이학석사 학위논문

Regulation of $CD4^+$ T cell differentiation by *Bifidobacterium* species-derived metabolites

Bifidobacterium species 유래 대사물에 의한

$CD4^+$ T cell 분화 조절

2023년 8월

서울대학교 대학원

생명과학부

박주연

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지도교수 박 주 홍

이 논문을 이학석사 학위논문으로 제출함

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박주연의 이학석사 학위논문을 인준함

2023년 7월

위 원 장 노 성 훈 (인)

부위원장 박 주 홍 (인)

위 원 김 형 (인)

Abstract

Regulation of $CD4^+$ T cell differentiation by *Bifidobacterium* species-derived metabolites

Juyeon Park

Biological Science

The Graduate School

Seoul National University

Microbiota-derived metabolites play a vital role in mediating the interaction between microbes and host immunity. The *Bifidobacterium* genus is critical to the regulation of the immune system. Specifically, *Bifidobacterium pseudolongum*, *Bifidobacterium adolescentis*, and *Bifidobacterium longum* subsp. *longum* have been identified as regulators of $CD4^+$ T cell subsets, including T helper 17 cells (Th17) and regulatory T cells (Treg) involved in adaptive immunity. However, it remains unclear whether their metabolites are involved in immune modulation. Therefore, this study aimed to examine the possibility that the metabolites derived from these three *Bifidobacterium* species play a role in regulating the differentiation of Treg and Th17 cells. The results of this study demonstrated that the conditioned media of *B. pseudolongum*

promoted the differentiation of Treg cells and upregulated the expression of genes associated with Treg functions. Furthermore, the conditioned media of both *B. pseudolongum* and *B. adolescentis* significantly enhanced the differentiation of IL−17A^{+} Th17 cells. These findings suggest that *B. pseudolongum* and *B. adolescentis* may produce immunomodulatory metabolites that regulate the differentiation of CD4^{+} T cells.

Keywords :

Bifidobacterium, CD4^{+} T cells, Immune modulation, T helper 17 cell, Regulatory T cell

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Abbreviations

Th17 cell, T helper 17 cell

Treg cell, regulatory T cell

AhR, Aryl hydrocarbon receptor

OD, optical density

DMEM, Dulbecco's Modified Eagle's Medium

FBS, Fetal Bovine Serum

CM, Conditioned media

B. pseudolongum, Bifidobacterium pseudolongum

B. adolescentis, Bifidobacterium adolescentis

B. longum subsp. *longum, Bifidobacterium longum* subspcies *longum*

Chapter I. Introduction

1. Study Background

1.1. $CD4^+$ T cell differentiation in immune system

$CD4^+$ T cells are essential lymphocytes that control the overall immune response. They are matured in the thymus and enter the secondary lymphoid organ, where the T cell receptors (TCRs) recognize antigen-derived peptides displayed on the antigen-presenting cells (APCs), bound to major histocompatibility complex class II (MHCII). (Robey and Fowlkes, 1994; Itano and Jenkins, 2003) They are activated with additional co-stimulatory signals via CD28 and cytokines, and then transmit the signal to B cells and $CD8^+$ T cells to activate them, which stimulates humoral and cellular immunity. (Shahinian *et al.,* 1993) Depending on the cytokine milieu, activated $CD4^+$ T cells are differentiated into subsets of $CD4^+$ T cells, including T helper 1 (Th1), T helper 2 (Th2), T helper 17cells (Th17), which protect the host from pathogens, and regulatory T cells (Treg), which inhibit excessive inflammation mediated by T helper cells to maintain immune homeostasis. (Schmitt and Ueno, 2015)

1.2. Gut Microbiota and immune response

1.2.1. Overview of gut microbiota

Microbiota is defined as collective microbial communities living in a specific environment. (Marchesi and Ravel, 2015) In a human, the gut microbiota is composed of various microorganisms, including bacteria, fungi, and viruses. Among them, bacteria make up the majority of the gut microbiota, exerting profound impacts on host health. They

contribute to digestion, nutrient metabolism, immune regulation, and defense against pathogens.

In the intestine of a human, about 3.8×10^{13} commensal bacteria establish mutualism with approximately 70% of human immune cells. (Sender *et al.,* 2016) Over millions of years, commensal bacteria have co-evolved with humans to modulate the host immune system. (Moeller *et al.,* 2016) Accumulating evidence suggests that the gut microbiota plays a crucial role in developing immune structures. For instance, Peyer's patches, which are lymphoid tissues of the gut-associated lymphoid tissue (GALT), had a limited number of T cells in germ-free (GF) mice. (Yipp, 2012) Furthermore, the number of intraepithelial lymphocytes (Takiishi *et al.*, 2017) substantially decreased in GF mice but increased upon administration of fecal suspension from conventionally housed mice. (Umesaki *et al.,* 1993) These findings suggest that gut microbiota has a close relationship with host immunity.

1.2.2. Immune modulation by gut bacteria

There is some evidence supporting gut bacteria-mediated immune modulation. IL-17-producing Th17 cells, which protect host mucosa from the invasion of pathogens, are absent in GF mice, suggesting that the microbiota is a major inducer of the Th17 cells. (Ivanov *et al.,* 2008) Specifically, segmented filamentous bacteria (SFB) and *B. adolescentis* can induce Th17 cell differentiation via adhesion to epithelial cells. (Ivanov *et al.,* 2009; Atarashi *et al.,* 2015; Tan *et al.,* 2016) On the other hand, Treg cells suppress excessive pro-

inflammatory effector T cells to maintain intestinal homeostasis. A study reported that Treg cells are impaired in germ-free mice. (Östman *et al.,* 2006) In addition, capsular polysaccharide A (PSA) of *Bacteroides fragilis* induces the development of $FOXP3^+$ IL-10-producing Treg, which can alleviate dextran sulfate sodium (DSS)-induced colitis in mice. (Round and Mazmanian, 2010) These studies suggest that gut bacteria exert critical influences on the regulation of the host immune system, maintaining homeostasis.

1.2.3. Immunomodulatory bacterial metabolites

It is revealed that commensal bacteria produce diverse immunomodulatory metabolites that are promising factors for transmitting messages to the host immune system. (Rooks and Garrett, 2016) Recent research demonstrated that patients suffering from inflammatory bowel disease have substantially different metabolite profiles compared with those of healthy individuals, indicating a strong correlation between metabolites and the pathogenesis of immune diseases. (Heinken *et al.,* 2021). These findings emphasize the importance of understanding microbial metabolite-mediated immune modulation.

There are largely three types of bacterial metabolites that modulate immune responses. The first type is produced from the catabolism of dietary components: short-chain fatty acids (SCFAs), and tryptophan catabolites. In the colon, indigestible fiber from diet is fermented by microbes to produce SCFAs, including acetate, propionate, and butyrate. (Lewis *et al.,* 2019). Tryptophan is

catabolized by various gut bacteria, and the metabolites include indole lactic acid (ILA), indoleacetic acid (IAA), and indole aldehyde (Iald). The second type is generated by modifying host-derived bile acid, forming secondary bile acid. Lastly, the third type is produced by de novo synthesis: polyamines and branched-chain amino acids (e.g., valine, leucine, and isoleucine,) (Rooks and Garrett, 2016; Yang and Cong, 2021)

1.2.3.1. Immune regulation via bacterial tryptophan catabolites

Gut commensal bacteria can degrade tryptophan into indole derivatives in the colon. Recently, immune regulation by microbial tryptophan metabolites has been actively investigated. It has been well known that *Lactobacillus, Bacteroides,* and *Clostridium* can produce tryptophan catabolites. (Roager and Licht, 2018) Representative indole derivatives produced via tryptophan catabolism include indole lactic acid (ILA), indole aldehyde (IAld), indole propionic acid (IPA), and indole acetic acid (IAA). (Roager and Licht, 2018; Smith and Macfarlane, 1996)

These tryptophan metabolites have been reported to regulate immune responses by binding to the ligand-activated transcription factor aryl-hydrocarbon receptor (AhR), expressed in diverse cells, including lymphocytes. (Zhou, 2016) Notably, IAA, IAld, ILA, and tryptamine were demonstrated to bind AhR. (Roager and Licht, 2018) IAld produced by *Lactobacillus* was shown to induce IL-22 from innate-like cell 3 (ILC3) through an AHR-dependent pathway, which induces the secretion of antimicrobial peptides from colonic epithelial

cells to protect the mucosal barrier from bacterial pathogens. (Lee *et al.,* 2011; Zheng *et al.,* 2008; Jeong *et al.,* 2012; Zelante *et al.,* 2013)

1.3. *Bifidobacterium* and immune response

Bifidobacterium is a genus of Actinobacteria that is abundant in the human gut and colonizes mammals. In humans, three phylogenetic groups of *Bifidobacterium* primarily reside in the colon: the *B. pseudolongum* group, the *B. longum* group, and the *B. adolescentis* group. (Lugli *et al.,* 2014) Several *Bifidobacterium* species support the development of immature immunity in infants by colonizing within the first week of life. (Boesten *et al.,* 2011) For example, *B. longum* subsp. *infantis* catabolizes tryptophan and produces indole-lactic acid (ILA) in the presence of human milk oligosaccharides (HMOs), which downregulates the pro-inflammatory cytokine IL-8. (Sakurai *et al.,* 2019; Ehrlich *et al.,* 2020) Despite a decrease with age, *Bifidobacterium* species stably reside throughout life, interacting with intestinal immunity. (Lay *et al.,* 2005)

In the *Bifidobacterium* genus, most studies on immune regulation through tryptophan catabolism have primarily focused on *B. longum* subsp. *infantis*, when it utilizes human milk. However, there is limited research investigating whether the metabolites of other *Bifidobacterium* species also mediate the regulation of the immune response. For instance, *B. adolescentis* induces and accumulates Th17 differentiation in the gut through epithelial adhesion. However, it remains elusive whether their metabolites are involved in Th17

differentiation. (Tan *et al.,* 2016) In addition, *B. pseudolongum* has been reported to activate Th1 cells by producing inosine, a purine nucleoside modified from adenosine in RNA, exhibiting anti-tumor effects (Mager *et al.,* 2020; Nascimento *et al.,* 2021) while it has not been investigated whether the *B. pseudolongum*-derived metabolites regulate immunity.

Furthermore, *B. longum* subsp. *longum* is relatively well-known for its various effects on immunity. When *B. longum* BL-10, isolated from healthy infants, was orally administered to BALB/c mice, pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-17 decreased, and the anti-inflammatory cytokine IL-10 increased. (Dong *et al.,* 2022) However, it is not evident whether the mechanism is mediated by its antigens or metabolites.

