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Possibility of a Product of Awamori Moromi Vinegar Fermented by *Lactobacillus plantarum* K-3 as a Prebiotic

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Abstract: Awamori moromi vinegar (AMV) contains essential amino acids and citric acid; however, its peculiar flavor prevents its acceptance as a functional food material. In a previous study, a fermented product of AMV (FP-AMV) was prepared using *Lactobacillus plantarum* K-3 to resolve the peculiar flavor of AMV, and its possibility to improve lipid metabolism through an approach to gut microbiota was suggested. In this study, using *in vitro* and *in vivo* experiments, it was aimed to determine whether FP-AMV could be used as a prebiotic to improve gut microbiota. The *in vitro* prebiotic assay showed increased turbidity for eight lactic acid bacteria and five bifidobacteria with the addition of FP-AMV, suggesting the comprehensive bacterial growth-promoting effect of FP-AMV. In contrast, the growth of *Clostridium* perfringens was greatly suppressed by FP-AMV. Therefore, an animal experiment was conducted to investigate the relationship between FP-AMV ingestion and the gut microbiota. Gut microbiota analysis of fecal samples in animal experiments proved that FP-AMV induced not only an increase in the prevalence of probiotic species, such as *Lactobacillus* and *Bacteroides*, but also a decrease in the prevalence of pathogenic species, such as *Clostridium*, in the gut microbiota of male C57BL/6JJcl mice. These results suggest that FP-AMV contributes to the improvement of the gut microbiota and the gut environment. Thus, it can be used as a potential prebiotic food.

Keywords: Fermented Product of Awamori Moromi Vinegar (FP-AMV), *Lactobacillus plantarum* K-3, Gut Microbiota, Prebiotics

1. Introduction

Awamori-distilled lees are produced as a by-product of the brewing process of Awamori, which is a traditional Okinawan distilled liquor. In 1973, a method for producing awamori moromi vinegar (AMV) from awamori-distilled lees has been developed [1]. Essential amino acids and citric acid present in AMV enable its use as a potential functional food material. However, the peculiar flavor of AMV limits its widespread applicability.

Lactobacillus plantarum is the most versatile species with beneficial properties and is frequently present in several fermented food products [2-4]. Therefore, AMV has been fermented earlier using *Lactobacillus plantarum* K-3 to address the issues with flavor [5]. Sensory analysis of flavor showed a decrease in sour taste in the fermented product of AMV (FP-AMV), while sweetness and mellowness increased greatly. Furthermore, this fermentation approach reduced furfural, the main product causing the burnt odor of AMV, suggesting a considerable improvement in the AMV flavor.

In feeding experiments on mice, this easy-to-consume FP-AMV has shown an inhibitory effect on cholesterol synthesis by suppressing the mRNA expression of sterol regulatory element-binding protein-2 and

3-hydroxy-3-methyl-glutaryl-CoA reductase [5]. Since the gut microbiota and lipid metabolism are closely related [6-9], in relation to its effect on lipid metabolism, this study aimed to determine the potential of FP-AMV as a prebiotic food for improving gut microbiota through *in vitro/vivo* prebiotic experiments.

2. Materials and Methods

2.1. Materials

AMV was obtained from Ishikawa Distillery Inc. (Okinawa, Japan). Gifu anaerobic medium (GAM) broth and de Man, Rogosa, and Sharpe (MRS) broth were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Becton Dickinson and Company (NJ, USA), respectively. All the other reagents used in this study were high-quality analytical-grade materials.

2.2. Preparation of FP-AMV

FP-AMV was prepared in Ishikawa Distillery Inc., as previously described [5]. Briefly, AMV was inoculated with *L. plantarum* K-3 and incubated at 30°C for 24 h. The resulting solution was sterilized by heating it at 85°C for 15 min. In this study, AMV and its fermented product, FP-AMV, were eventually lyophilized using a freeze dryer, FDU-1200 (TOKYO RIKAKIKAI Co., Ltd., Tokyo, Japan).

2.3. In vitro Prebiotics Assay

Lyophilized FP-AMV powder was dissolved in distilled water. The obtained solution was subjected to filtration through a 0.45-μm filter. Ten types of lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactococcus lactis* subsp. *lactis*, and *Pediococcus pentosaceus*), five types of *Bifidobacterium* species (*Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, and *Bifidobacterium longum* subsp. *infantis*), and *Clostridium perfringens* were precultured at 34°C for 24 h in GAM or MRS broth. These preculture broths were diluted 200-fold using GAM or MRS broth. An aliquot of each bacterial suspension (90 μL) was mixed with a sterilized solution of FP-AMV (10 μL) in a 96-well microplate. The mixture was incubated at 34°C for 24 h and its turbidity was measured at 660 nm. A similar experiment was conducted with AMV.

