pectin, hemicellulose (such as xyloglucan and alpha-glucans), amylopectin, acetyl-Co A, biotin, beet molasses, yeast extracts, and resistant starch.

<Method for Inhibiting Proliferation or Accumulation of Regulatory T Cells>

As will be shown in Example 5 to be described later, Gram-positive commensal bacteria have principal roles in the proliferation or accumulation of regulatory T cells. Accordingly, the present invention can also provide a method for inhibiting proliferation or accumulation of regu-

latory T cells in an individual, the method comprising a step of administering an antibiotic against Gram-positive bacteria to the individual.

In the present invention, the "antibiotic against Gram-positive bacteria" is not particularly limited, and examples thereof include cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem).

As described above, the "individual" in the present invention is not particularly limited, and examples thereof include humans, various kinds of livestock, poultry, pets, experimental animals, and the like. The "individual" may be in a healthy state or a diseased state. Such a diseased state is not particularly limited, and examples thereof include states of being subjected to cancer immunotherapy and of suffering from an infectious disease.

Moreover, as another mode of the "method for inhibiting proliferation or accumulation of regulatory T cells," the present invention can provide a method for inhibiting proliferation or accumulation of regulatory T cells in an individual, the method comprising a step of administering, to the individual, any one of an antibody, an antibody fragment, and a peptide, which are against an antigen that is at least one substance selected from the group consisting of the following (a) to (c):

(a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;

(b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and

(c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

<Vaccine Composition and Method for Treating or Preventing Infectious Disease or Autoimmune Disease by Using the Vaccine Composition>

As described above, and as will be shown in Example 15 to be described later, the induction of Treg cells in the colon by the *Clostridium* has an important role in local and systemic immune responses. Accordingly, the present invention can also provide a "vaccine composition comprising at least one substance selected from the group consisting of the following (a) to (c): (a) bacteria belonging to the genus *Clostridium*; (b) a spore of bacteria in a spore-forming fraction of a fecal sample obtained from a mammal; and (c) bacteria in a chloroform-treated fraction of a fecal sample obtained from a mammal" and a "method for treating, aiding in treating, reducing the severity of, or preventing at least one disease selected from infectious diseases and autoimmune diseases in an individual, the method comprising administering the vaccine composition to the individual."

Note that such "autoimmune diseases" are not particularly limited, and examples thereof include those described as the "specific examples of target diseases" in <Composition Having Effect of Inducing Proliferation or Accumulation of Regulatory T cells>. The "infectious diseases" are also not particularly limited, and examples thereof include infectious diseases associated with "infectious pathogens" described as the "example of infectious pathogens" in <Composition Having Effect of Inducing Proliferation or Accumulation of Regulatory T cells>.

<Method for Screening for Compound Having Activity to Promote Proliferation or Accumulation of Regulatory T Cells>

The present invention can also provide a method for screening for a compound having an activity to promote proliferation or accumulation of regulatory T cells, the method comprising:

(1) preparing a test substance from at least one substance selected from the group consisting of the following (a) to (c):

(a) bacteria belonging to the genus *Clostridium* or a physiologically active substance derived from the bacteria;

(b) a spore-forming fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction; and

(c) a chloroform-treated fraction of a fecal sample obtained from a mammal or a culture supernatant of the fraction.

(2) preparing non-human mammals in which a reporter gene is to be expressed under control of IL-10 gene expression;

(3) bringing the test substance into contact with the non-human mammal;

(4) after the contact with the test substance, detecting cells expressing the reporter gene in a CD4⁺ Foxp3⁺ cell group of the non-human mammal, and determining the number of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene or a ratio of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene to cells in the CD4⁺ Foxp3⁺ cell group not expressing the reporter gene;

(5) detecting cells expressing the reporter gene in a CD4⁺ Foxp3⁺ cell group of the non-human mammal which has not been in contact with the test substance, and determining the number of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene or a ratio of cells in the CD4⁺ Foxp3⁺ cell group expressing the reporter gene to cells in the CD4⁺ Foxp3⁺ cell group not expressing the reporter gene; and

(6) comparing the absolute numbers or the ratios determined in steps (4) with the number or the ratio determined in (5), and determining, when the number or the ratio determined in (4) is greater than that determined in (5), that the test substance is a compound that promotes proliferation or accumulation of Treg cells.

The “test substance” according to the present invention is not particularly limited, as long as the test substance is a substance prepared from at least one substance selected from the group consisting of the substances (a) to (c). Examples of the test substance include proteins, polysaccharides, lipids, and nucleic acids which are derived from at least one substance selected from the group consisting of the above described substances (a) to (c).

The “non-human mammal in which a reporter gene is to be expressed under control of IL-10 gene expression” according to the present invention is not particularly limited, as long as the non-human mammal is a non-human mammal having a reporter gene whose expression is controlled by an IL-10 gene expression control region (for example, a promoter, or an enhancer). Examples of such a reporter gene include genes encoding fluorescent proteins (for example, GFP), and genes encoding luciferase. As the “non-human mammal in which a reporter gene is to be expressed under control of IL-10 gene expression” according to the present invention, an IL10^{enus} mouse to be shown later in Examples can be preferably used.

The “contact” according to the present invention is not particularly limited, and examples thereof include adminis-

tration of the test substance to the non-human mammal orally or parenterally (for example, intraperitoneal injection, or intravenous injection).

The present invention can also provide a non-human mammal which is used for the method, and in which the reporter gene is to be expressed under the control of the IL-10 gene expression.

Furthermore, the present invention can also provide a method for isolating, from a sample of bacteria belonging to the genus *Clostridium*, a compound having an activity to promote proliferation or accumulation of regulatory T cells, the method comprising the following steps (1) to (3):

(1) preparing a genomic DNA from the sample of bacteria belonging to the genus *Clostridium*;

(2) inserting the genomic DNA into a cloning system, and preparing a gene library derived from the sample of bacteria belonging to the genus *Clostridium*; and

(3) isolating a compound having an activity to promote proliferation or accumulation of regulatory T cells, by use of the gene library obtained in step (2).

In such steps, methods for the preparation and the isolation are not particularly limited, and known techniques for an in-vitro or in-vivo system can be used as appropriate. Moreover, the compound isolated by this method is not particularly limited, and examples thereof include nucleic acids (for example, a DNA, a mRNA, and a rRNA) derived from bacteria belonging to the genus *Clostridium*, as well as polypeptides and proteins derived from the bacteria belonging to the genus *Clostridium*.

<Other Embodiment Modes according to Present Invention>

In addition to the above-described embodiment modes, the present invention can also provide the following embodiment modes.

Specifically, the present invention can also provide a method for determining the composition of a microbiota in an individual, wherein the increase in the ratio or the absolute number of bacteria belonging to the genus *Clostridium* after the administration of the composition of the present invention to the individual with respect to the ratio or the absolute number before the administration is used as an index of increased immunosuppression. In such a method, the method for determining the composition of the microbiota is not particularly limited, and known techniques (for example, 16S rRNA sequencing) can be used as appropriate.

The present invention can also provide a method for measuring differentiation of Treg cells, wherein the increase in differentiation of Treg cells in an individual after administration of the composition of the present invention to the individual with respect to that before the administration is used as an index of increased immunosuppression (or immunoregulation).

Moreover, the composition of the present invention can also be administered to an individual under an antibiotic treatment. The timing of the administration is not particularly limited, and the composition of the present invention can be administered before or simultaneously with the antibiotic treatment, for example. Meanwhile, the composition of the present invention is preferably administered in the spore form from the viewpoint of resistance to antibiotics.

Moreover, in a preferred mode of such administration, the composition of the present invention is administered after or simultaneously with administration of an antibiotic against Gram-positive bacteria, for example. Note that such an “antibiotic against Gram-positive bacteria” is not particu-

25

larly limited, and examples thereof include cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem).

Meanwhile, in another preferred mode of such administration, the composition of the present invention is administered after (or simultaneously with) a treatment using vancomycin, metronidazole, linezolid, ramoplanin, or fidaxomicin, for example.

EXAMPLES

Hereinafter, the present invention is described more specifically on the basis of Examples. However, the present invention is not limited to Examples below.

Note that mice used in Examples were prepared or produced as follows. In the following description, mice may be referred to with "SPF" or "GF" attached in front thereof. These "SPF" and "GF" indicate that the mice were maintained in the absence of specific pathogenic bacteria (specific pathogen-free, SPF), and that the mice were maintained under Germ-Free (GF) conditions, respectively.

<Mice>

C57BL/6, Balb/c, and IQ1 mice maintained under SPF or GF conditions were purchased from Sankyo Labo Service Corporation, Inc. (Japan), JAPAN SLC, INC. (Japan), CLEA Japan, Inc. (Japan), or The Jackson Laboratory (USA). GF mice and gnotobiotic mice were bred and maintained within the gnotobiotic facility of The University of Tokyo, Yakult Central Institute for Microbiological Research, or Sankyo Labo Service Corporation, Inc. *Myd88*^{-/-}, *Rip2*^{-/-}, and *Card9*^{-/-} mice were produced as described in Non-Patent Documents 1 to 3, and backcrossed for 8 generations or more, so that a C57BL/6 genetic background was achieved. *Foxp3*^{eGFP} mice were purchased from the Jackson Laboratory.

<II10^{Venus} Mice>

To form a bicistronic locus encoding both II10 and Venus under control of an II10 promoter, a targeting construct was first created. Specifically, a cassette (IRES-Venus-SV40 polyA signal cassette, refer to Non-Patent Document 4) which was made of an internal ribosome entry site (IRES), a yellow fluorescent protein (Venus), and a SV40 polyA signal (SV40 polyA) and which was arranged next to a neomycin-resistant gene (neo), was inserted between a stop codon and a polyA signal (Exon 5) of a II10 gene. Next, the obtained targeting construct was used to cause homologous recombination with the II10 gene region in the genome of mice. Thus, II10^{Venus} mice having an II10^{Venus} alleles were produced (refer to FIG. 1). Note that in FIG. 1 "tk" represents a gene coding thymidine kinase, "neo" represents the neomycin-resistant gene, and "BamH1" represents a cleavage site by the restriction enzyme BamH1.

Genomic DNAs were extracted from the II10^{Venus} mice, treated with BamH1, and Southern blotted by use of a probe shown in FIG. 1. FIG. 2 shows the obtained results. Wild-type and II10^{Venus} alleles were detected as bands having sizes of 19 kb and 5.5 kb, respectively. Hence, as is apparent from the results shown in FIG. 2, it was found that the homologous recombination shown in FIG. 1 occurred in the genome of the II10^{Venus} mice.

26

Further, CD4⁺ Venus cells or CDC⁺ Venus⁺ cells in the colonic lamina propria of the II10^{Venus} mice were sorted by use of a FACSARIA. Then, real-time RT-PCR was carried out on an ABI 7300 system by a method to be described later, to determine the amount of IL-10 mRNA expressed. FIGS. 3 and 4 show the obtained results. As is apparent from the results shown in FIGS. 3 and 4, it was found that, since the development of the IL-10 mRNA was detected only in the CDC4⁺ Venus⁺ cells, the expression of IL-10 mRNA in the II10^{Venus} mice was correctly reflected in the expression of Venus. Note that the germ-free states of such II10^{Venus} mice were established in Central Institute for Experimental Animals (Kawasaki, Japan). The II10^{Venus} mice in the germ-free states were maintained in vinyl isolators in Sankyo Labo Service Corporation, Inc. (Tokyo, Japan), and used in the following Examples.

Meanwhile, experiments and analyses in Examples were carried out as follows.

<Method for Colonization of Mice with Bacteria and Analysis Thereof>

According to the description in Non-Patent Documents 5 and 6, mice in which SFB or *Clostridium* were colonized were produced. Cecal contents or feces of the obtained gnotobiotic mice were dissolved in sterile water or an anaerobic dilution solution. The dissolved cecal contents or feces as they were or after a chloroform treatment were orally administered to GF mice. Three strains of the *Lactobacillus* and 16 strains of the *Bacteroides* were cultured separately from each other in a BL or EG agar medium in an anaerobic manner. The cultured bacteria were harvested, suspended in an anaerobic TS broth, and orally administered forcibly to GF mice. The state of the colonization of the bacteria in the mice was assessed by microscopic observation conducted on a smear preparation of fecal pellets.

<Cell Separation and Flow Cytometry>

In order to isolate lymphocytes from the colonic lamina propria and the small intestinal lamina propria, the small intestine and the colon were collected, and cut open longitudinally. Then, fecal content and the like therein were washed to remove. Subsequently, the small intestine and the colon were shaken in HBSS containing 5 mM of EDTA at 37° C. for 20 minutes. After removal of epithelium and fat tissue, the intestinal tissues were cut into small pieces. To the small pieces, RPMI 1640 (4% fetal bovine serum (FBS), 1 mg/ml of collagenase D, 0.5 mg/ml of dispase, and 40 µg/ml of DNaseI (all of which were manufactured by Roche Diagnostics K.K.)) were added, and the mixture was shaken in a water bath kept at 37° C. for 1 hour. The digested tissues were washed with HBSS containing 5 mM of EDTA, and resuspended in 5 ml of 40% PERCOLL® (GE Healthcare). The suspension was overlaid on 2.5 ml of 80% PERCOLL® in a 15-ml Falcon tube. Then, centrifugation was carried out at room temperature and at 2000 rpm for 20 minutes to conduct cell separation by PERCOLL® density gradient centrifugation. Cells at the interface were collected, and used as lamina propria lymphocytes. The collected cells were suspend in a staining buffer (PBS, 2% FBS, 2 mM EDTA, and 0.09% NaN₃), and stained by use of an anti-CD4 antibody (RM4-5, BD Biosciences) labeled with PE or PE-Cy7. After the staining of CD4, *Foxp3* in the cells were stained by use of CYTOFIX/CYTOPERM® Kit Plus with GOLGISTOP® (BD Biosciences) or *Foxp3* Staining Buffer Set (eBioscience), as well as an anti-*Foxp3* antibody (FJK-16s, eBioscience) labeled with Alexa647. Flow cytometry was performed by use of a FACScant II, and the data were analyzed by FLOWJO® software (TreeStar Inc.). The sorting of the cells were performed by use of a FACSARIA™.

<Real-Time RT-PCR>

From an RNA prepared by using RNeasy Mini Kit (Qiagen), a cDNA was synthesized by use of a MMV reverse transcriptase (Promega KK). The obtained cDNA was analyzed by real-time RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) and ABI 7300 real time PCR system (Applied Biosystems), or real-time RT-PCR using SYBR Premix Ex Taq (TAKARA) and Light Cycler 480. For each sample, a value obtained was normalized for the amount of GAPDH. A primer set was designed by using Primer Express Version 3.0 (Applied Biosystems), and those exhibiting a 90% or higher sequence identity at an initial evaluation were selected. The primer set used was as follows:

Foxp3	(SEQ ID NO: 1)
5' -GGCAATAGTTCCTTCCCAGAGTT-3'	
	(SEQ ID NO: 2)
5' -GGGTCGCATATTGTGGTACTTG-3'	
CTLA4	(SEQ ID NO: 3)
5' -CCTTTTGTAGCCCTGCTCACTCT-3'	
	(SEQ ID NO: 4)
5' -GGGTCACTGTATGGCTTCAG-3'	
GITR	(SEQ ID NO: 5)
5' -TCAGTGCAAGATCTGCAAGCA-3'	
	(SEQ ID NO: 6)
5' -ACACCGGAAGCCAAACACA-3'	
IL-10	(SEQ ID NO: 7)
5' -GATTTTAATAAGCTCCAAGACCAAGGT-3'	
	(SEQ ID NO: 8)
5' -CTTCTATGCAGTTGATGAAGATGTCAA-3'	
GAPDH	(SEQ ID NO: 9)
5' -CCTCGTCCCGTAGACAAAATG-3'	
	(SEQ ID NO: 10)
5' -TCTCCACTTTGCCACTGCAA-3'	
Mmp2	(SEQ ID NO: 11)
5' -GGACATTGTCTTTGATGGCA-3'	
	(SEQ ID NO: 12)
5' -CTTGTCACGTGGTGTCACTG-3'	
Mmp9	(SEQ ID NO: 13)
5' -TCTCTGGACGTCAAATGTGG-3'	
	(SEQ ID NO: 14)
5' -GCTGAACAGCAGAGCCTTC-3'	
Mmp13	(SEQ ID NO: 15)
5' -AGGTCTGGATCACTCCAAGG-3'	
	(SEQ ID NO: 16)
5' -TCGCCTGGACCATAAAGAA-3'	
Ido1	(SEQ ID NO: 17)
5' -AGAGGATGCGTGACTTTGTG-3'	
	(SEQ ID NO: 18)
5' -ATACAGCAGACCTTCTGGCA-3'	

<Preparation and Culturing of Large Intestinal Epithelial Cells (IECs)>

First, the colon was collected, cut open longitudinally, and rinsed with PBS. Subsequently, the colon was treated with 1 mM dithiothreitol (DTT) at 37° C. for 30 minutes on a shaker, and then vortexed for one minute to disrupt the epithelial integrity. The released IECs were collected, and suspended in 5 ml of 20% percoll. The suspension was overlaid on 2.5 ml of 80% percoll in a 15-ml Falcon tube. Then, the tube was centrifuged at 25° C. and 780 g for 20 minutes to conduct cell separation by percoll density gradient centrifugation. Cells at the interface were collected, and used as colonic IECs (purity: 90% or higher, viability: 95%). The obtained IECs thus collected were suspended in RPMI containing 10% FBS, and 1×10⁵ cells of the IECs were cultured in a 24-well plate for 24 hours. Thereafter, the culture supernatant was collected, and measured for active TGF-β1 level by ELISA (Promega).

Meanwhile, for culturing T cells in vitro, 1.5×10⁵ MACS-purified splenic CD4⁺ T cells were cultured in each well of a round-bottomed 96-well plate, together with a 50% conditioned medium in which IECs isolated from GF mice or *Clostridium*-colonized mice were cultured, and with 25 ng/ml of hIL-2 (PeproTech), in the presence or absence of 25 μg/ml of an anti-TGF-β antibody (R&D). Note that 10 μg/ml of an anti-CD3 antibody and an anti-CD28 antibody (BD Bioscience) were bound to the round-bottomed plate. After a 5-day culture, the CD4⁺ T cells were collected, and subjected to a real-time PCR.

<Colitis Experimental Model>

A fecal suspension of *Clostridium*-colonized mice was orally administered to C57BL/6 mice (2-week old), and grown in a conventional environment for six weeks.

For preparing a DSS-induced colitis model, 2% (wt/vol) DSS (reagent grade, DSS salt, molecular weight=36 to 50 kD, manufactured by MP Biomedicals), together with drinking water, was given to the mice for six days.

Meanwhile, for preparing an oxazolone-induced colitis model, the mice were presensitized by transdermally applying, onto the mice, 150 μl of a 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich)/100% ethanol solution. Five days after that, 150 μl of a 1% oxazolone/50% ethanol solution was intrarectally administered again to the presensitized mice under a light anesthesia. Note that the intrarectal administration was conducted by using a 3.5 F catheter.

Each mouse was analyzed daily for body weight, occult blood, bleeding visible with the naked eyes (gross blood), and the hardness of stool. Moreover, the body weight loss percentage, intestinal bleeding (no bleeding, occult blood (hemocult+), or bleeding visible with the naked eyes), and the hardness of stool (normal stool, loose stool, or diarrhea) were evaluated numerically, and the disease activity index (DAI) was calculated in accordance with the description in "S. Wirtz, C. Neufert, B. Weigmann, M. F. Neurath, Nat Protoc 2, 541 (2007)."

<OVA Specific IgE Reaction>

BALB/c SPF mice were inoculated with a fecal suspension of *Clostridium*-colonized mice (2-week old), and grown in a conventional environment. Then, 1 μg of OVA (grade V, Sigma) and 2 mg of alum (Thermo Scientific), 0.2 ml in total, were intraperitoneally injected to the mice (at their ages of 4 weeks and 6 weeks). Sera were collected every week from the mice at the root of their tail, and OVA-specific IgE was measured by ELISA (Chondrex). Then, at their ages of 8 weeks, splenic cells were collected, inoculated in a 96-well plate at 1×10⁶ cells per well, and stimu-

29

lated with OVA (100 µg/ml) for three days. Thereafter, the culture supernatant was collected, and measured for IL-4 and IL-10 levels by ELISA (R&D).

<Statistical Analysis>

The difference between control and experimental groups was evaluated by the Student's t-test.

Example 1

First, it was investigated whether or not accumulation of regulatory T cells (Treg cells) in the colonic lamina propria was dependent on commensal bacteria. Specifically, lymphocytes were isolated from peripheral lymph nodes (pLN) of Balb/c mice bred in the absence of specific pathogenic bacteria (SPF) or from lamina propria of the colon or the small intestine (SI) of the mice. The CD4 and Foxp3 were stained by antibodies. Then, the ratio of Foxp3⁺ cells in CD4⁺ lymphocytes was analyzed by flow cytometry. FIG. 5 shows the obtained results. As is apparent from the results shown in FIG. 5, it was found that Foxp3⁺ Treg cells were present at a high frequency in the lamina propria of the gastrointestinal tracts, especially in the colonic lamina propria, of the mice kept under the environment free from specific pathogenic microorganisms (SPF). In addition, it was also found that the number of the Foxp3⁺ Treg cells in the colonic lamina propria gradually increased up to three months after their birth, whereas the number of the Foxp3⁺ Treg cells in the peripheral lymph nodes was basically constant from the time of two weeks after their birth.

Example 2

Next, it was investigated whether or not the temporal accumulation of the Treg cells in the colon as found in Example 1 had a relationship with the colonization of intestinal commensal microbiota. Specifically, the expression of CD4 and the expression of Foxp3 in lymphocytes isolated from the small intestine, the colon, and the peripheral lymph nodes of mice bred under a germ-free (GF) or SPF environment (8 weeks old: Balb/c mice, IQI mice, and C57BL/6 mice) were analyzed. Similar results were obtained in three or more independent experiments. FIGS. 6 and 7 show the obtained results. Note that, in FIG. 7, each white circle represents the absolute number of CD4⁺ Foxp3⁺ cells in an individual mouse, and the error bars represent standard deviations (SDs).

In addition, lamina propria lymphocytes were collected from SPF mice and GF mice (Balb/c mice or C57BL/6 mice). CD4 and Foxp3 were stained with antibodies. Then, the lamina propria lymphocytes were analyzed by FACS. FIG. 8 shows the obtained results. Note that in FIG. 8 each white circle represents the absolute number of CD4⁺ Foxp3⁺ cells in an individual mouse, ** indicates that "P<0.001", and indicates that "P<0.01."

Further, lymphocytes were isolated from the lamina propria of the colon, the lamina propria of the small intestine (SI), Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) of mice (SPF C57BL/6 mice) to which antibiotics were orally administered with water for eight weeks. CD4 and Foxp3 were stained with antibodies. Then, the lymphocytes were analyzed by FACS. Similar results were obtained in two or more independent experiments. FIG. 9 shows the obtained results (the ratio of the Foxp3⁺ cells in the CD4⁺ cells of an individual mouse). Note that the following antibiotics were used in combination in accordance with the description in the following document:

30

ampicillin (A; 500 mg/L, Sigma)
vancomycin (V; 500 mg/L, NACALAI TESQUE, INC.)
metronidazole (M; 1 g/L, NACALAI TESQUE, INC.)
neomycin (N; 1 g/L, NACALAI TESQUE, INC.)

Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, R. Medzhitov, *Cell* 118, 229 (Jul. 23, 2004)

Fagarasan et al., *Science* 298, 1424 (Nov. 15, 2002)

In FIG. 9, each white circle represents the absolute number of the CD4⁺ Foxp3⁺ cells in an individual mouse, each horizontal bar represents the average value of the absolute numbers, * indicates that "P<0.01," and "AVMN" represents the kinds of the administered antibiotics by using the first letters of the antibiotics.

As is apparent from the results shown in FIGS. 6 to 9, the frequencies and the absolute numbers of Foxp3⁺ CD4⁺ cells in the small intestine and the peripheral lymph nodes of the GF mice were equal to or greater than those of the SPF mice (refer to FIGS. 6 to 8). In addition, the numbers of the Treg cells in the small intestinal lamina propria, Peyer's patches, and mesenteric lymph nodes of the SPF mice to which the antibiotics were orally administered for eight weeks were equal to or greater than those of the SPF mice (refer to FIG. 9). Meanwhile, the number of the Foxp3⁺ CD4⁺ cells in the colonic lamina propria of the GF mice was decreased significantly in comparison with that of the SPF mice (refer to FIGS. 6 and 7). This decrease was commonly observed among mice of different genetic backgrounds (Balb/c, IQI, and C57BL/6), as well as among mice bred in different animal facilities (refer to FIG. 7 for the data regarding the different genetic backgrounds, the data regarding the mice bred in the different animal facilities are not shown in the drawings). In addition, it was also shown that the number of Treg cells in the colonic lamina propria of the SPF C57BL/6 mice to which the antibiotics were administered was decreased significantly (refer to FIG. 9).

Example 3

Next, it was directly checked whether or not the decrease in the number of the Treg cells in the colonic lamina propria of the GF mice shown in Example 2 was attributed to the absence of microbiota. Specifically, a fecal suspension of B6 SPF mice purchased from The Jackson Laboratory was orally administered to GF-IQI mice (conventionalization). Three weeks after the administration, lymphocytes were isolated from the colonic lamina propria, and the expression of Foxp3 in CD4⁺ lymphocytes was analyzed. FIGS. 10 and 11 show the obtained results. Note that each white circle in FIG. 11 represents the absolute number of CD4⁺ Foxp3⁺ cells in an individual mouse, the error bars represent standard deviations (SD), * indicates that "P<0.01" in Student's t-test, and ** indicates that "P<0.001." As is apparent from the results shown in FIGS. 10 and 11, the number of Treg cells in the small intestinal lamina propria did not change. However, the number of the Treg cells in the colonic lamina propria increased significantly. Hence, it was shown that host-microbial interaction played an important role in the accumulation of Foxp3⁺ Treg cells in the colonic lamina propria, while the accumulation of the Treg cells in the small intestinal lamina propria had a different mechanism.

Example 4

Next, the relationship between the gut-associated lymphoid tissues of mice and the number of Foxp3⁺ cells in the colonic lamina propria of the mice was investigated in accordance with the method described in M. N. Kweon et al., *J Immunol* 174, 4365 (Apr. 1, 2005). Specifically, 100 µg

31

of an extracellular domain recombinant protein (a fusion protein (LT β R-Ig) between a lymphotoxin β receptor (LT β R) and a Fc region of human IgG1, refer to Honda et al., J Exp Med 193, 621 (Mar. 5, 2001)) was injected intraperitoneally into pregnant C57BL/6 mice 14 days after conception. The LT β R-Ig was again injected intraperitoneally into fetuses obtained from such mice, so that mice from which isolated lymphoid follicles (ILFs), Peyer's patches (PPs), and colonic-patches (CPs) were completely removed were produced. Then, the ratios of Foxp3⁺ cells in CD4⁺ cells in the colonic lamina propria of the mice treated with the LT β R-Ig, and mice treated with rat IgG (control) were analyzed by FACS. FIG. 12 shows the obtained results. Note that in FIG. 12 each white circle represents the ratio of Foxp3⁺ cells in an individual mouse, and the error bars represent standard deviations. As is apparent from the results shown in FIG. 12, it was found that the ratio of the Foxp3⁺ cells in the colonic lamina propria of the mice deficient in isolated lymphoid follicles, Peyer's patches, and the colonic-patches (the mice treated with the LT β -Ig) rather increased. Accordingly, it was suggested that the decrease in the number of the Treg cells in the colonic lamina propria of the GF mice and the mice treated with the antibiotics was caused because the transmission of specific signals which promotes the accumulation of Treg cells in the colonic lamina propria and which is caused by the intestinal microbes did not occur, rather than simply because of a secondary effect of disorganized gut-associated lymphoid tissues.

Example 5

To investigate whether or not a specific intestinal flora induced the accumulation of colonic Treg cells, vancomycin as an antibiotic against Gram-positive bacteria or polymyxin B as an antibiotic against Gram-negative bacteria was administered to SPF mice (from 4 weeks of age) for four weeks, and analyzed for the ratio of Foxp3⁺ cells in the CD4⁺ cell group ([%] Foxp3⁺ in CD4). FIG. 30 shows the obtained results. Note that, in FIG. 30, "SPF" indicates the result of SPF mice (control), "poly B" indicates the result of the SPF mice to which polymyxin B was administered, and "Vanco." indicates the result of the SPF mice to which vancomycin was administered. Meanwhile, indicates that "P<0.01."

As is apparent from the results shown in FIG. 30, the number of Treg cells in the colon of the mice to which vancomycin was administered was markedly decreased in comparison with that of the control. In contrast, no influence was observed on the number of Treg cells of the mice to which polymyxin B was administered. Those facts suggested that Gram-positive commensal bacteria played a major role in accumulation of Treg cells.

Example 6

A recent report has suggested that spore-forming bacteria play an important role in intestinal T cells response (see V. Gaboriau-Routhiau et al., Immunity 31, 677 (Oct. 16, 2009)). In this respect, fecal microorganisms (spore-forming fraction) resistant to 3% chloroform were orally administered to GF mice, which were then analyzed for the ratio of Foxp3⁺ cells in the CD4⁺ cell group ([%] Foxp3⁺ in CD4). FIG. 31 shows the obtained results. Note that, in FIG. 31, "GF" indicates the result of GF mice, and "+chloro" indicates the result of the GF mice to which the chloroform-treated feces were administered. Meanwhile, ** indicates that "P<0.001."

32

As is apparent from the results shown in FIG. 31, three weeks after the administration of the chloroform-treated feces, the number of Treg cells in the administered mice was markedly increased to the same level as those of the SPF mice and the GF mice to which the untreated feces was forcibly administered (see FIGS. 7 and 11).

Accordingly, considering the results shown in Example 5 in combination, it was revealed that the specific components of the indigenous microbiota were highly likely to belong to the Gram-positive group, and that the spore-forming fraction played an important role in the induction of Treg cells.

Example 7

Next, the species of the intestinal microbiota which induced the accumulation of Treg cells in the colon as suggested in Examples 4 to 6 were identified. Specifically, segmented filamentous bacteria (SFB), 16 strains of the Bacteroides spp. (Bactero. (6 strains of *B. vulgatus*, 7 of the *B. acidifaciens* group 1, and 3 of the *B. acidifaciens* group 2)), 3 strains of the *Lactobacillus* (Lacto. (*L. acidophilus*, *L. fermentum*, and *L. murinum*)), and 46 strains of *Clostridium* spp. (Clost., refer to "Itoh, K., and Mitsuoka, T. Characterization of clostridia isolated from faeces of limited flora mice and their effect on caecal size when associated with germ-free mice. Lab. Animals 19: 111-118 (1985)"), or microbiota collected from mice (SPF) bred under a conventional environment was orally administered to GF-Balb/c mice or GF-IQI mice. The mice were maintained in vinyl isolators for three weeks. Then, CD4 cells were isolated from the colon and the small intestine of these mice. The numbers of Treg cells in the colon and the small intestine were analyzed by flow cytometry.

FIG. 13 shows FACS dot-plots obtained when a gate was set on CD4⁺ cells of the Balb/c mice. FIG. 14 shows the ratio of Foxp3⁺ cells in CD4⁺ cells of each mouse.

Note that, the bacteria belonging to the genus *Clostridium* are classified by sequencing of 16S rRNA gene, as follows. Specifically, the 16S rRNA genes of the bacteria were amplified by PCR using 16S rRNA gene-specific primer pairs: 5'-AGAGTTTGATCMTGGCTCAG-3' (SEQ ID NO: 19) and 5'-ATTACCGCGGCKGCTG-3' (SEQ ID NO: 20) (see T. Aebischer et al., Vaccination prevents *Helicobacter pylori*-induced alterations of the gastric flora in mice. FEMS Immunol. Med. Microbiol. 46, 221-229(2006)). The 1.5-kb PCR product was then introduced into pCR-Blunt Vector. The inserts were sequenced and aligned using the ClustalW software program. The resulting sequences of 16S rRNA genes derived from strain 1-41 of 46 strains of *Clostridium* spp. were shown in SEQ ID NO: 21-61. Phylogenetic tree which was constructed by the neighbor-joining method with the resulting sequences of the 41 strains of *Clostridium* and those of known bacteria obtained from Genbank database using Mega software was shown in FIG. 49.

As is apparent from the results shown in FIGS. 13 and 14, no effect on the number of the Treg cells in the colon was observed in the GF mice in which the segmented filamentous bacteria (SFB) were colonized (refer to FIG. 14). Moreover, mice in which the cocktail of three strains of *Lactobacillus* was colonized gave similar results (refer to FIG. 14). On the other hand, it was shown that the accumulation of Foxp3⁺ cells in the colonic lamina propria was strongly induced in the mice in which 46 strains of *Clostridium* spp. were colonized. Importantly, such accumulation was promoted irrespective of the genetic backgrounds of the mice, and led to the increase in number similar to that in the SPF mice although intestinal microbiota

33

of only a single genus were colonized. It was also shown that the colonization of the *Clostridium* did not change the number of Treg cells in the small intestinal lamina propria (refer to FIG. 14). Note that, when the 16 strains of *Bacteroides* spp. were colonized, the number of Treg cells in the colon was increased significantly. However, the extent of the increase varied depending on the genetic background of the mice in which the bacteria were colonized (refer to FIGS. 13 and 14).

Example 8

Next, CD4 expression, Foxp3 expression, and Helios expression in LP lymphocytes of the thymuses and the colons of SPF mice, GF mice, *Lactobacillus*-colonized mice, and *Clostridium*-colonized mice were analyzed by flow cytometry.

FIGS. 32 and 33 show the obtained results. Note that, in FIGS. 32 and 33, "GF" or "Germ Free" indicates the results of the GF mice, "SPF" indicates the results of the SPF mice, "Lacto." indicates the results of the *Lactobacillus*-colonized mice, and "Clost." indicates the results of the *Clostridium*-colonized mice. In FIG. 32, the vertical axis represents the ratio of Helios cells in the Foxp3⁺ cell group ([%] Helios⁺ in Foxp3⁺), and ** indicates that "P<0.001."

As is apparent from the results shown in FIGS. 32 and 33, most Foxp3⁺ cells found in the SPF mice or the *Clostridium*-colonized mice did not express Helios. Note that Helios is a transcription factor known to be expressed in thymic-derived natural Treg cells (see A. M. Thornton et al., J Immunol 184, 3433 (Apr. 1, 2010)). Accordingly, it was suggested that most of the Treg cells in the SPF mice and the *Clostridium*-colonized mice were Treg cells induced in peripheral portions, i.e., so-called iTreg cells.

Example 9

Next, it was investigated whether or not the colonization of the *Clostridium* or the like had an influence on other T cells. Specifically, SFB, 16 strains of *Bacteroides* spp. (Bactero.), 46 strains of *Clostridium* spp. (Clost.), or microbiota collected from mice bred under a conventional environment (SPF) was colonized in GF IQI mice. Three weeks later, lymphocytes in the colonic lamina propria were isolated from these mice, and stimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) for four hours in the presence of GOLGISTOP® (BD Bioscience). After the stimulation was given, intracellular cytokines were stained by using an anti-IL-17 PE antibody (TC11-18H10) and an anti-IFN-γ FITC antibody (BD Bioscience) in accordance with the manual of a CYTOFIX/CYTOPERM® kit (BD Bioscience). Then, the ratio of IFN-γ⁺ cells or IL-17⁺ cells in CD4⁺ leucocytes was analyzed by flow cytometry. FIGS. 15 and 16 show the obtained results. Note that, in FIGS. 15 and 16, each white circle represents the absolute number of CD4⁺ IFN-γ⁺ cells or the absolute number of CD4⁺ IL-17⁺ cells in each individual mouse, and the error bars represent standard deviations (SD). As is apparent from the results shown in FIGS. 15 and 16, the colonization of the *Clostridium* did not have any influence on Th1 cells (CD4⁺ IFN-γ⁺ cells) in the colon, and caused only a slight increase of Th17 cells (CD4⁺ IL-17⁺ cells). Accordingly, it was suggested that the genus *Clostridium* was a genus of bacteria which specifically induced Treg cells.

Example 10

It has been reported that 46 strains of *Clostridium* spp. exert an influence on the accumulation of CD8⁺ intestinal

34

tract intraepithelial lymphocytes (IELs) in the colon. Accordingly, it is conceivable that *Clostridium* regulates the immune system in various aspects, and that *Clostridium* exhibits a marked ability to induce and maintain Treg cells especially in the colon, as described above. In addition, a kind of cytokines, transforming growth factor-β (TGF-β, is known to play an important role in regulation of Treg cell generation.

In this respect, it was examined whether or not the colonization of *Clostridium* provided a colonic environment rich in TGF-β. Specifically, first, the whole colons of GF mice, *Clostridium*-colonized mice, and *Lactobacillus*-colonized mice were cultured for 24 hours, and the culture supernatants thereof were measured for the concentration of active TGF-β (TGF-β1) by ELISA (the number of mice analyzed was four per group). FIG. 34 shows the obtained results. Note that, in FIG. 34, "GF" indicates the result of the GF mice, "Clost." indicates the result of the *Clostridium*-colonized mice, and "Lacto." indicates the result of *Lactobacillus*-colonized mice. Meanwhile, * indicates that "P<0.02," and ** indicates that "P<0.001."

As is apparent from the results shown in FIG. 34, the amount of TGF-β produced in the colons of the *Clostridium*-colonized mice was significantly larger than those of the GF mice and the *Lactobacillus*-colonized mice.

Next, intestinal epithelial cells (IECs) of GF mice and *Clostridium*-colonized mice were cultured for 24 hours, and the culture supernatants thereof were measured for the concentration of active TGF-β (TGF-β1) by ELISA (the number of mice analyzed was four per group). FIG. 35 shows the obtained results. Note that, in FIG. 35, "GF" indicates the result of the GF mice, and "Clost." indicates the result of the *Clostridium*-colonized mice. Meanwhile, ** indicates that "P<0.001."

As is apparent from the results shown in FIG. 35, TGF-β was detected in the culture supernatant of the IECs isolated from the *Clostridium*-colonized mice, whereas no TGF-β was detected in the culture supernatant of the IECs isolated from the GF mice.

Next, as described above, splenic CD4⁺ T cells were cultured for five days together with a 50% conditioned medium in which IECs isolated from the GF mice or the *Clostridium*-colonized mice were cultured, and with the anti-CD3 antibody, in the presence or absence of an anti-TGF-β antibody. Then, the T cells were collected, and analyzed for expression of Foxp3 by real-time RT-PCR. FIG. 36 shows the obtained results. Note that, in FIG. 36, "Medium" indicates the result of a medium in which no cells were cultured, "GF" indicates the result of the conditioned medium in which the IECs of the GF mice were cultured, "Clost." indicates the result of the conditioned medium in which the IECs of the *Clostridium*-colonized mice were cultured, and "Clost.+αTGFβ" indicates the result of the conditioned medium to which the anti-TGF-β antibody was added and in which the IECs of the *Clostridium*-colonized mice were cultured. Meanwhile, * indicates that "P<0.001."

As is apparent from the results shown in FIG. 36, when the culture supernatant of the IECs derived from the *Clostridium*-colonized mice was added to the splenic CD4⁺ T cells, the differentiation into Foxp3-expressing cells was accelerated. Meanwhile, the differentiation into the Treg cells was inhibited by the anti-TGF-β antibody.

Moreover, the expression of MMP2, MMP9, and MMP13, which are thought to contribute to the activation of latent TGF-β was investigated. The expression of indoleamine 2,3-dioxygenase (IDO), which is thought to be involved in the induction of Treg cells, was also investigated. Spe-

35

cifically, 46 bacterial strains of the genus *Clostridium* (Clost.), or three bacterial strains of the genus *Lactobacillus* (Lacto.) were orally administered to C57BL/6 germ-free mice. Three weeks after the administration, IECs were collected, and analyzed for relative mRNA expression levels of MMP2, MMP9, MMP13, and IDO genes by real-time RT-PCR (the number of mice analyzed was three per group). FIGS. 37 to 40 show the obtained results. Note that, in FIGS. 37 to 40, "GF#1 to 3" indicate the results of GF mice, "Clost.#1 to 3" indicate the results of the *Clostridium*-colonized mice, and "Lacto.#1 to 3" indicate the results of the *Lactobacillus*-colonized mice.

For the relationship between the activation of latent TGF- β and the above-describe MMP, see D→Angelo et al., J. Biol. Chem. 276, 11347-11353, 2001; Heidinger et al., Biol. Chem. 387, 69-78, 2006; Yu et al., Genes Dev. 14, 163-176, 2000. For the relationship between IDO and the induction of Treg cells, see G. Matteoli et al., Gut 59, 595 (May, 2010).

As is apparent from the results shown in FIGS. 37 to 39, in agreement with the production of TGF- β described above, transcription products of the genes encoding MMP2, MMP9, and MMP13 were expressed at higher levels in the IECs derived from the *Clostridium*-colonized mice than those in the GF mice and in the *Lactobacillus*-colonized mice.

Moreover, as is apparent from the results shown in FIG. 40, IDO was expressed only in the *Clostridium*-colonized mice.

Accordingly, it was revealed that the *Clostridium* activated the IECs, and led to the production of TGF- β and other Treg cell-inducing molecules in the colon.

Example 11

Next, it was investigated whether or not the Treg cell accumulation induced by the colonization of the *Clostridium* was dependant on signal transmission by pathogen-associated molecular pattern recognition receptors. Specifically, the numbers of Treg cells in the colonic lamina propria of each of SPF mice of *Myd88*^{-/-} (deficient in Myd88 (signaling adaptor for Toll-like receptor)), *Rip2*^{-/-} (deficient in Rip2 (NOD receptor adaptor)), and *Card9*^{-/-} (deficient in Card9 (essential signal transmission factor for Dectin-1 signal transmission)) were examined. In addition, *Clostridium* spp. were caused to be colonized in the *Myd88*^{-/-} GF mice, and the change in the number of Treg cells was investigated. FIGS. 17 and 18 show the obtained results. As is apparent from the results shown in FIGS. 17 and 18, the number of Treg cells of each kind of the SPF mice deficient in the associated factors of the pathogen-associated molecular pattern recognition receptors did not change relative to that of wild-type mice of the same litter, which served as a control. In addition, it was found that also when *Clostridium* spp. were colonized in GF mice deficient in Myd88, the accumulation of Treg cells in the colonic lamina propria was induced. Accordingly, it has been suggested that the mechanism of inducing the accumulation of Treg cells in the colonic lamina propria relies not on activation of recognition pathway for major pathogen-associated molecular patterns as is caused by most of bacterium, but on specific commensal bacterial species.

Example 12

Intestinal tract Foxp3⁺ Treg cells are known to exert some immunosuppressive functions through IL-10 production (re-

36

fer to Non-Patent Document 9). Meanwhile, animals having CD4⁺ Foxp3⁺ cells from which IL-10 is specifically removed are known to develop inflammatory bowel disease (refer to Non-Patent Document 18). In this respect, first, the expression of IL-10 in lymphocytes of various tissues was examined. Specifically, lymphocytes were isolated from various tissues of SPF *Il10*^{venus} mice, and the expression of CD4 and the expression of Venus were analyzed by flow cytometry. FIG. 19 shows the obtained results. Note that each numeric value in FIG. 19 represents the ratio of cells within the corresponding one of regions divided into four.

Moreover, lymphocytes in the colonic lamina propria were isolated from *Il10*^{venus} mice, and the expression of T cell receptor β chain (TCR β) on the surfaces of the cells was detected by FACS. FIG. 20 shows the obtained results (FACS dot-plots obtained when a gate was set on CD4⁺ cells). Note that each numeric value in FIG. 20 represents the ratio of cells within the corresponding one of regions divided into four.

Furthermore, lymphocytes in the colonic lamina propria were isolated from *Il10*^{venus} mice. The lymphocytes were stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for four hours in the presence of GOLGISTOP® (BD Bioscience). Then, after the stimulation was given, intracellular cytokines were stained by using an anti-IL-17 PE antibody, an anti-IL-4 APC antibody (11B11), and an anti-IFN- γ FITC antibody (BD Bioscience) in accordance with the manual of a CYTOFIX/CYTOPERM® kit (BD Bioscience). FIG. 21 shows the obtained results (FACS dot-plots obtained when a gate was set on CD4⁺ cells). Note that each numeric value in FIG. 21 represents the ratio of cells within the corresponding one of regions divided into four.

In addition, Foxp3⁺ CD4⁺ cells and Foxp3⁻ CD4⁺ cells were isolated from the spleen (Spl) of Foxp3^{eGFP} reporter mice, and Venus⁺ cells were isolated from the colonic lamina propria and the small intestine (SI) lamina propria of *Il10*^{venus} mice. Then, the obtained cells were analyzed in terms of the expression of predetermined genes. The gene expression was analyzed by real-time RT-PCR using a Power SYBR Green PCR Master Mix (Applied Biosystems) and an ABI 7300 real time PCR system (Applied Biosystems). Here, the value for each cell was normalized for the amount of GAPDH. FIG. 22 shows the obtained results. Note that in FIG. 22 the error bars represent standard deviations.

As is apparent from the results shown in FIGS. 19 to 22, almost no Venus⁺ cells (IL-10-producing cells) were detected in the cervical lymph nodes (peripheral lymph nodes), thymus, peripheral blood, lung, and liver of mice kept under the SPF conditions. Meanwhile, in the spleen, Peyer's patches, and mesenteric lymph nodes thereof, Venus⁺ cells were slightly detected (refer to FIG. 19). On the other hand, many Venus⁺ cells were found in the lymphocytes in the small intestine lamina propria and colonic lamina propria. In addition, most of the Venus⁺ cells in the intestines were positive for CD4, and also positive for T cell receptor β chain (TCR β) (refer to FIGS. 19 and 20). Moreover, it was found that the Venus⁺ CD4⁺ T cells expressed Foxp3 and other Treg cell-associated factors such as a cytotoxic T-Lymphocyte antigen (CTLA-4) and a glucocorticoid-induced TNFR-associated protein (GITR) although the Venus⁺ CD4⁺ T cells showed none of the phenotypes of Th2 (IL-4-producing) and Th17 (IL-17-producing) (refer to FIGS. 21 and 22). In addition, it was shown that the expression level of CTLA-4 in the intestinal Venus⁺ cells was higher than that in the splenic GFP⁺ Treg cells isolated from the Foxp3^{eGFP} reporter mice (refer to FIG. 22).

