Exploring the Role of the Microbiota Member Bifidobacterium in Modulating Gamma Amino Butyric Acid Production

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(ヒト腸内フローラによるガンマアミノ酪酸生産におけるビフィズス菌の役割)

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Dissertation summary

GABA is a four-carbon amino acid produced by the irreversible decarboxylation of glutamate. It is the major inhibitory neurotransmitter in the central nervous system. GABA has been widely studied because of its numerous health benefits including both physiological and psychological benefits.

Bifdobacteria are important probiotic bacteria inhabiting the gut of all mammals including both animal and human. Recently, specific strains of *bifidobacterum* were reported as GABA producers. The study of GABA production ability of bifidobacteria can open the way for new insights in these bacteria. In this study, I investigate GABA production ability of *Bifidobacterium* for the industrial benefit and for the host benefit. In addition, I developed a useful cloning strategy which supported the study.

In the first chapter, I have developed a useful tool for molecular cloning. A series of new *Escherichia coli* entry vectors (pIIS18-*Sap*I, pIIS18-*Bsm*BI, pIIS18-*Bsa*I, pIIS18-*Bfu*AI-1, and pIIS18-BfuAI-2) was constructed based on a modified pUC18 backbone, which carried newly designed multiple cloning sites, consisting of two facing type IIS enzyme cleavage sites and one blunt-end enzyme cleavage site. These vectors are useful for seamless gene cloning. I also proposed a good strategy for precise single and multi-gene cloning, applicable in all bacteria

including bifidobacteria. This strategy was used in the second chapter of this study.

In the second chapter, I elucidated the machinery responsible for GABA production in wild type and recombinant *Bifidobacterium* strains to maximize GABA productivity. *B. adolescentis 4-2*, a human fecal isolate, was identified as a high GABA producer. GABA-producing genes of this strain, glutamate decarboxylase (*gadB*) and glutamate-GABA antiporter (*gadC*), were introduced to non-GABA-producing *Bifidobacterium* hosts. Expression was monitored through two high-expressing promoters (*gap* and *BLt43*) in addition to the original *gadB* promoter. Fermentation conditions, including media type, substrate amount (Mono sodium glutamate, MSG), and pH, were adjusted. Two model strains had efficient productivity and unique characteristic features: *B. adolesentis* JCM1275/*gadBC* with the gap promoter, in which GABA production reached about 377mM in fed batch fermentation, and *B. adolescentis* JCM 1275/*gadBC* with the original promoter, in which an interesting pH induction phenomenon was found when grown on medium with an acidic initial pH. To the best of our knowledge, this is the first introduction of *Bifidobacterium* as an emerging microbial cell factory for enhanced GABA production.

In the third and fourth chapter, I analyzed the relation between fecal GABA

content and microbial composition of more than 70 human volunteers. A further approach was applied to those with low GABA content aiming to improve production ability. The study revealed that the microbiome of the high GABA group had lower alpha diversity than low and medium groups. Interestingly, Bifidobacteriaceae exhibited high abundance in their microbiome. To validate this finding, a fecal isolate-GABA producer *Bifidobacterium adolescentis* 4-2 was co-cultured with low GABA producing microbiomes, it enhanced GABA productivity significantly. Further, a collection of oligosaccharides, as an efficient prebiotic, enhanced both GABA productivity and *Bifidobacterium* abundance in fecal cultures of low GABA producer microbiomes. Sensationally, a combination of mannooligosaccharides (MOS) and *B. adolescentis* 4-2 exhibited a maximal fecal GABA content. This study demonstrated that *Bifidobacterium* abundance is co-related with high fecal GABA content in apparently healthy human subjects. Further, *B. adolescentis* 4-2 and MOS are a new symbiotic mixture, able to tune up fecal GABA level in *in vitro* culture.

論文要旨

GABA は、グルタミン酸の不可逆的な脱炭酸によって生成され、中枢神経系の主要な抑制性神経伝達物質である。 GABA には、生理学的および心理的な健康機能があり、広く研究されてきた。

ビフィズス菌は、ヒトの腸に生息する重要なプロバイオティクスである。近年、ビフィズス菌の特定の菌株が GABA 生産菌として報告された。ビフィズス菌の GABA 産生能力の研究は、本菌の新たな活用方法につながる。本研究では、産業利用、またヒト宿主の健康のために、ビフィズス菌の GABA 産生能力を調査する。さらに、研究をサポートする有用なクローニング戦略を開発した。

最初の章では、分子クローニングに役立つツールを開発した。以下に記述する新しい大腸菌エントリーベクター（pIIS18-SapI、pIIS18-BsmBI、pIIS18-BsaI、pIIS18-BfuAI-1、および pIIS18-BfuAI-2）は、pUC18 のバックボーンに基づいて構築され、2 つの向かい合う IIS 型制限酵素切断部位と 1 つの平滑末端制限酵素切断部位からなる。これらのベクターは、インサートに制限酵素切断部位などの余分な配列を付加する必要がある。また、これらのベクターを用いた遺伝子発現ベクターの構築戦略は、本研究の第 2 章で使用した。

第 2 章では、GABA の生産性を向上させるために、野生株および組換えビフィズス菌株における GABA 産生のメカニズムを解明した。ヒトの糞便分離ビフィズス菌株である *Bifidobacterium adolescentis* 4-2 を、高 GABA 産生株とし

て同定した。この菌株の GABA 産生遺伝子であるグルタミン酸デカルボキシラーゼ遺伝子（*gadB*）とグルタミン酸/GABA 対向輸送体遺伝子（*gadC*）を、GABA 非産生ビフィズス菌株に導入した。元の *gadB* プロモーターに加えて、2 つの高発現プロモーター（*gap, BLt43*）を介して発現量を測定した。また、培地の種類、基質量（グルタミン酸ナトリウム、MSG）、pH などの発酵条件を調整した。その結果、2 つのモデル株は効率的な生産性と独特の特徴を持っていた。*gap* プロモーターを備えた *B. longum* 105-A / *gadBC* の GABA 産生は、5% MSG および pH 6.7 で約 23 g /L に達し、元のプロモーターを備えた *B. adolescentis* JCM 1275 /*gadBC* は、pH 4.4 の 4% MSG 含有 MRS 培地で増殖させると、興味深い pH 誘導が見られた。本研究は、GABA 高生産へ向けた新たな微生物 Cell Factory 系を、ビフィズス菌で初めて構築した例となる。

第 3 章では、70 人以上のボランティアの糞便中の GABA 含有量と細菌叢組成の関係を分析した。この研究により、高 GABA 生産グループの腸内細菌叢は低･中 GABA 生産グループよりもα多様性が低いことが明らかになった。また、興味深いことに、高 GABA 生産グループでは、Bifidobacteriaceae 科が細菌叢に豊富に含まれていた。この現象を詳しく検証するために、*B. adolescentis* 4-2 を低 GABA 産生細菌叢と共培養したところ、GABA 生産性が大幅に向上した。そこで、次に、GABA 含有量の少ないヒト腸内での GABA 生産能力の向上を目指し、さらなるアプローチを試みた。本研究では、ビフィズス菌の資化できるオ

リゴ糖をプレバイオティクスとして用いた。低 GABA 生産細菌叢の糞便培養に各種オリゴ糖を添加した結果、GABA 生産性とビフィズス菌の存在比率の両方が向上した。その中で最も GABA 生産の向上に効果的だったのは、マンノオリゴ糖（MOS）と *B. adolescentis* 4-2 の組み合わせた場合だった。本研究は、ビフィズス菌の存在量が、健康なヒトの糞便中の GABA 含有量と相互に関連していることを示している。さらに、*B. adolescentis* 4-2 と MOS は、*in vitro* 培養系で糞便の GABA レベルを向上させることができることから、新規シンバイオティクスへの利用が今後期待される。

General introduction

1. *Bifidobacterium*

Bifidobacteria are member of the phylum *Actinobacteria,* order *Bifidobacteriales,* genus *Bifidobacterium* [1]. They are gram positive, anaerobic, non-motile, non-spore-forming, non-gas producing, catalase-negative bacteria. Morphologically, they have bifid or irregular V- or Y-shaped rods like branches. Bifidobacterial genome is characterized by high GC content ranging from 59.2% (*B. adolescentis*) to 64.6 % (*B. scardovii*) [2]. The average size of its genome is 2.2 mega base pairs (Mb) with a considerable size variation between species. Bifidobacteria were first isolated from the feces of breast-fed infants in 1899 by Henri Tissier, and later, it was isolated from other sources. *Bifidbacterium* is a beneficial symbiotic colonizer of mammal's intestinal tract, mainly in the colon.

Metagenomic analysis studies of intestinal microbiome has revealed that human intestinal microbiota consists of two major phyla, *Bacteroidetes* and *Firmicutes*, and four other prominent phyla, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Tenericutes* with a significant individual variation [3]. These studies also indicate that the *Actinobacteria* phylum, including *Bifidobacterium*, is relatively abundant.

Bifidobacteria have several health benefits for the carrier host, animal and

human, through production of numerous beneficial materials named as postbiotics. Postbiotics are metabolites secreted by live bacteria or released after bacterial lysis providing physiological benefits to the host [4]. One of the recently focused postbiotic materials is gamma amino butyric acid (GABA). Specific strains of *Bifdobacterium* have been reported as GABA producers including *B. adolescentis*, *B. dentium*, *B. angulatum* and *B. longum subsp. Infantis* [5]. Through GABA production *Bifidobacterium* is thought to affect gut-brain communication. Gut microbiota-Brain axis refers to a complex network of communication between intestine, intestinal microflora and brain through signaling between central nervous system (CNS) and enteric nervous system (ENS) [6]. The impact of gut microbial communities on human mental health is one of the emerging topics of research. The study of GABA production ability of *Bifidobacterium* can reveal a new insight for these bacteria

2. Gamma Amino Butyric Acid (GABA)

GABA is a ubiquitous non-protein amino acid that is widely distributed among microorganisms, plants and animals [7]. In bacteria, GABA acts for energy production and acid tolerance [8]. In plants, it acts as growth stimulator and involved in stress response [9]. In mammals, it is the chief inhibitory neurotransmitter in the central nervous system [10]. It also has a numerous

potential health benefits such as antihypertensive, immune stimulant and antidiabetic [11, 12]. Due to its great benefit, GABA production by various ways has gained a great attention. GABA biosynthesis pathway is universal. It occurs through decarboxylation of glutamate to GABA by the action of glutamate decarboxylase [13]. Recently, several lactic acid bacteria have exhibited a relevant ability for GABA production. These bacteria were named psychobiotics.

3. Psychobiotics

Psychobiotics defined as mind-altering germs or the specific bacteria (probiotic), that when consumed in adequate amount results in beneficial effects on mood, motivation, and cognition [14]. This definition later was overlooked to include any exogenous influence whose effect on the brain is bacterially mediated [15]. These exogenous influencers can be whether postbiotic, prebiotic.

4. Prebiotics

Prebiotics are a group of nutrients that are not digestible by the host but, it is degraded by gut microbiota [16]. They act like fertilizers for gut friendly bacteria, and their degradation products are short-chain fatty acids that are released into blood circulation, consequently, its effect extend beyond the gastrointestinal tract to affect also distant organs [17]. One of the most important prebiotics are oligosaccharides. It can rearrange our gut microbiota towards

increase of beneficial bacteria and decrease of pathogenic bacteria.

5. Aim of the study

In our study, we have three main targets. The first, study the machinery of GABA production from *Bifidobacterium* aiming to maximize its productivity. The second, clarify the microbial diversity between human volunteers of different fecal GABA content and the possible implication of *Bifidobacterium* in this diversity. The third, we aim to find out a suitable formula of probiotic and prebiotic for improving microbial GABA productivity.

Chapter one

A New *Escherichia coli* Entry Vector Series (pIIS18) for Seamless Gene Cloning Using Type IIS Restriction Enzymes

1.1 Introduction

The plasmid construction in Escherichia coli is one of the essential routine-works in the field of molecular biology [18, 19]. The seamless (or scarless) gene cloning technique is an important tool for precise assembly of DNA fragments which leaves no additional linker sequence between assembled fragments. This method enables the creation of an ideal condition for precise functional studies, such as mutation study, gene fusion, and genome engineering [20, 21]. Recently, the seamless cloning techniques, such as Golden Gate cloning (GGC) [22, 23] and Gibson assembly [24], have been developed and widely used in various genetic engineering applications.

Type IIS restriction enzymes recognize a 5- to 8-bp asymmetrical sequence and cleave outside the recognition sequence [25]. This unique feature fits for the seamless cloning method and is used in GGC. Usually, PCR-amplified fragments are used for GGC or other seamless cloning techniques. However, each fragment needs 10 or more excess bases to be added at the 5′end of each primer (Fig. 1C), which may disturb PCR amplification. Using a PCR fragment, it also needs to confirm the DNA sequence to obtain a correct clone because the DNA polymerases do not have 100% fidelity. Several expression vectors have become available for GGC and other seamless cloning techniques. In this paper, we focused on

constructing a new series of entry vectors, pIIS18-*Sap*I, pIIS18-*Bsm*BI, pIIS18-*Bsa*I, pIIS18-*Bfu*AI-1, and pIIS18-*Bfu*AI-2. Each vector carries a newly designed multiple cloning site (MCS) on a modified pUC18 backbone [18, 26]. We constructed this plasmid series as described below.

1.2 Materials and methods

1.2.1 Linker design

Five different series of DNA linkers were designed to include two facing type IIS enzyme cleavage sites (*Sap*I, *Bsm*BI, *Bsa*I, or *Bfu*AI) and one blunt-end enzyme cleavage site (*Eco*RV, *Bsp*68I, or *Swa*I) (Fig. 1A). Each design retains the same reading frame of the ß-galactosidase gene (*lacZ*). pUC18 carries two *Bsm*BI sites, one *Sap*I site, and one *Bsa*I site within its backbone. Linkers sequences are listed in table 1.

Table 1 Oligonucleotides used in the study.

Oligo's Name	Sequence 5` to 3`
*Sap*I linker 1	GGC TCT TCG CGA AGA GCG AG
*Sap*I linker 2	GAT CCT CGC TCT TCG CGA AGA GCC TGC A
*Bsa*I Linker1	GCG GTC TCG CGA GAC CG
*Bsa*I Linker2	GAT CCG GTC TCG CGA GAC CGC TGC A
*Bsm*BI Linker1	GCC GTC TCG CGA GAC GG
*Bsm*BI Linker2	ACG TCG GCA GAG CGC TCT GCC CTA G
*Bfu*A1 Linker1	GCA CCT GCA GAT ATC TGC AGG TG
*Bfu*A1 Linker2	GAT CCA CCT GCA GAT ATC TGC AGG TGC TGC A
Beta3 Linker1	GCC ACC TGC ATT TAA ATG CAG GTG CG

Beta3 Linker2	GAT CCG CAC CTG CAT TTA AAT GCA GGT GGC TGC A
pREGO18BsmBI_1 Fw	tctaagaaaccattaGAGCAGACAAGCCCGTCA
pLEGO18BsmBI_1Rv_ tag	gtcagtgagcgaggaagCGGAAtAGCGCCCAATAC
pLEGO18BsmBI_2Fw	TCCTCGCTCACTGACTCG
pLEGO18BsmBI_2Rv	gctctcgcggtatCATTGCAGCACTGG
pLEGO18BsmBI_3Fw	ATGATACCGCGAGAgC
pLEGO18BsmBI_3Rv	TAATGGTTTCTTAGACGTCAGG
pUC seq Fw	GCAAGGCGATTAAGTTGGGTA
pUC seq Rv	CCTCCGGCTCGTATGTTGTGT

Small under lined sequence are the infusion tags.
Small red sequence is a replaced nucleotide.

1.2.2 Site directed mutagenesis

Type IIS sites within pUC18 backbone was removed using site-directed mutagenesis. Three primer sets were used for the process of Type IIS removal Table 1. PCR amplification performed using KOD plus new, plasmid parts was then ligated by In-Fusion® HD Cloning Kit (Clontech, Japan). The obtained construct was introduced to *E. coli* DH5α. Positive colonies were selected on LB media containing x-gal and ampicillin (25 ug/ml). This will prevent jamming

(incorrect) re-ligation in case of the one-pot reaction of GGC (Fig. 1D) [22].

1.2.3 Plasmids construction

For pIIS18-*Sap*I construction, pUC18 was doubly digested with *Pst*I and *Bam*HI. The linearized pUC18 was purified and ligated with the *Sap*I DNA linker (Fig. 1A). The obtained ligation product was introduced to *E. coli* DH5α chemically competent cells (Nippon Gene, Japan), following the standard protocol, and colonies were selected on an LB agar plate supplemented with ampicillin (100 µg/ml) and 2% X-Gal (5-bromo-4-chloro-3-indolyl-ß-d-galactopyranoside).

1.2.4 Construct validation

The modified construct (within blue colonies) was confirmed by successful cleavage with *Sap*I or *Bsp*68I and whole-plasmid sequencing using the BigDye Terminator ver. 3.1 cycle sequencing kit. The sequence data were analyzed using an ABI 3130xl genetic analyzer (Thermo Fisher Scientific, Inc.). The sequence primers are listed in table 1. The other four pIIS18 vector models have been constructed in the same way as pIIS18-*SapI*.

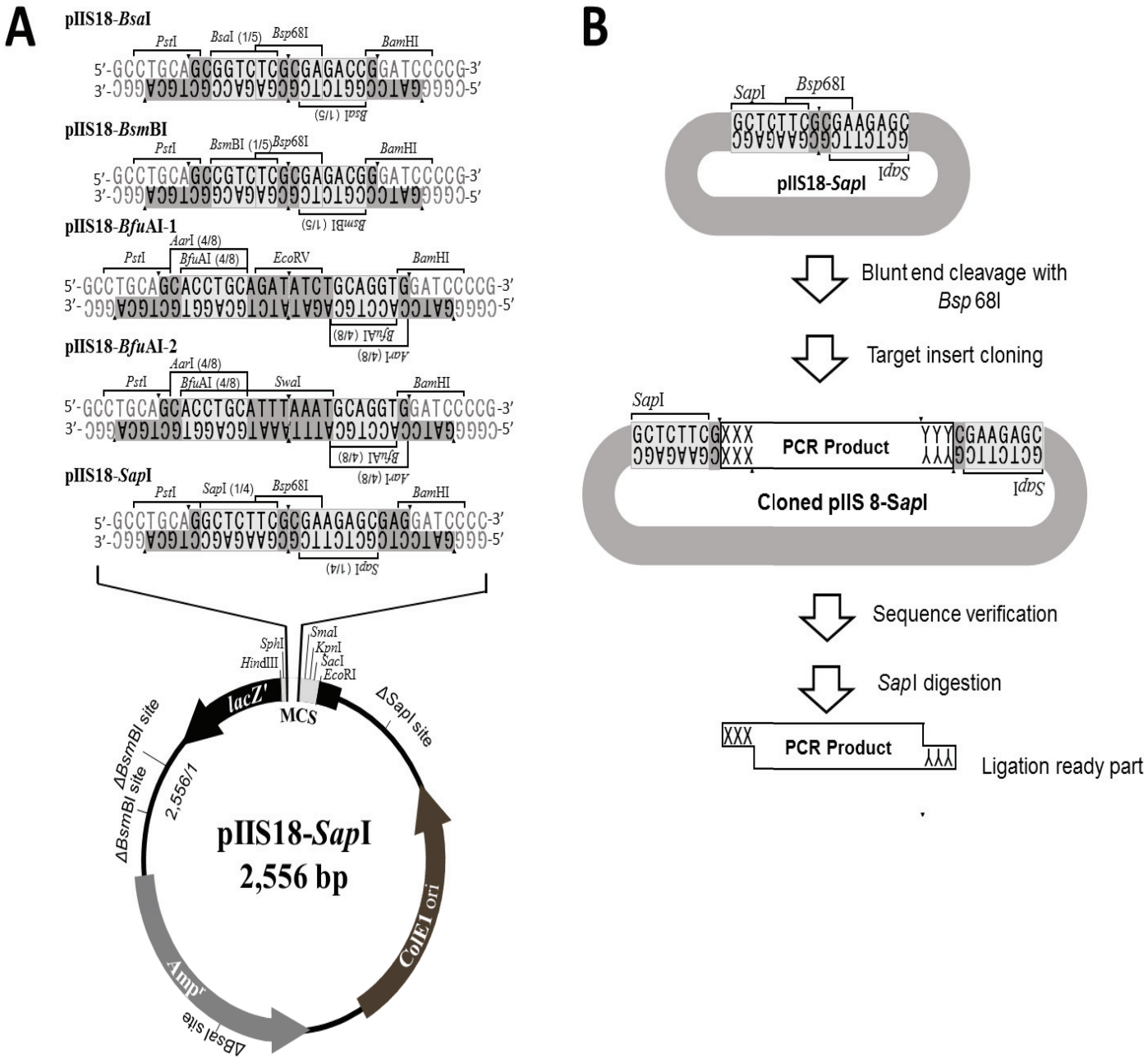
1.3 Result and discussion

pIIS18 series are designed for type IIS restriction enzyme-mediated seamless gene fusion, such as GGC (Fig. 1D). The cohesive ends resulting from type IIs enzymes digestion are typically matching for golden gate cloning. The occurrence of variant type IIs restriction sites over lapping or divided by one blunt ended restriction site allows for convenient rapid direct cloning and sub cloning of DNA insert into the MCS. The tagged PCR fragments can be directly inserted into restricted pUC18 with one variant of blunt ended site and subsequently removed as *Sap*I, *Bsa*I, *BfuA*I or *BsmB*I related fragment. Once the DNA fragment inserted in the entry vector, it can be used as a template for sequencing using the same primers for any insert. They allow for direct sequence analysis of a cloned DNA fragment with just a single primer set annealing to the plasmid backbone before seamless ligation (Fig. 1B). The mutation rate during the ligation and transformation reactions is practically ignorable. The pIIS18 series will be helpful for molecular biologists, especially in experiments requiring DNA sequence verification, such as multigene fusion (Fig. 1D), and systematic construction of mutants.

