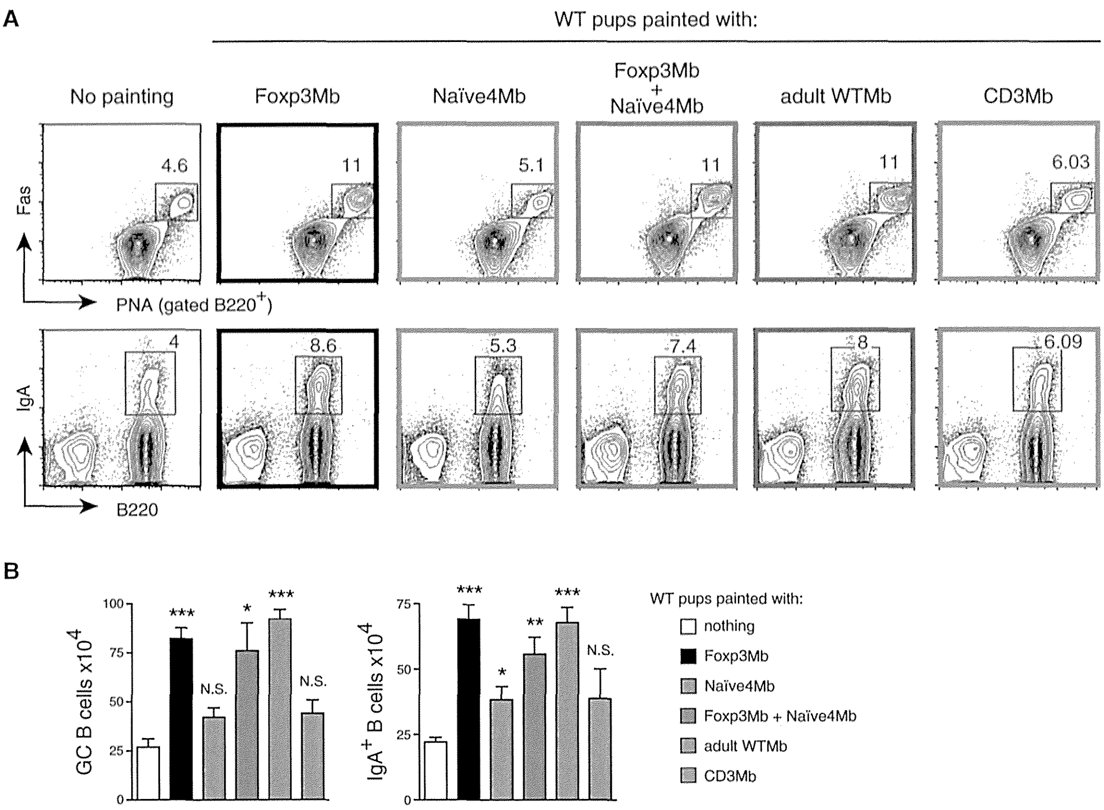

Figure 7. Dominant Effect of Foxp3-Regulated Microbiota

(A) Representative flow cytometric profiles of cells from PPs of WT pups "painted" with fecal extracts from adult WT mice (adult WTMb), nontransferred *Cd3e*$^{-/-}$ mice (CD3Mb), or the *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells (Naive4Mb) or Foxp3$^+$ T cells (Foxp3Mb). Fecal extracts from two to four mice per group were pooled 10–12 weeks after T cell transfer, and host mice were analyzed 2 weeks after the painting. Numbers indicate the frequency of cells in the gates. Data represent one of the three experiments with consistent results. At least four mice per group were analyzed.

(B) Total numbers of GC B cells and IgA$^+$ B cells from the PPs of indicated mice. Mean ± SEM for three to five mice per group. Two-tailed unpaired Student's t test was used to compare between nonpainted and the indicated mouse groups; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; N.S., no significant difference.

B cells switching not only to IgA but also to IgG1, and possibly to IgE). These observations suggest that the immune system recognizes complex and balanced microbial communities as "gut Mb signature" and respond by adaptations that foster the maintenance of such complex bacterial structures. An increased diversity probably enhances the stability of Mb potentiating its metabolic capacity, in parallel with exerting a constant yet controllable pressure for diversification and fitness of the immune system. In contrast, poor and skewed bacterial communities might be recognized as having pathogenic treats and as such elicit systemic type of responses that in certain conditions could lead to autoimmune diseases or allergies. Thus, the acquisition of Foxp3, IgA, and its secretory mechanisms, and the development of complex GALT structures that facilitate coordinated and controlled immune-receptor diversification seem adaptations that allowed vertebrates to establish symbiotic relationships with Mb and to become an evolutionary success.

The results presented here should be useful when considering strategies to reestablish symbiosis in intestinal pathologies caused by various immunodeficiencies and associated with gut inflammation.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 germ-free (GF) mice were initially purchased from Sankyo Laboratories Japan and were bred and maintained in vinyl isolators in the animal facility at IMS-RCAI, RIKEN Yokohama. 5-week-old GF mice were used for microbiota transplantation experiments. Other mice, like wild-type (WT), *Cd3e*$^{-/-}$ (Malissen et al., 1995), *Ighm*$^{-/-}$, *Rag1*$^{-/-}$, *Foxp3*^{EGFP} (Ly5.1 or 5.2) (Wang et al., 2008), *Bcl6*$^{yfp/yfp}$ (Kitano et al., 2011), and *Aicda*$^{-/-}$, were on a C57BL/6 background, bred and maintained in SPF facility at IMS-RCAI. All animal experiments were performed in accordance with approved protocols from the Institutional Animal Care at RIKEN. Littermate information of immunodeficient mice is described in Supplemental Experimental Procedures.

IgA$^+$ Cell Sorting, IgA Heavy Chain Gene Sequencing, and Mutational Analyses

Single B220$^-$IgA$^+$ plasma cells from lamina propria of small intestine (SILP) were sorted into 96-well PCR plates containing 10 μl of 50 μg/ml yeast tRNA as carrier, using FACS Aria cell sorter (Becton Dickinson). The method for IgA heavy chain gene sequence analyses was previously described (Hershberg et al., 2008; Kawamoto et al., 2012).

Histological Analysis

For immunohistochemical analysis, small intestine samples were fixed and stained as previously described (Kawamoto et al., 2012). Before Foxp3 staining, formaldehyde-fixed sections were treated with HistoVT One (Nacalai Tesque) at 70°C for 20 min for antigen retrieval. For hematoxylin-eosin (HE) staining, large intestine samples were fixed and stained with HE (Muto Pure Chemicals) according to the manufacturer's protocol. The stained slides were examined with a Zeiss Axioplan 2 fluorescence microscope.

Assessment of Intestinal Inflammation

Mice were sacrificed usually around 10–12 weeks after the transfer of T cells. Distal colons were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin. The degree of intestinal inflammation was graded from 0 to 3 for the four following criteria: degree of epithelial hyperplasia and goblet cell depletion; leukocyte infiltration in the lamina propria; area of tissue affected; and the presence of markers of severe inflammation such as crypt abscesses and submucosal inflammation (Izcue et al., 2008). Scores for each of the criteria were added to give a total score of 0 to 12 for each sample. The total colonic score was calculated as the average of the individual scores from several sections per mouse.

Fecal Suspension for Microbiota Reconstitution

Fecal pellets were collected from C57BL/6 or from T-cell-transferred *Cd3e*$^{-/-}$ mice and suspended with sterile PBS (5–7 feces/3 ml PBS). Bacteria number in fecal suspension was counted by Flow-Check Fluorospheres (Beckman Coulter) with FACS CantoII (Becton Dickinson) and adjusted to same number in each sample. The spore fraction was prepared as described previously (Atarashi et al., 2013).

Germ-free WT Mice Experiments

Germ-free C57BL/6 mice were transferred into autoclaved sterile cages and gavaged with 100 μl of fecal suspension from the *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells, naive CD4$^+$ T cells, or nontransferred *Cd3e*$^{-/-}$ mice. At 2 weeks after gavage, mice were analyzed and cecum contents were collected for bacterial DNA sequencing.

Germ-free *Cd3e*$^{-/-}$ Mice Experiments

Germ-free *Cd3e*$^{-/-}$ mice were gavaged with fecal suspension from the *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells or naive CD4$^+$ T cells. After 1 week, 2×10^5 CD4$^+$GFP$^+$ (Foxp3$^+$) T cells sorted from spleen and LNs of Foxp3^{EGFP} mice were injected intravenously. The recipient mice were analyzed 2 weeks after the Foxp3$^+$ T cell transfer.

Fur-Painting of SPF Mice

For painting experiments, 3-week-old female C57BL/6 mice purchased from CLEA Japan had fecal suspension from different mice painted on their fur. Mice were analyzed 2 weeks after the painting.

Evaluation of IgA-Coated Bacteria by Flow Cytometry

Flow cytometric analysis of bacteria was performed as described (van der Waaij et al., 1996) with some modifications. Detailed procedure is described in Supplemental Experimental Procedures.

Sorting of IgA-Coated Fecal Bacteria

Fecal bacteria were stained as described above and purified on a FACSAria (Becton Dickinson) cell sorter as IgA^{neg} (IgA$^-$Igκ$^-$), IgA^{int} (IgA^{int}Igκ^{int}), or IgA^{hi} (IgA^{hi}Igκ^{hi}) cells. Sorted bacteria were centrifuged at 12,000 × *g* for 10 min and bacterial pellet was stored at −80°C until use. Bacterial genomic DNA was purified with conventional phenol:chloroform extraction followed by ethanol precipitation. 16*S* rRNA genes were amplified and analyzed as described below.

Preparation of DNA and Pyrosequencing

Cecum and stool samples were stored at −80°C until use. DNA was purified with the QIAamp DNA stool mini kit (QIAGEN) according with manufacturer's instructions with a high-temperature incubation option. DNA samples were amplified using V1–V2 region primers targeting bacterial 16*S* rRNA genes with Roche 454 Lib-L pyrosequencing adaptor and barcode sequence as previously described (Kawamoto et al., 2012). PCR products were cleaned by Wizard SV Gel and PCR Clean-up system (Promega) and sequencing was carried out by with a 454 GS Junior pyrosequencer (Roche).

16*S* rRNA Data Processing and Analysis

Sequences were processed and analyzed with QIIME pipeline (Caporaso et al., 2010). Detailed procedure is described in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The bacterial 16S rRNA amplicon sequence data are available in DNA Data Bank of Japan (DDBJ) under the accession number PRJDB2881.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.05.016.

AUTHOR CONTRIBUTIONS

S.K. conducted all mice experiments and analysis. M.M. conducted the bacterial 16S rRNA pyrosequencing, data processing, and analysis. L.M.K. conducted IgA mutational analyses, evaluation, and sorting of IgA-coated fecal bacteria. W.S. and M.H. provided help for 16S rRNA data analysis. K.A. and K.H. provided help for isolation and culture of bacterial strains from mouse cecum. Y.D., Y.T., and H.Q. provided help for mice experiment and Y.D. provided help with IgA heavy chain gene sequencing. T.O. provided *Bcl6*$^{yfp/yfp}$ mice. S.K., M.M., L.M.K., and S.F. interpreted the data and wrote the manuscript. S.F. designed and supervised the study.

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REFERENCES

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J.R., Pfeffer, K., Coffer, P.J., and Rudensky, A.Y. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature *504*, 451–455.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science *331*, 337–341.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature *500*, 232–236.