Venus⁺ cells can be classified into at least two subsets, namely, Venus⁺ Foxp3⁺ double positive (DP) Treg cells and Venus⁺ Foxp3⁻ Treg cells on the basis of intracellular Foxp3 expression. Cells of the latter subset correspond to type 1 regulatory T cells (Tr1) (refer to Non-Patent Documents 8 and 9). In this respect, the Venus⁺ cells (IL-10-producing cells) observed in Example 8 were investigated in terms of the expression of Foxp3. Specifically, the expression of CD4, Foxp3, and Venus in the lamina propria of the colon and the lamina propria of the small intestine of *Il10^{venus}* mice kept under GF or SPF conditions was analyzed by FACS, and the numbers of Venus⁺ cells in the intestinal tract lamina propria were compared between SPF and GF *Il10^{venus}* mice. FIG. 23 shows the obtained results (dot-plots obtained when a gate was set on CD4⁺ cells).

In addition, the intracellular expression of Venus and Foxp3 in CD4 cells in various tissues of SPF *Il10^{venus}* mice was analyzed by flow cytometry. FIG. 24 shows the obtained results (dot-plots obtained when a gate was set on CD4⁺ cells). Note that each numeric value in FIG. 24 represents the ratio of cells within the corresponding one of regions divided into four.

Moreover, in order to investigate whether or not the presence of commensal bacteria had any influence on the expression of IL-10 in regulatory cells in the gastrointestinal tracts, germ-free (GF) *Il10^{venus}* mice were prepared. Then, predetermined species of bacteria were caused to be colonized in the obtained GF *Il10^{venus}* mice. Three weeks after the species of bacteria were colonized, a CD4⁺ cell group (V⁺F⁻, Venus⁺ Foxp3⁻ cells; V⁺F⁺, Venus⁺ Foxp3⁺ cells; and V⁻F⁺, Venus⁻Foxp3⁺ cells) in which Foxp3 and/or Venus were expressed in the colon and the small intestine was analyzed by flow cytometry. FIG. 25 shows dot-plots obtained when a gate was set on colonic CD4⁺ cells, and FIGS. 26 and 27 show the ratios in the CD4⁺ cell group of each mouse. Note that each numeric value in FIG. 25 represents the ratio of cells within the corresponding one of regions divided into four. Meanwhile, the error bars in FIGS. 26 and 27 represent standard deviations, indicates that “P<0.02,” and ** indicates that “P<0.001.”

Moreover, in order to check whether or not the presence of commensal bacteria had any influence on the expression of IL-10 in regulatory cells in the gastrointestinal tracts, antibiotics were orally given with water to five or six *Il10^{venus}* mice per group for 10 weeks. The following antibiotics were used in combination.
ampicillin (A; 500 mg/L Sigma)
vancomycin (V; 500 mg/L NACALAI TESQUE, INC.)
metronidazole (M; 1 g/L NACALAI TESQUE, INC.)
neomycin (N; 1 g/L NACALAI TESQUE, INC.)

Then, CD4 and Foxp3 of lymphocytes in the lamina propria of the colon, the lamina propria of the small intestine (SI), mesenteric lymph nodes (MLN), and Peyer's patches (PPs) were stained with antibodies, and analyzed by FACS. The results were obtained from two or more independent experiments which gave similar results. FIG. 28 shows the obtained results (the ratio of Venus⁺ cells in CD4⁺ cells in each sample). Note that each white circle in FIG. 28 represents an individual sample, each horizontal bar represents an average value, * indicates that “P<0.02,” and “AVMN” represents the kinds of the administered antibiotics by using the first letters of the antibiotics.

As is apparent from the results shown in FIGS. 23 and 24, it was shown that the small intestinal lamina propria was rich in Venus⁺ Foxp3⁻ cells, namely, Tr1-like cells, and that the

Venus⁺ Foxp3⁺ DP Treg cells were present at a high frequency in the colon of the SPF mice (refer to FIGS. 23 and 24). In contrast, although sufficient numbers of Foxp3⁺ cells were observed also in other tissues, the expression of Venus was not observed in almost all of the cells (refer to FIG. 24).

In addition, as is apparent from the results shown in FIGS. 23 and 25 to 28, it was shown that all regulatory T cell fractions of Venus⁺ Foxp3⁻, Venus⁺ Foxp3⁺, and Venus⁻ Foxp3⁺ in the colon significantly decreased under the GF conditions (FIGS. 23 and 26 to 27). Moreover, similar decrease in Venus⁺ cells was observed also in the SPF *Il10^{venus}* mice treated with the antibiotics (refer to FIG. 28).

Moreover, as is apparent from the results shown in FIGS. 25 to 27, the colonization of *Clostridium* spp. strongly induced all regulatory T cell fractions of Venus⁺ Foxp3⁻, Venus⁺ Foxp3⁺, and Venus⁻ Foxp3⁺ in the colon, and the degrees of the induction thereof were equal to those in the SPF mice (refer to FIGS. 25 and 27). In addition, it was found that the colonization of the three strains of *Lactobacillus* or the colonization of SFB had an extremely small influence on the number of Venus⁺ and/or Foxp3⁺ cells in the colon (refer to FIGS. 25 and 27). Moreover, the colonization of 16 strains of *Bacteroides* spp. also induced Venus⁺ cells, but the influence of the colonization was specific to Venus⁺ Foxp3⁻ Tr1-like cells (refer to FIGS. 25 and 27). On the other hand, it was found that none of the bacterial species tested exerted any significant influence on the number of IL-10-producing cells in the small intestinal lamina propria (refer to FIG. 26).

Hence, it was shown that the genus *Clostridium* colonized in the colon or a physiologically active substance derived from the bacteria provided a signal for inducing the accumulation of IL-10⁺ regulatory T cells in the colonic lamina propria or the expression of IL-10 in T cells. Meanwhile, it was shown that the number of Venus⁺ cells in the small intestine was not significantly influenced by the situation where no commensal bacteria were present or commensal bacteria were decreased (refer to FIGS. 23 and 26 to 28), and that IL-10⁺ regulatory cells (Tr1-like cells) accumulated in the small intestinal lamina propria independently of commensal bacteria.

Example 14

It was investigated whether or not Venus⁺ cells induced by the genus *Clostridium* had an immunosuppressive function similar to that of Venus⁺ cells in the colon of SPF mice. Specifically, CD4⁺ CD25 cells (effector T cells, Teff cells) isolated from the spleen were seeded in a flat-bottomed 96-well plate at 2×10⁴/well, and cultured for three days together with 2×10⁴ splenic CD11c⁺ cells (antigen-presenting cells) subjected to 30 Gy radiation irradiation treatment, 0.5 μg/ml of an anti-CD3 antibody, and a lot of Treg cells. In addition, for the last six hours, the CD4⁺ CD25⁻ cells were cultured, with [³H]-thymidine (1 μCi/well) was added thereto. Note that, Treg cells used in Example 14 were CD4⁺ GFP⁺ T cells isolated from the spleen of Foxp3^{eGFP} reporter mice, or CD4⁺ Venus⁺ T cells in the colonic lamina propria of GF *Il10^{venus}* mice in which *Clostridium* spp. were colonized or SPF *Il10^{venus}* mice. Then, proliferation of the cells was determined based on the uptake amount of [³H]-thymidine, and represented by a count per minute (cpm) value.

As is apparent from the results shown in FIG. 29, Venus⁺ CD4⁺ cells of the mice in which the genus *Clostridium* was colonized suppressed in vitro proliferation of CD25 CD4⁺ activated T cells. The suppression activity was slightly

inferior to that of GFP⁺ cells isolated from the Foxp3^{eGFP} reporter mice, but equal to that of Venus⁺ cells isolated from the SPF II10^{Venus} mice. Accordingly, it has been shown that the genus *Clostridium* induces IL-10-expressing T cells having sufficient immunosuppressive activities, and thereby plays a critical role in maintaining immune homeostasis in the colon.

Example 15

Next, the influence, on the local immune response, of the colonization of a large number of *Clostridium* and the resultant proliferation of Treg cells was investigated.

<Dextran Sulfate Sodium (DSS)-Induced Colitis Model>

First, the DSS-induced colitis model was prepared as described above, and the influence, on the model mice, of the inoculation of the *Clostridium* and the proliferation of Treg cells was investigated. Specifically, control mice and *Clostridium*-inoculated mice were treated with 2% DSS, then observed and measured for six days for the body weight loss, the hardness of stool, and bleeding, and then were evaluated numerically. In addition, on day 6, the colons were collected, dissected, and analyzed histologically by HE staining. FIGS. 41 to 43 show the obtained results. Note that, in FIGS. 41 to 43, “SPF+Clost.” or “SPF+Clost.#1 to 3” indicate the results of C57BL/6 mice inoculated with a fecal suspension of *Clostridium*-colonized mice, and grown in a conventional environment for six weeks, and “SPF” or “SPF#1 to 3” indicate the results of C57BL/6 mice (control mice) grown in a conventional environment for six weeks without being inoculated with the fecal suspension. In addition, in FIG. 41, the vertical axis “Disease score” represents the disease activity index (DAI) described above, and the horizontal axis “post 2% DSS(d)” represents the days elapsed after the initial administration of 2% DSS to the mice. Moreover, in FIG. 41, * indicates that “P<0.02,” and ** indicates that “P<0.001.” Meanwhile, Treg cells induced by regulatory dendritic cells are known to play a preventive role in a DSS-induced colitis model (see S. Manicassamy et al., Science 329, 849 (Aug. 13, 2010)).

As is apparent from the results shown in FIGS. 41 to 43, the symptoms of the colitis such as body weight loss and rectal bleeding were significantly suppressed in the mice having a large number of *Clostridium* (hereinafter also referred to as “*Clostridium*-abundant mice”) in comparison with the control mice (see FIG. 41). All the features typical for colonic inflammation, such as shortening of the colon, edema, and hemorrhage, were observed markedly in the control mice in comparison with the *Clostridium*-abundant mice (see FIG. 42). Moreover, histological features such as mucosal erosion, edema, cellular infiltration, and crypt loss were less severe in the DSS-treated *Clostridium*-abundant mice than in the control mice (see FIG. 43).

<Oxazolone-Induced Colitis Model>

Next, the oxazolone-induced colitis model was prepared as described above, and the influence, on the model mice, of the inoculation of *Clostridium* and the proliferation of Treg cells was investigated. Specifically, control mice and *Clostridium*-inoculated mice were sensitized with oxazolone, and subsequently the inside of the rectums thereof were treated with a 1% oxazolone/50% ethanol solution. Then, the body weight loss was observed and measured. In addition, the colons were dissected, and analyzed histologically by HE staining. FIGS. 44 and 45 show the obtained results. Note that, in FIGS. 44 and 45, “SPF+Clost.” indicates the results of C57BL/6 mice (*Clostridium*-abundant mice) inoculated with a fecal suspension of

Clostridium-colonized mice, and grown in a conventional environment for six weeks, and “SPF” indicates the results of C57BL/6 mice (control mice) grown in a conventional environment for six weeks without being inoculated with the fecal suspension. In addition, in FIG. 44, the vertical axis “Weight (% of initial)” represents the body weight after the administration of 1% oxazolone where the body weight before the administration was taken as 100%, and the horizontal axis “post 1% oxazolone (d)” represents the days elapsed after the administration of 1% oxazolone to the mice. Meanwhile, it is known that Th2-type T cells are involved in colitis induced by oxazolone. (see M. Boirivant, I. J. Fuss, A. Chu, W. Strober, J Exp Med 188, 1929 (Nov. 16, 1998)).

As is apparent from the results shown in FIGS. 44 and 45, the colitis proceeded along with persistent body weight loss in the control mice. Meanwhile, the body weight loss of the *Clostridium*-abundant mice was reduced (see FIG. 44). In addition, it was also revealed that portions having histological diseases such as mucosal erosion, edema, cellular infiltration, and hemorrhage were reduced in the colon of the *Clostridium*-abundant mice (see FIG. 45).

Example 16

Next, the influence, on the systemic immune response (systemic IgE production), of the colonization of a large number of *Clostridium* and the resultant proliferation of Treg cells was investigated. Specifically, as described above, control mice and *Clostridium*-inoculated mice were immunized by administering alum-adsorbed ovalbumin (OVA) twice at a 2-week interval. Then, sera were collected from these mice, and the OVA-specific IgE level thereof was investigated by ELISA. In addition, splenic cells were collected from the mice in each group, and IL-4 and IL-10 production by in-vitro OVA restimulation was investigated. FIGS. 46 to 48 show the obtained results. Note that, in FIGS. 46 to 48, “SPF+Clost.” indicates the results of BALB/c SPF mice (*Clostridium*-abundant mice) inoculated with a fecal suspension of *Clostridium*-colonized mice, and grown in a conventional environment, “SPF” indicates the results of BALB/c SPF mice (control mice) grown in a conventional environment without being inoculated with the fecal suspension, and ** indicates that “P<0.001.” Meanwhile, in FIG. 46, the vertical axis “OVA-specific IgE (ng/ml)” represents the concentration of OVA-specific IgE in the sera. Moreover, in FIG. 46, the horizontal axis represents the days elapsed after the initial administration of the alum-adsorbed ovalbumin to the *Clostridium*-abundant mice or the control mice (4-week old), and “OVA+Alum” indicates the timing of the administration of the alum-adsorbed ovalbumin. In addition, in FIGS. 47 and 48, “OVA” on the horizontal axis indicates the results in the case where the in-vitro OVA restimulation was performed, and “-” indicates the results in the case where no in-vitro OVA restimulation was performed. Moreover, in FIGS. 47 and 48, the vertical axes “IL-4 (μg/ml)” and “IL-10 (μg/ml)” show the IL-4 concentration and the IL-10 concentration in culture supernatants of splenic cells, respectively.

As is apparent from the results shown in FIGS. 46 to 48, the IgE level was significantly lower in the *Clostridium*-abundant mice than in the control mice (see FIG. 46). Moreover, the IL-4 production by the OVA restimulation was reduced (see FIG. 47) and the IL-10 production thereby was increased (see FIG. 48) in the splenic cells of the *Clostridium*-abundant mice sensitized with OVA and alum, in comparison with those of the control mice.

Accordingly, in consideration of the results shown in Example 15 in combination, it has been revealed that the

41

induction of Treg cells by *Clostridium* in the colon plays an important role in local and systemic immune responses.

Example 17

Next, GF Balb/c were colonized with three strains of *Clostridium* belonging to cluster IV (strains 22, 23 and 32 listed in FIG. 49). Three weeks later, colonic Foxp3⁺ Treg cells were analyzed by FACS. FIG. 50 shows the obtained results. As is apparent from the results shown in FIG. 50, gnotobiotic mice colonized with three strains of *Clostridium* showed an intermediate pattern of Treg induction between GF mice and mice inoculated with all 46 strains.

Example 18

Next, it was investigated whether or not a spore-forming (for example, a chloroform resistant) fraction of a fecal sample obtained from humans had the effect of inducing proliferation or accumulation of regulatory T cells similar to the spore-forming fraction of the fecal sample obtained from mice.

Specifically, human stool from a healthy volunteer (Japanese, male, 29 years old) was suspended with phosphate-buffered saline (PBS), mixed with chloroform (final concentration 3%), and then incubated in a shaking water bath for 60 min. After evaporation of chloroform by bubbling with N₂ gas, the aliquots containing chloroform-resistant (for example, spore-forming) fraction of human intestinal bacteria were orally inoculated into germ-free (GF) mice (IQI, 8 weeks old). The treated mice were kept in a vinyl isolator for 3 weeks. The colon was collected and opened longitudinally, washed to remove fecal content, and shaken in Hanks' balanced salt solution (HBSS) containing 5 mM EDTA for 20 min at 37° C. After removing epithelial cells and fat tissue, the colon was cut into small pieces and incubated with RPMI1640 containing 4% fetal bovine serum, 1 mg/ml collagenase D, 0.5 mg/ml dispase and 40 µg/ml DNase I (all manufactured by Roche Diagnostics) for 1 hour at 37° C. in a shaking water bath. The digested tissue was washed with HBSS containing mM EDTA, resuspended in 5 ml of 40% PERCOLL® (manufactured by GE Healthcare) and overlaid on 2.5 ml of 80% PERCOLL® in a 15-ml Falcon tube. PERCOLL® gradient separation was performed by centrifugation at 780 g for 20 min at 25° C. The interface cells were collected and suspended in staining buffer containing PBS, 2% FBS, 2 mM EDTA and 0.09% NaN₃ and stained for surface CD4 with Phycoerythrin-labeled anti-CD4Ab (RM4-5, manufactured by BD Biosciences). Intracellular staining of Foxp3 was performed using the Alexa647-labeled anti-Foxp3 Ab (FJK-16s, manufactured by eBioscience) and Foxp3 Staining Buffer Set (manufactured by eBioscience). The percentage of Foxp3 positive cells within the CD4 positive lymphocyte population was analyzed by flow cytometry. FIGS. 51 and 52 show the obtained results.

In figures, representative histograms (FIG. 51) and combined data (FIG. 52) for Foxp3 expression by CD4 positive lymphocytes from GF mice (GF) or GF mice gavaged with chloroform-treated human stool (GF+Chloro.) are shown. In addition, numbers in FIG. 51 indicate the percentages of cells in the gate. Each circle in FIG. 52 represents a separate animal, error bars indicate the SD, and ** indicates that "P<0.001."

As is apparent from the results shown in FIGS. 51 and 52, it was found that also when the spore-forming (for example, the chloroform resistant) fraction of human intestinal bacteria was colonized in GF mice, the accumulation of Foxp3⁺ regulatory (Treg) cells in the colonic lamina propria of the mice was induced.

Next, it was investigated what species of bacteria grew by gavaging with chloroform-treated human stool.

Specifically, using a QIAamp DNA Stool mini kit (manufactured by QIAGEN), bacterial genomic DNA was isolated

42

from the human stool from a healthy volunteer as described above (human stool) or fecal pellets from GF mice gavaged with chloroform-treated human stool (GF+Chloro). Quantitative PCR analysis was carried out using a LightCycler 480 (manufactured by Roche). Relative quantity was calculated by the ΔCt method and normalized to the amount of total bacteria, dilution, and weight of the sample. The following primer sets were used:

10 total bacteria (SEQ ID NO: 62)
5'-GGTGAATACGTTCCCGG-3'
and
15 5'-TACGGCTACCTTGTTACGACTT-3' (SEQ ID NO: 63)
Clostridium cluster XIVa (*Clostridium coccoides* subgroup) (SEQ ID NO: 64)
5'-AAATGACGGTACCTGACTAA-3'
20 and (SEQ ID NO: 65)
5'-CTTTGAGTTTCATTCTTGCGAA-3'
Clostridium cluster IV (*Clostridium leptum*) (SEQ ID NO: 66)
25 5'-GCACAAGCAGTGGAGT-3'
and (SEQ ID NO: 24)
5'-CTTCCTCCGTTTGTCAA-3'
30 *Bacteroides* (SEQ ID NO: 67)
5'-GAGAGGAAGGTCCCCAC-3'
and (SEQ ID NO: 68)
35 5'-CGCTACTTGGCTGGTTCAG-3'.

FIG. 53 shows the obtained results.

As is apparent from the results shown in FIG. 53, mice gavaged with chloroform-treated human stool exhibited high amounts of spore-forming bacteria, such as *Clostridium* clusters XIVa and IV, and a severe decrease of non-spore-forming bacteria, such as *Bacteroides*, compared with the human stool before chloroform treatment.

INDUSTRIAL APPLICABILITY

As has been described above, the present invention makes it possible to provide an excellent composition for inducing proliferation or accumulation of regulatory T cells (Treg cells) by utilizing bacteria belonging to the genus *Clostridium* or a physiologically active substance or the like derived from the bacteria. Since the composition of the present invention has immunosuppressive effects, the composition can be used, for example, to prevent or treat autoimmune diseases or allergic diseases, as well as to suppress immunological rejection in organ transplantation or the like. In addition, healthy individuals can easily and routinely ingest the composition as a food or beverage, such as a health food, to improve their immune functions.

SEQUENCE LISTING

SEQ ID NO:1 to 20, 62 to 69
<223> Artificially synthesized primer sequence
65 SEQ ID NO:21 to 61
<223> 16S rRNA coding gene sequence of each *Clostridium* strain

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 69

<210> SEQ ID NO 1
 <211> LENGTH: 23
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 1

ggcaatagtt ccttcccaga gtt 23

<210> SEQ ID NO 2
 <211> LENGTH: 22
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 2

gggtcgcata ttgtgttact tg 22

<210> SEQ ID NO 3
 <211> LENGTH: 23
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 3

cctttttagt ccttgctcac tct 23

<210> SEQ ID NO 4
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 4

gggtcacctg tatggcttca g 21

<210> SEQ ID NO 5
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 5

tcagtgcaag atctgcaagc a 21

<210> SEQ ID NO 6
 <211> LENGTH: 19
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 6

acaccggaag ccaaacaca 19

<210> SEQ ID NO 7
 <211> LENGTH: 27
 <212> TYPE: DNA
 <213> ORGANISM: Artificial

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 7

gattttaata agctccaaga ccaaggt 27

<210> SEQ ID NO 8
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 8

cttctatgca gttgatgaag atgtcaa 27

<210> SEQ ID NO 9
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 9

cctcgtcccg tagacaaaat g 21

<210> SEQ ID NO 10
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 10

tctccacttt gccactgcaa 20

<210> SEQ ID NO 11
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 11

ggacattgtc tttgatggca 20

<210> SEQ ID NO 12
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 12

cttgtcacgt ggtgtcactg 20

<210> SEQ ID NO 13
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 13

tctctggacg tcaaatgtgg 20

-continued

<210> SEQ ID NO 14
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 14

gctgaacagc agagccttc 19

<210> SEQ ID NO 15
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 15

aggtctggat cactccaagg 20

<210> SEQ ID NO 16
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 16

tcgcctggac cataaagaa 19

<210> SEQ ID NO 17
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 17

agaggatgcg tgactttgtg 20

<210> SEQ ID NO 18
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 18

atacagcaga ccttctggca 20

<210> SEQ ID NO 19
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 19

agagtttgat cmtggctcag 20

<210> SEQ ID NO 20
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

-continued

<400> SEQUENCE: 20

attaccgcgg ckgtg

16

<210> SEQ ID NO 21

<211> LENGTH: 1460

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1460)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 1

<400> SEQUENCE: 21

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac 60
 gagaaccatt ggatcgagga ttctgtccaag tgaaggtggg gaaagtggcg gacgggtgag 120
 taacgcgtga gcaatctgcc ttggagtggg gaataacggc tggaaacagc cgctaatacc 180
 gcatgataca gctgggaggc atctccctgg ctgtcaaaga tttatcgctc tgagatgagc 240
 tcgcgtctga ttagctagtt ggcggggtaa cggccacca aggcgacgat cagtagccgg 300
 actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca 360
 gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgccgc gtgaagggaag 420
 aaggctttcg ggttgtaaac ttcttttgtc agggacgaag caagtgcagg tacctgacac 480
 ggctactacg gtcagcagcg cgtatacgta ggtgccagcg tatccggaat tacctgggtt 540
 aaggcgtggt agccggactg cagtcagatg tgaatcacgg gctcaacttg tgctgcattg 600
 gaactgtagt tctgagtact gagagcagac ggaattctag gtagcggtag atgcgtagat 660
 ataggaggac acagtgcgag gcgtctgctg acagcaactg acgctgaggc ggggaagcgtg 720
 ggggagccaa caggattaga tacctggtag ttcacgcctg gtaaaacgat ggatactagg 780
 tgtgggggga ctgacccctc cgtggccgcc agttaacacc aataaagtat cccacctggg 840
 agtacgatcg caaggttgaa actcaaagga attgacgggg cccgcacaag cggtaggagta 900
 tgtggtttaa ttcgaagcaa cgcgaagaac cttaccaggg cttgacatcc cgaggaccgg 960
 actagagata gtcttttctc ttccggagacc tcggtgacag gtggtgcatg gttgtcgtca 1020
 gctcgtgtcg taagatgttg ggttaagtcc cgcaacgagc gcaaccctta ttgttagttg 1080
 ctacgcaaga gcactctagc gagactgccg ttgacaaaac ggaggaaggt ggggacgacg 1140
 tcaaatcatc atgcccctta tgccttgggc cacacacgta ctacaatggt ggtcaacaga 1200
 gggaagcaat accgcgaggt ggagcaaatc cctaaaagcc atcccagttc ggatcgcagg 1260
 ctgcaacccg cctgcgtgaa gttggaatcg ctagtaatcg cggatcagca tgcgcgggtg 1320
 aatacgttcc cgggccttgt acacaccgcc cgtcacacca tgagagtcgg gaacacccca 1380
 agtccgtagc ctaaccgcaa gggggggcgc ggcggaaggt gggttcgata attgggggtga 1440
 agtcgtaaca aggtagccgt 1460