1.4 Data availability.

The complete sequences of pIIS18-*SapI*, pIIS18-*BsmBI*, pIIS18-*BsaI*, pIIS18-BfuAI-1, and pIIS18-BfuAI-2 have been deposited in the DNA Data Bank of Japan under the accession numbers LC459971 to LC459975, respectively. The resource can be obtained from the Addgene depository (https://www.addgene.org/) and the GCMR library of Gifu University (https://www1.gifu-u.ac.jp/~g_cmr/index.html). The raw sequencing reads are available at https://www1.gifu-u.ac.jp/~suzuki/pIIS_plasmids/.

Figure 1


A
pIIS18-BsaI
PstI
BsaI (1/5)
Bsp68I
BamHI
5′-GCCTGCAGCGGTCTCGCGAGACCGGATCCCCG-3′
3′-CGGGGATCGCCAGAGCGCTCTGGCCTAGGGC-5′
pIIS18-BsmBI
PstI
BsmBI (1/5)
Bsp68I
BamHI
5′-GCCTGCAGCCGTCTCGCGAGACGGGATCCCCG-3′
3′-CGGGGATCGCCAGAGCGCTCTGCCCTAGGC-5′
pIIS18-BfuAI-1
AarI (4/8)
PstI
BfuAI (4/8)
EcoRV
BamHI
5′-GCCTGCAGCACCTGCAGATATCTGCAGGTGGATCCCCG-3′
3′-CGGGGATCCACCTGCAGATATCTGCAGCTGCTGCCTAGGC-5′
pIIS18-BfuAI-2
AarI (4/8)
PstI
BfuAI (4/8)
SwaI
BamHI
5′-GCCTGCAGCACCTGCATTTAAATGCAGGTGGATCCCCG-3′
3′-CGGGGATCCACCTGCATTTAAATGCAGCTGCTGCCTAGGC-5′
pIIS18-SapI
PstI
SapI (1/4)
Bsp68I
BamHI
5′-GCCTGCAGGCTCTTCGCGAAGAGCGAGGATCCCC-3′
3′-CGGGGATCGCCTCTTCGCGAAGAGCCTGCTAGGC-5′
SphI
HindIII
SmaI
KpnI
SacI
EcoRI
lacZ′
MCS
ΔSapI site
ΔBsmBI site
ΔBsmBI site
2,556/1
pIIS18-SapI
2,556 bp
ColE1 ori
Ampr
ΔBsaI site
B
SapI
Bsp68I
pIIS18-SapI
Blunt end cleavage with
Bsp 68I
Target insert cloning
SapI
PCR Product
Cloned pIIS 8-SapI
Sequence verification
SapI digestion
PCR Product
Ligation ready part

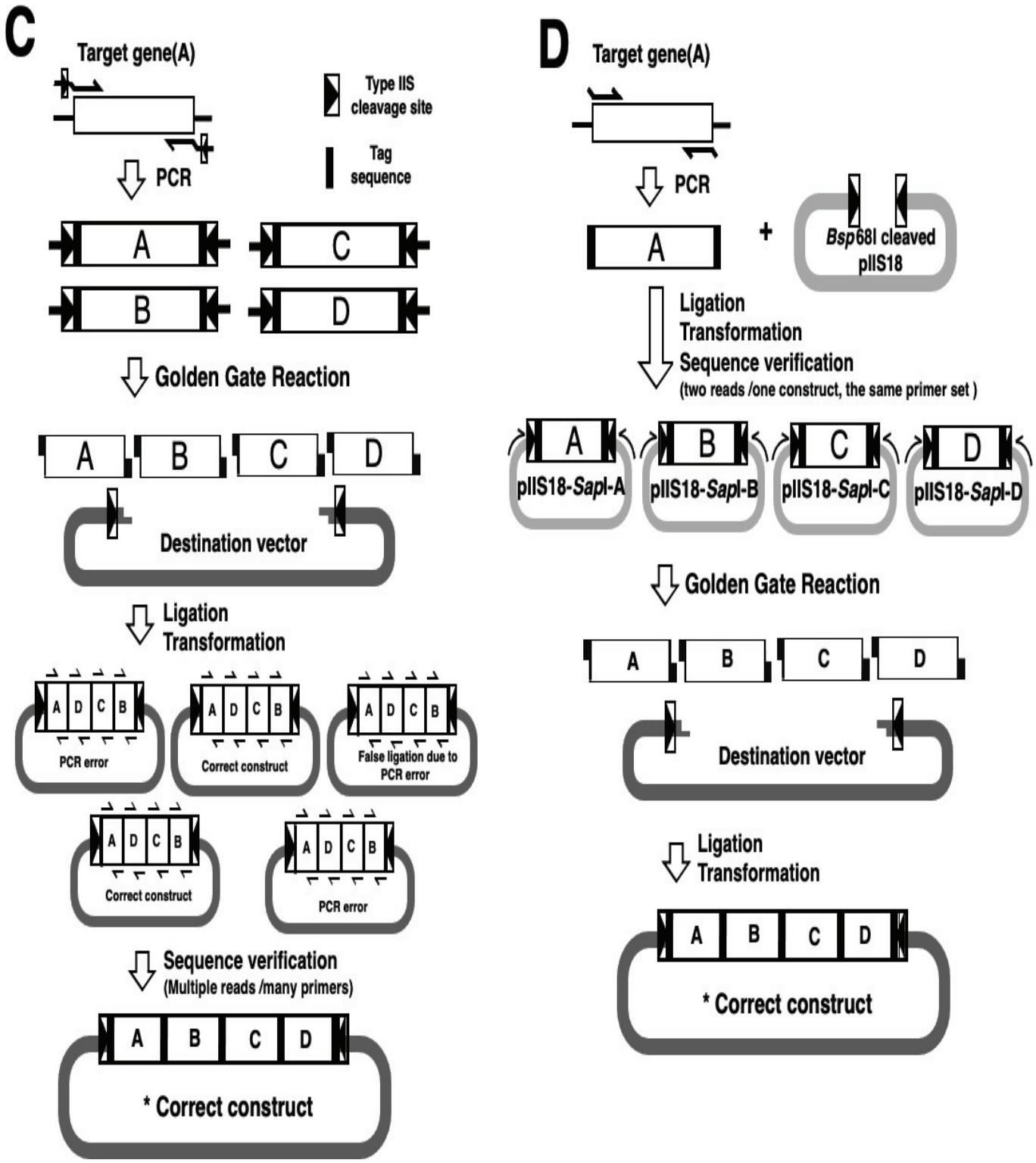


Fig. 1. A: Molecular structure of pIIS18-*Sap*I cloning vector series, showing genes on the plasmid backbone. The removed Type IIS enzyme cleavage sites from pUC18 are marked with parenthesis on plasmid map. MCS structure is

illustrated within the *lacZ'* gene. The DNA linkers inserted into the MCS of each plasmid construct is demonstrated. Each linker carries one blunt-end enzyme and two facing Type IIS enzyme cleavage sites located between *Pst*I and *BamH*I sites. **B:** Model for usage of the pIIS18 entry vector, demonstrating the insertion of a three base pair tagged PCR product within the blunt-end cleaved pIIS18-*Sap*I. Once a fragment is inserted in pIIS18, it can be sequence verified then become a ligation ready part. **C:** Original GGC, in which PCR errors make it possible to get a mutated construct, hence it requires multiple proof sequence reads to find out the correct construct and requires multiple primer design. **D:** GGC with pIIS18, in which PCR errors eliminated through additional cloning and sequencing step before GGC. The additional step removes the possibility of PCR error construct and allows the multiple usage of sequence verified DNA part several times. Few sequence reads will be needed just to confirm fragment order in the final construct.

Chapter two

Cell factory for gamma amino butyric acid production using bifidobacteria

2.1 Introduction

Microbial cell factories are a bioengineering approach in which microbial cells are used for the cost-efficient production of valuable chemicals such as vitamins and essential amino acids [27, 28]. GABA is a four-carbon amino acid produced by the irreversible decarboxylation of glutamate [29]. It is the major inhibitory neurotransmitter in the central nervous system [30]. Moreover, GABA has been reported as a promising supplement in many conditions including depression, poor sleep quality [31, 32], immunity [33], and diabetes, as it is a strong insulin secretagogue [34, 35]. Thus, various approaches for GABA production have gained great attention in recent years [36]. Chemical synthesis and biotransformation (microbial synthesis) are two major methods for GABA production. Biotransformation based on microbial biosynthesis is an eco-friendly source of GABA compared to chemical biosynthesis. Lactic acid bacteria (LAB) have been used extensively in this field [36, 37].

The LAB, *Bifidobacterium,* are anaerobic gram-positive symbiotic bacteria that are well-known probiotic and health-promoting supplements used in the food industry. Some *Bifidobacterium* species have a strain-specific capacity for GABA production, including *B. adolescentis* [38], *B. dentium* [39] and *B. angulatum* [40]. Importantly, anaerobic fermentation requires no shaking or air sterilization, thus

reducing the final production cost. Hence, the anaerobic nature and simple GABA biosynthesis in *Bifidobacterium* make it a cost-effective candidate for GABA cell factories.

Metabolic engineering can be used to produce new phenotype-carrying microbes that are optimized to function as microbial cell factories [41]. Previously, there were limited gene manipulation tools for *Bifidobacterium*. However, several new tools have been developed in the last few years [42] and have opened the door for metabolic engineering. Our research group previously reported the construction of a *Bifidobacterium-Escherichia coli* shuttle vector pKKT427 [43], and the development of a gene knockout technique, temperature-sensitive plasmids [44], and a plasmid artificial modification method (PAM) [43]. We also conducted a recent analytic study of core promoters in *Bifidobacterium* [45]. In the current study, we aimed to use the available genetic tools for producing an industrially relevant strain of *Bifidobacterium*. We focused on GABA as a model compound. Hence, we aimed to elucidate the machinery of GABA production from *Bifidobacterium* recombinant strains aiming to maximize GABA productivity.

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids and cultivation condition

The bacterial strains, plasmids, and promoters used in this study are listed in table 1. Luria-Bertani (LB) was used for the cultivation of *E. coli* TOP10 competent cells (Invitrogen, life science, USA). MRS (de Man, Rogosa, Sharpe medium) (Becton, Dickinson and company sparks, MD 21152, USA) was used for the standard cultivation of *Bifidobacterium*. Other media used were: GAM (Gifu Anaerobic Medium) (Nissui Pharmaceutical Co., Ltd., Code/05422), developed for general culture and susceptibility testing of anaerobic bacteria, and BMM (*Bifidobacterium* Minimal Medium) [49], a chemically defined medium containing inorganic salts, glucose, vitamins, isoleucine, and tyrosine.

Bifidobacterium strains were manipulated under anaerobic conditions on a BUG Box (Dual gas, Ruskinn Technology, Ltd., UK) using mixed gas supplement (80% N_2, 10% CO_2, and 10% H_2). Monosodium glutamate (MSG) (Sigma Aldrich, France) was added to liquid culture as a substrate for GABA production. Standard cultivation was performed by direct inoculation of the frozen stock (−80 °C) to liquid culture, followed by incubation at 37 °C for 24 hours (h). Bacteria were then sub-cultured in MRS containing 1% MSG (v/v). After 48 h of incubation, bacteria were centrifuged, and the supernatant was used for HPLC analysis.

Spectinomycin (Sp) (75 μg/mL) was added to the culture of both *Bifidobacterium* and *E. coli* recombinant strains.

Table 1 *Bifidobacterium* strains and promoters used in this study

Strain	Characteristic feature	Origin	References
B. adolescentis 4-2	Wild type GABA producer	Human feces	This study
B. longum 105-A	High transformation efficiency	Adult gut	(46)
B. adolescentis JCM 1275	Low transformation efficiency	Intestine of adult	(43)
B. Longum infantis JCM 1222	High oxygen sensitivity	Infant gut	(47)
B. minimum JCM 5821	Unique oxygen tolerance	Sewage	
E. coli top10	Chemically competent cells	Thermo Fisher Scientific	
Promoter	Characteristic feature	Origin	References
P_{gap}	The promoter of Glyceraldehydes-3-phosphate dehydrogenase gene	*B. Longum* 105A	(48)
P_{BLt43}	The promoter of tRNA gene	*B. Longum* NCC2705	(unpublished data)
P_{ori}	The promoter of glutamate decarboxylase gene	*B. adolescentis* 4-2	This study
Plasmid	Characterestic feature	References	
pKKT427	A shuttle vector between *Escherachia coli* and *Bifidobacterium* shuttle vector	(44)	

	Sp^r, 3.9kb modified of pBRATA101	
pBCMAT_P*gap*_TdppA2	A plasmid construct for Chloramphenicol assay based on pKKT427 backbone, including gap-promoter	(unpublished data)
pBCMAT_P*Blt43*_TdppA2	A plasmid construct for Chloramphenicol assay based on pKKT427 backbone, including *BLt43*-promoter	(unpublished data)
pKKT427::P*ori*_*gadBC*	pKKT427 carrying *gadB* and *gadC* genes with the original promoter *gadB* gene	This study
pKKT427::P*gap*_*gadBC*	pKKT427 carrying *gadB* and *gadC* genes with the P_{gap}	This study
pKKT427::P*BLt43*_*gadBC*	pKKT427 carrying *gadB* and *gadC* genes with P_{Blt43}	This study
pPAM1233-1283	pBAD33 carrying BAD_1233 and BAD_1283 PAM plasmid, used for methylation of pKKT427	(43)

Underlined sequences are *Sap*I recognition site

2.2.2 Optimization of fermentation parameters for GABA production

The optimal combination of media type, MSG amount, initial pH, incubation time and bacterial growth was determined by measuring the extracellular GABA under each condition. Three culture media was tested. Different amount of MSG was added to each of the tested culture media and GABA productivity was estimated. The effect of the initial pH was assessed by adjusting the pH to values ranging from (4.4 to 7.0) with HCL or NaOH. Different

amounts of MSG were added to MRS media of low pH to examine original promoter activity under pH stress. GABA production and bacterial growth were estimated within the course of each tested parameter. Batch fermentation was performed in volume of 100ml MRS, pH 4.4, congaing 4% MSG. preculture volume was 10% of the whole batch culture volume. During the cultivation time, when MSG glutamate were reduced to 67mM additional 67mM MSG was added to growth media. Pyridoxal 5-phosphate (PLP) was added to the culture media at 0h and after 72h. One ml of the culture was withdrawn every 6 hour, 300ul was used for OD590 and 300ul was used for GABA and glutamate measurement.

2.2.3 Molecular cloning and DNA manipulations

Oligo-primers used in this study are listed in Table 2. Genomic DNA was extracted from *B. adolescentis* 4-2 using the isofecal DNA extraction kit (Nippon Gene, Japan). Plasmids were extracted from *E. coli* using the QIAprep spin mini kit (QIAGEN, Germany). Amplification of *gadB* and *gadC* genes was performed using KOD -*Plus*- neo (Nippon Gene, Japan) following the manufacturer's instructions. Golden Gate cloning (GGC), a type IIS enzymes-based strategy [50], was used for plasmid cloning. The cleavage site of *sap*I (Type IIS restriction enzyme [51] was added to the oligo-primers amplifying both *gadBC* (insert) and pKKT427 (plasmid backbone). A three base pair tag was added following the *Sap*I

site for precise ligation in the GGC reaction. GGC cycles were performed as described in [22]. The cloned plasmid was transformed into *E. coli* cells. Selection of positive colonies was based on antibiotic sensitivity. Plasmids were extracted and sequences were confirmed using the BigDye Terminator ver. 3.1 Cycle sequencing kit. The sequence data were analyzed using an ABI 3130xl genetic analyzer (Thermo Fisher Scientific, Inc.). The correct plasmid construct was introduced into *Bifidobacterium* using electroporation (MicroPulser, Bio-Rad, California, USA) as previously described [44]. Then, the transformants were selected on MRS (Sp) plates. The transformation of *B. adolescentis* JCM 1275 was performed using the PAM method as previously described [43].

Table 2 Oligoprimers used in the study

Primer	Coding sequence **5` to 3`**	Template	Purpose
gadBC_OP_Fw	ccagctcttcgACAacctgcccatcgtagc	*B. adolescentis* 4-2 genomic DNA	Amplify *gadBC* gene with the original promoter
gadBC_OP_RV	ccagctcttcgCTAtcagtattccggattcactagc		
pKKT427 Fw	caagctcttcgTAGgccaccgtcgccaagg	pKKT427	Amplify Pkkt427 plasmid backbone
pKKT427 Rv	caagctcttcgTGTgcctgcatgcaagctt		
gadBC Fw	ccagctcttcgatgtcagaaacacattccacc	*B. adolescentis* 4-2 genomic	Amplify *gadBC*

gadBC Rv	caagctcttcgtcagtattccggattcactagc	DNA	gene without the original promoter
pKKT427_ter_Fw	ccagctcttcgTGActgactcactgaacgg	pBCMAT_P*gap*_TdppA2	Amplify pKKT427 plasmid backbone including the promoter and terminator
pKKT427_pro_rv	ccagctcttcgCATgatgttctccttggtca		
gadB_RT1_Fw	catgttcctgcgtttgggat	*B. adolescentis* 4-2 genomic DNA	*gadB* quantitative expression
gadB_RT1_Rv	ccgtcgttccacagcgta		
gadC_RT1_Fw	cgtcggtttcgtcgctt		*gadB* quantitative expression
gadC_RT1_Rv	Cacaagaatcgcatatgaaacgcta		
16srRNA_FW	Cacattccaccgttacacc		Normalize gene expression in *B. adolescentis* 4-2
16srRNA_FW	Cgttatccggaattattggg		

2.2.4 Real time PCR and mRNA manipulations

Total RNA was extracted from *Bifidobacterium* using the TRIzol reagent as previously described [50]. The quality of extracted mRNA was estimated using a Bioanalyzer Agilent2100 (Agilent Technologies, Germany). Reverse transcription was performed using the iScript™ cDNA synthesis kit (Bio-Rad, USA). The resulting cDNA was assessed with real-time PCR on an ABI StepOnePlus system (Applied Biosystems, Singapore) using theΔΔCt method [52]. The real-time PCR reaction was performed using Thunder Bird™ SYBR® qPCR mix (Toyobo, Japan). The *B. adolescentis* 4-2 16S rRNA gene was used as an internal standard for expression normalization. The primers used for 16S rRNA, *gadB*, and *gadC* genes are listed in Table 2. Primers were designed using Oligo ver. 7 software.

2.2.5 High performance liquid chromatography (HPLC) analysis

GABA concentrations were quantified through HPLC (Agilent series 1100, Shimadzu, Japan) equipped with a fluorescence detector (Ex 350 nm EM 450 nm) and a Cosmosil packed column $5C_{18}$-MS-II (3.0ID X 150 mm). Prior to analysis, each sample was derivatized with the reagent O-phthalaldehyde (OPA) [53]. The mobile phase composed of A ($CH_3CN/CH_3OH/H_2O$ 45/40/15, v/v/v) and B (20 mM KH_2PO_4 (pH6.9), H_3PO_4). Compounds were eluted using gradient program: 0-9

min, 100% B; 9-12 min, 89% B; 12-21 min, 78% B. The column temperature was maintained at 35 °C with a flow rate of 0.7 mL/min. GABA and glutamate were identified and quantified by their characteristic retention times and standard curves, respectively. GABA and glutamate were purchased from Wako (Japan) and Sigma, respectively.

2.2.6 Statistical analysis

All data are expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA). Data were deemed significant when $P < 0.05$, unless otherwise indicated. Analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) and SPSS (Statistical Package for Social Sciences, USA). Multi factorial design, surface plot and contour plot were created using MINITAB® software (v17.1.0, 2002) (Minitab Inc, Co., Pine Hall RdState College, PA 16801-3008, USA).

