

Doctoral Dissertation

Academic Year 2018

Multionics analysis of microbiota-metabolites
relations and meta-analysis for the effects of
probiotics on children with atopic dermatitis

Graduate School of Media and Governance

Keio University

Yuri Yamamoto

Keio University



**Multionics analysis of microbiota-metabolites relations and
meta-analysis for the effects of probiotics on children with
atopic dermatitis**

Yuri Yamamoto

A dissertation for the degree in the Graduate School of Media and Governance
Systems Biology Program

KEIO UNIVERSITY

2018

Multionics analysis of microbiota-metabolites relations and meta-analysis for the effects of probiotics on children with atopic dermatitis

Abstract

The gastrointestinal microbiota colonize the digestive tract and are composed of approximately 10^{14} microbes and more than 400 species. These microbes are responsible for maintaining the gastrointestinal environment by facilitating digestion and interacting with the host immune system. In this study, the relationships among microbiota, their host and metabolites are analyzed from the viewpoint of molecular biology and public health. First, the effect of microbiota on atopic dermatitis is reviewed and analyzed. The results of a meta-analysis examining the effects of probiotics on children with atopic dermatitis make it difficult to conclude that probiotics have a suppressing effect on atopic dermatitis (AD) symptoms based on some qualified papers. Among these studies, the differences of patient age and country region produced the strongest effects on AD. On the other hand, it is difficult to discuss the effect of probiotics considering the differences in probiotics, as well as the differing dynamics of microbial profiles, which proved to be a major limitation of this study. Second, metabolic analysis and microbial population analysis of the murine intestinal tract components are conducted to understand the ecosystem among the host, metabolites and microbiota. Metabolic profiles have differences among each part of the intestine. Particularly, sugar shows the largest differences, and sugars are clustered into seven groups.

Keywords:

Microbiota, Atopic dermatitis, Meta-analysis, Metabolome analysis, Microbial population analysis

論文題目

腸内細菌叢-代謝物間のマルチオミクス解析と小児アトピー性 皮膚炎プロバイオティクス効果のメタアナリシス

論文要旨

腸内細菌は腸内に生息しており、量にして 10^{14} 個、400 種だと言われている。腸内環境を維持するのにとても重要であると言われており、宿主の消化作用や免疫をサポートしていると知られている。本研究では、腸内細菌を分子生物学と公衆衛生学の観点から解析し、腸内細菌と代謝物、細菌間、宿主との相互作用について調べた。まず、腸内細菌は小児アトピー性皮膚炎へのプロバイオティクス効果が期待されているので、宿主に対してどのような効果があるのかを解析した。小児アトピー性皮膚炎患者に対するプロバイオティクスのメタアナリシスの結果、本研究で扱ったいくつかの論文の場合には、プロバイオティクスの維持・改善効果があるとは言えないことを示した。本研究のリミテーションは、使用した論文の臨床試験の対象者の年齢や実施国、さらには投与した菌種が異なるため、一様に議論を行うことが難しい。また、次に SPF マウスと GF マウスの腸管を用いた部位別のメタボローム解析と細菌叢解析を行い、腸内のエコシステム内でどのような代謝物と細菌が存在するのか解析した。結果、メタボローム解析では、腸管の部位ごとに代謝物のプロファイルが大きく異なることを示した。大きく変化していたアミノ酸のと糖のプロファイルを観察したところ、アミノ酸のプロファイルは部位ごとに大きな変化はなく、ほとんどのアミノ酸が同じ挙動を示した。一方で、糖のプロファイルは、糖のサイズによって部位ごとに濃度が大きく変化していることが分かった。

キーワード:

腸内細菌、アトピー性皮膚炎、メタアナリシス、細菌叢解析、メタボローム解析

Contents

1. Introduction	1
1.1. Relationship between humans and bacteria.....	1
1.2. Objective.....	4
2. The effects of probiotics on children with atopic dermatitis.....	5
2.1. Introduction	5
2.1.1. Atopic dermatitis.....	5
2.1.2. SCORAD.....	6
2.1.3. Probiotics, prebiotics, and synbiotics.....	7
2.1.4. Systematic review	7
2.2. Methods.....	9
2.2.1. Investigated papers.....	9
2.2.2. Statistical Analysis	10
2.3. Results	14
2.4. Discussion.....	26
3. Molecular approaches for understanding murinal intestinal microbiota profiles	31
3.1. Introduction	31
3.2. Material and Methods.....	33
3.2.1. Experimental design.....	33
3.2.2. Sample collection and preparation for metabolome analysis	33
3.2.3. Instrument parameters for CE-TOFMS.....	36
3.2.4. LC-MS/MS Conditions	36
3.2.5. CE-TOFMS Conditions.....	37
3.2.6. Processing of metabolome data.....	39
3.2.7. Metabolome data analysis.....	39
3.2.8. DNA extraction, PCR amplification, and pyrosequencing of fecal samples	40
3.2.9. Analysis of microbial 16S rRNA gene sequences	42
3.2.10. Statistical analysis	42
3.3. Results	42

3.3.1. Characteristics of gastrointestinal luminal metabolites in SPF and GF mice	42
3.3.2. Comparison of luminal metabolome profiles among each section of the gastrointestinal tracts	47
3.3.3. Comparison of gastrointestinal luminal metabolites between SPF and GF mice	53
3.3.4. Features of sugars and amino acids within the gastrointestinal lumen of SPF and GF mice.....	59
3.4. Discussion.....	65
4. Conclusion.....	69
<i>Acknowledgements</i>	76
<i>References</i>	80
<i>Abbreviations</i>	86

List of Tables

Table 2.1 Checklist of CONSORT.....	12
Table 2.2 Evidence table of review.....	17
Table 2.3 CONSORT checklists for evaluating studies' quality.....	19
Table 2.4 Studies included in meta-analysis.....	23
Table 2.5 Estimator of changes of SCORAD index mean.....	25
Table 3.1 Characteristic luminal metabolites from each section of the gastrointestinal tract.....	57

List of Figures

Figure 1.1	The area of bacteria living.....	3
Figure 2.1	Evaluation sheet for SCORAD.....	13
Figure 2.2	Flow diagram of the process of selecting relevant trials.....	15
Figure 2.3	Includes the forest plots and statistics of shifts of SCORAD.....	24
Figure 3.1	Collected sample position of gastrointestinal tract.....	35
Figure 3.2	Luminal metabolome profiles of the gastrointestinal in SPF and GF mice	44
Figure 3.3	Differences of metabolites kinds in SPF and GF mice.....	45
Figure 3.4	PCA of gastrointestinal luminal metabolome profiles in SPF and GF mice.	46
Figure 3.5	Differences in luminal metabolites among parts of the gastrointestinal tract in SPF and GF mice.....	52
Figure 3.6	OPLS-DA of the luminal metabolome profiles of the gastrointestinal in SPF and GF mice.....	56
Figure 3.7	Composition of gastrointestinal microbiota in SPF mice.....	58
Figure 3.8	Clustered luminal carbohydrate profiles of the gastrointestinal in SPF mice	61
Figure 3.9	Luminal carbohydrate profiles of the gastrointestinal in SPF and GF mice	63
Figure 3.10	Luminal amino acid profiles of gastrointestinal in SPF and GF mice.....	64
Figure 4.1	Outline of experiment.....	72
Figure 4.2	Result of modeling based on LVM.....	74

Chapter 1

Introduction

1.1. Relationship between humans and bacteria

Bacteria are relevant and important to human life. Animals and plants coexist with bacteria in various ways. Animals retain bacteria on their skin to maintain the skin environment and within the intestinal tract for obtaining secondary metabolites such as vitamins (Figure 1.1). In addition, humans produce fermented food and drinks such as yogurt, miso, cheese, kimchi, wine and Japanese sake. The intestinal microbiota are a research target particularly in relation to the immune system and health. Some bacteria have functions in the management of the intestinal tract. The function is dependent on probiotics, prebiotics, and synbiotics. The most notable definition of probiotics is, “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance,” defined by Fuller (Fuller, 1989). Another notable definition states, “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host” as defined by FAO/WHO (Group, 2002). Two widely known terms that are also related to probiotics are prebiotics and synbiotics (Schrezenmeir and de Vrese, 2001). One definition of

prebiotics states, “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Synbiotics is known as the mixture of probiotics and prebiotics.

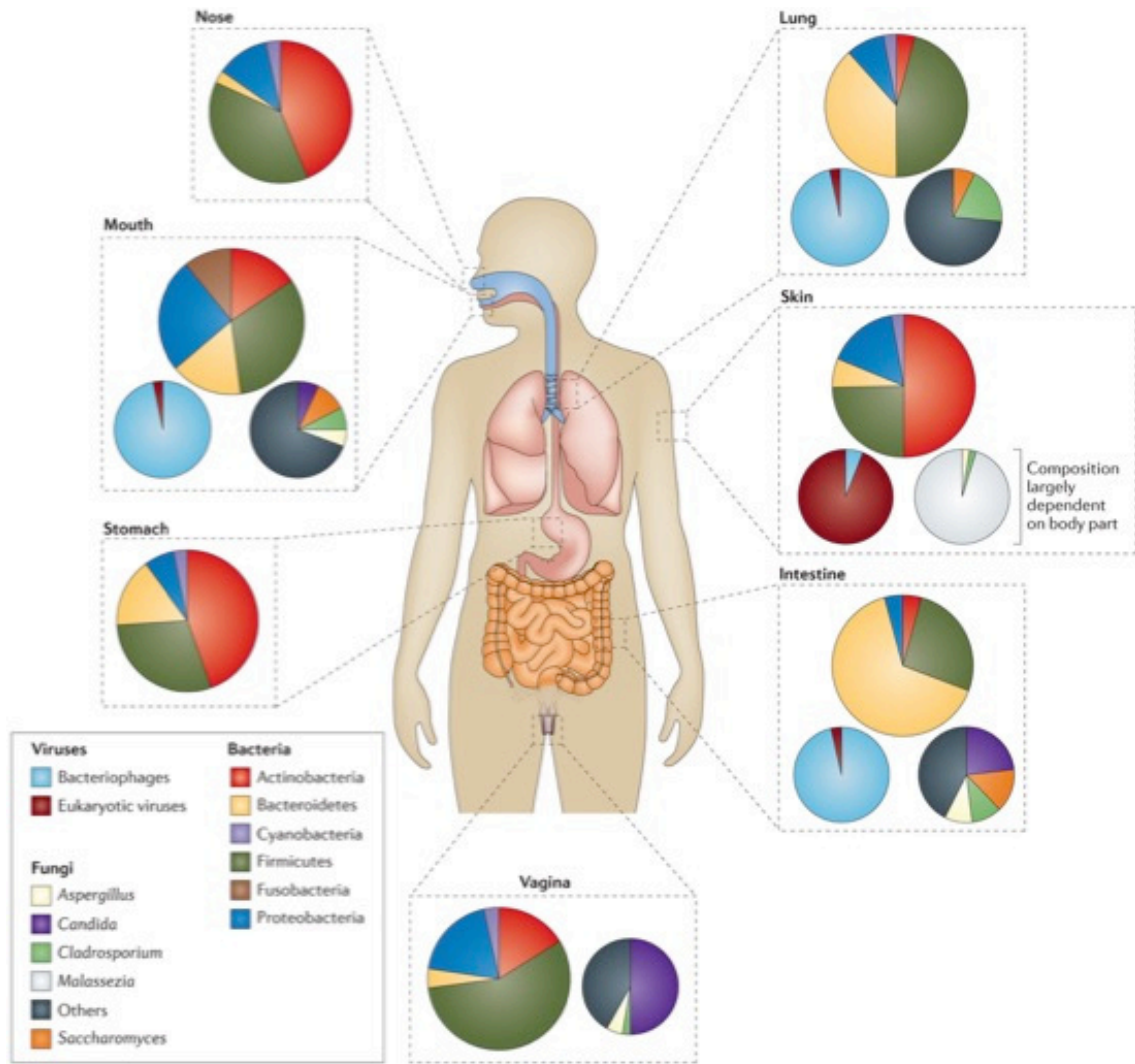


Figure 1.1 The area of bacteria living

This figure show the area of human body where microbiota live. Large circles: Bacteria, Small circle right: Fungi, Small circles left: Viruses (Marsland and Gollwitzer, 2014)

1.2. Objective

As microbiota function, they have a relationship with the host through metabolites. In this study, the relationships among microbiota, their host and metabolites are analyzed from the viewpoint of molecular biology and public health to understand the effect of intestinal microbiota. In the first section, a systematic review and meta-analysis of the effect of probiotics on children with atopic dermatitis (AD) is conducted. The purpose of this systematic review and meta-analysis is to determine the effects of probiotics on AD children. In the second research study, a luminal metabolic evaluation for each part of the intestinal tract is performed to understand the differences among them. We measured the gastrointestinal luminal metabolites across different parts of the intestinal tract in specific-pathogen-free (SPF) and germ-free (GF) mice by using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The final research study aims to apply the previous study's method (Stein et al., 2013) to this experimental data to understand the relationship between bacteria.

Chapter 2

The effects of probiotics on children with atopic dermatitis

2.1. Introduction

2.1.1. Atopic dermatitis

Atopic dermatitis (AD) is one of the most pervasive chronic allergic diseases with itching and redness (Kay et al., 1994). One of the features of this disease is that its prevalence in children is higher than that of adults. It is known that up to 10–20% of children and 1-3% of adults have AD in Europe (Leung and Bieber, 2003). Although many causes have been speculated for AD, including genetic factors, environmental factors, nutrition and intestinal microbiota, the mechanism of this disease is still unclear. As a genetic factor, it has been reported that the genetic mutation of filaggrin may cause the underlying symptoms of AD, while reducing the barrier function of the skin (Hoffjan and Stemmler, 2015). Filaggrin is an essential protein for skin barrier function, in particular, the formation of a horny layer. When the skin barrier becomes weak due to a mutation on a gene coding filaggrin, the environment is also important factor for inducing stimulation. Dysbiosis, a microbial imbalance on our body, is said

to be one of the causes of AD. Intestinal microbiota are bacteria that live inside animal intestinal tracts, and support the host's digestive function, and provide nutrition by producing secondary metabolites, as well as maintaining the homeostasis of the host's intestinal tract (Holmes et al., 2012). Additionally, it became clear that there are relationships between intestinal microbiota and some diseases, including AD (Björkstén et al., 1999; 2001; Kalliomäki et al., 2001).

2.1.2. SCORAD

The primary outcome was the change of SCORAD index (STALDER and TAIEB, 1993) from baseline to post-treatment. SCORAD, which was evaluated by 10 trained clinicians, is the major index for evaluating AD symptoms (Figure 2.1). The worst symptom corresponds to 103 points, and this score is evaluated from by extent, intensity and subjective. The severity of symptom is categorized into six fields: erythema, edema/papulation, oozing/crusts, excoriations, lichenification, and dryness. Considering the characteristic of AD symptom, the score weighting is different between under/over two years.

2.1.3. Probiotics, prebiotics, and synbiotics

Microbiota is used as probiotics for improving our health. The most notable definition of probiotics is, “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance,” defined by Fuller (Fuller, 1989).

Another notable definition states, “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host” as defined by FAO/WHO (Group, 2002). Two widely known terms that are also related to probiotics are prebiotics and synbiotics (Schrezenmeir and de Vrese, 2001). One definition of prebiotics states, “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Synbiotics is known as the mixture of probiotics and prebiotics.

2.1.4. Systematic review

For a given research target, a variety of approaches and techniques are used for investigation; however, the quality of researches is dependent upon the study design.

In the epidemiology field, two types of observational study, case-control study and cohort study, are mainly used. Case-control study identifies factors that may contribute

to a medical condition by comparing groups differing in outcome, retrospectively. On the other hand, cohort study compares outcome of two groups differing in condition by following them for a certain period of time. In addition to these designs, there is other kind of study known as intervention study. This study has two methods, nonrandomized and randomized control trials (RCT). A randomized control trial produces more quality data considering its lack of subject bias.

Furthermore, systematic review is one of the highest quality research methods used to collect and examine research data. Systematic review is different from conventional reviews at the point that evaluation is systematically conducted. Systematic review is an essential process of evidence-based medicine. The method for integrating this data and evaluating the result is called meta-analysis (Sacks et al., 1987). Meta-analysis enables to overcome the problem of subject size and the obstacle of bias among clinical trials. Summarizing the relation between outcomes of clinical trials related to AD children and probiotics is important for understanding the effect of probiotics and their mechanisms. Recently, Kim reported a meta-analysis for the relationship between AD and the effect of probiotics (Kim et al., 2014). They conducted a comprehensive study, from infant to adult, considering the AD severity and patient age. As a result, they concluded that probiotics were effective for

moderating severe AD in patients of all ages. However, as the interaction with the host intestinal environment was not considered, the children and home country were focused on in order to take into account the diversity of host intestinal microbiota. The children's profiles have less diversity than the adults' profiles (Kostic et al., 2013). The purpose of this systematic review and meta-analysis was to determine the effects of probiotics on AD children.

