Studies on astragaloside IV metabolism in lactic acid bacteria and bifidobacteria

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Contents

Abbreviations

AIV	Astragaloside IV
BraB	Brachyoside B
CycB	Cyclogaleginoside B
CA	Cycloastragenol
CA-2H	20*R*,24*S*-epoxy-6α,-16*β*,-25-trihydroxyl-9,19-cycloartan-3-one
KPB	Potassium phosphate buffer
GAM	Gifu anaerobic media
LAB	Lactic acid bacteria
HPLC	High performance liquid chromatography
LCMS	Liquid chromatography mass spectrometry
CFE	Cell free extract
US	Soluble fraction
UP	Insoluble fraction

General Introduction

Research studies centering around traditional herbal medicine have been steadily increasing. Although, the use of herbal medicine and its effectiveness among the general population has been controversial. The growing concern for their safety and quality control has long been an issue (Ekor 2014). However, the potential of new chemical compounds derived from plant extract, especially from traditional herbal medicine that has beneficial physiological effects in humans is still high. After the findings that intestinal bacteria play a significant role in biotransforming crude natural products derived from plant extracts to more potent bioactive compounds (Kobashi and Akao 1997), the research looking into specific chemistry of digestion and how these chemicals are absorbed in human bodies have been rising.

In this series of research objectives, we examine astragaloside IV (AIV) a biomarker for the herbal medicine *Astragalus membranaceus* commonly known as Mongolian milkvetch. It is one of the 50 fundamental herbs used in traditional Mongolian medicine. Various research has been carried out on AIV and its beneficial physiological effects on humans. However, AIV is a compound not easily absorbed in our digestive system and has a low bioavailability (Huang *et al*. 2006). Its aglycone cycloastragenol (CA) is more readily absorbed and various research on CA has shown potent telomere activation and telomere elongation that play a significant role in our immune system (Fauce *et al*. 2008). Pharmacokinetic evidence has shown that AIV is metabolized to CA by a consortium of rat derived intestinal bacteria (Zhou *et al*. 2012). However, no specific intestinally derived bacteria have been shown to metabolize AIV to CA to our knowledge.

This study focuses on screening and finding specific gut microbes derived from human intestines and understanding how AIV is possibly metabolized by different microbes in our digestive system (Chapter 1). Furthermore, by utilizing the positive AIV metabolizing microbes found in chapter 1 the author attempts to develop a method to produce CA efficiently from AIV using washed cells of LAB and bifidobacteria (Chapter 2). Lastly, the author focuses on the enzymatic activity of *Bifidobacterium pseudocatenulatum* JCM 7041 and attempts to identify the enzyme responsible for the AIV metabolism (Chapter 3).

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Chapter 1

Analysis of astragaloside IV metabolism to cycloastragenol in human gut microorganism, bifidobacteria, and lactic acid bacteria

Abstract

This study investigated different gut bacteria in an anaerobic environment to identify specific candidates that could transform astragaloside IV (AIV) to cycloastragenol (CA). Two representative gut microbes, lactic acid bacteria (LAB) and bifidobacteria, could metabolize AIV to CA. Multiple screenings showed two metabolic pathways to metabolize AIV in two groups of bacteria. LAB metabolized AIV initiated by removing the C-6 glucose, whereas bifidobacteria indicated the initial removal of C-3 xylose. The final products differed between the two groups as bifidobacteria showed the production of CA, whereas LAB demonstrated preferential production of 20*R*,24*S*-epoxy-6α,-16*β*,-25-trihydroxy-9,-19-cycloartan-3-one (CA-2H).

Introduction

Herbal medicines and extracts have been used as traditional medicine worldwide since ancient times. It has been well established that the intestinal bacterium plays a vital role in determining the absorption of most herbal medicine through structural modification of the active components in the gut after oral intake (Kobashi and Akao 1997; Feng *et al*. 2019). A well-known example is the decomposition of ginsenosides, the active compound in ginseng. Ginsenoside is metabolized in stepwise deglycosylation and dehydration by gut microbes, forming a more permeable active compound (Liu *et al.* 2009; Liu *et al.* 2006; Qi, Wang and Yuan 2011). Astragaloside IV (AIV) is a cyclo-artane-type triterpene glycoside (Zhou *et al.* 2012). It is the main component of *Astragalus membranaceus* and the main saponin discovered in Astragali Radix (Gou *et al.* 2019; Ma *et al.* 2002), another herbal medicine used for various health benefits (Zhang *et al*. 2003; Zhang *et al*. 2006; Zhao *et al.* 2012 Du *et al.* 2005). However, the bioavailability of AIV after oral administration is poor (Gu *et al*. 2004). Transport studies of AIV using the Caco-2 cell model have shown poor permeability of the compound through the intestinal wall (Huang *et al*. 2006). Zhou *et al*. (2012) conducted a pharmacokinetic study of a sapogenin compound, cycloastragenol (CA), the aglycone of AIV, producing it in rats' intestinal system and proposed the metabolic pathway of AIV to CA as follows (Figure 1.1). Removing the xylose at the C-3 position and glucose at the C-6 position from AIV produces CA. CA is efficiently absorbed through the intestinal epithelium by passive diffusion (Zhu *et al*. 2010) and therefore has a higher bioavailability than AIV. There is proof that CA acts as a telomerase activator to modestly slow telomere shortening in $CD8^+$ T lymphocytes from HIV-infected human donors leading to antiviral activity (Fauce *et al*. 2008). CA is a telomerase activator and anti-inflammatory to improve wound healing and delay the onset of age and disease progression (Wan *et al*. 2018; Shen *et al*. 2017; Sevimli-Gür *et al*. 2011). Several studies have shown CA production, including chemical conversion using the Smith degradation and mild acid hydrolysis (Feng *et al*. 2014), microbial conversion through *Bacillus* sp. (Wang *et al*. 2017), and enzymatically using sugar-stimulated *β*-glucosidase and *β*-xylosidase from *Dictyoglomus thermophilum* (Li *et al*. 2019). Nevertheless, no studies have investigated specific bacteria in the intestinal environment that metabolizes the AIV.

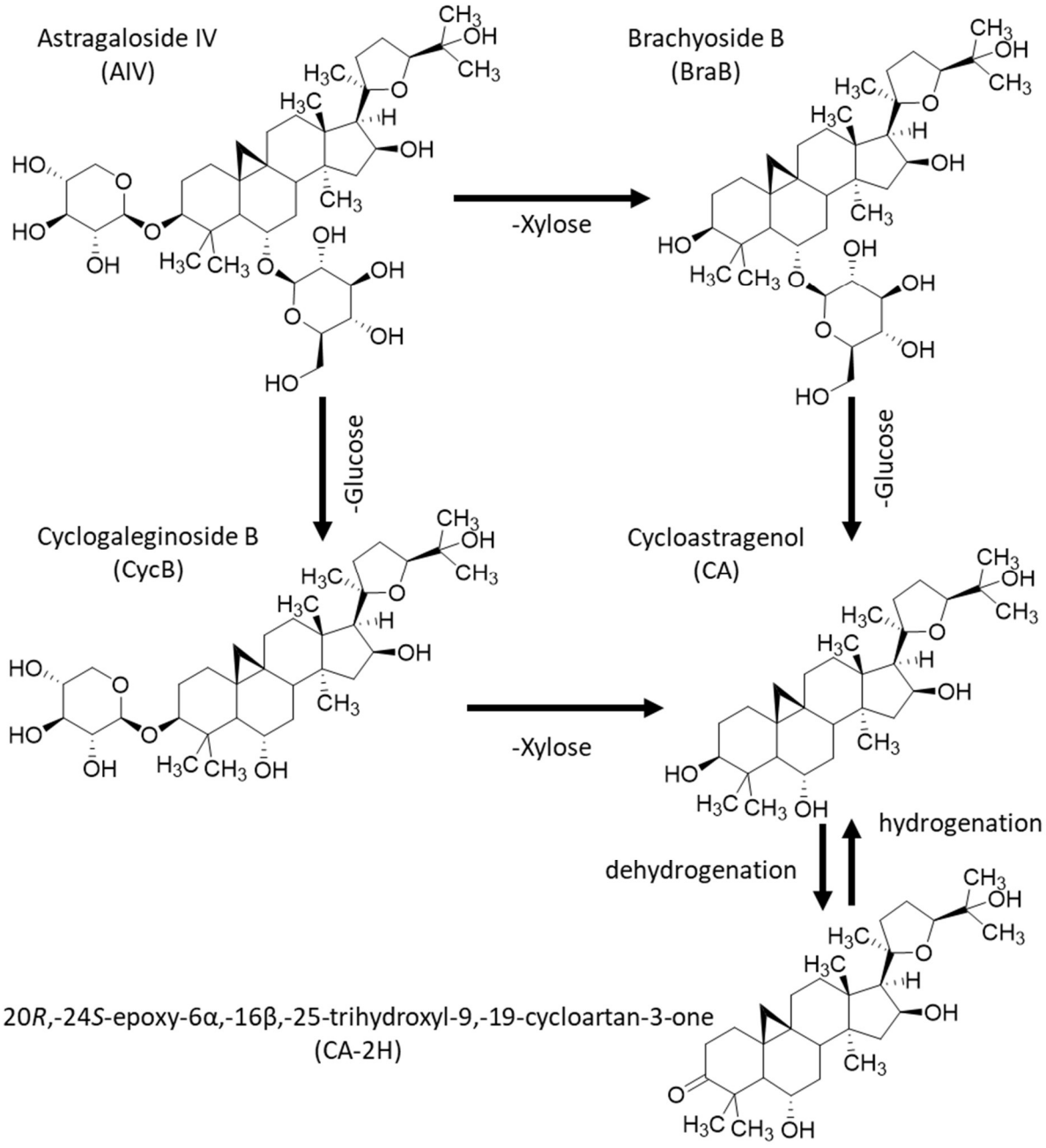

Figure 1.1: Hypothesized metabolic pathway of AIV by intestinal bacteria

Materials and Method

Preparation of AIV and CA solutions

AIV (98% purity) and CA (98% purity) were purchased from Carbosynth (Berkshire, UK). AIV was reconstituted in dimethyl sulfoxide (DMSO) and sterile filtered through a 0.22 μm syringe filter for a stock solution (19 mM) used in cell reaction screening of microorganisms. AIV and CA were reconstituted in methanol for a stock solution (1.3 mM) used in the high-performance liquid chromatography (HPLC) analysis. To produce AIV and CA concentration standards when analyzing screening samples with HPLC, serially diluted 1.3 mM stock solution solutions were used.

Gut-derived microorganisms screening with resting-cell reaction

Microorganisms derived from the human intestinal environment were purchased from Japan collection of microorganisms (JCM) (Table S1). Each strain was inoculated and grown in 15 mL screw cap glass tubes with 15 mL of sterilized (115°C for 15 min) ½ strength Gifu anaerobic media (GAM) broth (29.5 g/L) at 37°C and shaken at 130 strokes per min for 24 h under anaerobic condition. After cultivation, the cells were harvested by centrifugation at 1,500 *g* for 20 min. Next, the cells were washed with 0.85% NaCl, transferred to a 2 mL centrifuge tube, and harvested by centrifugation at 20,000 *g* for 20 min. The harvested cells were reacted with 1 mL of 0.1 M potassium phosphate buffer (KPB) (pH 6.5) with 13.3 μl of AIV stock solution for a final AIV concentration of 0.25 mM. The resting-cell reaction was incubated at 37°C and shaken at 1,000 strokes per min for 72 h on a bench top shaker Deep Well Maximizer (Taitec, Saitama, Japan). Finally, samples were lyophilized and reconstituted in 1 mL methanol for analysis.

HPLC analysis of metabolites

Quantitative analysis of CA and its intermediate components was performed by HPLC using a Shimadzu LC 10A system (Shimadzu, Kyoto, Japan) equipped with a UV detector, vacuum degasser, a binary pump, an autosampler, and a column oven. The samples were eluted on a $5C_{18}$-AR-II column (4.6 ID × 150 mm; COSMOSIL). The column temperature was maintained at 35°C. 20 μl of filtered supernatant (0.45 μm) was injected into the HPLC. The mobile phase for gradient elution contained degassed Milli-Q Water (A) and acetonitrile (B). The gradient elution program was as follows; 30% B, 0–1.5 min; 30%–80% B, 1.5–8 min; 80%–100% B, 8–8.01 min; 100% B, 8.01–12.5 min; 100%–80% B, 12.5–12.51 min; 80%–

30% B, 12.51–13.01 min; 30% B, 13.01–18 min. The flow rate was set at 1.5 mL/min. The effluent was monitored with a UV detector at an excitation wavelength of 203 nm.

HPLC-MS confirmation of metabolites

Before eluate was directed into a mass spectrometer, samples were separated using HPLC using a Shimadzu LC 20A system (Shimadzu, Kyoto, Japan) equipped with a vacuum degasser, a binary pump, an autosampler, and a column oven. Samples were eluted on a 2.5C_{18}-MS-II column (3.0 ID × 100 mm; COSMOSIL). The column temperature was kept at 35°C. 1 µl of filtered supernatant (0.45 µm) was injected into HPLC. The mobile phase for gradient elution contained 0.1 formic acid (A) and acetonitrile/0.1% formic acid (B). The gradient elution program was as follows; 30% B, 0–3.19 min; 30%–80% B, 3.19–17 min; 80%–100% B, 17–17.03 min; 100% B, 17.03–24.46 min; 100%–80% B, 24.46–24.48 min; 80%–30% B, 24.48–25.52 min; 30% B, 25.52–38.5 min. The flow rate was set at 0.2 mL/min. The effluent was monitored with a UV detector at an excitation wavelength of 203 nm. Additionally, mass spectrometry was performed on a triple quadrupole mass spectrometer (LCMS-8045; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface. The instrument was operated in positive ion mode (Figure S1).

Bifidobacteria and lactic acid bacteria (LAB) screening with resting-cell reaction

All bifidobacteria strains were purchased from Japan collection of microorganisms (JCM). Each strain was grown three times for 24 h before being analyzed according to the initial screening technique. The initial LAB screening was performed using the LAB library consisting of purchased LAB strains from the Biological Resource Center, NITE (NBRC) culture catalog. The microorganisms used for the additional screening consist of new LAB strains purchased from NBRC and most of the strains purchased from the RD screening strains provided by the National Institute of Technology and Evaluation (NITE). Following the previous screens, strains were grown, processed, and analyzed using HPLC.

Time-course analysis of bifidobacteria and LAB

All three strains were grown three times in 15 mL screw cap glass tubes with 15 mL sterilized (115°C for 15 min) ½ strength GAM broth (29.5 g/L) at 37°C and shaken at 130 strokes per min for 14 h. Furthermore, 150 µl broth of the initial seed sample was transferred to another 15 mL sterilized sample of ½ strength GAM, grown at 37°C, and shaken at 130 strokes per minute for 24 h. Cells were harvested and reacted with the same procedure as during the screening process. Time course samples were taken and frozen at the 0-h, 24-h, 48-h, and

72-h time points (−80°C). For HPLC analysis, samples were lyophilized and reconstituted in 1 mL of methanol for analysis utilizing HPLC.

Results

Screening of bacteria derived from human gut flora

The initial screening of bacteria derived from human gut flora using a resting-cell reaction indicated that multiple strains of bacteria possess the characteristics to metabolize AIV (Table 1.1). 14 strains out of 84 bacterial strains derived from the human gut flora indicated AIV metabolism to brachyoside B (BraB), cyclogaleginoside B (CycB), CA, and CA-2H. Half the positive strains were derived from the *Bifidobacterium* genus. Also, the initial screening indicated that various gut microbial strains metabolize AIV into intermediate BraB or CycB at numerous concentrations. No strains showed the presence of both BraB and CycB intermediates during the metabolism of AIV. *Bifidobacterium pseudocatenulatum* JCM 7041 showed high biotransformation of AIV to BraB with considerable amounts of CA and CA2-H production. AIV and its metabolites were confirmed by LCMS (Figure S1).

Screening of bifidobacteria for AIV metabolism

Because half of the positive organisms from the initial screen reflecting human gut flora were from the *Bifidobacterium genus*, the second screening focused on bifidobacteria. Six new strains out of the 38 indicated the capacity to metabolize AIV and produce BraB (Figure 1.2). No production of CycB was detected, suggesting that bifidobacteria prefer the metabolism of AIV initiated by removing the C-3 xylose sugar molecule. *Bifidobacterium pseudocatenulatum* JCM 7041 showed complete metabolism of AIV under screening conditions with some production of CA. Two new strains, *Bifidobacterium angulatum* JCM 7096 and *Bifidobacterium catenulatum* JCM 1194, also indicated the production of CA.

Screening of LAB for AIV metabolism

Because *Lactobacillus johnsonii* JCM 1022 indicated high metabolism of AIV and production of CycB during the initial human gut flora screening, the third screening focused on LAB. Multiple LAB strains in the screening showed the metabolism of AIV to CycB, an intermediate of CA (Figure 1.3). No LAB strain indicated BraB production, suggesting LAB metabolizes AIV differently from bifidobacteria and initially removes the C-6 glucose instead of the C-3 xylose. Four strains indicated the production of CA from AIV. The remaining strain showed the biotransformation of AIV to CA-2H, but CA was undetected with the HPLC during analysis. *Pediococcus acidilactici* NBRC 3885 and *Streptococcus citrovorum* NBRC 3397 metabolized nearly half of AIV and produced CA and CA-2H. *Pediococcus pentosaceus* NBRC 3182 and *Pediococcus pentosaceus* NBRC 12229 showed metabolism of AIV and

production of CA-2H with no detectable CA. An additional screening of 157 LAB strains derived from fermented vegetables and fruits revealed 74 strains that metabolize AIV and produce CycB (Figure S2). 14 of the 74 strains showed the production of CA. Still, the remaining strains did not show detectable CA production. LAB strains showed no BraB production in the additional screen.

Time-course analysis of LAB and bifidobacteria metabolism of AIV

With many of the LAB strains producing CA-2H, with no detectable CA during analysis, a time-course study was conducted on *B. pseudocatenulatum* JCM 7041, *S. citrovorum* NBRC 3397, and *P. pentosaceus* NBRC 3182, shown in Figure 1.4, 1.5 and 1.6. The time-course profile of *B. pseudocatenulatum* JCM 7041 showed complete AIV decomposition at 72 h and high production of BraB intermediate with no CycB production during the entire time course. CA production is detectable starting at 24 h and increases through 72 h. *S. citrovorum* NBRC 3397 showed metabolism of AIV through to 72 h. However, compared with *B. pseudocatenulatum,* JCM 7041 indicated a lag of 24 h before metabolizing AIV and producing the intermediate CycB along with CA-2H. CA production of *S. citrovorum* NBRC 3397 was seen after the 48-h time point. *P. pentosaceus* NBRC 3182 like *S. citrovorum* NBRC 3397, showed a 24-h lag before producing CycB at 24 h. Both LAB strains showed no production of BraB during the time-course analysis. Nevertheless, *P. pentosaceus* NBRC 3182 produces CA-2H with no detectable CA produced during the entire time-course study.