2.3 Results

2.3.1 GABA production in wild type *B. adolescentis* 4-2.

Annotation analysis revealed that GABA production from the wild type *B. adolescentis* 4-2 genome is a function of two genes, *gadB* and *gadC*, encoding glutamate decarboxylase and GABA-glutamate antiporter, respectively. Wild type *B. adolescentis* 4-2 strain produced approximately 14 mM GABA during 72 h fermentation with an average production rate of 0.0194 g/L/h. GABA accumulation in the medium occurred slowly over a period of 48–72 h (Fig. 1A). Moreover, *gadB* and *gadC* expression increased in the early stages of growth and decreased in the late stages. At 24 h, *gadB* and *gadC* was 0.7-fold lower than that at 12 h. At 36 h, *gadB* and *gadC* was 0.9 and 0.8-fold lower than at 12 h, respectively (Fig.1B).

Figure 1

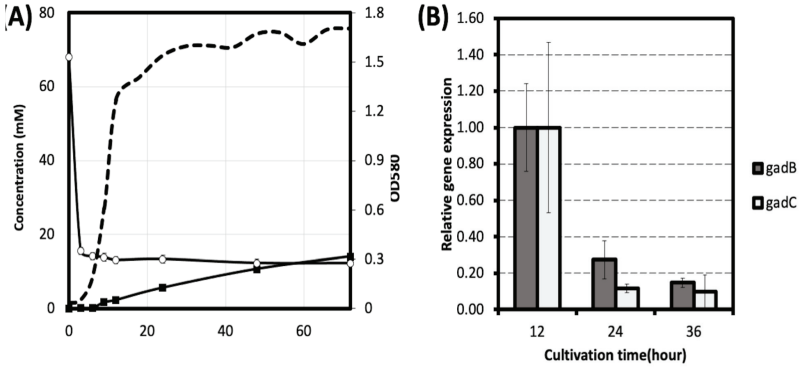


Figure 1. GABA productivity in wild type *B. adolescentis* 4-2. (A) GABA/glutamate conversation pattern and bacterial growth over the course of 72 h. Bacterial growth, GABA production and glutamate consumption are displayed. (B) Expression of GABA production genes, *gadB* and *gadC*, in different growth stages of *B. adolescentis* 4-2

2.3.2 GABA productivity from recombinant *Bifidobacterium*.

A total of nine recombinants were constructed in this study using five different *Bifidobacterium* strains. These five strains represent four different characteristic features of *Bifidobacterium* Table 1. The wild-type strain was used for self-cloning. Three different promoters were examined for expression, including two constitutive promoters (*gap* and *BLt43*) and the original *gadB* promoter (Fig. 2A). Both *gap* and *s* promoter were reported as high-expressing promoters in *Bifidobacterium* (22). GABA production genes, *gadB* and *gadC*, were over expressed in *B. adolescentis* JCM1275 as well as three other *Bifidobacterium* species (*B. longum* 105-A, *B. longum infantis* JCM 1222, and *B. minimum* JCM 5821). (Fig. 2B-C). Recombinant strains successfully produced GABA from available MSG in growth media. Conversion ratio of glutamate to GABA ranged from 93±6 to 100% when grown on MRS medium containing 67mM MSG (Fig. 2B-C). The conversion ratio was calculated using the following equation:

$$\text{Conversion ratio} = \frac{final\ GABA\ production}{Initial\ glutamate\ concentration} \times 100$$

As all produced recombinants had similar degree of expression. We randomly chose two strains to further investigate: one carrying the original promoter (*B. adolescentis* JCM 1275/pKKT427::P_{ori}-*gadBC*) and the other carrying the *gap* promoter (*B. adolescentis* JCM 1275/pKKT427::P_{gap}-*gadBC*). The first strain had a slow production pattern that reach its maximum after 48–72 h (Fig. 3A). In contrast, the second strain had a speedy

production pattern that maximized at 18–24 h (Fig. 3B). In the exponential phase, GABA produced per hour was higher in *B. adolescentis* JCM1275/pKKT427::P_{ori}-*gadBC*, compared to *B. adolescentis* JCM 1275/pKKT427::P_{ori}-*gadBC* (0.2 g/L/h versus 0.1 g/L/h, respectively).

Figure 2

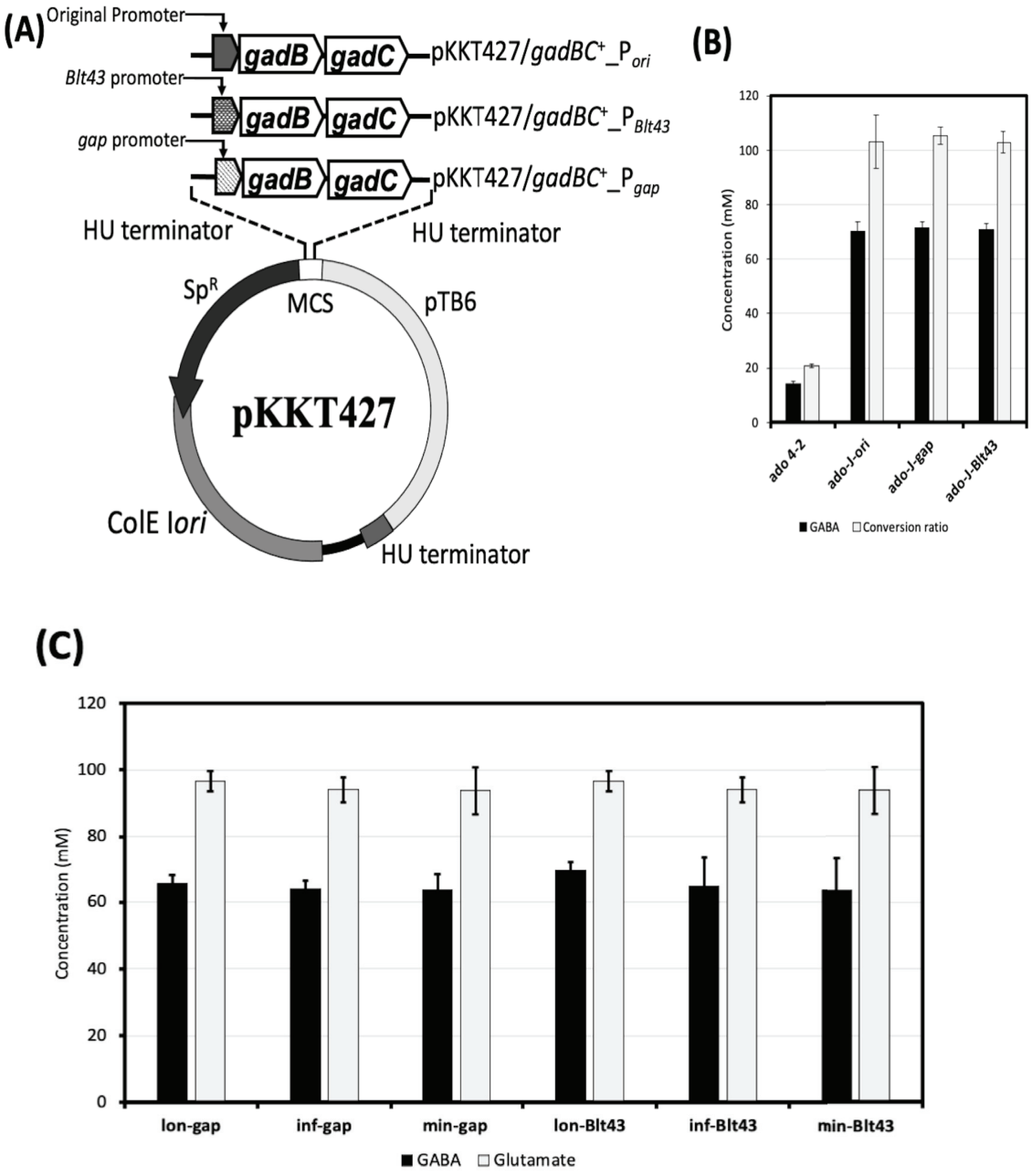


Figure 2. (A) Diagram of expression vector construction displaying the backbone of pKKT427, a *Bifidobacterium-E. coli* shuttle vector, in which GABA producing genes were inserted within the multiple cloning site (MCS). The names of three plasmid constructs with different promoters are illustrated in the upper part of MCS. (B) GABA production and Glutamate/GABA conversion ratio by *B. adolescentis* strains; *B. adolescentis* 4-2 wild (ado4-2), *B. adolescentis* JCM 1275/pKKT427::P_{ori}-*gadBC* (ado-J-ori), *B. adolescentis* JCM 1275/pKKT427::P_{gap}-*gadBC* (ado-J-gap) and *B. adolescentis* JCM 1275/pKKT427::P_{Blt43}-*gadBC* (ado-J-Blt43). (C) GABA production and Glutamate/GABA conversion by other *Bifidobacterium* recombinant strains; *B. longum*105-A (lon.), *B. longum* subsp *infantis* JCM 1222 (inf.), *B. minimum* JCM 5821 (min.), each cloned with both *gap* and *Blt43* promoters. The promoter names are displayed with the corresponding strains. Values are presented as means ± SD. Analysis was performed on three independent bacterial cultures.

Figure 3

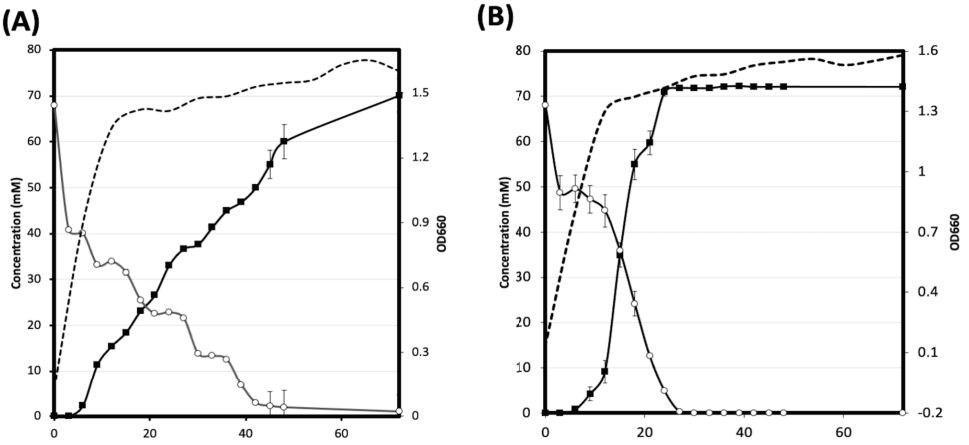


Figure 3. Bacterial growth and glutamate/GABA conversion pattern in two recombinant strains. (A) *B. adolescentis* JCM 1275/ pKKT427::P_{ori} -*gadBC* . (B) *B. adolescentis* JCM 1275/pKKT427::P_{gap}-*gadBC*. Bacterial growth, GABA production and optical density are presented by black separated line, black line with rectangle and gray line with circles, respectively. Values are presented as means ± SD. Analysis was performed on three independent bacterial cultures.

2.3.3 GABA production from *B. adolescentis* JCM 1275/pKKT427::P_{ori}-*gadBC*

2.3.3.1 The effect of different media on GABA productivity

The effects of three different media on GABA productivity were tested. Both MRS and BMM exhibited equal GABA productivity in 2% MSG. MSG concentrations higher than 2% suppressed both GABA production and bacterial growth in BMM (Fig. 4A and B). GAM medium had the lowest conversion rate of glutamate to GABA (Fig. 4A). Bacterial growth on MRS was better than that on GAM and BMM medium (Fig. 4B). MSG addition suppressed bacterial growth at the early exponential growth phase (Fig. 4C). Further, statistical analysis revealed that media had an extremely significant effect ($P < 0.0001$), accounting for 53.55% of the total variance between the groups. The media directly affected bacterial growth but did not enhance GABA productivity (Fig. 4B).

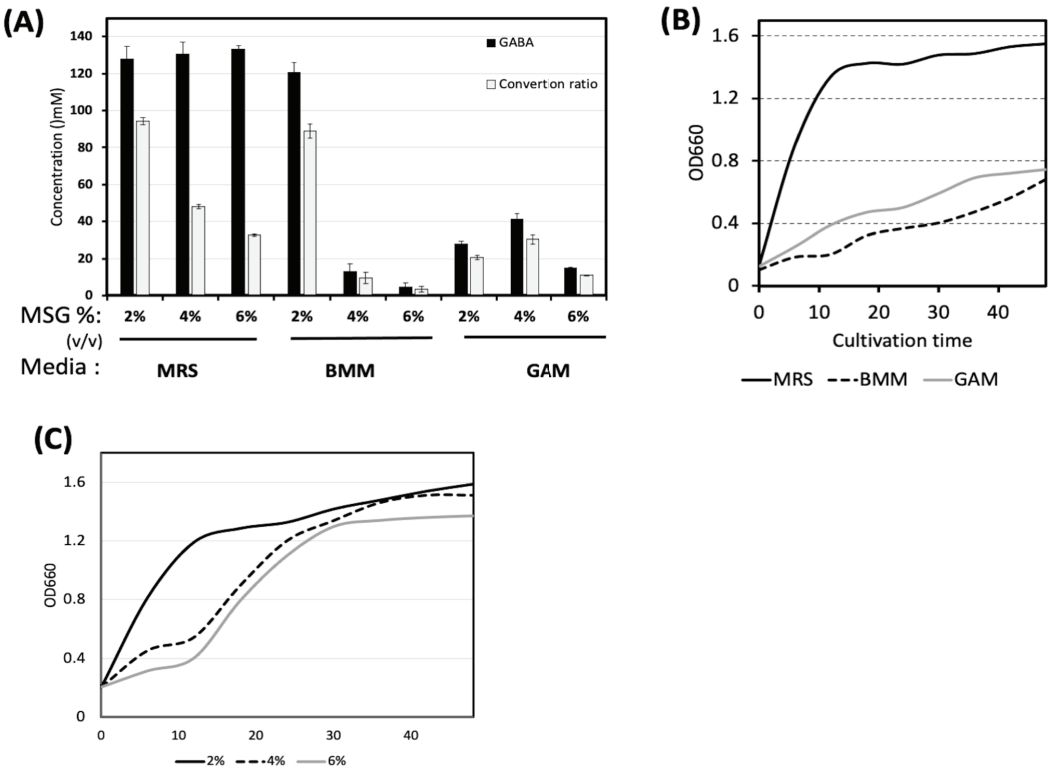


Figure 4. GABA production from *B. adolescentis* JCM 1275/pKKT427::P$_{ori}$-*gadBC* in different fermentation conditions. (A) The effect of media on glutamate/GABA conversion. Three media and three different concentrations of MSG (%, v/v) were used. Media names and MSG % are displayed at the bottom of the graph. (B) Bacterial growth curves for different media types are displayed. (C) The effect of substrate (MSG) concentration on GABA production. (D) The effect of MSG concentration on bacterial growth. Bacterial growth is represented as optical density. Values are presented as means of ± SD. Analysis was performed on three independent bacterial cultures.

2.3.3.2 The effect of pyridoxal 5-phosphate on GABA productivity

Since, Pyridoxal 5-phosphate is an essential co-factor for GABA production. We hypothesized that it may recover gad activity especially at late stages of bacterial growth. Hence, we investigated the effect of PLP addition on extracellular GABA. PLP was added at different time points during bacterial growth 0h, 24h and 48h. When PLP was added at 24h and 48h of fermentation, the GABA production was much higher than that of which PLP was added at the 0h Fig 5A. The result suggests that PLP addition partially recovered gad activity. However, PLP could be denaturized when added at the early stages of growth (0h). Therefore, it is more efficient to add PLP at 24- 48h to enhance GABA production.

2.3.3.3 The effect of culture pH and substrate concentration on GABA productivity

Multi- factorial design was constructed to test the effect of both culture pH and MSG concentration on extracellular GABA production. To estimate the efficiency of PLP addition to enhance GABA productivity, two models were investigated with and without PLP addition.

As a substrate for GAD, MSG was an important element for GABA production. But, extra glutamate may inhibit cell growth and decrease GABA production [54]. The same thing has occurred at pH 6.0. However, additional MSG was eventually

converted to GABA at pH4.4 (Fig.5B-C). These results suggest that the optimum pH for extracellular GABA production was pH 4.4. PLP addition improved extracellular GABA production double times as that of no PLP fermentation. From 211 mM to 408 mM (Fig.5D-E).

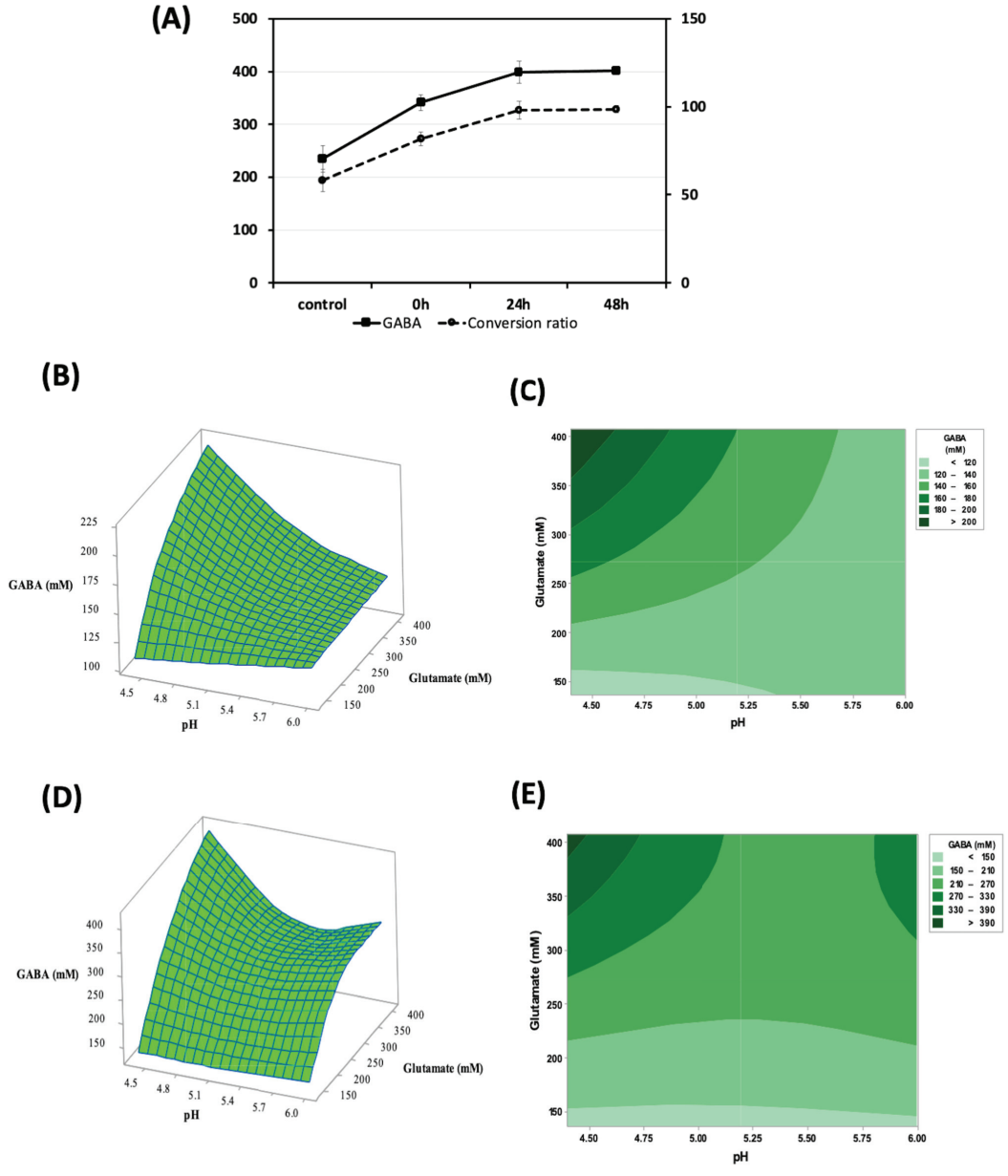


Figure 5. Effect of pH, MSG concentration and PLP addition on extracellular GABA production from *B. adolescentis* JCM 1275/pKKT427::P*ori-gadBC*. (A) Effect of

PLP addition at different time point compared to control, with no PLP. GABA production is displayed by black line with closed circles (●) and conversion ratio is displayed by black separated line with opened circles (○). (B-C) Surface plot and Contour plot illustrating the effect of culture pH and MSG concentration on extracellular GABA production without addition of PLP. (D-E) Surface plot and Contour plot demonstrating the effect of culture pH and MSG concentration on extracellular GABA production with addition of PLP.

2.3.4 GABA production from *B. adolescentis* JCM 1275/pKKT427::P_{gap}-*gadBC*

2.3.4.1 The effect of pyridoxal 5-phosphate on GABA productivity

As an imperative co-factor for GABA production, PLP addition improved GABA productivity. The addition of PLP at 24h was better than 0h and 48h. As this strain seemed to produce GABA in shorter time. It can be hypnotized that 24h is the point reduction of GAD activity in this strain. Addition of PLP at this point seemed to recover GAD activity.

2.3.4.2 The effect of culture pH and substrate concentration on GABA productivity

Multi- factorial design was constructed to test the effect of both culture pH and MSG concentration on extracellular GABA production. To estimate the efficiency

of PLP addition to enhance GABA productivity, two models were investigated with and without PLP addition.