Additionally, it has been reported that cell lysates of *B. longum* subsp. RAPO, isolated from a healthy human, reduced Th17 and the expression of associated genes, including RORC, IL17A (interleukin 17A), and IL23R (interleukin 23 receptor), in vitro when treating the lysates with peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis patients. (Jeong *et al.,* 2021) Although these findings suggest the possibility that *B. longum*-derived antigens affect immunity, the function of metabolites of *B. longum* on immune response is rarely known. In conclusion, there has been a lack of research dealing with the direct effects of metabolites produced by the three bacteria, *B. pseudolongum, B. longum,* and *B. adolescentis*, on the immune cells.

2. The aim of this study

The gut microbiota significantly contributes to both disease and health in the host by regulating the immune system and immune homeostasis. Among the various bacteria, the *Bifidobacterium* genus is important in immune development and modulation, colonizing the human gut throughout life. However, there has been limited research performed on the immunomodulatory effects of specific *Bifidobacterium* species, such as *B. adolescentis*, *B. pseudolongum,* and *B. longum* subsp. *longum*, through their metabolites, despite of their ability to regulate adaptive immune cells, including Th17 and Treg. The aim of this study is to identify the influence of *Bifidobacterium species*, especially *B. pseudolongum*, *B. longum* subsp. *longum*, and *B. adolescentis*, on the differentiation of Th17 and Treg cells via the bacterial conditioned media that may contain metabolites.

Chapter II. Materials and Methods

1.1. Bacterial strains

In this study, four bacterial strains were used. Two strains, *B. adolescentis* ATCC15703, and *B. longum* ATCC15707 were purchased from the American Type Culture Collection (ATCC) and isolated from adult humans. *B. pseudolongum* KGMB04129 was purchased from the Korean Gut Microbiome Bank (KGMB) and isolated from an adult human. Another *B. pseudolongum* strain was isolated from the feces of a wild-type C57BL/6 mouse.(Orientbio.) Except for the *B. pseudolongum* strains, the used bacteria were type strains. The origin of each bacterium was indicated at the end of its name.

1.2. Conditioned media preparation

All strains were incubated in an anaerobic chamber at 37℃. For propagation, all bacterial strains were grown in MRS broth and cultured overnight. DMEM (Welgene.) supplemented with 5% Fetal Bovine Serum (FBS, Welgene.) was used as a bacterial culture medium. To identify the growth curves, the optical density values were measured at 600nm and at intervals of 2~3 hours for 24 hours. To acquire bacterial conditioned media(Woodmansey *et al.*, 2004), the culture supernatant of each bacterial strain was acquired during some phases; mid-exponential phase, early-stationary phase, late-stationary phase (24 hours after cultivation), and 48 hours after cultivation. The supernatant was filtered using 0.2um syringe filters and used as the bacterial conditioned media (CM). The conditioned

media were stored in a deep freezer at about −80℃.

1.3. Aryl hydrocarbon receptor(AhR) reporter assay

1.3.1. Cell line

The HT29−Lucia AhR cell line was purchased from InvivoGen. They are derived from the human HT29 colorectal adenocarcinoma cell line. In the cell line, Lucia luciferase genes are placed under the promoter of *Cyp1a1,* one of AhR downstream genes. Therefore, when the Ahr ligands bind to the endogenous AhR of the cells, the Lucia luciferase gene is expressed, allowing for quantitative assessment via luminescence measurement. To propagate the cells, they were cultured in growth media composed of DMEM supplemented with 10% FBS, penicillin−streptomycin (Welgene.), and 100 μg/ml Normocin.

1.3.2. AhR reporter assay

To examine if the conditioned media of *Bifidobacterium* species contain AhR ligands that can activate AhR. For the reporter assay, the HT29−Lucia AhR reporter cells were resuspended in the assay medium, whose composition is the same as that of the growth medium without Normocin at a concentration of 3~4X10^5/ml. The cells were treated with the CM of *Bifidobacterium* species and incubated at 37℃. After 24 hours, QUANTI−Luc™ 4 Reagent (luciferase substrate) solution was added, and the luminescence was measured using a microplate luminometer.

The data were normalized to the average of the negative control (DMEM+FBS), and the relative activation levels were represented.

1.4. $CD4^+$ lymphocytes Isolation

Wild-type C57BL/6 mice were purchased from Orientbio, Korea. From the spleen of the mice, $CD4^+$ T lymphocytes were isolated using Dynabeads™ Untouched™ Mouse CD4 Cells Kit (Invitrogen) and differentiated into Treg and Th17 cells.

Mice were sacrificed, and their spleens were collected. The tissues were digested in 1X dPBS (Welgene) and centrifuged at 2000rpm for 5 minutes at 4℃. The pellet of spleen cells was treated with red blood cell lysis buffer (RBC buffer) for 5 minutes and washed with PBS. The cells were resuspended in the isolation buffer (Invitrogen). From the cells, $CD4^+$ T cells were purified using Dynabeads™ Untouched™ Mouse CD4 Cells Kit (Invitrogen) following the manufacturer's protocol, which uses an antibody mix that binds to other lymphocytes except for $CD4^+$ T cells and removes the other cells using antibody-binding beads. The purity of the isolated $CD3^+$ $CD45^+$ $CD4^+$ T lymphocytes among total FCS-SSC subsets was 92~93%, and viabilities were 98~99%, as detected by FACS analysis.

1.5. In vitro T cell differentiation

For *in vitro* Treg and Th17 differentiation, the purified $CD4^+$ T cells were cultured at a density of $0.95\sim1X10^6$/ml in 96-well plates, resuspended in RPMI 1640 media supplemented with 10% FBS, 1X penicillin, and streptomycin, 10mM HEPES, and 50μM β-mercaptoethanol.

To activate TCR-mediated T cell activation, 5ug/ml of anti-CD3 (BD

Horizon™ BB700 Armenian Hamster Anti-Mouse CD3e, 566494) in PBS were coated onto the 96-well plates and incubated overnight for Th17 differentiation. For Treg differentiation, in addition to the previous conditions, anti-CD28 was added. The $CD4^+$ T cells were then cultured on the plates under the following conditions:

For Th17 differerntiation: 1ug/ml of soluble anti-CD28(BD, 553294), 0.3ng/ml of recombinant mouse (rm) TGF-β1 (R&D systems, 7666-MB-005), 30ng/ml of rmIL-6 (Biolegend, 575702), 1ug/ml of anti-IFNγ (BD Biosciences, 554409), 1ug/ml of anti-IL4 (Invitrogen, 14-7041-81) with 10% bacterial conditioned media(Woodmansey *et al., 2004*) and 4-day cultivation in a CO2 incubator at 37℃ .

For Treg differentiation: 2ug/ml of soluble anti- CD28 (BD, 553294), 1ng/ml of the recombinant mouse (rm) TGF-β 1(R&D systems, 7666-MB-005), 10ng/ml of rmIL-2 (Biolegend, 575702), 2ug/ml of anti-IFNγ (BD Biosciences, 554409), 1ug/ml of anti-IL4 (Invitrogen, 14-7041-81) with 10% bacteria cultured media and 3-day cultivation in a CO2 incubator at 37℃.

1.6. Fluorescence-activated cell sorting (FACS)

To determine the differentiation of cultured cells, fluorescence-activated cell sorting (FACS) was performed using LSR Fortessa (BD Biosciences). The cells were treated with reagents sequentially and washed before adding the next reagent. For intracellular cytokine staining, only the cells cultured under the Th17 skewing condition were restimulated by incubating them in a solution including PMA

(50ng/ml), Ionomycin (1ug/ml), Brefeldin (5ug/ml) (all Sigma Aldrich) for 4 hours prior to surface staining.

First, the cells were incubated with Near-IR fluorescent reactive dye (Invitrogen) for 20 minutes at 4℃ to stain live cells. CD16/32 was then treated for 15 minutes at 4℃ to block non-specific binding by Fc receptors. Next, surface marker staining was performed. To detect $CD4^+$ T cells, a solution containing CD3-Pecy7 (BioLegend), CD45.2-FITC (Biolegend), and CD4-V500 (BD) was treated for 30 minutes at 4℃. Subsequently, fixation buffer (eBioscience) was added. After overnight incubation, intracellular transcription factors staining was performed for an hour incubation at room temperature. The followings are used antibodies: Th17, IL-17A-PECF594(BD), RORγt-BV650 (BD) and Treg, FOXP3-Percp/cy5.5 (Invitrogen), RORγt-BV650 (BD), Helios-Pacific Blue (BioLegend). The data were analyzed using Flowjo software (BD).

1.7. RNA preparation and qRT-PCR

1.7.1. RNA extraction

To detect the gene expression level, total RNA was extracted from the differentiated cells using the Rneasy mini kit (Qiagen), according to the manufacturer's protocol. (Keller *et al.,* 1993)

1.7.2. qRT-PCR

The isolated RNA was mixed with random hexamers (Sigma) and incubated for 5 minutes at 65℃ in a PCR machine. After cooling on ice, a mixture containing 5X reaction buffer, 20U SUPERase RNase

inhibitor (Invitrogen), 1mM dNTP (Thermo Scientific), and 200U RevertAid Reverse Transcriptase (Thermo Scientific) was added to synthesize cDNA. The cDNA synthesis was conducted at the following temperatures: 25℃ for 10 minutes, 42℃ for 60 minutes, and 70℃ for 10 minutes in a PCR machine.

For quantitative real-time PCR, the following genes were analyzed: *Ctla4, Il10, Cd25, Rorc, Foxp3, Maf, Cd39, Tgfb1, Gapdh, Hprt*. SYBR green qPCR master mix (RR420A, TaKaRa) was used, and the primer sequences are listed in Table 1.

The thermocycling process is composed of three stages: 1) initial denaturation at 95℃ for 30 seconds, 2) additional denaturation at 95℃, for 5 seconds 3) annealing at 60℃ for 30 seconds (repeated for 40 cycles), and 4) dissociation stage at 95℃ 15 seconds, 60℃ for a minute, and 95℃ for 15 seconds for measurement of amplification and fluorescence.