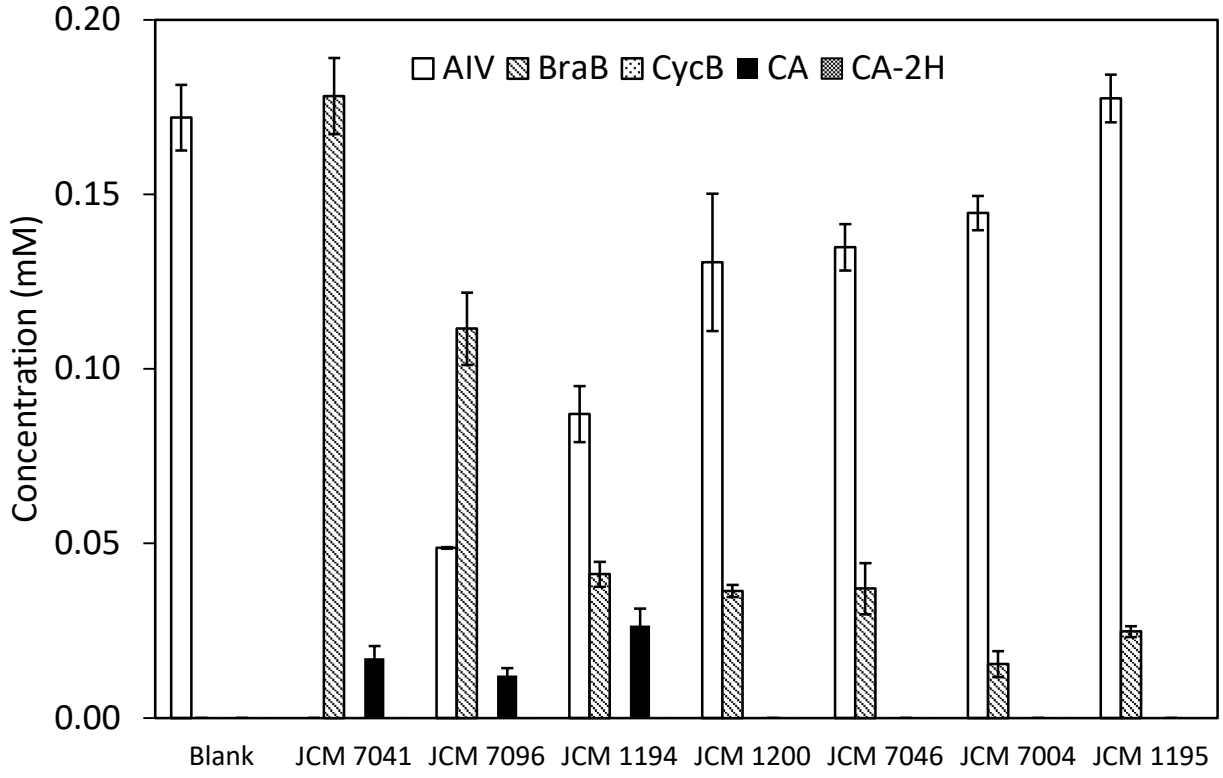

Figure 1.2: Bifidobacteria strains showing metabolism of AIV

Bifidobacteria strains show positive metabolism of AIV to increase AIV remaining after a 72 h cell reaction. Error bars show the SD (n = 3).
Bifidobacterium pseudocatenulatum JCM 7041, *Bifidobacterium angulatum* JCM 7096, *Bifidobacterium catenulatum* JCM 1194, *Bifidobacterium pseudocatenulatum* JCM 1200, *Bifidobacterium adolescentis* JCM 7046, *Bifidobacterium dentium* JCM 1195, *Bifidobacterium bifidum* JCM 7004

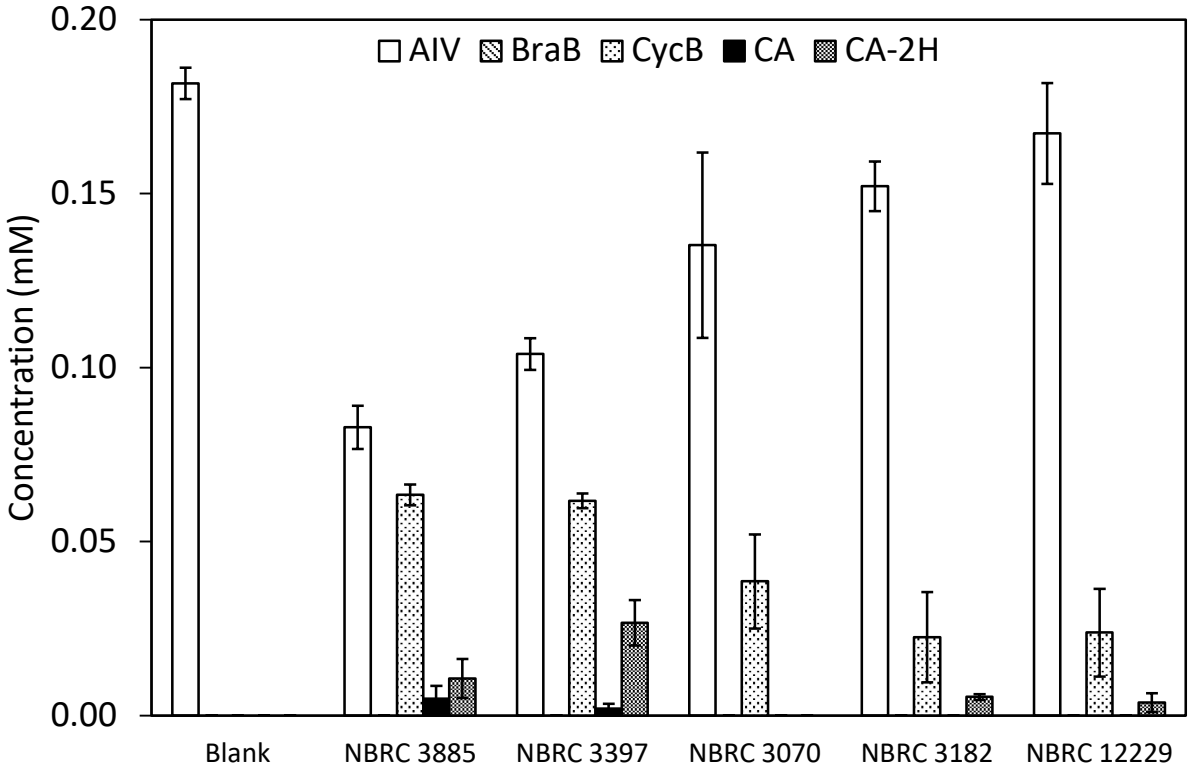

Figure 1.3: LAB strains showing metabolism of AIV

Lactic acid bacteria strains show positive metabolism of AIV to increase AIV remaining after a 72 h cell reaction. Error bars show the SD (n = 3).
Pediococcus acidilactici NBRC 3885, *Streptococcus citrovorum* NBRC 3397, *Lactobacillus plantarum* NBRC 3070, *Pediococcus pentosaceus* NBRC 3182, *Pediococcus pentosaceus* NBRC 12229

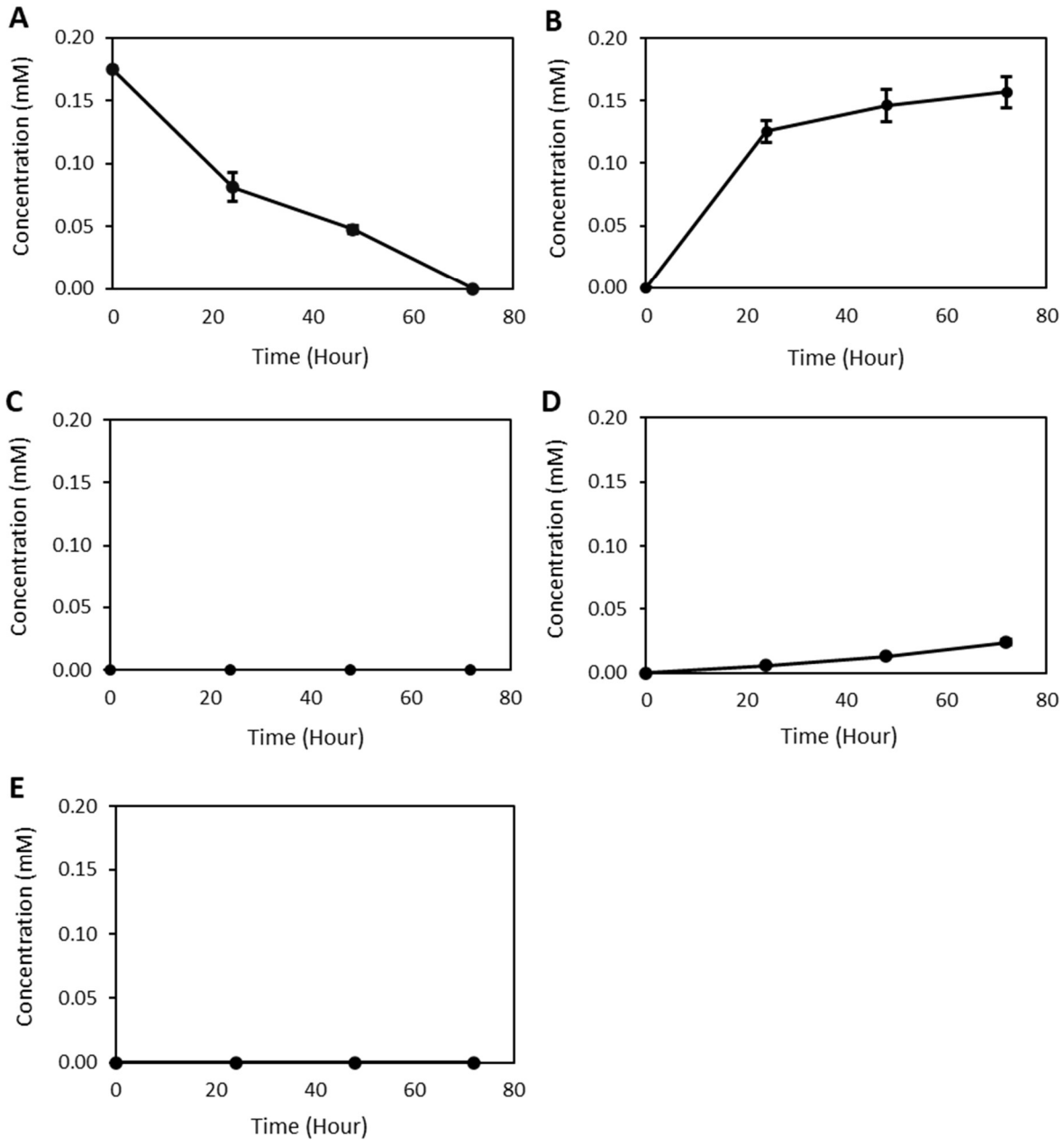

Figure 1.4

Bifidobacterium pseudocatenulatum JCM 7041 time-course profile of AIV metabolism (A) and production of BraB (B), CycB (C), CA (D), and CA-2H (E) over 72 h. Error bars show the SD (n = 3)

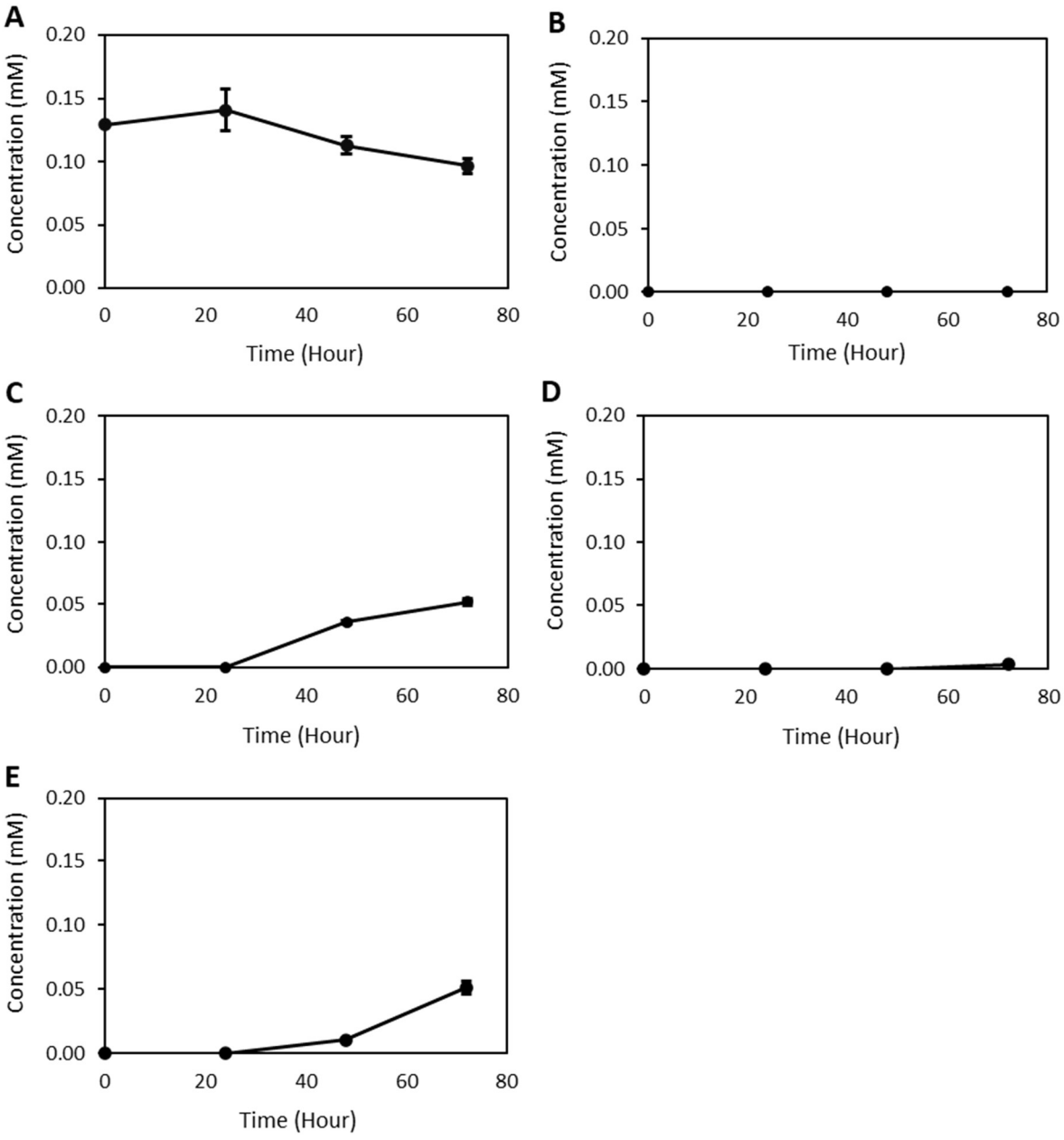

Figure 1.5

Streptococcus citrovorum NBRC 3397 time-course profile of AIV metabolism (A) and production of BraB (B), CycB (C), CA (D), and CA-2H (E) over 72 h. Error bars show the SD (n = 3)

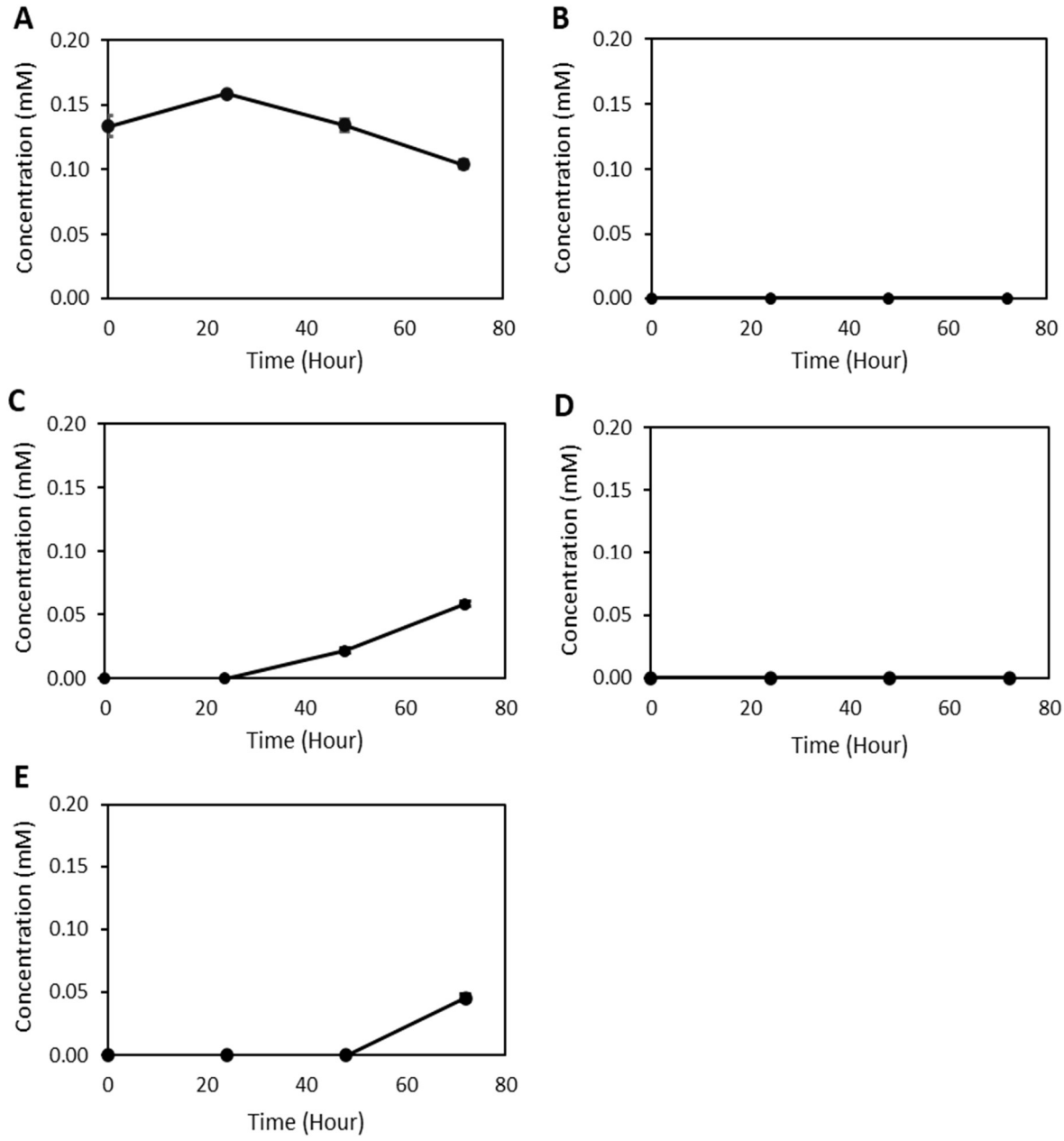

Figure 1.6

Pediococcus pentosaceus NBRC 3182 time-course profile of AIV metabolism (A) and production of BraB (B), CycB (C), CA (D), and CA-2H (E) over 72 h. Error bars show the SD (n = 3)