2.2. Methods

2.2.1. Investigated papers

PubMed and Cochrane Library were searched in June of 2015 with the following keywords: "Dermatitis, Atopic"[Mesh] OR atopic dermatitis AND "Probiotics"[Mesh], OR "Lactobacillus"[Mesh], OR "Bifidobacteriales Infections"[Mesh], OR "Lactococcus"[Mesh], OR "Saccharomyces" [Mesh], OR "Streptococcus thermophilus" [Mesh], OR "Bacillus Subtilis" [Mesh], OR "Enterococcus faecalis"[Mesh], OR "Clostridium"[Mesh]. The type of article was filtered to include only Randomized control trials. After a systematic search, a manual search was conducted. Included (1) articles of the RCT type, (2) patients of age (< 10 years) with AD, (3) cases with intervention conducted by using probiotics, (4) articles

written in English. Excluded (1) cases in which intervention was conducted by using prebiotics or synbiotics, (2) cases in which the main target was an allergy such as food allergy. WHO's definition of probiotics was used for this study, and if indigestible sugar or fibers were included as part of the intervention, these were regarded as prebiotics. Before conducting the meta-analysis, the article qualities were checked using CONSORT (Moher et al., 2010)(Table 2.1) to compare the quality of the RCTs. The nine studies that satisfied these requirements included crossover trials. When the quality of an article is checked, CONSORT does not accept crossover trials; however, CONSORT was used because the checklist for crossover trials has not yet published.

2.2.2. Statistical Analysis

The primary outcome was the change of SCORAD index (STALDER and TAIEB, 1993) from baseline to post-treatment. SCORAD index is the major index for evaluating AD symptoms (Figure 2.1). A small index size indicated that the symptom was mild, and a large index size indicated that the symptom was severe. In order to combine the results from these RCTs, the standard deviation (S.D.) of each study was calculated and incorporated. The requirement of this research was written as the average and S.D. or standard error (S.E.) of SCORAD from baseline to after treatment

in both group control and intervention. Data syntheses were based upon, either the fixed or the random effect model, according to the extent of heterogeneity (p-value of Cochrane Q-test < 0.05). All of the statistical analyses were performed by R 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria. <http://www.r-project.org/index.html>). A two sided p-value < 0.05 was set as the significant level.

Table 2.1 Checklist of CONSORT

表 ランダム化比較試験を報告する際に含まれるべき情報の CONSORT 2010 チェックリスト*
CONSORT 2010 checklist of information to include when reporting a randomized trial

章/トピック (Section/Topic)	項目番号 (Item No)	チェックリスト項目 (Checklist Item)	報告頁 (Reported on page No)
タイトル・抄録 (Title and Abstract)			
	1a	タイトルにランダム化比較試験であることを記載。	
	1b	試験デザイン (trial design), 方法 (method), 結果 (result), 結論 (conclusion) の構造化抄録 (詳細は「雑誌および会議録でのランダム化試験の抄録に対する CONSORT 声明」 ^{21, 31)} を参照)。	
はじめに (Introduction)			
背景・目的 (Background and Objective)			
	2a	科学的背景と論拠 (rationale) の説明。	
	2b	特定の目的または仮説 (hypothesis)。	
方法 (Method)			
試験デザイン (Trial Design)			
	3a	試験デザインの記述 (並行群間, 要因分析など), 割付け比を含む。	
	3b	試験開始後の方法上の重要な変更 (適格基準 eligibility criteria など) とその理由。	
参加者 (Participant)			
	4a	参加者の適格基準 (eligibility criteria)。	
	4b	データが収集されたセッティング (setting) と場所。	
介入 (Intervention)			
	5	再現可能となるような詳細な各群の介入。実際にいつどのように実施されたかを含む。	
アウトカム (Outcome)			
	6a	事前に特定され明確に定義された主要・副次的アウトカム評価項目。いつどのように評価されたかを含む。	
	6b	試験開始後のアウトカムの変更とその理由。	
症例数 (Sample size)			
	7a	どのように目標症例数が決められたか。	
	7b	あてはまる場合には, 中間解析と中止基準の説明。	
ランダム化 (Randomization)			
順番の作成 (Sequence generation)			
	8a	割振り (allocation) 順番を作成 (generate) した方法。	
	8b	割振りのタイプ: 制限の詳細 (ブロック化, ブロックサイズなど)。	
割振りの隠蔽機構 (Allocation concealment mechanism)			
	9	ランダム割振り順番の実施に用いられた機構 (番号付き容器など), 各群の割付けが終了するまで割振り順番が隠蔽されていたかどうかの記述。	
実施 (Implementation)			
ブラインディング (Blinding)			
	10	誰が割振り順番を作成したか, 誰が参加者を組入れ (enrollment) たか, 誰が参加者を各群に割付けた (assign) か, ブラインド化されていた場合, 介入に割付け後, 誰がどのようにブラインドかされていたか (参加者, 介入実施者, アウトカムの評価者など)。	
	11a	関連する場合, 介入の類似性の記述。	
	11b	関連する場合, 介入の類似性の記述。	
統計学的手法 (Statistical method)			
	12a	主要・副次的アウトカムの群間比較に用いられた統計学的手法。	
	12b	サブグループ解析や調整解析のような追加的解析の手法。	
結果 (Results)			
参加者の流れ (Participant flow)			
	13a	各群について, ランダム割付けされた人数, 意図された治療を受けた人数, 主要アウトカムの解析に用いられた人数の記述。	
	13b	各群について, 追跡不能例とランダム化後の除外例を理由とともに記述。	
募集 (Recruitment)			
	14a	参加者の募集期間と追跡期間を特定する日付。	
	14b	試験が終了または中止した理由。	
ベースライン・データ (Baseline data)			
	15	各群のベースラインにおける人口統計学的 (demographic), 臨床的な特性を示す表。	
解析された人数 (Number analyzed)			
	16	各群について, 各解析における参加者数 (分母), 解析が元の割付け群によるものであるか。	
アウトカムと推定 (Outcome and estimation)			
	17a	主要・副次的アウトカムのそれぞれについて, 各群の結果, 介入のエフェクト・サイズの推定とその精度 (95% 信頼区間など)。	
	17b	2項アウトカムについては, 絶対エフェクト・サイズと相対エフェクト・サイズの両方を記載することが推奨される。	
補助的解析 (Ancillary analysis)			
	18	サブグループ解析や調整解析を含む, 実施した他の解析の結果。事前に特定された解析と探索的解析を区別する。	
害 (Harm)			
	19	各群のすべての重要な害 (harm) または意図しない効果 (詳細は「ランダム化試験における害のよりよい報告: CONSORT 声明の拡張」 ²⁸⁾ を参照)。	
考察 (Discussion)			
限界 (Limitation)			
	20	試験の限界, 可能性のあるバイアスや精度低下の原因, 関連する場合は解析の多重性の原因を記載。	
一般化可能 (Generalisability)			
	21	試験結果の一般化可能性 (外的妥当性, 適用性)。	
解釈 (Interpretation)			
	22	結果の解釈, 有益性と有害性のバランス, 他の関連するエビデンス。	
その他の情報 (Other information)			
登録 (Registration)			
	23	登録番号と試験登録名。	
プロトコール (Protocol)			
	24	可能であれば, 完全なプロトコールの入手方法。	
資金提供者 (Funding)			
	25	資金提供者と他の支援者 (薬剤の供給者など), 資金提供者の役割。	

*本声明は, 各項目についての重要な解説を記載した CONSORT 2010 解説と詳細¹³⁾ とともに用いることを強く推奨する。クラスターランダム化比較試験¹¹⁾, 非劣性・同等性試験¹²⁾, 非薬理的治療³²⁾, ハーブ療法³³⁾, 実用的試験³⁴⁾ については, CONSORT 声明拡張版を推奨する。そのほかの拡張版も近日発表予定 (それらと本チェックリスト関連の最新情報は www.consort-statement.org を参照)。

**SCORAD
EUROPEAN TASK FORCE
ON ATOPIC DERMATITIS**

Last Name: First Name:

Date of Birth: DD/MM/YY

Date of Visit:

INSTITUTION:

PHYSICIAN:

Topical Steroid used:

Potency (brand name):

Amount / Month: (E)

Number of Flares / Month:

(8.5)

(8.5)

Figures in parentheses for children under two years

A: EXTENT Please indicate the area involved:

B: INTENSITY

CRITERIA	INTENSITY
Erythema	
Edema/Papulation	
Crusting/ooze	
Excoration	
Lichenification	
Dryness *	

MEANS OF CALCULATION

INTENSITY (ITS)

Coverage representative area

0= absence

1= mild

2= moderate

3= severe

* Dryness is evaluated on uninvolved areas

C: SUBJECTIVE SYMPTOMS

PRURITUS+SLEEP LOSS

SCORAD $A/5 + 7B/2 + C$

Visual analog scale (average for the last 3 days or nights)

PRURITUS (0 to 10) 0 10

SLEEP LOSS (0 to 10) 0 10

TREATMENT:

REMARKS:

Figure 2.1 Evaluation sheet for SCORAD

Symptom of AD is evaluated conforming to this evaluation sheet (original figure from Stalder *et al.*, 1993).

2.3. Results

Sixty-four trials were identified in total from PubMed and Cochrane Library, and nine RCTs were included in the meta-analysis (Figure 2.2, Supplementary List 1: <http://tinyurl.com/znrpexg>). 2 studies not written in English were excluded, 4 studies that were not original papers and 27 studies that differed from our purpose. Finally, 24 studies were separated into three groups, infant and mother, child, and adult. Thirteen studies categorized into the child group included 2 studies focused on infants. It is known that the profile of intestinal microbiota differs between neonates and infants, so 2 studies were excluded from the child group. Additionally, the outcome of 2 studies did not employ the SCORAD index. Although a hand search was performed for references from these nine articles, no articles satisfied the requirement (Supplementary List 2: <http://tinyurl.com/hmgmj5>).

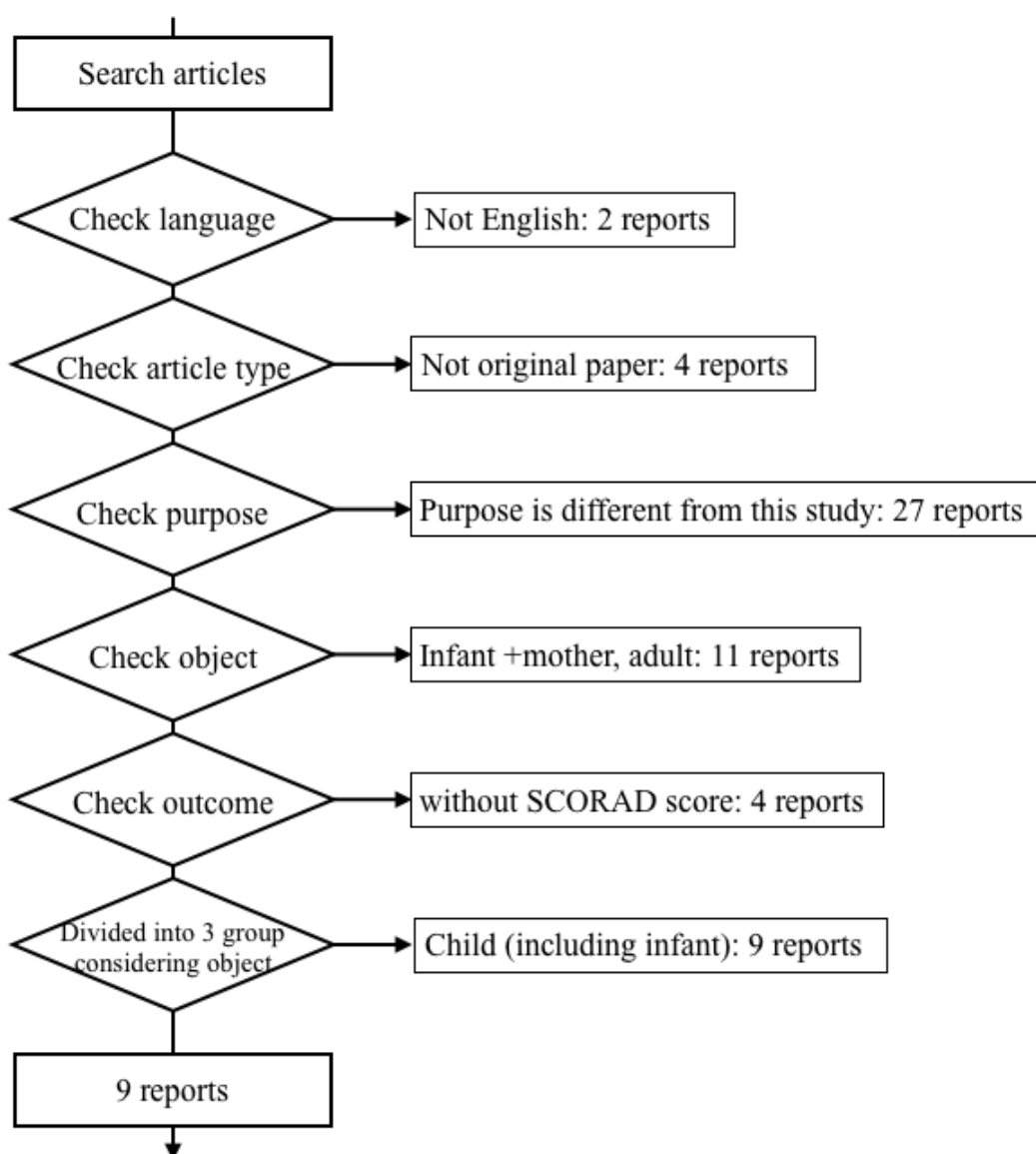


Figure 2.2 Flow diagram of the process of selecting relevant trials

In total, 64 articles were satisfactory using these keywords at the first review.

The summary of these 9 studies is shown in Table 2.2. The maximum and minimum intervention terms were six months and six weeks, respectively. There were two types of interventions, powder and capsule. When prescribed the powder, the amount and type of liquid were limited. Thirteen species of intestinal microbiota were used as probiotics. Probiotics were composed of one specific species of bacteria in five studies and two species of bacteria in four other studies. Bacteria from the *Lactobacillus* genus were used as probiotics in all the nine studies. The most frequently used bacteria in these studies were the *Lactobacillus rhamnosus* strain GG (LGG). The minimum amount of bacteria was 1×10^9 Count-Forming Units (CFU), twice a day, and the maximum amount of bacteria was 2×10^{10} CFU / g once a day. In these studies, allergy existence was also considered as baseline data, along with personal data. As usual, a steroid was used as a main treatment. Although the amount of steroid was limited, steroid uses in these studies were permitted. Within these nine studies, four studies showed improvement of the symptom outcome, and the other five studies showed no improvement. In addition, the four studies showing the effects of probiotics were focused on children > 6 months.

Table 2.2 Evidence table of review

Article	Study size	Duration	Proiotics	Amount	Intervention term (weeks)	Follow up (weeks)	Outcome		
							A	B	C
1 Følster-Holst <i>et al</i> 2006	n = 53	1-55 months	<i>Lactobacillus rhamnosus</i> strain GG	5×10 ¹⁰ CFU, twice/day	8	4	W		I
2 Gore <i>et al</i> 2011	n = 208	3-6 months	<i>Lactobacillus paracasei</i> CNCM I-2116 <i>Bifidobacterium lactis</i> CNCM I-3446	1×10 ¹⁰ CFU, everyday	12	3 years	W		I
3 Nermes <i>et al</i> 2010	n = 39	2.2-13 months	<i>Lactobacillus rhamnosus</i> strain GG ATCC 53103	3.4×10 ¹⁰ CFU, everyday	12	-	W	W	I
4 Weston <i>et al</i> 2005	n = 56	6-18 months	<i>Lactobacillus fermentum</i> VRI-033 PCC	1×10 ¹⁰ CFU, twice/day	8	8	W		E
5 Rose <i>et al</i> 2010	n = 121	6-24 months	<i>Lactobacillus rhamnosus</i> strain GG ATCC 53103	1×10 ¹⁰ CFU, twice/day	6	6	W		I
6 Larsen <i>et al</i> 2011	n = 50	7-24 months	<i>Lactobacillus acidophilus</i> NCFM ATCC SD5220 <i>Bifidobacterium animalis</i> subsp. <i>lactis</i> Bi-07	1×10 ¹⁰ CFU, everyday	8	-		W	E
7 Sistek <i>et al</i> 2006	n = 59	1-10 years	<i>Lactobacillus rhamnosus</i> <i>Bifidobacteria lactis</i>	Total 2×10 ¹⁰ CFU/g, everyday	12	4			E
8 Han <i>et al</i> 2012	n = 118	1-13 years	<i>Lactobacillus plantarum</i> CJLP133	5×10 ¹⁰ CFU, twice/day	16	2	W		E
9 Rosenfeldt <i>et al</i> 2004	n = 43	1-13 years	<i>Lactobacillus rhamnosus</i> 19070-2 <i>Lactobacillus reuteri</i> DSM 12246	1×10 ¹⁰ CFU, twice/day	8	8	W		I

Outcome A: molecular outcome, B: microbial outcome, C: Symptom outcome. W: written, I: ineffective, E: effective

Molecular outcome represented as amount of IgA. Microbial outcome included amount of microbial population. Symptom outcome included SCORAD index.

After screening, an evaluation on the quality of RCTs was conducted (Table 2.3). Only five studies within the nine studies estimated the 95% confidence interval (CI) within the SCORAD index. Weston's study reported statistic results and baseline data separately; therefore, these two reports were used for evaluation. As a result, no studies significantly lacked the necessary data. For this reason, sub-group analysis was not conducted in this study.