Baumjohann, D., Preite, S., Reboldi, A., Ronchi, F., Ansel, K.M., Lanzavecchia, A., and Sallusto, F. (2013). Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. Immunity *38*, 596–605.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods *7*, 335–336.

Chung, Y., Tanaka, S., Chu, F., Nurieva, R.I., Martinez, G.J., Rawal, S., Wang, Y.H., Lim, H., Reynolds, J.M., Zhou, X.H., et al. (2011). Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat. Med. *17*, 983–988.

Cong, Y., Feng, T., Fujihashi, K., Schoeb, T.R., and Elson, C.O. (2009). A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc. Natl. Acad. Sci. USA *106*, 19256–19261.

Elson, C.O., and Cong, Y. (2012). Host-microbiota interactions in inflammatory bowel disease. Gut Microbes *3*, 332–344.

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., and Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science *298*, 1424–1427.

Fagarasan, S., Kawamoto, S., Kanagawa, O., and Suzuki, K. (2010). Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu. Rev. Immunol. *28*, 243–273.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature *504*, 446–450.

Geuking, M.B., Cahenzli, J., Lawson, M.A., Ng, D.C., Slack, E., Hapfelmeier, S., McCoy, K.D., and Macpherson, A.J. (2011). Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity *34*, 794–806.

Good-Jacobson, K.L., Szumilas, C.G., Chen, L., Sharpe, A.H., Tomayko, M.M., and Shlomchik, M.J. (2010). PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nat. Immunol. *11*, 535–542.

Hershberg, U., Uduman, M., Shlomchik, M.J., and Kleinstein, S.H. (2008). Improved methods for detecting selection by mutation analysis of Ig V region sequences. Int. Immunol. *20*, 683–694.

Hirota, K., Turner, J.E., Villa, M., Duarte, J.H., Demengeot, J., Steinmetz, O.M., and Stockinger, B. (2013). Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. Nat. Immunol. *14*, 372–379.

Hooper, L.V., Littman, D.R., and Macpherson, A.J. (2012). Interactions between the microbiota and the immune system. Science *336*, 1268–1273.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell *139*, 485–498.

Izcue, A., Coombes, J.L., and Powrie, F. (2006). Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. Immunol. Rev. *212*, 256–271.

Izcue, A., Hue, S., Buonocore, S., Arancibia-Cárcamo, C.V., Ahern, P.P., Iwakura, Y., Maloy, K.J., and Powrie, F. (2008). Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. Immunity *28*, 559–570.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science *325*, 1006–1010.

Josefowicz, S.Z., Niec, R.E., Kim, H.Y., Treuting, P., Chinen, T., Zheng, Y., Umetsu, D.T., and Rudensky, A.Y. (2012). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature *482*, 395–399.

Kamada, N., Seo, S.U., Chen, G.Y., and Núñez, G. (2013). Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. *13*, 321–335.

Karlsson, F.H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C.J., Fagerberg, B., Nielsen, J., and Bäckhed, F. (2013). Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature *498*, 99–103.

Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L., and Gordon, J.I. (2011). Human nutrition, the gut microbiome and the immune system. Nature *474*, 327–336.

Kawamoto, S., Tran, T.H., Maruya, M., Suzuki, K., Doi, Y., Tsutsui, Y., Kato, L.M., and Fagarasan, S. (2012). The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. Science *336*, 485–489.

Kitano, M., Moriyama, S., Ando, Y., Hikida, M., Mori, Y., Kurosaki, T., and Okada, T. (2011). Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. Immunity *34*, 961–972.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Almeida, M., Arumugam, M., Batto, J.M., Kennedy, S., et al.; MetaHIT consortium (2013). Richness of human gut microbiome correlates with metabolic markers. Nature *500*, 541–546.

Linterman, M.A., Pierson, W., Lee, S.K., Kallies, A., Kawamoto, S., Rayner, T.F., Srivastava, M., Divekar, D.P., Beaton, L., Hogan, J.J., et al. (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nat. Med. *17*, 975–982.

Littman, D.R., and Pamer, E.G. (2011). Role of the commensal microbiota in normal and pathogenic host immune responses. Cell Host Microbe *10*, 311–323.

Lochner, M., Peduto, L., Cherrier, M., Sawa, S., Langa, F., Varona, R., Riethmacher, D., Si-Tahar, M., Di Santo, J.P., and Eberl, G. (2008). In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. J. Exp. Med. *205*, 1381–1393.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. EMBO J. *14*, 4641–4653.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., et al. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut *55*, 205–211.

Maynard, C.L., Elson, C.O., Hatton, R.D., and Weaver, C.T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. Nature *489*, 231–241.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., and Pettersson, S. (2012). Host-gut microbiota metabolic interactions. Science *336*, 1262–1267.

Nishikawa, J., Kudo, T., Sakata, S., Benno, Y., and Sugiyama, T. (2009). Diversity of mucosa-associated microbiota in active and inactive ulcerative colitis. Scand. J. Gastroenterol. *44*, 180–186.

Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.H., and Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. Science *325*, 1001–1005.

Ott, S.J., Musfeldt, M., Wenderoth, D.F., Hampe, J., Brant, O., Fölsch, U.R., Timmis, K.N., and Schreiber, S. (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut *53*, 685–693.

Peterson, D.A., McNulty, N.P., Guruge, J.L., and Gordon, J.I. (2007). IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe *2*, 328–339.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature *490*, 55–60.

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly-Y, M., Glickman, J.N., and Garrett, W.S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science *341*, 569–573.

Strugnell, R.A., and Wijburg, O.L. (2010). The role of secretory antibodies in infection immunity. Nat. Rev. Microbiol. *8*, 656–667.

Sutherland, D.B., and Fagarasan, S. (2012). IgA synthesis: a form of functional immune adaptation extending beyond gut. Curr. Opin. Immunol. *24*, 261–268.

Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T., and Fagarasan, S. (2004). Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc. Natl. Acad. Sci. USA *101*, 1981–1986.

Takahashi, H., Kanno, T., Nakayamada, S., Hirahara, K., Sciumè, G., Muljo, S.A., Kuchen, S., Casellas, R., Wei, L., Kanno, Y., and O'Shea, J.J. (2012). TGF-β and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells. Nat. Immunol. *13*, 587–595.

Tsuji, M., Komatsu, N., Kawamoto, S., Suzuki, K., Kanagawa, O., Honjo, T., Hori, S., and Fagarasan, S. (2009). Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science *323*, 1488–1492.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature *444*, 1027–1031.

van der Waaij, L.A., Limburg, P.C., Mesander, G., and van der Waaij, D. (1996). In vivo IgA coating of anaerobic bacteria in human faeces. Gut *38*, 348–354.

van der Waaij, L.A., Kroese, F.G., Visser, A., Nelis, G.F., Westerveld, B.D., Jansen, P.L., and Hunter, J.O. (2004). Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. Eur. J. Gastroenterol. Hepatol. *16*, 669–674.

Vinuesa, C.G., Fagarasan, S., and Dong, C. (2013). New territory for T follicular helper cells. Immunity *39*, 417–420.

Wang, Y., Kissenpfennig, A., Mingueneau, M., Richelme, S., Perrin, P., Chevrier, S., Genton, C., Lucas, B., DiSanto, J.P., Acha-Orbea, H., et al. (2008). Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells. J. Immunol. *180*, 1565–1575.

Wei, M., Shinkura, R., Doi, Y., Maruya, M., Fagarasan, S., and Honjo, T. (2011). Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. Nat. Immunol. *12*, 264–270.

Wollenberg, I., Agua-Doce, A., Hernández, A., Almeida, C., Oliveira, V.G., Faro, J., and Graca, L. (2011). Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J. Immunol. *187*, 4553–4560.

Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., et al. (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity *31*, 457–468.

Zhou, L., Lopes, J.E., Chong, M.M., Ivanov, I.I., Min, R., Victora, G.D., Shen, Y., Du, J., Rubtsov, Y.P., Rudensky, A.Y., et al. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature *453*, 236–240.

ARTICLE ADDENDUM

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Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia

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Keywords: Clostridia, Treg, gut microbiota

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The gut microbiota plays important roles in the development of the host immune system. We have previously shown that a combination of 46 strains of commensal Clostridia isolated from conventionally reared mice can induce the accumulation of CD4$^+$Foxp3$^+$ regulatory T (Treg) cells in the mouse colonic lamina propria. Subsequently, we succeeded in isolating and selecting 17 strains of Clostridia from a healthy human fecal sample that can significantly increase the number and function of colonic Treg cells in colonized rodents, thereby attenuating symptoms of experimental allergic diarrhea and colitis. Here we characterize each of the 17 strains of human-derived Clostridia in terms of sensitivity to antibiotics and ability to produce short chain fatty acids and other metabolites, and discuss their potential as biotherapeutics to correct dysbiosis and treat immune-inflammatory diseases.

Seventeen Strains of Treg-Inducing Clostridia Isolated From the Human Intestine

The intestinal microbiota is composed of ~100 trillion commensal bacteria and has co-evolved with the host by participating in many essential physiologic and metabolic functions. There is also abundant evidence that the gut microbiota affects the host immune status. Immunological effects of the microbiota are not simply due to the presence of innocuous bacteria, but to the biological activities of the gut microbiota consortium.[1] In many cases, regulation of development and/or effector functions of different immune cell populations (such as Treg cells vs Th17 cells) depends on the activity of different members of the commensal community. The relative abundance of these different immunomodulatory members can direct the general nature of host mucosal and systemic immunity. Previously, we referred to such immunomodulatory members of the microbiota as "autobionts".[2] However, currently there are relatively few specific examples of autobionts.

We and other groups have shown that segmented filamentous bacteria can potently induce interleukin-17-producing CD4$^+$ T cells (Th17 cells) in the small intestine of mice.[3] We have also shown that a combination of 46 strains of Clostridia indigenous to conventionally reared mice can induce Treg cells in the mouse colonic lamina propria and thereby contribute to protecting mice against colitis and allergic responses.[4] In the most recent publication by our group, we identified 17 strains of human-derived Clostridia as potent inducers of Treg cells.[5] Starting from a complete healthy human fecal sample, a sequence of selection steps was applied to obtain Treg cell-inducing

Cluster	strain#	Closest species/strain		Similarity (%)	Benzylpenicillin	Ampicillin	Ampicillin /sulbactam	Amoxicillin /clavulanate	Tazobactam /piperacillin	Cefmetazole	Vancomycin	Imipenem	Meropenem	Metronidazole	Clindamycin
Cluster XVIII	St.1	*Clostridium*	*saccharogumia*	99.46											
	St.8	Clostridiaceae	JC13	99.16											
	St.18	*Clostridium*	*ramosum*	100.00											
Cluster IV	St.3	Lachnospiraceae	7_1_58FAA	100.00											
	St.13	*Anaerotruncus*	*colihominis*	100.00											
Cluster XIVa	St.4	*Clostridium*	*hathewayi*	99.06											
	St.6	*Blautia*	*producta*	99.79											
	St.7	*Clostridium*	*bolteae*	99.53											
	St.9	*Clostridium*	*indolis*	99.26											
	St.14	*Ruminococcus*	*sp.* ID8	98.59											
	St.15	*Clostridium*	*asparagiforme*	99.73											
	St.16	*Clostridium*	*sp.* 7_3_54FAA	100.00											
	St.21	*Eubacterium*	*contortum*	99.58											
	St.26	*Clostridium*	*scindens*	99.72											
	St.27	Lachnospiraceae	3_1_57FAA_CT1	97.54											
	St.28	Clostridiales	1_7_47FAA	99.73											
	St.29	Lachnospiraceae	3_1_57FAA_CT1	99.60											
CLSI break points (µg/ml)				Susceptible	0.25	≤0.25	≤4/8	≤4/2	≤4/32	≤16		≤4	≤4	≤8	≤2
				Intermediate	1	1	8/16	8/4	4/64	32		8	8	16	4
				Resistant	≥2	≥2	≥16/32	≥16/8	≥4/128	≥64		≥16	≥16	≥32	≥8