Table 1.1 Concentration of AIV-derived metabolites produced by intestinal bacteria

Microorganism	Concentration (mM)				
	AIV[a]	Bra B[b]	Cyc B[c]	CA[d]	CA-2H[e]
Bifidobacterium breve JCM 7016	0.04	0.12	ND[f]	ND	ND
Bifidobacterium pseudocatenulatum JCM 7041	0.06	0.11	ND	0.01	0.02
Ruminococcus productus JCM 1471	0.07	ND	0.11	0.02	0.03
Bifidobacterium longum subsp. *infantis* JCM 1222	0.07	ND	0.02	0.02	ND
Lactobacillus johnsonii JCM 1022	0.08	ND	0.08	ND	0.02
Clostridium difficile JCM 5243	0.08	ND	0.07	ND	0.07
Ruminococcus gnavus JCM 6515	0.08	ND	0.06	0.01	0.01
Bacteroides uniformis JCM 5828	0.12	0.05	ND	0.03	0.01
Bifidobacterium breve JCM 1273	0.12	0.03	ND	0.01	ND
Bifidobacterium adolescentis JCM 1275	0.13	0.03	ND	ND	ND
Bifidobacterium breve JCM 7020	0.14	0.02	ND	ND	0.01
Bifidobacterium animalis subsp. *lactis* JCM 10602	0.14	ND	0.01	ND	ND
Bifidobacterium breve JCM 7019	0.14	0.01	ND	ND	ND
Pseudoflavonifractor capillosus JCM 30894	0.16	0.02	ND	ND	ND

[a]AIV, Astragaloside IV.
[b]BraB, Brachyoside B.
[c]CycB, Cyclogaleginoside B.
[d]CA, Cycloastragenol.
[e]CA-2H, 20*R*,-24*S*-epoxy-6α,-16*β*,-25-trihydroxy-9,-19-cycloartan-3-one.
[f]ND, Not detected.

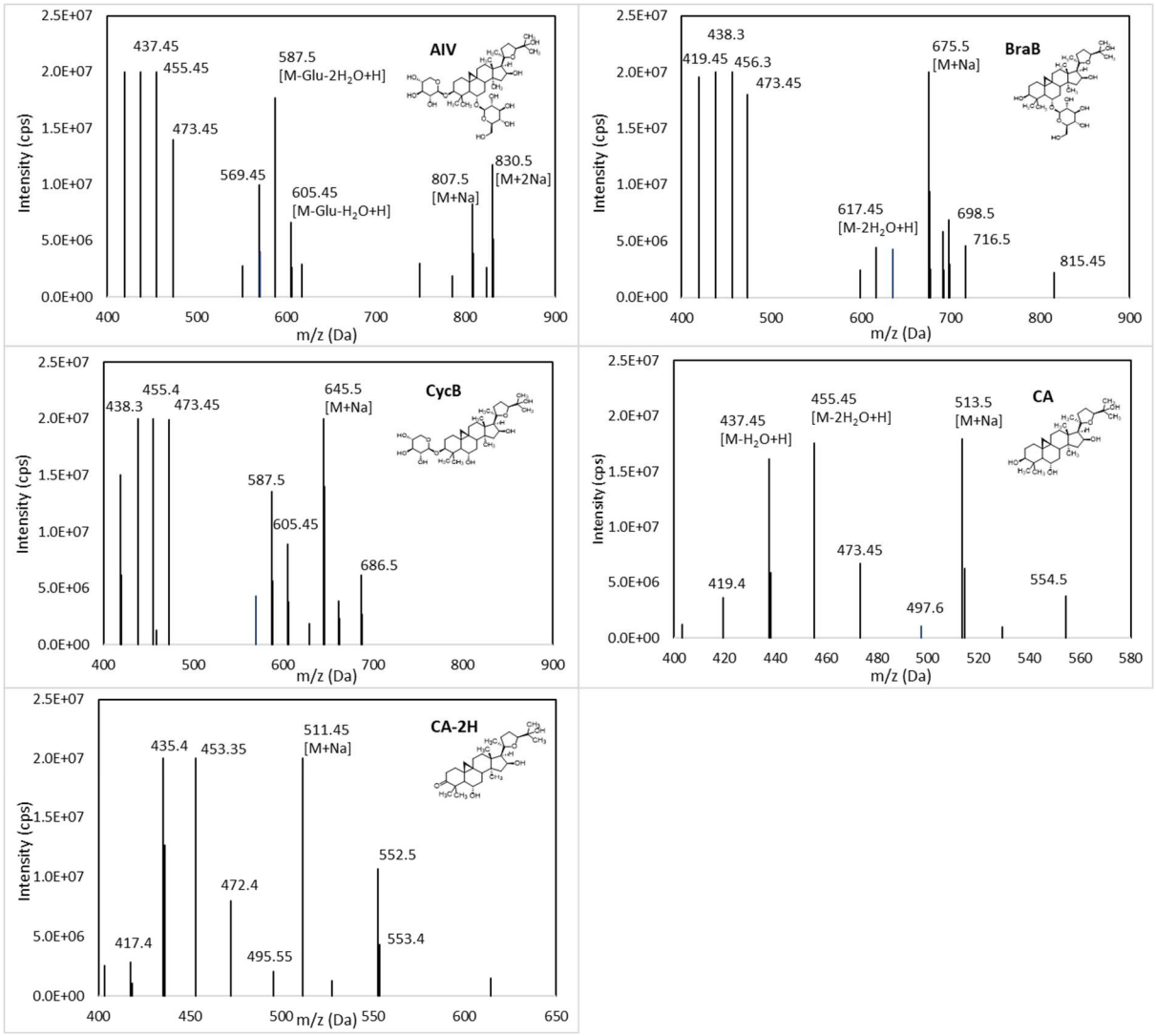

Figure S1

Chemical structures of AIV, BraB, CycB, CA, and CA-2H and their mass spectra

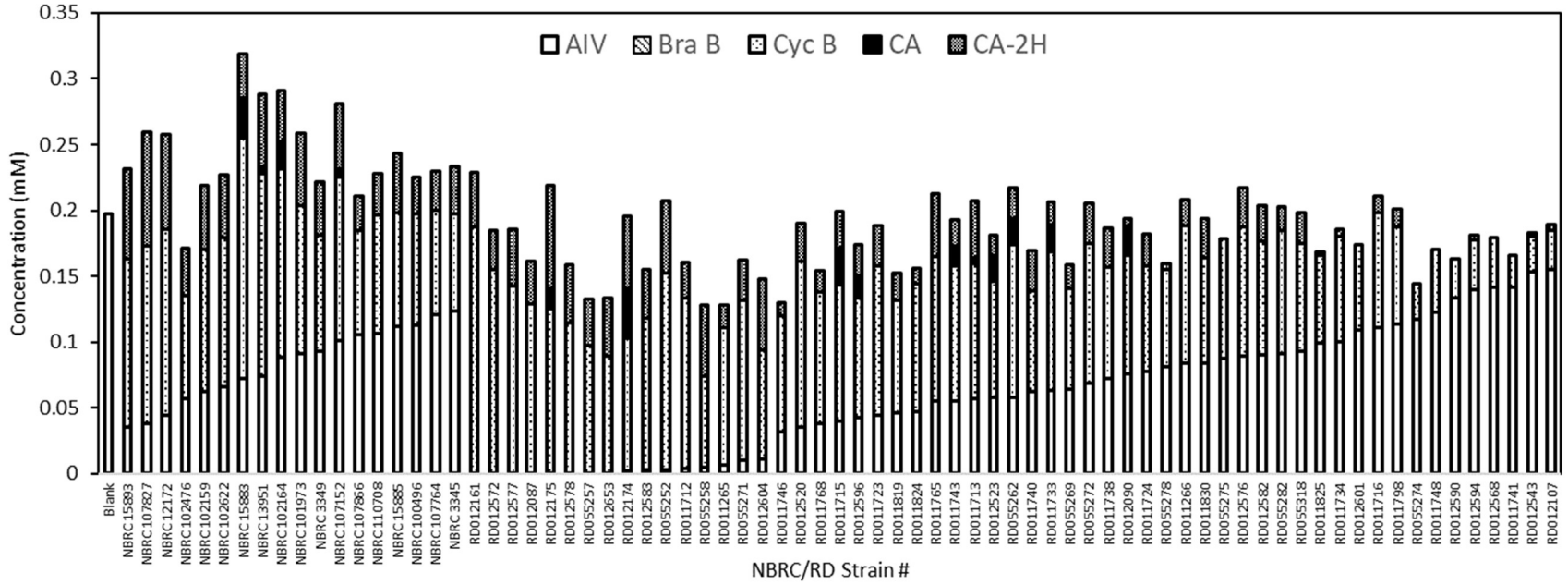

Figure S2 Additional screening results of LAB derived from fermented vegetables and fruits

Lactobacillus sakei subsp. *sakei* NBRC 15893, *Pediococcus argentinicus* NBRC 107827, *Tetragenococcus halophilus* NBRC 12172, *Leuconostoc citreum* NBRC 102476, *Lactobacillus mali* NBRC 102159, *Lactobacillus delbrueckii* subsp. *lactis* NBRC 102622, *Lactobacillus casei* NBRC 15883, *Lactobacillus acidophilus* NBRC 13951, *Lactobacillus acidipiscis* NBRC 102164, *Lactobacillus plantarum* NBRC 101973, *Leuconostoc mesenteroides* subsp. *dextranicum* NBRC 3349, *Lactobacillus paralimentarius* NBRC 107152, Leuconostoc lactis NBRC 107866, *Lactobacillus parabuchneri* NBRC 110708, *Lactobacillus fermentum* NBRC 15885, *Leuconostoc mesenteroides* subsp. *mesenteroides* NBRC 100496, *Lactobacillus buchneri* NBRC 107764, *Lactobacillus brevis* NBRC 3345

RD: un-identified LAB library for screening purpose

Table S1. The intestinal bacteria screening for metabolism of AIV

Microorganism name	JCM Number
Bacteroides dorei	13471
Bacteroides finegoldii	13345
Bacteroides fragilis	11019
Bacteroides intestinalis	13265
Bacteroides stercoris	9496
Bacteroides thetaiotaomicron	5827
Bacteroides uniformis	5828
Bacteroides vulgatus	5826
Bacteroides xylanisolvens	15633
Bifidobacterium adolescentis	1275
Bifidobacterium animalis subsp. *lactis*	10602
Bifidobacterium breve	1192
Bifidobacterium breve	1273
Bifidobacterium breve	7016
Bifidobacterium breve	7019
Bifidobacterium breve	7020
**Bifidobacterium breve*	-
Bifidobacterium longum subsp. *infantis*	1222
Bifidobacterium longum subsp. *longum*	1217
Bifidobacterium longum subsp. *longum*	7052
Bifidobacterium longum subsp. *longum*	7053
Bifidobacterium longum subsp. *longum*	7054
Bifidobacterium longum subsp. *longum*	7055
Bifidobacterium longum subsp. *longum*	7056
Bifidobacterium longum subsp. *longum*	7060
Bifidobacterium longum subsp. *longum*	11343
Bifidobacterium pseudocatenulatum	1200
Bifidobacterium pseudocatenulatum	7041
Bifidobacterium pseudocatenulatum	11661
Blautia hansenii	14655
Clostridium butyricum	7834
Clostridium difficile	1296
Clostridium difficile	5243
Clostridium indolis	1380
Clostridium nexile	31500
Collinsella aerofaciens	7790
Collinsella aerofaciens	10188
Dorea longicatena	11232
Eubacterium cylindroides	10261
Fusobacterium varium	6320

Microorganism name	JCM Number
Lactobacillus acidophilus	1028
Lactobacillus acidophilus	1034
Lactobacillus acidophilus	1132

Lactobacillus acidophilus	1229
Lactobacillus acidophilus	2124
Lactobacillus acidophilus	11047
Lactobacillus amylovorus	2125
Lactobacillus amylovorus	5811
Lactobacillus gallinarum	1036
Lactobacillus gallinarum	8782
Lactobacillus gallinarum	8783
Lactobacillus gallinarum	8784
Lactobacillus gallinarum	8785
Lactobacillus gallinarum	8786
Lactobacillus gasseri	1025
Lactobacillus gasseri	1130
Lactobacillus gasseri	1131
Lactobacillus johnsonii	1022
Lactobacillus johnsonii	2012
Lactobacillus johnsonii	8791
Lactobacillus johnsonii	8794
Lactobacillus paracasei subsp. *paracasei*	1109
Lactobacillus paracasei subsp. *paracasei*	1133
Lactobacillus reuteri	1112
Lactobacillus salivarius	1040
Lactobacillus salivarius	1042
Lactobacillus salivarius	1044
Lactobacillus salivarius	1045
Lactobacillus salivarius	1046
Lactobacillus salivarius	1231
Lactobacillus antri	15950
Lactobacillus crispatus	1185
Lactobacillus crispatus	8778
Lactobacillus farraginis	14108
Lactobacillus gallinarum	2011
Lactococcus lactis	1158
Parabacteroides johnsonii	13406
Phascolarctobacterium faecium	30894
Ruminococcus gnavus	6515
Ruminococcus productus	1471
Ruminococcus torques	6553

Microorganism name	JCM Number
Streptococcus salivarius	5707
Streptococcus thermophilus	17834
Streptococcus thermophilus	20026

* *Bifidobacterium breve* is not labeled with JCM number but is cataloged as *Bifidobacterium breve* UCC2003 or *Bifidobacterium breve* NCIMB 8807 in NCBI.

Discussion

The initial screening found specific bacterial strains from intestinal bacteria to study the metabolism of AIV and the production of CA and its intermediates. The metabolic pathway of AIV metabolism into BraB and CycB and eventually to CA and CA-2H were studied using a mixture of intestinal bacteria obtained from laboratory rats (Zhou *et al.* 2012). Screening for specific intestinal bacteria strains that metabolize AIV would allow a better understanding of intestinal bacteria's physiological role in human health. The initial 84 strains for screening came from a library of bacteria based on the data from Qin *et al.* 2010 to represent the general gut flora found in most humans (Table S1). Half strength GAM was used instead of the recommended concentration to balance bacterial growth, promote the bacteria to scavenge for carbon sources, and ultimately remove the sugar molecules off AIV to produce the target aglycone. Although the bacteria were grown in anaerobic chambers to ensure growth for the gut-derived microbes, the cell reaction metabolism of AIV reacted under non-anaerobic conditions. The sugar removal from AIV was assumed to be a hydrolysis reaction with no oxygen dependence. An initial experiment (no data present) ensured no significant differences in AIV metabolism during cell reaction under aerobic and anaerobic reactions. The high strokes per minute for the cell reaction was to get proper mixing of the substrate with cells due to AIV's low solubility.

Multiple bacteria strains indicated the metabolism of AIV (Table 1.1). Seven specific strains, *B. pseudocatenulatum* JCM 7041, *Bifidobacterium breve* JCM 7016, *Ruminococcus productus* JCM 1472, *Bifidobacterium longum* subsp. *infantis* JCM 1222, *Ruminococcus gnavus* JCM 6515, *Bifidobacterium uniformis* JCM 5828, and *Bifidobacterium breve* JCM 1273, indicated CA production when analyzed with HPLC and LCMS. The main goal of the initial screening using the intestinal bacteria was to find a trend among the genus or higher-order taxonomic classification of bacteria that can metabolize AIV. Various strains that metabolized AIV discovered in the gut microbe screen were derived from bifidobacteria. Bifidobacteria are known probiotics, and a follow-up screening looked for new strains to metabolize AIV. A secondary objective for the screening was to search for any bifidobacteria that could produce CycB instead of BraB during the transformation of AIV.

The second screening, including only bifidobacteria, indicated a relatively high frequency of strains metabolizing AIV (6/48). No CycB-producing bifidobacteria were

discovered in this screening, suggesting that bifidobacteria prefer removing the C-3 xylose from AIV. There is proof of different biotransformation of AIV using bacteria and enzymes, but both processes initially remove glucose to produce CA (Wang *et al*. 2017; Li *et al*. 2019). Nevertheless, the pharmacokinetic metabolism of AIV in rat intestines (Zhou *et al.* 2012) suggests that BraB is produced first before the removal of C-6 glucose in producing CA. Six new strains showed the metabolism of AIV from the bifidobacteria screening, and no strains showed detectable CA-2H production during the screening (Figure 1.2). This research demonstrates that bifidobacteria are a beneficial microorganism in producing CA in an environment similar to the intestine.

LAB is a representative gut microbe and a unique probiotic used in many industries. Although only one strain, *L. johnsonii* JCM 1022, showed the metabolism of AIV in the gut microbe screen, the strain showed the production of CycB and preferential removal of C-6 glucose instead of C-3 xylose in the metabolism of AIV. This metabolic pathway of AIV was different from that of bifidobacteria, and a third screening confirmed this metabolic pathway. The third screen using the purchased LAB strains showed new strains of LAB metabolized AIV to CycB (Figure 1.3). No strains indicated detectable production of BraB, and six of the eight strains showed the production of CA-2H. However, only four strains produced CA at a detectable level. To better understand the trend of LAB preferential production of CA-2H and the small production of CA, an additional 157 LAB strains obtained from fermented vegetables and fruits were screened. Seventy-four new strains metabolized AIV and produced CycB; nevertheless, only 14/74 strains showed detectable production of CA in the additional screen. Furthermore, LAB strains saw no BraB production in the additional screening of 157 strains. Unlike bifidobacteria, LAB prefers the removal of C-6 glucose over C-3 xylose in the metabolism of AIV. However, unlike bifidobacteria, LAB does not stably produce CA and prefers the production of CA-2H.