<210> SEQ ID NO 22

<211> LENGTH: 1485

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1485)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 2

-continued

<400> SEQUENCE: 22

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
ggagaccccc tgaaggagtt ttcggacaac ggaagggaact gcttagtggc ggacgggtga    120
gtaacgcgtg aggaacctgc cttggagtgg ggaataacag ctggaacag ctgctaatac    180
cgcataatat atctgggccc catggctctg gatatacaag atttatcgct ctgagatgga    240
ctcgcgtctg attagctagt tggcggggta acggcccacc aaggcgacga tcagtagccg    300
gactgagagg ttggccggcc acattgggac tgagacacgg ccagactcc tacgggaggc    360
agcagtgggg aatattgggc aatgggcgca agcctgaccc agcaacgccg cgtgaaggaa    420
gaagccttcc ggggtgtaaa cttcttttgt cagggacgaa gcaagtgcac gtacctgacg    480
aataagccac ggctaactac gtgccagcag ccgcggtaat acgtaggtag caagcgttat    540
ccggatttac tgggtgtaaa gggcgtgtag gcgggactgc aagtcagatg tgaaccac    600
gggctcaacc tgtgggcctg catttgaaac tgtagttctt gactactgga gaggcagacg    660
gaattctagt tgtagcgtga aatgcgtaga tatagaagaa cacagttgcg gagccggtct    720
gcaactgacg ctgagcgcga aagcgtgggg agcaaacagg attagatacc ctggtagtcc    780
acgctgtaaa cgatggatta ctagggtgg ggggactgac cccctccgtg ccgcagttaa    840
cacaataagt atcccactg gggagtacga tcgcaaggtt gaaactcaaa aggaattgac    900
gggggccccg acaagcgggt gagtatgtgg tttaaatcgg aagcaacgcg aagaacctta    960
ccagggcttg acatcccggg gaccgtccta gagataggat tttcccttcg gggacactgg   1020
agacaggtag tgcattggtg tcgtcagctc gtgtcgtgag atgttgggtt aagtcccgca   1080
acgagcgcga cccttattgt tagttgctac gcaagagcac tctagcgaga ctgccgttga   1140
caaaacggag gaagggtggg acgacgtcaa atcatcatgc cccttatgtc ctgggccaca   1200
cacgtactac aatggtgggc aacagaggga agcaaagccg cgagggtggag caaatcccta   1260
aaagccatcc cagttcggat cgcaggctgc aaccgcctg cgtgaagttg gaatcgctag   1320
taatcgcgga tcagaatgcc gcggtgaata cggtcccggg ccttgtagac accgcccgtc   1380
acaccatgag agtcgggaac acccgaaagtc cgtagcctaa ccgcaagggg ggcgcggccg   1440
aaggtagggt cgataattgg ggtgaagtcg taacaaggta gccgt                      1485

```

<210> SEQ ID NO 23

<211> LENGTH: 1491

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1491)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 3

<400> SEQUENCE: 23

```

agagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
ggagacacct cgaaagagac ttcggtcaat ggaaaagaat gcttagtggc ggacgggtga    120
gtaacgcgtg aggaacctgc ctttcagtgg gggacaacag ttggaaacga ctgctaatac    180
cgcataacgt acgggtatcg catggtatct gtaccaaaga tttatcgctg agagatggcc    240
tcgcgtctga ttagctagtt ggtagggtaa cggcctacca aggcgacgat cagtagccgg    300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca    360
gcagtgggga atattgggca atgggcgaaa gcctgaccca gcaacgccg gtgaaggaa      420

```

-continued

```

aaggctttcg ggttgtaaac ttcttttgac ggggaagagc agaagacggt acctgtcgaa 480
taagccacgg ctaactacgt gccagcagcc gcggtaatat gtaggtggca agcgttggtcc 540
ggatttactg ggttgtaaagg gcgtgtagcc gggctgacaa gtcagatgtg aaatccgggg 600
gctcaacccc cgaactgcat ttgaaactgt tggctttgag tatcggagag gcaggcggaa 660
ttcctagtgt agcggtgaaa tgcgtagata ttagggggaa caccagtggc gaagcggcct 720
gctggacgac aactgacggt gaggcgcgaa agcgtgggga gcaaacagga ttagataccc 780
tggtagtcca cgctgtaaac gatggatact aggtgtgcgg ggactgaccc ctgcgtgccg 840
cagctaacgc aataagtatc ccacctgggg agtacgatcg caagggtgaa actcaaagga 900
attgacgggg gcccgacaaa gcggtggatt atgtggttta attcgatgca acgcgaagaa 960
ccttaccagg gcttgacatc ctactaacga agtagagata cattaggtac ccttcggggg 1020
aagtagagac aggtggtgca tggttgtcgt cagctcgtgt cgtgagatgt tgggttaagt 1080
cccgcaacga gcgcaacct tattgttagt tgctacgcaa gagcacteta gcgagactgc 1140
cgttgacaaa acggaggaag gtggggacga cgtcaaatca tcatgcccc tatgtcctgg 1200
gtacacacg taatacaatg gcggtcaaca gagggatgca aaaccgcgag gtggagcgaa 1260
ccccataaag ccgtcccagt tcagatcgca gtctgcaacc cgactgcgtg aagtcggaat 1320
cgctagtaat cgcggtatcg catgccgagg tgaatacgtt cccgggcctt gtacacaccg 1380
cccgtcacac catgagagtc gggaacaccc gaagtccgta gcctaaccgc aaggaggggc 1440
cggccgaagg tgggttcgat aattgggggtg aagtcgtaac aaggtagccg t 1491

```

```

<210> SEQ ID NO 24
<211> LENGTH: 1491
<212> TYPE: DNA
<213> ORGANISM: Clostridium coccoides
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1491)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 4

```

```

<400> SEQUENCE: 24

```

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac 60
gggtgtacgg ggaggaaggc ttccggcggg aaacctgtgc atgagtggcg gacgggtgag 120
taacgcgtgg gcaacctggc ctgtacaggg ggataaacct tagaaatagg tgctaatacc 180
gcataaacggg ggaagccgca tggcttttcc ctgaaaactc cgggtgtaca ggatggggcc 240
gcgtctgatt agccagttgg cagggtaacg gcctacccaa gcgacgatca gtagccggcc 300
tgagaggggc gacggccaca ctgggactga gacacggccc agactcctac gggaggcagc 360
agtgggggat attgcacaat ggggggaaac cctgatgcag cgacgcgcgc tgagtgaaga 420
agtatttcgg tatgtaaagc tctatcagca ggaagaaaa tgacggtacc tgactaagaa 480
gccccggcta actacgtgcc agcagccgcg gtaatacgtg gggggcaagc gttatccgga 540
tttactgggt gtaaaggagg cgtagacggc agcgcaagtc tgagtgaat cccatggctt 600
aaccatggaa ctgctttgga aactgtgcag ctggagtgca ggagagtaag cggaaattcct 660
agtgtagcgt gaaatgcgta gattatagga ggaacaccag tggcgaaggc ggctaactga 720
actgtaactg acgttgaggc tcgaaagcgt ggggagcaaa caggattaga taccctggta 780
gtccacgcgc taaacgatga ttactagtg ttgggggacc aaggtcttcg gtgccggcgc 840
aaacgcatta agtaatccac ctggggagta cgttcgcaag aatgaaactc aaaggaattg 900

```


-continued

```

acgggggaccc gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc gaagaacctt 960
acctggtctt gacatcccgga tgcagagtga gcaaagtcac ttcccttcg gggcattgga 1020
gacaggtggt gcatggttgt cgtcagctcg tgcctgaga tgttgggtta agtcccgcaa 1080
cgagcgcaac ccctatttcc agtagccagc aggtagagct gggcactctg gagagactgc 1140
ccgggataac cgggaggaag gcgggggatga cgtcaaatca tcatgcccc tatgatcagg 1200
gtacacacg tgctacaatg gcgtaacaa agggaagcga gacggtgacg ttgagcaaat 1260
cccaaaaata acgtcccagt tcggattgta gtctgcaact cgactacatg aagctggaat 1320
cgctagtaat cgcaatcag aatgtcgcgg tgaatacgtt cccgggtctt gtacacaccg 1380
cccgtcacac catgggagtc ggaatgccc gaagtcagtg acctaaccga aaggaaggag 1440
ctgccgaagg tggagccggt aactggggtg aagtcgtaac aaggtagccg t 1491

```

<210> SEQ ID NO 25

<211> LENGTH: 1467

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1467)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 5

<400> SEQUENCE: 25

```

agagtttgat cctggctcag gacgaacgct ggcggcacgc ctaacacatg caagtcgaac 60
ggagtgaaga tgctcgcac tgaacttagt ggcggacggg tgagtcaac gtgagcaacc 120
tgcccttcag aggggggatta cgtttggaaa cgaacgctaa taccgcataa aatatcgag 180
tcgcatggca ctgatatcaa aggagcaatc cgctgaaaga tgggctcgcg tccgattagg 240
cagttggcgg ggtatcggcc caccaaaccg acaatcggtg gccggactga gaggttgaa 300
ggccacattg ggactgagac gcggcccaga ctctacggg aggcagcagt gggggatatt 360
gcacaatggg ggaaaccttg atgcagcgat gccgcgtgaa tgaagacggc cttcgggttg 420
taaagtctcg tcgcagggga cgaataatgac ggtaccctgc aagaaagctc cggctaacta 480
cgtgccagca gcccggttaa tacgtaggga gcaagcgttg tccggaatta ctgggtgtaa 540
agggagcgta ggcgggagga taagtgaat gtgaaatcta tgggctcaac ccatagctgc 600
gttcaaacgt ttcttcttga gtgaagtaga ggcaggcgga attcctagt tagcggtgaa 660
atgcgtagat attaggagga caccagtggc gaaggcgggc tgctgggctt tactgacgct 720
gaggctcgaa agcgtgggta gcaaacagga ttagataccc tggtagtcca cgcggtaaac 780
gatgattact aggtgtgggt ggactgacct catccgtgcc ggagttaaca caataagtaa 840
tccacctggg gagtacggcc gcaaggttga aactcaaagg aattgacggg ggcccgcaca 900
agcagtggag tatgtggttt aattcgacgc aacgcgaaga acctaccag gtcttgacat 960
cgagtgcagg acatagagat atgtctttcc ttcgggacac gaagacaggt ggtgcatggt 1020
tgctgtcagc tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aacccttacc 1080
attagttgct acgcaagagc actctaattg gactgccgtt gacaaaacgg aggaagggtg 1140
ggatgacgtc aaatcatcat gcccttatg acctgggcga cacacgtact acaatggcgg 1200
tcaacagagg gaggcaaagc cgcgaggcag agcaaacccc taaaagccgt ctgagttcgg 1260
attgcaggct gcaactcgcc tgcataagat cggaattgct agtaatcgcg gatcagcatg 1320
ccgcggtgaa tacgttcccg ggccttgtag acaccgccc tcacaccatg agagccggta 1380

```

-continued

```
acacccgaag tcaatagtct aaccgcaagg aggacattgc cgaaggtggg attggttaatt 1440
ggggtgaagt cgtaacaagg tagccgt 1467
```

```
<210> SEQ ID NO 26
<211> LENGTH: 1474
<212> TYPE: DNA
<213> ORGANISM: Clostridium coccoides
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1474)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 6
```

```
<400> SEQUENCE: 26
```

```
agagtttgat catggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac 60
gggtgtacgg gaaggaaggc ttccggccga aaacctgtgc atgagtggcg gacgggtgag 120
taacgcgtgg gcaacctggc ctgtacaggg ggataacact tagaaatagg tgctaatacc 180
gcataacggg ggaagccgca tggcttttcc ctgaaaactc cggtggtaca ggatggggccc 240
gcgtctgatt agccagttag cagggtaacg gcctacaaa gcgacgatca gtagccggcc 300
tgagaggcg gacggccaca ctgggactga gacacggccc agactcctac gggaggcagc 360
agtgggggat attgcacaat ggggggaacc ctgatgcagc gacgccgct ggggtgaagaa 420
gcgcctcgcc gcgtaaagcc ctgtcagcag ggaagaaaat gacggtagct gaagaagaag 480
ccccggctaa ctactgtcca gcagccgagg taatacgtag gggggcaagc gttatccgga 540
tttactgggt gtaaggggg cgagacggc gatgcaagcc aggagtgaag gcccggggcc 600
caaccccggg actgctcttg ggaactgctg ggctggagtg cagagggcag cggaattcct 660
ggtgaaatgc gtagatatca gaagacacgg tgcgaggcgg cctgctgact gcaactgacgt 720
tgagccgaag cgtggggagc aaacaggatt agataccgtg gtagtcacgc cgtaaacgat 780
gattactagg tgtcggggag cagagactgc ccggtgccgc agccaacgca ttaagtaatc 840
cacctgggga gtacgttcgc aagaatgaaa ctcaaaggaa ttgacgggga cccgcacaag 900
cgggtggagca tgtggtttaa ttcgaagcaa cgcgaagAAC cttacctggt cttgacatcc 960
cgatgacgag tgagcaaaagt cactttccct tcggggcatt ggagacaggt ggtgcatggt 1020
tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aaccctatt 1080
tccagtagcc agcaggtaga gctgggcact ctggagagac tgcccgggat aaccgggagg 1140
aaggcgggga tgacgtcaaa tcatcatgcc ctttatgatc agggctacac acgtgctaca 1200
atggcgtaaa caaagggaag cgagacggtg acgttaagca aatcccaaaa ataactgcc 1260
agttcggatt gtagtctgca actcgactac atgaagctgg aatcgctagt aatcgcgat 1320
cagaatgtcg cggtaatac gttcccggt cttgtacaca ccgcccgtca caccatggga 1380
gtcggaaatg cccgaagtca gtgacctaac cgaaagggaag gagctgccga aggtggagcc 1440
ggtaactggg gtgaagtcgt aacaaggtag ccgt 1474
```

```
<210> SEQ ID NO 27
<211> LENGTH: 1484
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1484)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 7
```

```
<400> SEQUENCE: 27
```

-continued

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
gagaatccag tgaaggagtt ttcggacaac ggatctggag gaaagtggcg gacgggtgag    120
taacgcgtga gcaatctgcc ttggagtggg gaataacggt tggaaacagc cgctaatacc    180
gcatgatgcg tctgggaggc atctctctgg acgccaaaga tttatcgctc tgagatgagc    240
tcgcgtctga ttagcttggt ggcggggtaa aggccaccca aggcgacgat cagtagccgg    300
actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca    360
gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgccgc gtgaagggaag    420
aaggctttcg ggttgtaaac ttcttttctg agggacgaag aaagtgacgg tacctcagga    480
ataagccacg gctaactacg tgccagcagc cgcggtaata cgtagggtggc aagcgttatc    540
cggatttatt ggggtgaaag ggcgtgtagg cgggaaagca agtcagatgt gaaaactcag    600
ggctcaaccg tgagcctgca tttgaaactg tttttcttga gtgctggaga ggcaatcgga    660
attccgtgtg tagcgggtga atcgcttagat atacggagga caccagtggc gagcggattg    720
ctggacagta ctgacgtgca agcgcgaaaag cgtggggagca aacagataga tacctggtag    780
tcacgcgtaa acgatggata ctagggtgtg ggggactgac cccctccgtg ccgcagctaa    840
cgcaataagt atcccacctg gggagtacga tcgcaaggtt gaaactcaaa ggaattgacg    900
ggggcccgca caagcgttgg agtatgttgt ttaattcgaa gcaacgcgaa gaaccttacc    960
agggcttgac atcctgctaa cgaaccagag atggattagg tgcccttcgg ggaaagcaga   1020
gacaggttgt gcattggtgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa   1080
cgagcgcaac ccttattgtt agttgctacg caagagcact ctacgcgagc tgccgttgac   1140
aaaacggagg aaggtgggga cgacgtcaaa tcatcatgcc ccttacgtcc tgggccacac   1200
acgtactaca atggcggcca acaagagagag gcaagaccgc gagtgaggc aaatctcaaa   1260
aagccgtccc agttcggatc gcaggctgca acccgctgc gtgaagtgg aatcgctagt   1320
aatcgcggat cagcatgccg cggatgaatac gttcccgggc cttgtacaca ccgccgtca   1380
caccatgaga gtcgggaaca cccgaagtcc gtagcctaac cgcaaggggg gcgcggccga   1440
aggtgggttc gataattggg gtgaagtcgt aacaaggtag ccgt                    1484

```

```

<210> SEQ ID NO 28
<211> LENGTH: 1483
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1483)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 8

```

```

<400> SEQUENCE: 28

```

```

agagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
ggagcacccc tgaaggagtt ttcggacaac ggatgggaat gcttagtggc ggactgggtga   120
gtaacgcgtg aggaacctgc cttccagagg gggacaacag ttgaaacga ctgctaatac   180
cgcatgatgc gttggagccg catgactccg acgtcaaaga tttatcgctg gaagatggcc   240
tcgcgtctga ttagctagtt ggtgaggtaa cggcccacca aggcgacgat cagtagccgg   300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca   360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaagggaag   420
aaggctttcg ggttgtaaac ttcttttaag ggggaagagc agaagacggt accccttgaa   480

```

-continued

taagccacgg ctaactacgt gccagcagcc gcggtaatat gtaggtggca agcgttgtcc	540
ggatttactg ggtgtaaagg gcgtgcagcc ggagagacaa gtcagatgtg aaatccacgg	600
gctcaaccgg tgaactgcat ttgaaactgt ttcctttgag tgctggagag gtaatcggga	660
ttccttgtgt agcgggtgaat gcgtagatat agagaccaca gtgccgacgc cgaataactga	720
cgatactgac ggtgagcgcg aaagcgtggg gagcaaacag gattagatac cctggtagtc	780
cacgctgtaa acgatcgata ctagggtgtgc ggggactgac ccctgcgtgc cggagttaac	840
acaataagta tcgcacctgg ggagtacgat cgcaagggtg aaactcaaag gaattgacgg	900
gggcccgcac aagcgggtgga ttatgtggtt taattcgaag caacgcgaag aaccttacca	960
gggcttgaca tcctgctaac gaagtagaga tacattaggt gcccttcggg gaaagcagag	1020
acagtggtg catggtgtgc gtcagctcgt gtcgtgagat gttgggttaa gtcccgaac	1080
gagcgcaacc cctattgtta gttgtacgc aagagcactc tagcgagact gccgttgaca	1140
aaacggagga aggcggggac gacgtcaaat catcatgccc cttatgtcct gggctacaca	1200
cgtaatacaa tggcggttaa caaaggatg caaagccgcg aggcagagcg aacccccaaa	1260
agccgtccca gttcggatcg caggctgcaa cccgcctgcg tgaagtcgga atcgctagta	1320
atcgcgatc agcatgcgc ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac	1380
accatgagag tcgggaacac ccgaagtcg tagcctaacc gcaaggaggg cgcggccgaa	1440
ggtgggttcg ataattgggg tgaagtcgta acaaggtagc cgt	1483

<210> SEQ ID NO 29

<211> LENGTH: 1480

<212> TYPE: DNA

<213> ORGANISM: Clostridium coccoides

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1480)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 9

<400> SEQUENCE: 29

ggagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
gggctcatat tgaaacctag tgatgtatga gttagtggcg gacgggtgag taacgcgtgg	120
agaacctgcc gtatactggg ggataaacct tagaaatagg tgctaatacc gcataagcgc	180
acagcttcgc atgaagcagt gtgaaaaact ccggtggtat acgatggatc cgcgtctgat	240
tagctggttg gcggggtaac agcccaccaa ggcgacgac agtagccggc ctgagagggt	300
gaacggccac attgggactg agacacggcc caaactccta cgggaggcag cagtggggaa	360
tattgcacaa tgggggaaac cctgatgcag cgacgccgcg tgagtgaaga agtatttcgg	420
tatgtaaagc tctatcgca ggaagaaat actgacctta cggtcagcag acggtacctg	480
actaagaagc cccgggctaa ctacgtgcc aacacccgcg taatacgtag gggcaagcgt	540
tatccgatt tactgggtgt aaagggggcg cagacggcga tgcaagccag gagtgaagc	600
cggggcccaa cccgggact gctcttgac tgcgtggctg gagtgcagag ggcagcgaat	660
tcctgtgtag cgtgaatgcg tagattcaga ggacacgtgc gagcgctgc tgactgcact	720
gacgtgagcc cgaagcgtgg ggagcaacaa ggattagata cctggtagtc cacgcgtaa	780
acgatgatta ctagggtgcg gggagcagag actgcccgtt gccgcagcca acgcattaag	840
taatccacct ggggagtagc ttgcgaagaa tgaaactcaa aggaattgac ggggacccgc	900
acaagcgggt gagcatgtgg tttaattcga agcaacgcga agaaccttac caggccttga	960

-continued

```

catccccctg gatggccgt aacgggggtca gccttttcggg gcaggggaga caggtggtgc 1020
atggttgctg tcagctcgtg tcgtgagatg ttgggttaag tcccgcacg agcgcaaccc 1080
ctgcccgcag tagccagcat tttagatggg gactctgcgg ggactgccgg ggacaacccg 1140
gaggaagcgg gggatgacgt caaatcatca tgccccttat ggectgggct acacacgtgc 1200
tacaatggcg ccgacagagg gaggcgaagc ggcgacgcgg agcgaacccc aaaaacggcg 1260
tcccagttcg gattgtagtc tgcaacccga ctacatgaag ccggaatcgc tagtaatcgc 1320
ggatcagaat gcccggtga atacgttccc ggggtcttga cacaccgccc gtcacaccat 1380
gggagccggg aatgcccgaa gtctgtgacc gaaccgtaa ggggaggggc agccgaaggc 1440
aggcccggtg actggggtga agtcgtaaca aggtagccgt 1480

```

<210> SEQ ID NO 30

<211> LENGTH: 1489

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1489)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 10

<400> SEQUENCE: 30

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgagc 60
gaagcacttt tttagaactc ttcggaggga agagagggtg acttagcggc ggacgggtga 120
gtaacgcgtg ggcaacctgc cttacacagg gggataacaa ttagaaatga ttgctaatac 180
cgcataagac caccgtactg catggtacag tggtaaaaac tgagggtggtg taagatgggc 240
ccgcgtctga ttaggtagtt ggtggggtag aagcctacca agccgacgat cagtagccga 300
cctgagaggg cgaccggcca cattgggact gagacacggc ccaaactcct acgggaggca 360
gcagtgggga atattgcaca atgggggaaa ccctgatgca gcgacgccgc gtgagtgagg 420
aagtatttgc gtatgtaaag ctctatcagc agggaagaaa atgacggtag ctgactaaga 480
agcccccgcc taactacgtg ccagcagccg cggtaatacg tagggggcaa gcgttatccg 540
gatttactgg gtgtaaaggg agcgtagacg gacttgcaag tctgatgtga aaatccgggg 600
cccaaccggt gactgcattg aaactgtatt ttttgagggt gtccgaggag gcaagtggaa 660
tcctgggtag cggtgaaatg gcgtagaatt caggagggaa caccagtggc ggaaggcgaa 720
ttactggacg ataactgacg gtgaggcgcg aagcgtggga gcaaacaaga attagatacc 780
ctggtagtcg cgctgtaacg atcgatacta ggtgtgcggg gactgacccc tgcgtgccgg 840
agttaacaca ataagtatcg cactggggag tacgatcgca aggttgaaac tcaaaggaat 900
tgacgggggc ccgcacaagc ggtggattat gtggtttaat tcgaagcaac gcgaagaacc 960
ttaccagggc ttgacatcct gctaacgaag tagagataca ttaggtgccc ttcgggggaa 1020
gcagagacag gtggtgcatg gttgtcgtca gctcgtgtcg tgagatgttg ggtaagtcc 1080
cgcaacgagc gcaaccccta ttgttagttg ctacgcaaga gactctagc gagactgccg 1140
ttgacaaaac ggaggaaggc ggggacgacg tcaaatcatc atgcccctta tgcctggggc 1200
tacacacgta atacaatggc ggttaacaaa gggatgcaaa gccgcgaggc agagcgaacc 1260
ccaaaaagcc gtcccagttc ggatcgacgg ctgcaaccgg cctgcgtgaa gtcggaatcg 1320
ctagtaatcg cggatcagca tgcccggtg aatacgttcc cgggccttgt acacaccgcc 1380
cgtcacacca tgagagtcgg gaacaccgga agtccgtagc ctaaccgcaa ggagggcgcg 1440