As Lower pH is not the optimum pH for bacterial growth and higher glutamate concentration may also suppress bacterial growth. GABA was not efficiently produced at lower pH and higher initial glutamate concentration 407mM (Fig.6B-C). However, additional MSG was eventually converted to GABA at pH6.0 (Fig.6B-C). These results suggest that the optimum pH for extracellular GABA production was directed toward neutral pH (pH 6.0), possibly due to change in promoter activity from *ori* to *gap*. In contrast, PLP addition improved GABA production at both low (pH4.4) and near neutral pH (6.0) compared to no PLP fermentation, from 233 mM to 375 mM at pH6.0 and from 101 mM to 286 mM at pH4.4(Fig.6B-D).

Figure 6

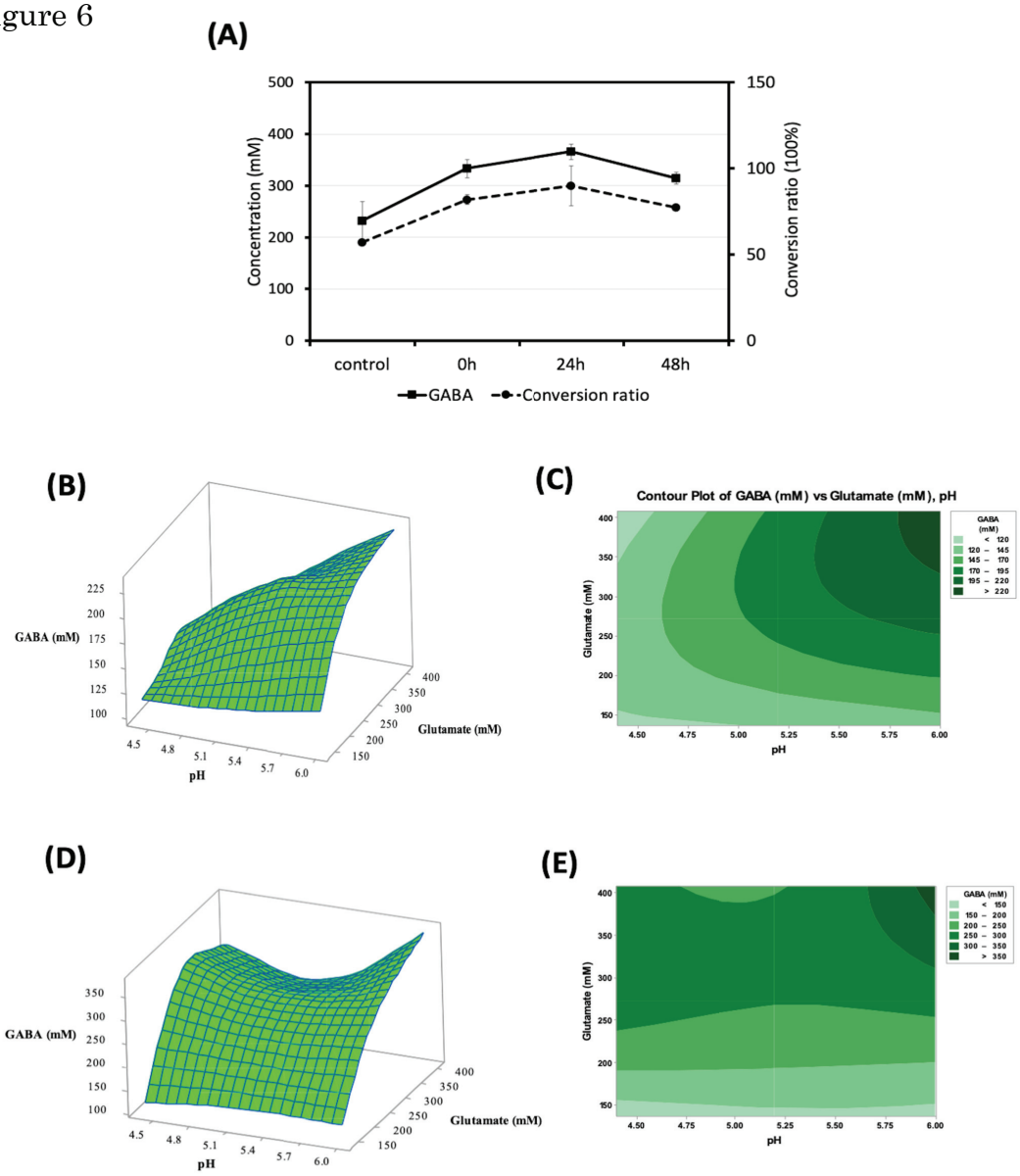


Figure 6. Effect of pH, MSG concentration and PLP addition on extracellular GABA production from *B. adolescentis* JCM 1275/pKKT427::P*gap*-*gadBC.* (A) Effect of PLP addition at different time point compared to control, with no PLP. GABA production is displayed by black line with closed circles (●) and conversion ratio is displayed by black separated line with opened circles (○). (B-C) Surface plot and Contour plot illustrating the effect of culture pH and MSG concentration on

extracellular GABA production without addition of PLP. (D-E) Surface plot and Contour plot demonstrating the effect of culture pH and MSG concentration on extracellular GABA production with addition of PLP.

2.3.5 Fermentation model for enhanced GABA production from both recombinants.

Batch fermentation was performed for *B. adolescentis* JCM 1275/pKKT427::P*ori-gadBC* on MRS containing 270 mM MSG (Fig. 7A). Culture pH was maintained at pH4.4 during the course of fermentation. When glutamate level was reduced to 67mM, additional glutamate was added dissolved in MRS medium of pH4.4. As PLP improved GABA production in small scale experiments (Fig.5A). Two times PLP addition were decided 0 and 72 h. Total added MSG estimated as 408mM which were totally converted to GABA using this model of fermentation producing 415mM GABA after 96h incubation. Another model was performed for *B. adolescentis* JCM 1275/pKKT427::P*gap-gadBC* on MRS containing 270 mM MSG (Fig. 7B). Culture pH was maintained around pH6.0 during the first 12 hours. When glutamate level was reduced to 30 mM, additional glutamate was added dissolved in MRS medium of pH 6.0. Two times PLP addition were decided 0 and 36 h.

Figure 7

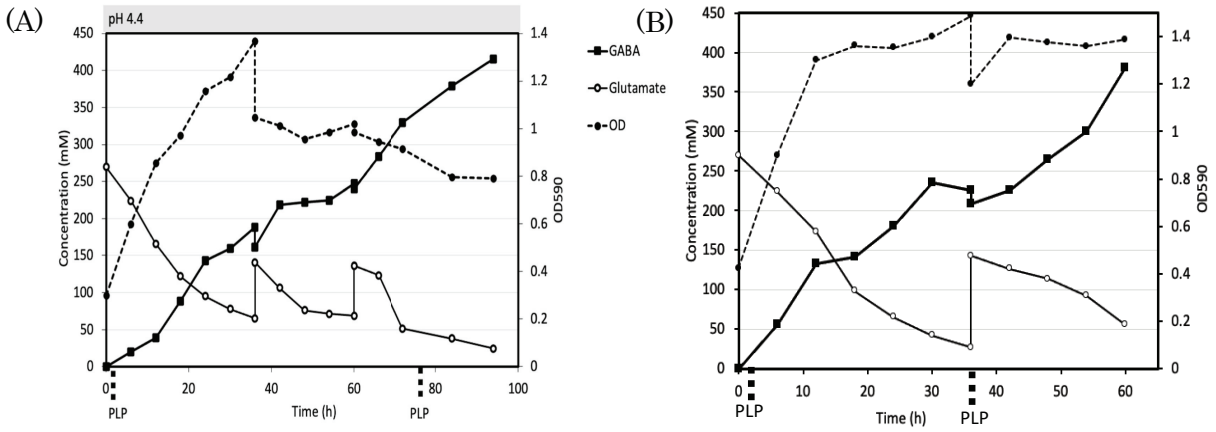


Figure 7. Evolution of GABA production (mM;■), glutamate concentration (mM; ○) and biomass production (OD580,●) during growth of two recombinants of *Bifidobacterium*. (A)*B. adolescentis* JCM 1275/pKKT427::P*ori-gadBC* (A) in MRS containing 270 mM of initial MSG concentration. pH was maintained at 4.4 during the course of fermentation. Two additions of approximately 70 mM MSG were added at 39 and 60 h of fermentation. Two addition of 0.05mM PLP were added at 0 and 72 h. (B)*B. adolescentis* JCM 1275/pKKT427::P*gap-gadBC* in MRS containing 270 mM of initial MSG concentration. pH was maintained aound pH 6 only during the first 12 hours. Single additions of approximately 110 mM MSG were added at 39 and 60 h of fermentation. Two addition of 0.05mM PLP were added at 0 and 36 h.

2.4 Discussion

Bifidobacteria are important symbiotic bacteria widely used in the probiotic industry [55]. GABA production has been reported in some

Bifidobacterium species [34]. Recently, the production of GABA using microbial cell factories has gained a lot of interest owing to its eco-friendly nature [27, 28]. In this study, we examined the ability of bifidobacteria to function as a cell factory for GABA production. We screened more than 20 strains, belonging to nine species of *Bifidobacterium*, for GABA production (data shown in chapter 4). *B. adolescentis* 4–2 was selected as a high GABA producer. We proceeded by estimating the productivity of the wild-type GABA producer *B. adolescentis* 4–2, in which the GABA conversion ratio was not sufficient to fit for cell factory production (Fig. 1A). Hence, we constructed a total of 9 recombinant strains of *Bifidobacterium* overexpressing *gadB* and *gadC* genes (Fig. 2). We used four non-GABA-producing *Bifidobacterium* strains, belonging to three different species with different characteristic features (Table 1). The differences in characteristic features, [43, 47, 56], did not have a noticeable effect on GABA production, indicating that any *Bifidobacterium* species would be is a suitable host for biotransformation. The limiting step of low transformation efficiency in *B. adolescentis* JCM 1275 was eliminated using the PAM system [43].

Two of the nine recombinants were focused on, *B. adolescentis* JCM 1275/ pKKT427:: P_{ori}-*gadBC* and *B. adolescentis* JCM 1275/ pKKT427:: P_{gap}-*gadBC* owing to efficient Conversion ratio which exceeded 100%. Fermentation

parameters to improve GABA production was optimized using multi factorial design. Three different media, MRS, GAM, and BMM, were examined. GABA production was higher on MRS medium than on the other two. MRS is a nutrient rich medium compared to the other two used media, which favor the condition for bacterial growth and hence for GABA production.

In *B. adolescentis* JCM 1275/ pKKT427:: P_{ori}-*gadBC,* MSG concentrations of more than 2% suppressed GABA production at pH6.0. The reduction in the initial pH of the culture media improved GABA production to approximately 220mM. It has been reported that lower pH improves *gadB* activity in LAB and *E. coli* [57]. Hence, the effect of pH was examined using MRS with a modified pH. In *B. adolescentis* JCM 1275/ pKKT427:: P_{ori}-*gadBC*, reducing the initial MRS pH improved GABA productivity, especially at higher concentrations of the substrate. This enhancement denotes that *gadB* expression was induced by lower pH. The pH of the growing *B. adolescentis* JCM 1275/ pKKT427:: P_{ori}-*gadBC* culture was reduced as the bacteria proliferated. This reduction possibly enhanced GABA production in resting cells, especially in the late stationary phase.

B. adolescentis JCM 1275/ pKKT427:: P_{gap}-*gadBC* exhibited higher GABA production in a shorter time with minimal modifications in the culture

media, which should reduce the total production cost. A GABA production level of approximately 280mM was achieved on MRS containing 408mM MSG. The difference noticed between both recombinants possibly refer to varying the promoter, *ori* and *gap*, which control GAD genes activity.

PLP addition was previously reported to improve GABA production in *L. plantarum* 90sk and not for *B. adolescentis* 150 nor *B. angulatum* GT102 [40]. However, in our study PLP improved GABA production from recombinant *bifidobacterium* strains, when added at 24 or 48h, approximately double times as that with no PLP. This result indicates that PLP may recover GAD activity at late growth stages. The effectiveness of PLP addition at late growth stages could refer to the easy denaturation of PLP during the fermentation and lose the role as a co-enzyme of GAD. Therefore, it may be more efficient for improved GABA production to add PLP at 24 or 48h of fermentation.

Previously, *Bifidobacterium* was a difficult host for use in metabolic engineering. In particular, the usage of *B. adolescentis* was impractical in this field because of its high oxygen sensitivity. In this study, we applied the available genetic tools and techniques to use it as a cell factory host. We succeeded in improving GABA production from *Bifidobacterium* by

recombination. The GABA producing ability of *B. adolescentis* recombinants can be considered high compared to other reported microbial cell factories as the conversion ratio reached 100%. The findings of the current study indicate that bifidobacteria are a promising candidate for use in biotransformation.

Chapter three

Bifidobacteriaceae abundance among gut microbiota is correlated with high fecal GABA content

3.1. Introduction

The gut microbiota comprises several microorganisms, including bacteria, archaea, and fungi, which inhabit the gastrointestinal tract (GIT) of mammals. The number of microorganisms in the GIT can exceed 10^{14}, which is ten times the number of cells in the human body [58, 59]. The gut microbiota is often called the "forgotten organ" owing to its broad spectrum of health benefits for the host [60]. Gut microbiota acts as a key modulator of host digestion, metabolism, and immune response. Recent research has shown that the effect can extend beyond the gastrointestinal tract to affect the mental health of the host through bidirectional communication between the gut and brain, which is referred to as the microbiota-gut-brain axis [61, 62]. Signals transfer between the gut and the brain via neural, endocrine, immune, and humoral links [63]. One important link is the neural pathway in which the gut microbiota mediates the production of active neurotransmitters that pass from the gut to its target organs, including the brain.

Gamma-aminobutyric acid (GABA) is the most abundant neurotransmitter in the central nervous system (CNS) [64]. It is a non-protein amino acid, biosynthesized by the decarboxylation of glutamate through the

action of glutamate decarboxylase. Glutamate is an excitatory neurotransmitter in the CNS [65]. GABA is found in a wide range of organisms, from prokaryotes to vertebrates. GABA production has been reported in several species belonging to the families Bifidobacteriaceae, Lactobacillaceae, Bacteroidaceae, Enterococcaceae, Propionibacteriaceae, and Streptococcaceae [66, 67]. *Bifidobacterium* exhibits the ability to produce GABA from specific strains belonging to the species *B. dentium*, *B. angulatum*, *B. adolescentis*, and *B. longum subsp. infantis* [68, 69]. GABA-producing bacteria are considered glutamate consumers as glutamate activates enzymatic conversion using microbial glutamate decarboxylases.

Microbial GABA can pass from the gut to other organs through several pathways, including the blood or vagal pathways [70]. It has been reported that mental disorders, such as depression, are negatively correlated with the abundance of GABA-producing Bacteroides [71]. Further, accumulating evidence from animal trials suggests that the ingestion of GABA-producing bacteria supports relief from psychiatric illnesses, such as depression, and physical ailments, such as diabetes [72, 73, 74]. As, the majority of available evidence for GABA relation to microbial composition has been performed in animals, there is a need for more evidence from human cohorts to encourage

the ap-plication of these microbes as probiotic agents.

Understanding the relationship between microbial composition and the level of fecal neurotransmitters, GABA and glutamate, can highlight the vital role of some microbes which can redirect the microbiome activity towards GABA or glutamate production. In this study, we aimed to assess microbial diversity among human subjects with different fecal GABA and glutamate levels.

3.2. Materials and Methods

3.2.1 Study subjects

From March 2020 to August 2020, fecal samples were obtained from 77 participants. Eligible participants were those who did not receive antibiotic treatment, at least three months before sample collection. Participants were from different geographical origins. Their ages ranged from 1 month to 80 years. All were apparently healthy with no sys-temic or psychiatric illnesses.

3.2.2 Ethical statement

All experimental protocols were approved by the Institutional Ethics Review Board of Gifu University (certificate number: 2019–283), approved on March 3, 2020. Written informed consent was obtained from each participant.

2.3 Fecal sample manipulations

Stool samples were collected in sterile 12 mL tubes with tight caps. Samples were frozen immediately at −20 °C and delivered to the laboratory using cool containers. Thereafter, samples were stored at –80 °C, directly after being obtained, until used for amino acid quantification and DNA extraction.

3.2.3 High-performance liquid chromatography (HPLC)

Fecal samples were diluted 10 times with pure water (w/v), homogenized, and the liquid fraction filtered through a 0.45-µm membrane filter and subsequently derivatized with o-phthalaldehyde (OPA) (Wako, Osaka, Japan) using the OPA method [75]. Derivatization performed at room temperature for 2 min. The derivatization product was analyzed using HPLC (Agilent Technologies, Waldbronn, Germany) with a fluorescence detector (Ex 350 nm EM 450 nm) and a Cosmosil packed column 5C18-MS-II (3.0ID × 150 mm). The mobile phase was composed of reagents A (CH3CN/CH3OH/H2O 45/40/15, v/v/v) and B (20 mM KH2PO4 (pH 6.9), H3PO4). Compounds were eluted using a gra-dient program: 0–9 min, 100% B; 9–12 min, 89% B; 12–21 min, 78% B at a flow rate of 0.7 mL/min. Potassium dihydrogen phosphate, methanol and acetonitrile (HPLC grade) were selected from (Wako). The column temperature was maintained at 35 °C. GABA (Wako) and glutamate (Sigma, Louis, MO, USA) were used for standard curve preparation.

3.2.4 DNA manipulation and next-generation sequencing (NGS)

Genomic DNA was extracted from fecal samples using ISOFECAL kit for Beads Beating (Nippon Gene, Tokyo, Japan). The polymerase chain reaction (PCR) was per-formed with the barcoded primers, Fw (5' GTGCCAGCMGCCGCGGTAA 3') and Rv (5' GGACTACHVGGGTWTCTAAT 3'), targeting the V3-V4 region of the bacterial 16S ri-bosomal RNA gene. It produced a fragment length of approximately 550 base pairs. The PCR was performed using 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Woburn, MA, USA) according to the manufacturer's instructions. Subsequently, the PCR amplicons were purified using AgencourtR AMPureR XP beads (Beckman Coulter, Beverly, MA, USA). Dual indices and Illumina sequencing adapters were attached using Nextera XT (Illumina, San Diego, CA, USA) in the index PCR step. The concentration of PCR am-plicons was measured using a Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, US). Quality control for the created library was performed using a Bioanalyzer (Agilent Technologies). Pooled libraries were denatured with NaOH, diluted with hy-bridization buffer, and subsequently heat denatured prior to MiSeq sequencing. PhiX 5% was used as an internal control in each run. The NGS of amplicons was carried out on Illumina MiSeq (Illumina)

using the MiSeq Reagent Kit v3, following which 300-bp paired-end reads were produced.

3.2.5 Bioinformatics and statistical analysis tools

Preprocessing of sequences obtained by NGS and extraction of operational taxonomic units (OTUs) was performed using the software *mothur* (version 1.41.0) [76]. OTUs of amplicons were designated at 97% sequence similarity. Taxonomic assignments were performed with *mothur*, based on non-redundant SILVA datasets (release 132) [77]. A phylogenetic tree for the *phyloseq* object was calculated using the *clearcut* function implemented in *mothur* [78]. OTUs that occurred only once (singletons) or twice (doubletons) among all samples were removed. Next, the number of reads of all samples was rarefied to be equal in size at the minimum read within samples using the *phyloseq* package of R.

Alpha diversity of samples was measured using Shannon, Observed, and Chao1 in-dices. Non-metric multidimensional scaling (NMDS), an unconstrained and distance-based ordination method, was performed with Bray-Curtis dissimilarity matrices and produced using the *phyloseq* and *vegan* packages of R software v2.4-1 [79, 80, 81]. Differences in the microbial community structure, calculated using Bray-Curtis distances, were analyzed

statistically using permutational multivariate analysis of variance with distance matrices (PERMANOVA using the ADONIS command implemented in the *vegan* package). OTUs designated at the family level of classification were used for heatmap and cluster analyses. Bray-Curtis dissimilarity distance was applied to these analyses. A heatmap combined with a dendrogram was generated using the gplot [82] and cluster [83] packages of R. Distance-based redundancy analysis (db-RDA) was performed with the Bray-Curtis distance matrix of family-level taxonomy of OTUs using the vegan package of R. Species scores of abundant taxa (top 10) were also displayed on db-RDA plots. Linear discriminant analysis (LDA) effect size (LEfSe) [84] was performed using the microbiome Marker package of R under default settings except for the LDA cutoff, which was set to 4 in this study [85]. The results of LEfSe were further analyzed with the "test_multiple_groups" function implemented in the microbiome Marker package of R to assess the biological relevance of the obtained results.