LAB screening results suggest the potential of LAB producing CA-2H without using CA as an intermediate, as suggested in Zhou *et al.* 2012. A time-course analysis performed on the three representative strains from the screens confirmed the metabolite production profile of these microbes over time. The time-course profile of *B. pseudocatenulatum* JCM 7041 showed bifidobacteria metabolize AIV quickly and produce BraB within 24 h. These results corroborate with that of Zhou *et al.* (2012), showing the production of BraB within the first 24 h when rat intestinal bacteria metabolize AIV. The increasing production of CA and CA-2H of

the *B. pseudocatenulatum* JCM 7041 suggests that the bacteria can eventually cleave the C-6 glucose molecule off the BraB intermediate, but at a much slower rate compared to xylose.

The LAB *S. citrovorum* NBRC 3397 and *P. pentosaceus* NBRC 3182 showed two different productions of CA-2H over 72 h. The time-course profile of *S. citrovorum* NBRC 3397 indicated an initial removal of the glucose sugar to produce the intermediate CycB and cleaving of the xylose to produce the CA and, eventually CA-2H. However, *P. pentosaceus* NBRC 3182 showed the production of CycB and CA-2H like *S. citrovorum* NBRC 3397, but throughout the entire period of the study, CA was undetectable. These findings suggest that certain LAB that produces CycB and CA-2H but not CA are using a different intermediate and possibly bypassing the CA production to produce CA-2H. This putative intermediate may be a novel compound undiscovered in the current literature about the metabolism of AIV. However, another hypothesis is that LAB produces CA through the deglycosylation of C-6 glucose and C-3 xylose, and the rate of converting CA to CA-2H exceeds the rate of C-3 xylose removal. Further investigation is needed to confirm the two hypotheses of the metabolic profile of LAB.

Lastly, LAB strains *S. citrovorum* NBRC 3397 and *P. pentosaceus* NBRC 3182 indicated delayed metabolism of AIV and production of CycB compared to the time-course of the bifidobacteria strain. Furthermore, these findings support the proof of Zhou *et al.* (2012), who found that CycB synthesis is delayed compared with BraB. The trend of screening bifidobacteria and LAB and its time-course analysis suggests that bifidobacteria and LAB metabolize AIV using two different pathways to produce CA. The individual metabolic profile of microbes from this study indicates that the decomposition of AIV in the gut environment is a collaborative effort of multiple strains indicating various rates of AIV-derived metabolite production. Although further experimentation is necessary to solidify evidence of collaborative metabolism of AIV in the intestinal system, two representative probiotics, bifidobacteria, and LAB, seem to be candidate strains in the metabolism of AIV into a more active compound in the human gut environment.

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Appendix A

Further analysis of CA-2H produced by LAB indicating a new intermediate derived from CycB

At the end of chapter 1, LAB showed a metabolic pathway not following the hypothesized metabolic pathway of AIV. Previous research had shown that AIV is metabolized by removing the C-3 xylose to produce the intermediate BraB, and C-6 glucose to produce another intermediate CycB. By then removing the complementary sugar moiety the aglycone CA is produced. Furthermore, by dehydrogenating CA, the compound CA-2H is formed. However, the results from the time course analysis of lactic acid bacteria *Pediococcus pentosaceus* NBRC 3182 suggested that LAB may be producing a CA-2H without producing any CA using a secondary intermediate CycB-2H(Figure A1). Further investigation into *P. pentosaceus* NBRC 3182 using LCMS and mass spectrometry scanning (CycB-2H) of the 72-hour sample from the time course profile showed a small peak with the mass spectrometry profile of CycB-2H (Figure A2).

These findings suggest that LAB metabolizes AIV through a putative intermediate not mentioned by other studies. However, this new intermediate CycB-2H raises another question. The dehydrogenated CA, CA-2H has been shown to have a structure with the C-3 hydroxyl group being converted into a carbonyl group. The CycB-2H intermediate cannot be dehydrogenated at the C-3 position due to the xylose moiety and can only be dehydrogenated at the C-6 and C-16 hydroxyl groups. If the CycB-2H is produced through a dehydrogenation process, the prediction that if the C-16 hydroxyl group is dehydrogenated an AIV-2H should exist in the sample arises. However, mass scanning showed no peaks of AIV-2H, suggesting that the C-16 hydroxyl group is not readily dehydrogenated. This suggests the CycB-2H is tentatively CycB with the C-6 hydroxyl group dehydrogenated to a carbonyl group. The subsequent removal of the C-3 xylose moiety would produce a compound with the same molecular weight as the CA-2H but with a different structure (Agricon-2H in Fig. A1).

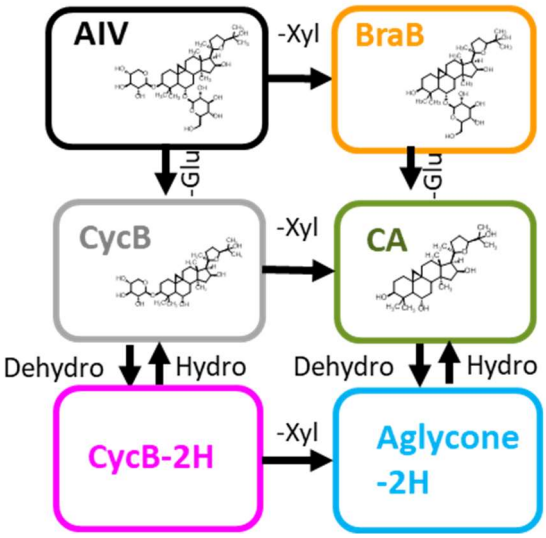

Figure A1 Hypothetical pathway of *Pediococcus pentosaceus* NBRC 3182

Abbreviations: Glu; glucose, Xyl; xylose, Dehydo; dehydrogenation, Hydro; hydrogenation.

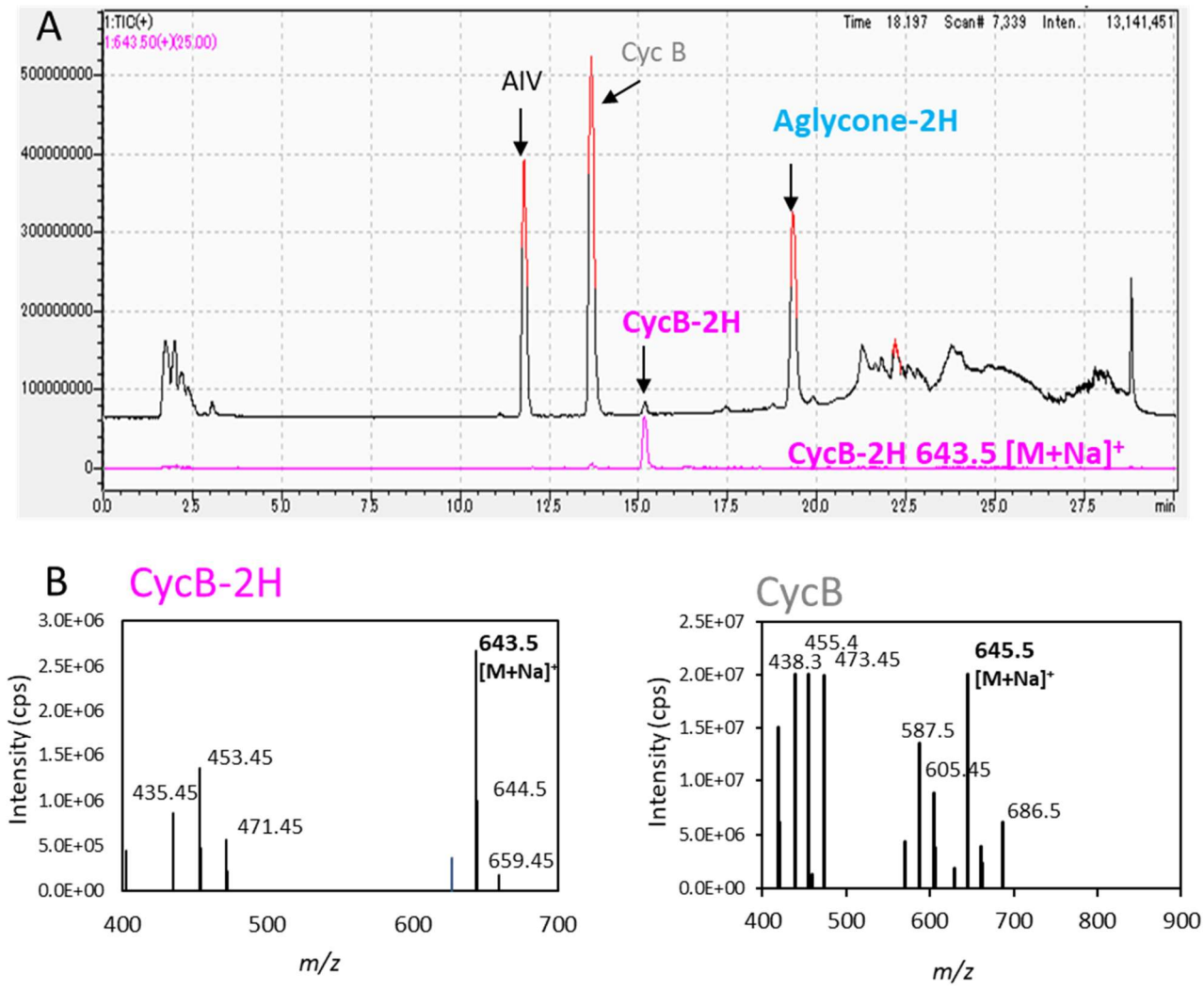

Figure A2 (A) LCMS chromatogram of *P. pentosaceus* NBRC 3182 at 72 hours. The black line is the TIC. The pink line is the ion chromatogram of *m/z* 643.5 at 25 times the standard intensity. (B) Mass spectrometry profile of CycB-2H and CycB.

Chapter 2

Efficient biotransformation of astragaloside IV to cycloastragenol through a two-step reaction catalyzed by washed cells of lactic acid bacteria and bifidobacteria

Abstract

Cycloastragenol (CA) is a telomerase activator with potential antiaging properties that are applicable to the food and medical industries. Efficient biotransforming microbes from representative gut microbe, lactic acid bacteria and bifidobacteria were selected through a screening process. Fermentative production using single strain with AIV-transforming activity to CA in the resting cell reaction showed C-6 xylose-removed intermediate, brachyoside B (Bra B), production but no CA production. Thus, a rationally designed two-step resting cell reaction, that combines the individual deglycosylation activity of lactic acid bacteria and bifidobacteria and removes C-6 glucose and C-3 xylose by respective strains from astragaloside IV (AIV) to produce CA, was investigated. Through this method, 0.25 mM of AIV was converted to 0.21 mM of CA with a yield of 82%. This study provides a feasible, effective method utilizing two microbial strains to produce CA from AIV.

Introduction

Several studies have shown CA production, including chemical conversion using the Smith degradation and mild acid hydrolysis (Feng *et al*. 2014), microbial conversion by *Bacillus* sp. (Wang *et al*. 2017), and enzymatic conversion using *β*-glucosidase and *β*-xylosidase (Cheng *et al*. 2020, Li *et al*. 2019). Herein, the author developed a microbial based production method to improve CA production. By combining the deglycosylating abilities of both lactic acid bacteria and bifidobacteria, a novel two step microbial biotransformation showing an efficient conversion of AIV to CA was established.

Materials and Methods

Preparation of AIV and CA solution

AIV (98% purity) and CA (98% purity) were purchased from Carbosynth (Berkshire, UK). AIV was reconstituted in dimethyl sulfoxide (DMSO) and sterile filtered through a 0.22 µm syringe filter for a stock solution (15 mg/mL) utilized in cell reactions screening of microorganisms. AIV and CA were reconstituted in methanol for a stock solution (1 mg/mL) use in HPLC analysis. Serially diluted solutions of 1 mg stock solution were used to produce concentration standards of AIV and CA when analyzing screening samples with HPLC.

Bifidobacteria and lactic acid bacteria screening with resting-cell reaction

All strains of bifidobacteria were purchased from Japan collection of microorganisms (JCM). LAB strains were purchased from Biological Resource Center, NITE (NBRC) and culture catalog. Each strain was inoculated and grown in 15 mL screw cap glass tubes with 15 mL of sterilized (115 °C for 15 minutes) ½ strength Gifu anaerobic media (GAM) broth (29.5 g/L) at 37°C and shaken at 130 strokes per minute for 24 hours under anaerobic condition. After cultivation, the cells were harvested by centrifugation at 1,500 g for 20 minutes. The cells were washed with 0.85 % NaCl and transferred to a 2 mL centrifuge tube and harvested by centrifugation at 20,000 g for 20 minutes. The harvested cells were reacted with 1 mL of 0.1 M solution of potassium phosphate buffer (KPB) (pH 6.5) with 13.3 µl of AIV stock solution for a final AIV concentration of 0.2 mg/mL. The resting-cell reaction was incubated at 37°C and shaken at 1,000 strokes per minute for 72 hours on bench top shaker Deep Well Maximizer (Taitec, Saitama, Japan). Samples were lyophilized and reconstituted in 1 mL of methanol for analysis.

HPLC analysis of metabolites

Quantitative analysis of CA and its intermediate components was performed by high-performance liquid chromatography (HPLC) by using a Shimadzu LC 10A system (Shimadzu, Kyoto, Japan) equipped with a UV detector, vacuum degasser, a binary pump, an autosampler, and a column oven. Samples were eluted on a 5C18-AR-II column (4.6 ID × 150 mm ; COSMOSIL). The column temperature was kept at 35 °C. 20 µl of filtered supernatant (0.45 µm) was injected into the HPLC. The mobile phase for gradient elution contained degassed Milli-Q Water (A) and acetonitrile (B). The gradient elution program was as follows; 30 % B, 0-1.5 min; 30-80 % B, 1.5-8 min; 80-100 % B, 8-8.01 min; 100 % B, 8.01-12.5 min; 100-80 % B, 12.5-12.51 min; 80-30 % B, 12.51-13.01 min; 30 % B, 13.01-18 min. Flow rate was set at

1.5 mL/min. The effluent was monitored with a UV detector at excitation wavelength of 203 nm.

HPLC-MS confirmation of metabolites

Before eluate was directed into the mass spectrometer, samples were separated using HPLC using a Shimadzu LC 20A system (Shimadzu, Kyoto, Japan) equipped with a vacuum degasser, a binary pump, an autosampler, and a column oven. Samples were eluted on a 2.5C18-MS-II column (3.0 ID × 100 mm ; COSMOSIL). The column temperature was kept at 35°C. 1 µl of filtered supernatant (0.45 µm) was injected into HPLC. The mobile phase for gradient elution contained 0.1 formic acid (A) and acetonitrile/0.1% formic acid (B). The gradient elution program was as follows; 30% B, 0-3.19 min; 30-80% B, 3.19-17 min; 80-100% B, 17-17.03 min; 100% B, 17.03-24.46 min; 100-80% B, 24.46-24.48 min; 80-30% B, 24.48-25.52 min; 30% B, 25.52-38.5 min. Flow rate was set at 0.2 mL/min. The effluent was monitored with a UV detector at excitation wavelength of 203 nm. Mass spectrometry was performed on a triple quadrupole mass spectrometer (LCMS-8045; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface. The instrument was operated in positive ion mode.

Fermentative transformation of AIV by LAB and bifidobacteria

All strains were grown in 15 mL screw cap glass tubes with 15 mL of sterilized (115 °C for 15 minutes) GAM broth (59 g/L) and (121 °C for 15 minutes) MRS broth (55 g/L) at 37°C and shaken at 130 strokes per minute for 14 hours. 150 µl of broth of the initial seed sample were transferred to another 15 mL sterilized sample of GAM and MRS broth and grown at 37°C and shaken at 130 strokes per minute for 3 weeks in anaerobic conditions. 1 mL culture broth was removed at 1-week intervals under anaerobic conditions and frozen (-80°C). Samples were lyophilized and reconstituted in 1 mL of methanol for analysis utilizing HPLC.

Extended fermentative transformation of AIV by LAB and bifidobacteria

All strains were grown in 15 mL screw cap glass tubes with 15 mL of sterilized (115 °C for 15 minutes) ½ strength GAM broth (29.5 g/L) at 37°C and shaken at 130 strokes per minute for 14 hours. 150 µl of broth of the initial seed sample were transferred to another 15 mL sterilized sample of ½ strength GAM and grown at 37°C and shaken at 130 strokes per minute for 4 weeks in anaerobic conditions. 1 mL culture broth was removed at a 2-week interval under anaerobic conditions and frozen (-80°C). Samples were lyophilized and reconstituted in 1 mL of methanol for analysis utilizing HPLC.

Simultaneous dual strain cell reaction for AIV conversion to CA

Bifidobacteria pseudocatenulatum JCM 7041 and two LAB candidate strains were grown in 15 mL screw cap glass tubes with 15 mL of sterilized (115 °C for 15 minutes) ½ strength GAM broth (29.5 g/L) at 37°C and shaken at 130 strokes per minute for 14 hours. 150 µl of broth of the initial seed sample were transferred to another 15 mL sterilized sample of ½ strength GAM and grown at 37°C and shaken at 130 strokes per minute for 24 hours in anaerobic conditions. The cells were washed with 0.85 % NaCl and transferred to a 2 mL centrifuge tube and harvested by centrifugation at 20,000 x g for 20 minutes. The harvested cells were reconstituted with 500 µl of 0.1 M solution of potassium phosphate buffer (KPB) (pH 6.5). The resting cells of one LAB strain were combined with the resting cells of one bifidobacteria strain to make a 1.0 mL dual strain cell reaction mixture. 13.3 µl of AIV stock solution for a final AIV concentration of 0.2 mg/mL was added to the cell reaction mixture. The resting-cell reaction was incubated at 37°C and shaken at 1,000 strokes per minute for 72 hours on a bench top. Samples were lyophilized and reconstituted in 1 mL of methanol for analysis.

Sequential resting cell reactions using LAB and bifidobacteria in one pot

Three candidate bifidobacteria strains were grown in 15 mL screw cap glass tubes with 15 mL of sterilized (115 °C for 15 minutes) ½ strength GAM broth (29.5 g/L) at 37°C and shaken at 130 strokes per minute for 14 hours. 150 µl of broth of the initial seed sample were transferred to another 15 mL sterilized sample of ½ strength GAM and grown at 37°C and shaken at 130 strokes per minute for 24 hours in anaerobic conditions. The cells were washed with 0.85 % NaCl and transferred to a 2 mL centrifuge tube and harvested by centrifugation at 20,000 x g for 20 minutes. The harvested cells were reconstituted with 700 µl of 0.1 M solution of potassium phosphate buffer (KPB) (pH 6.5) with 13.3 µl of AIV stock solution. The resting-cell reaction was incubated at 37°C and shaken at 1000 strokes per minute for 72 hours on a bench top. Using the same protocol as bifidobacteria, five candidate LAB strains were grown 38 hours, prior to bifidobacteria resting cell reaction reaching 72 hours. LAB cells were washed using above mentioned protocol and added with 500 µl of 0.1 M solution of potassium phosphate buffer (KPB) (pH 6.5) to 700 µl bifidobacteria cell reaction and re-incubated at 37°C and shaken at 1000 strokes per minute for an additional 72 hours on the bench top (total 144 hours). Samples were lyophilized and reconstituted in 1 mL of methanol for analysis.