The relative quantification was determined by comparing the Ct values of each target gene to the mean Ct of the housekeeping gene. (*gapdh*, and *hprt*)

Table 1. The list of primers used in qRT−PCR

Target gene	Forward(5’−3’)	Reverse(5’−3’)	Reference
Ctla4	ACTCATGTACCCACCGCCATA	GGGCATGGTTCTGGATCAAT	Kong KF *et al.*, 2014
Il10	CCCTTTGCTATGGTGTCCTT	TGGTTTCTCTTCCCAAGACC	Murai M *et al.*, 2009
Cd25	CTGTGGTGGTTATGGGGCAG	GGGAAAACGGGGTGGACTC	Permanyer M *et al.*, 2021
Rorc	TTTGGAACTGGCTTTCCATC	AAGATCTGCAGCTTTTCCACA	Cretney E *et al.*, 2011
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT	Cretney E *et al.*, 2011
Maf	AGCAGTTGGTGACCATGTCG	TGGAGATCTCCTGCTTGAGG	Chen X *et al.*, 2022
Cd39	AGCTGCCCCTTATGGAAGAT	TCAGTCCCACAGCAATCAAA	Haas CB *et al.*, 2021
Tgfb1	ATCCTGTCCAAACTAAGGCTCG	ACCTCTTTAGCATAGTAGTCCGC	Lainé A *et al.*, 2021
Gapdh	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCGC	
Hprt	CACAGGACTAGAACACCTGC	GCTGGTGAAAAGGACCTCT	Keller *et al.*, 1993

Chapter III. Results

III. Results

1. *B. pseudolongum*-conditioned media enhance Treg differentiation and gene expression.

Metabolites are intermediates or products produced through metabolism. (Medina-Carmona *et al.,* 2023) Bacteria generally produce primary metabolites from carbon, nitrogen sources in the exponential phase, (Sanchez and Demain, 2008), and secondary metabolites from products of primary metabolites in the stationary phase. (Seyedsayamdost, 2019) In order to find out the phase during which *Bifidobacterium* species produce immunomodulatory metabolites, bacterial conditioned media (CM) from *B. pseudolongum* (mouse), *B. adolescentis* (human), and *B. longum* subsp. *longum* (human) were harvested at three distinct time points: mid-exponential phase, early-stationary phase, and late-stationary phase (24 hours after cultivation), and subsequently $CD4^+$ T cells were treated with the bacterial CM under the Treg cell polarizing condition. **(Figure 1)**

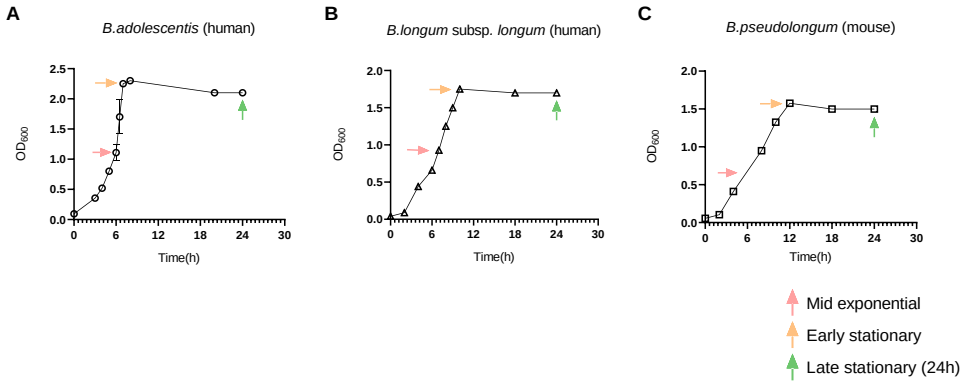

Figure 1. The growth curves and three time-points at which conditioned media of *Bifidobacterium species* were acquired.

The growth curves of **(A)** *B. adolescentis* (human), **(B)** *B. longum* subsp. *longum* (human), and **(C)** *B. pseudolongum* (mouse). Bacteria were grown on DMEM with 5% FBS medium and OD values were measured at 600nm at intervals of 2~3 hours for 24 hours. The conditioned media of the indicated bacteria were obtained at the mid-exponential phase (pink), early-stationary phase (orange), and late-stationary phase (green).

FOXP3, the master transcription factor expressed in Treg cells, (Fontenot *et al.,* 2003; Hori *et al.,* 2003) regulates the expression of genes required for the development and inhibitory functions of Treg. (Hori *et al.,* 2003) Consequently, it was observed that only the CM of *B.pseudolongum* (mouse) harvested during early- and late-stationary phases increased the differentiation of $FOXP3^+$ Treg cells. **(Figure 2A)**

In the human intestine, $ROR\gamma t^+$ $Helios^-$ $FOXP3^+$ Treg cells differentiate from $CD4^+$ T cells under the influence of gut microbiota. (Atarashi *et al.,* 2011) Based on the previous finding, the effect of *Bifidobacterium* species-CM on the differentiation of $ROR\gamma t^+$ $Helios^-$ Treg cells was examined. However, all bacterial CM failed to significantly increase the differentiation of $ROR\gamma t^+$ Treg cells. **(Figure 2B)**

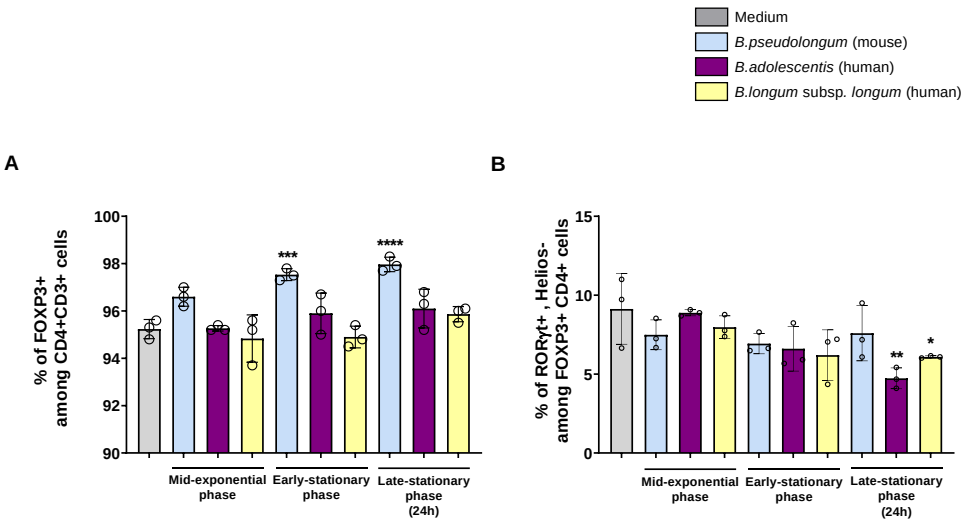

Figure 2. Mouse-derived *B.pseudolongum*-conditioned media promote FOXP3^{+} Treg under the Treg-polarizing condition.

To identify the role of *Bifidobacterium* species-CM in Treg differentiation, Treg skewing was performed. CD4^{+} T cells isolated from the spleen of wild-type C57BL/6 mice were cultured with the bacterial CM harvested at indicated phases under the condition for Treg differentiation (1ng/ml of mTGF-β1 and 10ng/ml rIL-2) for 3 days. The growth phases when the bacteria cultured media were acquired were shown on the X-axis. FACS analysis was performed and show the frequency of **(A)** Foxp3^{+} cells among the CD4^{+} CD3^{+} cell population. **(B)** Rorγt^{+} Helios^{-} cells among FOXP3^{+} CD4^{+} cell population. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). *p value < 0.05, **p value < 0.01, ***p value < 0.001, **** p value < 0.0001.

While most Treg cells express FOXP3, previous studies reported that activated $CD4^+$ T cells can also express FOXP3 without regulatory function. (Wang *et al.,* 2007; Kmieciak *et al.,* 2009) Considering these findings, multiple criteria were required to accurately examine Treg cells differentiated by the bacterial CM. Therefore, the impacts of the bacterial CM on the expression level of genes involved in Treg differentiation and regulatory function were measured via quantitative real-time polymerase chain reaction (qRT-PCR). Identified genes included *Foxp3*, *Rorγt*, and *Maf* which is involved in the final differentiation of $ROR\gamma t^+$ Treg cells and the production of IL-10 in Treg cells. (Neumann *et al.,* 2019) As a result, the CM of *B. pseudolongum* (mouse) obtained during early and late stationary phases upregulated the transcription level of *Maf*. **(Figure 3C)** However, none of the bacterial CM significantly enhanced the expression of *Foxp3* and *Rorc*. **(Figure 3A,B)**

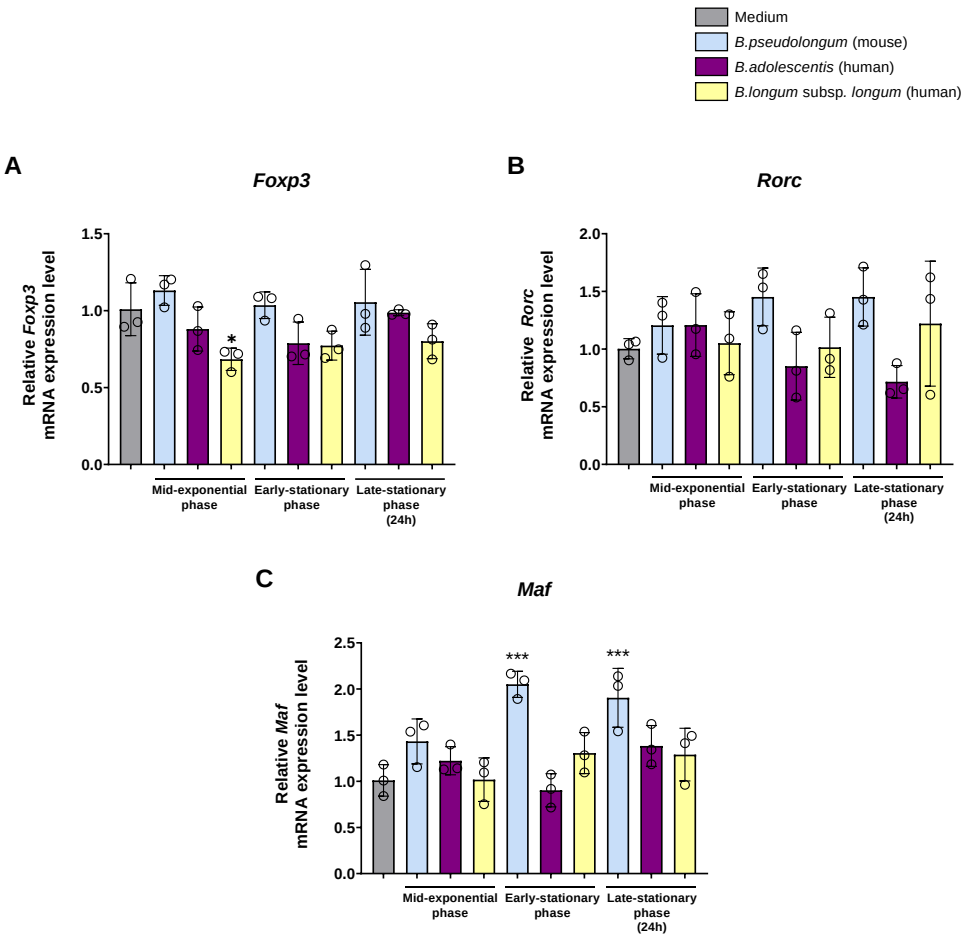

Figure 3. The impacts of the conditioned media of *Bifidobacterium* species on the expression of genes encoding transcription factors involved in differentiation.