Figure 1. Antibiotic sensitivity of the 17 strains. The closest known species/strains for each of the 17 strains are shown. Antibiotic sensitivities are categorized as susceptible (white), intermediate (light orange) or resistant (dark orange). Antimicrobial susceptibility testing was performed using the broth microdilution method with dry plates (Eiken Chemical, Japan) according to the Clinical and Laboratory Standards institute (CLSI) guidelines M11-A8 and M100-S23. Briefly, each strain grown on Eggerth-Gagnon agar was harvested and suspended in ABCM broth (Eiken Chemical, Tokyo, Japan). The number of colony-forming units (CFU) was adjusted to 1×10^5 CFU/mL and an aliquot (100 µL) of the suspension was inoculated into each well of the plates. After incubation at 37 °C for 48 h, bacterial growth was visually assessed to determine the minimal inhibitory concentrations (MICs). The Etest (Sysmex-bioMérieux, Japan) was also employed to test for susceptibility to metronidazole and vancomycin.

human-derived bacterial strains using gnotobiotic techniques.[5] We first observed full Treg cell induction in the colon of ex-germ-free mice orally inoculated with a chloroform-treated human fecal sample. Then the cecal contents from these mice were treated with chloroform, diluted, and serially transplanted into other germ-free mice, while monitoring Treg induction capability. We succeeded in obtaining colonized mice in which the complexity of the gut microbiota was greatly decreased without sacrificing Treg-inducing potency. From these mice, we cultured and selected 17 strains which, when mixed together and orally administered to gem-free mice and rats, were able to induce a significant accumulation of CD4$^+$Foxp3$^+$ Treg cells in the colon. Furthermore, repeated oral ingestion of the mixture of 17 strains rendered specific-pathogen-free mice resistant to experimental allergic diarrhea and trinitrobenzene sulfonic acid (TNBS)-induced colitis.[5] Therefore, the 17 strains have at least a prophylactic effect in mouse colitis models.

Characterization of the 17 Strains

The 17 Treg-inducing strains isolated in our study all belong to the class Clostridia.[5] Clostridia species are gram-positive anaerobic rods and typically can form endospores. Some Clostridia species, including *Clostridium tetani*, *C. botulinum*, and *C. perfringens*, are well-known pathogens that are often isolated as a singular cause of infectious disease, and *C. difficile* is responsible for antibiotic-associated diarrhea and colitis. On the other hand, Clostridia species are extremely heterogeneous and many of them inhabit the large intestine of human and animals as predominant symbiotic microbes. Clostridia species can be classified into 19 clusters (I to XIX),[6] and many of the pathogenic species listed above belong to

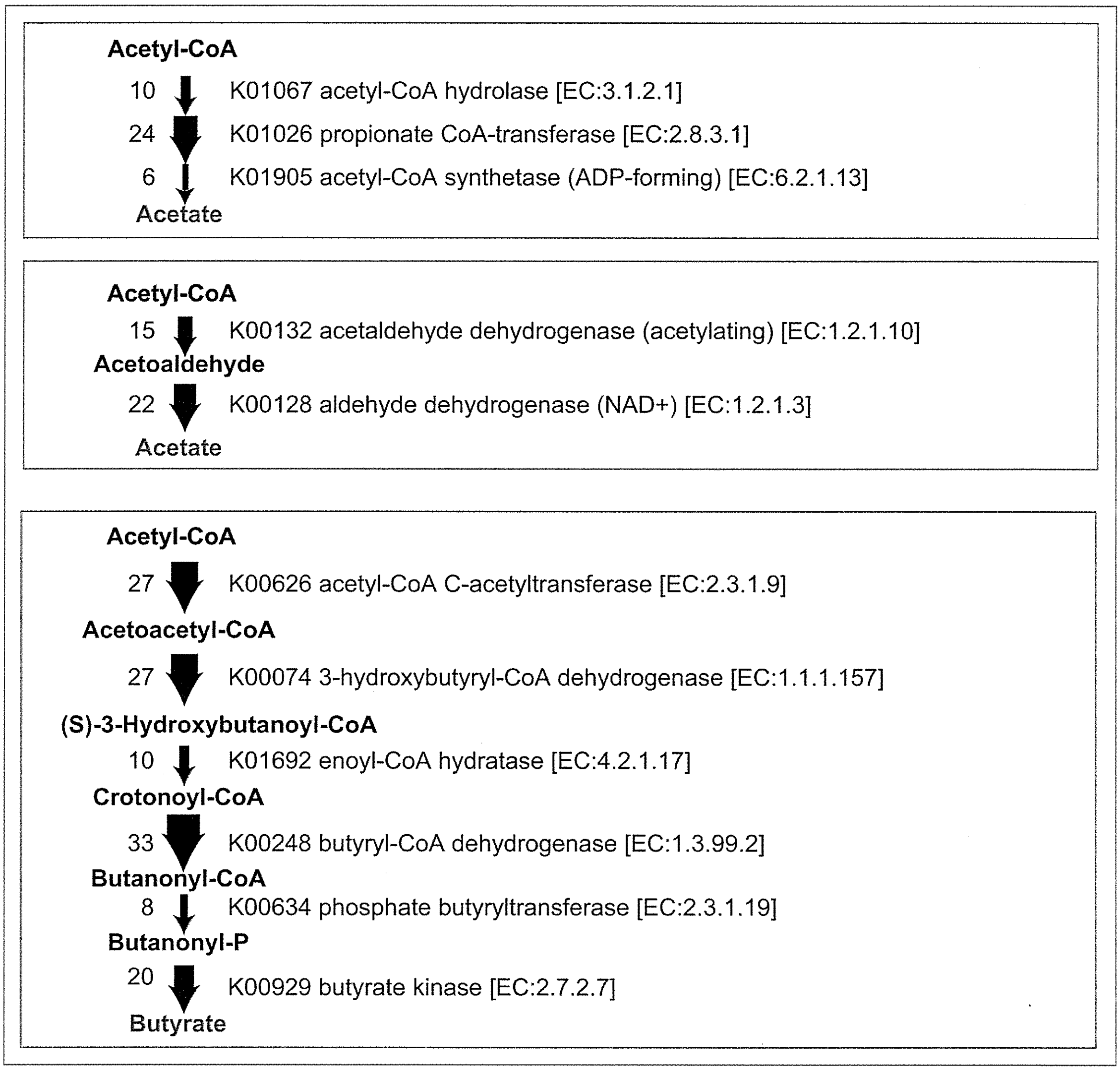

Figure 2. SCFA biosynthesis pathways and corresponding gene copy numbers in the 17 strains. Potential metabolic pathways leading to the production of acetate and butyrate from acetyl-CoA are shown. The thickness of the arrow and the number on the left of the arrow indicates the copy number of genes identified in the genomes of the 17 strains.

cluster I. The 17 Treg-inducing strains fall within clusters IV, XIVa, and XVIII of Clostridia (**Fig. 1**). It has been reported that species within clusters XIVa and IV are indispensable for various physiological host functions. For instance, colonization with Clostridia clusters XIVa and IV normalizes the enlarged cecum found in germ-free mice and supports epithelial growth and turnover.[7] It was also shown that colonization with Clostridia cluster XIVa strains renders mice resistant to *C. difficile* colonization.[8]

It should be noted that there have been several reports of Clostridia clusters IV, XIVa, and XVIII species (*Clostridium clostridioforme*, *Clostridium innocuum*, and *Clostridium ramosum*, in particular) in clinical specimens of opportunistic infections.[9] However they are rarely single isolates, but rather a fraction of the multiple organisms in these clinical samples. Considering that commensal Clostridia species colonize in close proximity to the gut epithelial surface,[10] they may translocate when the barrier is compromised, not necessarily because of specific pathogenic properties, but simply because of their local abundance. Sequencing of the genomes of the 17 strains revealed that they lack known toxins and virulence factors.[5] Some of the 17 strains possess genes encoding putative sialidase, hyaluronidase, flagella-related protein, and fibronectin binding protein, but with low similarity to genes found in pathogenic Clostridia species. Furthermore, ingestion of a mixture of the 17 strains of Clostridia was effective in preventing colitis induced by TNBS[5] and even by dextran sulfate sodium (DSS) (data not shown), which chemically disrupts the epithelial barrier. Given the lack of major virulence factors and toxins in their genomes and the lack of any toxicity upon dosing to animals,

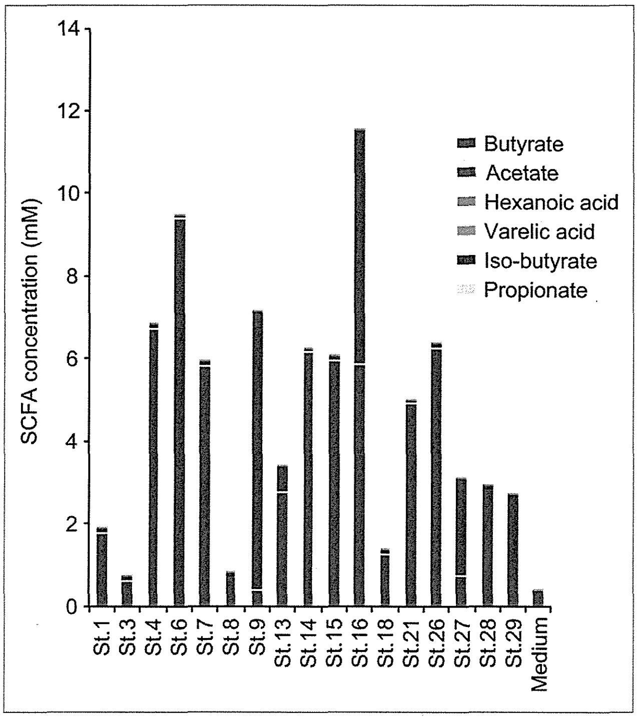

Figure 3. SCFA production by each of the 17 strains. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) analysis of short chain fatty acid metabolites was performed on 48 h cultures of peptone-yeast extract-Fildes solution supplemented with glucose at a concentration of 1% (w/v). Fatty acids in the culture supernatant were derivatized with 2-nitrophenylhydrazine and purified by liquid/liquid extraction.[28] Short chain fatty acid peaks were identified by both their specific MS/MS ion-transitions and comparison of the retention times with those of known short chain fatty acids in a standard solution.

the Clostridia strains isolated in our study appear to be safe for clinical use.