```

-continued

gccgaaggtg gggtcgataa ttgggggtgaa gtcgtaacaa ggtagccgt 1489

<210> SEQ ID NO 31
 <211> LENGTH: 1490
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium leptum
 <220> FEATURE:
 <221> NAME/KEY: rRNA
 <222> LOCATION: (1)..(1490)
 <223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 11

<400> SEQUENCE: 31

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
gagaatccag tgaaggagtt ttcggacaac ggatctggag gaaagtggcg gacgggtgag    120
taacgcgtga gcaatctgcc ttggagtggg gaataacggt tggaacacgc cgctaatacc    180
gcatgatgcg tctgggaggc atctctctgg acgccaaaga tttatcgctc tgagatgagc    240
tcgcgtctga ttagcttgtt ggcggggtaa aggccaccca aggcgacgat cagtagccgg    300
actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca    360
gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgcgcg gtgaagggaag    420
aaggctttcg ggttgtaaac ttcttttctg agggacgaag aaagtgcagg tacctcagga    480
ataagccacg gctaactacg tgccagcagc cgcggtaata cgtaggtggc aagcgttatc    540
cggatttatt ggggtgtaaag ggcgtgtagg cgggaaagca agtcagatgt gaaaactcag    600
ggctcaaccc tgagcctgca tttgaaactg tttttcttga gtgctggaga ggcaatcgga    660
attccgtggt gtagcgtgta aatgcgtaga ttataccgga ggaaccacca gtggcggaag    720
gcggtattgt ggaacagtaa ctgacgctga ggcgcgcaaa gcgtggggag caaacaggat    780
agataccctg gtagtccacg ccgtaaacga tggatactaa gtgtggggga ctgaccctt    840
cgtgcccagc taagcaataa gtttcccacc tggggagtac gatcgcagg gaaactcaaa    900
ggaattgacg ggggcccgcc caagcgggtg gagtaggggt taattggagc aacgggaaga    960
accttaccag ggcttgacat cctgtaacga accagaagag ggattaggtg ccttcgggga   1020
aagcagagac aggtggtgca tggttgcgt cagctcgtgt cgtgagatgt gggtaaagtc   1080
ccgcaacgag cgcaaccctt attgttagtt gctacgcaag agcactctag cgagactgcc   1140
gttgacaaaa cggaggaagg tggggacgac gtcaaatcat catgccctt acgtcctggg   1200
ccacacacgt actacaatgg cggccaacaa agagaggcaa gaccgcgagg tggagaaaat   1260
ctcaaaaagc cgtcccagtt cggatcgag gctgcaaccc gcctgcgtga agttggaatc   1320
gctagtaatc gcggatcagc atgcccggt gaatacgttc ccgggccttg tacacaccgc   1380
ccgtcacacc atgagagtcg ggaacacccg aagtccttag cctaaccgca agggggggcg   1440
ggccgaaggt gggttcgata attgggggtga agtcgtaaca aggtagccgt   1490

```

<210> SEQ ID NO 32
 <211> LENGTH: 1489
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium coccoides
 <220> FEATURE:
 <221> NAME/KEY: rRNA
 <222> LOCATION: (1)..(1489)
 <223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 12

<400> SEQUENCE: 32

-continued

agagtttgat catggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
gggctcatat tgaaacctag tgatgtatga gttagtggcg gacgggtgag taacgcgtgg	120
agaacctgcc gtatactggg ggataacact tagaaatagg tgctaatacc gcataagcgc	180
acagcttcgc atgaagcagt gtgaaaaact ccggtgggtat acgatggatc cgcgtctgat	240
tagctggttg gcggggaac agcccaccaa ggcgacgac agtagccggc ctgagaggggt	300
gaacggccac attgggactg agacacggcc caaactccta cgggaggcag cagtggggaa	360
tattgcacaa tgggggaaac cctgatgcag cgacgccgcg tgagtgaaga agtatttcgg	420
tatgtaaagc tctatcagca ggaagaaat actgacctta cggtcagcag acggtacctg	480
actaagaagc cccggctaac tacgtgccag cagccgcggt aatacgtagg gggcaagcgt	540
tatccggtatt tactgggtgt aaagggagcg tagacggcag cgcaagtctg aagtgaatc	600
ccatggctta accatggaac tgctttggaa actgtgcagc tggagtgcag gagaggtaag	660
cgggaattcct agtgtagcgg tgaatgcgta gatattagag gacaccagtg gcgatgcggc	720
ttactggact gtactgacgt tgagctcgaa agcgtgggga gcaccagaat tagaatactg	780
tagtcacgcc gtaaccgatg atactagggt tgggggacca aggtctctgt ccggcggcaa	840
acgcattaag taatccacct ggggagtacg ttcgcaagaa tgaaactcaa aggaattgac	900
ggggaccgcg acaagcgggt gagcatgtgg tttaattcga agcaacgcga agaaccttac	960
ctggtcttga catcccgatg acgagtgagc aaagtcactt tcccttcggg gcattggaga	1020
cagggtggtgc atggtgtctg tcagctcgtg tcgtgagatg ttgggttaag tcccgaacg	1080
agcgcaacc cttattccag tagccagcag gtagagctgg gcaactctga gagactgccc	1140
gggataaccg ggaggaaggc ggggatgacg tcaaatcacc atgcccctta tgatcagggc	1200
tacacacgtg ctacaatggc gtaacaaaag ggaagcgaga cggtgacgtt aagcaaatcc	1260
caaaaataac gtcccagttc ggattgtagt ctgcaactcg actacatgaa gctggaatcg	1320
ctagtaatcg cgaatcagaa tgctcgggtg aatacgttcc cgggtcttgt acacaccgcc	1380
cgtcacacca tgggagtcgg aaatgcccga agtcagtgac ctaaccgaaa ggaaggagct	1440
gccgaagggt gagccggtaa ctggggtgaa gtcgtaacaa ggtagccgt	1489

<210> SEQ ID NO 33

<211> LENGTH: 1456

<212> TYPE: DNA

<213> ORGANISM: Clostridium coccoides

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1456)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 13

<400> SEQUENCE: 33

agagtttgat catggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
gaagcacttg agaacgattc ttcggatgag gacttttgtg actgagtggc ggacgggtga	120
gtaacgcgtg ggtaacctgc cctatacagg gggataacag ttagaaatga ctgctaatac	180
cgcataagcg cactaaaacc gcatggttcg gtgtgaaaaa ctgaggtggt ataggatgga	240
cccgcgtctg attagcttgt tgggtgggta acggctcacc aaggcgacga tcagtagccg	300
gcctgagagg gcgaccggcc acattgggac tgagacacgg cccaaactcc tacgggaggc	360
agcagtgggg gatattgcac aatgggggga accctgatgc agcgacgccg cgtgggtgaa	420
gaagcgcttc ggcgcgtaaa gccctgtcag cagggaagaa aatgacggta cctgaagaag	480

-continued

aagccccgcg taactacgtg ccagcagccg cggtaatacg taggggcaag cgttattccg	540
ggatttactg ggtgtaaagg gggcgagac ggcatgcaa gccaggagtg aagccccggg	600
ccccccggg actgctcttg gactgcgtgc tggagtgcag aaggggcagc gatcctgtgt	660
accgtgaatt gcgtagatat cagagacacg ttgcgagcgc tgcgtactgc actgacgtga	720
gcgaagctgg agcacagata gatactgtag tcagcgtaac gatgatacta gtgtcgggag	780
cagagactgc ccgttgccgc agcccaacgc attagtattc cacttgggga gtacgtttcg	840
cagaatgaac ttcaaggaaa tgacggggac ccgcacaagg cgggtggagca tgtgggttaa	900
ttcgaagcaa cgcaagaac cttaccaggc cttgacatcc cccctggatg gcccgtaacg	960
gggtcagcct ttcggggcag gggagacagg tgggtcatgg ttgtcgtcag ctcgtgtcgt	1020
gagatgttgg gttaagtccc gcaacgagcg caaccctgc ccgcagtagc cagcatttta	1080
gatggggact ctgcggggac tgccggggac aaccggagg aagcgggga tgacgtcaaa	1140
tcacatgcc ccttatggcc tgggtacac acgtgctaca atggcgccga cagagggagg	1200
cgaagcggcg acgcggagcg aaccccaaaa acggcgcccc agttcggatt gtagtctgca	1260
acccgactac atgaagccgg aatcgctagt aatcgcgat cagaatgccg cgggtgaatac	1320
gttccccggg cttgtacaca ccgccgtca caccatggga gccgggaatg cccgaagtct	1380
gtgaccgaac ccgtaagggg aggggcagcc gaaggcaggc tcggtgactg ggggtgaagtc	1440
gtaacaaggt agccgt	1456

<210> SEQ ID NO 34

<211> LENGTH: 1475

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1475)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 14

<400> SEQUENCE: 34

agagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
ggagaccccc tgaaggagtt ttcggacaac ggatgggaat gcttagtggc ggactgggtga	120
gtaacgcgtg aggaacctgc cttccagagg gggacaacag ttggaaacga ctgctaatac	180
cgcattgatgc gttggagccg catgactccg acgtcaaaga tttatcgctg gaagatggcc	240
tcgcgtctga ttacttagtt ggtgaggtaa cggcccacca aggcgacgat cagtagccgg	300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca	360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaagggaag	420
aaggctttcg ggttgtaaac ttcttttaag ggggaagagc agaagacggt accccttgaa	480
taagccacgg ctaactacgt gccagcagcc gcggtaatac gtagggtggca agcgttgtcc	540
ggatttactg ggtgtaaagg gcgtgcagcc ggagagacaa gtcagatgtg aaatccacgg	600
gtcacaacccg tgaactgcat ttgaaactgt ttcccttgag tgctgggagag gtaatcgga	660
tttccttggtg tagcggtgaa tgcttagata taaggaagga cacagtggcg agcggattac	720
tggacgatac tgacgtgagc gcgaaagcgt gggggagcaa cagaaattag atactgtagt	780
gcagctgtaa cgatcgatac tagttgcggg actgaccctc tcgctgcgag ttacacaata	840
agtatcgcac ctgggagtag gatcgcaagg ttggaactca aaggaattga cggggcccg	900
acaagcgttg gattatgtgg ttaattcga agcaacgcga agaaccttac cagggttgga	960

-continued

catcctgcta acgaagtaga gatacattag gtgcccttcg gggaaagtag agacaggtgg	1020
tgcattggtg tgcagctc gtgtcgtgag atgttgggtt aagtcgccga acgagcgcaa	1080
cccctattgt tagttgctac gcaagagcac tctagcgaga ctgccgttga caaaacggag	1140
gaaggcgggg acgacgtcaa atcatcatgc cccttatgtc ctgggctaca cacgtaatac	1200
aatggcgggt aacaaaggga tgcaaagcgg cgaggcagag cgaaccccaa aaagccgtcc	1260
cagttcggat cgcaggctgc aacccgcctg cgtgaagtcg gaatcgctag taatcgcgga	1320
tcagcatgcc gcggtgaata cgttcccggt ccttgtagac accgccgctc acaccatgag	1380
agtcgggaac acccgaagtc cgtagcctaa ccgcaaggag ggccgcccgc aaggtgggtt	1440
cgataattgg ggtgaagtcg taacaaggta gccgt	1475

<210> SEQ ID NO 35

<211> LENGTH: 1480

<212> TYPE: DNA

<213> ORGANISM: Clostridium coccoides

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1480)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 15

<400> SEQUENCE: 35

agagtttgat cctggctcag gatgaacgct ggccggcgtgc ttaacacatg caagtcgaac	60
gggctcatat tgaaacctag tgatgtatga gttagtggcg gacgggtgag taacgcgtgg	120
agaacctgcc gtatactggg ggataacact tagaaatagg tgctaatacc gcataagcgc	180
acagcttcgc atgaagcagt gtgaaaaact ccgggtggtat acgatggatc cgcgtctgat	240
tagctggttg gcggggtaac agcccaccaa ggccgacgac agtagccggc ctgagagggg	300
gaacggccac attgggactg agacacggcc caaactccta cgggaggcag cagtggggaa	360
tattgcacaa tgggggaaac cctgatgcag cgacgccgcg tgagtgaaga agtatttcgg	420
tatgtaaagc tctatcagca gggaagaaat actgacctta cggtcagcag acggtacctg	480
actaagaagc cccggctaac tacgtgccag cagccgcggt aatacgtagg ggcaagcgtt	540
atccggattt actgggtgta aagggagcgt agacggcagc gcaagtctga agtgaaatcc	600
catggcttaa cccatggaac tgctttggaa actgtgcagc tggagtgcag gagaggtaa	660
cggaaattcct agtgtagcgt gaaatgcgta gattattagg aggacaacag tgcgagcgct	720
actgacgtga ggctcgaagc gtggggagcaa acaggattag atacctggta gtcacgcgta	780
aacgatgatt actaggggtg tgggggacca aggtcttcgg tgccggcgca aacgcattaa	840
gtaatccacc tggggagtag gttcgcaaga atgaaactca aaggaattga cggggacccg	900
cacaagcggg ggagcatgtg gtttaattcg aagcaacgcg aagaacctta cctggtcttg	960
acatcccgat gacgagttag caaagtcact ttcccttcgg ggcatggag acaggtgggtg	1020
catggttgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc	1080
cctattttcca gtatccagca ggtagagctg ggcactctgg agagactgcc cgggataacc	1140
gggaggaagg cggggatgac gtcaaatcat catgcccctt atgatcaggg ctacacacgt	1200
gctacaatgg cgtaaacaaa gggaagcgag acggtgacgt taagcaaatc ccaaaaaata	1260
cgtcccagtt cggattgtag tctgcaactc gactacatga agctggaatc gctagtaatc	1320
gcgaatcaga atgtcgcggt gaatacgttc ccgggtcttg tacacaccgc ccgtcacacc	1380
atgggagtcg gaaatgccg aagtcagtga cctaaccgaa aggaaggagc tgccgaaggt	1440

-continued

ggagccggta actggggtga agtcgtaaca aggtagccgt 1480

<210> SEQ ID NO 36
 <211> LENGTH: 1486
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium papyrosolvens
 <220> FEATURE:
 <221> NAME/KEY: rRNA
 <222> LOCATION: (1)..(1486)
 <223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 16

<400> SEQUENCE: 36

```

agagtttgat cctggctcag gataaacgct ggccggcgac ataagacatg caagtcgaac    60
ggacttaact cattcttcta gattgagagc ggttagtgcc ggactggtga gtaacacgta    120
agcaacctgc ctatcagagg ggaataacag tgagaaatca ttgctaatac cgcataatgct    180
cacagtatca catgatacag tgaggaaagg agcaatccgc tgatagatgg gcttgcgcct    240
gattagttag ttggtggggt aacggcctac caagacgacg atcagtagcc ggactgagag    300
gttgaacggc cacattggga ctgagatacg gccagactc ctacgggagg cagcagtcgg    360
gaatattgag caatggagga aactctgacg cagtgcgccc gcgtatagga agaaggtttt    420
cggattgtaa actattgtcg ttagggaaga taaaagactg tacctaagga ggaagccccg    480
gctaactatg tgccagcagc cgcggtaata catagggggc aagcgttacc cggaattatt    540
gggtgtaaag ggtgcgtaga cggaagaaca agttggttgt gaaatccctc ggctcaactg    600
aggaactgca accaaaacta ttctccttga gtgtcggaga ggaaagtgga attcctagtg    660
tagcgggtgaa atgcgtagat attaggagga acaccagtg cgaaggcgac tttctggacg    720
ataactgacg ttgaggcacg aaagtgtggg gagcaaacag gattagatac cctggtagtc    780
cacactgtaa acgatggata ctagggttag ggtgtattaa gcactctgtg ccgccgctaa    840
cgcattaagt atcccacctg gggagtagca ccgcaagggt gaaactcaaa ggaattgacg    900
ggggcccgca caagcagtg agtatgtggt ttaattcgaa gcaacgcgaa gaaccttacc    960
agggcctgac atataccgga atatactaga gatagtatag tccttcggga ctggtataca   1020
gggtgtgcat ggtgtgtgtc agctcgtgtc gtgagatggt gggttaagtc ccgcaacgag   1080
cgcaaccctc atcgttagtt gctagcaggt aatgctgaga actctagcga gactgccggt   1140
gataaatcgg aggaaggtgg gtagtagctc aaatcatcat gccctttatg tcctgggcta   1200
cacacgtact acaatggcgc taacagaggg aagcaatata gtgatatgga gcaaacacct   1260
aaaagcggtc tcagttcgga ttgaaggctg aaattcgctc tcatgaagcc ggaattgcta   1320
gtaatggcag gtcagcatac tgccgtgaat acgttcccgg gccttgtaga caccgcccgt   1380
cacaccatga gagttggaaa taccggaagc ctgtgagcta actgtaaaga ggcagcagtc   1440
gaaggtagag ccaatgattg ggggaagtc gtaacaaggt agccgt                    1486
  
```

<210> SEQ ID NO 37
 <211> LENGTH: 1493
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium leptum
 <220> FEATURE:
 <221> NAME/KEY: rRNA
 <222> LOCATION: (1)..(1493)
 <223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 17

<400> SEQUENCE: 37

```

agagtttgat catggctcag gatgaacgct ggccggcgtg ttaacacatg caagtcgaac    60
  
```

-continued

gagaaccaac ggattgagga ttcgtccaaa tgaagttggg gaaagtggcg gacgggtgag	120
taacgcgtga gcaatctgcc ttggagtggg gaataacggt tggaacagc cgctaatacc	180
gcatgatcgc tctgggaggc atctctctgg acgccaaga tttatcgctc tgagatgagc	240
tcgcgtctga ttagctagtt ggcggggcaa cggcccacca aggcgacgat cagtagccgg	300
actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca	360
gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgccgc gtgaaggaa	420
aaggctttcg ggttgtaaac ttcttttaag ggggacgaac aaatgacggt accccttgaa	480
taagccacgg ctaactacgt gccagcagcc gcggtatac gtaggtggca agcgttatcc	540
ggattttatg ggtgtaaagg gcgtgtaggc gggaatgcaa gtcagatgtg aaaactatgg	600
gctcaaccca tagcctgcat ttgaaactgt atttcttgag tgctggagag gcaatcgga	660
ttccgtgtgt agcggtgaaa tgcgtagata tacggaggaa caccagtggc gaagcggatt	720
gctggacagt aactgacgct gaggcgcgaa agcgtgggga gcaaacaggg attagatacc	780
ctggtagtca cgcctgaaac gatggatact aggtgtgggg ggactgacct cctccgtgcc	840
gcagctaacg caataagtat cccacctggg gagtacgac gcaagggttg aaactcaaag	900
gaattgacgg gggcccgcac aagcggtgga gtatgtggtt taattcgaag caacgcgaag	960
aaccttacca gggcttgaca tctgctaac gaaccagaga tggatcaggt gcccttcggg	1020
gaaagcagag acaggtgggtg catggtgtgc gtcagctcgt gtcgtgagat gttgggttaa	1080
gtcccgaac gagcgcaacc cctattgtta gttgctacgc aagagcactc tagcgagact	1140
gccgttgaca aaacggagga aggtggggac gacgtcaaat catcatgccc cttacgtcct	1200
gggccacaca cgtactacaa tggcgcccaa caaagagagg caagaccgcg aggtggagca	1260
aatctcaaaa agccgtccca gttcggatcg caggctgcaa cccgcctgcg tgaagttgga	1320
atcgctagta atcgcgatc agcatgccgc ggtgaatacg ttcccgggac ttgtacacac	1380
cgcccgtcac accatgagag tcgggaacac ccgaagtcg tagcctgacc gcaagggggg	1440
cgcgccgcaa ggtgggttcg ataattgggg tgaagtagta acaaggtagc cgt	1493

<210> SEQ ID NO 38

<211> LENGTH: 1493

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1493)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 18

<400> SEQUENCE: 38

agagtttgat cctggctcag gacgaacgct ggcggcgcgc ctaacacatg caagtcgaac	60
ggagcttata ttccagaagt ttccggatgg acgagagata agcttagtgg cggacgggtg	120
agtaacacgt gagcaacctg cctttcagag ggggataaca gttggaaacg actgctaata	180
ccgcataacg ctgcgatggg gcatccgat gcagccaaag gagcaatccg ctgaaagatg	240
ggctcgcggc cgattagcta gttggtgggg caacggccca ccaaggcgac gatcggtagc	300
cggactgaga ggttgatcgg ccacattggg actgagacac ggcccagact cctacgggag	360
gcagcagtgg gggatattgc acaatggagg aaactctgat gcagcgacgc cgcgtgaggg	420
aagacggtct tcgattgta aacctctgtc ttgggggaag aaaatgacgg taccctaaaga	480
ggaagctccg gctaactacg tgccagcagc gcggtataa cgtacggagc gagcgttgc	540

-continued

```

cgggaattact ggggtgtaaag ggagcgtacg cgggcgagaa agttgaatgt taaatctacc    600
ggcttaactg gtagctgcgt tcaaaacttc ttgtcttgag tgaagtagag gcaggcgga    660
ttcctagtgt agcggtgaaa tgcgtagata taggaggaca ccagtggcg aagccgcctg    720
ctgggccttta actgacgctg aggcctcgaaa gcgtggggag caaaccagga ttagataccc    780
tggtagtcaa cgctgtaaac gatgattact aggtgtgggg gggactgacc ccctccgtgc    840
cgcagttaac acaataagta tccacctggg gagtacggcc gcaaagtttg aaaactcaaa    900
aggaatgacg ggggcccgcg caaagcagtg gagtatgtgg tttaatctcg aagcaacgcg    960
aagaacctta ccaggtcttg acatcgtgcg catagcctag agataggtga agcccttcgg   1020
ggcgcacaga caggtgtgtc atggttgtcg tcagctcgtg tcgtgagatg ttgggttaag   1080
tcccgcacag agcgcaaccc ttattattag ttgctacgca agagcactct aatgagactg   1140
ccgttgacaa aacggaggaa ggtggggatg acgtcaaac atcatgcccc ttatgacctg   1200
ggctacacac gtactacaat ggcactgaaa cagaggggaag cgacatcgcg aggtgaagcg   1260
aatcccaaaa aagtgtccca gtccggattg caggctgcaa ctgcctgca tgaagtcgga   1320
attgctagta atcgcgatc agcatgccgc ggtgaatacg ttcccgggcc ttgtacacac   1380
cgcccgtcac accatgggag tcggtaacac ccgaagccag tagcctaacc gcaaggaggg   1440
cgctgtcgaa ggtgggattg atgactgggg tgaagtcgta acaaggtagc cgt         1493

```