To identify the relative mRNA expression levels of genes encoding transcription factors involved in differentiation, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. **(A)** *Foxp3*, **(B)** *Rorc*, and **(C)** *Maf*. RNA was extracted from the cells differentiated under the Treg-skewing condition(**Figure 2**) with the bacterial CM harvested at indicated phases. The growth phases when the bacteria cultured media were acquired were shown in the X-axis. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean

of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). *p value < 0.05, ***p value < 0.001.

There are representative surface molecules involved in the inhibitory functions of Treg cells. Firstly, CTLA-4 encoded by *Ctla4* (Cytotoxic T-lymphocyte antigen 4) competes with CD28 for binding to CD80 and CD86 on antigen-presenting cells to prevent the activation of nearby T cells. (Walker, 2013). Secondly, CD25 encoded by *Cd25* is an alpha chain of the IL-2 receptor and essential cytokine for the survival of T cells. (Kelly *et al.,* 2002) Highly expressed IL-2Rα inhibits the activation of other immune cells by using the cytokine, which results in the death of cells. (Thornton and Shevach, 1998; Chinen *et al.,* 2016) Lastly, CD39 encoded by *Cd39*, namely the enzyme named Ectonucleoside triphosphate diphosphoytolase-1 (ENTPD), suppresses the maturation of the dendritic cell and one the of antigen-presenting cells by hydrolyzing ATP to AMP. (Borsellino *et al.,* 2007) Also, this hydrolysis contributes to inhibiting effector T cells. (Zarek *et al.,* 2008)

To identify the effects of *Bifidobacterium* species-CM on the gene expression of surface molecules, the expression levels of genes including *Ctla4, Cd25, and Cd39* were measured. **(Figure 4A,B,C)** However, no significant increase in the expression of the genes was detected in the response to bacterial CM.

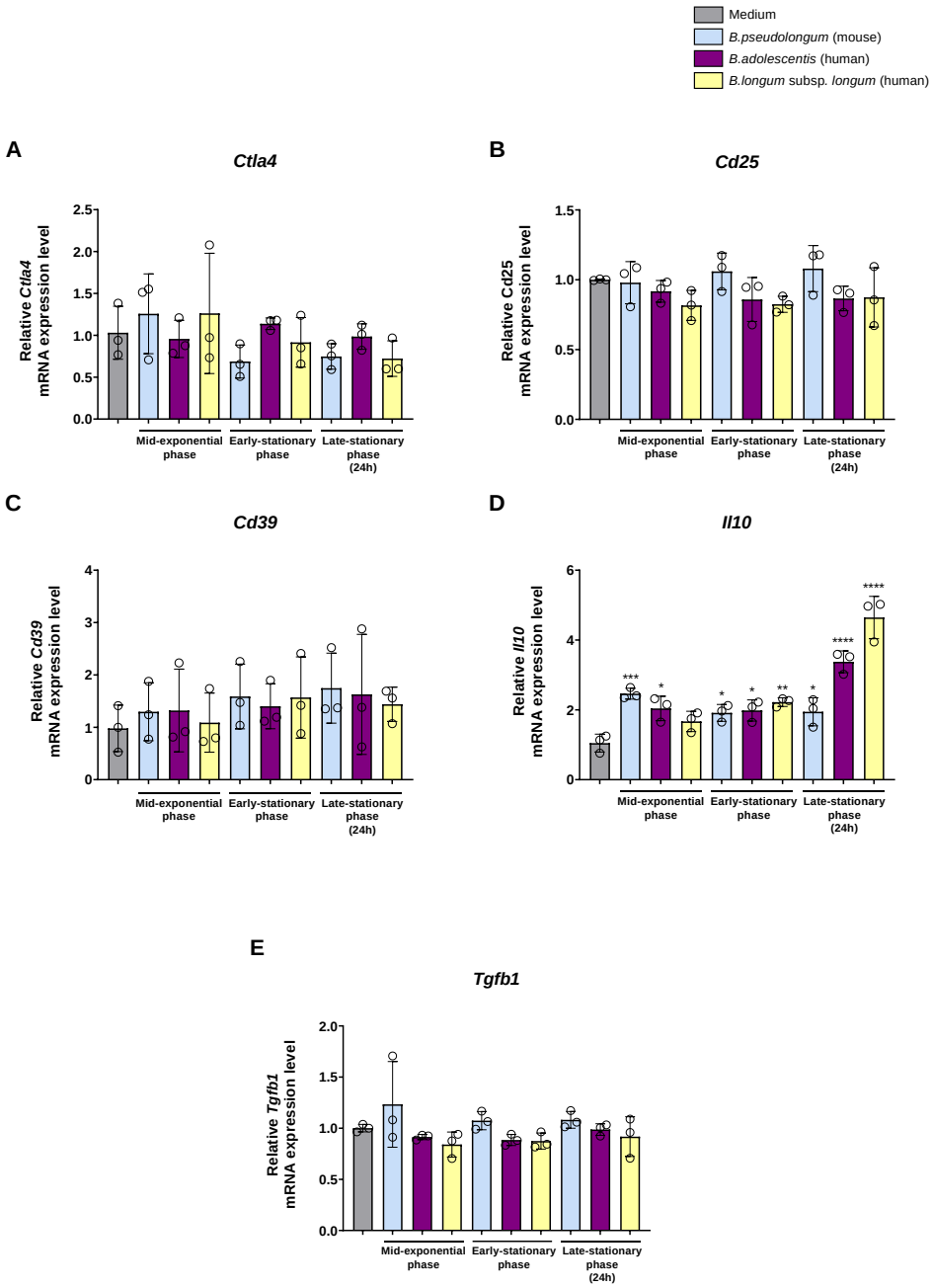

Figure 4. The impacts of the conditioned media of *Bifidobacterium* species on the expression of genes encoding regulatory molecules.

To identify the relative mRNA expression levels of genes encoding regulatory molecules, qRT-PCR was performed. (A) *Ctla4*, (B) *Cd25,* (C) *Cd39,* (D) *Il10,* (E) *Tgfb1*. RNA was extracted from the cells differentiated

under the Treg-skewing condition **(Figure 2)** with the bacterial CM harvested at indicated phase. The growth phases when the bacteria cultured media were acquired were shown in the X-axis. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). $*p$ value < 0.05, $**p$ value < 0.01, $***p$ value < 0.001, $****p$ value < 0.0001.

Next, the expression of genes encoding inhibitory cytokines expressed in Treg cells was examined: IL-10 encoded by *Il10* suppresses CD28 singling responsible for T cell activation (Taylor *et al.,* 2006); TGF-β1 encoded by *Tgfb1* inhibits T cell proliferation and differentiation. (Gorelik *et al.,* 2002) As a result, all the bacterial CM enhanced the expression of *Il10*, except for the CM of *B.longum* subsp. *longum* acquired during the mid-exponential phase. **(Figure 4D)** However, any bacterial CM did not significantly increase the expression of *Tgfb1*. **(Figure 4E)** Collectively, these findings suggest the possibility that *B.pseudolongum* (mouse) produces metabolites that promote the differentiation of $FOXP3^+$ Treg cells, and all three *Bifidobacterium* species generate metabolites that enhance *Il10* expression.

Although *B.pseudolongum* (mouse)-CM increased the differentiation of FOXP3+ Treg cells, it limitedly enhanced the expression of the genes. It is known that when gut commensal bacteria enter the stationary phase from the exponential phase, their metabolism is

shifted from saccharolysis to proteolysis. (Roager and Licht, 2018) Moreover, it has been reported that specific *Bifidobacterium* species upregulate the expression of genes involved in the transport and metabolism of amino acids during the stationary phase. (Veselovsky *et al.,* 2022) In addition, a previous study revealed that it takes 48 hours for some bacteria to deplete all tryptophan in minimal media supplemented with tryptophan, with the duration depending on bacterial species. (Mindt *et al.,*2022) Considering the results of previous findings, it can be inferred that when bacteria are incubated for 48 hours, more metabolites are expected to be accumulated, which may lead to different effects on Treg differentiation.

To confirm the expectation, *B.pseudolongum* (mouse)-CM was acquired after 48 hours of cultivation. In addition, to examine whether human-isolated *B.pseudolongum,* indicated as *B.pseudolongum* (human), exerts similar effects to *B. pseudolongum* (mouse), *the B. pseudolongum* (human) was cultured for 48 hours and its CM was obtained. The $CD4^+$ T cells were cultured with the CM of the two strains of *B.pseudolongum* under the Treg-polarizing condition. As a result, CM of the two strains of *B.pseudolongum* significantly enhanced $FOXP3^+$ Treg cell differentiation **(Figure 5B),** and especially *B.pseudolongum* (mouse)-CM increased $ROR\gamma t^+$ $Helios^-$ Treg cell differentiation. **(Figure 5A,C)** The results imply that *B. pseudolongum* species may produce metabolites that can increase Treg differentiation.

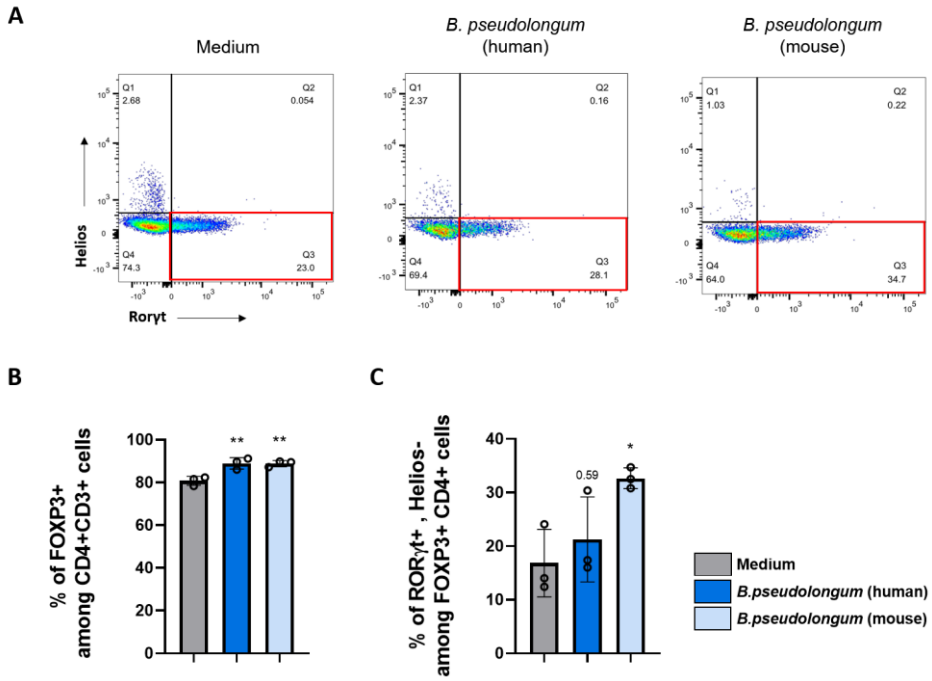

Figure 5. *B.pseudolongum*−conditioned media promote FOXP3^{+} Treg in the Treg−polarizing condition.