Table 2.3 CONSORT checklists for evaluating studies' quality

Check list		1	2	3	4	5	6	7	8	9	
Title, abstract	Title	1a	Y	n.r.	Y	n.r.	n.r.	I	Y	n.r.	
	Abstract	1b	Y	Y	Y	Y	n.r.	Y	Y	Y	
Introduction	Background	2a	Y	Y	Y	Y	Y	Y	Y	Y	
	Objectives	2b	Y	Y	Y	Y	Y	Y	Y	Y	
Methods	Trial Design	3a	Y	Y	Y	I	Y	I	Y	Y	
	Changes to trial design	3b	-	-	-	-	-	-	-	-	
	Participants	4a	Y	Y	Y	Y	n.r.	Y	Y	Y	
	Study settings	4b	n.r.	n.r.	n.r.	Y	Y	n.r.	n.r.	n.r.	
	Interventions	5	Y	Y	Y	Y	Y	Y	Y	Y	
	Outcomes	6a	Y	I	Y	Y	I	I	Y	Y	
	Changes to outcomes	6b	-	-	-	-	-	-	-	-	
	Sample size	7a	Y	n.r.	n.r.	Y	n.r.	Y	Y	Y	
	Interim analyses and stopping guidelines	7b	-	-	-	-	-	-	-	-	
	Randomisation: sequence generation	8a	n.r.	n.r.	n.r.	n.r.	n.r.	Y	n.r.	n.r.	
	Randomization: type	8b	Y	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	Y	
	Randomisation: allocation concealment mechanism	9	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	Y	n.r.	
	Randomisation: implementation	10	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	Y	n.r.	
	Blinding	11a	Y	Y	Y	Y	n.r.	Y	Y	Y	
	Similarity of interventions	11b	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	
	Statistical methods	12a	Y	Y	Y	Y	Y	n.r.	Y	Y	
	Additional analyses	12b	Y	-	-	-	-	-	-	-	
	Result	Participant Flow	13a	Y	Y	Y	Y	n.r.	Y	Y	Y
		Losses and exclusions	13b	Y	Y	Y	Y	n.r.	Y	Y	Y
Recruitment		14a	n.r.	n.r.	I	I	n.r.	I	n.r.	I	
Reason for stopped trial		14b	n.r.	Y	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	
Baseline Data		15	n.r.	Y	Y	Y	n.r.	Y	Y	Y	
Numbers analyzed		16	Y	Y	Y	Y	Y	Y	Y	Y	
Outcomes and estimation		17a	Y	Y	Y	Y	I	Y	I	Y	
Binary outcomes		17b	-	-	I	Y	-	Y	-	-	
Ancillary analyses		18	-	-	-	-	-	-	-	-	
Harms	19	n.r.	n.r.	Y	n.r.	n.r.	n.r.	n.r.	n.r.		

2. The effects of probiotics on children with atopic dermatitis

Discussion	Limitations	20	Y	n.r.	Y	Y	Y	Y	n.r.	n.r.	Y
	Generalizability	21	Y	Y	Y	Y	n.r.	n.r.	n.r.	Y	Y
	Interpretation	22	Y	Y	Y	Y	Y	Y	Y	Y	Y
Other information	Registration	23	Y	Y	n.r.	Y	n.r.	n.r.	Y	n.r.	n.r.
	Protocol	24	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
	Funding	25	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y: Written, I: Written partially, n.r.: Not reported, -: Not reported (not necessary)

1-9: Corresponded to the article's number from Table 2.2

Although the nine studies were included for evaluation, only five studies were used for meta-analysis (Table 2.4, Figure 2.3A) due to the limitation of statistical data. Four studies were not sufficiently significant to mention the results of their SCORAD index. Figure 2.3 shows the results of meta-analysis based on the changes of SCORAD index. Although heterogeneity was large in a randomized effect model, meta-analysis of entire studies did not show a significant decrease in the SCORAD index within the probiotics group compared to that within the control group (estimator: -0.27, 95% CI: -3.69 to 3.15, $p = 0.88$; Table 2.5, Figure 2.3A). This 95% CI was extended across 0; therefore, the effect of probiotics was not observed in this meta-analysis. Additionally, Han's study was the only study showing the effects of probiotics for AD, and there were no commonalities between other studies in terms of probiotics type, intervention term, or patients.

Next, sub-group analysis was conducted by age group. These nine studies were separated by the patients age, which was six months (Figure 2.3B). The heterogeneity of studies that focused on children < 6 months was small ($p = 0.96$). As the result of sub-group analysis, the estimator of SCORAD was 2.01 (95% CI: 0.89 to 3.13, $p < 0.001$; Table 2.5, Figure 2.3B) and the effects of probiotics in increasing the index are shown. The heterogeneity of studies that focused on children > 6 months

was large ($p < 0.001$). As the result of sub-group analysis, the estimator of SCORAD was -2.04 (95% CI: - 6.06 to 1.99, $p = 0.32$; Table 2.5, Figure 2.3C) and the effects of probiotics in decreasing the index are shown (Figure 2.3C). 95% CI was extended across 0; therefore, the effects of probiotics were not shown in this sub-group analysis.

As the other sub-group analysis, the patient's country of origin was focused on. In this analysis, it was found that the studies conducted in Europe showed a similar result among each trial (Figure 2.3D). The heterogeneity of the sub-group analysis was not significant ($p = 0.14$). As the result of sub-group analysis, the estimator of SCORAD was 1.53 (95% CI: 0.60 to 2.47, $p < 0.001$; Table 2.5, Figure 2.3D) and the effects of probiotics in significantly increasing the index were shown. As the result of these sub-group analyses, the common factor toward showing large heterogeneity came from including Han and Sistek's studies

2. The effects of probiotics on children with atopic dermatitis

Table 2.4 Studies included in meta-analysis

	Article(Year)	Study size	Duration	Country	Proiotics	Amount	Intervention term (weeks)	Follow up	Outcome		
									A	B	C
1	Fölster-Holst <i>et al</i> (2006)	n = 53	1-55 months	Germany	<i>Lactobacillus rhamnosus</i> strain GG	5×10 ⁹ CFU, twice/day	8	4 weeks	W	I	
2	Gore <i>et al</i> (2011)	n = 208	3-6 months	England	<i>Lactobacillus paracasei</i> CNCM I-2116	1×10 ¹⁰ CFU, everyday	12	3 years	W	I	
3					<i>Bifidobacterium lactis</i> CNCM I-3446						
4	Larsen <i>et al</i> (2011)	n = 50	7-24 months	Denmark	<i>Lactobacillus acidophilus</i> NCFM ATCC SD5220	1×10 ¹⁰ CFU, everyday	8	-	W	E	
5					<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> Bi-07						
6	Sistek <i>et al</i> (2006)	n = 59	1-10 years	New Zealand	<i>Lactobacillus rhamnosus</i> <i>Bifidobacteria lactis</i>	Total 2×10 ¹⁰ CFU/g, everyday	12	4 weeks			E
7	Han <i>et al</i> (2012)	n = 118	1-13 years	Korea	<i>Lactobacillus plantarum</i> CJLP133	5×10 ⁹ CFU, twice/day	16	2 weeks	W	E	

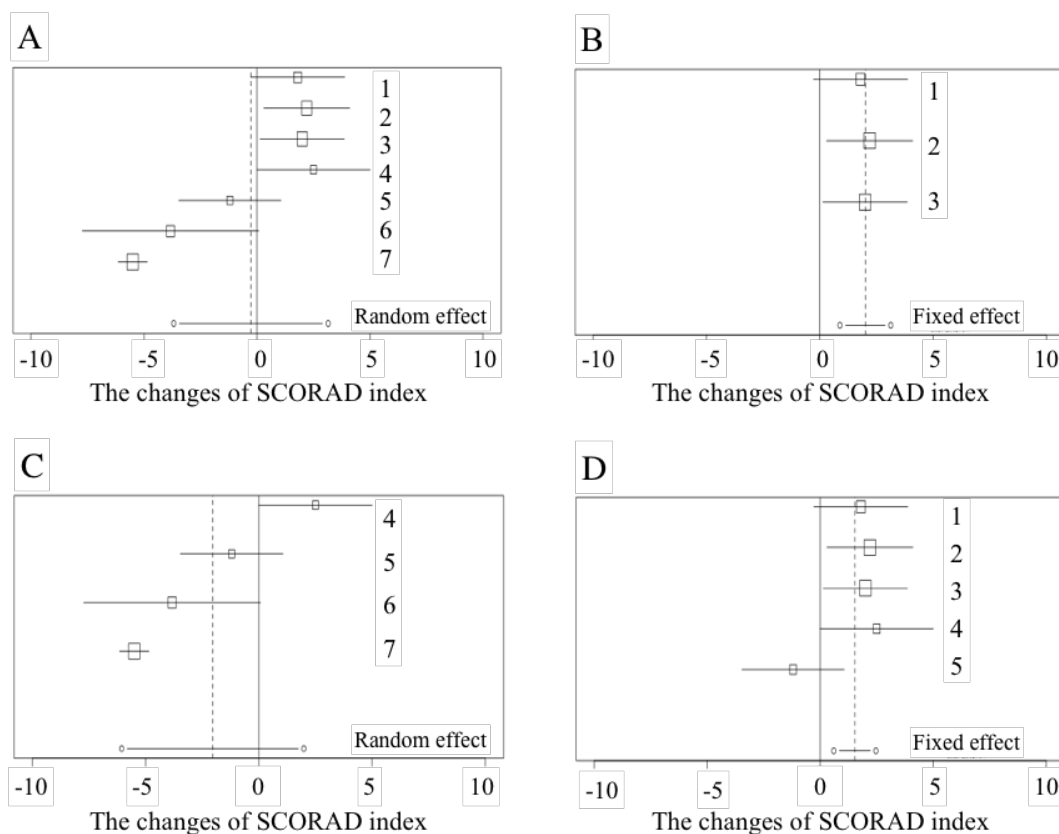


Figure 2.3 Includes the forest plots and statistics of shifts of SCORAD

A-D: Left side means negative and right side means positive. The horizontal axis shows the estimator of changes of SCORAD index mean and the box size depends on the size of population. Dotted line indicates the estimator of statistical model. (A) All, (B) < 6months, (C) > 6 months, (D) Europe.

Table 2.5 Estimator of changes of SCORAD index mean

Object	Included trial numbers	F/R	Estimator (P-value)	95% CI (Estimator)	Heterogeneity P-value
A All	7	R	-0.27 (P = 0.88)	- 3.69 / 3.15	< 0.001
B < 6 months	3	F	2.01 (P < 0.001)	0.89 / 3.13	0.96
C > 6 months	4	R	-2.04 (P = 0.32)	- 6.06 / 1.99	< 0.001
D Europe	5	F	1.5341 (P = 0.001)	0.60 / 2.47	0.14

F: Fixed effect, R: Random effect

A-D: Corresponded to the sub-group's number from Figure 2.3.

2.4. Discussion

In this study, meta-analyses was conducted and reviewed papers included five trials considering age and country region. Five trials conducted in Europe were gathered as one sub-group. The result of the sub-group analysis showed that the symptoms became worse when the European AD children took probiotics. The heterogeneity of these data was large and 95% CI was extended across 0, even when using a randomized effect model. This result suggests that it is difficult to conclude if the probiotics had an effect on AD children, as this result is consistent with Kim's previous research (Kim et al., 2014). Björkstén reported that the amount of *Enterococcus* and *Bifidobacterium* was significantly small between one-month-old and one-year-old AD children's intestinal tract (Björkstén et al., 2001), as it is said that probiotics rearrange the profiles of intestinal microbiota and suppress symptoms. Although the method of treatment was discussed, the effect was not clearly shown in the case of AD. There remains causal ambiguity, as many reports and books do not refer to the effect of probiotics for AD, and the effects were dependent on each clinical trial (Van Der Aa et al., 2010). The reasons for variation in the effects of bacteria on patients were thought to dependent on patient age, gender, race, life style, original profiles of intestinal microbiota, amount of probiotics, kinds of species, and the clinical trial design. The

features of intestinal microbiota were dependent on kinds and strains, which served to produce the disparity in data regarding the effect of these microbiota.

LGG, used in many reports, was bacillus and produced lactate. It was reported to give benefits for hosts and resistance to stomach acid and bile acid. Unfortunately, the trials that used LGG had the following three results: probiotics had effects, no effects were observed, and unclear. Therefore, LGG's effects as probiotics are still unclear. In addition, some trials did not refer to the strain of bacteria, and because the species were different among these trials, it is difficult to discuss the effect of the probiotics.

Sub-analysis was conducted according to the patient's age and country, which were thought to be a cause of heterogeneity. In the case of sub-group analysis including trials with patient age < 6 months, the changes in SCORAD index increased. This suggested that the probiotics did not have the effect of suppressing the AD symptoms, as AD became worse. In the case of sub-group analysis including trials with object age > 6 months, the heterogeneity was small and the changes in SCORAD index did not increase. This suggested that probiotics had the effect of suppressing the AD symptoms, as AD did not become worse. The clinical use of probiotics is defined as intake of bacteria known to benefit us, and the effects of probiotics were thought to

depend on the host intestinal environment and immune system. It is known that the host microbial profiles change relative to the host age, and it was reported that aerobic bacteria increased first, followed by anaerobic bacteria that increased through oxygen consumption, as reported by the study focused on the host's growth (Turroni et al., 2012). Smith reported that microbial patterns were almost the same and not dependent on the host animal (Smith, 1965).

Additionally, the result of sub-group analysis considering the patient's country showed that the heterogeneity was small. The changes in SCORAD index increased. Comparing the heterogeneity of meta-analysis including whole trials and sub-analysis by considering the country, the size of heterogeneity decreased. This sub-group segmentation, which considered patient country regions, was different from Kim's research, and suggested that the country regions are a possible factor in changing the size of heterogeneity. Considering the results of the sub-group analysis, it seems that one of the causes for effects of bacteria on patients was the host intestinal environment. There were already some reports focused on the relationship among the microbial profiles, diet, and country, and the intestinal microbial profiles were divided into three types and the differences were dependent on the diet (Arumugam et al., 2011). It may also be noted that Japanese intestinal microbiota have a potential to

digest seaweed such as laver (Hehemann et al., 2010). The country region and race are important factors in understanding the details of systems related to intestinal microbiota.

There were some similarities within the large heterogeneity. For example, when data were integrated and the heterogeneity was large, studies by Han and Sistek were included for analysis. From this result, it was suggested that these two trials most directly affected the size of heterogeneity. These two trials were difficult to integrate because there were many discrepancies in the data, because the reliability was low, as the countries in which the trials were performed were Korea and New Zealand. *Lactobacillus plantarum* CJLP133, used in Han's study, was isolated from Kimchi and suggested to control the immune system (Lee et al., 2011). In addition, this strain was also reported to have the effect of suppressing AD in an AD mouse by oral intervention (Won et al., 2011). This strain also demonstrated the effect of curing dermatitis-like AD by inducing house dust and mites on an NC/Nga mouse (Won et al., 2012).

In summary, clinical trials that focused on the effects of probiotics on infant AD were screened systematically. Among these studies, the differences of patient age and country region produced the strongest effects on AD. On the other hand, it is difficult to discuss the effect of probiotics considering the differences in probiotics, as

well as the differing dynamics of microbial profiles, which proved to be a major limitation of this study. As the number of trials using the same strain of probiotics increases around the world, it will become easier to analyze and discuss the effect of probiotics. In addition, the effect of host microbial profiles was considered in this study; therefore, it is important to focus on these profiles, as well as changes to the host environment.

Chapter 3

Molecular approaches for understanding murinal intestinal microbiota profiles

3.1. Introduction

Intestinal microbiota mainly refer to the bacteria inhabiting the intestinal tract of mammals; these bacteria support various functions in the host ranging from digestion to the maintenance of a robust immune function (Holmes et al., 2012). Recently, there have been many reports regarding the metabolites secreted by intestinal microbiota. For example, Japanese people possess specific bacteria that have the necessary gene for digesting laver and soft seaweed (Hehemann et al., 2010). Intestinal microbiota have also been reported in relation to host diseases such as obesity (Ridaura et al., 2013; Turnbaugh et al., 2006), diabetes (Forslund et al., 2015; Le Chatelier et al., 2013; Qin et al., 2012), atherosclerosis (Koeth et al., 2013; Wang et al., 2011; 2016), atopic dermatitis (Björkstén et al., 1999; 2001; Kalliomäki et al., 2001), immune system disorders (Cahenzli et al., 2013; Thorburn et al., 2015), and brain function disorders (Buffington et al., 2016; Hsiao et al., 2013; Kadowaki et al., 2016; Sampson et al., 2016). The human intestinal tract is a long organ, and microbial diversity is distinct

within each section of the intestinal tract and dependent on its corresponding function. In the case of humans, *Lactobacillus* accounts for a large percentage of microbes within the stomach, and *Enterobacteriaceae* spp. and *Bacteroides* are observed at high levels in the upper small intestine. In the lower small intestine, *Bifidobacterium* spp. and *Enterococcus* spp. exist at a high percentage. Additionally, *Enterococcus* spp., *Enterobacteriaceae* spp., *Clostridium* spp., and *Bacteroides* spp. are observed at a high percentage within the cecum and colon (Metges, 2000). Although there are many reports focused on the differences among varying parts of the intestine, these reports primarily focused on the stomach, cecum and colon or only included information solely about either the metabolite or microbial profile (Devaraj et al., 2013; Gu et al., 2013; Matsumoto et al., 2012; Tian et al., 2012). In reports that focused on the intestinal differences with regard to location, pyrosequencing was used to evaluate the differences in intestinal contents derived from the stomach, the three parts of the small intestine, the cecum, the colon and fecal samples. However, metabolome profiling throughout the gastrointestinal tract has not been conducted. Therefore, in this study, we performed luminal metabolic evaluation for each section of the intestinal tract to understand the differences among each part of intestinal tract. We measured gastrointestinal luminal metabolites across different parts of the intestinal tract in

specific-pathogen-free (SPF) and germ-free (GF) mice using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

3.2. Material and Methods

3.2.1. Experimental design

The animal experiment was performed using protocols approved by Central Institute for Experimental Animals (CIEA) (Tokyo, Japan). Germ-free (GF) male C57BL/6J mice and specific pathogen-free (SPF) male mice were purchased from CIEA Japan Inc. These GF mice were maintained in germ-free isolators at CIEA during experiment. GF and SPF mice were housed individually in each cage and fed with normal diet (CA-1, CIEA Japan) *ad libitum*. Diet and water were both irradiated and autoclaved. Ethics numbers were 09043 and 12036.