The reverse sequence starting with LAB strain followed by bifidobacteria using the above protocol was also performed to acquire the LAB to bifidobacteria sequential dual strain resting cell reaction data.

Results

Screening of bifidobacteria and LAB

Bifidobacterium pseudocatenulatum JCM 7041 and *Pediococcus pentosaceus* NBRC 3182 were used as representable controls for bifidobacteria and LAB, respectively, from previous screening efforts. Through further screening of various bifidobacteria and LAB, multiple strains of both bifidobacteria and LAB were found that can transform all AIV added to the reaction mixture to some metabolites (Figure 2.1). *B. pseudocatenulatum* JCM 7041 and JCM 1200 metabolized AIV completely to CA and one of its intermediate BraB. *Bifidobacterium angulatum* JCM 7096 did not show complete metabolism of AIV, however, showed the next highest level of metabolism of AIV. *Weissella confusa* RD 12161 and *Weissella cibaria* RD 12578 showed complete metabolism of AIV to CycB and CA-2H with no detectable production of CA. *Leuconostoc carnosum* RD 12174, and *Lactobacillus sakei* RD 11715 showed high level metabolisms of AIV and productions of CycB. Both these strains also showed the production of CA and CA-2H.

Fermentative transformation of AIV by bifidobacteria and LAB in GAM and MRS media

Fermentative transformation of AIV was monitored in time course to observe if CA was produced during fermentation. *B. pseudocatenulatum* JCM 7041 during a 3-week fermentation period shows metabolism of AIV and production of BraB in GAM media (Figure 2.2). However, the metabolism of AIV dropped and the production of BraB significantly was less when *B. pseudocatenulatum* JCM 7041 was fermented in MRS media. Significant CA production by *B. pseudocatenulatum* JCM 7041 was not observed under both conditions. LAB strains showed no significant metabolism of AIV, and no intermediates and CA were detected over a 3-week fermentation time course in both GAM and MRS media (Figure 2.2).

Long term fermentation of bifidobacteria to produce CA

Using representative four bifidobacterial strains, AIV conversion to CA was monitored during extended fermentation periods (Figure 2.3). Although at a much slower rate compared to the cell reaction, bifidobacteria show the ability to metabolize AIV and production of BraB. *Bifidobacterium catenulatum* JCM 1194 and *B. angulatum* JCM 7096 showed production of CA during the 4-week fermentation period (Figure 2.3). Other than above mentioned bifidobacteria, *B. pseudocatenulatum* JCM 1200 and *Bifidobacterium adolescentis* JCM 1192 showed slight production of CA during long term fermentation as well (data not shown).

Simultaneous dual strain cell reaction for AIV conversion to CA

B. pseudocatenulatum JCM 7041 and two candidate LAB strains were applied to dual strain resting cell reactions (Figure 2.4). The dual combination of bifidobacteria and each LAB resulted in BraB accumulation but not CycB. The combination of the two bacterial types also showed no production of CA, suggesting that simply combing the two bacterial types in one reaction will not produce CA. The dual strain combination also showed no production of CA-2H that was seen with the LAB single cell reaction.

Sequential resting cell reaction using bifidobacteria and LAB in this sequence in one pot for AIV conversion to CA

Sequential resting cell reactions using firstly bifidobacteria and secondly LAB in one pot were carried out with three candidate bifidobacteria and five candidate LAB in different combinations (Figure 2.5). The sequential dual strain reaction resulted in the production of BraB intermediate and some production of CA. However, the reaction did not show any production of CycB and CA-2H. In this way, the addition of LAB to the existing bifidobacteria cell reaction showed the same results as the single bifidobacteria strains. In the first stage reaction, *B. pseudocatenulatum* JCM 7041 and *B. angulatum* JCM 7096 showed near complete AIV conversion to BraB and CA, with BraB being the most product. In the second stage reaction, the addition of LAB to the bifidobacteria cell reaction after 72 hours showed no additional production of CA in 72 h reaction. In the case of *B. pseudocatenulatum* JCM 1200 followed by LAB, AIV was converted to BraB in lower yield and CA was not produced. Overall, the addition of LAB to bifidobacteria showed no significant difference compared to single cell reactions of bifidobacteria.

Sequential resting cell reaction using LAB and bifidobacteria in this sequence in one pot for AIV conversion to CA

Sequential resting cell reactions using firstly LAB and secondly bifidobacteria in one pot were carried out with five candidate LAB and three candidate bifidobacteria in different combinations (Figure 2.6). The dual resting cell reaction with *W. cibaria* RD 12578 and *B. pseudocatenulatum* JCM 7041 resulted in the highest CA production of 0.21 mM concentration with 82% yield to 0.25 mM AIV as substrate.

These results along with the other results show significant production of CA by sequentially combining the resting cell reaction of LAB and bifidobacteria. Although the highest production of CA was observed with the combination of the *W. cibaria* RD 12578 and *B. pseudocatenulatum* JCM 7041, *W.confusa* RD12161 showed consistent production of CA in all three bifidobacteria combinations. There are variations among the combination of LAB

and bifidobacteria. However, throughout the experiments, the production of CycB was not seen. These results suggest that bifidobacteria have the activity to remove C-3 xylose moiety of the intermediate CycB. Also, the combination of the LAB to bifidobacteria showed high production of CA, and the production of CA-2H was relatively inhibited, suggesting that bifidobacteria have some inhibiting effects of LAB catalytic activity.

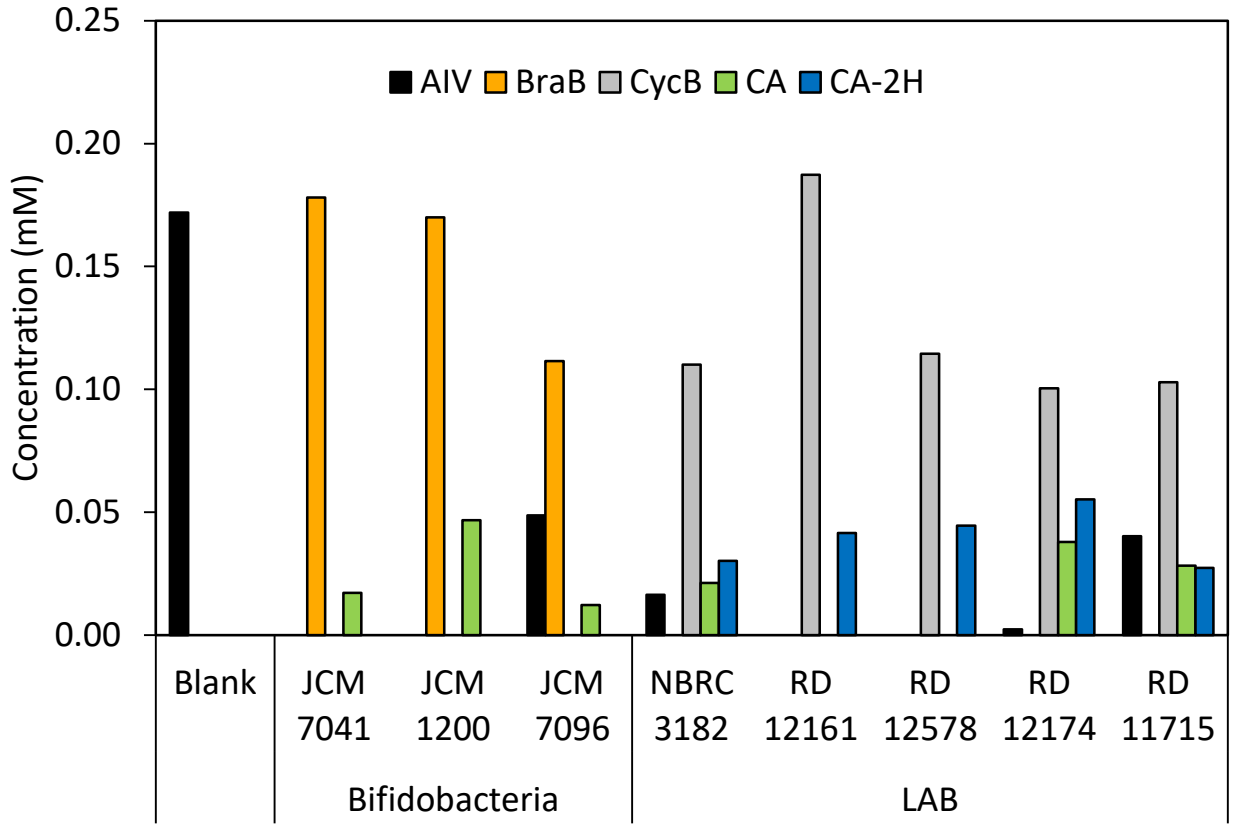

Figure 2.1 Screening of AIV-metabolizing Bifidobacteria and LAB

List of microorganisms; Bifidobacterium pseudocatenulatum JCM 7041, *Bifidobacterium pseudocatenulatum* JCM 1200, *Bifidobacterium angulatum* JCM 7096, *Pediococcus pentosaceus* NBRC 3182, *Weissella confusa* RD 12161, *Weissella cibaria* RD 12578, *Leuconostoc carnosum* RD 12174, *Lactobacillus sakei* RD 11715

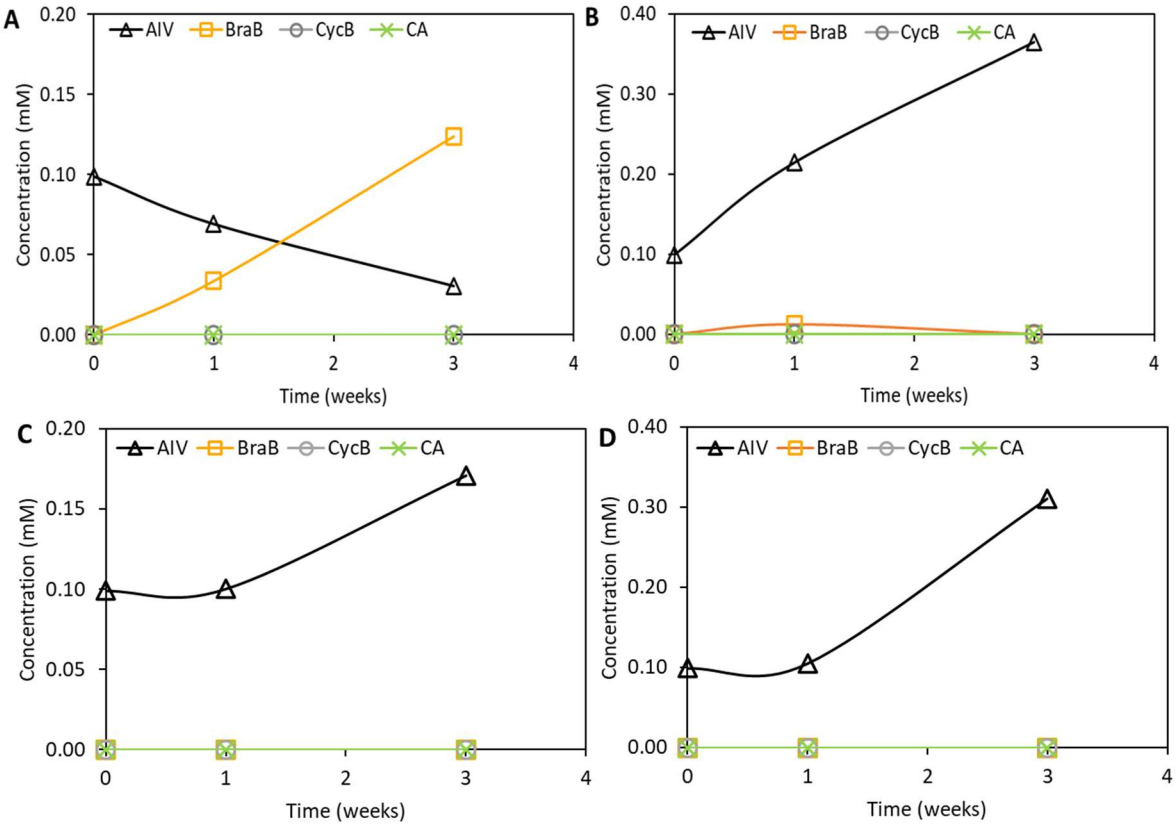

Figure 2.2 Fermentative AIV-transformation by Bifidobacteria and LAB in GAM and MRS media

(A) and (B) show *Bifidobacterium pseudocatenulatum* JCM7041 fermentation profile (3 weeks) in GAM and MRS, respectively. (C) and (D) show *Pediococcus pentosaceus* NBRC 3182 fermentation profile (3 weeks) in GAM and MRS, respectively.

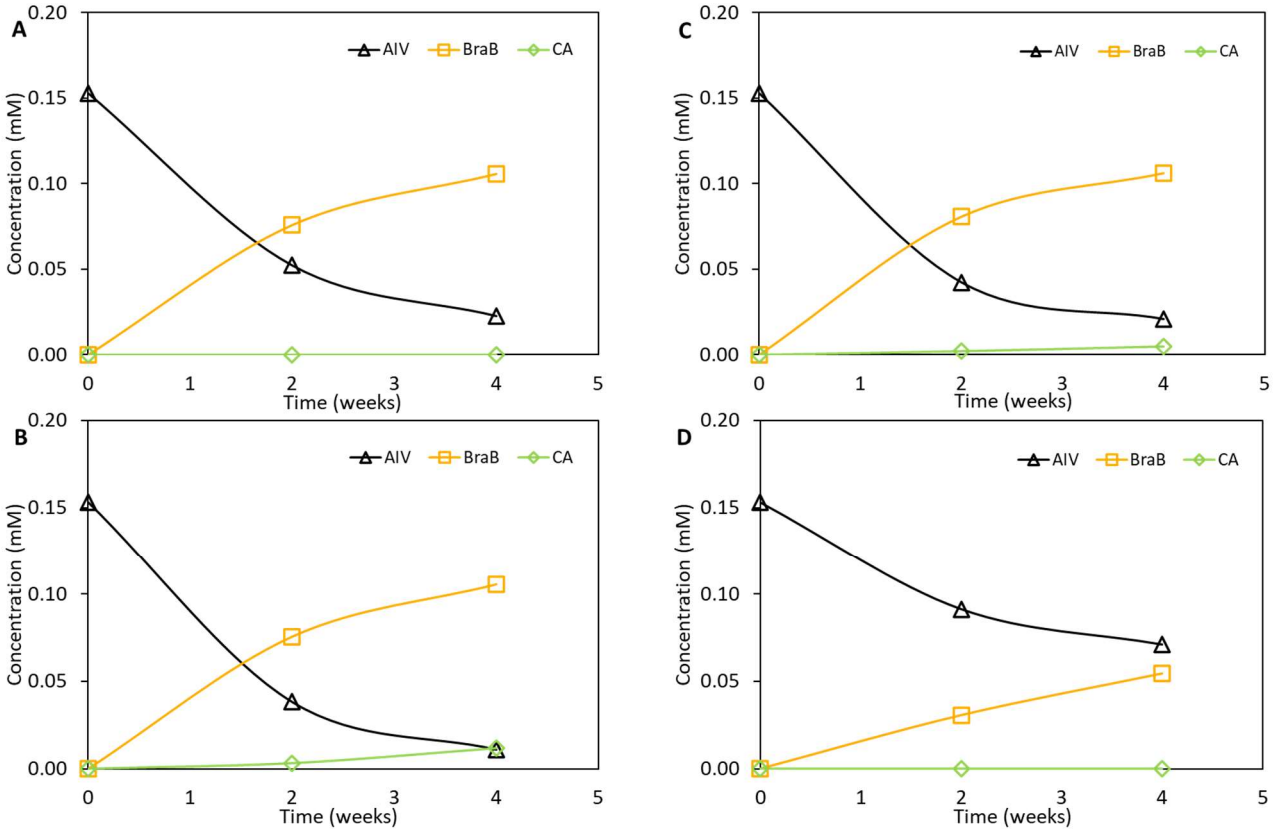

Figure 2.3 Long term fermentative AIV-transformation by bifidobacteria in ½ GAM medium

Time-course profile of AIV transformation over a 4-week period. (A) *Bifidobacterium pseudocatenulatum* JCM 7041, (B) *Bifidobacterium catenulatum* JCM 1194, (C) *Bifidobacterium angulatum* JCM 7096, (D) *Bifidobacterium adolescentis* JCM 7046

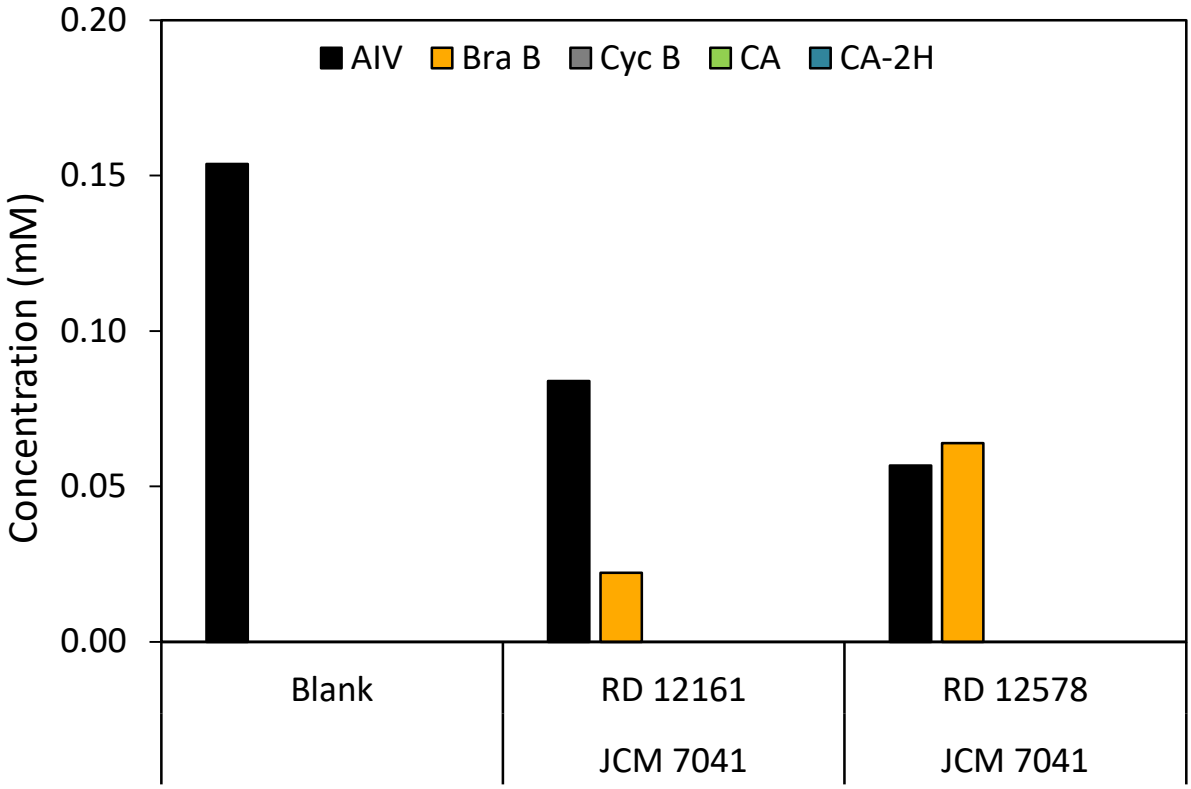

Figure 2.4 Simultaneous dual strain resting cell reaction for AIV transformation

The combined resting cell reactions of *B. pseudocatenulatum* JCM 7041 with *Weissella confuse* RD 12161 or *Weissella cibaria* RD 12578.