To further confirm that the isolated Clostridia strains are not harmful, antibiotic sensitivity of each strain was tested. All strains were susceptible to ampicillin-sulbactam, piperacillin-tazobactam, amoxicillin-clavulanate, metronidazole, and chloramphenicole, although some strains were resistant to penicillin G and ampicillin alone, and strains 1, 3, 8, and 18 showed low level resistance to vancomycin with MIC values of 4 to 8 μg/ml (**Fig. 1**). Since *C. ramosum* and *C. innocuum* are reported to have intrinsic resistance to glycopeptides and lipopeptides (vancomycin),[9] and genes identical to VanB2 ligase of *Enterococcus* spp. can be found in *C. hathewayi*, *C. boltae*, and *C. inocuum*-like bacteria,[11] it is not surprising that strains 1, 3, 8 and 18, which have 16S sequence similarities with those species, showed low level resistance to vancomycin. Overall, none of the 17 strains have high-level resistance to antibiotics tested; rather they show a benign safety profile. Further careful characterization of the strains and pre-clinical studies will be required before clinical translation.

Mechanisms of Treg Accumulation by the 17 Strains

The precise mechanism underlying how the 17 strains of Clostridia stimulate the induction of colonic Treg cells remains to be further elucidated. One suggested mechanism is the production of short chain fatty acids (SCFAs), which have multiple metabolic and immune functions.[12] In the context of Treg induction, SCFAs can elicit a TGF-β1 response in epithelial cells, which can contribute to de novo induction of peripheral Treg cells (pTreg).[5] SCFAs, particularly butyrate, can suppress dendritic cell activation through suppression of expression of the NFκB component RelB.[13] It has also been shown that butyrate activates signaling pathways through GPR109a to induce anti-inflammatory genes in dendritic cells.[14] In addition to its effects on dendritic cells, butyrate can directly stimulate thymic Treg cell (tTreg) proliferation through activation of GPR43[15] and the differentiation of naïve $CD4^+$ T cells into pTreg cells through histone H3 acetylation of the *Foxp3* gene intronic enhancer by inhibition of histone deacetylase (HDAC).[13,16]

The genomes of the 17 strains contain abundant genes predicted to be involved in the biosynthesis of acetate and butyrate (**Fig. 2**). SCFA production by each of the 17 strains when cultured in vitro in glucose-supplemented medium was analyzed by a validated liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) system[17] (**Fig. 3**). Among SCFAs, acetate and butyrate were detected at high concentrations in most of the culture supernatants except those of strains 1, 3, 8, and 18. Notably, strains 9, 13, 16, 27, and 29 showed very high butyrate production (**Fig. 3**). These findings are consistent with previous reports showing that bacterial strains belonging to Clostridia cluster IV and XIVa were positive for butyryl-CoA:acetate CoA transferase, an enzyme responsible for butyrate production.[18] Importantly, mono-colonization of GF mice with one of each of the 17 strains was insufficient to induce Treg cells in vivo[5]; however, the mixture of 17 strains is effective, suggesting synergistic effects in a microbial community-dependent manner.

We also examined enzymatic properties of the 17 strains using API ZYM, Rapid ID 32A, and API 20A systems in vitro (**Fig. 4**). Enzyme activities related to virulence such as trypsin, α-chymotrypsin, and gelatin hydrolase, which have been implicated in infective endocarditis, and β-glucuronidase, which may release toxic substances by

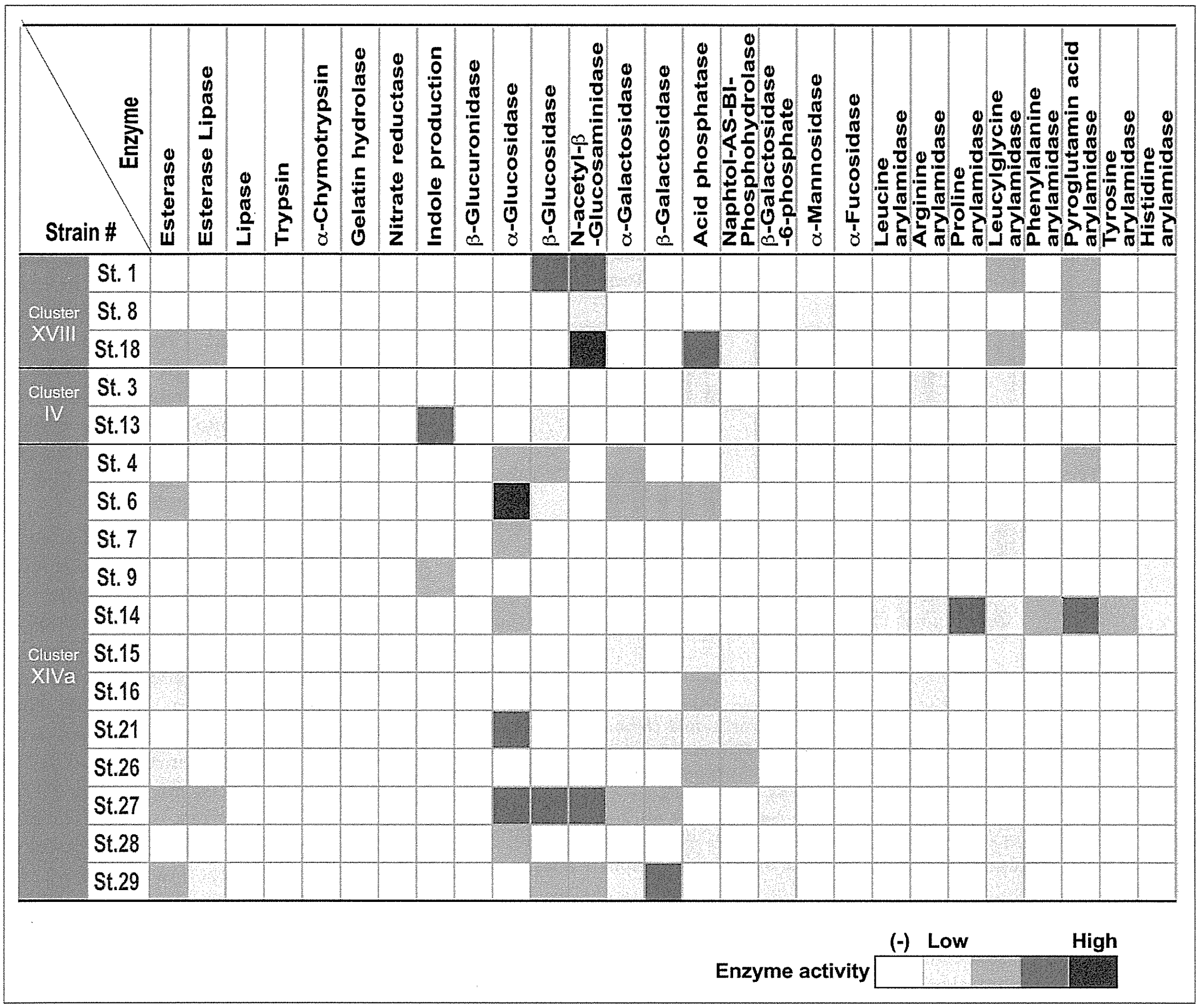

Figure 4. Enzymatic activity of the 17 strains. APIZYM and Rapid API 32A tests (Sysmex BioMérieux) were used for determination of enzymatic activities of each of the 17 strains. Cells from cultures grown on Eggerth-Gagnon blood agar plates for 48 h at 37 °C in an anaerobic chamber were suspended in saline and the turbidity was adjusted to 5–6 in the McFarland scale (approx. 1.5–1.9 × 10^9 CFU/ml). Aliquots of 65 μl of the suspensions were inoculated into cupules in the strip. In the case of Rapid API 32A, the turbidity was matched to 4 in the McFarland scale and aliquots of 55 μl were applied to the cupules. The strips were incubated at 37 °C for 4 h and the reactions were determined according to the manufacturer's instructions.

cleaving glucuronic acid-conjugation in the intestine, were very low or absent in the 17 strains. Of note, 17 strains showed variety of enzyme activities. Some strains were positive for the activity of principal mucin degrading enzymes, such as β-galactosidase and N-acetyl-β-glucosaminidase, and many strains exhibited strong activity of α-glucosidase, which is an intestinal enzyme that breaks down a variety of carbohydrates. Strain 9 and 13 could convert tryptophan into indole, which is reported to enhance epithelial barrier function and contribute to suppression of colitis.[19] Strain 14 was positive for proline arylamidase and pyroglutamic acid arylamidase activities. Together with SCFA production profiles, the 17 strains are diverse and heterogeneous in terms of enzymatic and metabolic properties. Considering that the dysbiotic microbiota associated with inflammatory bowel disease (IBD) is frequently accompanied by significantly decreased diversity,[20] the use of a functionally diverse mixture like the 17 strains of Clostridia described here may be reasonable for clinical applications to correct dysbiosis.

Future Directions

Imbalance in the gut microbiota, termed dysbiosis, significantly contributes to various human diseases. Several studies have reported decreased levels of Clostridia and Bacteroidetes in fecal samples from IBD patients.[21] Marked decreases in the prevalence of Clostridia have been associated with increased risk of airway hypersensitivity and atopic dermatitis.[22,23] Animal studies have also shown that bacterial dysbiosis helps perpetuate the cycle of chronic inflammation characteristic of IBD.[24] Therefore, manipulation of the gut microbiota as a therapeutic strategy holds great promise for immuno-inflammatory diseases including IBD and allergy.

Alteration of the gut microbiome via fecal microbiota transplantation (FMT), which involves placing stool from a healthy donor via duodenal tubing, colonoscopy, or enema, has been shown effective in patients with pseudomembranous colitis induced by *Clostridium difficile* infection,[25] and researchers and patients are interested in testing the potential of FMT for the treatment of other disease associated with disruption of the intestinal microbiota, including insulin resistance, multiple sclerosis, and IBD.[26] While FMT has been established as a proof of principle for the feasibility of manipulating the human microbiome as a therapeutic strategy, the development of commercial products based on fecal transplants faces a number of hurdles from manufacturing, quality assurance, pathogen contamination risk, donor selection, and patient acceptance perspectives. Therefore, treatment with a composite of well-characterized benign microorganisms is more desirable from many perspectives for medical purposes.

There are many probiotics with a history of safe use in humans for medicinal purposes. However, probiotics currently in use have generally been selected based on properties such as ease of culture and tolerance to acid and oxygen and are not among the major colonizers of the human gut.[27] In other words, they have not been isolated based on their ability to correct microbiome dysbiosis associated with human disease or to boost specific arms of the host immune system. Presumably as a result, the dysbiotic microbiota are refractory to treatment with currently available individual probiotic strains, and most probiotics tested to date have demonstrated, at best, mediocre effects in the clinic. Thus, there is a compelling need to identify more robust therapeutic organism compositions that are compatible and symbiotic to the host and, ideally, able to induce broader changes to the microbial ecosystem to correct dysbiosis and drive the immune system to normal homeostasis.

In this context, we believe that the 17 strains of Clostridia isolated in our study can form the basis for a future live biotherapeutic product to treat certain forms of IBD, allergy, and other immune-inflammatory diseases. Compositions based on these strains could (1) help correct microbiome imbalances; (2) be easily administered orally and not necessarily frequently, providing patients with a convenient therapeutic option; (3) be safe for human use, since they are commensal strains that are long-term colonizers of the healthy human gut, do not have prominent virulence factors, and are sensitive to antibiotics; (4) be viewed favorably by patients given that they are natural compositions; and (5) be manufactured with traditional fermentation methods and developed under the live biotherapeutics route outlined by the FDA, thus circumventing the manufacturing, regulatory, and patient acceptance hurdles that hamper commercialization of fecal transplants.