```

<210> SEQ ID NO 39
<211> LENGTH: 1483
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1483)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 19

```

```

<400> SEQUENCE: 39
agagtttgat cctggctcag gacgaacgct ggcggcacgc ctaacacatg caagtcgaac    60
ggagtgaaga tgcttgcatc tgaacttagt ggcggacggg tgagtaacac gtgagcaacc   120
tgcctttcag agggggataa cgtttgaaa cgaacgctaa taccgcataa aatatcgga    180
tcgcatggca ctgatataa aggagcaatc cgctgaaaga tgggctcgcg tccgattagg    240
cagttggcgg ggtaacggcc caccaaaccg acaatcggtg gccggactga gaggttgaac   300
ggccacattg ggactgagac acggcccaga ctctacggg aggcagcagt gggggatatt   360
gcacaatggg ggaacccttg atcgacgat gccgcgtgaa tgaagacggc cttcgggttg   420
taaagttctg tcgcagggga cgaaaatgac ggtaccctgc aagaaagctc cggttaacta   480
cgtgccagca gcccggttaa tacgtaggga gcaagcgttg tccggaatta ctgggtgtaa   540
agggagcgta ggcgggagga taaagttgaa tgtgaaatct atgggctcaa cccatagctg   600
cgttcaaaac tgttcttctt gactgaagta gaggcaggcg gaattcctag tgtagcggtg   660
aaatgcgtag atattaggag gaacaccagt ggcgaaagcg gcctgctggg cttttactga   720
cgctgaggct cgaaagcgtg ggtagcaaac agaattagat taccctgtta ttcacggcgg   780
taaacgatga ttactaggtt tgggttgacc tgacccccat tcgtgccgga agtaaacacca   840
taaagtaatc cacctggggg agtacggcgg ccaggttgaa acttcaaaag gaattgacgg   900
gggcccgcac aagcagtgga ggtatgtggt ttaatttcga cgcaaacgcg aagaacctta   960
ccagggtctt gacatcgagt gacggacata gagatatgtc tttcctttcg ggacacgaag  1020

```

-continued

```

acaggtggtg catggtgtgc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac 1080
gagcgcaacc cttaccatta gttgctacgc aagagcactc tgatgggact gccgttgaca 1140
aaacggagga aggtggggat gacgtcaaat catcatgccc cttatgacct gggcgacaca 1200
cgtactacaa tggcgggtcaa cagagggagg caaagccgag aggcagagca aaccctaaa 1260
agccgtctca gttcggattg caggctgcaa ctgcctgca tgaagtcgga attgctagta 1320
atcgcggtac agcatgcgcg ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac 1380
accatgagag ccggtaacac ccgaagtaa tagtctaacc gcaaggagga cattgccgaa 1440
ggtgggattg gtaattgggg tgaagtcgta acaaggtagc cgt 1483

```

```

<210> SEQ ID NO 40
<211> LENGTH: 1511
<212> TYPE: DNA
<213> ORGANISM: Clostridium coccoides
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1) .. (1511)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 20

```

```

<400> SEQUENCE: 40
agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac 60
ggggtcatat tgaaacctag tgatgtatga gttagtggcg gacgggtgag taacgcgtgg 120
agaacctgcc gtatactggg ggataacact tagaaatagg tgctaatacc gcataagcgc 180
acagcttcgc atgaaacagt gtgaaaaact ccggtggtat acgatggatc cgcgtctgat 240
tagctggttg gcggggtaac agcccaccaa ggcgacgac agtagccggc ctgagagggg 300
gaacggccac attgggactg agacacggcc caaactccta cgggaggcag cagtggggaa 360
tattgcacaa tgggggaaac cctgatgcag cgacgccgag tgagtgaaga agtatttcgg 420
tatgtaaagc tctatcagca ggaagaaat actgacctta cggtcagcag acggtacctg 480
actaagaagc cccggctaac tacgtgccag cagccgcggg aatacgtagg ggcaagcgtt 540
atccggattt actgggtgta aaggagcgt agacggcagc gcaagtctga gtgaaatccc 600
atggcttaac catggaactg ctttggaac tgtgcagctg gagtgcagga gaggtaaagc 660
ggaattccta gtgtagcggg tgaatgctg agatatagga ggaacaacag tggcgaagg 720
cggctactgg gactgtaact gacgttgagg ctgaaagcg tggggagcaa acaggattag 780
ataccctggt agtcacgcg taaacgatga ttactaggtg ttgggggacc ataggtcttc 840
ggtgccggcg caaacgcaat taagtaatcc acctggggga gtacgttcgc aagaatgaaa 900
ctcaaagga ttgacgggga ccgcacaaa gcggtggagc atgtggttta attcgaaagc 960
aaacgcgaag aaaccttacc tggctctgac atcccgatga cgagtgcgca aagtcacttt 1020
cccttcgggg caattggaga cagggtggtg atgggttgtc gtcagctcgt gtcgtgagat 1080
gttgggttaa gtcccgcaac gagcgcaacc cctatttcca gtagccagca ggtagagctg 1140
ggcactctgg agagactgcc cgggataacc gggaggaagg cgggatgac gtcaaatcat 1200
catgcccctt atgatcaggg ctacacacgt gctacaatgg cgtaaacaaa ggggaagcgag 1260
acggtgacgt taagcaaat ccaaaaaataa cgtcccagtt cggattgtag tctgcaactc 1320
gattacatga agctggaatc gctagtaatc gcgaatcaga atgtcgcggg gaatacgttc 1380
ccgggtcttg tacacaccgc ccgtcacacc atgggagtcg gaaatgcccg aagtcagtga 1440
cctaaccgaa aggaaggagc tgccgaaggt ggagccggtg actggggtga agtagataac 1500

```

-continued

aaggtagcgcg t 1511

<210> SEQ ID NO 41
 <211> LENGTH: 1495
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium leptum
 <220> FEATURE:
 <221> NAME/KEY: rRNA
 <222> LOCATION: (1)..(1495)
 <223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 21

<400> SEQUENCE: 41

```

agagtttgcg cctgcgcgtca ggacgaacgc tggcggcgcg cctaacacat gcaagtcgaa    60
cgggactatt ttgggagaag ttttcggatg gatctcggga tagtttagtg gcggacgggt    120
gagtaacgcg tgggcaacct gccttacaca gggggataac aattagaaat gattgctaata    180
accgcataag accacggtac tgcattgtac agtggtaaaa actgaggtgg tgtaagatgg    240
gccccgcgtct gattaggtag ttggtggggg agaagcctac caagccgacg atcagtagcc    300
gacctgagag ggcgaccggc cacattggga ctgagacacg gcccaaacct ctacgggagg    360
cagcagtggtg gaattattgca caatggggga aacctgatg cagcgacgcc gcgtgagtga    420
ggaagtattt cggtatgtaa agctctatca gcagggaaga aaatgacggg acctgactaa    480
gaagcccccg ctaactacgt gccagcagcc gcggtaatac gtagggggca agcgttatcc    540
ggatttactg ggtgtaaagg gagcgtagac ggacttgcaa gtctgatgtg aaaatccggg    600
ggcccaaccc cggaactgca ttggaaactg tatatctaga gtgtcggaga ggcaagtgga    660
atttcctggt gtagcgtgta aatgcgtaga tatcagagga acaccagtgg cgaaggcgct    720
tgcttgacga tgactgacgt tgaagctcga aaagcgtggg tagcaaacag aattagatac    780
cctggtaagt caaccggta aacgatgatt actaggtttt ggttggaactg accccatccg    840
tgccggagta acaccaataa gttatccaac ctgggaagta cggccggcag gttgaaactc    900
aaaaggaaat gacggggggc cgcacaagca gttgaagtat gtgggttaat tcgacgcaaa    960
cgcaagaac cttaccaggt cttgacatcg agtgacggac atagagatat gtctttcctt   1020
cgggacacga agacaggtgg tgcattgttg tcgtcagctc gtgtcgtgag atgttggtt   1080
aagtcccgcg acgagcgcaa ccottacat tagttgtac gcaagagcac tctgatggga   1140
ctgccgttga caaacggag gaaggtgggg atgacgtcaa atcatcatgc cccttatgac   1200
ctgggcgaca cacgtactac aatggcggtc aacagagga ggcaagccg cgaggcagag   1260
caaaccccta aaagcgtct cagttcggat tgcaggctgc aactgcctg catgaagtcg   1320
gaattgctag taatcgcgga tcagcatgcc gcggtgaata cgttccggg ccttgtacac   1380
accgcccgtc acaccatgag agccggtaac accgaagtc aatagtctaa ccgcaaggag   1440
gacattgccg aaggtgggat tggtaattgg ggtgaagtcg taacaaggta gccgt      1495

```

<210> SEQ ID NO 42
 <211> LENGTH: 1491
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium leptum
 <220> FEATURE:
 <221> NAME/KEY: rRNA
 <222> LOCATION: (1)..(1491)
 <223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 22

<400> SEQUENCE: 42

-continued

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
gagaatccag tgaaggagtt ttcggacaac ggatctggag gaaagtggcg gacgggtgag	120
taacgcgtga gcaatctgcc ttggagtggg gaataacggt tggaaacagc cgctaatacc	180
gcatgatgcg tctgggaggg atctctcttg acgccaaaga tttatcgctc tgagatgagc	240
tcgcgtctga ttagcttggtt ggcggggtaa aggccacca aggcgacgat cagtagccgg	300
actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca	360
gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgccgc gtgaagggaag	420
aaggctttcg ggttgtaaac tttttttctg agggacgaag aaagtgacgg tacctcagga	480
ataagccacg gctaactacg tgccagcagc cgcggtaata cgtaggtggc aagcgttatc	540
cggatttatt ggggtgtaaag ggcgtgtagg cgggaaagca agtcagatgt gaaaactcag	600
ggctcaaccc tgagcctgca tttgaaactg tttttcttga gtgctggaga ggcaatcgga	660
attccgtgtg tagcgggtga atgcgtagat atacggagga caccagtggc gaagcggatt	720
gctggacagt aactgacgct gaggcgcgaa gcgtggggag caaacaggat tagataccct	780
ggtagtccac gccgtaaacg atggatacta ggtgtggggg gactgacccc ctccgtgccg	840
cagctaacgc aataagtatc ccacctgggg agtacgatcg caaggttgaa actcaaagga	900
attgacgggg gcccgacaaa gcggtggagt atgtggttta attcgaagca acgcgaagaa	960
ccttaccagg gcttgacatc ctgctaacga accagagatg gattaggtgc ccttcgggga	1020
aagcagagac aggtggtgca tggttgtcgt cagctcgtgt cgtgagatgt tgggttaagt	1080
cccgcaacga gcgcaaccct tattgttagt tgctacgcaa gagcactcta gcgagactgc	1140
cgttgacaaa acggaggaag gtggggacga cgtcaaatca tcatgcccct tacgtcctgg	1200
gccacacacg tactacaatg gcggccaaca aagagaggca agaccgcgag gtggagcaaa	1260
tctcaaaaag ccgtcccagt tcggatcgca ggctgcaacc cgctgcgtg aagttggaat	1320
cgctagtaat cgcggatcag catgccgcgg tgaatacgtt cccgggcctt gtacacaccg	1380
cccgtcacac catgagagtc gggaaacccc gaagtccgta gcctaaccgc aaggggggcg	1440
cggccgaagg tgggttcgat aattggggtg aagtcgtaac aaggtagccg t	1491

<210> SEQ ID NO 43

<211> LENGTH: 1495

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1495)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 23

<400> SEQUENCE: 43

agagtttgat cctgtgcctc aggatgaacg ctggcggcgt gcttaacaca tgcaagtcga	60
acgagaacca acggaattgag gattcgtcca aatgaagttg gggaaagtgg cggacgggtg	120
agtaacgcgt gagcaatctg ccttgagtg ggaataacg gttggaaca gccgctaata	180
ccgcatgatg cgtctgggag gcatctctct ggacgcaaaa gatttatcgc tctgagatga	240
gctcgcgtct gattagctag ttggcggggc aacggccccc caaggcgacg atcagtagcc	300
ggactgagag gttggccggc cacattggga ctgagacacg gccagactc ctacgggagg	360
cagcagtggt gaattattgg caatgggcgc aagcctgacc cagcaacgcc gcgtgaagga	420
agaaggcttt cgggttgtaa acttctttta agggggacga acaaatgacg gtaccccttg	480

-continued

aataagccac ggctaactac gtgccagcag ccgcggtaat acgtaggtgg caagcgttat	540
ccggattttat tgggtgtaaa gggcgtgtag gcgggaatgc aagtcagatg tgaaaactat	600
gggctcaacc catagcctgc atttgaaact gtatttcttg agtgctggag aggcaatcgg	660
aattccgtgt gtagcgtgta aatgcgtaga tatacggagg aacaccagtg gcgaaggcgg	720
attgtcggac agtaactgac gctgagggcg gaaagcgtgg ggagcaaaca ggattagata	780
ccctggtagt ccacgcgcta aacgatggat actaagtgtg gggggactga cccctccgt	840
gccgcagcta acgcaataag tatcccacct ggggagtagc atcgcaaggt tgaaactcaa	900
aggaattgac gggggcccg cacaagcgtg gagtatgttg tttaattcga agcaacgcga	960
agaaccttac cagggcttga catcctgcta acgaaccaga gatggatcag gtgcccttcg	1020
gggaaagcag agacaggttg tgcattggtg tcgtcagctc gtgtcgtgag atgttgggtt	1080
aagtcgccca acgagcgcaa cccctattgt tagttgctac gcaagagcac tctagcgaga	1140
ctgccgttga caaaacggag gaaggtgggg acgacgtcaa atcatcatgc cccttacgtc	1200
ctgggccaca cacgtactac aatggcggcc aacaaagaga ggcaagaccg cgaggtggag	1260
caaattctca aaagccgtcc cagttcggat cgcaggtcgc aaccgcctg cgtgaagtgt	1320
gaatcgctag taatcgcgga tcagcatgcc gcggtgaata cgttcccggt ccttgtagac	1380
accgcccgtc acaccatgag agtcgggaac acccgaagtc cgtagcctga ccgcaagggg	1440
ggcgcggccg aaggtgggtt cgataattgg ggtgaagtcg taacaaggta gccgt	1495

<210> SEQ ID NO 44

<211> LENGTH: 1440

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1440)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 24

<400> SEQUENCE: 44

agagtttgat cctggctcag gacgaacgct ggcggcacgc ctaacacatg caagtcgaac	60
ggagtgaaaga tgctcgcatc tgaacttagt ggcggacggg tgagtaacac gtgagcaacc	120
tgccctttcag agggggataa cgtttggaaa cgaacgctaa taccgcataa aatatcggag	180
tcgcatggca ctgatataca aggagtaatc cgtgaaaga tgggctcgcg tccgattag	240
cagttggcgg ggttaacggc caccaaaacc acaatcggtg gccggactga gaggttgaac	300
ggccacattg ggactgagac acggcccaga ctccctacggg aggcagcagt gggggatatt	360
gcacaatggg ggaacccttg atgcagcgat gccgcgtgaa tgaagacggc cttcgggttg	420
taaagtcttg tcgcagggga cgaaaatgac ggtaccctgc aagaaagctc cggctaacta	480
cgtgccagca gccgcggtaa tacgtaggga gcaagcgttg tccggaatta ctgggtgtaa	540
agggagcgta ggcgggagga taagtgaat gtgaaatcta tgggctcaac ccatagttgc	600
gttcaaaact gttcttcttg agtgaagtag aggcaggcgg aattcctagt gtagcgtgta	660
aatgcgtaga tatttagagga acaccagtgg cgaagcggcc tgctgggctt ttactgacgc	720
tgagctcgaa agcgtgggta gcaacaggat tagataccct ggtagtccac gcggtaaacg	780
atgattacta gtgtgggttg actgacccat ccatgccgga gttaacacaa tagtaatcca	840
cctggggagt acgcgcagtg aactcaaagg attgacgggg ccgcacaag cagtgagtat	900
gtggtttatt cgacgcacgc gagactacag tcttgacatc gatgacggac tagagatatg	960

-continued

tctttctcgg acacgaagac aggtggtgca tggttgtcgt cagctcgtgt cgtgagatgt	1020
tgggttaagt cccgcaacga gcgcaaccct taccattagt tgttacgcaa gagcactcta	1080
atgggactgc cgttgacaaa acggaggaag gtggggatga cgtcaaatca tcatgccct	1140
tatgacctgg gcgacacacg tactacaatg gcggtcaaca gagggaggca aagccgcgag	1200
gcagagcaaa cccctaaaag ccgtctcagt tcggattgca ggctgcaact cgcctgcatg	1260
aagtcggaat tgctagtaat cgcggatcag catgccgcgg tgaatacgtt cccgggcctt	1320
gtacacacgc cccgtcacac catgagagcc ggtaacaccc gaagtcaata gtctaaccgc	1380
aaggaggaca ttgccgaagg tgggatggta attgggggtga agtagtaaca aggtagccgt	1440

<210> SEQ ID NO 45

<211> LENGTH: 1495

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1495)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 25

<400> SEQUENCE: 45

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
gagaaccatt ggatcgagga ttcgtccaag tgaagggtgg gaaagtggcg gacgggtgag	120
taacgcgtga gcaatctgcc ttggagtggg gaataacggc tggaaacagc cgctaatacc	180
gcatgataca gctgggaggc atctccctgg ctgtcaaaga tttatcgctc tgagatgagc	240
tcgcgtctga ttactagtt ggcggggtaa cggcccacca aggcgacgat cagtagccgg	300
actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca	360
gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgccgc gtgaaggaa	420
aaggctttcg ggttgtaaac ttcttttgc agggacgaag caagtgcgag tacctgacga	480
ataagccacg gctaactacg tgccagcagc cgcggttaata cgtagtggca agcgttatcc	540
ggatttattg ggggtgaaag ggcgtgtagg cgggaatgca agtcagatgt gaaaactatg	600
gggctcaacc catagcctgc atttgaact gtatttcttg agtgctggag aggcaatcga	660
attccgtgtg tagcgggtga aatgcgtaga tatacggagg aacaccagtg gcgaagcgga	720
ttgctggaca agtaactgac gctgagggcg gaaagcgtgg ggagcaaaca ggattagata	780
ccctggtagt ccacgccgta aacgatggat actaggtgtg ggggggactga cccctccgt	840
gccgcagcta acgcaataag tatccacct ggggagtacg atcgcaaggt tgaaactcaa	900
aggaattgac gggggcccg acaagcggtg gagtatgtgg tttaattcga cgcaacgcga	960
agaaccttac cagggcttga cactacta acgaaccaga gatggattag gtgcccttcg	1020
gggaaagtag agacaggtgg tgcattggtg tcgtcagctc gtgtcgtgag atgttgggtt	1080
aagtcgccga acgagcgcaa cccctattgt tagttgtac gcaagagcac tctagcgaga	1140
ctgccgttga caaaacggag gaagggtggg acgacgtcaa atcatcatgc ccttacgtc	1200
ctgggccaca cacgtactac aatggcggcc aacaaagaga ggcaaagccg cgagggtgag	1260
caaattctca aaagccgtcc cagttcggat cgcaggctgc aaccgcctcg cgtgaagttg	1320
gaatcgctag taatcgcgga tcagcatgcc gcggtgaata cgttccggg ccttgtagac	1380
accgccgctc acaccatgag agtcgggaac acccgaaatc cgtagcctaa ccgcaagggg	1440
ggcgcggccg aagggtgggt cgataattgg ggtgaagtcg taacaaggta gccgt	1495

-continued

```

<210> SEQ ID NO 46
<211> LENGTH: 1495
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1495)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
        strain 26

<400> SEQUENCE: 46

agagtttgat catggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac      60
ggagcacccc tgaaggagtt ttcggacaac ggatgggaat gcttagtggc ggactgggta      120
gtaacgcgtg aggaacctgc cttccagagg gggacaacag ttggaaacga ctgctaatac      180
cgcatgatgc gttggagccg catgactccg acgtcaaaga tttatcgctg gaagatggcc      240
tcgcgtctga ttagctagtt ggtgaggtaa cggcccacca aggcgacgat cagtagccgg      300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca      360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaagggaag      420
aaggctttcg ggttgtaaac ttcttttaag ggggaagagc agaagacggt accccttgaa      480
taagccacgg ctaactacgt gccagcagcc gcggtaatat gtaggtggca agcgttgtcc      540
ggatttactg ggtgtaaagg gcgtgcagcc ggagagacaa gtcagatgtg aaatccacgg      600
gctcaaccgc tgaactgcat ttgaaactgt ttcccttgag tgctggagag ggtaatcgga      660
attcctttgt gtagcgtgta aatgcgtaga tataagaaga acaccagtgg cgaaggcgga      720
ttactggaag ataactgacg gtgaggcgcg aaagcgtggg ggagcaacag attaaatacc      780
ctggtagtcc acgctgttaa cgatcgatac taggtgtgcc gggactgacc ccctgcgtgc      840
ccggagttaa ccacaataag tatcgacact ggggagtacg atcgcaaggt gaacttcaaa      900
ggaattgacg ggggcccgcc ccaagccgtg gattatgtg ttaattcgaa gcaacgcgaa      960
gaacctaccc agggccttgac atcctgctaa cgaagtagag atacattagg tgccctttcg      1020
gggaaagcag agacaggtgg tgcattgttg tcgtcagctc gtgtcgtgag atgttgggtt      1080
aagtcccgca acgagcgcaa cccctattgt tagttgtac gcaagagcac tctagcgaga      1140
ctgccgttga caaacggag gaaggcgggg acgacgtcaa atcatcatgc cccttatgtc      1200
ctgggctaca cacgtaatac aatggcgggt aacaaaggga tgcaaagccg cgaggcagag      1260
cgaaccccaa aaagccgtcc cagttcggat cgcaggctgc aaccgcctg cgtgaagtcg      1320
gaatcgctag taatcgcgga tcagcatgcc gcggtgaata cgttccggg ccttgtagac      1380
accgccgctc acaccatgag agtcgggaac acccgaagtc cgtagcctaa ccgcaaggag      1440
ggcgcgccg aaggtgggtt cgataattgg ggtgaagtcg taacaaggta gccgt      1495

```

```

<210> SEQ ID NO 47
<211> LENGTH: 1509
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1509)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
        strain 27

```