3.3. Results

3.3.1. Analysis of fecal GABA and glutamate levels from 77 participants

To investigate the microbiome activity for GABA production, fecal GABA and glutamate levels were evaluated in 77 participants. These

participants were from different geo-graphical Origins, including Northeast Africa, Southeast Asia, South Asia, and East Asia. There were 55 participants from Japan and 22 from other geographical areas. The GABA and glutamate levels were detected in a broad range (0–330 µg/g feces), (55-475 µg/g feces), respectively. Correlation coefficient between GABA and glutamate concentrations was estimated as - 0.402 with 95 percent confidence interval [-0.596, -0.162] which indicates negative co-relation between both neurotransmitters. Participants' samples were divided into high, medium, and low, based on their fecal GABA content. The high, medium, and low groups were defined as those with productivity (µg-GABA/g-feces) ≥100, 10–100, and <10, respectively (Figure 1). Notably, the high GABA group samples had low glutamate content and vice versa, indicating that the microbiome was actively involved in con-verting the available glutamate in the gut to GABA. Samples data are summarized in table 1.

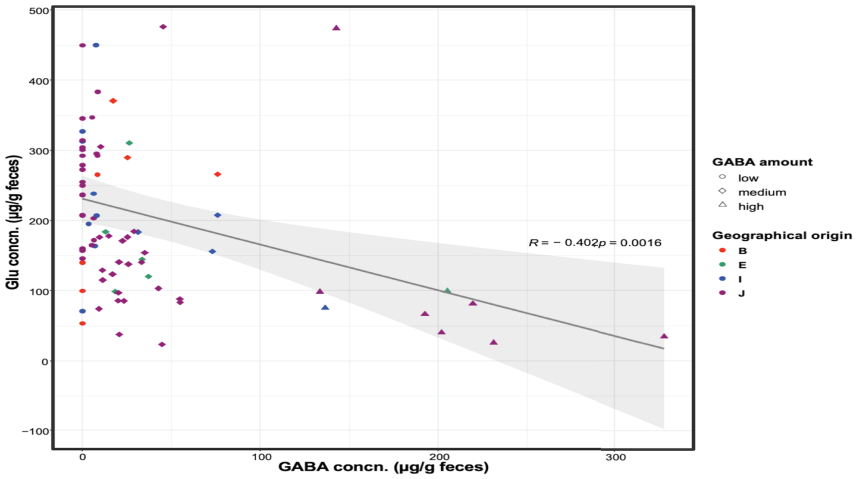


Figure 1. Fecal GABA and glutamate concentrations. Fecal GABA and glutamate contents were analyzed among healthy human participants. Participants were divided based on GABA productivity into high, medium, and low groups. Circle, diamond, and triangle symbols represent participants defined at low, medium, and high GABA productivity groups, respectively. Different colors represent the geographical origin of each participant: B, South Asia; E, Northeast Africa; I, Southeast Asia; J, East Asia. Regression curve is displayed on the figure in deep gray line, showing a negative correlation between fecal GABA and glutamate concentrations. Confidence interval (95%) is expressed in light gray color. Correlation coefficient (R) and p-value of the regression curve are also shown on the plot.Table 1 Summary of human volunteers' data

Table 1

Serial number	**ID**	**GABA ug/gram**	**Glutamate ug/gram**	**Geographical origin**	**Age**	**Gender**
1	I1	7.6	450.2	Southeast Asia	26 years	F
2	I2	3.5	195.4	Southeast Asia	26 years	M
3	I3	31.3	183.6	Southeast Asia	11months	F

4	I4	6.2	238.4	Southeast Asia	12 years	F
5	I5	ND	312.6	Southeast Asia	10 years	F
6	I6	ND	327.3	Southeast Asia	28 years	F
7	I7	136.37	75.8	Southeast Asia	27 years	M
8	I8	ND	70.3	Southeast Asia	4 months	F
9	I9	7.3	156.3	Southeast Asia	27 years	F
10	I10	7.6	207.8	Southeast Asia	23 years	F
11	B1	ND	100.0	South Asia	28 years	M
12	B2	7.6	265.9	South Asia	29 years	M
13	B3	17.2	370.8	South Asia	29 years	F
14	B4	ND	140.2	South Asia	1 month	F
15	B5	25.2	289.8	South Asia	32 years	M
16	B6	ND	53.8	South Asia	36 years	M
17	E1	13.0	184.0	Northeast Africa	37 years	F
18	E2	205.2	99.8	Northeast Africa	12 months	F
19	E3	18.37	99.0	Northeast Africa	37 years	M
20	E4	37.2	120.6	Northeast Africa	2.5 years	M
21	E5	33.6	144.9	Northeast Africa	8 months	F
22	E6	26.4	310.6	Northeast Africa	25 years	F

23	J1	25.2	176.6	East Asia	55 years	F
24	J2	ND	207.2	East Asia	76 years	F
25	J3	ND	158.3	East Asia	46 years	F
26	J4	11.1	129.3	East Asia	21 years	F
27	J5	33.2	140.9	East Asia	23 years	F
28	J6	ND	237.9	East Asia	48 years	F
29	J7	14.7	178.0	East Asia	22 years	F
30	Y1	20.2	97.4	East Asia	41 years	M
31	Y2	142.5	474.1	East Asia	10 years	M
32	Y3	44.7	23.6	East Asia	41 years	F
33	Y4	23.2	85.8	East Asia	15 years	M
34	Y6	ND	272.4	East Asia	68 years	F
35	Y7	20.0	86.0	East Asia	71 years	M
36	Y8	10.2	305.2	East Asia	9 years	M
37	Y10	19.4	44.5	East Asia	40 years	F
38	Y12	35.0	154.5	East Asia	66 years	F
39	Y13	10.7	115.3	East Asia	12 years	M
40	Y14	10.5	174.8	East Asia	13 years	M
41	Y15	5.7	157.9	East Asia	32 years	F

42	Y16	6.5	201.9	East Asia	34 years	M
43	Y17	16.9	123.8	East Asia	54 years	F
44	Y24	219.5	81.7	East Asia	1 years	M
45	Y26	9.2	166.9	East Asia	27 years	M
46	Y27	328.0	34.7	East Asia	8 months	F
47	Y29	45.3	476.2	East Asia	3 years	F
48	Y30	ND	304.2	East Asia	33 years	M
49	Y31	ND	236.6	East Asia	28 years	M
50	Y32	19.8	253.7	East Asia	32 years	F
51	Y33	231.0	26.1	East Asia	1 year	M
52	Y34	52.8	82.6	East Asia	37 years	F
53	M36	80.5	339.4	East Asia	73 years	F
54	M37	5.4	347.1	East Asia	83 years	M
55	M38	25.8	138.1	East Asia	47 years	F
56	M39	ND	279.8	East Asia	47 years	F
57	M40	ND	313.9	East Asia	47 years	F
58	M41	ND	157.3	East Asia	79 years	F
59	M42	192.4	66.9	East Asia	30 years	F
60	M43	ND	208.2	East Asia	30 years	F

61	M44	ND	293.5	East Asia	49 years	F
62	M45	ND	145.9	East Asia	78 years	F
63	M46	28.9	184.8	East Asia	52 years	F
64	M47	ND	254.1	East Asia	24 years	F
65	M48	ND	248.0	East Asia	46 years	F
66	M49	53.3	88.2	East Asia	47 years	F
67	M50	201.8	40.8	East Asia	9 years	M
68	M51	133.5	98.64	East Asia	15 years	M
69	M52	29.9	134.7	East Asia	20 years	F
70	M53	11.3	75.98	East Asia	53 years	M
71	M54	ND	449.8	East Asia	46 years	F
72	M55	42.4	102.3	East Asia	49 years	M
73	M56	ND	345.76	East Asia	49 years	F
74	M57	ND	160.8	East Asia	26 years	M
75	M58	23.1	172.7	East Asia	21 years	F
76	M59	8.5	383.8	East Asia	76 years	F
77	M60	6.7	291.1	East Asia	23 years	F

Values are the average of duplicate or triplet analysis of each sample

3.3.2. Reduced alpha diversity of the high GABA group

Quality check and preprocessing of NGS data yielded a total of 1885 OTUs with a median of 171 OTUs per sample. To unify the depth of all sample data, singletons or doubletons were first removed and all samples were rarefied at even depth (n = 24,770). The total coverage of each sample ranged from 99.5 to 99.9%. The number of reads for all samples was rarefied to be equal to the minimum read within samples. Rarefaction plots, grouped by GABA level and nationality, are displayed in Figure 2A and B.

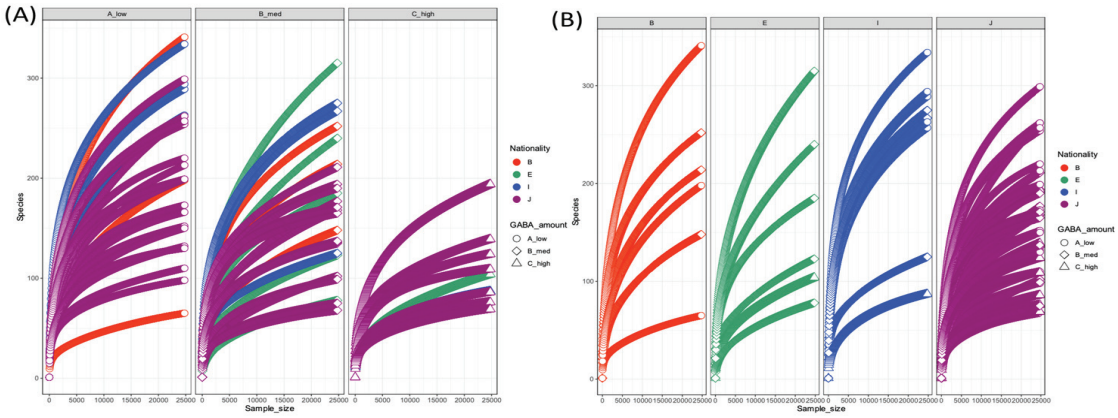


Figure 2. Rarefaction curves for analyzed samples. All samples were rarified to be equal in size. (A) Rarefaction plot for samples grouped by gamma-aminobutyric acid (GABA) level (B) Rarefaction plot for samples grouped by geographical origin (nationality) of sample donor. Both (A) and (B) showing the number of sequences' reads and their corresponding number of bacterial species.

Alpha diversity was subsequently quantified by the total number of

observed species, Chao1 richness, and Shannon diversity index, which estimates both OTU richness and evenness. Figure 3 shows the alpha diversity measurements for the high GABA group versus the medium and low groups. The alpha diversity of the high (H) GABA group compared to the medium (M) and low (L) groups were significantly reduced in observed species (H-L; $P_{Observed}$ = 0.0006), Shannon diversity (H-L; $P_{Shannon}$ = 0.001) (H-M; $P_{Shannon}$ = 0.04), and Chao1 richness (H-L; PChao1 = 0.002) (M-H; PChao1 = 0.03). Statistical comparison between the M and L groups showed no difference in Shannon diversity (M-L; $P_{Shannon}$ = 0.21), Chao1 richness (M-L; P_{Chao1} = 0.39), and observed species (M-L; $P_{Observed}$ = 0.07). A summary of alpha diversity indices is shown in Table 3.

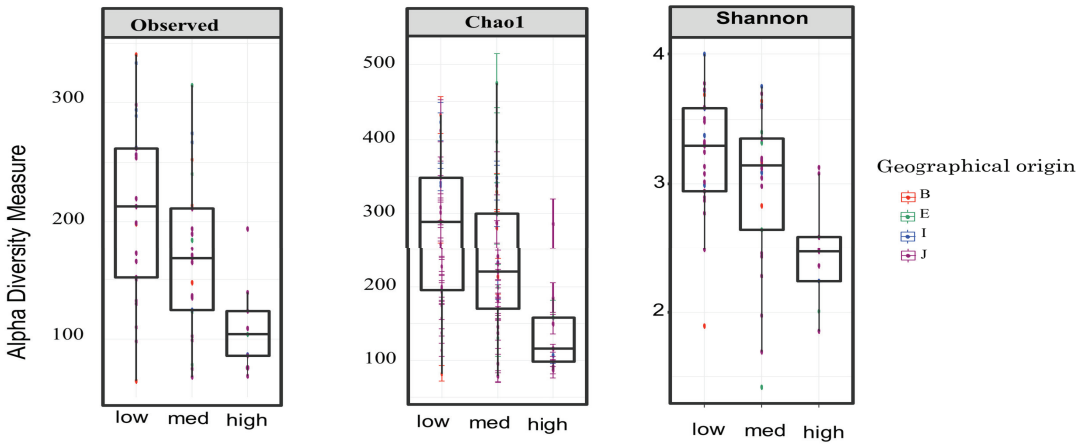


Figure 3. Alpha diversity and the relative abundance of gamma-aminobutyric acid (GABA)-producers among fecal GABA groups. Alpha diversity, measured by observed species and Chao1 and Shannon diversity indices, is plotted for

examined samples, i.e., in high, medium, and low GABA groups. Box plots depict microbiome diversity and abundance differences according to each test. The horizontal line inside the box represents the median. Outliers and individual sample values are represented by dots. Different colors represent the geographical origin of each partici-pant: B, South Asia; E, Northeast Africa; I, Southeast Asia; J, East Asia. All alpha diversity measurements shown here significantly decreased in the high GABA group compared to those in the low group.

Table 2. Alpha diversity between GABA groups.

			95% confidence level		
Model	Compared groups	diff	lwr	upr	Adjusted p-value
Invsimpson	High_Low	-6.9	-13	-1	0.01
	Medium_Low	-1.2	-5	3	0.76
	Medium_High	5.7	0.2	11	0.04
Shannon	High_Low	-0.8	-1	-0.3	0.001
	Medium_Low	-0.3	-0.6	0.1	0.210
	Medium_High	0.5	0.003	1	0.048
ACE	High_Low	-128	-218	-37	0.003

	Medium_Low	-35	-101	31	0.4
	Medium_High	93	2	184	0.04
Chao1	High_Low	-130	-219	-41	0.002
	Medium_Low	-35	-100	29	0.4
	Medium_High	94	6	183	0.03
Observed	High_Low	-103	-166	-40	0.0006
	Medium_Low	-43	-88	3	0.07
	Medium_High	60	-2	123	0.06

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

3.3.3. Microbial composition differed between GABA groups

As a distance-based discrimination approach, NMDS ordination was applied to investigate dissimilarities in the microbial composition between tested samples based on GABA production. The NMDS plot for community structure based on the Bray-Curtis matrix of family-level taxonomy is displayed in Figure 4A. The microbiome of individuals with high GABA content showed a shift to the right, which indicates compositional differences. This was confirmed by ADONIS test, which provided more precise information regarding the homogeneity of dispersion between the two sample groups. Significant differences ($p < 0.05$) were observed between groups

(Table 3).

Table 3. ADONIS test between each two sample groups.

Group	F.Model	R2	P-value	P-adjust	Significance
Low vs Medium	5	0.09	0.001	0.003	*
Low vs High	8	0.19	0.001	0.003	*
Medium vs High	3	0.09	0.004	0.012	.

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1"

Relationships among microbial community structures and environmental variables were examined with db-RDA based on the Bray-Curtis distance matrix of family-level taxonomy of OTUs. This revealed that the abundance of the families Bifidobacteriaceae and Streptococcaceae was closely associated with GABA levels, while that of the families Lachnospiraceae and Ruminococcaceae was closely associated with glutamate levels (Figure 4B).

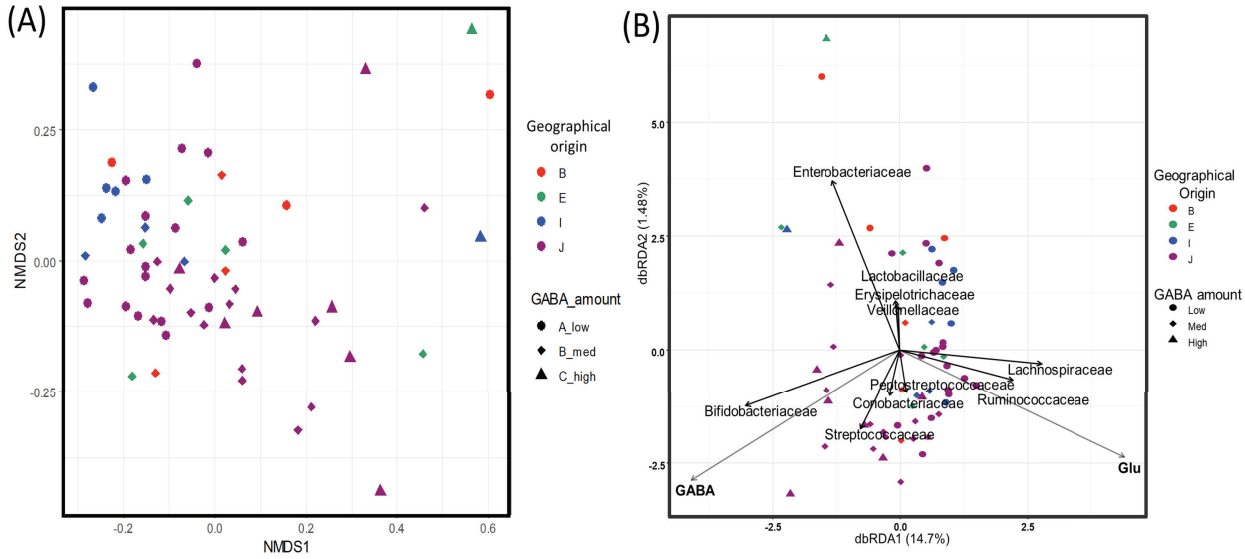


Figure 4. Beta-diversity and community similarity analysis among fecal gamma-aminobutyric acid (GABA) groups. (A) Non-metric multidimensional scaling (NMDS) plot based on the distance matrix of operational taxonomic units (OTUs) designated at the family level of taxonomic classification calculated using the Bray-Curtis model. (B) Distance-based redundancy analysis (db-RDA) using the Bray-Curtis dissimilarity matrix calculated with OTUs designated at the family level of taxonomy. The environmental variables were statistically significant ($p < 0.01$), and the top 10 most abundant taxa are displayed. Circle, diamond, and triangle symbols represent participants defined at low, medium, and high GABA productivity groups, respectively. Different colors rep-resent the geographical origin of each participant: B, South Asia; E, Northeast Africa; I, Southeast Asia; J, East Asia.

3.3.4 Trend toward clustering of the microbiome of individuals with high GABA content

We subsequently investigated whether fecal GABA content could reflect a notable difference in the microbial composition of individuals. The top 20 abundant taxa are displayed as a heatmap in Figure 5. A distant matrix computed with all OTUs was used to produce the dendrogram. Hierarchical cluster analysis revealed that the community structure profiles of most high-

fecal GABA samples were separated from other analyzed microbiomes (Figure 5). Six out of nine high GABA samples were clustered in the same group. All nine samples showed an abundance of Bifidobacteriaceae, except for one participant, ID: E2. This participant had a unique abundance of two other GABA-producer candidates, Lactobacillaceae and Leuconostocaceae. Participant, ID: Y33, showed a relative low abundance of Bifidobacteriaceae. Nonetheless, other GABA producer candidates were detectable in this microbiome, Y33, including Streptococcaceae, Bacteroidaceae and Enterococcaceae. Ruminococcaceae exhibited a relatively high abundance in the low and medium groups. Lachnospiraceae was abundant in most analyzed samples.

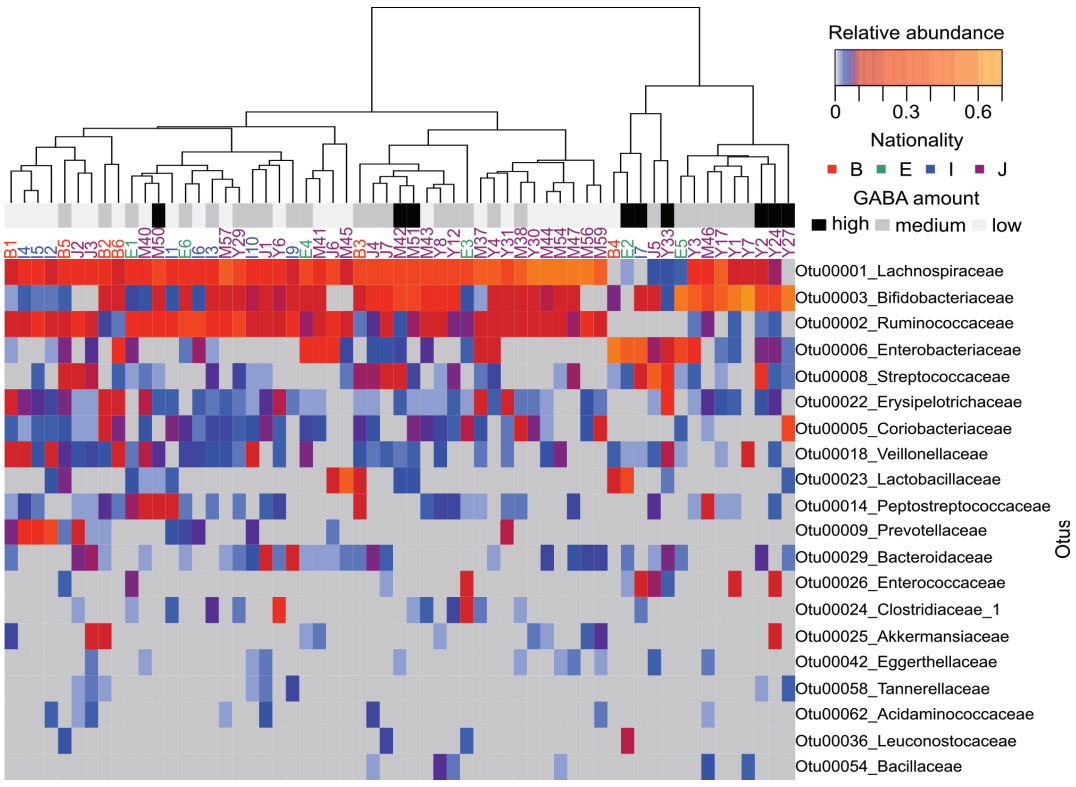


Figure 5. Heatmap of the top 20 abundant taxa in the examined human fecal samples. Operational Taxonomic units (OTUs) were collapsed at family-level taxonomy. A dendrogram was constructed with the beta flexible method based on the distance matrix of OTUs, calculated using the Bray-Curtis model. The geographical origins of each participants are presented by different colors: B, South Asia; E, Northeast Africa; I, Southeast Asia; J, East Asia.