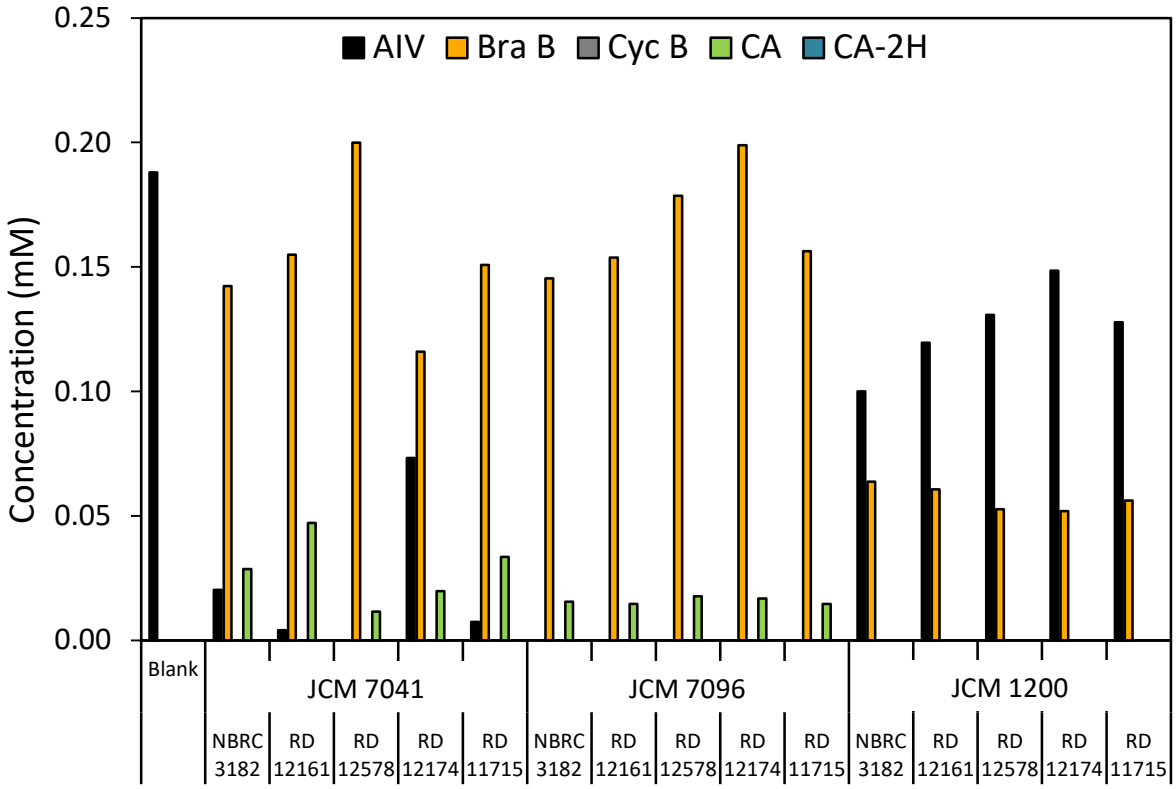

Figure 2.5 Sequential resting cell reactions using firstly bifidobacteria and secondly LAB in one pot

The sequential resting cell reactions for AIV transformation were carried out firstly with bifidobacteria for 72 hours and secondly with LAB strains for an additional reaction time of 72 hours (total 144 hour). List of strains; *B. pseudocatenulatum* JCM 7041, *B. angulatum* JCM 7096, *B. pseudocatenulatum* JCM 1200, *P. pentosaceus* NBRC 3182, , *W. confuse* RD 12161, *W. cibaria* RD 12578, *L. carnosum* RD 12174, and *L. sakei* RD 11715.

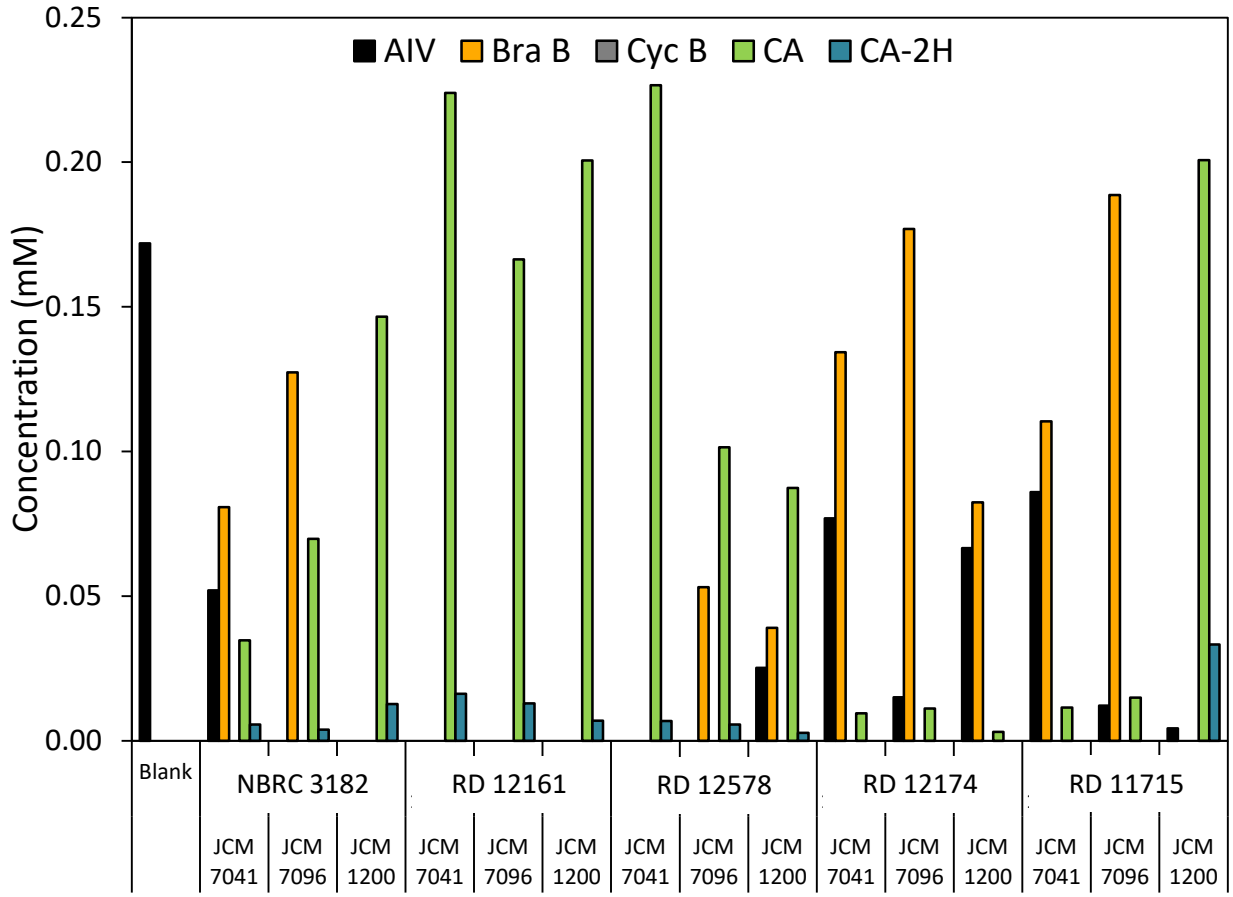

Figure 2.6 Sequential resting cell reactions using firstly LAB and secondly bifidobacteria in one pot

The sequential resting cell reactions for AIV transformation were carried out firstly with LAB for 72 hours and secondly with bifidobacteria strains for an additional reaction time of 72 hours (total 144 hours). List of strains; *P. pentosaceus* NBRC 3182, *W. confuse* RD 12161, *W. cibaria* RD 12578, *L. carnosum* RD 12174, *L. sakei* RD 11715, *B. pseudocatenulatum* JCM 7041, *B. angulatum* JCM 7096, and *B. pseudocatenulatum* JCM 1200.

Discussion

To find optimal microbial candidates for producing CA from AIV, multiple screens were performed to find high CA yielding microbes from bifidobacteria and LAB. As shown in Figure 2.1 several candidates from bifidobacteria and LAB were discovered. The fermentative transformation of AIV was tested for possible industrial production of CA with a small-scale anaerobic fermentation using LAB and bifidobacteria (Figure 2.2). Bifidobacteria showed the potential to metabolize AIV during in vivo fermentation after a 3-week fermentation, however, the LAB strains showed no AIV transformation during fermentation. *B. pseudocatenulatum* JCM 7041 exhibited high AIV metabolizing ability when grown in GAM media. However, when *B. pseudocatenulatum* JCM 7041 was fermented in MRS media containing AIV, little metabolism of AIV was observed. This difference is most likely due to the lower total carbohydrate concentration of GAM media.

In general, MRS media is optimized for LAB growth but during fermentation and the high level of carbohydrate availability in the media may be a factor for not showing metabolization of AIV in fermentation. However, LAB also did not metabolize AIV in the GAM media. The resting cell reactions showed the capability of LAB to metabolize AIV. Thus, further media optimization for LAB fermentation is needed to determine conditions for LAB to metabolize AIV during fermentation.

With bifidobacteria showing metabolism of AIV in fermentation, extended fermentations were tested in Figure 2.3 in half strength GAM with lower amounts of carbohydrates promoting quicker metabolism of the sugar moieties of AIV. Multiple strains of bifidobacteria including *Bifidobacterium pseudocatenulatum* JCM 7041, *Bifidobacterium catenulatum* JCM 1194, *Bifidobacterium angulatum* JCM 7096 showed near complete metabolization of AIV during a 4-week fermentation. Two stains *Bifidobacterium catenulatum* JCM 1194 and *Bifidobacterium angulatum* JCM 7096 showed CA production after the second week and onto the fourth week of fermentation, but the production amount was low, and most of the product remained in the intermediate BraB form after AIV was metabolized. Efficient production with single strain fermentation showed to be difficult with traditional fermentation methods.

It has been hypothesized in chapter 1, that the bifidobacteria and LAB take two different metabolic pathways to metabolize AIV. By combining the metabolic pathways, the author hypothesized that an efficient production method can be developed using multiple strains with

different metabolisms of AIV. The initial results using a simultaneous resting cell reaction using both bifidobacteria and LAB (Figure 2.4) showed that simultaneous resting cell reaction seems to favor bifidobacteria because only BraB intermediate generated by bifidobacteria was detected.

To test if either microbe can metabolize the intermediate CycB and BraB to the desired CA product, sequential resting cell reactions utilizing the two microbial types were tested (Figure 2.5 and 2.6). Although the LAB strains did not show the ability to convert BraB to CA, the bifidobacteria strains displayed an activity to convert CycB to CA with little CA-2H produced during the cell reaction. The dual strain resting cell reaction involving *W. cibaria* RD 12578 and *B. pseudocatenulatum* JCM 7041 showed the highest level of production of CA. The dual strain reaction involving *W. confusa* RD 12161 and all three bifidobacteria showed similar levels of production and conversion rate with the above-mentioned values. Furthermore, comparing the single cell reaction using LAB or bifidobacteria, the sequential dual strain resting cell reaction of bifidobacteria added to the LAB showed a significantly higher level of AIV metabolism and CA production. These results suggest a possible synergistic effect with LAB and bifidobacteria in the metabolism of AIV. In humans' digestive system, it is well known that LAB is readily more dominant in the small intestine (Walters 2008) and bifidobacteria are more abundant in the large intestine(Arumugam *et al.*). LAB likely metabolizes AIV into CycB intermediate in the small intestine and bifidobacteria sequentially metabolizes the CycB readily into CA in the gut environment. Utilizing a similar method found in nature and applying this synergetic effect of the LAB and bifidobacteria, a highly efficient production method may be developed in the future. Although conventional methods can be utilized for the production of CA, using natural probiotics such as LAB and bifidobacteria already found in our guts, a safe and target approach to producing CA could be industrially developed. Furthermore, by augmenting traditional Chinese medicine with microbes, a novel application scheme in the digestive tract can be developed for a more efficient way to consume herbal medicine.

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Chapter 3

Purification of astragaloside IV-hydrolyzing *β*-D-xylosidase from *Bifidobacterium pseudocatenulatum* JCM 7041

Abstract

Bifidobacterium pseudocatenulatum JCM 7041 was shown to possess the ability to readily remove the C-3 xylose from AIV. The catalytic activity was highest with the cells harvested at 48 hours of growth. The enzyme catalyzing the reaction was found to be a soluble protein, and ammonium sulfate precipitation resulted in the activity collection in the fraction obtained with 30-55 % ammonium sulfate saturation. During column chromatography purification, elution profiles of xylosidase activity detected with 4-nitrophenyl-*β*-D-xylopyranoside as the substrate well corresponded to those detected with BraB-producing activity from AIV analyzed by HPLC. SDS-PAGE analysis of the purified protein after three steps of purification through the ammonium sulfate precipitation and two column chromatography steps suggested that a protein band with M_r of roughly 80,000 was the target enzyme protein. LCMS analysis of peptide fragments of the candidate protein revealed a high score match of the amino acid sequences of the peptides with those of an uncharacterized protein derived from *Bifidobacterium pseudocatenulatum*.

Introduction

Related to the biotransformation of AIV, Wang *et al.* (2017) isolated *Bacillus* sp. from the soil and characterized the glucosidase and xylosidase activities but the enzymes were not purified. Cheng *et al.* (2020) and Li *et al.* (2019) both purified *β*-glucosidase and *β*-xylosidase from filamentous fungi and thermophilic bacteria, respectively. However, the purification and characterization of *β*-D-xylosidase (EC 3.2.1.37) from intestinal bacteria related to the metabolism of AIV has not been studied. Therefore, the author attempted to purify and characterize AIV hydrolyzing *β*-D-xylosidase from *Bifidobacterium pseudocatenulatum* JCM 7041, which was chosen as an AIV metabolizing strain derived from human gut flora.

Materials and Method

Preparation of AIV and CA solution

AIV (98% purity) and CA (98% purity) were purchased from Carbosynth (Berkshire, UK). AIV was reconstituted in dimethyl sulfoxide (DMSO) and sterile filtered through a 0.22 μm syringe filter for a stock solution (15 mg/mL) utilized in cell reactions screening of microorganisms. AIV and CA were reconstituted in methanol for a stock solution (1 mg/mL) use in HPLC analysis. Serial diluted solutions of 1 mg stock solution were used to produce concentration standards of AIV and CA when analyzing screening samples with HPLC.

Cultivation and resting-cell reaction using *Bifidobacterium pseudocatenulatum* JCM 7041

Bifidobacterium pseudocatenulatum JCM 7041 was purchased from Japan collection of microorganisms (JCM). 50 μL of *B. pseudocatenulatum* JCM 7041 was inoculated from a frozen glycerol stock and grow in 15 mL screw cap glass tubes with 15 mL of sterilized (115 °C for 15 minutes) Gifu anaerobic media (GAM) broth (59 g/L) at 37°C and shaken at 130 strokes per minute for 18 hours under anaerobic condition. The seed culture was transferred to sterilized ½ strength GAM media (29.5 g/L) in a 1 liter screw cap bottle and incubated at 37°C with agitation using a magnetic stirrer to mix media during cultivation at low speeds. After cultivation, the cells were harvested by centrifugation at 8,000 *g* for 20 minutes. The cells were washed with 0.85 % NaCl and transferred to a 50 mL centrifuge tube and harvested by centrifugation at 15,000 *g* for 20 minutes. The harvested cells were reconstituted with 10 mM solution of potassium phosphate buffer (KPB) (pH 6.5) at a ratio of 2 mL of buffer for every gram of wet cell weight. The resting cell reaction was performed with 500 μL of the above mixture with 13.3 μl of AIV stock solution for a final AIV concentration of 0.4 mg/mL. The resting-cell reaction was incubated at 37°C and shaken at 1000 strokes per minute for 4 hours on bench top shaker Deep Well Maximizer (Taitec, Saitama, Japan). Samples were extracted with 500 μL of methanol and centrifuged for analysis.

Bifidobacterium pseudocatenulatum JCM 7041 cells were lysed using 6 ultra-sonication cycles using Insonator 201M (Kubota, Tokyo, Japan) for 5 min with mixing between each cycle at 0°C. The supernatant after centrifugation at 8,000 g for 20 minutes was collected as cell free extract (CFE) solution. The CFE was then centrifuged at 100,000 g for 60 minutes to separate the CFE in ultracentrifuged supernatant (US/crude extract) and pellet (UP).