3.2.2. Sample collection and preparation for metabolome analysis

Twelve-week-old male GF and SPF mice (n=3) were sacrificed for resecting intestinal contents via an abdominal incision. The intestinal tract was separated into six parts; stomach (St), upper small intestine (Si1), lower small intestine (Si2), cecum (Ce),

upper colon (Co1) and lower colon (Co2) (Figure 3.1). Intestinal contents were stored at -80°C until use. Extraction for intestinal metabolites was as described previously with slight modifications (Nagao-Kitamoto et al., 2016). Intestinal contents were run with 1 mL of 0.957 mM PBS contained 200 mM of internal standards (methionine sulfone and D-camphor-10-sulfonic acid) for metabolome analysis by using syringe. 500 µL of sample solution was mixed with 500 µL of 100% methanol, then vortexed with 100 mg of 0.1 mm zirconia beads. The contents were centrifuged at 14,000 g for 5 min. Supernatant was transferred to a centrifugal filter tube (UltrafreeMC-PLHCC 250/pk for Metabolome Analysis, Human Metabolome Technologies) to remove protein and lipid molecules, and then centrifuged at 4,600 x g for 3 hours at 25 °C. Filtrates were centrifugally concentrated and dissolved in 30 µL of ultrapure water including reference compounds (200 µM each of 3-aminopyrrolidine and trimesic acid) immediately before capillary electrophoresis with electrospray ionization CE-TOFMS analysis. Concentrations of sugars in samples were determined by LC-MS/MS in negative multiple reaction monitoring mode on an API3000 triple quadrupole mass spectrometer (AB Sciex). All of LC-MS/MS data were acquired using Analyst Software (AB Sciex).

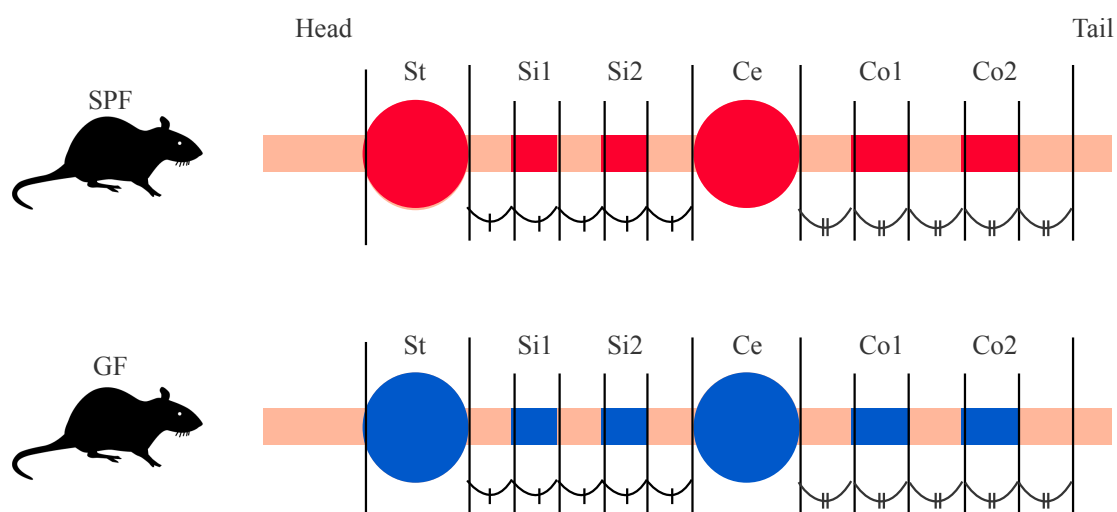


Figure 3.1 Collected sample position of gastrointestinal tract

Intestinal tract was separated some parts. Samples were obtained from colored parts. Red and blue indicate SPF and GF mice, respectively. Left side shows head and right side shows tail. St: stomach, Si1: upper small intestine, Si2: lower small intestine, Ce: cecum, Co1: upper colon, Co2: lower colon.

3.2.3. Instrument parameters for CE-TOFMS

Metabolome analysis was conducted as described previously with some modifications (Sugimoto et al., 2010). All CE-TOFMS experiments were based on Agilent CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), an Agilent G3250 AA LS/MSD TOF system (Agilent Technologies, Palo Alto, CA), an Agilent 1100 series binary HPLC pump which delivers sheath liquid, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The CE-MS adapter kit included a capillary cassette that facilitates thermostating of the capillary. The CE-ESI-MS sprayer kit simplifies the connecting CE system and the MS system, which were equipped with an electrospray ionization source. For system control and data acquisition, G2201AA Agilent Chem-Station software for CE and Analyst QS software for TOFMS were used.

3.2.4. LC-MS/MS Conditions

The mobile phase consisted of 0.5% (v/v) formic acid as solution A and acetonitrile as solution B. The gradient increased from 0% B to 99% for taking 20 minutes and then

retained at 99% until 23 minutes. The flow rate used was 0.2 ml/min and the injection volume was 1 μ l as described previously (Sugimoto et al., 2012).

3.2.5. CE-TOFMS Conditions

The CE-TOFMS conditions were as described previously with slight modification (Sugimoto et al., 2010). In the case of anionic metabolites, a commercially available COSMO(+) capillary (50 μ m i.d. \times 110 cm) (Nacalai Tesque, Kyoto, Japan), chemically coated with a cationic polymer, was used as the separation capillary. A 50 mM ammonium acetate solution (pH 8.5) was the electrolyte for CE separation. A new capillary was flushed successively with the running electrolyte, 50 mmol/L acetic acid (pH 3.4), and then the electrolyte again for 10 min each. Before each injection, the capillary was equilibrated for 2 min by flushing with 50 mM acetic acid (pH 3.4) and then for 5 min by flushing with the running electrolyte. In the case of cationic metabolites, fused-silica capillaries (50 μ m i.d. \times 100 cm total length) filled with 1 mol/L formic acid as the reference electrolyte was used as the separation capillary. After preparation the capillaries, sample solutions (30 nL) were injected at 50 mbar for 3 seconds, and a voltage of 30 kV, and -30 kV was applied. The capillary temperature was maintained at 20°C, and the temperature of the sample tray was kept

3. Molecular approaches for understanding murinal intestinal microbiota profiles

below 5°C. The sheath liquid, composed of methanol/water (50% v/v) and 0.1 µmol/L hexakis (2,2-difluoroethoxy) phosphazene (Hexakis), was delivered at 10 µL/min. ESI-TOF-MS was conducted in the positive ion mode and 5 mM ammonium acetate (50% v/v) methanol/water containing 0.1 µM Hexakis was derived at 10 µL/min in negative ion mode. The capillary voltage was set at 4 kV and 3500V, for cationic and anionic metabolites, respectively. In TOF-MS, the fragmenter, skimmer, and OCT RF voltage were set at 75, 50, and 125 V, respectively in positive ion mode, and at 100, 50, and 200 V, respectively in negative ion mode. A flow rate of drying nitrogen gas (heater temperature = 300°C) was maintained at 10 L/min. In the case of positive ion mode, automatic recalibration of each acquired spectrum was performed using reference masses of reference standards ($[^{13}\text{C}$ isotopic ion of protonated methanol dimer (2MeOH + H)]⁺, m/z 66.06371) and ([protonated Hexakis (M + H)]⁺, m/z 622.02896). In the case of negative ion mode, automatic recalibration of each acquired spectrum was performed using reference masses of reference standards ($[^{13}\text{C}$ isotopic ion of deprotonated acetic acid dimer (2CH₃COOH - H)]⁻, m/z 120.03841), and ([Hexakis + deprotonated acetic acid (CH₃COOH - H)]⁻, m/z 680.03554). Exact mass data were acquired at a rate of 1.5 spectra/s over a m/z 50-1000 range. The alignment

of detected peaks was performed according to the m/z value and normalized migration time.

3.2.6. Processing of metabolome data

Raw data were analyzed with our proprietary software: MasterHands (Sugimoto et al., 2010). Data were processed with noise-filtering, baseline correction, peak detection, and integration of the peak area from sliced electropherograms (m/z 0.02 width). Subsequently, the accurate m/z value for each peak detected within the time domain was calculated with Gaussian curve-fitting to the peak along the m/z axis. Spike noise, CE-specific noise showing small and narrow peaks, and low-quality (not peak-like shape) results were also removed. For the remaining features, metabolite identities were assigned by matching their m/z values and migration times with those of standard compounds.

3.2.7. Metabolome data analysis

SIMCA version 9.0.2 (SAS Institute, Cary, NC) and Multi Experiment Viewer (MeV TM4) (Saeed et al., 2003) were used for orthogonal projections to latent structures - discriminant analysis (OPLS-DA) and heat map analysis and clustering analysis and

K-means analysis, respectively. Metabolic pathway analysis was performed according to Kyoto Encyclopedia of Genes and Genomes (KEGG) and significant metabolites with p-value lesser than 0.1 were analyzed by MetaCore.

3.2.8. DNA extraction, PCR amplification, and pyrosequencing of fecal samples

DNA extraction was described as previously with slight modifications (Kato et al., 2014). Freeze-dried fecal samples were homogenized with mixture buffer [200 μ L 10% SDS/TE, 200 μ L 3M NaAc and 400 μ L phenol/chloroform/IAA] at 1,500 rpm for 5 min at 4°C and then centrifuged at 14,000 rpm for 5 min at 4°C. After transferring supernatant into new tubes and adding 400 μ L phenol chloroform/IAA and vortexed for 1 min. Samples were then centrifuged at 14,000 rpm for 10 min at 4°C. After transferring supernatant into new tubes again, 800 μ L 100% EtOH was added and placed on ice for 15 min. The liquid was then centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was discarded and 500 μ L 70% EtOH was added to desiccate the pellet. The suspension was centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was then discarded and the pellet left to dry after which 100 μ L TE was added and left at 4°C overnight. The V1-V2 region (310 bp) of 16S rRNA genes were amplified from genomic DNA samples using bacterial universal primer set 27F (5'-AGAGTTTGAT

3. Molecular approaches for understanding murinal intestinal microbiota profiles

CMTGGCTCAG-3') and 338R (5'-TGCTGCCTCC CGTAGGAGT-3') (Kim et al., 2013; Murakami et al., 2015). PCR was performed with Tks Gflex DNA Polymerase (Takara Bio Inc.) and amplification proceeded with an initial denaturation step at 96°C for 2 min, followed by 15 or 20 cycles of 96°C for 30 sec, 55°C for 45 sec and 72°C for 1 min, with a final extension step at 72°C for 10 min. They were visualized on agarose gels (2% in TE Buffer), purified with a QIAamp PCR Purification Kit. Purified PCR products were tagged with unique barcode labels using forward primer (5'-CCATCTCATC CCTGCGTGTC TCCGACTCAG NNNNNNNNNN agagtttgat cmtggctcag -3') containing the 454 primer A and reverse primer (5'- CCTATCCCCT GTGTGCCTTG GCAGTCTCAG tgctgcctcc cgtaggagt -3') containing the 454 primer B by PCR reaction that was 2 min at 96°C, 5 cycles of 96°C for 30 s, 55°C for 45 s, and 72°C for 1min, and a final extension of 72°C for 10 min. Prior to sequencing, 454 tagged PCR products were cleaned again with Agencourt AMPure XP (Beckman Coulter, Inc., Brea, CA, USA) and quantified using the Quant-iT PicoGreen DNA assay (Invitrogen, Carlsbad, CA). Following quantization, the amplicon from each reaction mixture were pooled in equimolar ratios based on concentration and subjected to emulsion PCR to generate amplicon libraries. Amplicon pyrosequencing was

performed on the Roche 454 GS Junior Titanium platform (Roche, Branford, CT, USA) according to the manufacturer's specifications.

3.2.9. Analysis of microbial 16S rRNA gene sequences

Sequence reads with an average Q-value < 25 were filtered out. Reads were then processed using quantitative insights into microbial ecology (QIIME) (v1.8.0) pipeline (Caporaso et al., 2010). Sequences were then clustered into operational taxonomic units (OTUs) using 97% sequence similarity and OTUs were assigned to taxonomy using Ribosomal Database Project (RDP) classifier (Wang et al., 2007).

3.2.10. Statistical analysis

Statistical analysis was performed with MATLAB version R2015a (The MathWorks, Inc., United States). P-values < 0.05 were considered as statistically significant, with $0.5 < p\text{-value} < 0.1$ as the tendency. The correlation network based on Spearman's correlation coefficient was constructed using R software.

3.3. Results

3.3.1. Characteristics of gastrointestinal luminal metabolites in SPF and GF mice

A metabolome analysis of gastrointestinal contents by CE-TOFMS and LC-MS/MS identified 344 and 308 metabolites, which were detected from SPF and GF mice, respectively (Figure 3.2). Additionally, 295, 269, 285, 300, 260, and 260 metabolites were detected in the stomach, upper small intestine, lower small intestine, cecum, upper colon and lower colon, respectively, from both SPF and GF mice (Figure 3.2). The numbers of metabolites were significantly different between SPF and GF mice in both the upper and lower colon (Figure 3.3). To visualize unique differences in metabolic data between GF and SPF mice, we performed principal component analysis (PCA). The PCA score plots were roughly separated by the presence of gut microbiota (Figure 3.4), suggesting that metabolites produced by gastrointestinal microbiota largely influence the luminal metabolome profile in each gastrointestinal section.

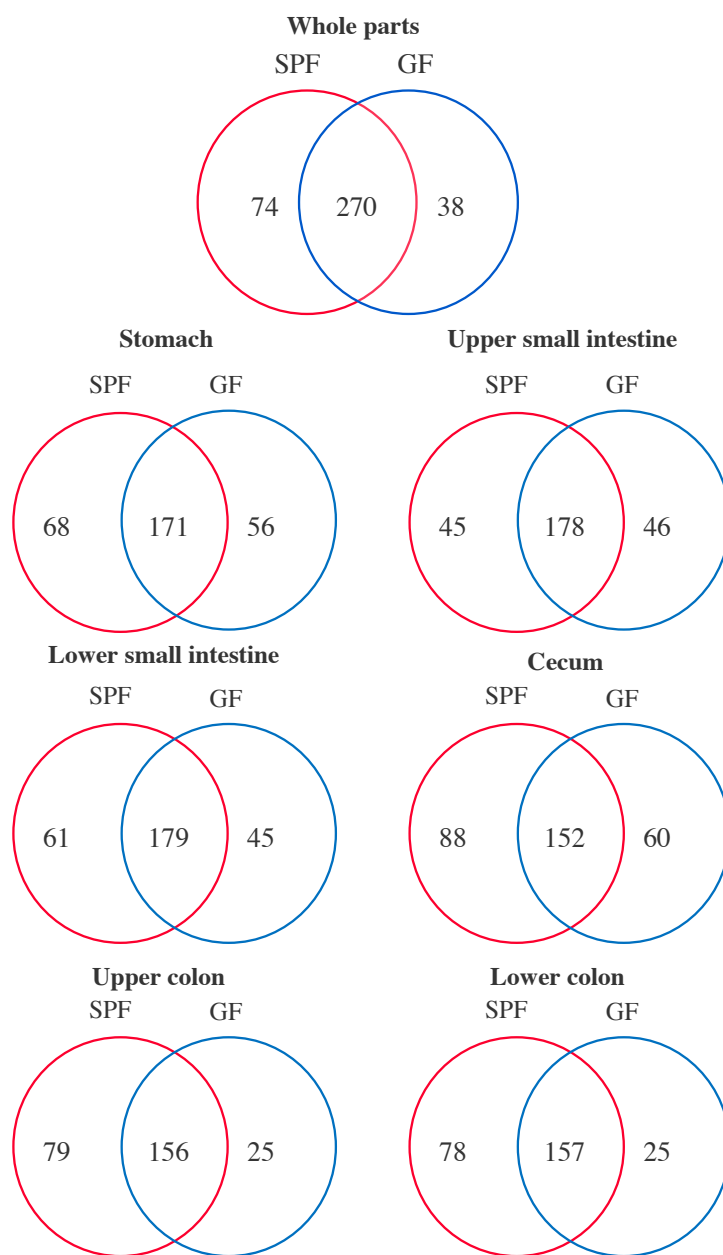


Figure 3.2 Luminal metabolome profiles of the gastrointestinal in SPF and GF mice

Venn diagram shows the number of detected identified gastrointestinal luminal metabolites in SPF and GF mice.