3.3.5 Bacterial taxonomic differences between GABA groups (specific OTUs)

To identify biomarkers from the high GABA group, we used LEfSe to comprehensively and accurately investigate compositional differences between the three GABA groups. Specific OTUs that showed the strongest effect for group differentiation were identified between the two sample groups (Figure 6). At the phylum level, Firmicutes was dom-inant in the microbiome of the low GABA group (Figure 7A), whereas the phylum Ac-tinobacteria was highly dominant in the microbiome of the high and medium groups (Figure 7B). The microbiomes of the low and medium groups were characterized by the dominance of the class Clostridia, which was primarily presented by the families Ru-minococcaceae and Lachnospiraceae. In contrast, the microbiome of the high GABA group was characterized by a high abundance of the class Actinobacteria and orders Bifidobacteriales and Lactobacillales, which were

principally presented by the families Bifidobacteriaceae, Enterococcaceae, and Streptococcaceae.

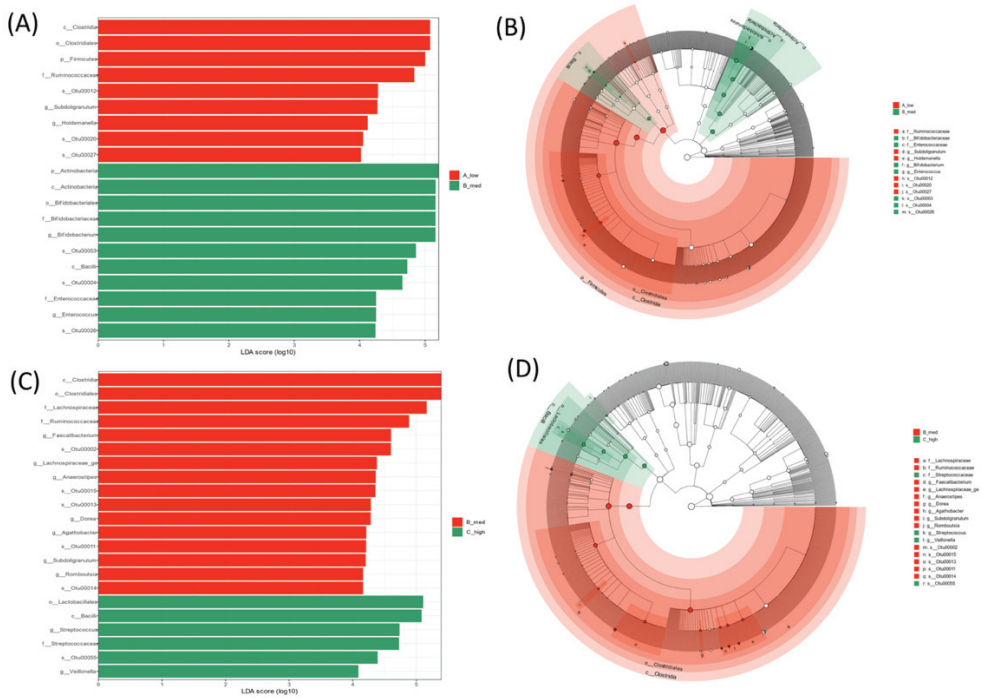


Figure 6. Linear discriminate analysis (LDA) Effect Size (LEfSe) showing the characteristics of the microbial community composition between the low and medium gamma-aminobutyric acid (GABA) groups in panels A and B as well as between the medium and high GABA groups in panels C and D. (A and D) LEfSe (LDA scores 10^4 and more) displaying statistical and differentially abundant taxa in each group. (B and C) Cladogram showing the microbiome differences at different phylogenetic levels. The central point represents the root of the tree (bacteria) and each ring displays the next (lower) taxonomic rank (p__, phylum; c__, class; o__ order; f__, family; g__, genus). The diameter of each circle

represents the relative abundance of each taxon.

The abundance of taxa that showed dominance in the high or low GABA group was compared between the three GABA groups. The degree of significance among the groups is displayed in Figures 8 and 9. The order Bifidobacteriales and family Bifidobacteriaceae were more abundant in the high and medium GABA groups than the low group. Sig-nificant (P <0.05) difference was noticed between low and medium groups and, larger difference was detected between low and high groups, while no significant difference was found between medium and high groups. The order Lactobacillales was more abundant in the high GABA group than other groups. Both the families Streptococaceae and Enterococaceae were more abundant in the high GABA group than in the low GABA group, as shown in Figure 8. The order Clostridiales and the two families Lachnospiraceae and Ruminococcaceae were dominant in the low GABA group (Figure 9).

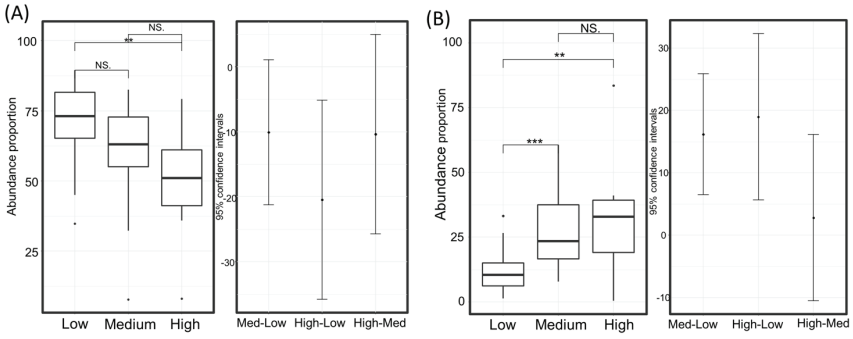


Figure 7. Comparison of the relative taxon abundance of the phyla Firmicutes

(panel A) and Actinobacteria (panel B) among the three gamma-aminobutyric acid (GABA) groups (high, medium, and low). All P values were <0.05.

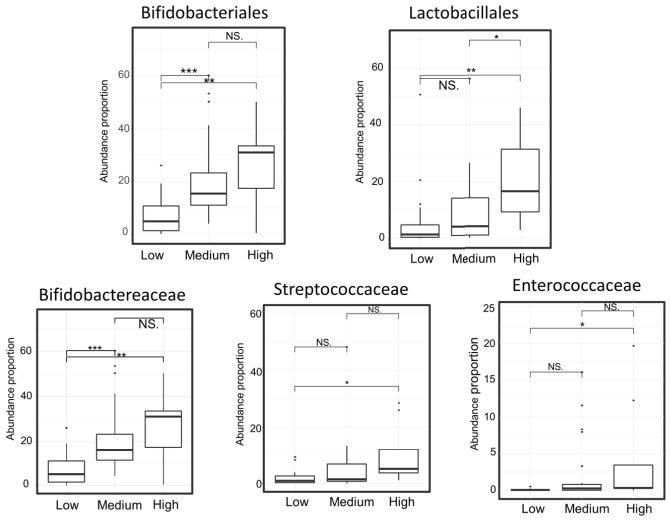


Figure 8. Proportion of the taxa that occurred abundantly in the high gamma-aminobutyric acid (GABA) group. Two taxa in the order level, comprising Bifidobacteriales (Panel A) and Lactobacillales (Panel B), are displayed. Three taxa at the family level are demonstrated incorporating Bifidobacteraceae (Panel C), Streptococcaceae (Panel D) and Entero-coccaceae (Panel E). Significance codes: < 0.001'***', < 0.01'**', < 0.05'*', <0.1'NS'. ..

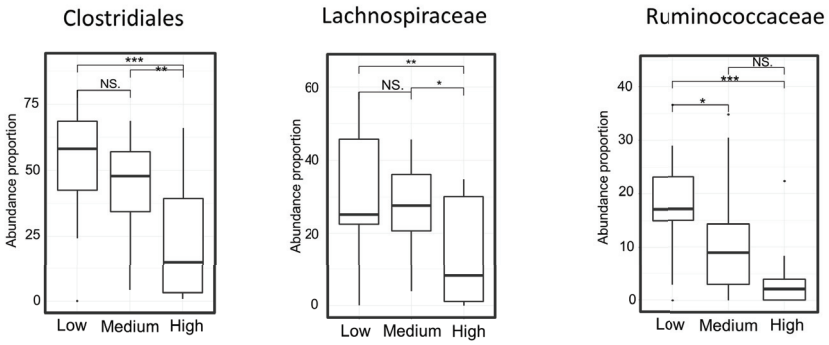


Figure 9. Proportion of taxa that occurred abundantly in the low gamma-aminobutyric acid (GABA) group. Statistical analysis was performed using analysis of variance (ANOVA) followed by the post-hoc test. All P values were <0.05.

To identify the microbiomes of individuals causing these differences, the abundance of the major taxa, associated with high or low GABA levels, was presented using a colored NMDS plot, as shown in Figure 9. The abundance of the dominant taxa in the high and low GABA groups is represented by gradient colors, which enables the clear visualization of taxa distribution among tested samples.

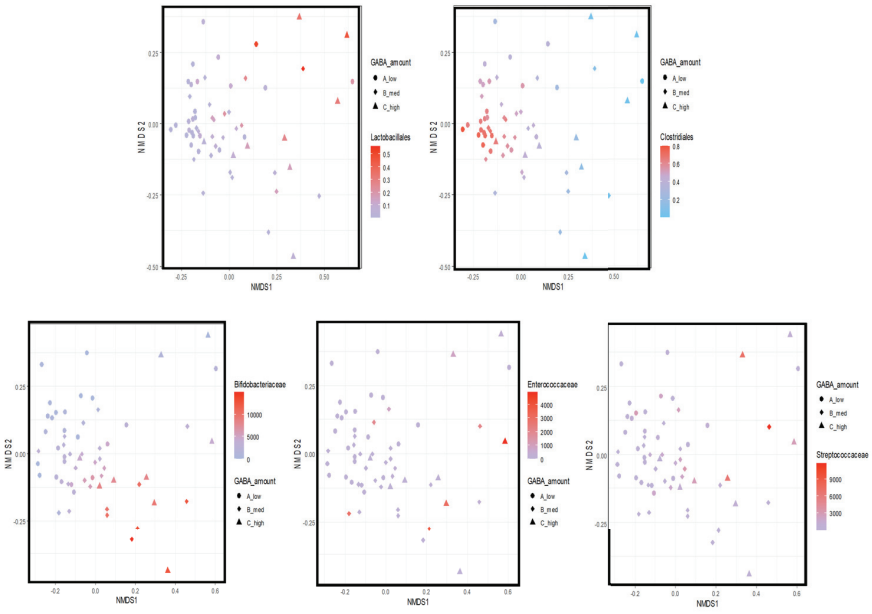


Figure 10. Non-metric multi-dimensional scaling (NMDS) of community structure for all tested samples. Gradient colors of the NMDS plot were based on the distribution of major taxa associated with high and low levels of gamma-

aminobutyric acid (GABA).

3.4. Discussion

Gut bacteria have the ability to produce numerous bioactive compounds including neurotransmitters, immune stimulants, and essential vitamins [86, 87]. Several studies emphasized the contribution of gut microbiota-derived materials in maintenance of host physical and psychological conditions [73, 74]. Assessing the neuroactive potential and composition of human gut microbiota can suggest the crucial role of some microbes [88, 89]. In the current study, we analyzed the relationship between microbial composition and the levels of the fecal neurotransmitters, GABA and glutamate. We found promising biomarker bacteria associated with high fecal GABA concentrations. Fecal GABA and glutamate concentrations showed substantial remodeling of gut microbiota at different levels.

Individuals with high fecal GABA concentration had a relatively low concentration of glutamate and vice versa, which indicates the existence of an active enzymatic process responsible for this conversion. GABA is biosynthesized by irreversible decarboxylation of glutamate via the action of glutamate decarboxylase [90]. In vitro studies revealed the ability of some bacteria to produce GABA [66]. To discover this ability in vivo, we examined

the microbial composition and diversity of participants with different fecal GABA and glutamate levels. Low microbial alpha diversity was associated with high fecal GABA levels compared to other samples of low and medium GABA concentrations. Further, ADONIS test confirmed the differences in homogeneity between GABA groups. Assuming a positive relationship, the reduced alpha diversity found in the high GABA group might reflect a bacterial community associated with GABA production.

db-RDA shifts the focus onto specific taxa associated with high GABA and glutamate levels. The families Bifidobacteriaceae and Streptococaceae were associated with high GABA production. The families Ruminococaceae and Lacnospiraceae were associated with high glutamate levels. Notably, several species of Bifidobacteriaceae and Streptococaceae have been reported to be high GABA-producers [68, 69, 91], indicating the contribution of GABA producing bacteria to the detected GABA levels.

The LEfSE results confirmed the relatively high abundance of the family Bifidobacteriaceae in the high GABA group. The (OTU0003) showed higher abundance in the medium GABA group compared to low group. Basic Local Alignment Search Tool analysis of this OTU revealed that it belonged to *B. adolescentis* species. A recent study has shown that this species is a key

member of the gut microbiota involved in GABA production [68]. Such capability is proposed to alter the classification of *Bifidobacterium* from ordinary probiotic bacteria to potential psychobiotic bacteria. The term psychobiotics was first introduced by Dinan [61] to describe mind-altering germs. It was subsequently broadened to include any exogenous influence whose effect on the brain is bacterially mediated [92].

These results indicate the important role of bifidobacteria in improving GABA production in the gut. Previous studies have shown that the gut microbiota affects the levels of excitatory and inhibitory neurotransmitters, such as serotonin, GABA, and dopamine [93]. Consistent with these previous findings, in the present study, we found a positive correlation between the abundance of the GABA-producer families, Bifidobac-teriaceae, Enterococcaceae, and Streptococcaceae, and fecal GABA concentrations. Com-pared to other GABA-producers in the gut, *Bifidobacterium* and *Streptococcus* have a simple GABA production system composed of two genes, *gadB*, which encodes glutamate de-carboxylase, and *gadC*, which encodes a glutamate-GABA anti-porter [69, 90, 94]. Other more complex systems exist in other GABA-producers, such as Enterococcus and lactoba-cillus [95, 96].

As GABA is an inhibitory neurotransmitter and glutamate is an excitatory one [70, 97], the existence of bacteria that can decarboxylate glutamate to GABA can produce a new therapeutic agent for supporting psychiatric illnesses. Analysis of both neurotransmitters is important for understanding the microbiome activity for the conversion of glutamate to GABA and vice versa. Our study revealed that gut microbiota seems to play a crucial role for balancing GABA- glutamate level, where GABA producing bacteria was positively co-related with high GABA levels and negatively co-related with high glutamate levels. Previous studies showed that balancing between both neurotransmitters, GABA and glutamate, was linked to several psychiatric disorders such as autism [98], multiple sclerosis (MS) [99] and neuro-Bechet's disease [100]. In autism patients, altered fecal concentration of GABA and glutamate was observed, where high fecal levels of glutamate were detected in children with autism and low fecal GABA was detected in other subtypes of autism [101]. The probiotic formulation with abilities to consume high levels of glutamate and convert them to GABA promises to aid in the development of new alternative therapy for autism and other related psychiatric disorders, the current study suggests that GABA producer bacteria, bifidobacteria, is a good candidate in this field. Nonetheless, our

study was limited to healthy participants, future studies will be warranted to include both healthy and diseased subjects.

3.5. Conclusions

The current study revealed that microbial diversity and composition differed based on fecal GABA level. This suggests the important role of some commensal gut microbes in mediating GABA production and glutamate consumption. This study also highlights the importance of assessing the neuroactive potential and composition of gut microbiota, which emphasized the imperative role performed by specific GABA producing microbes, GABA producing bifidobacteria. This finding may aid the development of potential probiotics to improve microbial GABA production which lead to new therapies for physical and psychiatric illnesses.

Chapter four

Synbiosis between *Bifidobacterium adolescentis* 4-2 and Mannooligosacharides enhances fecal GABA concentration in intestinal flora model.

4.1 Introduction

Bifidobacteria are symbiotic bacteria inhabiting the gut of all mammals including human. As the human grows up, the ration of bifidobacteria decreases while other bacteria such as E*. coli* and *Colisteridum* increases [102]. The unbalanced intestinal microflora composition has an impact on host health through variation in intestinal metabolite content [103]. Such evidence indicates that bifidobacterial supplementation is important for maintaining balanced intestinal flora composition for host benefit. Bifidobacteria have numerous health benefits which can extend beyond gastrointestinal tract to other organs [104]. Bifidobacteria can produce several bioactive compounds with physiological and psychological importance [105, 106]. One of these is gamma amino butyric acid (GABA). GABA is the chief inhibitory neurotransmitter in the central nervous system. GABA has numerous health benefits for the carrier host. It acts as an anti-depressant, hypotensive, immune stimulant and antidiabetic [107]. GABA reaches its target organ weather through blood circulation or nervous connection of the gut [108].

Prebiotics are a group of nutrients that are not digestible by the host but, it is degraded by gut microbiota [109]. They act like fertilizers for gut-friendly bacteria such as bifidobacteria, and their degradation products are short-chain

fatty acids that are released into blood circulation [110]. Prebiotics play a widely recognized role in modulating gut microbiota [111]. For example, seaweed polysaccharides were reported to increase abundance of certain bacteroides species while galactooligosaccharides were reported to increase certain *Bifidobacterium* species [112] [113]. The microbial shifts produced by oligosaccharides are thought to be reflected in all metabolic profiles in the gut, including postbiotics produced by gut bacteria [114]. GABA is one of the most important postbiotics produced by gut microbiota due to its broad-spectrum health benefits to the host [107].

In this study, I aim to elucidate the impact of GABA producing *Bifidobacterium*, and prebiotics (oligosaccharides) on fecal GABA content in *in vitro* fecal culture. Our study was established on Kobe University Human Intestinal Model (KUHIM). It is a cultured human colon flora model developed by Kobe University [115]. It allows performing serial related studies for the same microbiome under the same experimental condition.

4.2 Materials and methods

4.2.1 Bacterial strains and culture condition.

The *Bifidobacterium adolescentis* strains were isolated from the GIT of healthy people living in Japan. These strains belong to the collection of the laboratory of genome microbiology in Gifu university. Other commercial strains were directly obtained from depositaries of JCM company. All strains were cultivated in the MRS growth medium (BD, MD21152 USA) at 37 °C in anaerobic condition in BUG Box (Sony technology, UK), using mixed gas supplement (80%N2, 10%CO2 and 10%H2), Monosodium glutamate (Sigma) 1%(v/v) was added to liquid culture as a substrate for GABA. Standard cultivation was performed by the inoculation of 12 ml MRS broth with 30:50 µl of a frozen stock (– 80 °C) followed by incubation at 37 °C for 24 h. Bacteria then were sub-cultured to MRS with1% MSG followed by incubation at 37 °C for 48 h. Grown cells were then centrifuged (6000 x rpm) and supernatant were used for HPLC analysis.

4.2.2 Fecal samples manipulation

Fecal samples were collected from apparently healthy volunteers (no systematic or psychiatric illness) who did not receive antibiotic treatment until at least three months before sample uptake. BD BBL culture swab plus (BD Co.:212550) was used for sample collection. Samples were kept at 4 °C directly

after collection and were used for flora model within 12 hr. from the collection. A written informed consent was obtained from each volunteer. All experimental protocols were approved by the institutional ethics review board of Gifu University

4.2.3 Kope University Human Intestinal Microbiota model (KUHIM).

The operation of the batch fermentation system was performed using a pH-controlled multi-channel fermenter [116]. The simulator is composed of six parallel and independent vessels. The working volume per vessel was 100 mL of Gifu Anaerobic medium (GAM broth (Nissui Pharmaceutical Co., Ltd. Code:05422) with initial pH adjusted to 6.5. The anaerobic condition of the vessels was maintained in continuous supply with N_2 and CO_2 (80:20) gas (10 mL min^{-1}) through a 0.2 μm polytetrafluoroethylene membrane (Pall Corporation, Port Washington, NY, USA) at 37 °C for 30 min. before fermentation. The pH was continuously monitored for each vessel and continuous stirring was maintained at 300rpm. To prepare the inoculum, the fecal sample was suspended in 2 mL of physiological saline. It was later added to flora model through the side projection of each vessel. Four additives were prepared and added based on experimental purpose. Mannooligosaccharides (MOS), dextrin (Dex) and Fructooligosaccharides (FOS) were prepared on a final concentration 0.5% and

dissolved in 10 ml then added to test tanks. Sterilized water was added to the control tank. A fully-grown *B. adolescentis* 4-2, 0.1% of the total culture (v/v), was added to culture tank for testing ability of GABA production on fecal culture.