To identify the role of *Bifidobacterium* species−derived metabolites in Treg differentiation, Treg skewing experiments were performed. CD4^{+}T cells from the spleen of wild−type C57BL/6 mice were cultured with the 48−hour cultured media of indicated bacteria in the condition for Treg differentiation (1ng/ml of mTGF−β1 and 10ng/ml rIL−2) for 3 days. FACS analysis was performed and shows (A) the representative gating plots for Rorγt^{+} Helios^{-} cells and (B) the frequency of Foxp3^{+} cells among the CD4^{+} CD3^{+} cell population, (C) RORγt^{+} Helios^{-} cells among the FOXP3^{+} CD4^{+} population. Ordinary one−way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). *p value < 0.05, **p value < 0.01.

As for the genes encoding regulatory molecules, CM of both *B. pseudolongum* strains upregulated *Foxp3, Maf, Ctla4,* and *Il10* expression. **(Figure 6A, 6C, 7A and 7D)** *Rorc* was slightly upregulated by the *B. pseudolongum* (mouse)-CM. **(Figure 6B)** In addition, only *B. pseudolongum* (human)-CM increased *Cd39* expression. **(Figure 7C)** However, the expression levels of *Cd25* and *Tgfb1* were not raised by either of the CM of the two strains. **(Figure 7B,E)** These findings imply that *B. pseudolongum* species may generate metabolites that promote gene expression, and the effects are strain-specific.

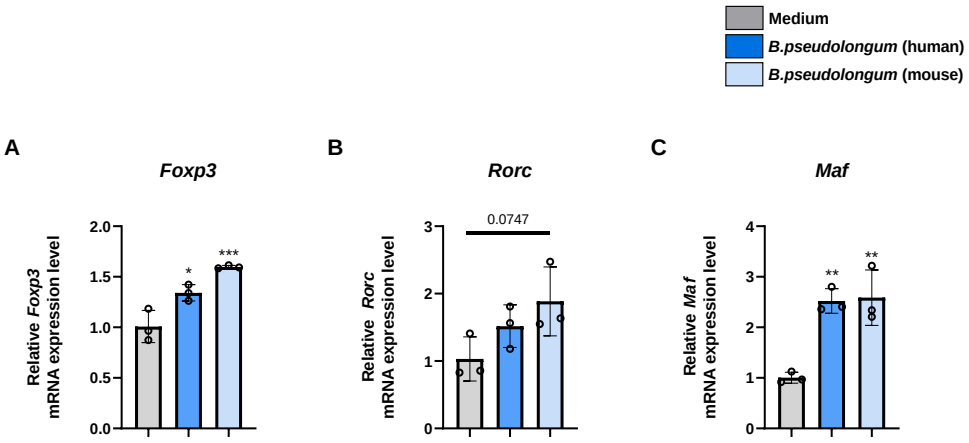

Figure 6. The impacts of *B. pseudolongum*-conditioned media on the expression of genes encoding transcription factors involved in differentiation.

To identify the relative mRNA expression levels of genes encoding transcription factors involved in differentiation, qRT-PCR was performed. (A) *Foxp3*, (B) *Rorc*, and (C) *Maf*. RNA was extracted from the cells differentiated under the Treg-skewing condition (Figure 5) with the 48-hour cultured media of indicated bacteria. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). $*p$ value < 0.05, $**p$ value < 0.01, $***p$ value < 0.001.

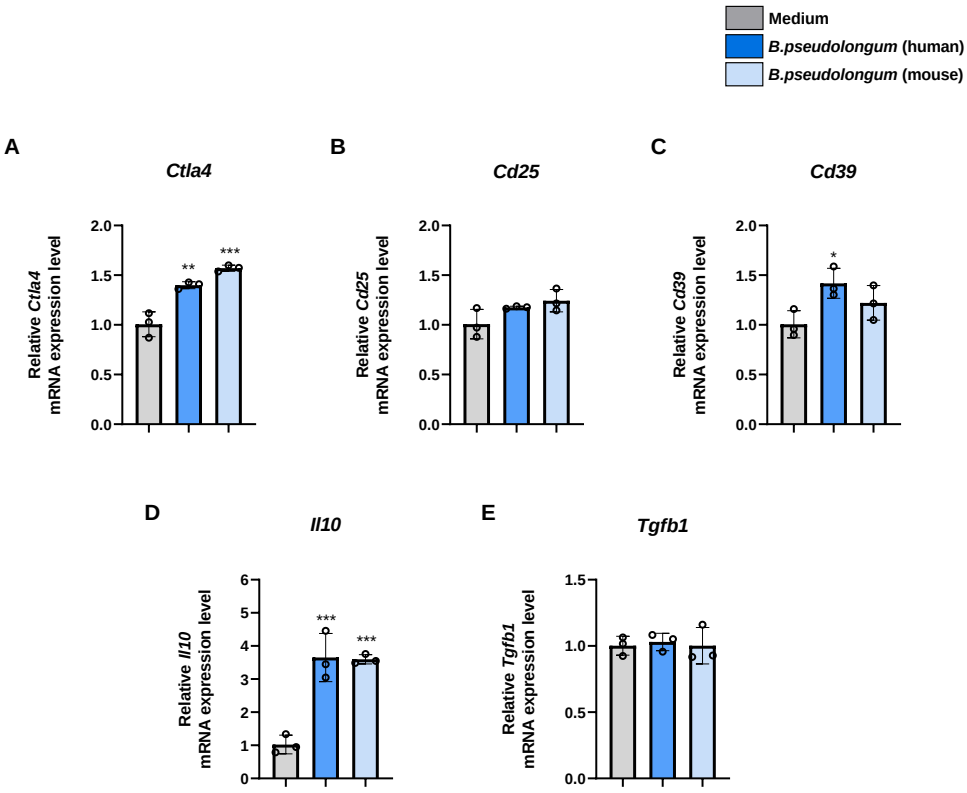

Figure 7. The impacts of *B. pseudolongum*-conditioned media on the expression of genes encoding regulatory molecules.

To identify the relative mRNA expression levels of genes encoding regulatory molecules, qRT-PCR was performed. (A) *Ctla4*, (B) *Cd25,* (C) *Cd39,* (D) *Il10,* and (E) *Tgfb1.* RNA was extracted from the cells differentiated under the Treg-skewing condition (**Figure 5**) with the 48-hour cultured media of the indicated bacteria. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of the samples is compared with the mean of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). $*p$ value < 0.05, $**p$ value < 0.01, $***p$ value < 0.001.

2. The conditioned media of *B. pseudolongum* and *B. adolescentis* promote Th17 differentiation.

To investigate whether metabolites of human-derived *Bifidobacterium* species exert influences on Th17 cell differentiation, each CM *of B.pseudolongum* (human), *B.adolescentis* (human), and *B.longum* subsp. *longum* (human) was obtained after 48-hour cultivation and treated with $CD4^+$ T cells under the Th17 cell polarizing condition.

RORγt is expressed as a master transcription factor in the Th17 cell and is responsible for producing IL-17A. (Ivanov *et al.,* 2006) IL-17A is mainly secreted from Th17 cells and plays a crucial role in the defense of the host against microbial infections on the mucosal surfaces by inducing inflammation. (Brabec *et al.,* 2023; Ishigame *et al.,* 2009)

The result showed that the CM of *B.adolescentis* (human), *B. pseudolongum* (human) promoted the differentiation of IL-$17A^+$ RORγt $^+$ Th17 cells. **(Figure 8B)** Additionally, it was observed that the IL-$17A^+$ $CD3^+$ $CD4^+$ T cells were significantly increased by the CM of human-derived *B.pseudolongum*, and *B.adolescentis*. **(Figure 8A,C)** These findings suggest that both *B.pseudolongum* (human) and *B.adolescentis* (human) may generate metabolites that promote the differentiation of IL-17A-producing Th17 cells.

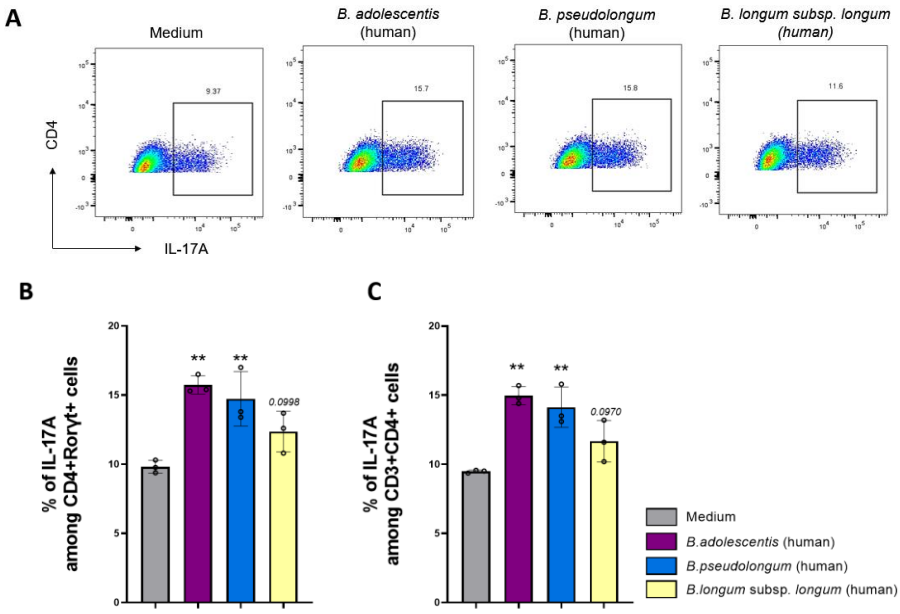

Figure 8. The conditioned media of *B. pseudolongum* and *B. adolescentis* promote Th17 cell differentiation under the Th17-polarizing condition.