Our next step should be optimization of the therapeutic mixture by elimination of nonessential components of the 17 strains for Treg cell induction. In addition to this, we need to identify and isolate other bacterial strains from human intestine that can regulate differentiation and activation of other immune cell subsets, such as Th17 cells. Our long-term goal is to offer a diverse but minimal consortium of microbes that can colonize the human gut and restore and maintain immune homeostasis to prevent and cure immune-inflammatory diseases.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

References

1. Honda K, Littman DR. The microbiome in infectious disease and inflammation. Annu Rev Immunol 2012; 30:759-95; PMID:22224764; http://dx.doi.org/10.1146/annurev-immunol-020711-074937
2. Ivanov II, Honda K. Intestinal commensal microbes as immune modulators. Cell Host Microbe 2012; 12:496-508; PMID:23084918; http://dx.doi.org/10.1016/j.chom.2012.09.009
3. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 2009; 139:485-98; PMID:19836068; http://dx.doi.org/10.1016/j.cell.2009.09.033
4. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science 2011; 331:337-41; PMID:21205640; http://dx.doi.org/10.1126/science.1198469
5. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 2013; 500:232-6; PMID:23842501; http://dx.doi.org/10.1038/nature12331
6. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JA. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 1994; 44:812-26; PMID:7981107; http://dx.doi.org/10.1099/00207713-44-4-812
7. Itoh K, 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 Anim 1985; 19:111-8; PMID:3889493; http://dx.doi.org/10.1258/002367785780942589
8. Reeves AE, Koenigsknecht MJ, Bergin IL, Young VB. Suppression of Clostridium difficile in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the family Lachnospiraceae. Infect Immun 2012; 80:3786-94; PMID:22890996; http://dx.doi.org/10.1128/IAI.00647-12
9. Alexander CJ, Citron DM, Brazier JS, Goldstein EJ. Identification and antimicrobial resistance patterns of clinical isolates of Clostridium clostridioforme, Clostridium innocuum, and Clostridium ramosum compared with those of clinical isolates of Clostridium perfringens. J Clin Microbiol 1995; 33:3209-15; PMID:8586704
10. Nava GM, Stappenbeck TS. Diversity of the autochthonous colonic microbiota. Gut Microbes 2011; 2:99-104; PMID:21694499; http://dx.doi.org/10.4161/gmic.2.2.15416
11. Ballard SA, Grabsch EA, Johnson PD, Grayson ML. Comparison of three PCR primer sets for identification of vanB gene carriage in feces and correlation with carriage of vancomycin-resistant enterococci: interference by vanB-containing anaerobic bacilli. Antimicrob Agents Chemother 2005; 49:77-81; PMID:15616278; http://dx.doi.org/10.1128/AAC.49.1.77-81.2005
12. Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 2002; 217:133-9; PMID:12480096; http://dx.doi.org/10.1111/j.1574-6968.2002.tb11467.x
13. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 2013; 504:451-5; PMID:24226773; http://dx.doi.org/10.1038/nature12726
14. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, Thangaraju M, Prasad PD, Manicassamy S, Munn DH, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 2014; 40:128-39; PMID:24412617; http://dx.doi.org/10.1016/j.immuni.2013.12.007

15. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 2013; 341:569-73; PMID:23828891; http://dx.doi.org/10.1126/science.1241165
16. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 2013; 504:446-50; PMID:24226770; http://dx.doi.org/10.1038/nature12721
17. Morikawa T, Kajimura M, Nakamura T, Hishiki T, Nakanishi T, Yukutake Y, Nagahata Y, Ishikawa M, Hattori K, Takenouchi T, et al. Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway. Proc Natl Acad Sci U S A 2012; 109:1293-8; PMID:22232681; http://dx.doi.org/10.1073/pnas.1119658109
18. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett 2009; 294:1-8; PMID:19222573; http://dx.doi.org/10.1111/j.1574-6968.2009.01514.x
19. Shimada Y, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, Takeda K. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. PLoS One 2013; 8:e80604; PMID:24278294; http://dx.doi.org/10.1371/journal.pone.0080604
20. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 2006; 55:205-11; PMID:16188921; http://dx.doi.org/10.1136/gut.2005.073817
21. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 2007; 104:13780-5; PMID:17699621; http://dx.doi.org/10.1073/pnas.0706625104
22. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, Gill N, Blanchet MR, Mohn WW, McNagny KM, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep 2012; 13:440-7; PMID:22422004; http://dx.doi.org/10.1038/embor.2012.32
23. Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, Masetti R, Ricci G, Pession A, Brigidi P. Unbalance of intestinal microbiota in atopic children. BMC Microbiol 2012; 12:95; PMID:22672413; http://dx.doi.org/10.1186/1471-2180-12-95
24. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, Glickman JN, Glimcher LH. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell 2007; 131:33-45; PMID:17923086; http://dx.doi.org/10.1016/j.cell.2007.08.017
25. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N Engl J Med 2013; 368:407-15; PMID:23323867; http://dx.doi.org/10.1056/NEJMoa1205037
26. Smits LP, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. Gastroenterology 2013; 145:946-53; PMID:24018052; http://dx.doi.org/10.1053/j.gastro.2013.08.058
27. O'Toole PW, Cooney JC. Probiotic bacteria influence the composition and function of the intestinal microbiota. Interdiscip Perspect Infect Dis 2008; 2008:175285; PMID:19277099
28. Miwa H, Yamamoto M. High-performance liquid chromatographic analysis of serum short-chain fatty acids by direct derivatization. J Chromatogr 1987; 421:33-41; PMID:3429573; http://dx.doi.org/10.1016/0378-4347(87)80376-6

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Dysbiosis of Salivary Microbiota in Inflammatory Bowel Disease and Its Association With Oral Immunological Biomarkers

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Abstract

Analysis of microbiota in various biological and environmental samples under a variety of conditions has recently become more practical due to remarkable advances in next-generation sequencing. Changes leading to specific biological states including some of the more complex diseases can now be characterized with relative ease. It is known that gut microbiota is involved in the pathogenesis of inflammatory bowel disease (IBD), mainly Crohn's disease and ulcerative colitis, exhibiting symptoms in the gastrointestinal tract. Recent studies also showed increased frequency of oral manifestations among IBD patients, indicating aberrations in the oral microbiota. Based on these observations, we analyzed the composition of salivary microbiota of 35 IBD patients by 454 pyrosequencing of the bacterial 16S rRNA gene and compared it with that of 24 healthy controls (HCs). The results showed that Bacteroidetes was significantly increased with a concurrent decrease in Proteobacteria in the salivary microbiota of IBD patients. The dominant genera, *Streptococcus*, *Prevotella*, *Neisseria*, *Haemophilus*, *Veillonella*, and *Gemella*, were found to largely contribute to dysbiosis (dysbacteriosis) observed in the salivary microbiota of IBD patients. Analysis of immunological biomarkers in the saliva of IBD patients showed elevated levels of many inflammatory cytokines and immunoglobulin A, and a lower lysozyme level. A strong correlation was shown between lysozyme and IL-1β levels and the relative abundance of *Streptococcus*, *Prevotella*, *Haemophilus* and *Veillonella*. Our data demonstrate that dysbiosis of salivary microbiota is associated with inflammatory responses in IBD patients, suggesting that it is possibly linked to dysbiosis of their gut microbiota.

Key words: Crohn's disease; ulcerative colitis; salivary microbiota; 16S rRNA; pyrosequencing

1. Introduction

Current advances of next-generation sequencing technologies (NGS) have enabled us to acquire massive DNA sequence data from any types of samples.[1] In particular, complex bacterial communities composed of numerous species in various environments including human body has become the practically feasible targets, and the analysis has been shifting to the DNA-based approach in conjugation with bioinformatics for enumerated data of metagenome and 16S rRNA gene (16S) produced by NGS.[2–5] Among these approaches, pyrosequencing-based 16S gene analysis is rapid and cost effective to comprehensively evaluate the overall structure of bacterial communities and to identify species present in them, irrespective of the yet-uncultured species.[6] This method includes targeted PCR amplification of 16S rRNA gene variable regions with appropriate primers, followed by sequencing of the 16S amplicons using 454 pyrosequencer.[7–10] We recently developed the improved analytical pipeline for pyrosequencing data of 16S rRNA gene V1–V2 variable region for human gut microbiota, by reassessing a PCR primer sequence, clustering conditions of error-prone 16S reads, and the quality check process to effectively remove low-quality data, and thereby the pipeline provided the high quantitative accuracy to estimation of the bacterial composition and abundance in the community.[10]

In this study, we applied our improved pipeline to the analysis of the human oral microbiota. The oral cavity is a large reservoir of bacteria of >700 species or phylotypes, and is profoundly relevant to host health and disease.[11–14] Current studies reported that various oral symptoms such as aphthous stomatitis, oral ulcer, dry mouth, and pyostomatitis vegetans are frequently observed in inflammatory bowel disease (IBD) patients.[15–20] IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, idiopathic, relapsing inflammatory disorder of the gastrointestinal tract.[21,22] The most widely accepted mechanism of IBD pathogenesis includes inflammation due to altered host immune response in association with continuous stimulation from the resident gut microbiota.[23–28] Many studies also revealed that the gut microbiota of IBD patients significantly differed from that of healthy controls (HCs), and is termed dysbiosis.[29–34]

Similarly, oral manifestations observed in IBD patients suggest the association of oral microbiota with such manifestations, yet-limited information exists about the oral microbiota of IBD patients. We characterized the salivary microbiota of IBD patients and HCs by barcoded pyrosequencing analysis of the bacterial 16S rRNA gene. We observed that the salivary microbiota in IBD patients significantly differed from that of HCs, and found particular bacterial species associated with dysbiosis. We also showed that the observed dysbiosis is strongly associated with elevated inflammatory response of several cytokines with depleted lysozyme in the saliva of IBD patients, some of which showed a strong correlation with the relative abundance of certain bacterial species. Thus, the present study demonstrates an association between dysbiosis of the salivary microbiota and change in the host's physiological state in IBD.

2. Material and methods

2.1. *Patients and control groups*

All participants of the CD, UC, and HC groups were informed of the purpose of this study, and written consent was obtained. This project was approved by the ethical committee of University of the Ryukyus. Metadata collected at the time of sampling included various demographics and a medication history for each patient (Supplementary Tables S1 and S2).

2.2. *Sample collection and DNA extraction*

Unstimulated saliva collected from subjects was immediately frozen by liquid nitrogen and stored in −80°C until use. Salivary genomic DNA was prepared according to the literature with minor modifications.[35]

Bacterial cells were harvested from 1 ml of saliva by centrifugation at 3300*g* for 10 min at 4°C. Bacterial pellets were suspended in 10 mM Tris–HCl/10 mM EDTA buffer and incubated with 15 mg/ml lysozyme (Sigma-Aldrich Co. LLC) for 1 h at 37°C. Purified achromopeptidase (Wako Pure Chemical Industries, Ltd.) was added to a final concentration of 2000 units/ml and samples were further incubated for 30 min. Ten percentage of (wt/vol) sodium dodecyl sulphate (SDS) and proteinase K (Merck Japan) were added to the suspension to final concentrations of 1% and 1 mg/ml, respectively, and samples were further incubated at 55°C for 1 h. The lysate was treated with phenol/chloroform/isoamyl alcohol (Life Technologies Japan, Ltd.) and centrifuged at 3300*g* for 10 min. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 4.5) and 2 volumes of ethanol (Wako Pure Chemical Industries, Ltd.) to the supernatant. DNA was pelleted by centrifugation at 3300*g* for 15 min at 4°C. DNA pellets were rinsed with 75% ethanol, dried and dissolved in 10 mM Tris–HCl/1 mM EDTA (TE) buffer. DNA was further treated with 1 mg/ml RNase A (Wako Pure Chemical Industries, Ltd.) at 37°C for 30 min, and precipitated by adding equal volumes of 20% PEG solution (PEG6000-2.5M NaCl). DNA was pelleted by centrifugation at 8060*g* at 4°C, rinsed twice with 75% ethanol, dried, and dissolved in TE buffer.