```

<400> SEQUENCE: 47

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac      60
ggagtacccc tgaaggagtt ttcggacaac tgatgggact acttagtggc ggacgggtga      120

```

-continued

gtaacgcgtg agtaacctgc cttggagtgg ggaataacag ctggaaacag ctgctaatac	180
cgcataatat gtctgtgtcg catggcactg gacatcaaag atttattcgt ctgagatgga	240
ctcgcgtctg attagctagt tggcggggta acggcccacc aaggcgacga tcagtagccg	300
gactgagagg ttggccggcc acattgggac tgagacacgg ccagactcc tacgggaggc	360
agcagtgggg aatattgggc aatgggcgca agcctgaccc agcaacgccg cgtgaaggaa	420
gaaggctttc gggttgtaaa cttcttttaa gggggaagag cagaagacgg tacccttga	480
ataagccacg gctaactacg tgccagcagc cgcggtaata cgtaggtggc aagcgttgtc	540
cggatttact ggggtgtaaag ggcgtgcagc cggagagaca agtcagatgt gaaatccacg	600
ggctcaaccc gtgaactgca ttgaaactg tttcctggag ttcggagggt atggaattct	660
tgttagcggg gaaatgctgt agatatggga gaaccaccag tgcgaggggg cttccgggac	720
tgtacttgac tgtagaggtc tcaaagctgg gggagcaccg aggaatgaga taccgtgata	780
gtcccacgcg gtaacggatg attactaggt gttgggggga cccaggctct ttcggtgccg	840
ggcgcgaaacc ctttaggaat tccacctggg gaattacgtt tggcaagaaa ggaacttcaa	900
agaaattgaa cgggggaccc cccaaccggg tggaggcatg gtgttttatt tcggaggaa	960
gggaagaacc tttacctgtg tctgaccttc cggatgacga agtgagcaaa gtcaacttcc	1020
cttcggggcc atggaggaca ggtggtggca tggttggtcg tcagctcgtg tcgtgagatg	1080
ttgggttaag tcccgcaacg agcgcgaacc ctatttccag tagccagcag gtagagctgg	1140
gcactctgga gagactgccc gggataaccg ggaggaaggc ggggatgacg tcaaatcatc	1200
atgcccctta tgatcagggc tacacacgtg ctacaatggc gtaaacaaag ggaagcgaga	1260
cggtgacgtt aagcaaatcc caaaaataac gtcccagttc ggattgtagt ctgcaactcg	1320
actacatgaa gctggaatcg ctagtaatcg cgaatcagaa tgcgcgggtg aatacgttcc	1380
cgggtcttgt acacacgcc cgtcacacca tgggagtcgg aaatgccga agtcagtgac	1440
ctaaccgaaa ggaaggagct gccgaagggt gagccggtaa ctgggggtgaa gtcgtaacaa	1500
ggtagccgt	1509

<210> SEQ ID NO 48

<211> LENGTH: 1583

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1583)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 28

<400> SEQUENCE: 48

agagtttgat cctggctcag gacgaacgct ggcggcgcgc ctaacacatg caagtcgaac	60
ggagcttata ttccagaagt ttccggatgg acgagagata agcttagtgg cggacgggtg	120
agtaacacgt gagcaacctg cctttcagag ggggataaca gttggaaacg actgctaata	180
ccgcataacg ctgcgatggg gcatccgat gcagccaaag gagcaatccg ctgaaagatg	240
ggctcgcggc cgattagcta gttggtgggg caacggccca ccaaggcgac gatcggtagc	300
cggactgaga ggttgatcgg ccacattggg actgagacac ggcccagact cctacgggag	360
gcagcagtgg gggatattgc acaatggagg aaactctgat gcagcgacgc cgcgtgaggg	420
aagacggtct tcgattgta aacctctgtc tttggggaag aaaatgacgg taccctaaaga	480
ggaagctccg gctaactacg tgccagcagc cgcggtaata cgtaggggag cgagcgttgt	540

-continued

```

ccggaattac tgggtgtaaa gggagcgtag cgggcgagaa agttgaatgt taaatctacc 600
ggcttaactg gtagctgcgt ccaaaacttc ttggtcttga gtgaaagtaa gaggccaggg 660
cggaaattct tagtgtaagc gggtgaaaat gcgttagata ttagggagga accaccaggt 720
gggcgaaggg cggttgctg ggctttaact ggacggctgg aggcttgga aaggcgtggg 780
gagagcaaac acagggaatt aagtataccc tggatatgt cacacgcttg taaagagtat 840
gattaactta ggggtggggg gggaaactga ccctttcgtg tgcgcgcagg ttaacacaca 900
tttagagtat atccaacttg gggagagtac ggccggcaaa gtttgaaact taaaaggga 960
aattgagacc ggggggcccg gccaccaagc acagtggaga gtatggtggg tttaatttcg 1020
agaagcaacc ggcggaagag aaactttacc agtccttgac atcggtggcg gcataagccc 1080
tagagattag gtgaagccct tcggggggcc caccagacag gtggtgcatg gttgtcgtca 1140
gctcgtgtcg tgagatgttg ggttaagtcc ccgcaaacga gcgcaaccct tattattagt 1200
ttgctacgca agagcactct aatgagactg ccgttgacaa aacggaggaa ggtgggggatg 1260
acgtcaaate atcatgcccc ttatgacctg ggctacacac gtactacaat ggcaactgaaa 1320
cagagggaag cgacatcgcg aggtgaagcg aatcccaaaa aagtgtccca gttcggattg 1380
caggctgcaa ctgcctgca tgaagtcgga attgctagta atcgcgatc agcatgccgc 1440
ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac accatgggag tcggtaaac 1500
ccgaagccag tagcctaacc gcaaggaggg cgctgtcgaa ggtgggattg atgactgggg 1560
tgaagtcgta acaaggtagc cgt 1583

```

<210> SEQ ID NO 49

<211> LENGTH: 1519

<212> TYPE: DNA

<213> ORGANISM: Clostridium coccoides

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1519)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 29

<400> SEQUENCE: 49

```

agagtttgat catggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac 60
gggtgtacgg gaaggaaggg ttccggccgga aaacctgtgc atgagtggcg gacgggtgag 120
taacgcgtgg gcaacctggc ctgtacaggg ggataaact tagaaatagg tgctaatacc 180
gcataacggg ggaagccgca tggcttttcc ctgaaaactc cgggtgtaca ggatggggcc 240
gcgtctgatt agccagttag cagggtaacg gcctacaaa gcgacgatca gtagccggcc 300
tgagagggcg gacggccaca ctgggactga gacacggccc agactcctac gggaggcagc 360
agtgggggat attgcacaat ggggggaacc ctgatgcagc gacgccgct ggggtgaagaa 420
gcgcctcggc gcgtaaagcc ctgtcagcag ggaagaaaa tgacggtacc tgaagaagaa 480
gccccgggct aactacgtgc cagcagccgg cggttaattac gtaggggggc aggcgttatc 540
cggatttact ggggtgtaaa ggggggcgca aacggcgatg gcaggccagg aatggaaagc 600
ccggggggcc aaccccgga cttgctcttg ggaaactggc ttggctggga gtggcaggag 660
gggcaggcgg aattctctgg tggtagcggg ggaatatggc taaaaatcaa gaagaaaaac 720
cgggtgggaa aggcggcctg gtgggactgc gaactgacgt tgaaggcccg aaagcgtggg 780
gaacaaacag gatagattcc ctggtagttc caccgccgta acgatgatta ctagggtgtc 840
gggagcagag actgcccggt gccgcagcca acgcattaag taatccacct ggggagtagc 900

```

-continued

```

ttcgcaagaa tgaactcaa aggaattgac ggggacccgc acaagcgggtg gagcatgtgg 960
tttaattcga agcaacgcga agaaccttac ctggtcttga catcccgatg acgagtgagc 1020
aaagtcactt tcccttcggg gcattggaga caggtgggtg atggttgctg tcagctcgtg 1080
tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc ctatttccag tagccagcag 1140
gtagagctgg gactctgga gagactgcc gggataaccg ggaggaaggc ggggatgacg 1200
tcaaatacat atgcccctta tgatcagggc tacacacgtg ctacaatggc gtaacaaaag 1260
ggaagcgaga cggtgacgtt aagcaaatcc caaaaaaac gtcccagttc ggattgtagt 1320
ctgcaactcg actacatgaa gctggaatcg ctagtaatcg cgaatcagaa tgctcgcggtg 1380
aatacgttcc cgggtcttgt acacacgcc cgtcacacca tgggagtcgg aaatgcccg 1440
agtcagtgc ctaaccgaaa ggaaggagct gccgaagggt gagccggtaa ctggggtgaa 1500
gtcgtaacaa ggtagccgt 1519

```

```

<210> SEQ ID NO 50
<211> LENGTH: 1497
<212> TYPE: DNA
<213> ORGANISM: Clostridium coccoides
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1) .. (1497)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 30

```

```

<400> SEQUENCE: 50
agagtttgat cctggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac 60
ggggtatata agcgaagtt tacgtaggga aggttatata cttagtggcg gacgggtgag 120
taacgcgtgg gcaacctgcc ccgtgccggg ggataccgcc tggaaacagg cgtaatacc 180
gcataagcgc atacagccgc atgggtgtat gcggaaagct ccggcggcac gggatgggcc 240
cgcccccgat tagccagttg gcggggtaac ggcccaccaa agcgacgacg ggtagccggc 300
ctgagagggc ggaaggccac attgggactg agacacggcc caaactccta cgggaggcag 360
cagtggggaa tattgcacaa tgggggaaac cctgatgcag caacgcgcgc tgggtgaagg 420
agcgtttcgg cgcgtaaagc cctgtcagcg gggaagaaga aagacggtag ccgaccaaga 480
agccccggct aactacgtgc cagcagccgc ggtaatacgt agggggcgag cgttatccgg 540
aattactggg tgtaaaggga gcgtagacgg cgaggtaacg ctgaagtgga agcccgcggc 600
ccaaccgcgg aactgctttg ggaactgttt tgctggagta tgggaggggt aagcggaatt 660
cctggtgtag cggtgaaatg cgtagatata aggaggaaca ccggtggcga aggcggctta 720
ctggaccata actgacgttg aggtcgcgaa gcgtggggag cgaacaggat tagataccct 780
ggtagtccac gcgtaaacga tgattaccag gtgtcgggtg tcgaaggacg gcccggtgcc 840
gcagcgaacg cagtaagtaa tccacctggg gagtacgttc gcaagaatga aactcaaagg 900
aattgacggg gacccgcaca agcgttgagg catgtggttt aattcgaagc aacgcgaaga 960
accttaccg gccttgacat cccttggaac gcatatgtaa tgtatgtttc cttcgggacc 1020
agggagacag gtggtgcatg gttgtcgtca gctcgtgtcg tgagatgttg ggtcaagtc 1080
cgcaacgagc gcaacccctg cccccagtag ccagcattta agatgggcac tctgggggga 1140
ctgccgggga taaccgcggg gaaggcgggg atgacgtcaa atcatcatgc cccttatggc 1200
cggggctaca cacgtgtac aatggcgtaa acagagggag gcgagacagc gatgttaagc 1260
gaaccccaaa aataacgtcc cagttcggat tgcagcctgc aactcggctg catgaagctg 1320

```

-continued

```

gaatcgctag taatcgcgga tcagaatgcc gcggtgaata cgttcccggg tcttgtagac 1380
accgcccgtc acaccatggg agtcgggaac gcccgaagcc ggtgaccgaa cccgaaaggg 1440
gaggagccgt cgaaggcggg cctgggtgact ggggtgaagt cgtaacaagg tagccgt 1497

```

```

<210> SEQ ID NO 51
<211> LENGTH: 1475
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1) .. (1475)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 31

```

```

<400> SEQUENCE: 51

```

```

gagtttgatc ctggctcagg ataaacgctg gcggcgacac taagacatgc aagtcgaacg 60
aacttaatac cttgcttgca aggtaaagcg ttagtggcgg actggtgagt aacacgtaag 120
aaatctgcct atcagagggg aataacagtg agaaatcact gctaataacc catatgccat 180
agttatcgca tgataatagt gggaaagaag caattcgctg atagatgagc ttgaggctga 240
ttagctagtt ggtggggtaa cgccctacca aggcgacgat cagtagccgg cctgagaggg 300
tgaacggcca cattgggact gagacacggc ccaaactcct acgggaggca gcagtgggga 360
atattgcaca atgggggaaa ccttgatgca gcgacgccgc gtgagtgaag aagtatttcg 420
gtatgtaaag ctctatcagc agggaagaaa atgacgggtac ctgactaaga aagccccggc 480
taactacgtg ccagcagccg cggtaatatc tagggggcaa gcgttatccg gatttactgg 540
tgtaaaggga gcgtagacgg cagcgcaagt ctgagtgaag tcccatggct taccatgaa 600
actgctttgg aaactgtgca gctggagtgc aggagaggta agcgggaatc tagttagcgc 660
gttgaaatgc gtagattatc agaaggaaca ccggtggccg aggcggcctg ctgggctttt 720
actgacgctg aggcctgaag cgtgggtgac aaacaggatt agataccctg gtagtccacg 780
cggtaaacga tgattactag gtgtgggtgg actgacccca tccgtgccgg agttaacaca 840
ataagtaac cactgggga gtacggccgc aagggtgaaa ctcaaaggaa ttgacggggg 900
cccgacaaag cagtggagta tgtggtttta ttcgacgcaa cgcaagaac cttaccaggt 960
cttgacatcg agtgacggac atagagatat gtctttcctt cgggacacga agacaggtgg 1020
tgcatggttg tcgtcagctc gtgtcgtgag atgttgggtt aagtccgca acgagcgcaa 1080
cccttaccat tagttgtac gcaagagcac tctaattgga ctgccgttga caaacggag 1140
gaagtgggg atgacgtcaa atcatcatgc cccttatgac ctgggcgaca cacgtactac 1200
aatggcggtc aacagaggga ggcaaagccg cgaggcagag caaaccccta aaagccgtct 1260
cagttcggat tgcaggctgc aactcgccct catgaagtcg gaattgctag taatcgcgga 1320
tcagcatgcc gcggtgaata cgttcccggg ccttgtagac accgcccgtc acaccatgag 1380
agccggtaac acccgaagtc aatagtctaa ccgcaaggag gacattgccg aaggtgggat 1440
tggttaattgg ggtgaagtcg taacaaggta gccgt 1475

```

```

<210> SEQ ID NO 52
<211> LENGTH: 1491
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1) .. (1491)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium

```

-continued

strain 32

<400> SEQUENCE: 52

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
gagaatcagt ggattgagga ttcgtccaaa tgaaactgag gaaagtggcg gacgggtgag    120
taacgcgtga gcaatctgcc ttggagtggg gaataacggc tggaacacgc cgctaatacc    180
gcatgataca gttgggaggc atctctctga ctgtcaaaga tttatcgctc tgagatgagc    240
tcgcgtctga ttagctagtt ggcggggtaa cggcccacca aggcgacgat cagtagccgg    300
actgagaggt tggccggcca cattgggact gagacacggc ccagactcct acgggaggca    360
gcagtgggga atattgggca atgggcgcaa gcctgaccca gcaacgcgcg gtgaagggaag    420
aaggctttcg ggttgtaaac ttcttttctg ggggacgaac aaatgacggg accccaggaa    480
taagccacgg ctaactacgt gccagcagcc gcggtaatac gtaggtggca agcgttatcc    540
ggattttatt ggtgtaaagg gcgtgtaggc gggaaatgcaa gtcagatgtg aaaactatgg    600
gctcaaccca tagcctgcac ttgaaactgt atttcttgag tgctggagag gcaatcgga    660
ttccgtgtgt agcggtgaaa tgcgtagata tacggaggaa caccagtggc gaagcggatt    720
gctggacagt aactgacgct gaggcgcgaa agcgtgggga gcaaacagga ttagataccc    780
tggtagtcca cgccgtaacg atggatacta gtgtgggggg actgaccccc tccgtgccgc    840
agctaacgca ataagtatcc ccacctgggg agtacgacgc caagggtgaa actcaaagga    900
attgacgggg gcccgacaaa gcgggtggagt atgtggttta attcgaagca acgcgaagaa    960
ccttaccagg gcttgacatc ctgctaacga accagagatg gattaggtgc ccttcgggga   1020
aagcagagac aggtggtgca tggttgtcgt cagctcgtgt cgtgagatgt tgggttaagt   1080
cccgcaacga gcgcaacccc tattgttagt tgctacgcaa gagcactcta gcgagactgc   1140
cgttgacaaa acggaggaag gtggggacga cgtcaaatca tcatgcccc ttcgtcctgg   1200
gccacacacg tactacaatg gcgggttaaca aagagaggca agaccgcgag gtggagcaaa   1260
tctcaaaaag ccgtcccagt tcggatcgca ggctgcaacc cgctgcgtg aagttggaat   1320
cgctagtaat cgcggatcag catgccgcgg tgaatacgtt cccgggcctt gtacacaccg   1380
cccgtcacac catgagagtc gggaaacacc gaagtccgta gcctaaccgc aaggggggcg   1440
cggcogaagg tgggttcgat aattgggggtg aagtcgtaac aaggtagccg t         1491

```

<210> SEQ ID NO 53

<211> LENGTH: 1495

<212> TYPE: DNA

<213> ORGANISM: Clostridium coccoides

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1495)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 33

<400> SEQUENCE: 53

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac    60
gggtgtacgg ggaggaaggc ttcggccgga aaacctgtgc atgagtggcg gacgggtgag    120
taacgcgtgg gcaacctggc ctgtacaggg ggataacact tagaaatagg tgctaatacc    180
gcataacggg ggaagccgca tggcttttcc ctgaaaactc cggtggtaca ggatgggccc    240
gcgtctgatt agccagttag cagggtaacg gcctacaaa gcgacgatca gtagccggcc    300
tgagagggcg gacggccaca ctgggactga gacacggccc agactcctac gggaggcagc    360

```

-continued

```

agtgggggat attgcacaat ggggggaacc ctgatgcagc gacgcccggt gggatgaagaa 420
gcgcctcggc gcgtaaagcc ctgtcagcag ggaagaaaat gacggtacct gaagaagaag 480
ccccggctaa ctacgtgcc a gcagccggc taatacgtag ggggcaagcg ttatccggat 540
ttactgggtg taaagggggc gcagacggcg atgcaagcca ggagtgaag cccggggccc 600
aaccgccgga ctgctcttgg aactgcgtgg ctggagtgcg ggaggggcag gcggaattcc 660
tggtgtagcg gtgaaatgcg tagatatcag aggaacacgc gtggcgaaag cggcctgctg 720
gactgcaact gacgttgagg ccgaaagcg gtgggagcaa acaggattag ataccctggt 780
agtccacgcc gtaaacgatg attactaggt gtcggggagc agagactgcc cgggtgccga 840
gcccacgcga ttaagtatcc acctggggag tacgttcgca agaataaac tcaaaggaat 900
tgacggggac ccgcacaagc ggtggagcat gtggtttaat tcgaagcaac gcgaagaacc 960
ttaccaggcc ttgacatccc cctggatggc ccgtaacggg gccagccctt tttgggcagg 1020
ggagacaggt ggtgcatggt tgtcgtcagc tcgtgctgct agatggtggg ttaagtcccg 1080
caacgagcgc aacccttgcc cgcagtagcc agcattttag atggggactc tgcggggact 1140
gccggggaca acccgaggga agcggggat gacgtcaaat catcatgcc cttatggcct 1200
gggctacaca cgtgctacaa tggcgccgac agagggagga gaagcgcgca cgcggagcga 1260
acccccaaaa cggcgctcca gttcggattg tagtctgcaa cccgactaca tgaagccgga 1320
atcgctagta atcgcgatc agaatgccgc ggtgaatacg ttcccgggtc ttgtacacac 1380
cgcccgctac accatgggag ccgggaatgc ccgaagtctg tgaccgaacc cgtaagggga 1440
ggggcagccg aaggcaggcc cggtgactgg ggtgaagtcg taacaaggta gccgt 1495

```

<210> SEQ ID NO 54

<211> LENGTH: 1493

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1493)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 34

<400> SEQUENCE: 54

```

agagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac 60
ggagaccccc tgaaggagtt ttccgacaac ggatgggaat gcttagtgtc ggactggtga 120
gtaacgcgtg aggaacctgc cttccagagg gggacaacag ttggaaacga ctgctaatac 180
cgcatgatgc gttggagccg catgactccg acgtcaaaga ttatcgtcg gaagatggcc 240
tcgcgtctga tttgttagtt ggtgaggtaa cggcccacca aggcgacgat cagtagccgg 300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca 360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaaggaa 420
aaggctttcg ggttgtaaac ttcttttaag ggggaagagc agaagacggt accccttgaa 480
taagccacgg ctaactacgt gccagcagcc cgggtaatac gtaggtggca agcgttgtcc 540
ggatttactg ggtgtaaagg gcgtgcagcc ggagagacaa gtcagatgtg aaatccacgg 600
gctcaacccg tgaactgcat ttgaaactgt ttcccttgag tgcggagag gtaatcgaa 660
ttccttggtg agcggtgaaa tgcgtagata taaggaagaa caccagtggc gaaggcggat 720
tactggacga taactgacgg tgaggcgcga aagcgtgggg agcaaacagg attagatacc 780
ctggtagtcc acgctgtaaa cgatcgatac taggtgtgcg gggactgacc ccctgcgtgc 840

```


-continued

cggagttaac acaataagta tcgcacctgg ggagtacgat cgcaaggttg aaactcaaag	900
gaattgacgg gggcccgcac aagcgggtgga ttatgtggtt taattcgaag caacgcgaag	960
aaccttacca gggcttgaca tctgctaac gaagtagaga tacattaggt gcccttcggg	1020
gaaagcagag acaggtgggtg catggtgtgc gtcagctcgt gtcgtgagat gttgggttaa	1080
gtcccgcaac gagcgcaacc cctattgtta gttgctacgc aagagcactc tagcgagact	1140
gccgttgaca aaacggagga aggcggggac gacgtcaaat catcatgccc cttatgtcct	1200
gggctacaca cgtaatacaa tggcgggttaa caaagggatg caaagccgcg aggcagagcg	1260
aaccccaaaa agccgtccca gttcgggatcg caggctgcaa cccgcctgcg tgaagtcgga	1320
atcgtagta atcgcggtac agcatgccgc ggtgaatacg ttccggggcc ttgtacacac	1380
cgcccgctac accatgagag tcgggaacac ccgaagtcgg tagcctaacc gcaaggaggg	1440
cgcggccgaa ggtgggttcg ataattgggg tgaagtcgta acaaggtagc cgt	1493

<210> SEQ ID NO 55

<211> LENGTH: 1498

<212> TYPE: DNA

<213> ORGANISM: Clostridium coccoides

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1)..(1498)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 35

<400> SEQUENCE: 55

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac	60
gggtgtacag aagggaagat tacggtcgga aggtctgtgc atgagtggcg gacgggtgag	120
taacgcgtgg gcaacctggc ctgtacaggg ggataaact tagaaatagg tgctaatacc	180
gcataacggg ggaagccgca tggcctttcc ctgaaaactc cgggtgtaca ggatggggcc	240
gcgtctgatt attttttttg tcagggtaac ggcctaccaa agcgacgac agtagccggc	300
ctgagagggc ggacggccac actgggactg agacacggcc cagactccta cgggaggcag	360
cagtgaggga tattgcacaa tggggggaac cctgatgcag cgacgccgcg tgggtgaaga	420
agcgctcgg cgcgtaaagc cctgtcagca gggaagaaaa tgacgggtacc tgaagaagaa	480
gccccggcta actacgtgcc agcagccgcg gtaatacgta aggggcaagc gttatccgga	540
tttactgggt gtaaaagggg cgcagacggc gatgcaagcc aggagtgaag gcccggggcc	600
caaccccggg actgctcttg ggaactgcgg tggctggagt gcaggagggg caggccggaa	660
ttcctggtgt agcggtgaaa tgcgtagata tcaggaggaa caccggtggc gaaggcggcc	720
tgctggactg caactgacgt tgaggcccg aagcgtgggg agcaaacagg attagatacc	780
ctggtagtca cgcgtaaac gatgattact aggtgtcggg gagcagagac tgcccggtgc	840
cgcagccaac gcattaagta atccacctgg ggagtacgtt cgcaagaatg aaactcaaag	900
gaattgacgg ggacccgcac aagcgggtgga gcatgtggtt taattcgaag caacgcgaag	960
aaccttacca ggccttgaca tccccctgga tggcccgtaa cgggggtcagc ctttcggggc	1020
aggggagaca ggtgggtgcat ggttgtcgtc agctcgtgtc gtgagatgtt gggttaagtc	1080
ccgcaacgag cgcaaccctt gcccgagta gccagcattt tagatgggga ctctgcgggg	1140
actgccgggg acaaccggga ggaaggcggg gatgacgtca aatcatcatg ccccttatgg	1200
cctgggctac acacgtgcta caatggcgcc gacagagggg ggcaagcgg cgacgcggag	1260
cgaaccccaa aaacggcgtc ccagttcgga ttgtagtctg caaccgcact acatgaagcc	1320

-continued