To identify the role of *Bifidobacterium* species-derived metabolites in Th17 differentiation, Th17 skewing experiments were performed. $CD4^+$ T cells from the spleen of wild-type C57BL/6 mice were cultured with 48-hour cultured media of indicated bacteria under the Th17 differentiation condition (0.3ng/ml of mTGF-β1 and 30ng/ml rIL-6) for 4 days. FACS analysis was performed and shows **(A)** the representative gating plots for IL-$17A^+$ $CD4^+$ cells **(B)** the frequency of IL-$17A^+$ cells among $CD4^+$ $ROR\gamma t^+$ cell population, and **(C)** IL-$17A^+$ cells among the $CD3^+$ $CD4^+$ cell population. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean of the medium (DMEM+FBS). Shown bars are the means and SD (n=3, independent measurements). **p value < 0.01.

3. Comparative effects of *Bifidobacterium*-conditioned media on immune cell differentiation with aryl hydrocarbon receptor activation.

It has been reported that some *Bifidobacterium* species metabolize tryptophan to indole derivatives, which enable them to activate the aryl hydrocarbon receptor (AhR) of immune cells as AhR ligands. The DMEM medium used in this study contains tryptophan. Therefore, to determine whether the metabolites derived from *Bifidobacterium* species would contain the AhR ligands, an AhR reporter assay was performed. The HT-29 AhR reporter cells were cultured with the CM of *Bifidobacterium* species used for $CD4^+$ T cell differentiation. Additionally, to verify that the activation was truly AhR-dependent, CH223191, an antagonist of AhR, was treated.

As a result, AhR activation was detected in response to *B. pseudolongum* (mouse)-CM harvested during the early stationary phase, which also promoted the differentiation of $FOXP3^+$ Treg cells and the expression of several genes, such as *Maf, Il10*. **(Figure 2A, 3C, 4D, 9A)** The CM of two *B.pseudolongum* strains obtained after 48-hour cultivation effectively induced Treg cells and activated AhR. **(Figure 5, 6, 7, 9B)** These findings imply that the CM of *B. pseudolongum* species may contain metabolites that act as AhR ligands and consistently affect $CD4^+$T cell differentiation and AhR activation.

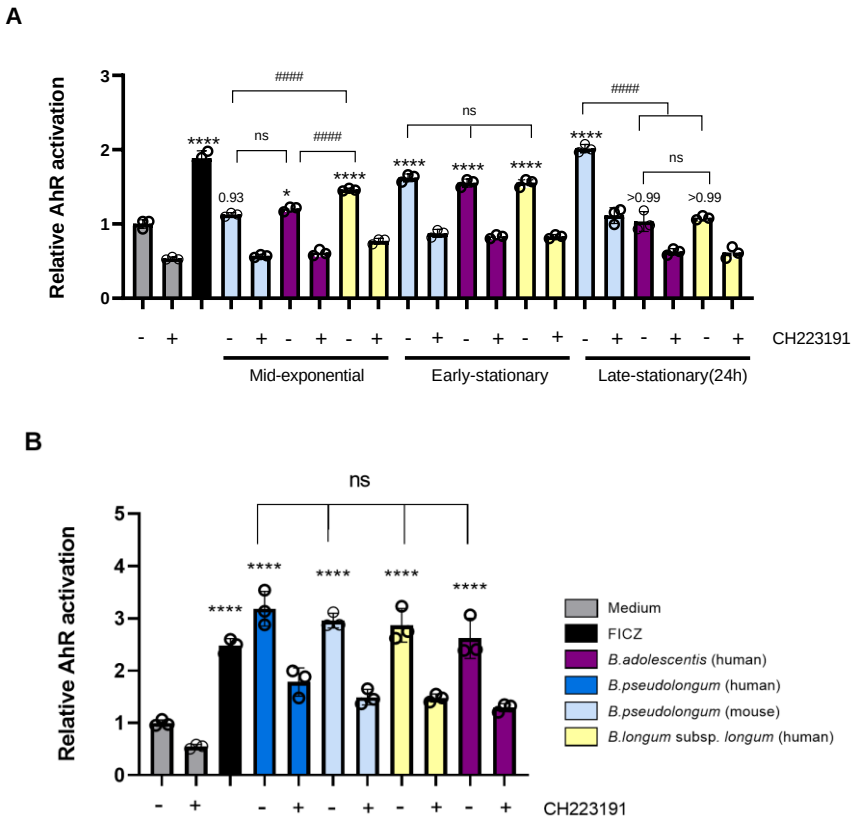

Figure 9. The conditioned media of *Bifidobacterium* species contain ligands for the aryl hydrocarbon receptor and are capable of activating the receptor. To identify whether the *Bifidobacterium* species-derived metabolites include the ligand for the aryl hydrocarbon receptor, the luciferase assay was performed using the HT29-AhR reporter cell line. The cells were cultured with the indicated bacterial cultured media, FICZ(an AhR agonist), and CH223191 (an AhR antagonist) for 24 hours. Treated bacterial cultured media are harvested at **(A)** three growth phases(mid-exponential, early-stationary, and late-stationary phases) and **(B)** 48-hour after cultivation. After that, the luminescence was assessed by a microplate luminometer, and the data were normalized to a medium-treated sample without CH223191. Ordinary one-way ANOVA was used for multiple comparisons. Each mean of samples is compared with the mean of the medium (DMEM+FBS). Shown

bars are the means and SD (n=3, independent measurements). ***p value < 0.001, **** p value < 0.0001, #### p value <0.0001.

On the contrary, the CM of *B. longum* subsp. *longum* (human) and *B. adolescentis* (human), obtained during the mid-exponential phase and early-stationary phase, activated AhR but did not affect $FOXP3^+$ Treg differentiation. **(Figure 2A, 9A)** These findings indicate that the effects of CM derived from *B. longum* subsp. *longum* (human) and *B. adolescentis* (human) on AhR activation and $CD4^+$ T cell differentiation are not consistent. In addition, the CM of the three *Bifidobacterium* species harvested after 48-hour cultivation showed similar levels of AhR activation. **(Figure 9B)** However, only *B. adolescentis* (human) and *B. pseudolongum* (human) promoted Th17 cell differentiation, implying that the CM of each *Bifidobacterium* species exhibits species-specific effects on immune regulation. **(Figure 8, 9B)**

Chapter IV. Discussion

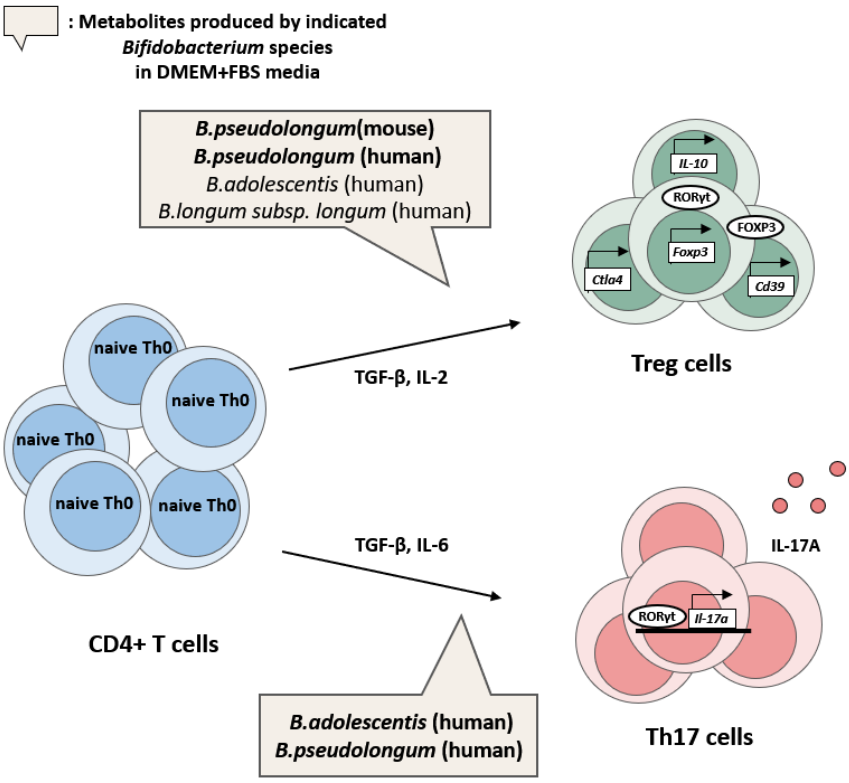

Figure 10. *Bifidobacterium* species may produce metabolites that have the ability to regulate $CD4^+$ T cell differentiation.

This model demonstrates that the metabolites derived from *Bifidobacterium* species may have impacts on $CD4^+$ T cell differentiation. *B.pseudolongum* (human) and *B.pseudolongum* (mouse) may produce metabolites increasing $FOXP3^+$ Treg differentiation and the expression of indicated genes. All the indicated *Bifidobacterium* species-derived metabolites may upregulate *Il10* expression. In addition, metabolites of *B.adolescentis* (human) and *B. pseudolongum* (human) may promote the differentiation of $ROR\gamma t^+$ $IL-17A^+$ Th17 cells.

This study shows the possibility that the *Bifidobacterium* species modulate $CD4^+$ T cell differentiation in a previously unknown way. Firstly, it was discovered that *B. pseudolongum* species-conditioned media (CM) upregulate the differentiation of $FOXP3^+$ Treg cells under the Treg-polarizing condition. However, the effects of the CM on $ROR\gamma t^+$ Treg differentiation and related gene expression were species- and strain-specific. In contrast, the CM produced by both *B. longum* subsp. *longum* (human) and *B. adolescentis* (human) did not induce Treg differentiation. Moreover, the CM generated by both human-isolated *B. pseudolongum* and *B. adolescentis* upregulated Th17 cell differentiation under the Th17-polarizing condition. These findings suggest that *B. pseudolongum* species and *B. adolescentis* (human) may produce immunomodulatory metabolites that regulate the differentiation of CD4+ T cells. **(Figure 10)**

Bifidobacterium species are important in humans. Several *Bifidobacterium* species are used in probiotic supplements due to their helpful impacts on host health, including digestion of dietary fibers, and anti-inflammatory effects. Furthermore, some species of this genus have been reported to play crucial roles in developing and modulating immune responses. Despite the importance of *Bifidobacterium* in human health, the influence of *Bfidiboacterium*-derived metabolites on adaptive immune responses has not been fully understood. For example, although *B. adolescentis* is well known to induce Th17 cells through epithelial cell attachment, (Tan *et al.,* 2016) the specific role of its metabolites in Th17 cell differentiation is

poorly understood. However, this study suggests that *B. adolescentis* (human)-derived metabolites containing AhR ligands may increase Th17 cell differentiation. In addition, there is a lack of research regarding the ability of *B. pseudolongum* to generate AhR ligands and promote immune cell development. However, this study illuminated the unexplored aspects. Moreover, previous studies have reported that *B. longum* subsp. *longum* alleviates Dextran Sulfate Sodium (DSS)-induced colitis by increasing IL-10, an inhibitory cytokine expressed by both dendritic cells and Treg cells, but the mediator was not investigated. (Srutkova *et al.,* 2015) This study suggests that *B. longum* subsp. *longum*-derived metabolites may upregulate IL-10 expression but not significantly promote Treg differentiation. In conclusion, this study expands our understanding of the potential role of metabolites of *Bifidobacterium* species in the differentiation of adaptive immune cells.