2.3. *Bacterial 16S rRNA gene-based analysis*

2.3.1. PCR amplification of the 16S rRNA gene V1–V2 region and barcoded 454 pyrosequencing The hypervariable V1–V2 region of the 16S rRNA gene was amplified by PCR with barcoded 27Fmod and 338R primers.[10] PCR was performed in 50 μl of 1 × Ex Taq PCR buffer composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ in the presence of 250 μM dNTP, 1 U Ex Taq polymerase (Takara Bio, Inc.), forward and reverse primers (0.2 μM) and ~20 ng template DNA. Thermal cycling consisted of initial denaturation at 96°C for 2 min, followed by 25 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min, and final extension at 72°C on a 9700 PCR system (Life Technologies Japan, Ltd.). Negative controls were treated similarly, except that no template DNA was added to the PCR reactions. PCR products of ~370 bp were visualized by electrophoresis on 2% agarose gels, while negative controls failed to produce visible PCR products and were excluded from further analysis. PCR amplicons were purified by AMPure XP magnetic purification beads (Beckman Coulter, Inc.), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan, Ltd.). Equal amounts of each PCR amplicon were mixed and then sequenced using either 454 GS FLX Titanium or 454 GS JUNIOR (Roche Applied Science).

2.3.2 Analysis pipeline for 16S data We developed and used an analysis pipeline for pyrosequencing data of the 16S rRNA gene V1–V2 region generated from oral microbiota. Based on sample specific barcodes, reads were assigned to each sample followed by the removal of reads lacking both forward and reverse primer sequences. Data were further denoised by removal of reads with average quality values <25 and possible chimeric sequences. For chimera checking and taxonomy assignment of the 16S rRNA data, we constructed our own databases from three publically available databases: Ribosomal Database Project (RDP) v. 10.27, CORE (http://microbiome.osu.edu/), and a reference genome sequence database obtained from the NCBI FTP site (ftp://ftp.ncbi.nih.gov/genbank/, December 2011). Reads having BLAST match lengths <90% with the representative sequence in the three databases were considered as chimeras and removed. Finally, filter-passed reads were used for further analysis after trimming off both primer sequences.

All of the 16S rRNA sequence data used in this study were deposited in DDBJ/GenBank/EMBL under accession numbers: DRA000984–DRA000986.

2.3.3. Operational taxonomic unit clustering and UniFrac analysis From the filter-passed reads, 3000 high-quality reads/sample were randomly chosen. The total reads (59 × 3000 reads) were then sorted according to average quality value and grouped into operational taxonomic units (OTUs) using UCLUST (http://www.drive5.com/) with a sequence identity threshold of 96%. Taxonomic assignments were made according to the best BLAST-hit phylotype. Weighted and unweighted UniFrac metrics[36] were used to assess the diversity of the salivary microbiota between the CD, UC, and HC groups. UniFrac distances were based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from the 16S rRNA gene sequences from all communities being compared.

2.4. *Immunoassays*

The centrifugal supernatant of unstimulated saliva was analyzed by the Luminex fluorescence technique, using the Bio-Plex Pro Human cytokine 27-Plex Assay (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. LL-37 (cathelicidin, hCAP-18) levels were measured by ELISA using the Human LL-37 ELISA Kit (Hycult Biotech, Uden, The Netherlands). IgA levels were measured using the EIA-sIgA Test (MBL, Nagoya, Japan). Salivary lysozyme levels were measured using turbidimetric technique (SRL Inc., Japan). Total protein concentrations were measured by the Bradford protein assay using bovine serum albumin as the standard. In this study, saliva samples of only 15 HC, 14 CD, and 10 UC subjects were used for the assay of biomarkers, because the saliva from the other subjects was insufficient for measurement of all the indicated biomarkers.

2.5. *Statistical analysis*

All statistical analyses were conducted with R version 2.15.2. Microbial richness, evenness, and diversity were assessed using the R Vegan package. Depending on the normality of the data, the Student's *t*-test or Mann-Whitney's U-test was used to perform statistical analysis. *P*-values were corrected for multiple testing using the Benjamini–Hochberg method. Correlations between relative abundance of genera and immunological markers in saliva were calculated by Pearson correlation coefficients.

3. Results

3.1. *Collection of 16S data*

We surveyed the salivary microbiota of 21 CD patients, 14 UC patients, and 24 HCs, all of whom (including their relatives) are residents, lasting at least three generations, of the Okinawa area in Japan. The general and clinical parameters of the study populations are given in Supplementary Table S1, and individual details are shown in Supplementary Table S2.

Sample-assigned pyrosequencing reads having both forward and reverse primer sequences accounted for ~60% of the total number of reads. The 16S reads having average quality values <25 and possibly chimeric sequences represented 0.75 and 0.46% of the selected dataset, respectively. Finally, 506 133 high-quality 16S reads were obtained from 59 salivary samples. Sorting of the 16S reads by average quality value prior to clustering enabled selection of the representative sequence with the highest quality value among the 16S reads grouped in each OTU. On the other hand, the primer check step for removing reads lacking both primer sequences[10] had the possibility to incorrectly remove reads containing V1–V2 regions longer than the maximum length of 431 bp in the filter-passed reads. This is because there are a few species with a V1–V2 region >431 bp (e.g. *Campylobacter rectus* has a length of 493 bp). Our primer check step did not significantly affect the present results because only one of the 177 000 raw reads examined hit to Campylobacter. However, to avoid the incorrect filtration of reads, we modified the primer check step so as not to remove reads having a length of >400 bp, even though they may not have both primer sequences.

3.2. *Overall composition of the salivary bacterial communities*

We evaluated the ecological features of the salivary bacterial communities of the CD, UC, and HC groups by a variety of indices at the OTU level.[37,38] The results are summarized in Table 1. Species richness is the observed number of bacterial species assigned by OTUs detected in the samples. Richness estimates were obtained from the observed number of species by the extrapolation method using estimators such as the Chao1 and ACE indices. Evenness is the degree of homogeneity of abundance of the species detected in the samples. Diversity estimates were obtained from species richness and evenness by using several different indices, which exhibit different sensitivities to given factors, to confirm our results. The results suggested that there were no significant differences in the overall configuration of the salivary microbiota among the three groups (Table 1).

Table 1. OTU-based microbial richness and diversity across the HC, CD and UC groups

	HC	CD	UC
Diversity estimates			
Shannon Index	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
Simpson Index	0.93 ± 0.01	0.93 ± 0.01	0.94 ± 0.01
Invsimpson Index	16.7 ± 1.1	16.7 ± 1.1	17.1 ± 1.4
Fisher alpha Index	26.8 ± 1.4	26.3 ± 1.4	24.8 ± 1.8
Evenness estimate			
Pielou's Index	0.7 ± 0.01	0.7 ± 0.01	0.71 ± 0.01
Richness estimates			
Number of OTUs	126 ± 5	124 ± 5	118 ± 7
chao1 Index	183 ± 8	183 ± 9	164 ± 13
ACE Index	182 ± 8	177 ± 8	165 ± 11

We then compared the overall bacterial community composition using the UniFrac distance metric, a phylogenetic tree-based metric ranging from 0 (distance between identical communities) to 1 (distance between totally different communities). A principal coordinate analysis (PCoA) plot based on the weighted UniFrac metric revealed clear clustering of most IBD samples apart from the HC samples, indicating the difference in microbial communities between the two groups (Fig. 1A). A bar chart more clearly shows the significant difference in microbiota composition between the IBD and HC groups (Fig. 1B). Comparison of the salivary microbiota of HCs with that of the CD and UC groups indicated that the microbiota of HCs significantly differs from both of them, and no significant difference was found between the UC and CD groups (Fig. 1C). Similar results were obtained using the unweighted UniFrac metric with lower statistical significance than that of the weighted UniFrac metric (Supplementary Fig. S1). These data suggest that species abundance, rather than species diversity, largely contributes to the observed differences in salivary microbiota between the HC and IBD groups.

Although the average age was considerably different between HCs and the IBD patients, weighted UniFrac distance analysis of 10 selected healthy subjects (average age 25.0 yr), 10 IBD patients (average age 28.7 yr, which matched the selected HC group), and the remaining 25 IBD patients (average age 54.6 yr) showed results similar to that of the total samples (Supplementary Fig. S2). Moreover, there was no significant difference between the two IBD subgroups. These data suggest that age might not affect the observed dysbiosis of the salivary microbiota of the IBD patients.

3.3. *Differences in salivary microbiota composition between the HC, CD, and UC groups*

The final dataset of the examined CD, UC, and HC groups ($n = 59$) consisted of 177 000 reads and included representatives of 12 bacterial phyla (Fig. 2; Supplementary Fig. S3 and Table S3). The majority of the 16S reads were classified into only five phyla: Firmicutes (46.5%), Bacteroidetes (22.3%), Actinobacteria (13.7%), Proteobacteria (12.5%), and Fusobacteria (4.2%). TM7, SR1, Spirochaetes, Synergistetes, Tenericutes, and Cyanobacteria were also detected and collectively represented <1% of the total reads analyzed. Analysis at the phylum level showed that the relative abundance of Bacteroidetes was significantly higher in both the CD

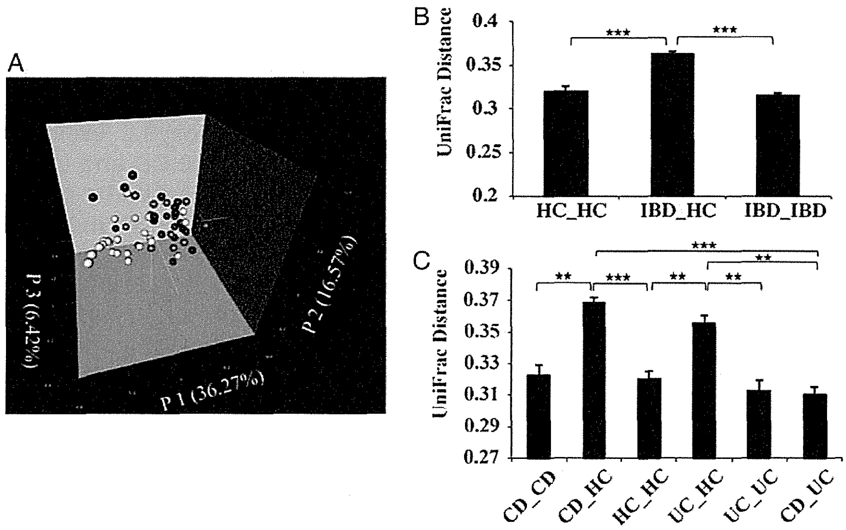


Figure 1. Analysis of the salivary microbiota of the HC, CD, and UC groups based on 16S data. (A) PCoA plot generated using weighted UniFrac metric. The three components explained 59.26% of the variance. White, grey, and black dots indicate HCs, UC, and CD samples, respectively. (B) Weighted UniFrac distance metric (a measure of differences in bacterial community structure) between HCs and the IBD (CD and UC) groups. (C) Weighted UniFrac distance metric between the HC, CD, and UC groups. Student's *t*-test was used; $*P < 0.01$, $**P < 10^{-5}$, and $***P < 10^{-10}$; mean ± S.E.M.