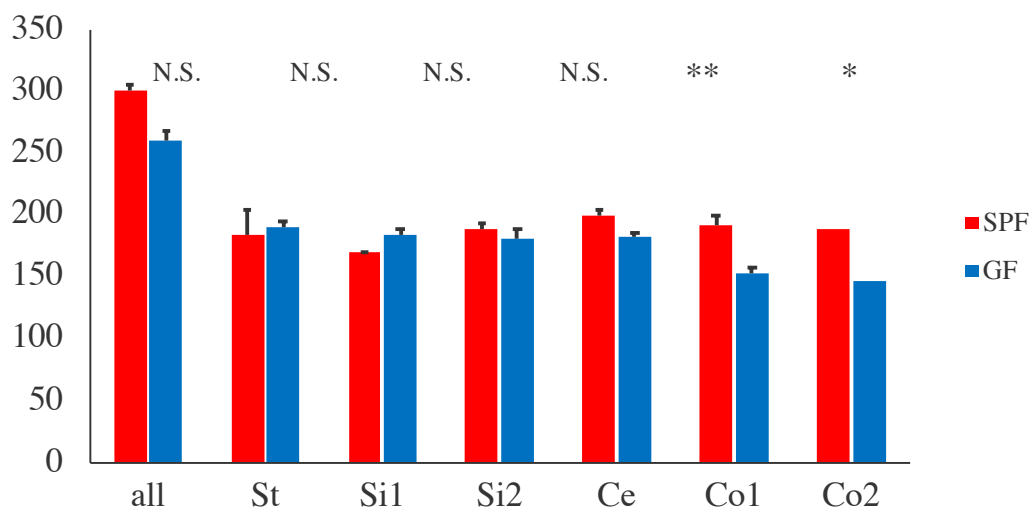


Figure 3.3 Differences of metabolites kinds in SPF and GF mice

Bar shows the number of mean of detected gastrointestinal luminal metabolites in SPF and GF mice. Student's t test, *:p < 0.05, **:p < 0.001

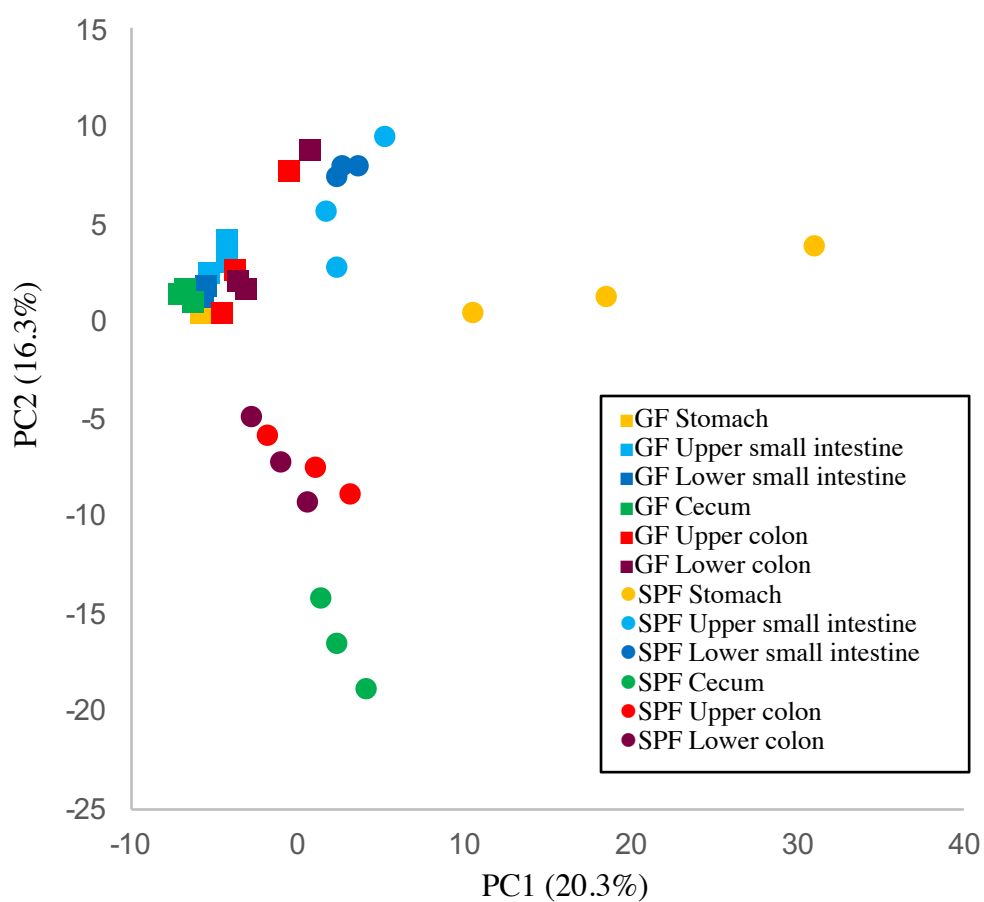


Figure 3.4 PCA of gastrointestinal luminal metabolome profiles in SPF and GF mice.

Error bars indicate standard deviation. Color designates the section of the intestinal tract, and shape denotes the type of mice used.

3.3.2. Comparison of luminal metabolome profiles among each section of the gastrointestinal tracts

To focus on features of the metabolome in each section of the gastrointestinal tract, we conducted K-means clustering based on z-score, and all metabolites in their respective clusters were categorized according to metabolic pathway designations from the KEGG database (Figure 3.5). In SPF mice, each cluster indicates the specific features of the gastrointestinal organ. A subset of metabolites at the highest concentrations in the stomach was clustered as cluster A; stomach or upper and lower small intestine as cluster B; upper and lower small intestine as cluster C; lower small intestine as cluster D and cecum as cluster E. On the other hand, metabolites observed in GF mice were not separated clearly into the specific intestinal categories listed above for SPF mice. Metabolites from GF mice observed at the highest concentrations in the stomach and small intestine were clustered as cluster F and cluster G, respectively. Those metabolites with the highest concentrations in the upper small intestine and colon or cecum and colon were clustered as cluster H and cluster I, respectively, while metabolites observed at highest concentrations in the colon were clustered as cluster J.

In the cecum and colon, the following metabolites—cholate, propionate, glucose, creatine and ribose—were detected at high concentrations only in SPF mice. Urea, inositol and dihydrouracil were detected only in GF mice. In the upper and lower small intestine, the numbers of identified metabolites were 32 and 58 in SPF mice and 33 and 63 in GF mice, respectively (Figure 3.5 Cluster B, C, G and H). The kinds of metabolites identified were independent of the existence of microbiota. The type of metabolites identified in the cecum and colon was different from the type in the stomach and small intestine. The population of metabolites that participate in carbohydrate and amino acid metabolism were more variable among the different parts of the intestine compared with metabolites in other pathways.

Furthermore, in the case of SPF mice, the metabolites that were significantly different from GF mice have been suggested to be related to amino acid metabolism, in addition to nucleotide metabolism and its regulation, based on MetaCore analysis. In stomach and small intestine, the significantly different metabolites were related to the metabolism and regulation of amino acids such as glycine, serine, cysteine and threonine metabolism (Betaine, Choline, Dimethylglycine, Choline phosphate, Creatine, 5-amino-levulinic acid); taurine and hypotaurine metabolism (Taurine); beta-alanine metabolism (Beta-alanine); and arginine metabolism (Urea,

Guanidinoacetate, Creatine, Spermidine). In the cecum and upper colon of SPF mice, significantly altered metabolites, compared to GF mice, were related to nucleotide metabolism and its regulation, such as TTP metabolism (uracil, thymine, 2'-deoxythymidine), CTP/UTP metabolism (Uridine, Uracil, Cytidine, Beta-alanine, UMP, CMP) and UMP biosynthesis (UMP). In the lower colon, significantly altered metabolites were related to amino acid metabolism and its regulation, such as Histidine-glutamate-glutamine metabolism (Urocanic acid, Succinic acid) and the tricarboxylic acid cycle (Succinic acid). In the case of GF mice, few significant metabolites were detectable. In the stomach and upper small intestine, significant metabolites were related to nucleotide metabolism and its regulation, such as GTP-XTP metabolism (guanosine, guanine) and dGTP metabolism (2'-deoxy-guanosine). In the lower small intestine, significant metabolites were related to nicotine action, such as nicotine metabolism in liver (S-Nornicotine). In the cecum and colon, significant metabolites were related to amino acid metabolism and its regulation, such as proline metabolism (4-Hydroxy-L-proline); arginine metabolism (Guanidinoacetate); and glycine, serine, cysteine and threonine metabolism (Guanidinoacetic acid). Although overlaps in metabolic pathways between SPF and GF mice were present, the differentiation in gastrointestinal location for specific

3. Molecular approaches for understanding murinal intestinal microbiota profiles

metabolites was different between the two types of mice, due to varying functions among gastrointestinal sections and gut microbiota colonization.

3. Molecular approaches for understanding murinal intestinal microbiota profiles

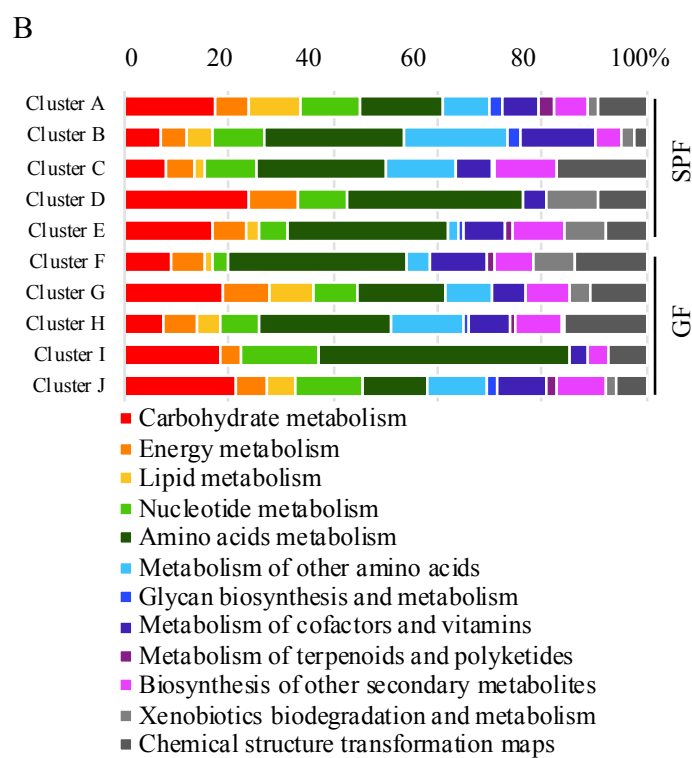
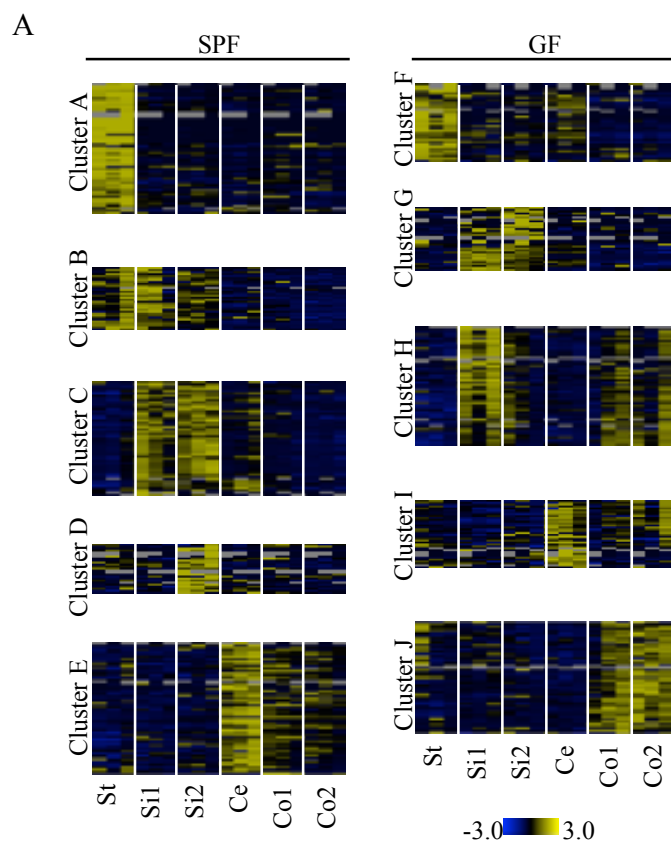


Figure 3.5 Differences in luminal metabolites among parts of the gastrointestinal tract in SPF and GF mice

(A) Heatmap of luminal metabolites categorized into five groups using K-means analysis. Columns correspond to gastrointestinal sections, while rows correspond to metabolites detected by CE-TOFMS and LC-MS/MS. Profiles were normalized to a relative value between -3 and 3. The colors indicate the z-score values calculated within SPF and GF mice separately. St: stomach, Si1: upper small intestine, Si2: lower small intestine, Ce: cecum, Co1: upper colon, Co2: lower colon. (B) Stack chart shows the percent distribution among pathways in which metabolites were detected within each cluster.

3.3.3. Comparison of gastrointestinal luminal metabolites between SPF and GF mice

We conducted OPLS-DA on each part of the luminal metabolome profiles of both SPF and GF mice (Figure 3.6A). S-plots showed that some metabolites contributed to the separation between SPF and GF mice (Figure 3.6B, Table 3.1). The following concentrations of metabolites were higher in SPF mice than those in GF mice: taurine (81.9-fold in stomach, 7.2-fold in upper small intestine), lactate (7.7-fold in stomach, 4.5-fold in upper colon), inositol (11.1-fold in stomach), maltose (811.0-fold in stomach), and butanoate (1374.2-fold in upper colon). Furthermore, taurine, butanoate and lactate appear to be characteristic metabolites within the upper small intestine, cecum, and upper colon, respectively, in SPF mice. On the other hand, raffinose (3680.3-fold in colon) was higher in the GF colon compared to that in the SPF colon. We conducted further microbiome analysis of the gastrointestinal tract in SPF mice and found that microbiome profiles were distinctive among the differing parts of the gastrointestinal tract (Figure 3.7). This result suggests that gastrointestinal luminal metabolome profiles may be related to the colonized microbiota, although further investigation is required to understand the detailed relationship between microbiota composition and metabolome profiles within the gastrointestinal tract of SPF mice. In

3. Molecular approaches for understanding murinal intestinal microbiota profiles

the case of unweighted, UniFrac analysis, the composition of gastrointestinal microbiota was similar within the same part of the intestine (Figure 3.7B)

3. Molecular approaches for understanding murinal intestinal microbiota profiles

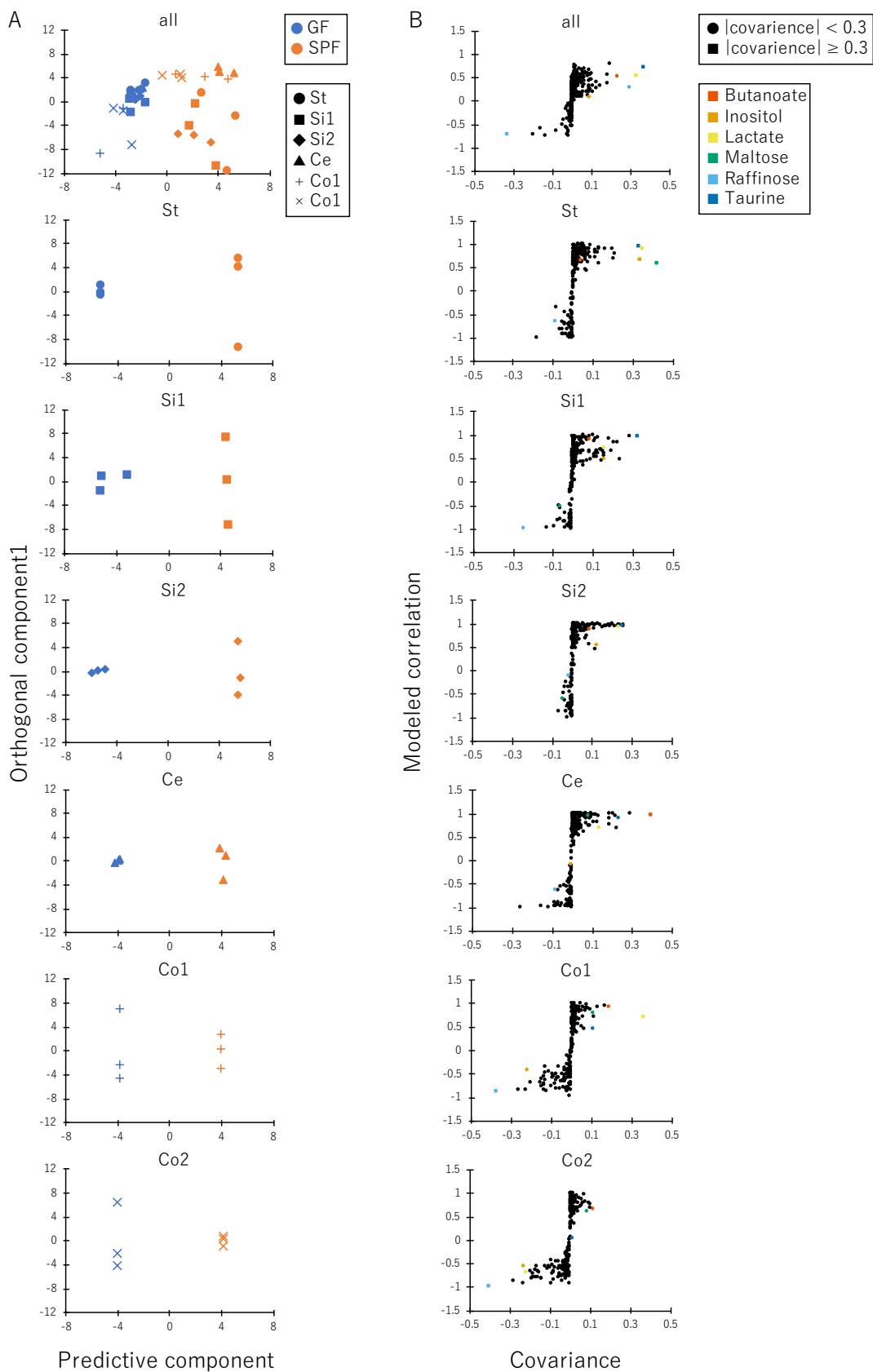


Figure 3.6 OPLS-DA of the luminal metabolome profiles of the gastrointestinal in SPF and GF mice

(A) The result of OPLS-DA. Red and blue indicate SPF and GF mice, respectively. (B) S-plot for OPLS-DA of each section of the intestinal tract. Each model represented SPF and GF mice in a single location: the stomach, upper small intestine, lower small intestine, cecum, upper colon and lower colon, with the cross-validated predicted ability $Q^2(Y) = 1$ and total explained variance $R^2(X) = 1$, $Q^2(Y) = 0.96$ and $R^2(X) = 0.93$, $Q^2(Y) = 0.99$ and $R^2(X) = 0.98$, $Q^2(Y) = 0.99$ and $R^2(X) = 0.89$, $Q^2(Y) = 1$ and $R^2(X) = 1$, $Q^2(Y) = 1$ and $R^2(X) = 1$, respectively.