Meanwhile, the CM of two *B. pseudolongum* strains effectively promoted immune cell differentiation, which correlated with the activation of the aryl hydrocarbon receptor (AhR). This suggests the possibility that the *B. pseudolongum* affect immune responses through the AhR pathway. On the contrary, *B. longum* subsp. *longum* (human) and *B. adolescentis* (human) showed inconsistency when it comes to the promotion of immune cell differentiation and AhR activation. Moreover, when comparing two *B. pseudolongum* strains, their CM exerted different effects on the differentiation of RORγt^+ Treg and the expression of *Rorc* and *Cd39*, despite similar levels of

AhR activation. These findings suggest that *Bifidobacterium* species exhibit species and strain-specific effects on the differentiation of Th17 and Treg cells. Especially, *B. pseudolongum* (mouse) showed a greater enhancement in the differentiation of RORγt^+ Treg and the expression of *Rorc* than *B. pseudolongum* (human).

One limitation of this study is that specific metabolites that mediate $CD4^+$ T cell differentiation were not identified. However, from previous studies, it is possible to guess the potential candidates for immunomodulatory metabolites contained in CM of *Bifidobacterium* species in this study. For example, a recent study found that breastfeeding-associated *Bifidobacterium* species, such as *B. longum* subsp. *longum*, *B. longum* subsp. *infantis, B. bifidum, and B. breve,* degrade aromatic amino acids (e.g., tryptophan, phenylalanine, and tyrosine) into aromatic lactic acids (e.g., indole acetic acid [ILA], phenyl lactic acid [IPA], and 4-hydroxyphenyl acetic acid [4-OH-PLA]) via aromatic lactate dehydrogenase (ALDH) when cultured in MRS media supplemented with human milk oligosaccharides (HMOs). (Laursen *et al.,* 2021)

In addition, another study found that the type strains of *B. pseudolongum*, *B. longum* subsp. *longum* and *B. adolescentis* possess the gene *ldh*, encoding the L-lactate dehydrogenase (LDH) enzyme that catabolizes tryptophan into indole-lactic acid (ILA) (Laursen *et al.,* 2020), one of the AhR ligands. Also, if the *Bifidobacterium* species

have the ability to degrade tryptophan, the metabolites may contain other tryptophan catabolites, including indole-acetic acid (IAA), indole acetaldehyde (IAld), and tryptamine, which can activate AhR. (Roager and Licht, 2018) These findings suggest that the metabolites derived from *Bifidobacterium* species may include amino acid catabolites, which can act as AhR ligands. To find out the precise mechanism, it is necessary to identify the exact composition and proportion of molecules in the cultured media through metabolomic analysis.

Finally, the findings of this study offer insights into the roles of metabolites of *Bifidobacterium* species on $CD4^+$ T cell responses by showing the effects of bacterial CM on Treg and Th17 cell differentiation *in vitro*. However, additional *in vivo* studies are needed to understand their immunomodulatory function in a dynamic and complex *in vivo* environment containing various factors that can affect immune cell differentiation and functions.

References

Robey, E., and Fowlkes, B. J. (1994). Selective Events in T Cell Development. Annual Review of Immunology 12, 675-705.

Itano, A.A., and Jenkins, M.K. (2003). Antigen presentation to naive CD4 T cells in the lymph node. Nature Immunology 4, 733-739.

Shahinian, A., Pfeffer, K., Lee, K.P., Kündig, T.M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P.S., Thompson, C.B. , and Mak, T.W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. Science 261, 609-612.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K., and Mescher, M.F. (1999). Inflammatory cytokines provide a third signal for activation of naive $CD4^+$ and $CD8^+$ T cells. J Immunol 162, 3256-3262.

Grewal, I.S., and Flavell, R.A. (1996). The role of CD40 ligand in costimulation and T-cell activation. Immunol Rev 153, 85-106.

Phares,T.W., Stohlman, S.A., Hwang, M., Min, B. , Hinton, D.R., and Bergmann, C.C. (2012). CD4 T cells promote CD8 T cell immunity at the priming and effector site during viral encephalitis. J Virol 86, 2416-2427.

Schmitt, N., and Ueno, H. (2015). Regulation of human helper T cell subset differentiation by cytokines. Curr Opin Immunol 34, 130-136.

Marie , J.C., Letterio , J.J., Gavin , M., and Rudensky , A.Y. (2005). TGF-β1 maintains suppressor function and Foxp3 expression in $CD4^+CD25^+$ regulatory T cells. Journal of Experimental Medicine 201, 1061-1067.

Hatton, Robin D. (2011). TGF-β in Th17 Cell Development: The Truth Is Out There. Immunity 34, 288-290.

Marchesi, J.R., and Ravel, J. (2015). The vocabulary of microbiome research: a proposal. Microbiome 3, 31.

Sender, R., Fuchs, S., and Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLOS Biology 14, e1002533.

Moeller, A.H., Caro-Quintero, A., Mjungu, D., Georgiev, A.V., Lonsdorf, E.V., Muller, M.N., Pusey, A.E., Peeters, M., Hahn, B.H., and Ochman, H. (2016). Cospeciation of gut microbiota with hominids. Science 353, 380-382.

Yipp, B.G. (2012). Microbiota and Immune Cells: Friends with Benefits. Science Translational Medicine 4, 141ec119-141ec119.

Umesaki, Y., Setoyama, H., Matsumoto, S., and Okada, Y. (1993). Expansion of alpha beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. Immunology 79, 32-37.

Ivanov, I.I., Frutos, R.d.L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., and Littman, D.R. (2008). Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. Cell Host & Microbe 4, 337-349.

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.

Atarashi, K., Tanoue, T., Ando, M., Kamada, N., Nagano, Y., Narushima, S., Suda, W., Imaoka, A., Setoyama, H., Nagamori, T., *et*

al. (2015). Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. Cell 163, 367–380.

Tan, T.G., Sefik, E., Geva-Zatorsky, N., Kua, L., Naskar, D., Teng, F., Pasman, L., Ortiz-Lopez, A., Jupp, R., Wu, H.-J.J., *et al.* (2016). Identifying species of symbiont bacteria from the human gut that, alone, can induce intestinal Th17 cells in mice. Proceedings of the National Academy of Sciences 113, E8141–E8150.

Östman, S., Rask, C., Wold, A.E., Hultkrantz, S., and Telemo, E. (2006). Impaired regulatory T cell function in germ-free mice. European Journal of Immunology 36, 2336–2346.

Round, J.L., and Mazmanian, S.K. (2010). Inducible $Foxp3^{+}$ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences 107, 12204–12209.

Rooks, M.G., and Garrett, W.S. (2016). Gut microbiota, metabolites and host immunity. Nature Reviews Immunology 16, 341–352.

Heinken, A., Hertel, J., and Thiele, I. (2021). Metabolic modelling reveals broad changes in gut microbial metabolism in inflammatory bowel disease patients with dysbiosis. npj Systems Biology and Applications 7.

Weng, Y.J., Gan, H.Y., Li, X., Huang, Y., Li, Z.C., Deng, H.M., Chen, S.Z., Zhou, Y., Wang, L.S., Han, Y.P., *et al.* (2019). Correlation of diet, microbiota and metabolite networks in inflammatory bowel disease. Journal of Digestive Diseases 20, 447–459.

Lewis, G., Wang, B., Shafiei Jahani, P., Hurrell, B.P., Banie, H., Aleman Muench, G.R., Maazi, H., Helou, D.G., Howard, E., Galle-Treger, L., *et al.* (2019). Dietary Fiber-Induced Microbial Short

Chain Fatty Acids Suppress ILC2-Dependent Airway Inflammation. Front Immunol 10, 2051.

Yang, W., and Cong, Y. (2021). Gut microbiota-derived metabolites in the regulation of host immune responses and immune-related inflammatory diseases. Cellular & Molecular Immunology 18, 866-877.

Roager, H.M., and Licht, T.R. (2018). Microbial tryptophan catabolites in health and disease. Nature Communications 9, 3294.

Smith, E.A., and Macfarlane, G.T. (1996). Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. Journal of Applied Bacteriology 81, 288-302.

Zhou, L. (2016). AHR Function in Lymphocytes: Emerging Concepts. Trends in Immunology 37, 17-31.

Lee, J.S., Cella, M., McDonald, K.G., Garlanda, C., Kennedy, G.D., Nukaya, M., Mantovani, A., Kopan, R., Bradfield, C.A., Newberry, R.D., and Colonna, M. (2011). AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nat Immunol 13, 144-151.

Zheng, Y., Valdez, P.A., Danilenko, D.M., Hu, Y., Sa, S.M., Gong, Q., Abbas, A.R., Modrusan, Z., Ghilardi, N., De Sauvage, F.J., and Ouyang, W. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nature Medicine 14, 282-289.

Jeong, K.T., Hwang, S.J., Oh, G.S., and Park, J.H. (2012). FICZ, a tryptophan photoproduct, suppresses pulmonary eosinophilia and

Th2-type cytokine production in a mouse model of ovalbumin-induced allergic asthma. Int Immunopharmacol 13, 377-385.

Zelante, T., Iannitti, Rossana G., Cunha, C., De Luca, A., Giovannini, G., Pieraccini, G., Zecchi, R., D' Angelo, C., Massi-Benedetti, C., Fallarino, F., *et al.* (2013). Tryptophan Catabolites from Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity via Interleukin-22. Immunity 39, 372-385.

Lugli, G.A., Milani, C., Turroni, F., Duranti, S., Ferrario, C., Viappiani, A., Mancabelli, L., Mangifesta, M., Taminiau, B. , Delcenserie, V., *et al.* (2014). Investigation of the evolutionary development of the genus Bifidobacterium by comparative genomics. Appl Environ Microbiol 80, 6383-6394.

Boesten, R., Schuren, F., Ben Amor, K., Haarman, M., Knol, J., and De Vos, W.M. (2011). Bifidobacterium population analysis in the infant gut by direct mapping of genomic hybridization patterns: potential for monitoring temporal development and effects of dietary regimens. Microbial Biotechnology 4, 417-427.