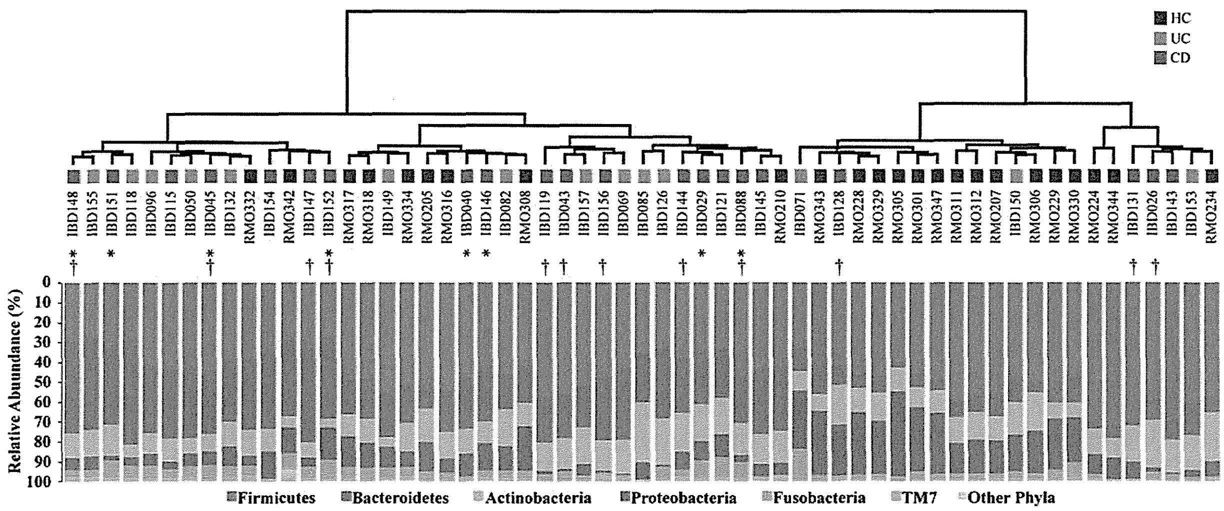


Figure 2. Cluster dendrogram generated using weighted UniFrac metric. Bar charts show the relative abundance of different phyla across the CD, UC and HC samples. Asterisks indicate samples taken during the active phase of CD. Dagger indicates anti-TNF-α antibody treated CD.

and UC groups as compared with HCs ($P < 0.01$), while that of Proteobacteria was significantly lower in both the CD and UC groups as compared with HCs ($P < 0.01$). No significant difference at the phylum level was observed between the UC and CD groups, which was consistent with the results of the UniFrac distance analysis.

In total, 107 bacterial genera were identified (at 95% identity), accounting for 97.8% of the total dataset. The remaining unclassified sequences (2.2%) were assigned to higher level taxa. Fourteen genera, including *Streptococcus*, *Prevotella*, *Rothia*, *Neisseria*, *Granulicatella*, *Actinomyces*, *Haemophilus*, *Veillonella*, *Gemella*, *Leptotrichia*, *Fusobacterium*, *Porphyromonas*, *Uncultured Lachnospiraceae*, and *Oribacterium*, predominated accounting for 92.7% of the total dataset. Other genera represented <0.5% each (Fig. 3; Supplementary Table S3). Two genera, *Prevotella* (phy. Bact.) and *Veillonella* (phy. Firm.), were significantly higher in both the CD and UC groups compared with HCs ($P < 0.01$). Two genera, *Streptococcus* (phy. Firm.) and *Haemophilus* (phy. Prot.), were significantly lower in both the CD and UC groups as compared with HCs ($P < 0.05$ and

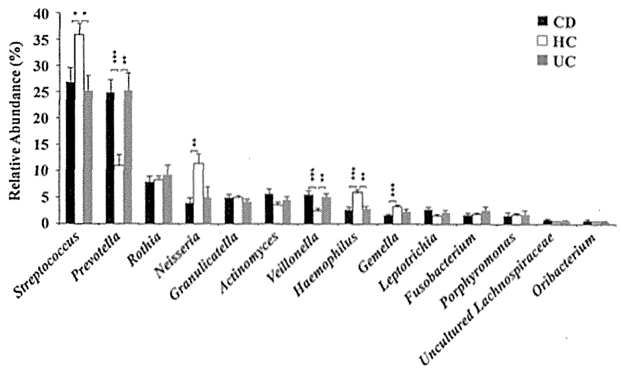


Figure 3. Mean genus abundance in the CD, UC and HC groups. Plotted values are the mean abundance of the 14 most abundant genera in each group. Welch's test with BH adjustment was used; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; mean ± S.E.M.

0.01, respectively). Two other genera, *Neisseria* (phy. Prot.) and *Gemella* (phy. Firm.), were also found to be significantly lower only in the CD group as compared with HCs ($P < 0.01$ and 0.001, respectively). These results indicate that the relative increase of Bacteroidetes in IBD patients was mainly due to the increase of *Prevotella*, and the relative decrease of Proteobacteria in IBD patients was mainly due to the decrease of *Neisseria* and *Haemophilus*. No significant difference in the relative abundance of either Gram-positive or Gram-negative bacteria was observed among the three groups (Supplementary Table S3).

Clustering of all reads using a 96% pairwise-identity cutoff generated 1257 OTUs, of which only 40 OTUs represented 67.2% of the total reads analyzed. The remaining OTUs were present at relative abundance levels <0.5% of the total dataset (Supplementary Table S4). The relative abundance of several OTUs belonging to the genera *Streptococcus*, *Prevotella*, *Veillonella*, *Nesisseria*, *Haemophilus*, and *Gemella* showed significant differences in IBD patients as compared with HCs. These results were concordant with those detected at the genus level. Among the abundant OTUs, those most closely assigned to *Prevotella melaninogenica*, *Veillonella* sp. oral taxon 158, *Streptococcus mitis*, *Gemella sanguinis*, *Neisseria mucosa*, and *Haemophilus parainfluenzae* showed significant differences in relative abundance between the HC and IBD groups (Supplementary Table S4).

3.4. *Salivary immunological biomarkers in the HC, CD, and UC groups*

We evaluated the inflammatory state, considering its influence on shaping the salivary microbiota, in saliva of the CD and UC patients as compared with that of HCs. The analysis was performed by measuring secretory IgA, cytokines, and enzymes including lysozyme in unstimulated saliva of 15 HC, 14 CD, and 10 UC individuals (Supplementary Table S5 and Fig. S4). There was no significant difference in the total protein concentration in saliva of the CD and UC patients as compared with that of HCs ($P = 0.112$ and 0.192, respectively). The lysozyme level was significantly lower in saliva of both the CD and UC groups as compared with HCs ($P < 0.01$). On the other hand, the levels of IgA and LL37 in both CD and UC groups were higher than that of HCs with statistical significance. The use of Luminex technology was highly sensitive in measuring cytokines from small volumes of saliva samples. In saliva of the CD and UC groups, the level of IL-1β was significantly higher as compared with HCs ($P < 0.05$ and <0.01, respectively). The levels of IL-6, IL-8, and MCP-1 were significantly higher only in saliva of the UC group, while elevated TNF-α level was found only in the CD group with statistical significance. The levels of IgA and MCP-1 in the UC group were significantly higher than those in the CD group. These data indicate that the oral cavity of IBD patients is usually in the inflammatory state, and the levels tend to be slightly higher in the UC group than the CD group.

3.5. *Composition of the salivary microbiota in relation to immunological biomarkers*

We searched for correlations between the relative abundance of dominant bacterial genera and the measured biomarkers in the saliva of 39 subjects (Supplementary Table S5). The results are shown in Fig. 4. The relative abundance of *Streptococcus* negatively correlates with IL-1β and IL-8 ($r = -0.54$ and -0.51, respectively, $P < 0.001$), while it positively correlates with lysozyme ($r = 0.63$, $P < 0.001$). On the other hand, the abundance of *Prevotella* positively correlates with IL-1β ($r = 0.58$, $P < 0.001$) but negatively correlates with lysozyme ($r = -0.54$, $P < 0.01$). The relative abundance of *Veillonella* negatively correlates with lysozyme ($r = -0.54$, $P < 0.001$), while *Haemophilus* positively correlates with lysozyme ($r = 0.58$, $P < 0.001$). Linear regressions also validated correlations between the relative abundance of *Streptococcus* and *Prevotella* and the levels of lysozyme and IL-1β, and between the relative abundance of *Veillonella* and *Haemophilus* and the level of lysozyme (Supplementary Fig. S5). On the whole, *Prevotella*, *Actinomyces*, *Veillonella*, and *Lachnospiracea* tended to positively correlate, while *Streptococcus*, *Rothia*, *Neisseria*, *Haemophilus*, and *Gemella* tended to negatively correlate with elevated cytokines in saliva of IBD patients.

3.6. *Validation of 16S pyrosequencing data by targeted quantitative PCR*

We designed specific PCR primers for quantitative PCR (qPCR) targeting genomes of *P. melaninogenica* and *H. parainfluenzae*, which showed significant differences

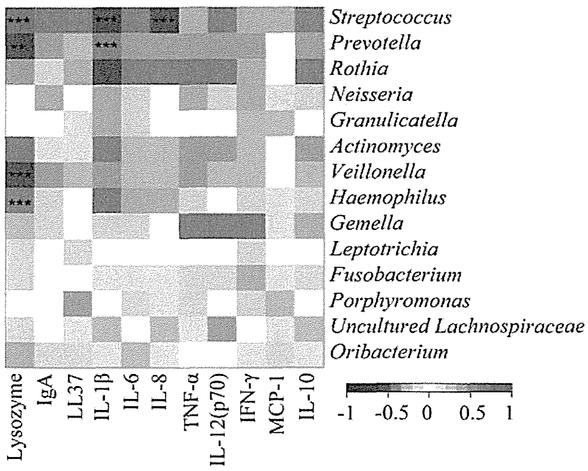


Figure 4. Correlation between the relative abundance of predominant genera and the level of immunological biomarkers in the saliva of IBD patients. Pearson product moment correlation coefficients are represented by colour ranging from blue, negative correlation (−1), to red, positive correlation (1). Normalized values of immunological biomarkers by total protein amount were used in this analysis. Significant correlations after *P*-value adjustment are marked by $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

between HCs and IBD patients by 16S pyrosequencing analysis (Supplementary Table S4). Using these primers, we found strong correlations between 16S-based and qPCR data for the quantification of *P. melaninogenica* ($r = 0.87$, $P < 0.001$) and *H. parainfluenzae* ($r = 0.86, P < 0.001$), indicating the quantitative accuracy of our 16S pyrosequencing-based results (Fig. 5).