HPLC analysis of metabolites

Quantitative analysis of CA and its intermediate components was performed by high-performance liquid chromatography (HPLC) by using a Shimadzu LC 10A system (Shimadzu, Kyoto, Japan) equipped with a UV detector, vacuum degasser, a binary pump, an autosampler, and a column oven. Samples were eluted on a $5C_{18}$-AR-II column (4.6 ID × 150 mm ; COSMOSIL). The column temperature was kept at 35°C. 20 µl of filtered supernatant (0.45 µm) was injected into to the HPLC. The mobile phase for gradient elution contained degassed Milli-Q Water (A) and acetonitrile (B). The gradient elution program was as follows; 30 % B, 0-1.5 min; 30-80 % B, 1.5-8 min; 80-100 % B, 8-8.01 min; 100 % B, 8.01-12.5 min; 100-80 % B, 12.5-12.51 min; 80-30 % B, 12.51-13.01 min; 30 % B, 13.01-18 min. Flow rate was set at 1.5 mL/min. The effluent was monitored with a UV detector at excitation wavelength of 203 nm.

Ammonium sulfate precipitation and ultrafiltration of crude extract

The crude extract was precipitated by increasing ammonium sulfate concentrations and the precipitates were redissolved in buffer (10mM HEPES buffer (pH 7.0). 10 mL of the resulting protein solution was centrifuged using a 30K MWCO concentration centrifuge tube 4 times to refine and clean the sample before further processing.

***p*-Nitrophenyl *β*-D-xylopyranoside to track AIV metabolic activity**

The *p*-Nitrophenol standard was produced by serial dilution of 0.1 M stock solution (139 mg in 10.0 mL of water). Standard concentration was as follows, 0.1 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM. The reaction mixture contained in 90 µL was added with 10 µL of substrate solution (40 mM *p*-nitrophenyl xylopyranoside). After incubation at 37 °C for 30 min, the reaction was stopped by adding 150 µL of 1 M Na_2CO_3. A standard curve was prepared by using the *p*-nitrophenol mentioned above. A_{405} was read using MULTISKAN sky (Thermo fisher scientific, MA, US) and 96 well plate with 200 µL of reaction mixtures added to each well.

Protein measurement

Protein quantification was performed by the Bradford method, using a bovine serum albumin as a standard.

Purification of *β*-D-xylosidase from *B. pseudocatenulatum* JCM 7041

The 30-55% ammonium sulfate precipitation fraction was applied on Hi-Trap Butyl - Sepharose column previously equilibrated with Buffer A (10 mM HEPES buffer (pH 7.0)

containing 1.5 M ammonium sulfate). The column was washed with the same buffer and the linear gradient elution was undertaken with Buffer B (10 mM HEPES buffer). All fractions obtained were assayed for *β*-D-xylosidase activity. The active fractions were analyzed using SDS-PAGE followed by Coomassie brilliant blue staining. The active fractions were collected, and the same volume of Buffer B was added. The highest active fraction was put on a Capto HiRes ion exchange column previously equilibrated with Buffer A (10 mM HEPES buffer (pH 7.0)). The column was washed with the same buffer and the linear gradient elution was undertaken with Buffer B containing 1.0 M NaCl Buffer A. Fractions obtained were assayed for *β*-D-xylosidase activity. The active fractions were concentrated, and SDS-PAGE analysis was performed using silver staining. The suspected band was applied to the LCMS peptide analysis.

Results

Evaluating enzymatic activity of *Bifidobacterium pseudocatenulatum* JCM 7041

The AIV metabolism of *Bifidobacterium pseudocatenulatum* JCM 7041 was monitored by following the BraB production to determine the optimal cultivation time for *B. pseudocatenulatum* JCM 7041 for enzyme purification (Figure 3.1). The resting cells obtained from the culture broth of 48 hours cultivation showed the highest production of BraB. Cell lysis through ultrasonication and ultra-centrifugation was performed on *B. pseudocatenulatum* JCM 7041 (Figure 3.2). The high BraB production found in the reactions with the cell free extract (CFE) suggests that the cell lysis using ultrasonication was successful and most of the enzymatic activity was conserved in CFE. The subsequent ultracentrifugation of the CFE solution showed the activity was conserved in the supernatant (US) and not in the pellet (UP). This suggests that the enzymatic activity is not membrane bound and primarily located intracellularly.

Ammonium sulfate precipitation and ultrafiltration of crude enzyme solution

The crude extract (US) of *B. pseudocatenulatum* JCM 7041 was purified using ammonium sulfate precipitation (Figure 3.3). Much of the activity was conserved in the ammonium sulfate precipitation fraction (ASP) with 30-45% saturation of ammonium sulfate.

Using *p*-nitrophenyl *β*-D-xylopyranoside as the substrate to characterize *B. pseudocatenulatum* JCM 7041 *β*-D-xylosidase activity.

p-Nitrophenyl *β*-D-xylopyranoside was utilized as the substrate to monitor the enzymatic activity of *B. pseudocatenulatum* JCM 7041 (Figure 3.4). The highest activity was found in ASP fraction 30-55% in both HPLC data monitoring the AIV metabolism and BraB production and in the colorimetric data using the *p*-nitrophenyl *β*-D-xylopyranoside. Thus, the colorimetric assay using the *p*-nitrophenyl *β*-D-xylopyranoside showed a high correlation when compared with BraB production using HPLC analysis.

Purification of astragaloside IV-hydrolyzing *β*-D-xylosidase from *Bifidobacterium pseudocatenulatum* JCM 7041

The ammonium sulfate precipitated 30-55% fraction was separated using hydrophobic interaction chromatography (Figure 3.5). Most of the activity was centered around fractions 22-25. Fraction 24 was then processed and was further purified using ion exchange chromatography (Figure 3.6). The highest activity was seen in fractions 30 and 31. Correlating

the SDS-PAGE analysis and *p*-nitrophenyl *β*-D-xylopyranoside assay results, the target protein band was hypothesized to have a M_r of 80,000. The candidate protein was purified 106-fold from a crude extract with a yield of 0.22% by the procedures shown in Table 3.1. The LCMS peptide analysis result of the candidate protein is shown in Table 3.2. The most likely candidate protein was 4-alpha-glucanotransferase derived from *Bifidobacterium pseudocatenulatum*. The nucleotide sequence and amino acid sequence of 4-alpha-glucanotransferase derived from *B. pseudocatenulatum* JCM7041 are shown in Table 3.3 and Table 3.4, respectively.

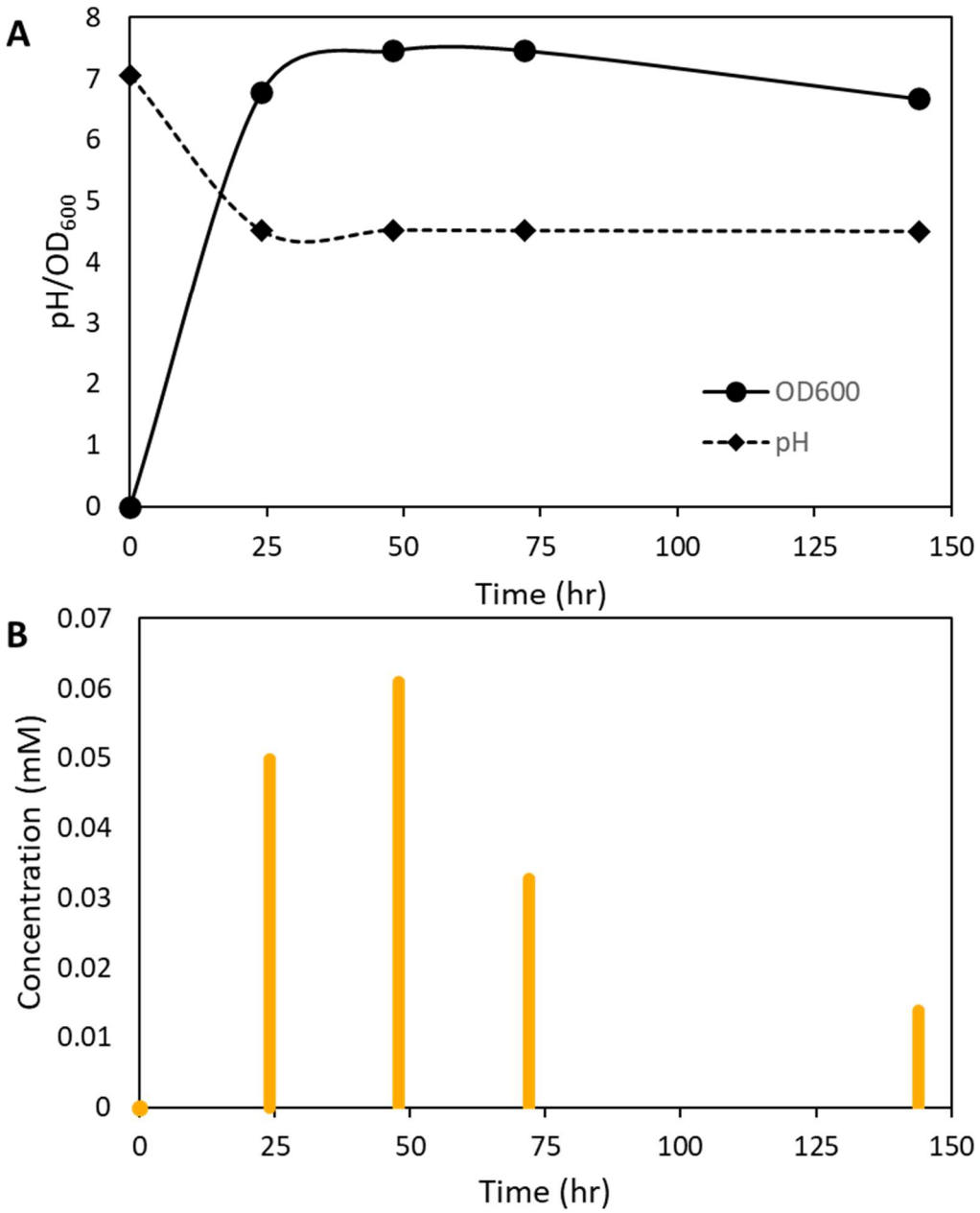

Figure 3.1

Evaluating enzymatic activity of *B. pseudocatenulatum* JCM 7041. (A) OD_{600} and pH time course profile of *B. pseudocatenulatum* JCM 7041. (B) BraB production by the resting cells taken during the cultivation time course.

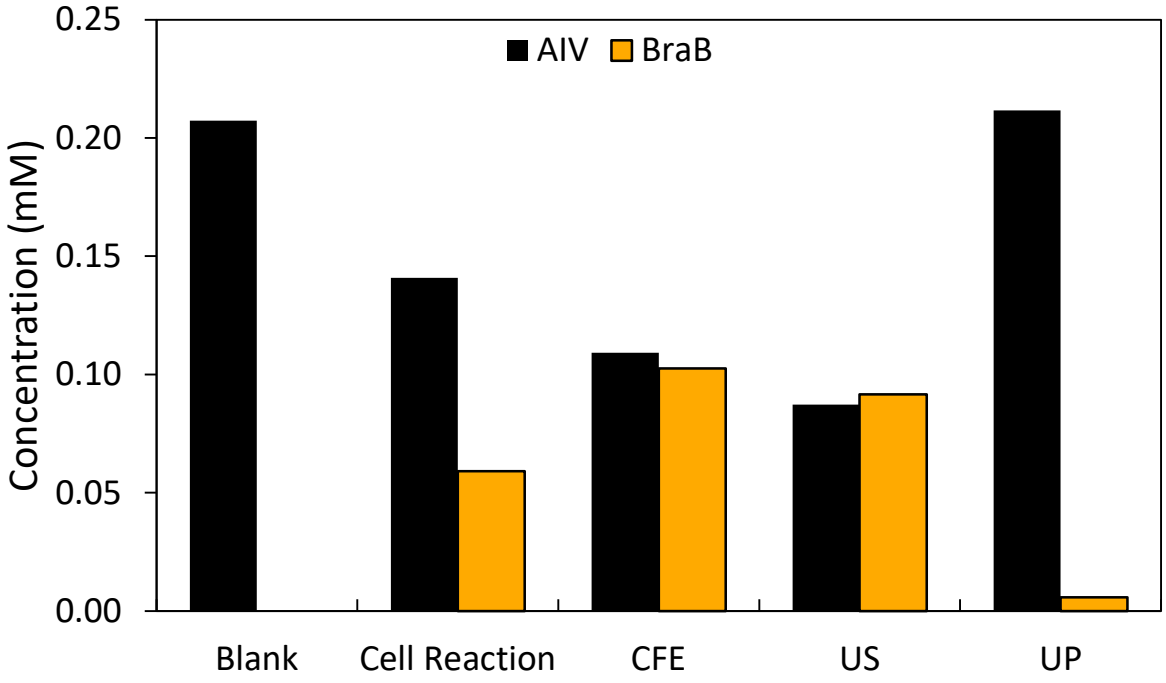

Figure 3.2 BraB production by cells of *B. pseudocatenulatum* JCM 7041, and those by CFE, US, and UP derived from *B. pseudocatenulatum* JCM 7041

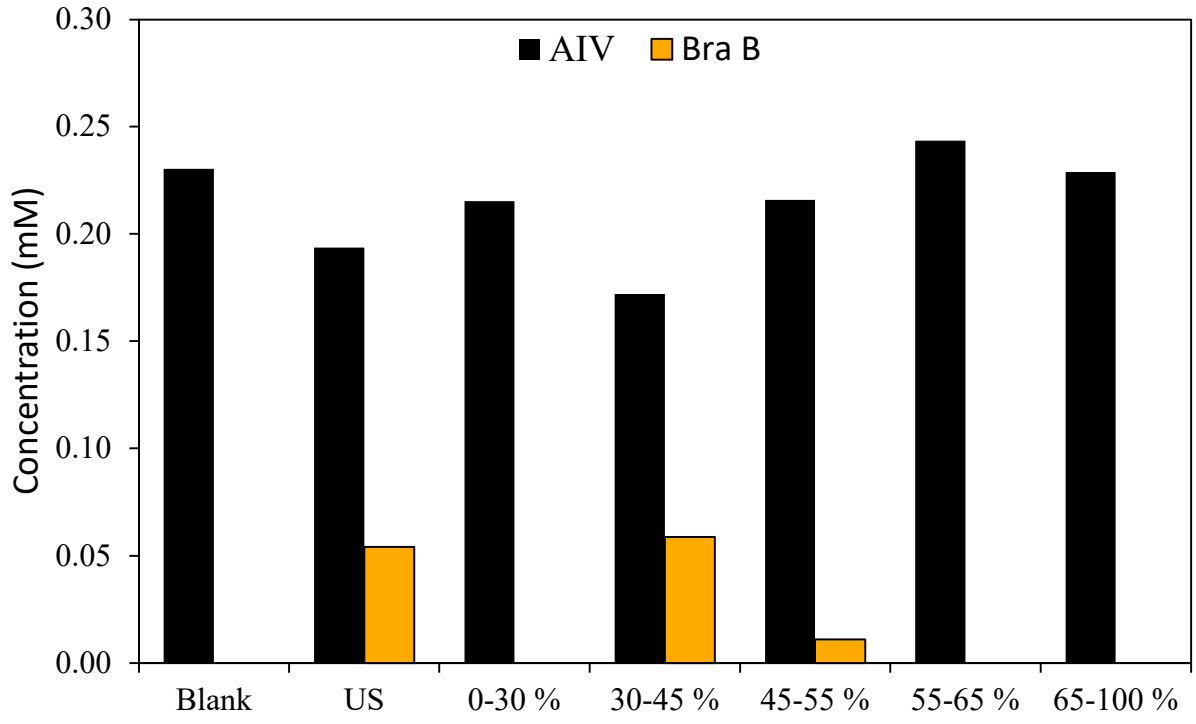

Figure 3.3 BraB production of ammonium sulfate precipitation fractions

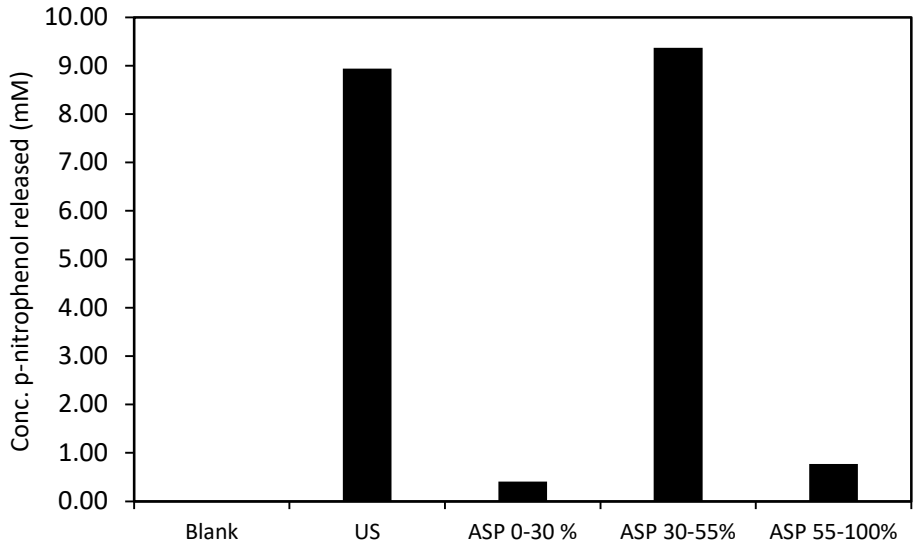

Figure 3.4 Xylosidase activity assay with *p*-nitrophenyl *β*-D-xylopyranoside as the substrate

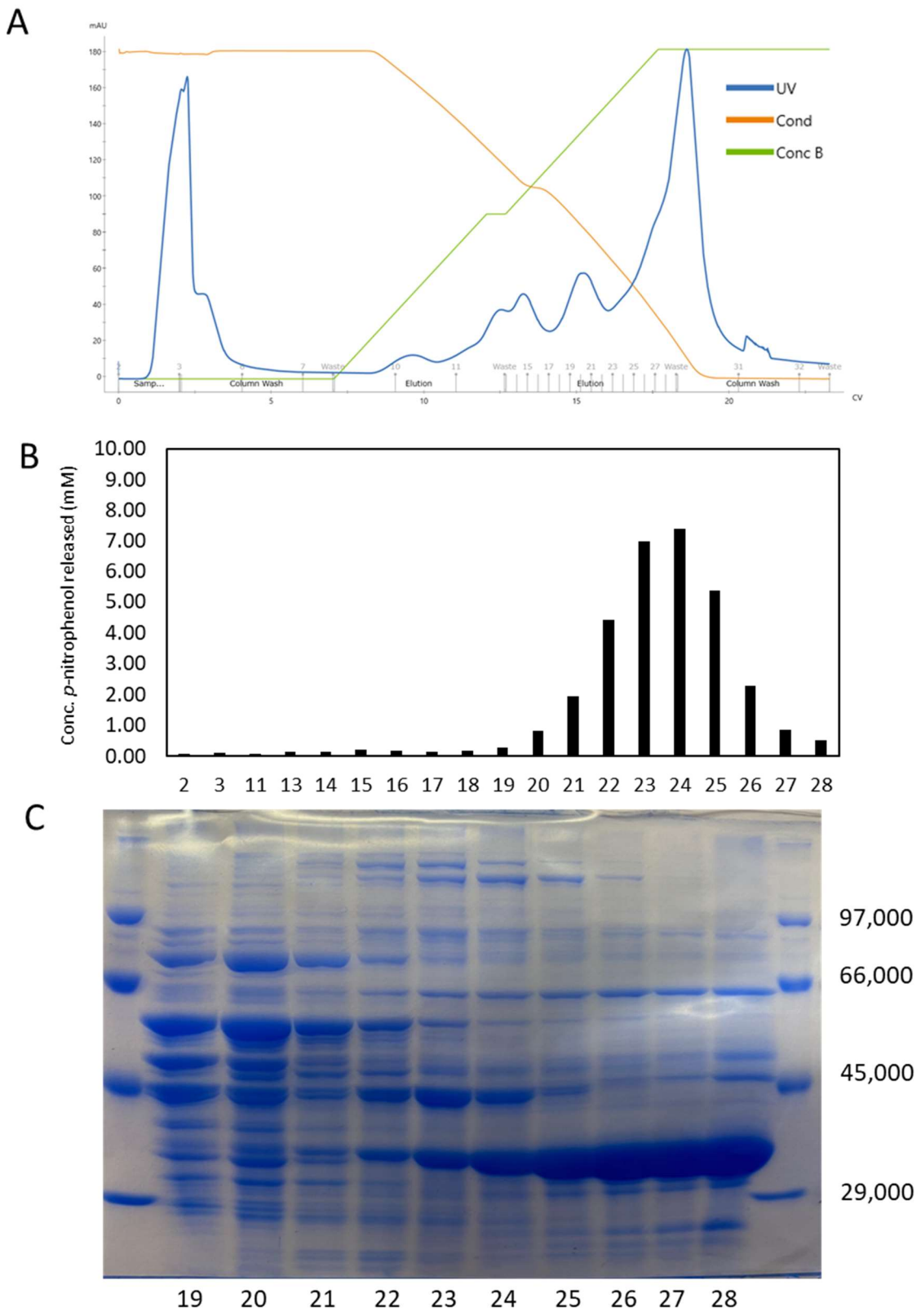

Figure 3.5 (A)FPLC chromatogram of Hi-Trap Butyl -Sepharose column. (B) *p*-nitrophenyl *β*-D-xylopyranoside assay of fractions from FPLC. (C) SDS-PAGE analysis of the fractions from FPLC.