4.2.4 HPLC analysis condition.

In case of bacterial culture and fecal cultures, the membrane-filtered liquid fraction (0.45 μm membrane filter) was directly used for HPLC analysis. GABA concentrations were quantified through HPLC (Agilent series 1100, Shimadzu, Japan) equipped with a fluorescence detector (Ex 350 nm EM 450 nm) and a Cosmosil packed column $5C_{18}$-MS-II (3.0ID X 150 mm). Prior to analysis, each sample was derivatized with the reagent O-phthalaldehyde (OPA) [75]. The mobile phase composed of A ($CH_3CN/CH_3OH/H_2O$ 45/40/15, v/v/v) and B (20 mM KH_2PO_4 (pH6.9), H_3PO_4). Compounds were eluted using gradient program: 0-9 min, 100% B; 9-12 min, 89% B; 12-21 min, 78% B. The column temperature was maintained at 35 °C with a flow rate of 0.7 mL/min. Quantification of unknown samples was achieved by comparing their peak area and retention time with a standard curve prepared with known standards and results were standardized to individual sample weights or dilution for both fecal samples and culture media, respectively.

4.2.5 DNA manipulation, NGS and real time PCR.

DNA manipulation and NGS analysis was performed as mentioned in chapter three.Quantitative PCR performed on gDNA to quantify the relative abundance of *B. adolescentis* 4-2 *gadB* gene within the KUHIM fecal culture extracted DNA. Standard quantification method was used for analysis. The primer set used for *gadB* amplification (Fw: 5` catgttcctgcgtttggga t 3`, Rv: 5` ccgtcgttccacagcgta 3`) were designed using oligo7 software. Quantification was operated on Step One Plus Real-Time PCR system (Applied Biosystems). The mixture of PCR reactions performed using Thunder Bird™ SYBR® qPCR mix (Toyobo) based on manufacturer instruction.

4.2.6 Bioinformatics tools

All row NGS data was processed using QIIME 2 software package [117]. The bar plot was prepared based on the extracted OTUs for both phylum and species level.

4.2.7 ß-mannosidase assay

One milli of KUHIM culture or liquid MRS culture was disrupted by sonication. The disrupted cells were then centrifuged at 13.000rpm for 15 min. 4 °C and the supernatant (cell-free extract) was used for ß -mannosidase assay. For the assay, a reaction mixture of 0.4 M sodium acetate buffer pH 6.0 (containing 4mM $CaCl_2$) 10mM PNPßM (p-nitrophenyl ß-D-mannopyranoside, Sigma),

distilled water (3:1:6) (v/v/v) was first mixed, 280 µL of the reaction mixture was mixed with 46.7 µL of cell free extract, the whole mixture was incubated at 37 °C for 1h, 0.2 M Na_2CO_3 490 µL was added to stop the reaction, 200 µL was used for spectrophotometric analysis at 450 nm wavelength. The concentration of the end product of B-manosidase digestion was calculated using standard curve equation. For standard curve preparation, the same protocol, mentioned above, was followed by varying standard samples. Standard samples were prepared from different concentrations of p-nitrophenol.

4.3 Results

4.3.1 A fecal isolate GABA producing *Bifidobacterium adolescentis* 4-2 significantly increase fecal GABA content in *invitro* fecal culture.

GABA production was surveyed between a collection of fecal isolates of *Bifidobacterium* species and commercially available species aiming to find a high GABA producer (Table 1). Our result confirmed that GABA production ability is strain rather than species specific ability as previously reported in [38]. Within the same species of *adolescentis* high, medium, low and non-producers were detected. *B. adolescentis* 4-2 strain was detected as the highest in GABA production among the *B. adolescentis* fecal isolates (1.4 ± 0.2 g/L); meanwhile, *B. adolescentis* JCM 1275 was detected as a non- producer strain (Table 1).

B. adolescentis 4-2 was tested in KUHIM fecal culture of low GABA producing microbiomes with addition of the substrate (MSG). *B. adolescentis* 4-2 enhanced GABA productivity compared to the control and MSG cultures and improved the assimilation of available glutamate towards high GABA production (Fig. 1).

Table 1. Bacterial strains used in the study

Bacterial strain	**GABA g L^{-1}**	**Source**	**Origin**
B. dentium JCM 1195	2	JCM company	Human
B. adolescentis* 4-2	1.4	Kobe, Japan	Adult human
B. adolescentis 12451*	0.6	Kobe ,Japan	Adult human

B. adolescentis 4-16*	0.3	Kobe ,Japan	Adult human
B. adolescentis 3-117*	0.2	Kobe ,Japan	Adult human
B. adolescentis JCM 7042	0.1	JCM company	Human
B. adolescentis 12-111*	0.1	Kobe ,Japan	Adult human
B. rumminantium JCM 8222	0.18	JCM company	Cattle Rumin
*B. Catenulatum**	0.2	Kobe ,Japan	Human
***B. adolesentis* JCM 1275**	**0**	Kobe ,Japan	Human
B. animalis animalis JCM1190	0	JCM company	Rat feces
B. animalis lactis JCM10602	0	JCM company	Fermented dairy product
B. indicum JCM 1302	0	JCM company	Human
B. longum 105A	0	JCM company	Human
B. longum infantis JCM 1222	0	JCM company	Human
B. breve JCM 1192	0	JCM company	Human
B. minimum JCM 5821	0	JCM	Human

Figure 1

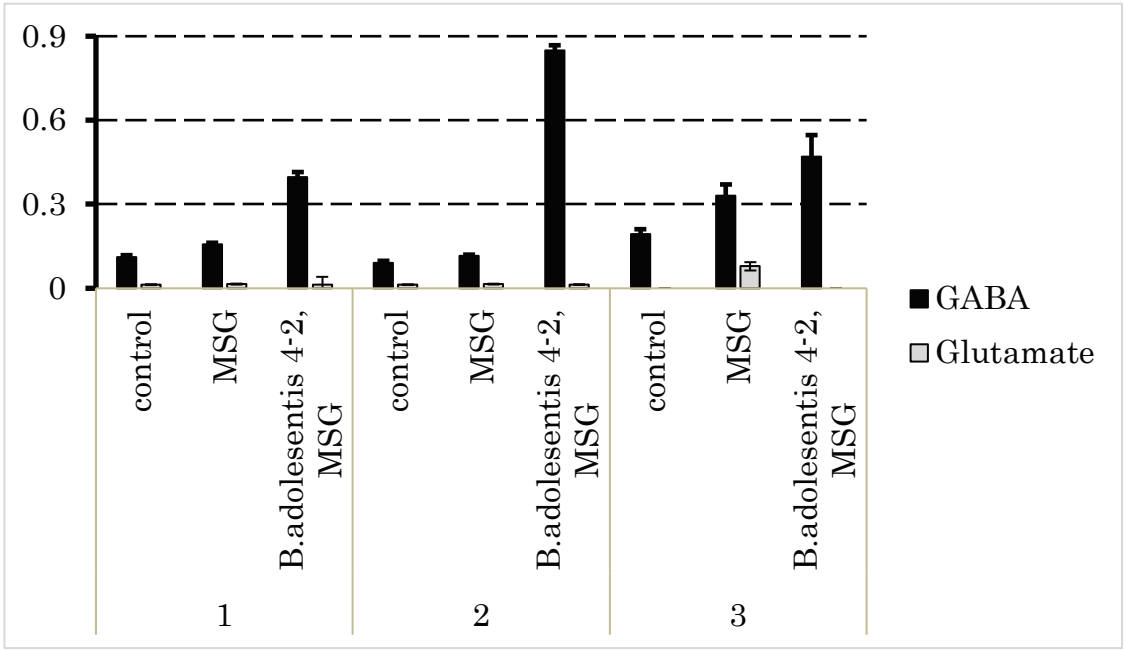


Figure 1. *B. adolescentis* 4-2 tunes up fecal GABA content in the KUHIM model.

Fecal GABA and glutamate content after 24 h co-cultivation of *B. adolescentis* 4-2 with fecal samples of three volunteers. Monosodium glutamate (MSG) was added as a substrate for GABA production.

4.3.2 Oligosaccharides alter vital parameters in fecal culture of low GABA producing microbiomes

Oligosaccharides, including MOS, Dex and FOS, were focused on our study to investigate their abilities as prebiotic enhancer for specific gut bacteria. The experiment was performed in five volunteers who provided their stools as representative for their microbiome. The culture was performed with the addition of 0.5% of each oligosaccharide separately. Interesting changes were observed in culture pH, microbial composition and GABA content.

4.3.2.1 GABA content

GABA was measured for the three oligosaccharide groups compared to the control group. Oligosaccharide groups revealed a several times increase in GABA production compared to the control. Especially MOS and FOS showed a higher tendency to increase GABA content (Fig. 2).

Figure 2

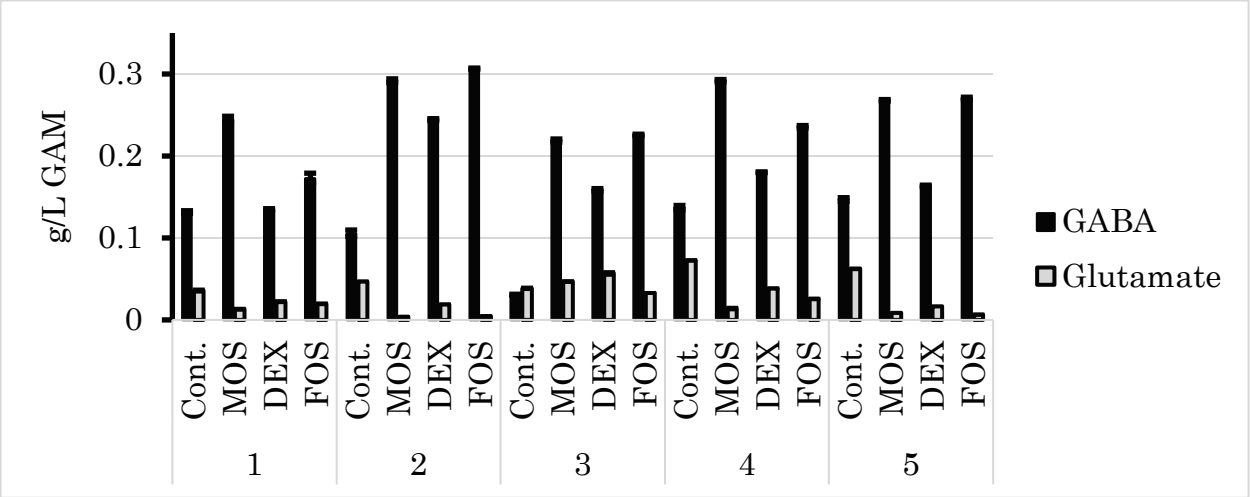


Figure 2. Oligosaccharides show a tendency towards increase fecal GABA content based on microbial produced glutamate. The figure displays fecal GABA and glutamate content on addition of oligosaccharides to fecal culture of five volunteers of low fecal GABA content. The added oligosaccharides include Mannooligosaccharides (MOS), dextrin (Dex) and fructooligosaccharides (FOS).

4.3.2.2 pH fluctuation during culture.

The pH was monitored through 24 h culture in the flora model. The pH of the control (Cont.) started to decrease around 5 hours from the beginning of the culture, gradually increased at 13 hours, and the final pH reached 6. The oligosaccharide-added groups showed a significant reduction in pH compared with the control. The reduction in pH was remarkable in MOS and FOS groups (Fig. 3A)

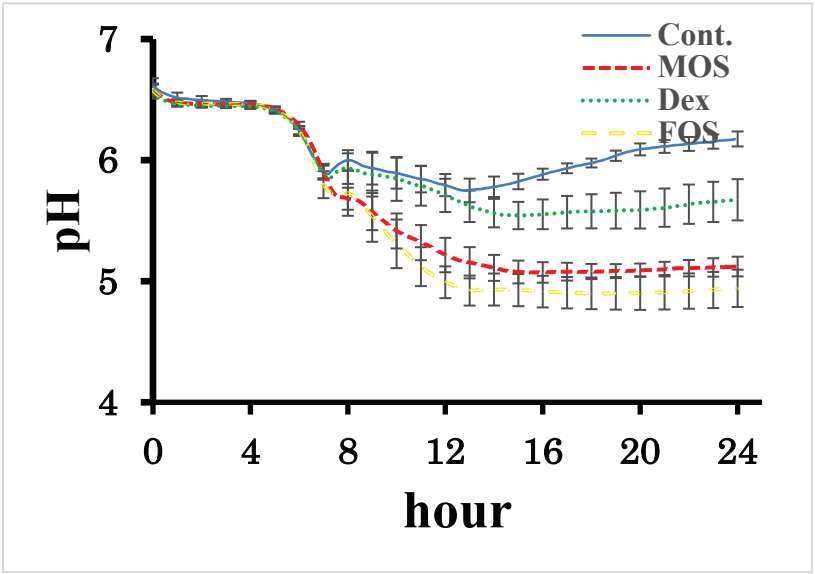
4.3.2.3 Constitutive analysis of intestinal flora using NGS

The microbial composition was analyzed for all groups after 24 h culture. The phylum level was compared between the four groups. The relative abundance of the phylum actinobacteria was increased in oligosaccharides groups including MOS, DEX, and FOS, compared to the control (Fig. 3B). Especially MOS and FOS groups revealed a substantial increase in most of tested samples.

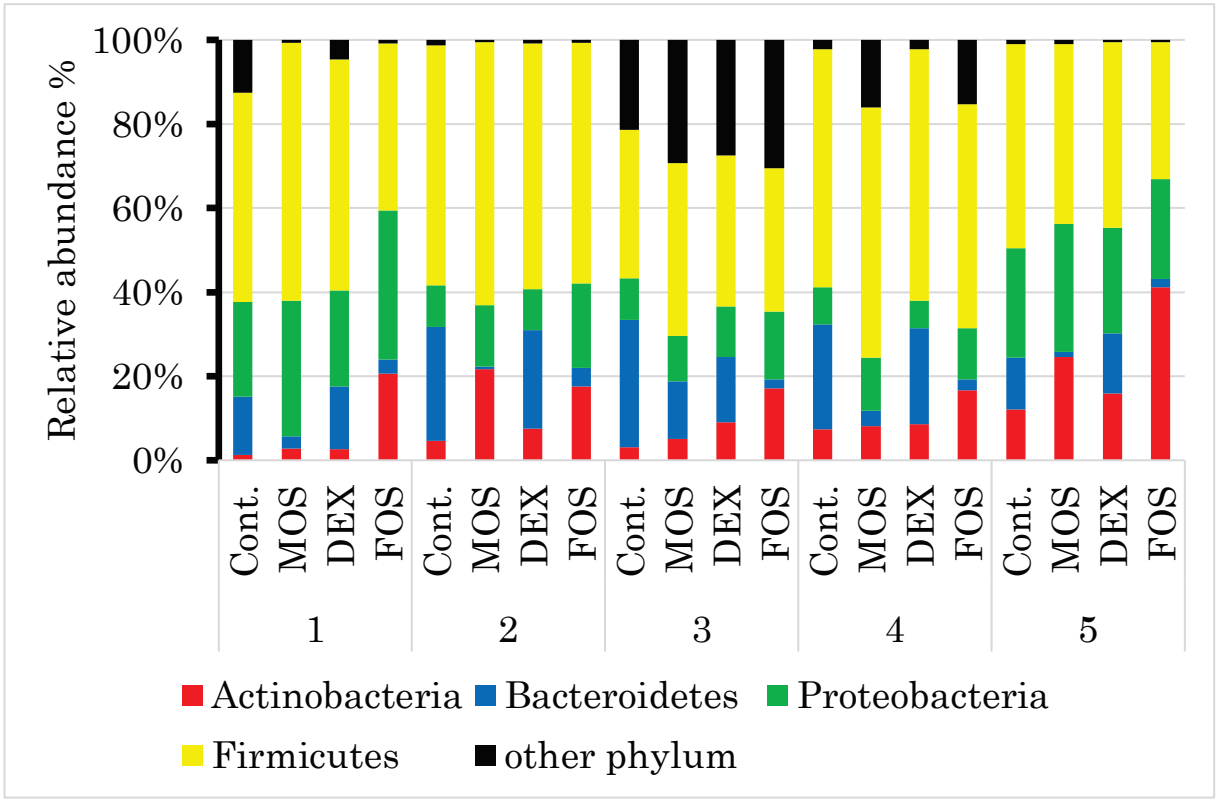
Bifidobacterium, as a central member of the phylum actinobacteria, was focused. I found that the genus *Bifidobacterium* represented most of the actinobacterium phylum in the tested groups (Fig. 3C). Three tested oligosaccharides were found to increase the genus *Bifidobacterium.* In volunteer one and two, a significant increase was observed in the species *B. adolescentis* which is known for their selective assimilation to oligosaccharides and their unique ability for GABA production [38, 118]

Figure 3

(A)



(B)


Relative abundance %
100%
80%
60%
40%
20%
0%
Cont.
MOS
DEX
FOS
1
2
3
4
5
Actinobacteria
Bacteroidetes
Proteobacteria
Firmicutes
other phylum

(C)

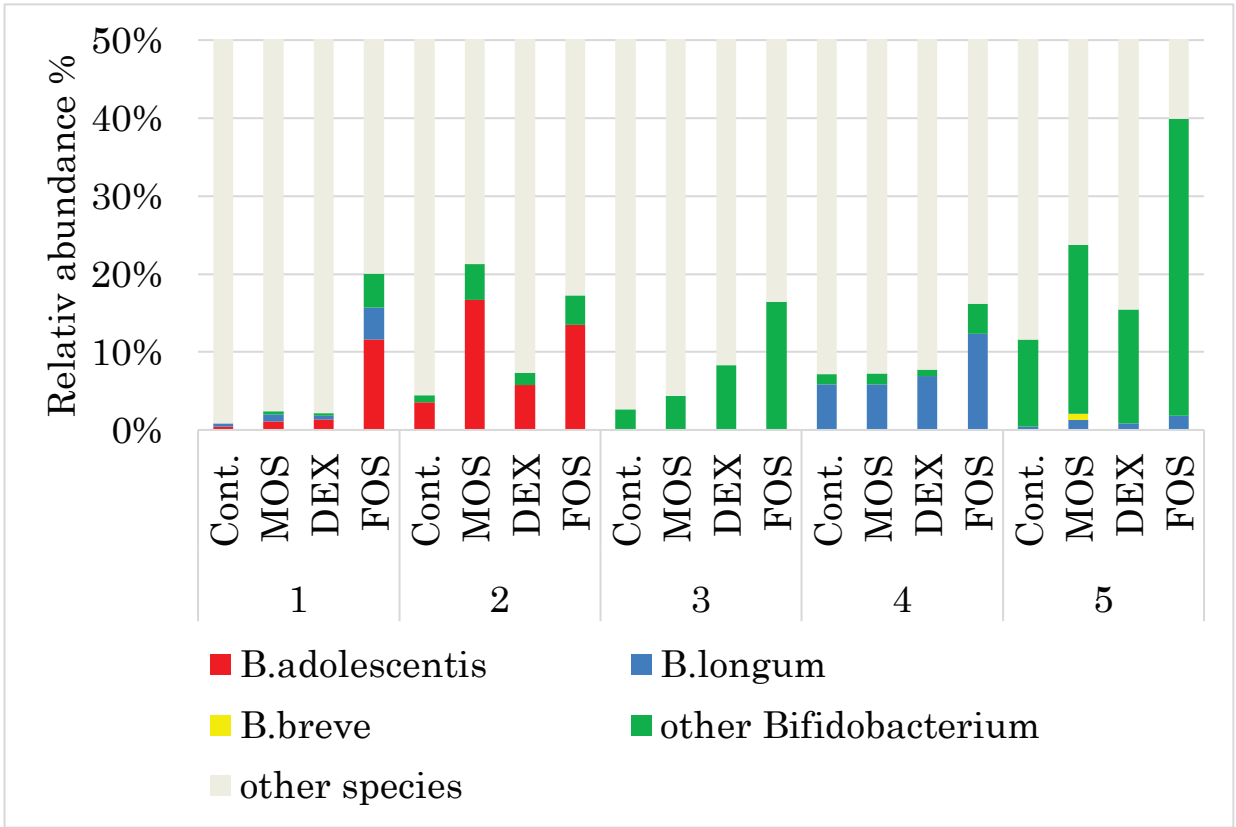
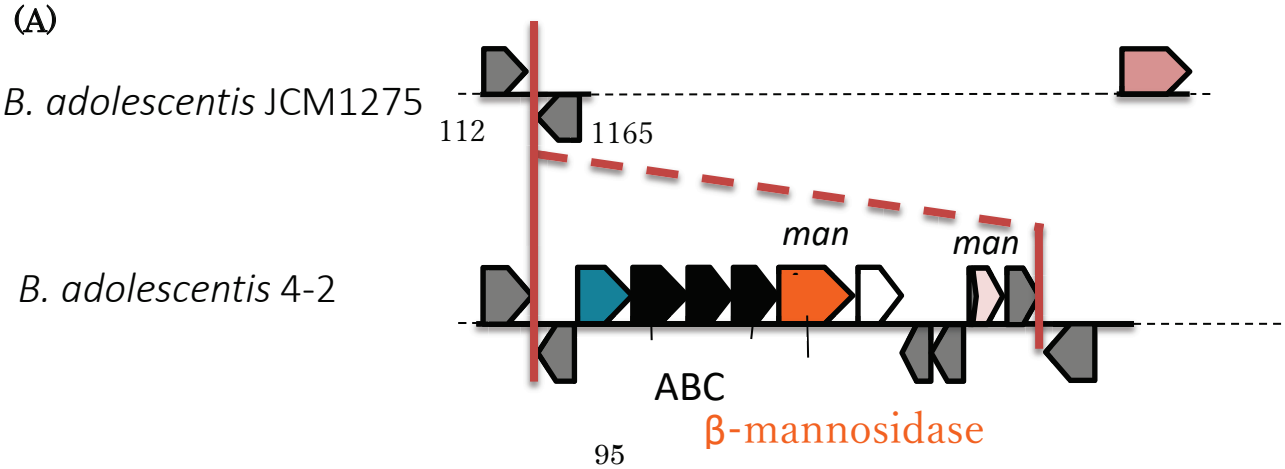

Relativ abundance %
50%
40%
30%
20%
10%
0%
Cont.
MOS
DEX
FOS
1
2
3
4
5
B.adolescentis
B.longum
B.breve
other Bifidobacterium
other species

Figure 3. Oligosaccharides alter vital parameters in the fecal culture of low GABA producing samples in KUHIM. (A) Culture media pH during the course of 24 h cultivation. (B) Phylum level classification of the most abundant taxa under cultivation with different oligosaccharides. (C) Species level classification of the phylum actinobacterium. It was almost presented by *Bifidobacterium*.