4. Discussion

4.1. Bacterial 16S rRNA-based pyrosequencing analysis

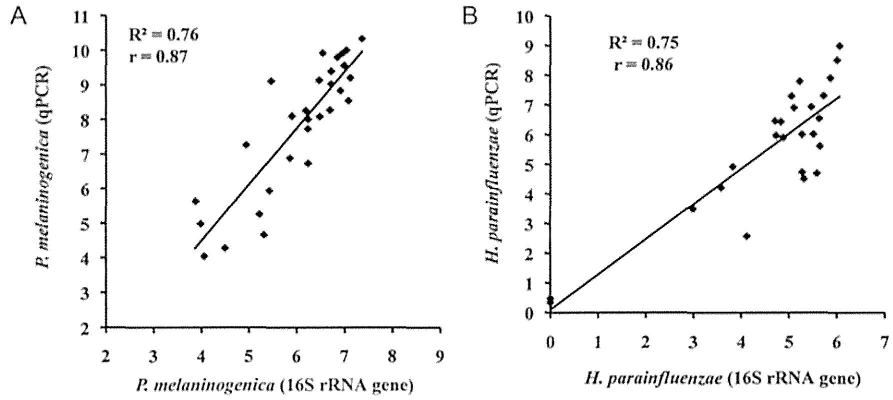
In this study, we used targeted amplicon sequencing of the 16S rRNA gene hypervariable V1–V2 region to evaluate bacterial composition at finer taxonomic levels. The use of primer 27Fmod enabled us to reduce underestimation of the relative abundance of *Bifidobacterium* species that predominate human microbiota, and thus the quantitative accuracy of the overall bacterial composition was greatly improved.[10,39] One limitation of clustering the 16S reads using the UCLUST program is selection of the representative sequence for each OTU. The quality of the representative sequence is not always the highest in the OTU, which affects the BLAST identity, *E*-value and score, sometimes providing inappropriate results for taxonomic assignment of the OTUs. We overcame this limitation by sorting the 16S reads by their average quality values prior to clustering, leading to 16S reads with the highest quality being selected as the representative sequence for each OTU. Our 16S-based results were also validated by strongly correlating with the qPCR data targeting bacterial species showing significant changes between HC and IBD samples (Fig. 5). In addition, clustering of the reads was performed with a 96% pairwise-identity cutoff to reduce overestimation of the number of bacterial species (or OTUs) largely due to 454 pyrosequencing errors.[10,40] Clustering with a 96% pairwise-identity cutoff should be applied for pyrosequencing reads obtained from other types of human microbiota.

4.2. Salivary microbiota composition in IBD patients

The abundant bacterial groups in the salivary microbiota detected in this study were similar to those previously reported,[41–44] but the compositions differed from those observed in plaque microbiota.[44] Our data clearly showed a significant difference in salivary microbiota composition between HCs and IBD patients. Shifts in oral microbiota composition were also observed in several oral manifestations such as dental caries,[45] periodontitis,[46] and oral squamous cell carcinoma.[47] Moreover, various components of the oral microbiota have been implicated in systemic diseases such as pancreatic disease including pancreatic cancer,[48] atherosclerosis,[49] bacteremia,[50] and endocarditis.[51]

Altered bacterial community structure in the gut microbiota of IBD patients is a common finding in comparison with that of healthy subjects. Previous studies showed overall structural changes as well as reduced species richness of the gut microbiota in IBD patients.[29–33] It is likely that the high microbial richness and diversity characterizing healthy microbiota may have a protective effect on humans. Unlike the gut microbiota of IBD patients, our estimates using several metrics revealed that microbial richness and diversity in the salivary microbiota of IBD patients was similar to that of HCs, despite significant changes in community structure (Fig. 1). These data suggest that the extent of the changes in the salivary microbiota is less than that in the gut microbiota of IBD patients.

Our data indicated a significant increase of the genus *Prevotella* in the salivary microbiota of IBD patients, in which its relative abundance was almost equivalent to that of reduced *Streptococcus*, which is most abundant in healthy salivary microbiota (Fig. 3). *Prevotella* is a Gram-negative, obligate anaerobe, and a member of the prevalent genera in the human microbiome.[52] Some *Prevotella* species were similarly increased, distinguishable from opportunistic infections, in bacterial vaginosis,[53] esophagitis,[54] antral gastritis,[55] and saliva of caries-active subjects.[45] These data suggest that the increase of *Prevotella,* with concurrently decreased *Streptococcus*, is clearly related with abnormal physiologies in IBD patients. The relative abundance of total Gram-positive and Gram-negative bacteria showed no significant difference between HCs and IBD patients



C. Bacterial genome specific primers for quantitative PCR.

Targeted species	Primer	Sequence (5'-3')	Tm (°C)	Product size (bp)	Annealing temp (°C)
Prevotella melaninogenica	Fw	GCTTTTGGAGCAAACAGAGG	60	234	55
	Rv	GTTGTGAATACGCGGTCCTT	60		
Haemophilus parainfluenzae	Fw	ACCGTGGTCGTTTAGCAATC	60	252	55
	Rv	GTCCGGGTTTACGTTTAGCA	60		

Figure 5. Correlation between the 16S rRNA pyrosequencing and qPCR data. The results are shown in (A) for *P. melaninogenica* and (B) for *H. parainfleuenzae*. The *y*-axis represents the copy number per nanogram of bacterial DNA obtained from qPCR data, transformed by the inverse hyperbolic sine method. The *x*-axis represents the number of reads assigned as bacterial spp. obtained from the pyrosequencing data, transformed by inverse hyperbolic sine method. Pearson product moment correlation coefficient (*r*) on transformed data (using inverse hyperbolic sine transformation) is shown. (C) Primer sequences and PCR conditions used for qPCR experiments are shown.

(Supplementary Table S3). From these results, Gram-stain properties of bacterial surface structures may not be related with dysbiosis of IBD salivary microbiota, unlike the association of Gram-negative oral bacteria with dysbiosis observed in subgingival microbiota in periodontitis.[56]

4.3. *Salivary microbiota associated with immunological biomarkers*

Saliva contains a variety of components such as cytokines, immunoglobulins, and antimicrobial proteins involved in host defence mechanisms for maintaining oral and systemic health.[57] Alteration of the salivary microbiota in IBD patients suggests the occurrence of inflammatory immune responses in the oral cavity of IBD patients as intestinal inflammation associated with aberrant gut microbiota of IBD.[23–26] Our data showed that the levels of many salivary cytokines and IgA were significantly higher in both CD and UC patients than those observed in HCs, indicating that inflammatory responses are elicited in the oral cavity of the patients. Similarly, elevated salivary IL-1β, IL-6, and TNF-α levels in CD patients and an elevated IL-8 level in the saliva of patients with bowel disease were also reported.[58,59] Unexpectedly, the elevated level of inflammatory biomarkers in UC patients was similar to or slightly higher than that observed in CD patients, regardless of differences in disease states between IBD patients (Supplementary Fig. S4 and Table S5). Salivary IgA induction was observed in CD patients with oral symptoms but not in those without oral symptoms.[60] The elevated level of IgA in most IBD patients' saliva examined suggests that those patients may have oral manifestations, however, we did not have access to their oral health clinical records.

Salivary lysozyme levels were significantly reduced in both CD and UC patients as compared with that of HCs. Lysozyme is an antimicrobial protein, expressed by various cells including neutrophils, macrophages, and epithelial cells. It is abundant in saliva and plays an important role in the host constitutive defence system.[61] It has been reported that salivary lysozyme was significantly lower in patients with gingivitis and periodontitis as compared with healthy subjects.[62] In contrast, faecal lysozyme levels were significantly elevated in IBD patients.[63] Further analysis will be required to elucidate the difference in lysozyme levels between saliva and the intestine.

Lysozyme exclusively catalyses hydrolysis of Gram-positive bacterial cell wall. However, lysozyme can also be bactericidal for Gram-negative bacteria *in vivo* through synergistic action with salivary lactoferrin in the normal state.[64] Therefore, this *in vitro* specificity of lysozyme activity may not be largely involved in the dysbiosis of salivary microbiota in IBD patients, in which the abundance of Gram-positive bacteria was not significantly different as compared with HCs (Supplementary Table S3).

There were several subgroups of patients dependent on different medical treatments, and patients with different states of disease (Supplementary Tables S1 and S2). In addition, Infliximab (anti-TNF-α antibody) therapy is commonly used for IBD patients, but up to one-third of the patients have been shown not to respond.[65] Therefore, it was very difficult to precisely evaluate the differences in microbiota structure and biomarker levels between the subgroups. Nevertheless, phylogenetic analysis based on the weighted UniFrac distance metric did not show discrete clustering of particular subgroups, such as CD patients with or without Infliximab treatment and active CD, or CD in remission, suggesting limited contributions from the patients' disease state or medical treatment to the overall microbiota structure (Fig. 2).

Strong correlations between some inflammatory biomarkers and salivary microbiota compositions were revealed (Fig. 4). The lower lysozyme and elevated IL-1β, IL-8, IgA and several other biomarkers were likely to be synergistically or interactively associated with the abundance of the four dominant genera, *Streptococcus*, *Prevotella*, *Veillonella*, and *Haemophilus*. Interactions between these microbes and other species may also be involved in the dysbiosis of salivary microbiota of IBD patients.

Finally, it is still unknown whether the inflammatory state in the oral cavity of IBD patients is the cause or a consequence of imbalances in the salivary microbiota, and which local (the oral cavity) or systemic (the gut) immune response is more responsible for the observed dysbiosis of salivary microbiota. Our results strongly suggest the existence of certain defined mechanisms by which aberrant, but similar, salivary microbiota among IBD patients is formed. The human gut microbiota is gradually shaped to its matured assemblage in a few years after birth, with temporal changes in the diversity and rank of dominant species largely dependent on diet and host physiological state.[66] Salivary microbiota may also be established similar to gut microbiota. Since $>$1000 ml of saliva is produced per day in the average adult and it always flows into the gastrointestinal tract, bacteria in saliva also have many opportunities to reach the intestine. Therefore, it can be postulated that salivary microbiota affects the development of gut microbiota to some extent. To evaluate this hypothesis, it is necessary to investigate the progression of infant salivary microbiota and the oral inflammatory state. Additionally, further studies such as comparison of the salivary microbiota between IBD and other diseases will provide informative sources for discovering non-invasive salivary biomarkers specific to IBD.

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Supplementary data: Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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References

1. Metzker, M.L. 2010, Sequencing technologies — the next generation, *Nat. Rev. Genet.*, **11**, 31–46.
2. Kunin, V., Copeland, A., Lapidus, A., Mavromatis, K. and Hugenholtz, P. 2008, A bioinformatician's guide to metagenomics, *Microbiol. Mol. Biol. Rev.*, **72**, 557–78.
3. Hamady, M. and Knight, R. 2009, Microbial community profiling for human microbiome projects: tools, techniques, and challenges, *Genome Res.*, **19**, 1141–52.
4. Kuczynski, J., et al. 2012, Experimental and analytical tools for studying the human microbiome, *Nat. Rev. Genet.*, **13**, 47–58.
5. Song, S., Jarvie, T. and Hattori, M. 2013, Our second genome – human metagenome – how next generation sequencer changes our life through microbiology, *Adv. Microb. Physiol.*, **62**, 119–44.
6. Jumpstart Consortium Human Microbiome Project Data Generation Working Group. 2012, Evaluation of 16S rDNA-based community profiling for human microbiome research, *PLoS ONE*, **6**, e39315.
7. Andersson, A.F., Lindberg, M., Jakobsson, H., Bäckhed, F., Nyrén, P. and Engstrand, L. 2008, Comparative analysis of human gut microbiota by barcoded pyrosequencing, *PLoS ONE*, **3**, e2836.
8. Wang, Y. and Qian, P.Y. 2009, Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies, *PLoS ONE*, **4**, e7401.