2.4. Ingestion of FP-AMV

Animal experiments were conducted in accordance with the "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 88, Ministry of the Environment, Government of Japan). The experimental protocol for animal experiments was prepared as described previously [10-12] and approved by the Ethics Review Committee of Nagasaki International University (Approval No. 119).

Four-week-old male C57BL/6JJcl mice (Kyudo Co., Ltd., Saga, Japan) were individually housed in a room with controlled temperature, relative humidity (RH), and light (23 ± 2°C, 60 ± 2% RH, and 7:00–19:00 h, respectively). The mice had *ad libitum* access to water and a normal diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan).

After a week of acclimatization, C57BL/6JJcl mice were divided into two groups of six mice each, based on their body weight (Figure 1). The mice in the control group (Group I) were fed *ad libitum* with the high-fat diet, HFD32 (CLEA Japan, Inc., Tokyo, Japan) for 56 days. For the experimental group (Group II), the normal diet was replaced 0 days after acclimatization with a high-fat diet supplemented with 3% lyophilized FP-AMV.

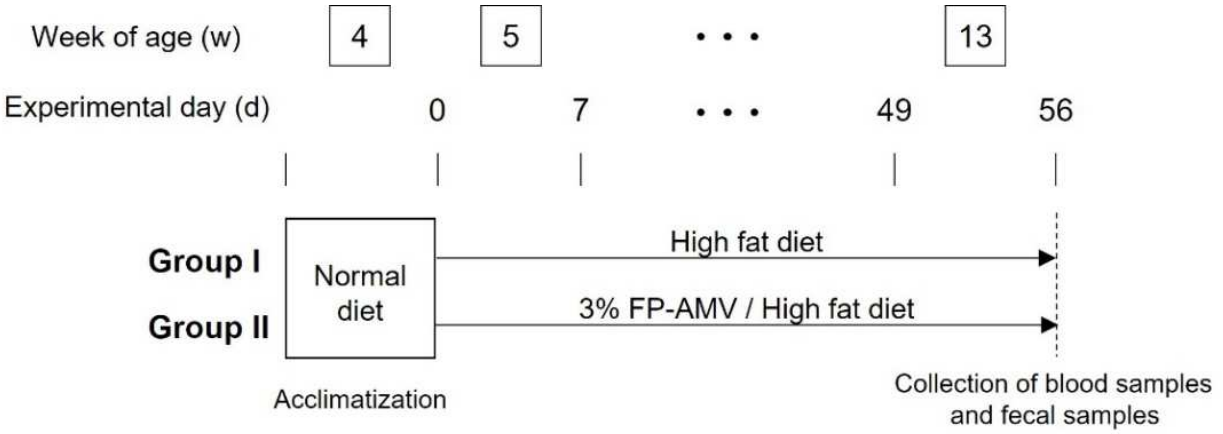

Figure 1. *The schedule for FP-AMV ingestion in the animal experiment. Male C57BL/6JJcl mice at 4 weeks of age were randomly divided into two groups (n = 6/group) after a week of acclimatization. The mice in Group I were fed a high-fat diet for 56 days after acclimatization. In Group II, a high-fat diet supplemented with 3% lyophilized FP-AMV was given to mice. Fecal samples were collected from all mice on the final day of the animal experiment.*

During the animal experiments, general symptoms were observed daily, and the body weight of each mouse was measured twice a week. The amount of feed ingested was estimated by weighing the leftovers weekly. Blood samples and fecal samples from all the mice were collected at the end of the animal experiment.

2.5. Biochemical Analysis of Serum

After blood samples were centrifuged to remove blood clots, the serum biochemical parameters, such as triglycerides (TG), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), were measured using LabAssay^{TM} kit series (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.6. Gut Microbiota Analysis

Gut microbiota analysis of fecal samples was performed according to 16S rRNA amplicon sequencing on an Illumina MiSeq platform, and the ratios of *Lactobacillus*, *Bacteroides*, and *Clostridium* in the gut microbiota were calculated.

2.7. Statistical Analyses

Each assay was performed in triplicates, and the resulting data were presented as the mean ± standard error. Statistical significance among treatment groups and the control group was evaluated using a two-tailed Student's *t*-test; $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Preparation of FP-AMV

AMV was fermented using *L. plantarum* K-3 to improve the flavor [5]. In this study, lyophilizing 1 mL of the liquid portions of AMV and its derivative beverage, FP-AMV, yielded 62.5 g and 82.5 g of powder, respectively. Considering the difference of 20.0 g between the two lyophilized powders, it was assumed that along with the derivatives of AMV, FP-AMV also contained the cell wall, bacterial components, and secreted metabolites from killed *L. plantarum* K-3. It is presumed that the substances include cell wall components of *L. plantarum* K-3, such as lipoteichoic acid and peptidoglycan and nucleic acid materials, such as unmethylated CpG motifs. In fact, barley *shochu* lees fermented using lactic acid bacteria are reported to contain substances that promote the growth of lactic acid bacteria and bifidobacteria [13]. Since many reports have indicated that substances obtained from fermentation using *L. plantarum* provide health benefits [14], the potential of FP-AMV as a prebiotic food was evaluated in this study.