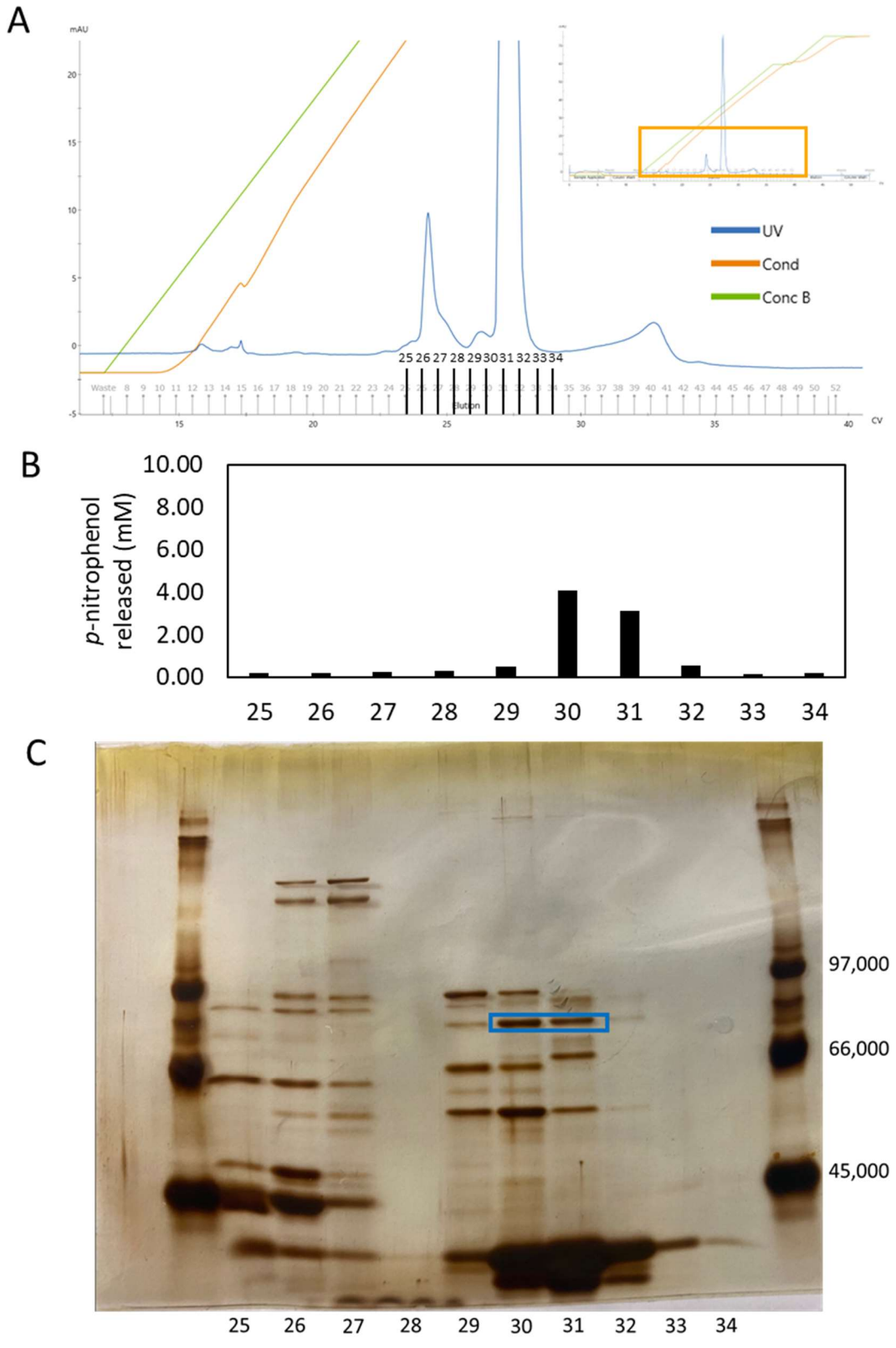

Figure 3.6 (A) FPLC chromatogram of Capto HiRes ion exchange column (B) *p*-nitrophenyl *β*-D-xylopyranoside assay of the fractions from FPLC. (C) SDS-PAGE assay of the fractions from FPLC with blue area highlighting suspected target band applied for LCMS peptide analysis

Table 3.1 Summary of purification of *β*-D-xylosidase from *B. pseudocatenulatum* JCM 7041

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)[A]	Recovery (%)	Fold purification
Crude Extract	293	130	2.25	100	1.00
Ammonium sulfate precipitation	308	47.4	6.50	36.4	2.89
Hi-Trap Butyl -Sepharose column chromatography	198	2.63	75.1	2.02	33.4
Capto HiRes ion exchange column chromatography	69.2	0.29	239	0.22	106

[A] One unit (U) of activity was defined as the amount of enzyme required to release one µM of *p*-nitrophenol per minute.

Table 3.2 Summary of LCMS peptide analysis of target band

Protein annotation	Protein accession #	Protein mass	Protein score	Protein matches
4-alpha-glucanotransferase	WP_226565626.1	79,514	2376	120
right-handed parallel beta-helix repeat-containing protein	WP_226556796.1	75,725	1651	80
choloylglycine hydrolase	WP_055063397.1	35,179	953	26

Table 3.3 Nucleotide sequence of 4-alpha-glucanotransferase in *B. pseudocatenulatum* JCM 7041

ATGACGGCAACCCAGCAAACCGAAAGCGCGGAACGCATCGCACGACCGCTTATTCAGCTCG
CCAAACTCAACGGCATCTCAACGTCGTATATCGACCAGCTCGGCACCTACGTGGAAATCCG
CGACGAAGTGCTCGTCTCCGTACTTGCGGCGCTCGGCGTGGACGCATCCAGCGATGAAGCG
ATCGCGGCATCGTACGAACTCACCAAGCAGCGCATCGCAGACACCCTTGTGGAACCGACCA
TCGTGAAGTTCATCGGCAAGGAAGCGACCACGCCGATCCGCGCGAAGGGCCACGACGTGAC
GCTACGCCTGTTGCTTGAGGACGGCACGCGGTATGAGGGCAACCTGTGCATGTATTTGGCC
CCGCAGACCGACGGCTCGCTCACGCTCACCCTGCCGGACGACATTCCCGCCGGCTACCACA
CGCTGCGCGTCAACGCGGGCCCGCTGCACGGCGAGGCGCGACTGATCTGCGCCCCGGCACG
CGTACCGCTGCCGCCTGCGGTTGCCGAAAAGCAGCGTTGGGGTTGGATGGCGCAAATGTAT
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GCCGGTCGAGCCGCTCGAACCGTCGCCGTACCTGCCGGAATCGCGCCGATTCATGAACGTC
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AAGTCGAGCGCTTGCACGCCGAAGTCGCTCCCGCCAACGACAATGCCGACGAACTCGACAT
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CCGCCGGACTTCTACAACCAGCAGGGCCAGGACTGGGGCCAGCCGCCGTTCAATCCGAACT
ATCTCGCCAAAACCGGTTATGGCGTGTACCGCGAAATGGTGCACAACATGTTCTCGCACGC
GGGCGCGGTGCGCATCGACCATGTGCTCGGCCTGTTCCGCCTGTGGTGGATTCCGCAGGGC
GAAGGCGCACGCGGCGGCGCATACGTCACCTACGACTATGAGGCGATGATCGCGATCCTCA
CGATCGAGGCTTCCCGCGTGAACGGCCTAGTGGTCGGCGAAGACCTCGGCACCGTGCCGGA
TTACGTGCGCACCGTGCTTGCCGAGCACGGTCTGCTCGGCTGCACGGTGGAATGGTTCGCC
CGTGTGGACGACTCCCCCAACGCCGGCGACCCGTATGCCGATCCGGCCGATTACCGCGAGT
ATGCGTTGGCATCCGTCACCACGCACGATCTGCCGCCGACCGCAGGCTACCTGCAGTTCGA
GCATGTCAAGCTGCGCGAAGAACTGAACCTGCTCACCGGTCCGGTCGAGGAATTCCAGGCG
TCCGCAACCGCGGAACGTCAGGCCATGCTTGACCGACTGGTCGAAAGCGAACTGATCACGC
CGGAAATCGCCGCGGACGTCGACGACCATATTCAAGAGATCGTCGAGGCGATGCACAAGAT
GCTACTCCGCTCGCCATCCGTGCTTCTGCAGGCGGCGCTGGTGGACGGCGTCGGCGAAACC
CGTTCGCAGAACCAGCCGGGCACGTCCAGCGAATACTGCAACTGGCGCGTGCCACTGGCAG
GCCCCGACCACAAGGTGGTGCACACCGACGAAGTGTTCGACCTGCCGCGCGTGAAGTCGCT
TTCCGCCATCATGAACGGCGAAAAGTAA

Table 3.4 Amino acid sequence of 4-alpha-glucanotransferase in *B. pseudocatenulatum* JCM 7041

MTATQQTESAERIARPLIQLAKLNGISTSYIDQLGTYVEIRDEVLVSVLAALGVDASSDE
AIAASYELTKQRIADTLVEPTIVKFIGKEATTPIRAKGHDVTLRLLLEDGTRYEGNLCMY
LAPQTDGSLTLTLPDDIPAGYHTLRVNAGPLHGEARLICAPARVPLPPAVAEKQRWGWMA
QMYSIRSAESWGVGDYGDLKLLLTDAAEKSHADFMLINPIHATAPVEPLEPSPYLPESRR
FMNVTYIRPQDIEEYAGLDEAALAEVERLHAEVAPANDNADELDINSAWWHKRQALQLVF
KVPRSAERQAAFEAFKEAAGPDLRAFAAWSVAFQMWGAPWEGTWFAETNRDSPEVAELMR
DHADMVEFECWLQWIADEQVTAAQNAARESGMALGLMQDMAVGVHSLGADVWWNPERFAV
GSVTVGCPPDFYNQQGQDWGQPPFNPNYLAKTGYGVYREMVHNMFSHAGAVRIDHVLGLF
RLWWIPQGEGARGGAYVTYDYEAMIAILTIEASRVNGLVVGEDLGTVPDYVRTVLAEHGL
LGCTVEWFARVDDSPNAGDPYADPADYREYALASVTTHDLPPTAGYLQFEHVKLREELNL
LTGPVEEFQASATAERQAMLDRLVESELITPEIAADVDDHIQEIVEAMHKMLLRSPSVLL
QAALVDGVGETRSQNQPGTSSEYCNWRVPLAGPDHKVVHTDEVFDLPRVKSLSAIMNGEK

Discussion

B. pseudocatenulatum JCM 7041 has shown consistent activity in metabolizing AIV into BraB through multiple screenings. This strain also showed the ability to transform CycB into CA in chapter 2. The enzymatic activity cleaving C-3 xylose off both AIV and CycB made the microorganism a favorable candidate to attempt to purify and characterize the *β*-D-xylosidase activity. Studies to determine the highest activity of *β*-D-xylosidase activity during the cultivation process were monitored using the resting cell reactions before the purification process. The highest xylosidase activity was seen at 48 hours of cultivation.

Cell lysis was performed on *B. pseudocatenulatum* JCM 7041 and the enzyme was deduced to be located intracellularly. The Ultra-centrifuge or high-speed centrifugal separation of the CFE showed the activity was not membrane bound and the activity was in the soluble fraction for further purification. Most of the enzymatic activity was obtained as protein precipitants in the 30-55% ammonium sulfate saturation fraction. These results, along with a very little decrease in the activity of metabolizing AIV and BraB production in ammonium sulfate precipitation (ASP) active fraction, suggest that ASP is a functionally useful procedure in the purification scheme.

Monitoring the enzymatic activity of *B. pseudocatenulatum* JCM 7041 by analyzing AIV metabolism and BraB production was causing a small bottleneck with a turnover time of 3 days between any experimentation, a quicker method utilizing spectrophotometry was developed using *p*-nitrophenyl *β*-D-xylopyranoside as a substrate and monitoring xylosidase activity by measuring the absorbance of the released *p*-nitrophenol. This method is often used to check the substrate specificity of purified enzymes, however, by correlating the AIV metabolic activity to pNP activity, a quick assay for activity confirmation was developed for further purification processing. The results using this method with the crude extract and ASP fraction of *B. pseudocatenulatum* JCM 7041 shown in Figure 3.4 suggested that the assay correlates well with the AIV-metabolizing activity.

Through purification shown in Figure 3.5 and Figure 3.6, a candidate band was selected to be analyzed by LCMS peptide analysis. The top three candidates were determined to be 4-alpha-glucanotransferase, right-handed parallel beta-helix repeat-containing protein, and choloylglycine hydrolase (Table 3.2). Although not shown here the band cut out for LCMS analysis showed two separate bands, the higher protein mass 4-alpha-glucanotransferase correlating the *β*-D-xylosidase activity was determined to be the likely candidate. The third

candidate can be ruled out due to its protein size and choloylglycine hydrolase in previous attempts by the author was hypothesized to be the protein seen in Figure 3.6 at the bottom of the SDS-PAGE through previous purification efforts. The author is working on confirming the enzymatic activity with the nucleotide and amino acid sequence derived from *B. pseudocatenulatum* JCM 7041 shown in Table 3.3, and Table 3.4, respectively.

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Conclusion

Looking at the results in chapter 1, multiple bacteria derived from human intestines showed the ability to metabolize AIV. Initial screening efforts showed that the bacteria show a preferential metabolism of AIV by either eliminating the C-3 xylose or C-6 glucose from the AIV rather than both simultaneously. Also, two representative groups of gut microbes, LAB and bifidobacteria showed mirroring metabolic pathways of AIV to CA production. Bifidobacteria showed preferential elimination of C-3 xylose to produce the intermediate BraB. Bifidobacteria also produced CA and showed no production of the intermediate CycB. LAB on the other hand showed preferential elimination of the C-6 glucose to produce CycB. LAB also showed preferential production of dehydrogenated product of CA, CA-2H rather than CA. LAB showed no production of intermediate BraB production through multiple screening efforts. These results suggest that various bacteria metabolize AIV in the intestinal system with different pathways, and when combined mimic previous research of AIV metabolic profile utilizing the consortium of intestinally derived bacteria.

Utilizing the information from chapter 1, a successful method to efficiently produce CA by harnessing the ability of LAB and bifidobacteria was achieved in chapter 2. Traditional single cell fermentation methods using either LAB or bifidobacteria resulted in long term fermentation and low concentration production of CA. However, successfully combing the AIV-metabolizing activity of LAB and bifidobacteria, specifically in that respective order allowed for significantly higher production of CA. When timed right there was little to no remaining intermediate of CycB and BraB produced in certain combinations of bacteria strains. Also, the production of dehydrogenated product of CA, CA-2H, was kept to a minimum by utilizing the washed cells of bifidobacteria to finish the biotransformation process. The dual resting cell reaction with *W. cibaria* RD 12578 and *B. pseudocatenulatum* JCM 7041 in this sequence showed the highest production of CA with 0.21 mM concentration with an 82% yield to 0.25 mM AIV as the substrate.

In chapter 2, the results suggested that *B. pseudocatenulatum* JCM 7041 can not only metabolize AIV to BraB but also convert the intermediate CycB to CA by eliminating the C-3 xylose. Through protein purification, the candidate protein band revealed a high score match

of the amino acid sequences of the peptides with those of 4-alpha-glucanotransferase derived from *Bifidobacterium pseudocatenulatum*.

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Daniel Makoto Takeuchi

Publications

1) Daniel M Takeuchi, Shigenobu Kishino, Yuuki Ozeki, Hiroyuki Fukami, and Jun Ogawa. Analysis of astragaloside IV metabolism to cycloastragenol in human gut microorganism, bifidobacteria, and lactic acid bacteria. *Biosci Biotechnol Biochem,* 86(10), 1467–1475 (2022).

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3) Daniel M Takeuchi, Yuuki Ozeki, Hiroyuki Fukami, Jun Ogawa, and Shigenobu Kishino. Purification of astragaloside IV-hydrolyzing *β*-D-xylosidase from *Bifidobacterium pseudocatenulatum* JCM 7041. In preparation.