4.3.2.4 The GABA producer *B. adolescentis* 4-2 is assimilative to MOS.

In order to hydrolyze mannan, several synergistic glycoside hydrolases are required (mannanase, mannosidase, glucosidase, galactosidase). *B. adolescentis* 4-2 has two genes encode for monosidase one is the precursor *manB* (ß-mannosidase precursor) and the other is the exo-acting ß-mannosidase encoded by *manA* (Fig. 4A). ß-mannosidase activity of *B. adolescentis* 4-2 was analyzed under different carbon source containing MRS (Fig. 4B). MOS was significantly higher than glucose to enhance ß -mannosidase activity which is an evident that MOS is a preferred substrate for ß-mannosidase of *B. adolescentis* 4-2. Hence, *B. adolescentis* 4-2 is actively assimilative to MOS.

Figure 4



(B)

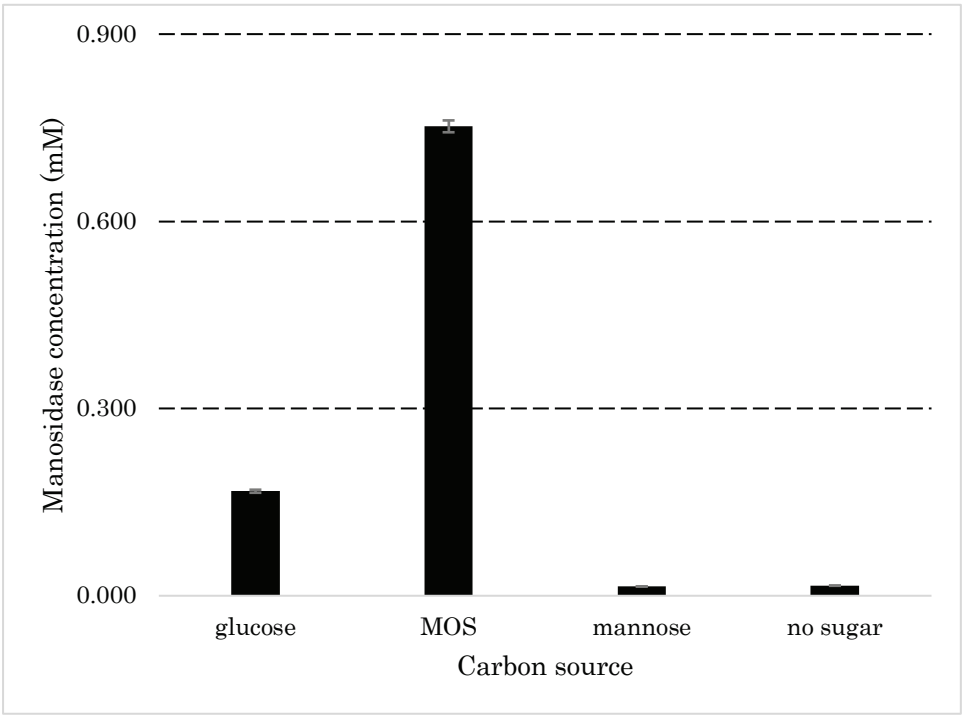


Figure 4. *B. adolescentis* 4-2 is assimilative to Mannooligosaccharides. (A) *B. adolescentis* 4-2 gene elements for MOS hydrolysis. (B) ß-mannosidase assay for *B. adolescentis* 4-2 was grown on MRS with different carbon sources.

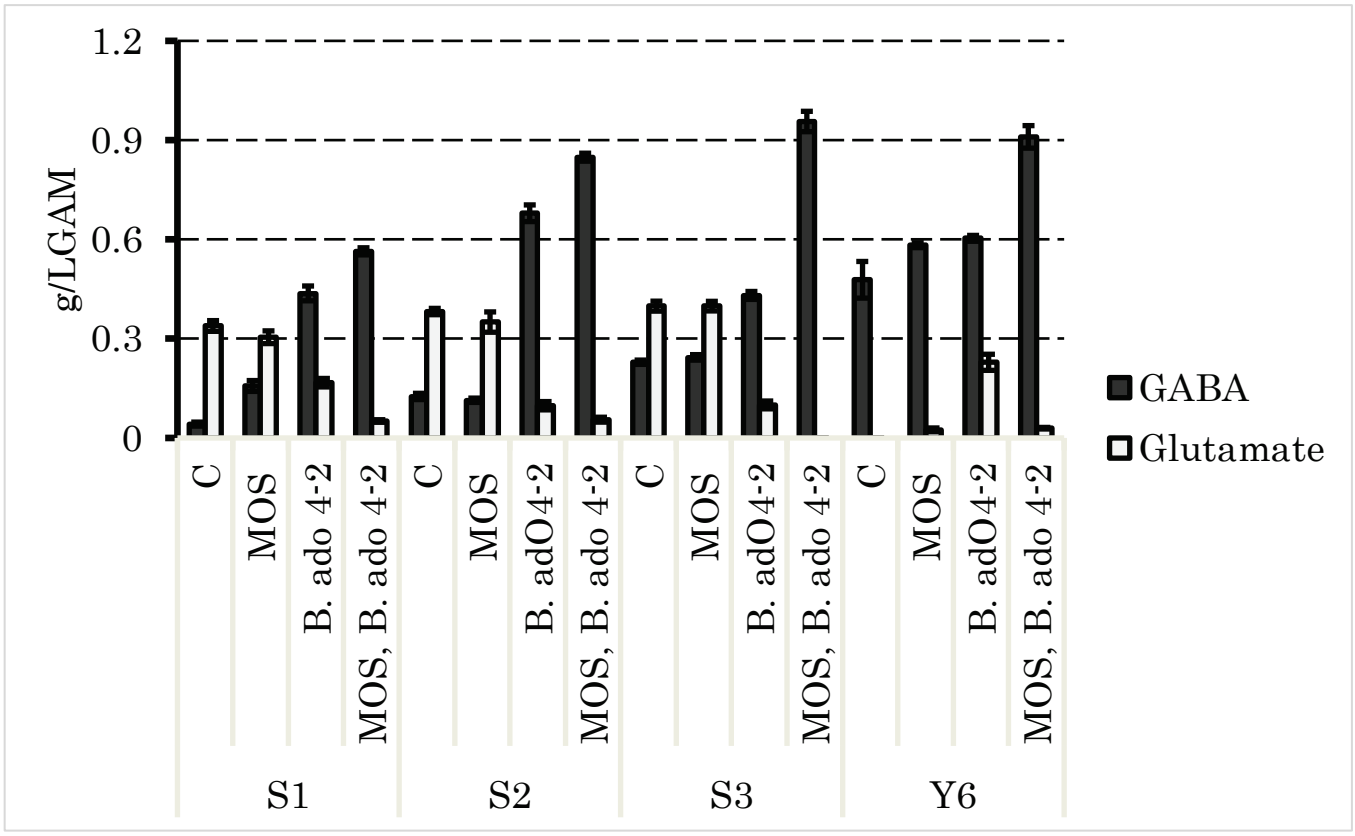
4.3.2.5 Symbiosis between *B. adolescentis* 4-2 and MOS tune up fecal GABA level in low producer volunteers.

The symbiosis between the two GABA enhancers *B. adolescentis* 4-2 and oligosaccharides was tested in the flora model using samples of low fecal GABA content. Five Conditions were tested, the control (no additives), 0.5% MOS, *B. adolescentis* 4-2 and 0.5% MOS and *B. adolescentis* 4-2. GABA content increased and glutamate content decreased in the following order MOS< *B. adolescentis* 4-

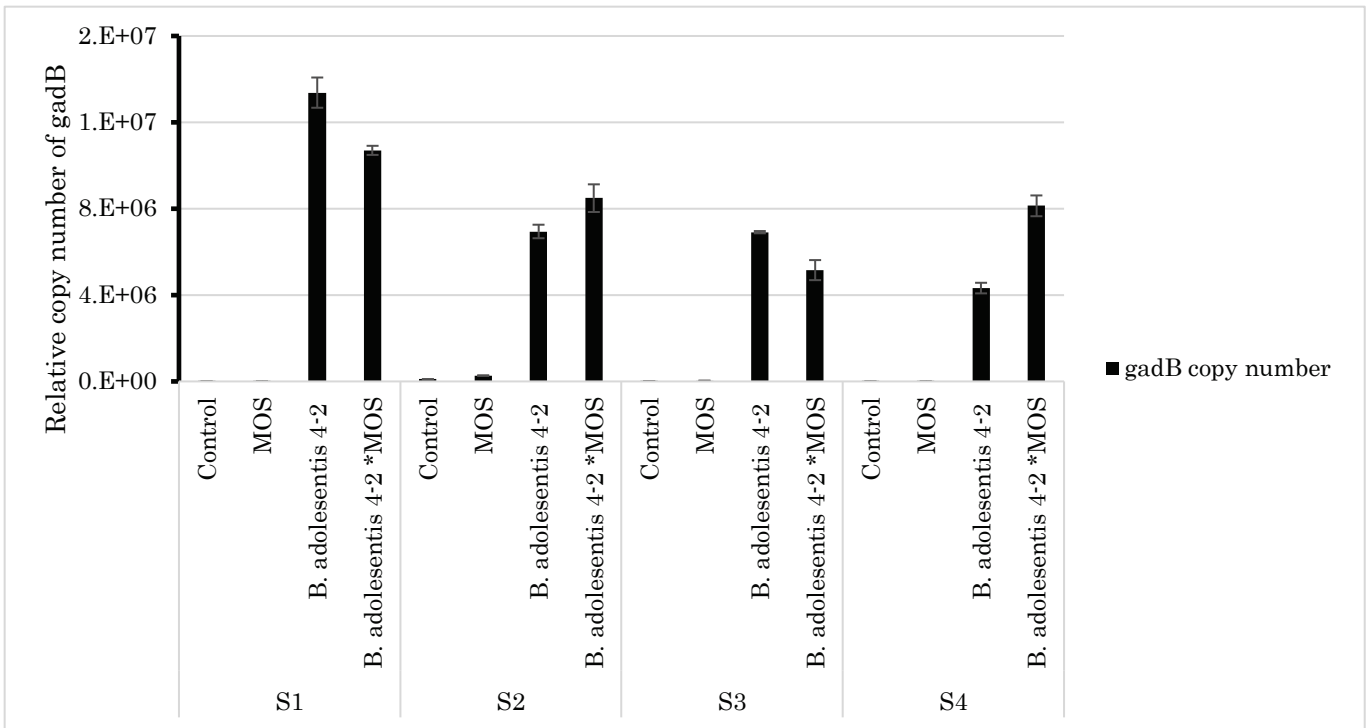
2< MOS and *B. adolescentis* 4-2 (Fig. 5A). The use of the symbiotic mixture enhanced GABA production more than the individual usage of each component. This implies the synergistic action between GABA producer *B. adolescentis* 4-2 and MOS for GABA production using a condition which mimics intestinal condition. To understand whether symbiosis is related to the increase in bacterial copy number, we analysed the copy number of *B. adolescentis* 4-2 *gadB* gene, as an indicator gene for this strain, using Absolut qPCR. The copy number of *gadB* gene was maximized in both *B. adolescentis* 4-2 and the mixture of *B. adolesentis* 4-2 and MOS. The mixture of *B. adolesentis* 4-2 and MOS exhibited a higher copy number of *gadB* gene in two out of four tested samples, indicating that MOS possibly enhanced the growth of *B. adolescentis* 4-2 (Fig. 5C). To confirm the occurrence of symbiosis within our formula, we analyzed β-mannosidase activity under each condition. Activity was markedly increased on the addition of MOS combined with *B. adolescentis* 4-2 (Fig. 5C). This denotes the ability of *B. adolescentis* 4-2 to assimilate MOS which can maximize the benefit from MOS besides enhancing GABA productivity as a positive symbiotic outcome for this formula.

Figure 5

(A)


1.2
0.9
0.6
0.3
0
g/LGAM
C
MOS
B. ado 4-2
MOS, B. ado 4-2
C
MOS
B. adO4-2
MOS, B. ado 4-2
C
MOS
B. adO4-2
MOS, B. ado 4-2
C
MOS
B. adO4-2
MOS, B. ado 4-2
S1
S2
S3
Y6
GABA
Glutamate

(B)


2.E+07
1.E+07
8.E+06
4.E+06
0.E+00
Relative copy number of gadB
Control
MOS
B. adolesentis 4-2
B. adolesentis 4-2 *MOS
Control
MOS
B. adolesentis 4-2
B. adolesentis 4-2 *MOS
Control
MOS
B. adolesentis 4-2
B. adolesentis 4-2 *MOS
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B. adolesentis 4-2 *MOS
S1
S2
S3
S4
gadB copy number

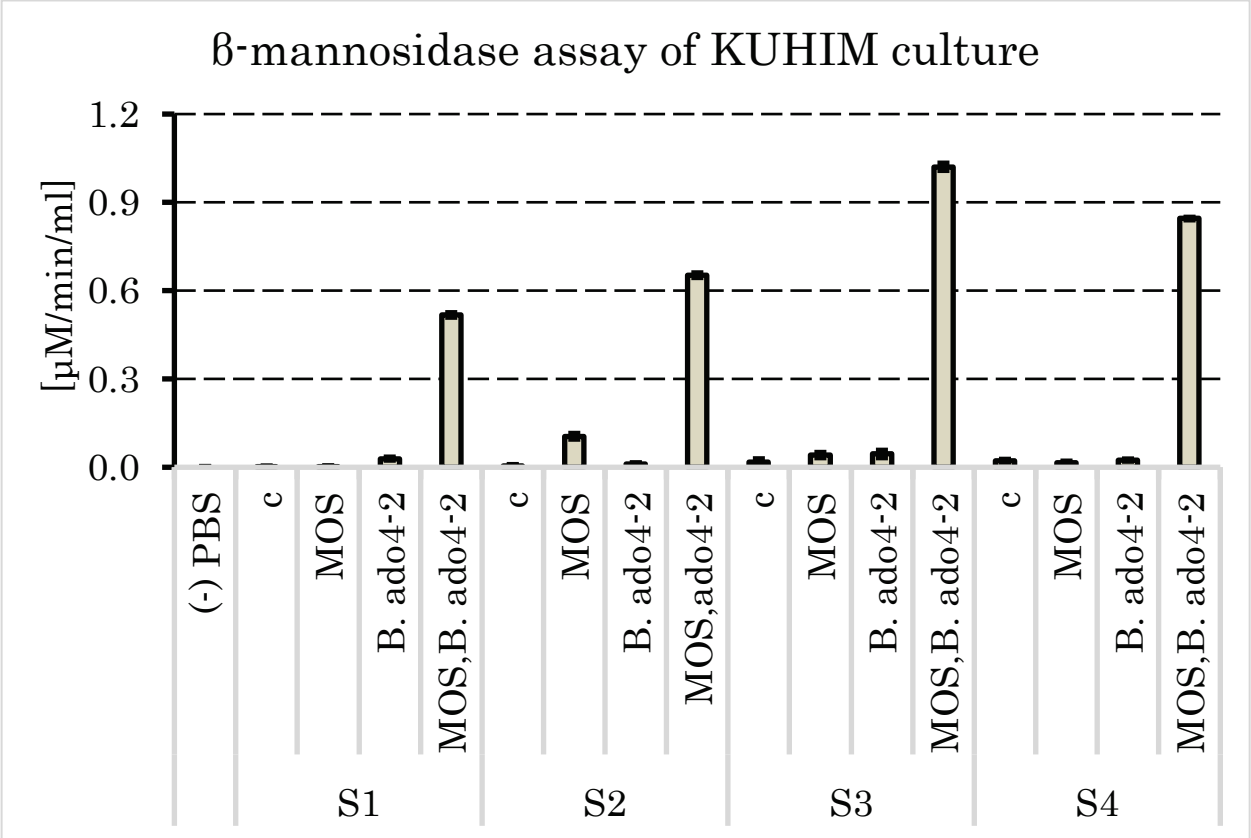

Figure 5. Symbiosis between mannooligosaccharides and *B. adolescentis* 4-2 maximizes fecal GABA content based on microbial glutamate. (A)Fecal GABA and glutamate after 24h co-culture with MOS, *B. adolescentis* 4-2 and a mixture of MOS and *B. adolescentis* 4-2. (B) *B. adolescentis* 4-2 copy number as estimated based on *gadB* gene as a unique gene for this strain. (C) ß-mannosidase assay of KUHIM model for volunteers S1, S2, S3, S4.

4.4 Discussion and Conclusion

For validation of the result obtained in the third chapter, we performed an *in vitro* survey of GABA productivity from numerous fecal isolated strains of bifidobacteria. The strain *B. adolescentis* 4-2 was found to be a high GABA producer among tested strain. This significantly improved the fecal GABA content in the *invitro* fecal culture of volunteers with low fecal GABA. The degree of enhanced GABA productivity varied between volunteers. We assume that it refers to the level of *B. adolesentis* 4-2 propagation. Thinking about how to improve their propagation invited the idea to test the effect of selected oligosaccharides, as potent prebiotics, on *Bifidobacterium* abundance. Supplementation of KUHIM with oligosaccharides resulted in rearrangement of intestinal flora. It mainly increased the relative abundance of actinobacteria phylum and specifically *Bifidobacterium* abundance. A significant increase in the species *B. adolescentis* was detected in two out of five volunteers. Besides to that, a relative reduction in pH was noticed which was parallel with *Bifidobacterium* abundance. I suggest that it occurs as a result of acid production from *Bifidobacterium*. In addition, oligosaccharides improved GABA productivity. We assume that its linked to the improved abundance of *Bifidobacterium* species. These results also suggest the bifidogenic effect of oligosaccharides, especially

MOS and FOS.

Aligning previous results together proposed the idea to combine both *B. adolescentis* 4-2 and its assimilative oligosaccharides in one mixture to maintain growth and GABA productivity. This formula maximized GABA content. For better understanding of the symbiosis between MOS and *B. adolescentis* 4-2, the activity of ß-mannosidase was assessed for *B. adolescentis* 4-2 growing on different soul carbon sources. MOS has revealed a remarkable ability to activate ß-mannosidase. ß-mannosidase are exo- acting enzyme which can degrade ß-1,4-linked mannan such as MOS releasing mannose. Hence, MOS is a candidate bifidogenic factor to improve propagation of *Bifidobacterium,* especially that having ß-mannosidase.

I assume that the symbiotic formula proposed in this study is a potential formula for enhanced GABA production. For future prospection, we encourage human trials of the proposed formula in specific cases in which GABA is prescribed as a supportive or main treatment. Further, studies will be needed for better understanding of bifidogenic ability of MOS.

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