Table 3.1 Characteristic luminal metabolites from each section of the gastrointestinal tract

	GF side			SPF side		
	Metabolite	x	y	Metabolite	x	y
Stomach				Maltose	0.42	0.56
				Lactate	0.35	0.89
				Inositol	0.34	0.66
				Taurine	0.33	0.93
Upper small intestine				Taurine	0.32	0.98
Lower small intestine						
Cecum				Butanoate	0.39	0.97
					0.29	0.98
Upper colon	Raffinose	-0.37	-0.88	Lactate	0.36	0.70
Lower colon	Raffinose	-0.40	-1.00			

This table shows the characteristic luminal metabolites detected by S-plot.

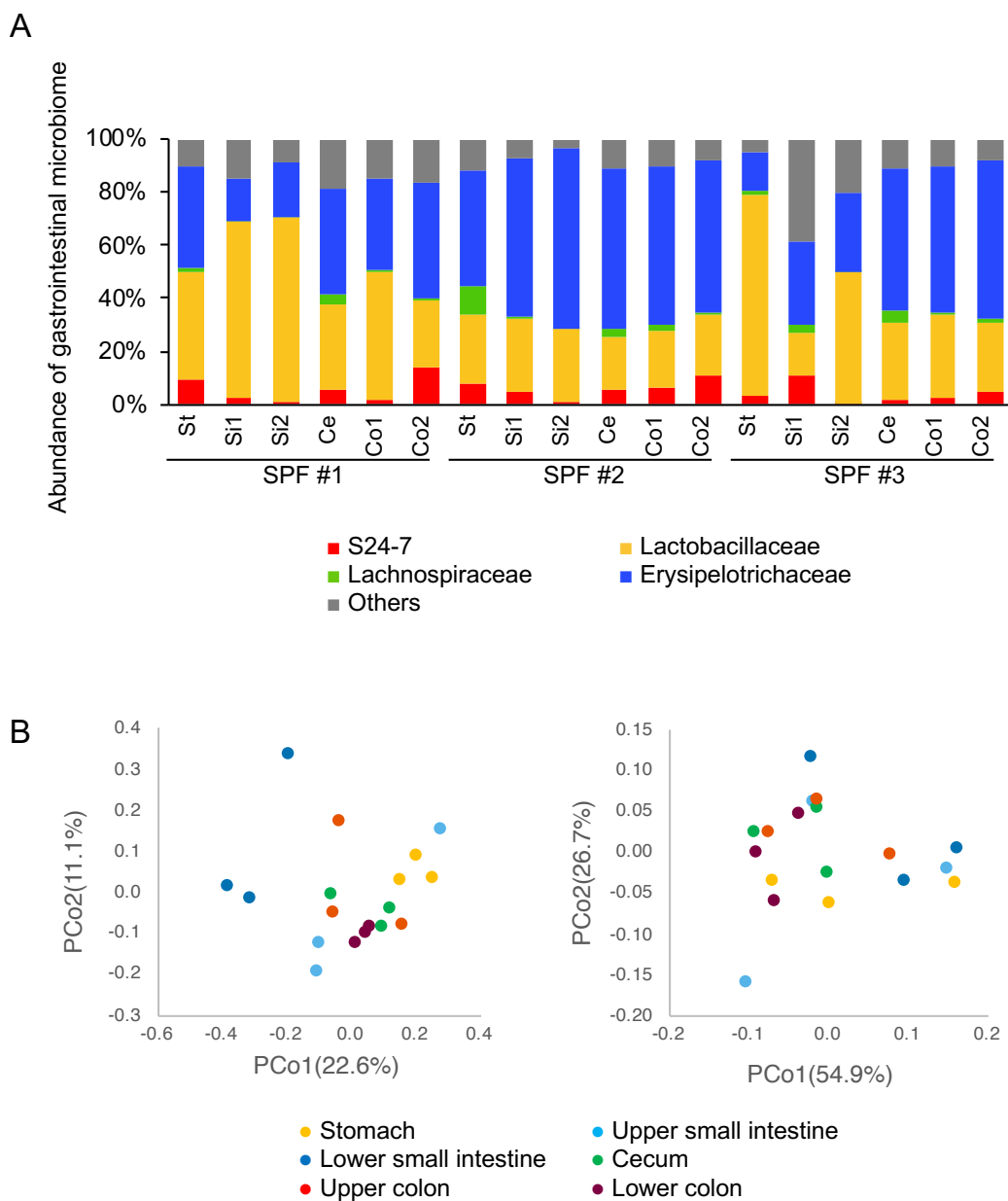


Figure 3.7 Composition of gastrointestinal microbiota in SPF mice

(A) Vertical bars indicate relative abundances of gastrointestinal microbiota colored by microbial family from binned OTU. Mice number was labeled at the bottom. St: stomach, Si1: upper small intestine, Si2: lower small intestine, Ce: cecum, Co1: upper colon, Co2: lower colon. (B) Principal coordinate analysis (PCoA) plot of sequence data. Left represents unweighted, and right represents weighted.

3.3.4. Features of sugars and amino acids within the gastrointestinal lumen of SPF and GF mice

We measured the concentrations of 19 sugars and amino acids (Figure 3.9, Figure 3.10). Sugars were separated into six groups using hierarchy clustering analysis to compare SPF and GF mice (Figure 3.9). These six categories displayed different metabolic patterns between each of the gastrointestinal locations. Panose was categorized as category A; this category includes metabolites observed at higher concentrations in the stomach of SPF mice compared to GF mice. Glucose, fructose, mannose and ribose were categorized as category B, which observed higher concentrations within the upper small intestine in SPF mice compared to GF mice. Mannitol, arabitol, sorbitol, raffinose, and trehalose were categorized as category C. The concentrations of these metabolites were higher within the upper colon in GF mice than concentrations in other GF gastrointestinal sections. Arabitol and trehalose had higher concentrations within the stomach and upper small intestine in SPF mice when compared with GF mice. In addition, category D includes inositol, maltotriose, sucrose, maltose and xylitol, which were more highly concentrated within the stomach in SPF mice when compared with GF mice. Categories E and F each included two metabolites,

galactose and rhamnose, and arabinose and xylose, respectively. These metabolites were all found to have high concentrations in the upper and lower colon in SPF mice. Amino acid profiles for each part of the gastrointestinal tract between SPF and GF mice were drastically different (Figure 3.10). In SPF mice, the concentrations of all amino acids were higher in the upper gastrointestinal tract than those of the lower intestine. However, in GF mice, the amino acid concentrations were higher in the lower intestine than those of the upper gastrointestinal tract. From these findings, the amino acids are considered to be intermediate metabolites in both protein digestion and absorption by the host, as well as microbial digestion and metabolization of amino acids.

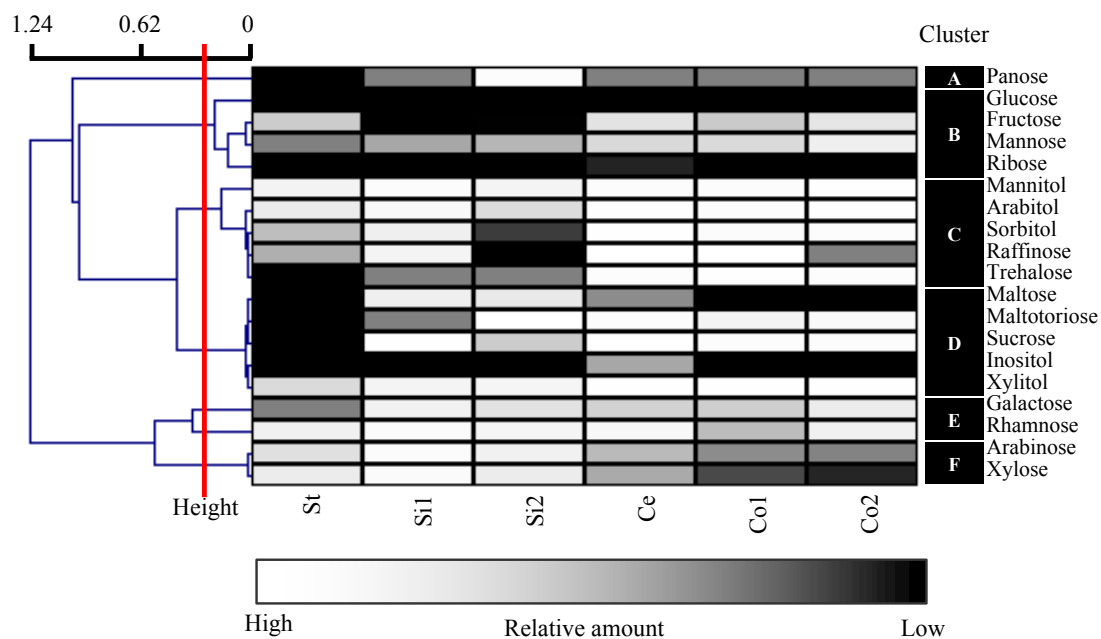
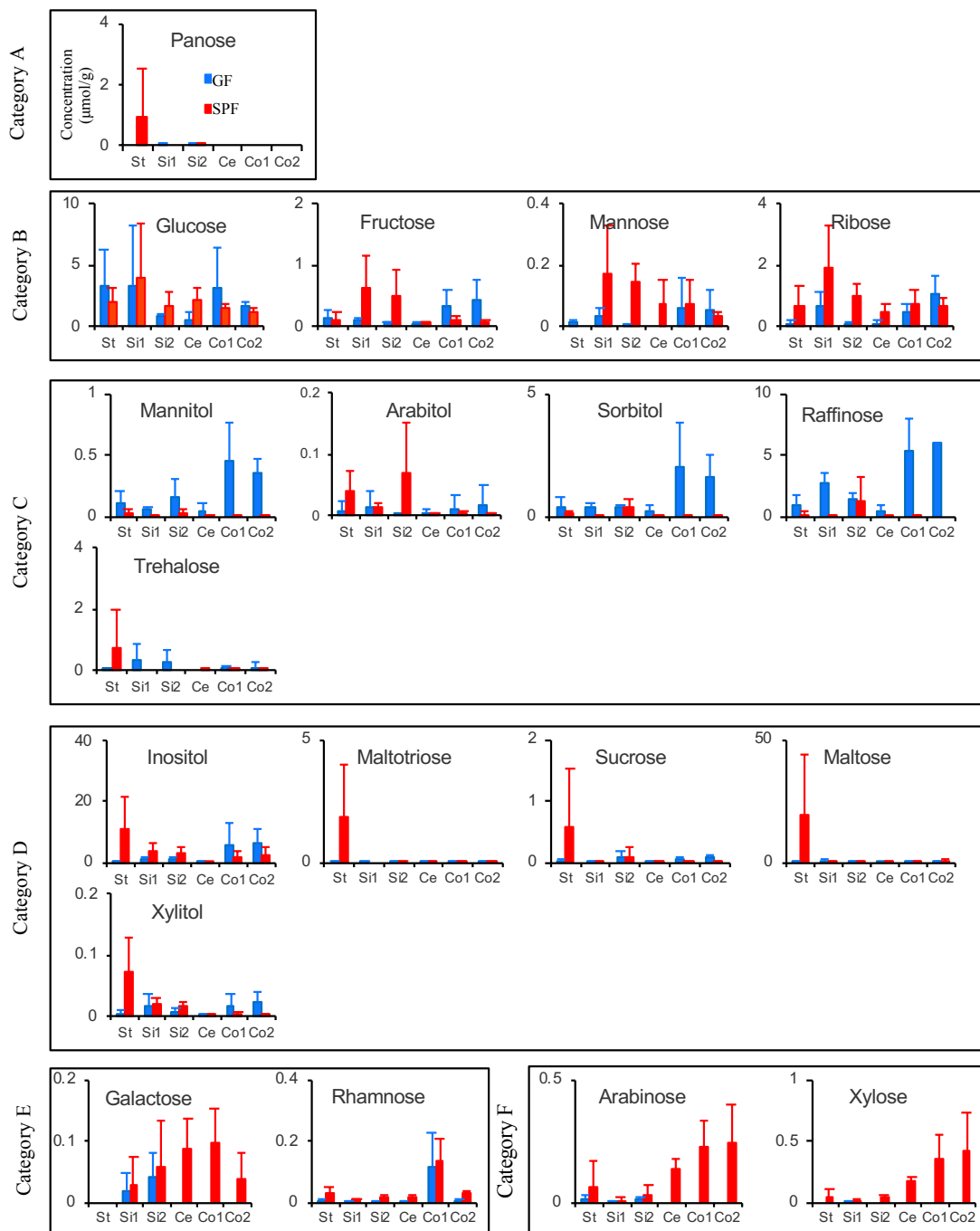


Figure 3.8 Clustered luminal carbohydrate profiles of the gastrointestinal in SPF mice

Carbohydrate profiles in SPF mice were clustered. St: stomach, Si1: upper small intestine, Si2: lower small intestine, Ce: cecum, Co1: upper colon, Co2: lower colon. These 6 clusters (the cut-off height of 1.7 shown as red line) further clustered by hierarchy clustering analysis based on Pearson's correlation.

3. Molecular approaches for understanding murinal intestinal microbiota profiles



**Figure 3.9 Luminal carbohydrate profiles of the gastrointestinal
in SPF and GF mice**

Bar graphs indicate the average concentration of carbohydrate in gastrointestinal contents. Red and blue indicate SPF and GF mice, respectively. Error bars indicate standard error. St: stomach, Si1: upper small intestine, Si2: lower small intestine, Ce: cecum, Co1: upper colon, Co2: lower colon.