Sakurai, T., Odamaki, T., and Xiao, J.-Z. (2019). Production of Indole-3-Lactic Acid by Bifidobacterium Strains Isolated from Human Infants. Microorganisms 7, 340.

Ehrlich, A.M., Pacheco, A.R., Henrick, B. M., Taft, D., Xu, G., Huda, M.N., Mishchuk, D., Goodson, M.L., Slupsky, C., Barile, D., *et al.* (2020). Indole-3-lactic acid associated with Bifidobacterium-dominated microbiota significantly decreases inflammation in intestinal epithelial cells. BMC Microbiology 20, 357.

Lay, C., Rigottier-Gois, L., Holmstrøm, K., Rajilic, M., Vaughan Elaine, E., de Vos Willem, M., Collins Matthew, D., Thiel, R., Namsolleck, P.,

Blaut, M., and Doré, J. (2005). Colonic Microbiota Signatures across Five Northern European Countries. Applied and Environmental Microbiology 71, 4153–4155.

Mager, L.F., Burkhard, R., Pett, N., Cooke, N.C.A., Brown, K., Ramay, H., Paik, S., Stagg, J., Groves, R.A., Gallo, M., *et al.* (2020). Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy. Science 369, 1481–1489.

Nascimento, F.P., Macedo-Júnior, S.J., Lapa-Costa, F.R., Cezar-dos-Santos, F., and Santos, A.R.S. (2021). Inosine as a Tool to Understand and Treat Central Nervous System Disorders: A Neglected Actor? Frontiers in Neuroscience 15.

Dong, J., Ping, L., Cao, T., Sun, L., Liu, D., Wang, S., Huo, G., and Li, B. (2022). Immunomodulatory effects of the Bifidobacterium longum BL-10 on lipopolysaccharide-induced intestinal mucosal immune injury. Frontiers in Immunology 13.

Yao, S., Zhao, Z., Wang, W., and Liu, X. (2021). Bifidobacterium Longum: Protection against Inflammatory Bowel Disease. Journal of Immunology Research 2021, 1–11.

Vighi, G., Marcucci, F., Sensi, L., Di Cara, G., and Frati, F. (2008). Allergy and the gastrointestinal system. Clin Exp Immunol 153 Suppl 1, 3–6.

Haque, S.Z., and Haque, M. (2017). The ecological community of commensal, symbiotic, and pathogenic gastrointestinal microorganisms – an appraisal. Clin Exp Gastroenterol 10, 91–103.

Alessandri, G., Ossiprandi, M.C., MacSharry, J., van Sinderen, D., and Ventura, M. (2019). Bifidobacterial Dialogue With Its Human Host

and Consequent Modulation of the Immune System. Front Immunol 10, 2348.

Jeong, Y., Jhun, J., Lee, S.Y., Na, H.S., Choi, J., Cho, K.H., Lee, S.Y., Lee, A.R., Park, S.J., You, H.J., *et al.* (2021). Therapeutic Potential of a Novel Bifidobacterium Identified Through Microbiome Profiling of RA Patients With Different RF Levels. Front Immunol 12, 736196.

Yu, R., Zuo, F., Ma, H., and Chen, S. (2019). Exopolysaccharide-Producing Bifidobacterium adolescentis Strains with Similar Adhesion Property Induce Differential Regulation of Inflammatory Immune Response in Treg/Th17 Axis of DSS-Colitis Mice. Nutrients 11.

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M.V. (1993). Hematopoietic Commitment During Embryonic Stem Cell Differentiation in Culture. Molecular and Cellular Biology 13, 473-486.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of $CD4^{+}CD25^{+}$ regulatory T cells. Nature Immunology 4, 330-336.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of Regulatory T Cell Development by the Transcription Factor Foxp3. Science 299, 1057-1061.

Williams, L.M., and Rudensky, A.Y. (2007). Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nature Immunology 8, 277-284.

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.

Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W., and Toes, R.E. (2007). Transient expression of FOXP3 in human activated nonregulatory $CD4^+$ T cells. Eur J Immunol 37, 129-138.

Kmieciak, M., Gowda, M., Graham, L., Godder, K., Bear, H.D., Marincola, F.M., and Manjili, M.H. (2009). Human T cells express CD25 and Foxp3 upon activation and exhibit effector/memory phenotypes without any regulatory/suppressor function. Journal of Translational Medicine 7, 89.

Neumann, C., Blume, J., Roy, U., Teh, P.P., Vasanthakumar, A., Beller, A., Liao, Y., Heinrich, F., Arenzana, T.L., Hackney, J.A., *et al.* (2019). c-Maf-dependent Treg cell control of intestinal TH17 cells and IgA establishes host-microbiota homeostasis. Nature Immunology 20, 471-481.

Walker, L.S. (2013). Treg and CTLA-4: two intertwining pathways to immune tolerance. J Autoimmun 45, 49-57.

Kelly, E., Won, A., Refaeli, Y., and Van Parijs, L. (2002). IL-2 and related cytokines can promote T cell survival by activating AKT. J Immunol 168, 597-603.

Thornton, A.M., and Shevach, E.M. (1998). $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 188, 287-296.

Chinen, T., Kannan, A.K., Levine, A.G., Fan, X., Klein, U., Zheng, Y., Gasteiger, G., Feng, Y., Fontenot, J.D., and Rudensky, A.Y. (2016). An essential role for the IL-2 receptor in Treg cell function. Nature Immunology 17, 1322-1333.

Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Höpner, S., Centonze, D., Bernardi, G., Dell'Acqua, M.L., *et al.* (2007). Expression of ectonucleotidase CD39 by Foxp3^{+} Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 110, 1225–1232.

Zarek, P.E., Huang, C.T., Lutz, E.R., Kowalski, J., Horton, M.R., Linden, J., Drake, C.G., and Powell, J.D. (2008). A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. Blood 111, 251–259.

Taylor, A., Verhagen, J., Blaser, K., Akdis, M., and Akdis, C.A. (2006). Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 117, 433–442.

Gorelik, L., Constant, S., and Flavell, R.A. (2002). Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. J Exp Med 195, 1499–1505.

Mindt, M., Beyraghdar Kashkooli, A., Suarez-Diez, M., Ferrer, L., Jilg, T., Bosch, D., Martins dos Santos, V., Wendisch, V.F., and Cankar, K. (2022). Production of indole by Corynebacterium glutamicum microbial cell factories for flavor and fragrance applications. Microbial Cell Factories 21, 45.

McGeachy, M.J., Bak-Jensen, K.S., Chen, Y., Tato, C.M., Blumenschein, W., McClanahan, T., and Cua, D.J. (2007). TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. Nature Immunology 8, 1390–1397.

Ivanov, I.I., McKenzie, B. S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL−17^{+} T Helper Cells. Cell 126, 1121−1133.

Brabec, T., Vobořil, M., Schierova, D., Valter, E., Splichalova, I., Dobeš, J., Brezina, J., Dobesova, M., Aidarova, A., Jakubec, M., *et al.* (2023). IL−17 driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum. Mucosal Immunol. S1933−0219(23)00005−3.

Ishigame, H., Kakuta, S., Nagai, T., Kadoki, M., Nambu, A., Komiyama, Y., Fujikado, N., Tanahashi, Y., Akitsu, A., Kotaki, H., *et al.* (2009). Differential Roles of Interleukin−17A and −17F in Host Defense against Mucoepithelial Bacterial Infection and Allergic Responses. Immunity 30, 108−119.

Hu, S., He, W., Du, X., Yang, J., Wen, Q., Zhong, X.−P., and Ma, L. (2017). IL−17 Production of Neutrophils Enhances Antibacteria Ability but Promotes Arthritis Development During Mycobacterium tuberculosis Infection. EBioMedicine 23, 88−99.

Chen, X., and Thibeault, S. (2013). Effect of DMSO concentration, cell density and needle gauge on the viability of cryopreserved cells in three dimensional hyaluronan hydrogel. Annu Int Conf IEEE Eng Med Biol Soc 2013, 6228−6231.

Srutkova, D., Schwarzer, M., Hudcovic, T., Zakostelska, Z., Drab, V., Spanova, A., Rittich, B. , Kozakova, H., and Schabussova, I. (2015). Bifidobacterium longum CCM 7952 Promotes Epithelial Barrier Function and Prevents Acute DSS−Induced Colitis in Strictly Strain−Specific Manner. PLOS ONE 10, e0134050.

Laursen, M.F., Sakanaka, M., von Burg, N., Mörbe, U., Andersen, D., Moll, J.M., Pekmez, C.T., Rivollier, A., Michaelsen, K.F., Mølgaard, C., *et al*. (2021). Bifidobacterium species associated with breastfeeding produce aromatic lactic acids in the infant gut. Nature Microbiology 6, 1367–1382.

Laursen, M.F., Sakanaka, M., Burg, N.v., Mörbe, U., Andersen, D., Moll, J.M., Pekmez, C.T., Rivollier, A., Michaelsen, K.F., Mølgaard, C., *et al*. (2020). Breastmilk-promoted bifidobacteria produce aromatic amino acids in the infant gut. bioRxiv, 2020.2001.2022.914994.

국문초록

Bifidobacterium species 유래 대사물에 의한 CD4^{+} T cell 분화 조절

장내미생물에서 유래한 대사물은 미생물과 숙주 면역계 간의 상호작용을 매개하는 데 중요한 역할을 담당한다. 비피도박테리움 속은 장내 세균 중 하나로 면역계 발달과 조절에 중요하다. 특히 *Bifidobacterium pseudolongum*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* subsp. *longum*은 Th17 세포와 조절 T 세포를 포함하는 적응 면역계의 CD4^{+} T 세포를 조절한다. 하지만 그들의 대사물이 이러한 면역 조절에 관여할 수 있는가에 대한 연구는 부족하다. 본 연구에서는 이 세가지 비피도박테리움 종의 배양액이 Th17 세포와 조절 T 세포에 분화에 미치는 영향을 조사했다. 그 결과, *B. pseudolongum*의 배양액이 조절 T 세포 분화를 촉진시키고, 관련된 유전자 발현을 증가시킬 수 있음을 관찰했다. 게다가, *B. pseudolongum*, *B. adolescentis*의 배양액이 인터루킨 17 A를 발현하는 Th17 세포의 분화를 향상시켰다. 따라서, *B. pseudolongum*, *B. adolescentis*의 대사물이 T 세포에 의한 면역 반응을 조절할 수 있는 가능성을 확인하였다.

주요어 :

비피도박테리움, CD4^{+} T 세포, 면역 조절, Th17 세포, 조절 T 세포

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