```

ggaatcgcta gtaatcgcgg atcagaatgc cgcggtgaat acgttcccgg gtcttgtaca 1380
caccccccgt cacaccatgg gagccgggaa tgcccgaagt ctgtgaccga acccgtaagg 1440
ggaggggcag ccgaaggcag gcccggtgac tgggggtgaag tcgtaacaag gtagccgt 1498

```

```

<210> SEQ ID NO 56
<211> LENGTH: 1491
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1491)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 36

```

```

<400> SEQUENCE: 56

```

```

agagtttgat catggctcag gacgaacgct ggcggcaagc ttaacacatg caagtcgaac 60
ggagcgccta tgaaggagat ttccgtcaac ggaataggct gcttagtggc tgacgggtga 120
gtaacgcgtg aggaacctgc ctttcagagg gggacaacag ttggaaacga ctgctaatac 180
cgcataacac ataggtgtcg catggcattt atgtcaaaga tttatcgctg aaagatggcc 240
tcgcgtctga ttagctagtt ggtgaggtaa cggctcacca aggcgacgat cagtagccgg 300
actgagaggt tagccggcca cattgggact gagatacggc ccagactcct acgggaggca 360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaagggaag 420
aaggctttcg ggttgtaaac ttcttttaag agggaagagc agaagacggg acctcttgaa 480
taagccacgg ctaactacgt gccagcagcc gcggtaatac gtagtggcaa gcgttgtccg 540
gatttactgg gtgtaaaggg cgtgtagccg ggctgacagt cagatgtgaa attccggggc 600
tcaaccccg accctgcatth gaaactgttg gtcttgagta tcggagaggc aggcggaatt 660
cctagtgtag cggtgaaatg cgtagatatt aggaggaaca ccagtggcga aggcggcctg 720
ctggacgaca actgacggtg aggcgcgaaa gcgtggggag caaacaggat tagataccct 780
ggtagtccac gctgtaaacg atggatacta ggtgtgcggg gactgacccc ctgcgtgccg 840
cagttaacac aataagtatc ccacctgggg agtacgatcg caagggtgaa actcaaagga 900
attgacgggg gccccgacaa gcgggtggatt atgtggttta attcgatgca acgcgaagaa 960
ccttaccagg gcttgacatc ctgctaacga ggtagagata cgtcagggtc ccttcgggga 1020
aagcagagac aggtggtgca tggttgctgt cagctcgtgt cgtgagatgt tgggttaagt 1080
cccgaacga gcgcaacctc tattgttagt tgctacgcaa gagcactcta gcgagactgc 1140
cgttgacaaa acggaggaag gtggggacga cgtcaaatac tcatgccccct tatgtcctgg 1200
gctacacacg taatacaatg gcggtaacaa gagggatgca atactgcgaa gtggagcgaa 1260
cccctaaaag ccgtcccagt tcagattgca gtctgcaact cgactgcatg aagtcggaat 1320
cgctagtaat cgcggatcag catgccgcgg tgaatacgtt cccgggcctt gtacacaccg 1380
cccgtcacac catgagagtc gggaacaccc gaagtcgcta gcctaaccgc aaggaggggc 1440
cggccgaagg tgggttcgat aattgggggtg aagtcgtaac aaggtagccg t 1491

```

```

<210> SEQ ID NO 57
<211> LENGTH: 1493
<212> TYPE: DNA
<213> ORGANISM: Clostridium coccoides
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1493)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 37

```

-continued

<400> SEQUENCE: 57

```

agagtttgat cctggctcag gatgaacgct ggcggcgtgc ctaacacatg caagtcgaac    60
gggtgtacgg ggaggaaggg ttccggccgga aaacctgtgc atgagtggcg gacgggtgag    120
taacgcgtgg gcaacctggc ctgtacaggg ggataaact tagaaatagg tgctaatacc    180
gcataacggg ggaagccgca tggcttttcc ctgaaaactc cgggtgtaca ggatgggccc    240
gcgtctgatt agccagttag cagggtaacg gcctacaaa gcgacgatca gtagccggcc    300
tgagaggggc gacggccaca ctgggactga gacacggccc agactcctac gggaggcagc    360
agtgggggat attgcacaat ggggggaaac cctgatgcag cgacgccgcg tgagtgaaga    420
agtatttcgg tatgtaaagc tctatcagca gggaagaaaa tgacggtacc tgactaagaa    480
gccccggcta actacgtgcc agcagccgcg gtaatacgtg gggggcaagc gttatccgga    540
tttactgggt gtaaaggagg cgtagacggc agcgcaagtc tgaagtgaaa tcccatggct    600
taaccatgga actgctttgg aaactgtgca gctggagtcg aggagaggta agcgggaattc    660
ctagtgtagc ggtgaaatgc gtagatatta ggaggaacac cagtggcgaa ggcggcttac    720
tggaactgtc tgacgttgag gctcgaaagc gtggggagca aacaggatta gataccctgg    780
tagtccacgc cgtaaacgat gattactagg tgttggggga ccaaggtctt cggtgccggc    840
gcaaacgcgt taagtaatcc acctggggag tacgttcgca agaataaac tcaaaggaat    900
tgacggggac ccgcacaagc ggtggagcat gtggtttaat tcgaagcaac gcgaagaacc    960
ttacctggtc ttgacatccc gatgacgagt gagcaaagtc actttccctt cggggcattg   1020
gagacagggt gtgcgtggtt gtcgtcagct cgtgtcgtga gatgttgggt taagtcgccg   1080
aacgagcgca acccctatct ccagtagcca gcaggtagag ctgggcactc tggagagact   1140
gccccgggata accgggagga aggcggggat gacgtcaaat catcatgccc cttatgatca   1200
gggctacaca cgtgctacaa tggcgtaaac aaagggaagc gagacggtga cgttgagcaa   1260
atccccaaaa taacgtccca gttcggattg tagtctgcaa ctcgactaca tgaagctgga   1320
atcgctagta atcgcaatc agaatgtcgc ggtgaatacg ttcccgggtc ttgtacacac   1380
cgcccgctac accatgggag tcggaaatgc ccgaagtcag tgacctaac gaaaggaagg   1440
agctgccgaa ggtggagccg gtaactgggg tgaagtcgta acaaggtagc cgt          1493

```

<210> SEQ ID NO 58

<211> LENGTH: 1493

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1493)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 38

<400> SEQUENCE: 58

```

aaagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac    60
ggagcacccc tgaaggagtt ttccgacaac ggatgggaat gcttagtggc ggactggtga    120
gtaacgcgtg aggaacctgc cttccagagg gggacaacag ttggaaacga ctgctaatac    180
cgcatgatgc gttggagccg catgactccg acgtcaaaga tttatcgctg gaagatggcc    240
tcgcgtctga ttagctagtt ggtgaggtaa cggccacaca aggcgacgat cagtagccgg    300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca    360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaaggaag    420

```

-continued

aaggctttcg ggttgtaaac ttcttttaag ggggaagagc agaagacggt accccttgaa	480
taagccacgg ctaactacgt gccagcagcc gcggtataac gtagtggaac gcgttggtccg	540
gatttactgg gtgtaaaggc cgtgcagccg gagagacaag tcagattgtga aatccacggg	600
ctcaaccctg gaactgcatt tgaactgtt tcccttgagt gtcggagagg taatcggaat	660
tccttggtga gcggtgaaat gcgtagatat aaggaagaac accagtggcg aaggcggatt	720
actggacgat aaactgacgg tgaggcgcca aagcgtggcg agcaaacagg attagatacc	780
ctggtagtcc acgctgtaaa cgatcgatac taggtgtgcg gggactgacc cctgcgtgc	840
cggagttaac acaataagta tcgcacctgg ggagtacgat cgcaagggtg aaactcaaag	900
gaattgacgg gggcccgcac aagcgtgga ttatgtggtt taattcgaag caacgcgaag	960
aaccttacca gggcttgaca tcctgctaac gaagtagaga tacattaggt gcccttcggg	1020
gaaagtagag acaggtggcg catggtgtgc gtcagctcgt gtcgtgagat gttgggttaa	1080
gtcccgaac gagcgcaacc cctattgtta gttgctacgc aagagcactc tagcgagact	1140
gccgttgaca aaacggagga agcgggggac gacgtcaaat catcatgccc cttatgtcct	1200
gggctacaca cgtaatacaa tggcgggtta caaagggatg caaagccgcg aggcagagcg	1260
aacccccaaa agccgtccca gttcggatcg caggctgcaa cccgcctgcg tgaagtcgga	1320
atcgctagta atcgcggtac agcatgccgc ggtgaatacg ttccggggcc ttgtacacac	1380
cgcccgtcac accatgagag tcgggaacac ccgaagtcgg tagcctaacc gcaaggaggg	1440
cgcgccgcaa ggtgggttcg ataattgggg tgaagtcgta acaaggtagc cgt	1493

<210> SEQ ID NO 59

<211> LENGTH: 1511

<212> TYPE: DNA

<213> ORGANISM: Clostridium leptum

<220> FEATURE:

<221> NAME/KEY: rRNA

<222> LOCATION: (1) .. (1511)

<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium strain 39

<400> SEQUENCE: 59

agagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac	60
ggagcaccoc tgaaggagtt ttccgacaac ggatgggaat gcttagtggc ggactggtga	120
gtaacgcgtg aggaacctgc cttccagagg gggacaacag ttggaacga ctgctaatac	180
cgcattgatg gttggagccg catgactccg acgtcaaaga tttatcgctg gaagatggcc	240
tcgcgtctga ttacttagtt ggtgaggtta cggcccacca aggcgacgat cagtagccgg	300
actgagaggt tggccggcca cattgggact gagatacggc ccagactcct acgggaggca	360
gcagtgggga atattgggca atggacgcaa gtctgaccca gcaacgccgc gtgaaggaa	420
aaggctttcg ggttgtaaac ttcttttaag ggggaagagc agaagacggt accccttgaa	480
taagccacgg ctaactacgt gccagcagcc gcggtataac gtagtggaac agcgttggtcc	540
ggatttactg ggtgtaaagg gcgtgcagcc ggagagacaa gtcagatgtg aaatccacgg	600
gtcaaccocg tgaactgcat ttgaaactgt ttcccttgag tgtcggagag gtaatcgga	660
ttccttggtg agcgggtgaaa tcgctagata taaggaagac accagtggcg aagcggatta	720
ctggacgata actgacgggt aggcgcgaaa gcgtggggag caaacaggat tagatacctg	780
ggtagtcaac gctgtaaacg atcgatacta ggtggtgcgg gggacttgac cccctgccgt	840
tgccggagtt aacaccaata aagtattcgg caccctgggg agtacgatcg caaaggttga	900

-continued

```

aaactcaaaa gaaatggacg gggggcccg cccaagcgg gtgggattat gttggtttat   960
ttcgaaagca acgcgaagaa ccctaacagg gcttgacatc ctgctaacga agtagagata  1020
cattaggtgc ccttcgggga aagtagagac aggtggtgca tggttgtcgt cagctcgtgt  1080
cgtgagatgt tgggttaagt ccgcgaacga gcgcaacccc tattgttagt tgctacgcaa  1140
gagcactcta gcgagactgc cgttgacaaa acggaggaag gcggggacga cgtcaaatca  1200
tcatgccctt tatgtcctgg gctacacacg taatacaatg gcggttaaca aagggatgca  1260
aagccgcgag gcagagcgaa ccccaaaaag ccgtccagc tgggatcgca ggctgcaacc  1320
cgctcgctg aagtgcgaat cgctagtaat cgcgatcag catgccgcgg tgaatacgtt  1380
cccgggcctt gtacacaccg ccgcgcacac catgagatgc gggaacccc gaagtcgta  1440
gcctaaccgc aaggaggcgg cgccgaagg tgggttcgat aattggggtg aagtcgtaac  1500
aaggtagccg t                                     1511

```

```

<210> SEQ ID NO 60
<211> LENGTH: 1499
<212> TYPE: DNA
<213> ORGANISM: Clostridium leptum
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1) .. (1499)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 40

```

```

<400> SEQUENCE: 60
agagtttgat cctggctcag gataaacgct ggcggcatgc ctaacacatg caagtcgaac   60
ggagcgcctt ggaaggagac ttcggtcaac ggaagaggag gcttagtggc ggacgggtga  120
gtaacgcgtg aggaacctgc ctacagagag gggataacac accgaaaggt gtgctaatac  180
cgcataacat atgagagggg catccctttc atatcaaaga tttattgctt tgagatggcc  240
tcgcgtccaa ttactagatt ggtgaggtaa cggcccacca aggcgacgat tggtagccgg  300
actgagaggt tgaacggcca cattgggact gagacacggc ccagactcct acgggaggca  360
gcagtgggga atattgcaca atggggggaa ccctgatgca gcaatgccgc gtgaaggatg  420
aaggttttcg gattgtaaac ttcttttgta cgggacgaag aaagtgcagg taccgtaaga  480
ataagccacg gctaactacg tgccagcagc cgcgtaata cgtaggtggc aagcgttatc  540
cggatttact ggggtgtaaag ggcgagtagg cgggattgca agtcagatgt gaaaactatg  600
ggctcaaccg atagagtgca ttgaaactg cagttcttga gtgatggaga ggacggcgga  660
attcccgttg tagcgggtga atgcgtagat atcgggaggg aacaccagtg gcgaaggcgg  720
cctgctggac attaactgac gctgatgcgc gaaagcgtgg ggagcaaaca ggattagata  780
ccctggtagt cacgctgtaa acgatgatta ctaggtgtgg ggggtactga ccccttccc  840
gtgccggagt taacacaata agtaatccac ctggggagta cggccgcaag gttgaaactc  900
aaaggaattg acggggggcc gcacaagcag tggagtatgt ggttttaatt cgaagcaacg  960
cgaagaacct taccagggct tgacatgggg atgaccgctt tagagataga gctttctctt  1020
cggagacatc ccacacaggt ggtgcatggt tgtcgtcagc tcgtgtcgtg agatgttggg  1080
ttaagtcccg caacgagcgc aacccttatt gttagtgtct acgcaagagc actctagcga  1140
gactgccgtt gacaaaacgg aggaagggtg ggacgacgct aaatcatcat gccctttatg  1200
tcctgggcta cacacgtact acaatggcgg acatacagag ggaagcaaga cagcgatgtg  1260
gagcaaatcc ctaaaagcgg tctcagttca gattgcaggc tgcaaccgcc ctgcataag  1320

```

-continued

```
tcggaattgc tagtaatcgc ggatcagcat gccgcggtga atacgttccc gggccttgta 1380
cacaccgccc gtcacaccat gagagtcgga aacacccgaa gcctgtagcc caaccgcaag 1440
gggggcgccag tcgaaggtgg gtctgataat tgggggtgaag tcgtaacaaa ggtagccgt 1499
```

```
<210> SEQ ID NO 61
<211> LENGTH: 1512
<212> TYPE: DNA
<213> ORGANISM: Clostridium coccooides
<220> FEATURE:
<221> NAME/KEY: rRNA
<222> LOCATION: (1)..(1512)
<223> OTHER INFORMATION: 16S rRNA coding gene sequence of Clostridium
strain 41
```

```
<400> SEQUENCE: 61
```

```
agagtttgat cctggctcag gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac 60
ggagatatca ttttcgaagc gattagttaa ctaagagcgg agatgttgct atcttagtgg 120
cggacgggtg agtaacgcgt gggtaacctg ccttgcaactg ggggataaca cttagaaata 180
ggtgctaata ccgcataaca gtaggagacg catgtctttt acttgaaaac tccggtggtg 240
taagatggac ccgcgtctga ttagcttggt ggcggggtaa cggcccacca aggcaacgat 300
cagtagccgg cctgagaggg tgaacggcca cattgggact gagacacggc ccaaactcct 360
acgggaggca gcagtgggga atattggaca atggggggaa ccctgatcca gcgacgccgc 420
gtgagtgaag aagtatttcg gtatgtaaag ctctatcagc agggaagaaa gaaatgacgg 480
tacctgacta agaagccccg gctaactacg tgccagcagc cgcggttaata cgtagggggc 540
aagcgttata cggatttact ggggtgtaaag ggagcgtaga cggcgatgca agtctgaagt 600
gaaaggcggg gggcccaacc ccggactgct ttggaaactg tatggctgga gtgcaggaga 660
ggtaagtgga attcctagtg tagcgtgtaa atgcgtagat attaggagga acaccagtgg 720
cgaaagcggc ttactggact gtaactgacg ttgaggctcg aaagcgtggg gagcaaacaa 780
gattagatac ctggtagtca cgccgtaaac gatgatcacc ggtttcgggt ggttatggac 840
ccatcggttg cgcagcaaac gcagtagtga tccacctggg gagtaacgtt cgcaagaatg 900
aaacttcaaa ggaaatgacg ggggaccccg cacaagcggg ggaggcatgt gtttaattcg 960
aagcaacgcg aagaacctta cccaagtctt gacatccgtg gacgagttag taacgtcact 1020
ttcccttcgg ggcagcggag acaggtggtg catggttgct gtcagctcgt gtcgtgagat 1080
gttggggtta gtcccgcaac gagcgcaacc cctatcctta gtaccagcg agttaggctc 1140
ggcactctag ggagactgcc ggggacaacc cggaggaagg tggggatgac gtcaaatcat 1200
catgcccctt atgatttggg ctacacacgt gctacaatgg cgtaaacaaa gggaagcgag 1260
cctgtgaagg taagcgaatc ccagaaataa cgtctcagtt cggattgtag tctgcaactc 1320
gactacatga agctggaatc gctagtaatc gcggatcaga atgccgcggt gaatacgttc 1380
ccgggtcttg tacacaccgc ccgtcacacc atgggagtcg gaaatgcccg aagtctgtga 1440
cccaacctga gaaggaggga gcagccgaag gcaggtcggg tgactggggg gaagtcgtaa 1500
caaggtagcc gt 1512
```

```
<210> SEQ ID NO 62
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence
```

-continued

<400> SEQUENCE: 62
ggtgaatacgt ttcccg 17

<210> SEQ ID NO 63
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 63
tacggctacc ttgttacgac tt 22

<210> SEQ ID NO 64
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 64
aatgacggt acctgactaa 20

<210> SEQ ID NO 65
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 65
ctttgagttt cattcttgcg aa 22

<210> SEQ ID NO 66
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 66
gcacaagcag tggagt 16

<210> SEQ ID NO 67
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 67
cttcctccgt tttgtcaa 18

<210> SEQ ID NO 68
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 68
gagaggaagg tccccac 18

<210> SEQ ID NO 69

-continued

<211> LENGTH: 19
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Artificially synthesized primer sequence

<400> SEQUENCE: 69

cgctacttgg ctggttcag

19

The invention claimed is:

1. A pharmaceutical composition, comprising a purified bacterial mixture of at least two live bacterial strains belonging to *Clostridium* clusters IV and/or XIVa, wherein the bacterial mixture induces proliferation and/or accumulation of regulatory T cells, wherein the bacterial strains are isolated from a human, wherein the pharmaceutical composition is formulated for delivery to the intestine, and wherein the pharmaceutical composition does not include *Bacteroides*.

2. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition does not include *Bifidobacterium*.

3. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition does not include *Lactobacillus*.

4. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition does not include gram-negative bacteria.

5. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition is formulated for oral administration.

6. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition further comprises a pharmacologically acceptable excipient.

7. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition further comprises a pH sensitive composition comprising one or more enteric polymers.

8. A method of treating a human subject having an infectious disease, an autoimmune disease or an allergic disease, the method comprising administering the pharmaceutical composition of claim 1.

9. The method of claim 8, wherein the human subject has an autoimmune disease.

10. The method of claim 9, wherein the autoimmune disease is organ transplant rejection, inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, sprue, rheumatoid arthritis, Type 1 diabetes, graft versus host disease, or multiple sclerosis.

11. The method of claim 8, wherein the subject has an infectious disease.

12. The method of claim 11, wherein the infectious disease is *Clostridium difficile* infection.

13. A pharmaceutical composition, comprising a purified bacterial mixture of at least two live bacterial strains belonging to *Clostridium* clusters IV and/or XIVa, wherein the bacterial mixture induces proliferation and/or accumulation of regulatory T cells, wherein the bacterial cells are isolated from a human, wherein the pharmaceutical composition is formulated for delivery to the intestine, and wherein the pharmaceutical composition does not include *Lactobacillus*.

14. The pharmaceutical composition of claim 13, wherein the pharmaceutical composition does not include *Bifidobacterium*.

15. The pharmaceutical composition of claim 13, wherein the pharmaceutical composition is formulated for oral administration.

16. The pharmaceutical composition of claim 13, wherein the pharmaceutical composition further comprises a pharmacologically acceptable carrier.

17. The pharmaceutical composition of claim 13, wherein the pharmaceutical composition further comprises a pH sensitive composition comprising one or more enteric polymers.

18. A method of treating a human subject having an infectious disease, an autoimmune disease or an allergic disease, the method comprising administering the pharmaceutical composition of claim 13.

19. The method of claim 18, wherein the human subject has an autoimmune disease.

20. The method of claim 19, wherein the autoimmune disease is organ transplant rejection, inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, sprue, rheumatoid arthritis, Type 1 diabetes, graft versus host disease, or multiple sclerosis.

21. The method of claim 18, wherein the subject has an infectious disease.

22. The method of claim 21, wherein the infectious disease is *Clostridium difficile* infection.

23. A pharmaceutical composition, comprising a purified bacterial mixture of at least two live bacterial strains belonging to *Clostridium* clusters IV and/or XIVa, wherein the bacterial mixture induces proliferation and/or accumulation of regulatory T cells, wherein the bacterial cells are isolated from a human, wherein the pharmaceutical composition is formulated for delivery to the intestine, and wherein the pharmaceutical composition does not include *Bifidobacterium*.

24. The pharmaceutical composition of claim 23, wherein the pharmaceutical composition is formulated for oral administration.

25. The pharmaceutical composition of claim 23, wherein the pharmaceutical composition further comprises a pharmacologically acceptable carrier.

26. The pharmaceutical composition of claim 23, wherein the pharmaceutical composition further comprises a pH sensitive composition comprising one or more enteric polymers.

27. A method of treating a human subject having an infectious disease, an autoimmune disease or an allergic disease, the method comprising administering the pharmaceutical composition of claim 23.

28. The method of claim 27, wherein the human subject has an autoimmune disease.

29. The method of claim 28, wherein the autoimmune disease is organ transplant rejection, inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, sprue, rheumatoid arthritis, Type 1 diabetes, graft versus host disease, or multiple sclerosis.

119

30. The method of claim **27**, wherein the subject has an infectious disease, and wherein the infectious disease is *Clostridium difficile* infection.

* * * * *

120