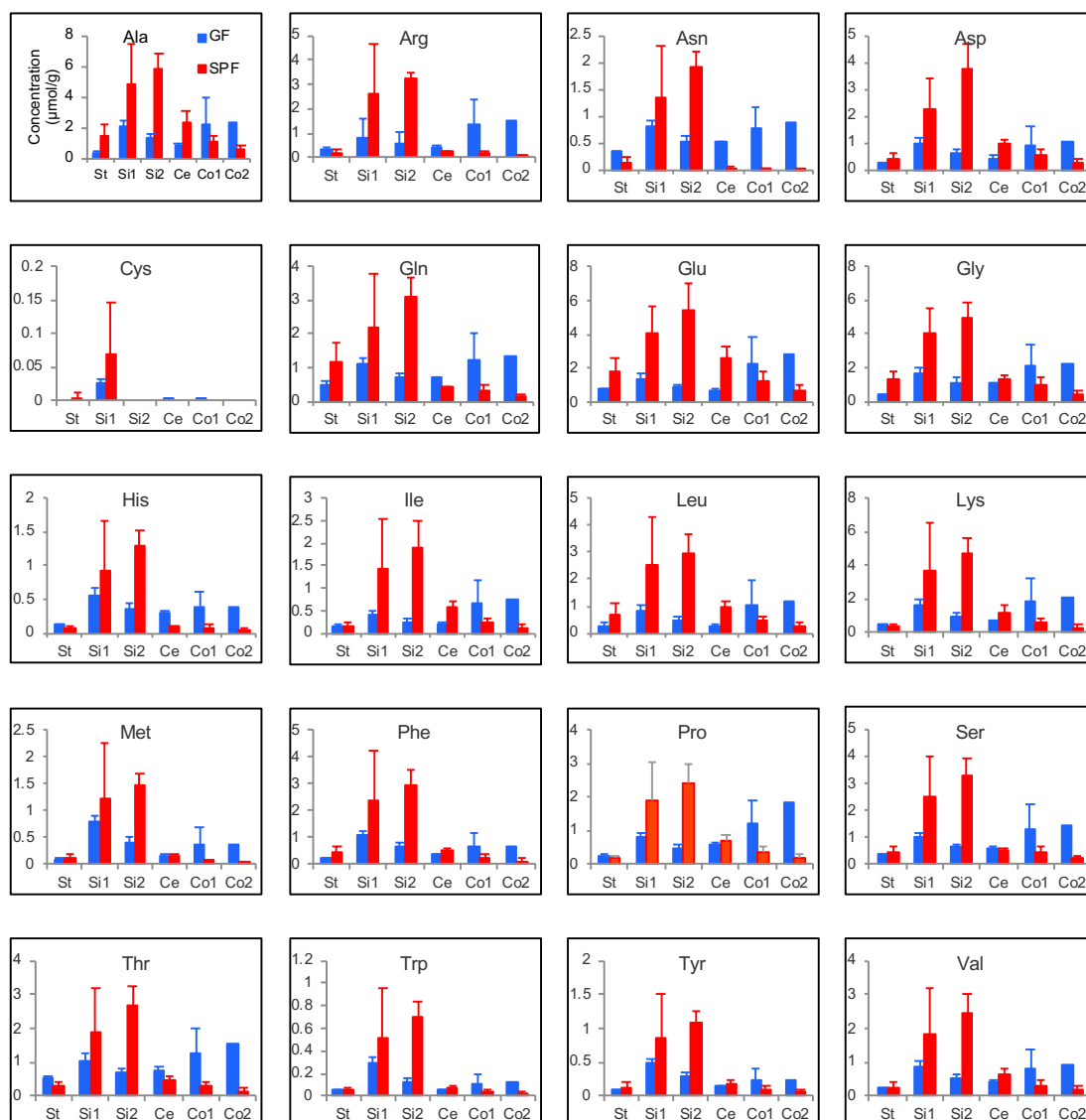


Figure 3.10 Luminal amino acid profiles of gastrointestinal in SPF and GF mice

Bar graphs indicate the average concentration of amino acids in gastrointestinal contents. Red and blue indicate SPF and GF mice, respectively. Error bars indicate standard error. St: stomach, Si1: upper small intestine, Si2: lower small intestine, Ce: cecum, Co1: upper colon, Co2: lower colon.

3.4. Discussion

The type and concentration of metabolites detected in this study were different between SPF and GF mice, although the quantity of identified metabolites was similar (Figure 3.2, Figure 3.3). PCA results showed that luminal metabolome profiles of SPF had higher diversity than GF mice (Figure 3.4). These differences seem to be dependent on the presence of microbiota. Cholate, propionate, glucose and ribose were detected only in SPF mice, while arabinol and trehalose were detected only in GF mice. Luminal metabolites were uniformly similar among each part of the intestinal tract in GF mice. On the other hand, some luminal metabolites were section-specific in the gastrointestinal tract of SPF mice, suggesting that these specific metabolites may be derived from gastrointestinal microbiota. In addition, PCA results showed that luminal metabolites in SPF and GF mice were categorized according to the location within the gastrointestinal tract (Figure 3.4). These profiles were expected due to the presence of gastrointestinal microbiota. When comparing luminal metabolome profiles between SPF and GF mice, higher amounts of organic acids, short-chain fatty acids (SCFA) and vitamins were detected in SPF mice (Table 3.1). Generally, it is well known that organic acids and SCFA are produced by intestinal microbiota (Furusawa et al., 2013; Matsumoto et al., 2012).

Carbohydrate digestive enzymes such as amylase, saccharase, and maltase are mainly secreted in the small intestine. To further understand carbohydrate profiles throughout the gastrointestinal tract, we classified the carbohydrates detected in this study into six groups by hierarchy cluster analysis: (A) panose (B) glucose, fructose, mannose, ribose (C) mannitol, arabitol, sorbitol, raffinose, trehalose (D) maltose, maltotriose, sucrose, inositol, xylitol (E) galactose, rhamnase (F) arabinose, xylose (Figure 3.9). The metabolites identified at the highest concentration level within the stomach of SPF mice were categorized as either group A or D. Maltotriose, which was categorized in group D, is metabolized from amylose and starch (Quezada-Calvillo et al., 2007). Sucrose, which is also categorized in group D, is digested into glucose and fructose in the small intestine (Davidson and Leese, 1977). We predict that these group D metabolites were further digested in the upper gastrointestinal by the host or microbiota, and then absorbed by host. As such, the Group D metabolites are not identified in the small intestine, cecum and colon. Group B consists of four monosaccharides detected at high levels in the upper small intestine of SPF mice; this outcome likely occurs because dietary food is digested in the stomach and then flows into the small intestine where it is absorbed there. Group C includes carbohydrates detected at higher concentrations in the lower intestine of GF mice than those of SPF

mice. Particularly, the concentrations of raffinose, sorbitol and mannitol were higher in the lower part of the colon in GF mice than those in SPF mice. It is estimated that these metabolites are accumulated in the upper colon because there are no bacteria present, and the host has little digestive use for them. Groups E and F contain four carbohydrates—arabinose, xylose, galactose and rhamnose—accumulated in the lower intestinal tract in SPF mice but are hardly produced in GF mice. In particular, the concentrations of arabinose, xylose and galactose gradually increased from the upper gastrointestinal tract to the lower in SPF mice. This result suggests that these metabolites were mainly produced from dietary food digestion by microbial carbohydrate enzymes and absorption by the small intestine may be difficult in mice.

Enzymes that digest protein, such as pepsin, trypsin, chymotrypsin and elastase, are secreted in the stomach and small intestine. Comparing the gastrointestinal luminal concentrations of the 20 amino acids detected in this study, we found that most amino acids profiles were similar (Figure 3.10), especially the finding of a relatively higher upper gastrointestinal tract concentration in SPF mice in opposition to those in GF mice. Based on these findings, we hypothesized that microbes contribute to protein digestion through their own protein digestive enzymes and/or enhance the secretion of protein digestion juice and enzymes from the host.

In this study, we revealed the differences in gastrointestinal luminal metabolome profiles between GF and SPF mice and highlighted metabolic features from each section of the gastrointestinal tract. The types of metabolites and characteristic metabolisms were similar between SPF and GF mice, but the metabolite concentrations and profiles throughout the gastrointestinal tract were different. This outcome could be partially attributed to the differences in function among sections of the intestine but could also simply be due to the colonization of microbiota. Particularly, carbohydrate, amino acid, and SCFA profiles were drastically different between SPF and GF mice. Although further investigation is required for a complete understanding of the contribution of gut microbiota to gastrointestinal luminal metabolome profiles, our findings are a progressive step toward understanding the relationship between commensal microbiota and gastrointestinal luminal metabolites.

Chapter 4

Conclusion

I conducted two research studies in molecular biology and public health, aimed at understanding the intestinal microbiota with which we live. In the first study, clinical trials focused on the effects of probiotics on infant AD were systematically screened. The result of this meta-analysis showed that symptoms worsened when European children with AD consumed probiotics. It is difficult to discuss the effect of probiotics considering their variability, as well as the differing dynamics of microbial profiles, which proved to be a major limitation of this study. As the number of trials using the same strain of probiotics increases around the world, it will become easier to analyze and discuss the effect of probiotics. In addition, the effect of host microbial profiles was considered in this study; therefore, it is important to focus on these profiles, as well as changes to the host environment. In the second study, the difference of metabolomics profiles from various parts of the intestinal tract was evaluated. These metabolic profiles were also evaluated using GF and SPF mice to examine the effect of gut microorganisms. Similar types of metabolites were identified in both GF and SPF mice.

In these two studies, it is difficult to discuss the role and mechanisms of microbiota because of the complexity of the bacterial community. The intestinal microbiota form a complicated community composed of many types of bacteria. In the case of human intestines, it is known that *E. coli* colonizes first, followed by *B. longum*. Other species colonize subsequently in a step-by-step fashion (Mitsuoka, 1978). It remains unclear how the bacteria construct this robust community. It is important to understand the dynamics of intestinal microbiota, as well as the metabolites derived by them, to understand the ecosystem within our intestinal tract.

Although details were not described here, I applied the method of a previous study (Stein et al., 2013) to this experimental data to simulate the dynamics of two kinds of intestinal microbes, *E. coli* and *B. longum*. Although some studies show the relationship between intestinal microbiota and metabolites, in addition to dynamics simulations of the bacterial community (Buffie et al., 2015; 2012; Coyte et al., 2015; Mishima et al., 2015), it is still unclear precisely how the dynamics of the bacteria and metabolites relate to one another. In addition, it is difficult to answer this question through a purely experimental approach.

Two groups of mice were prepared for microbiota implantation, one where *E. coli* served as the initial infection and the other where *B. longum* served as the initial

infection (Figure 4.1). Four BALB/c male germ-free mice were used in each group. When the mice were 16 weeks old, they were infected by oral treatment with the initial microbial species, concentrated at 1.0×10^8 CFU, and the second microbial species were introduced 56 days following the initial infection. Professor Fukuda at Keio Univ. and Professor Ono's group at RIKEN conducted this experiment. Five fecal samples were obtained at each of the following timepoints—0, 1, 2, 3, 6, 7, 14, 21, 28, 56, 57, 58, 59, 62, 63, 70, 77, 84, and 112 days—for microbial and metabolic analysis. The microbial experimental data were obtained as described in Fukuda's paper (Fukuda et al., 2011).

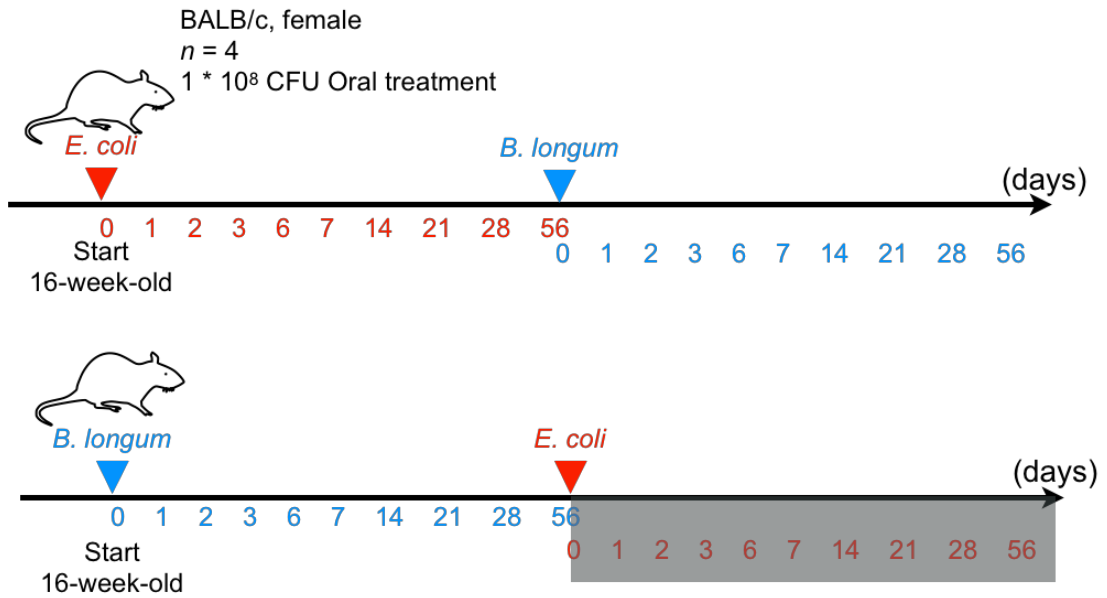


Figure 4.1 Outline of experiment

This figure presents the experiment. Samples were obtained at 0, 1, 2, 3, 6, 7, 14, 21, 28, 56, 57, 58, 59, 62, 63, 70, 77, 84, and 112 days following initial infection. Microbial abundance data were not collected during the time frame enclosed within the gray area.

We used the Lotka-Volterra Model (LVM) as the basic equation. Microbial data were used for simulation, and then metabolic data were used to expand the generalized Lotka-Volterra equations. In this term, we explored how to simulate using only microbial data on MATLAB. The quantity of bacteria E and B are explained by growth rate (μ_E and μ_B), the effect from themselves (M_{EE} and M_{BB}), and the effect from other species (M_{EB} and M_{BE}) (Equations 1, 2).

$$\frac{dE}{dt} = \mu_E E + M_{EE} E^2 + M_{EB} EB \quad (1)$$

$$\frac{dB}{dt} = \mu_B B + M_{BE} BE + M_{BB} B^2 \quad (2)$$

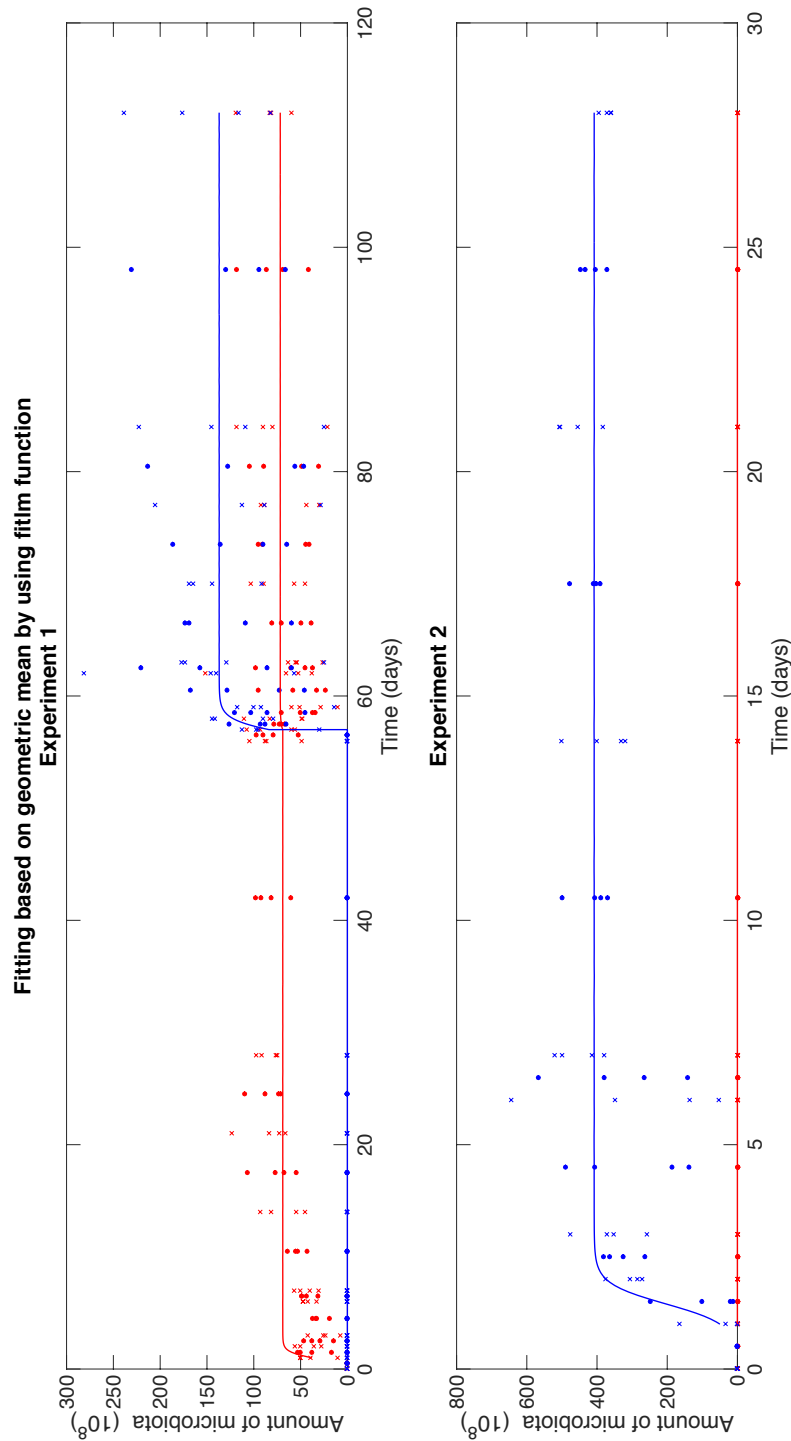


Figure 4.2 Result of modeling based on LVM

The line shows the result of simulation based on the plotted experimental data. Blue and red indicate *B. longum* and *E. coli*, respectively. x: experimental data, o: geometric mean based on experimental data.

From this research, we found mathematical modeling to be a key tool in understanding the behavior of bacteria in the first term of the human infant microbiota. Although the Lotka-Volterra model could be built, the drop shown in this model at < 5 days could not be explained using only microbial data (Figure 4.2). The results show that other such metabolites and immunoglobulins may have effects on the community among the bacteria.

Concluding the results of this research, we explored the complicated environment of microbiota and related metabolites within a host. Further experimental and medical investigations are required to understand the intestinal environment, including metabolites and microbiota. In addition, some field persons need to collaborate for understanding the object. In case of probiotics, although the effects of probiotics is shown by a molecular biological research, it is difficult to show the same effect by meta-analysis in medical field. It happened because the practical research method is not enough to share among the related field person. If the importance meta-analysis and any other methods is known by biological field researcher, the direction of research might be changed and become more effective research for other field and human. I hope researcher feel comfortable to collaborate with other field person and create the new values for themselves researches.

Acknowledgements

I would like to thank Prof. Masaru Tomita, the director of the Institute for Advanced Biosciences, Keio University (IAB), for providing an invaluable environment to execute my research.

Dr. Shinji Fukuda, a Project Associated Professor in IAB, and Dr. Yumiko Nakanishi provided immense support for my research. Furthermore, I spent a great deal of time in Tsuruoka with Shinnosuke Murakami, Chiharu Ishii, and Keita Kamezaki enjoying student life. They have supported my research, even until midnight, and provided advice.

Dr. Hitomi Sano taught me about modeling and simulations in the Doctoral course. I am thankful that she conveyed to me the importance of decision-making. Her words gave me a chance to make my decision.

I enjoyed talking with Akane Nishigata, Hikaru Inoue, and Tsubasa Watabe, and these conversations made me comfortable and relaxed. Sometimes they heard my worries and attempted to understand my situation, for which I am thankful.

Miho Tanaka, Shinkawa Haruka, Sayaka Saito, Yuriko Hasebe, Yuka Hirose, Hitoshi Iuchi, Megumi Uetaki, Mayuko Tsuruoka, Rena Fukuzawa, Fujitaka Baba, Haruka

Oba, Kaori Yamamoto, Miyu Noguchi, Mayu Kotake, and Kanuka Tanaka made my life in Tsuruoka comfortable.

Dr. Kazuharu Arakawa, Dr. Akio Kanai and Dr. Mitsuhiro Itaya provided useful words, which relayed many important things and encouraged me in my research.

Ayumi Mikami, Mizukami, Aya Saito, Chisa Namba, Miho Sato, Maki Oike, Miwa Miramoto, and Satomi Yokoi assisted with important documents and administrations.

The members of FC pharaoh and Spiber provided me with relaxation time in Tsuruoka. This time was important for me to refresh, in addition to the activities and exercise that kept me healthy.

I give many thanks to my first adviser, Midori Hashimoto, for teaching me the first step of research. I learned many things from her: how to read papers and summarize research, the importance of attending conferences, and how to make progress.

Dr. Toru Takebayashi and Dr. Daisuke Sugiyama taught me about public health and supported my master's thesis in the 2nd major. Midori Ishikawa and Miho Iida gave much advice.

Dr. Joao Xavier, Hilary Monaco, Dr. Jonas, Karen Chu, Nathaniel Campbell, Silja Heilmann, Kerry Boyle, Carlos Carmona-Fontaine, Maxime Deforet, and Jinyuan Yan accepted me as a member of Xavier's lab. They provided conversation and tried to help me think, even though my English was not perfect. Particularly, Dr. Xavier, Hilary and Jonas always conversed with me, providing many suggestions along with advice. These discussions were important for me to think about how to improve my research.

Furthermore, I would like to thank the Program for Leading Graduate School for its "Science for Development of Super Mature Society" in supporting my student life and research and teaching many things related to this world. My friends, Daisuke Ando, Takumi Kato, Masaki Sakamoto, Maki Dan, Hiroki Nagashima, Ken Nagao, Haoyang Yu, and Wataru Yoshiki gave me tremendous motivation for my research activities. I would further like to thank Prof. Dr. Fumihiko Kannari, Kenichi Hatori, Project Professor Masayuki Yamaguchi, and Project Professor Yoshiko Ishioka. Additionally, I would like to thank the members of this program for spending a great deal of time with me.

I would like to thank Mimi Matsuki, Naoto Nishizaka, the team members, Akihiro Nakamura and colleagues at IBM, who always try to understand my situation

and provide encouragement. Although managing work and being a student is hard, they understand and encourage me, making it possible to create a balance.

Nao Mizoe is one of my best friends, and we spent much time together during her undergraduate training. She helped maintain my motivation through her undergraduate work.

Hiroki Tanaka is special to me. He understood me and helped sustain my motivation. When I could not take it anymore, he gave me the chance to refresh. Sometimes, he persuaded me to face my research and taught me the methodology for simulation. Words cannot express how much I appreciate his kindness.

Finally, I thank my family so much for supporting me in my student life, believing in me, and encouraging me.

References

- Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.-M., et al. (2011). Enterotypes of the human gut microbiome. *Nature* 473, 174–180.
- Björkstén, B., Naaber, P., Sepp, E., and Mikelsaar, M. (1999). The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin. Exp. Allergy* 29, 342–346.
- Björkstén, B., Sepp, E., Julge, K., Voor, T., and Mikelsaar, M. (2001). Allergy development and the intestinal microflora during the first year of life. *J. Allergy Clin. Immunol.* 108, 516–520.
- Buffie, C.G., Bucci, V., Stein, R.R., McKenney, P.T., Ling, L., Gobourne, A., No, D., Liu, H., Kinnebrew, M., Viale, A., et al. (2015). Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nature* 517, 205–208.
- Buffie, C.G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., Ubeda, C., Xavier, J., and Pamer, E.G. (2012). Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infection and Immunity* 80, 62–73.
- Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., and Costa-Mattioli, M. (2016). Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* 165, 1762–1775.
- Cahenzli, J., Köller, Y., Wyss, M., Geuking, M.B., and McCoy, K.D. (2013). Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe* 14, 559–570.
- 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.
- Coyte, K.Z., Schluter, J., and Foster, K.R. (2015). The ecology of the microbiome: Networks, competition, and stability. *Science* 350, 663–666.
- Davidson, R.E., and Leese, H.J. (1977). Sucrose absorption by the rat small intestine in vivo and in vitro. *J. Physiol. (Lond.)* 267, 237–248.
- Devaraj, S., Hemarajata, P., and Versalovic, J. (2013). The human gut microbiome and body metabolism: implications for obesity and diabetes. *Clin. Chem.* 59, 617–628.
- Forslund, K., Hildebrand, F., Nielsen, T., Falony, G., Le Chatelier, E., Sunagawa, S., Prifti, E., Vieira-Silva, S., Gudmundsdottir, V., Pedersen, H.K., et al. (2015). Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* 528, 262–266.
-

- Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., Suzuki, T., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469, 543–547.
- Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365–378.
- 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.
- Gibson, G.R., and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125, 1401–1412.
- Group, J.F.W.W. (2002). Guidelines for the evaluation of probiotics in food (London Ontario).
- Gu, S., Chen, D., Zhang, J.-N., Lv, X., Wang, K., Duan, L.-P., Nie, Y., and Wu, X.-L. (2013). Bacterial community mapping of the mouse gastrointestinal tract. *PLoS ONE* 8, e74957.
- Hehemann, J.-H., Correc, G., Barbeyron, T., Helbert, W., Czjzek, M., and Michel, G. (2010). Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. *Nature* 464, 908–912.
- Hoffjan, S., and Stemmler, S. (2015). Unravelling the complex genetic background of atopic dermatitis: from genetic association results towards novel therapeutic strategies. *Arch. Dermatol. Res.* 307, 659–670.
- Holmes, E., Li, J.V., Marchesi, J.R., and Nicholson, J.K. (2012). Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. *Cell Metab.* 16, 559–564.
- Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J., Reisman, S.E., Petrosino, J.F., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155, 1451–1463.
- Kadowaki, A., Miyake, S., Saga, R., Chiba, A., Mochizuki, H., and Yamamura, T. (2016). Gut environment-induced intraepithelial autoreactive CD4(+) T cells suppress central nervous system autoimmunity via LAG-3. *Nature Communications* 7, 11639.
- Kalliomäki, M., Kirjavainen, P., Eerola, E., Kero, P., Salminen, S., and Isolauri, E. (2001). Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J. Allergy Clin. Immunol.* 107, 129–134.
- Kato, T., Fukuda, S., Fujiwara, A., Suda, W., Hattori, M., Kikuchi, J., and Ohno, H. (2014). Multiple omics uncovers host-gut microbial mutualism during prebiotic fructooligosaccharide supplementation. *DNA Res* 21, 469–480.

- Kay, J., Gawkrödger, D.J., Mortimer, M.J., and Jaron, A.G. (1994). The prevalence of childhood atopic eczema in a general population. *Journal of the American Academy of Dermatology* 30, 35–39.
- Kim, S.-W., Suda, W., Kim, S., Oshima, K., Fukuda, S., Ohno, H., Morita, H., and Hattori, M. (2013). Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. *DNA Res* 20, 241–253.
- Kim, S.-O., Ah, Y.-M., Yu, Y.M., Choi, K.H., Shin, W.-G., and Lee, J.-Y. (2014). Effects of probiotics for the treatment of atopic dermatitis: a meta-analysis of randomized controlled trials. *Annals of Allergy, Asthma & Immunology* 113, 217–226.
- Koeth, R.A., Wang, Z., Levison, B.S., Buffa, J.A., Org, E., Sheehy, B.T., Britt, E.B., Fu, X., Wu, Y., Li, L., et al. (2013). Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med.* 19, 576–585.
- Kostic, A.D., Howitt, M.R., and Garrett, W.S. (2013). Exploring host-microbiota interactions in animal models and humans. *Genes & Development* 27, 701–718.
- Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Almeida, M., Arumugam, M., Batto, J.-M., Kennedy, S., et al. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature* 500, 541–546.
- Lee, J., Yun, H.S., Cho, K.W., Oh, S., Kim, S.H., Chun, T., Kim, B., and Whang, K.Y. (2011). Evaluation of probiotic characteristics of newly isolated *Lactobacillus* spp.: Immune modulation and longevity. *International Journal of Food Microbiology* 148, 80–86.
- Leung, D.Y.M., and Bieber, T. (2003). Atopic dermatitis. *Lancet* 361, 151–160.
- Marsland, B.J., and Gollwitzer, E.S. (2014). Host-microorganism interactions in lung diseases. *Nat. Rev. Immunol.* 14, 827–835.
- Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Kurihara, S., Sawaki, E., Koga, Y., and Benno, Y. (2012). Impact of intestinal microbiota on intestinal luminal metabolome. *Sci Rep* 2, 233.
- Metges, C.C. (2000). Contribution of microbial amino acids to amino acid homeostasis of the host. *J. Nutr.* 130, 1857S–1864S.
- Mishima, E., Fukuda, S., Shima, H., Hirayama, A., Akiyama, Y., Takeuchi, Y., Fukuda, N.N., Suzuki, T., Suzuki, C., Yuri, A., et al. (2015). Alteration of the Intestinal Environment by Lubiprostone Is Associated with Amelioration of Adenine-Induced CKD. *J. Am. Soc. Nephrol.* 26, 1787–1794.
- Mitsuoka T. (1978). *Chonai saikin no hanashi*. Iwanami Shoten, Tokyo (in Japanese).
- Moher, D., Hopewell, S., Schulz, K.F., Montori, V., Gøtzsche, P.C., Devereaux, P.J., Elbourne, D., Egger, M., and Altman, D.G. (2010). CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. *Bmj* 340, c869–c869.

- Murakami, S., Goto, Y., Ito, K., Hayasaka, S., Kurihara, S., Soga, T., Tomita, M., and Fukuda, S. (2015). The Consumption of Bicarbonate-Rich Mineral Water Improves Glycemic Control. *Evid Based Complement Alternat Med* 2015, 824395.
- Nagao-Kitamoto, H., Shreiner, A.B., Gilliland, M.G., Kitamoto, S., Ishii, C., Hirayama, A., Kuffa, P., El-Zaatari, M., Grasberger, H., Seekatz, A.M., et al. (2016). Functional Characterization of Inflammatory Bowel Disease-Associated Gut Dysbiosis in Gnotobiotic Mice. *Cell Mol Gastroenterol Hepatol* 2, 468–481.
- 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.
- Quezada-Calvillo, R., Robayo-Torres, C.C., Opekun, A.R., Sen, P., Ao, Z., Hamaker, B.R., Quaroni, A., Brayer, G.D., Wattler, S., Nehls, M.C., et al. (2007). Contribution of mucosal maltase-glucoamylase activities to mouse small intestinal starch alpha-glucogenesis. *J. Nutr.* 137, 1725–1733.
- Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L., Griffin, N.W., Lombard, V., Henrissat, B., Bain, J.R., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341, 1241214–1241214.
- Sacks, H.S., Berrier, J., and Reitman, D. (1987). Meta-analyses of randomized controlled trials. ... *England Journal of*
- Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., et al. (2003). TM4: a free, open-source system for microarray data management and analysis. *BioTechniques* 34, 374–378.
- Sampson, T.R., Debelius, J.W., Thron, T., Janssen, S., Shastri, G.G., Ilhan, Z.E., Challis, C., Schretter, C.E., Rocha, S., Gradinaru, V., et al. (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* 167, 1469–1480.e12.
- Schrezenmeir, J., and de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics--approaching a definition. *Am. J. Clin. Nutr.* 73, 361S–364S.
- Smith, H.W. (1965). Observations on the flora of the alimentary tract of animals and factors affecting its composition. *The Journal of Pathology and Bacteriology* 89, 95–122.
- Stalder, J.F., and Taieb, A. (1993). Severity scoring of atopic dermatitis: the SCORAD index. *Journal of Dermatological Science* 6, 95.
- Stein, R.R., Bucci, V., Toussaint, N.C., Buffie, C.G., Räscher, G., Pamer, E.G., Sander, C., and Xavier, J.B. (2013). Ecological modeling from time-series inference: insight into dynamics and stability of intestinal microbiota. *PLoS Comput Biol* 9, e1003388.

- Sugimoto, M., Kaneko, M., Onuma, H., Sakaguchi, Y., Mori, M., Abe, S., Soga, T., and Tomita, M. (2012). Changes in the charged metabolite and sugar profiles of pasteurized and unpasteurized Japanese sake with storage. *J. Agric. Food Chem.* 60, 2586–2593.
- Sugimoto, M., Wong, D.T., Hirayama, A., Soga, T., and Tomita, M. (2010). Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics* 6, 78–95.
- Thorburn, A.N., McKenzie, C.I., Shen, S., Stanley, D., Macia, L., Mason, L.J., Roberts, L.K., Wong, C.H.Y., Shim, R., Robert, R., et al. (2015). Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. *Nature Communications* 6, 7320.
- Tian, Y., Zhang, L., Wang, Y., and Tang, H. (2012). Age-related topographical metabolic signatures for the rat gastrointestinal contents. *J. Proteome Res.* 11, 1397–1411.
- 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.
- Turroni, F., Peano, C., Pass, D.A., Foroni, E., Severgnini, M., Claesson, M.J., Kerr, C., Hourihane, J., Murray, D., Fuligni, F., et al. (2012). Diversity of Bifidobacteria within the Infant Gut Microbiota. *PLoS ONE* 7, e36957.
- Van Der Aa, L.B., Heymans, H.S., van Aalderen, W.M., Sillevius Smitt, J.H., Knol, J., Ben Amor, K., Goossens, D.A., Sprikkelman, A.B., Synbad Study Group (2010). Effect of a new synbiotic mixture on atopic dermatitis in infants: a randomized-controlled trial. *Clin. Exp. Allergy* 40, 795–804.
- Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73, 5261–5267.
- Wang, Z., Klipfell, E., Bennett, B.J., Koeth, R., Levison, B.S., Dugar, B., Feldstein, A.E., Britt, E.B., Fu, X., Chung, Y.-M., et al. (2011). Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 472, 57–63.
- Wang, Z., Roberts, A.B., Buffa, J.A., Levison, B.S., Zhu, W., Org, E., Gu, X., Huang, Y., Zamanian-Daryoush, M., Culley, M.K., et al. (2016). Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis. *Cell* 1–12.
- Won, T.J., Kim, B., Lee, Y., Bang, J.S., Oh, E.S., Yoo, J.-S., Hyung, K.E., Yoon, J., Hwang, S., Park, E.S., et al. (2012). Therapeutic potential of *Lactobacillus plantarum* CJLP133 for house-dust mite-induced dermatitis in NC/Nga mice. *Cellular Immunology* 277, 49–57.

Won, T.J., Kim, B., Oh, E.S., Bang, J.S., Lee, Y.J., Yoo, J.-S., Yu, H., Yoon, J., Hyung, K.E., Park, S.-Y., et al. (2011). Immunomodulatory activity of Lactobacillus strains isolated from fermented vegetables and infant stool. *Can. J. Physiol. Pharmacol.* 89, 429–434.

Abbreviations

AA	Amino Acids
AD	Atopic dermatitis
bp	Base pair
CE-TOFMS	Capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry
CFU	Count-Forming Units
CI	Confidence interval
CIEA	Central Institute for Experimental Animals
CMP	Cytidine monophosphate
CONSORT	Consolidated Standards of Reporting. Trials
CTP	Cytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization mass spectrometry
FAO	Food and Agriculture Organization of the United Nations
GF	Germ-free
GTP	Guanosine triphosphate
IAA	Indoleacetic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LGG	Lactobacillus rhamnosus strain GG
N.D.	Not detected
OCT	Optical coherence tomography
OPLS-DA	Orthogonal Partial Least Squares Discriminate Analysis
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
QIIME	Quantitative Insights Into Microbial Ecology
RCT	Randomized control trials
RDP	Ribosomal Database Project

SCFA	Short-chain fatty acid
SCORAD	Severity Scoring of Atopic Dermatitis
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
S.E.	Standard error
SPF	Specific pathogen free
TE	Tris- Ethylenediaminetetraacetic acid
UMP	Uridine 5'-monophosphate
UTP	Uridine triphosphate
WHO	World Health Organization