3.2. The Growth-Promoting Effect of FP-AMV on Lactic Acid Bacteria and Bifidobacteria

The turbidity of *L. gasseri* reached a plateau approximately 20 h after the start of the culture in the absence of FP-AMV and increased greatly with the addition of FP-AMV (Figure 2a). Comparative turbidities of *L. gasseri,* 24 h after the start of the culture, showed that turbidity significantly increased with the addition of FP-AMV but was unaffected by AMV (Figure 2b). In addition, maximum turbidity of *L. gasseri* was achieved when 500 μg/mL FP-AMV was used.

With the addition of FP-AMV, the turbidity of *B. longum* also increased 24 h after the start of culture in a concentration-dependent manner but remained constant with the addition of AMV (Figure 3).

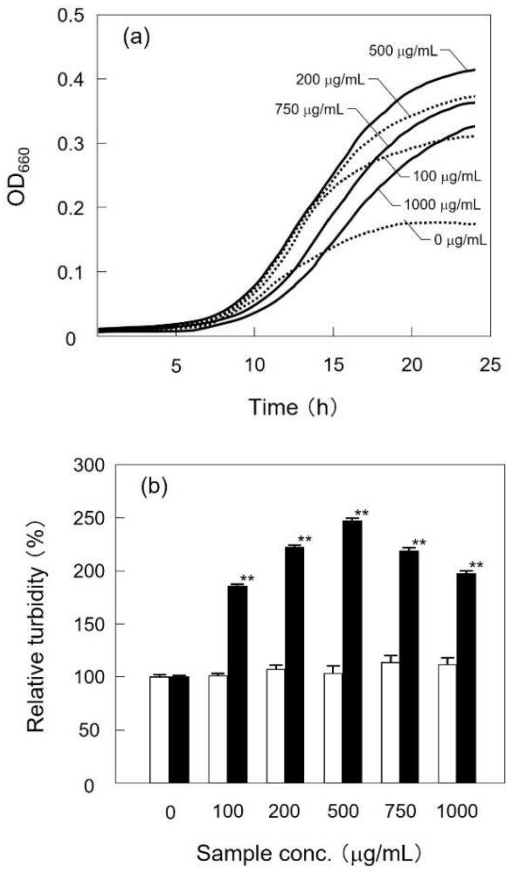

Figure 2. *The influence of FP-AMV on the growth of Lactobacillus gasseri. (a) The suspensions of L. gasseri were treated with FP-AMV and their turbidities at 660 nm were measured. The concentrations of FP-AMV acting on L. gasseri are indicated in the figure. (b) Based on Figure 2(a), the relative turbidities of the suspensions 24 h after the start of culture are shown in black bars. These were compared with the data obtained from the suspensions treated with AMV (white bars). The turbidity of the suspension incubated without both samples was taken as 100% (control). Experimental data are presented as mean ± standard error, and significant differences (using two-tailed Student's t-tests) from the values in control are indicated by **p < 0.01.*

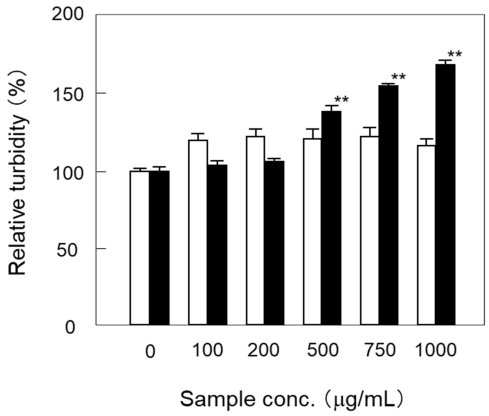

Figure 3. *The influence of FP-AMV on the growth of Bifidobacterium longum. The suspensions of B. longum were treated with FP-AMV and their turbidities were measured at 660 nm, 24 h after the start of culture (black bars). These were compared with the data obtained from the suspensions treated with AMV (white bars). The turbidity of the suspension incubated without samples was taken as 100% (control). Experimental data are presented as mean ± standard error, and significant differences (using two-tailed Student's t-tests) from the values in control are indicated by **p < 0.01.*

Since, in comparison to AMV, FP-AMV was found to potentially promote the growth of *L. gaserri* and *B. longum*, *in vitro* prebiotic assays were also performed on nine lactic acid bacteria and four bifidobacteria. It was found that the growth of most bacteria, except *L. casei* and *L. salivarius,* was significantly promoted. This proved the broad bacterial growth-promoting effect of FP-AMV (Table 1).

Based on these results, exopolysaccharides (EPS) were suggested as potential substances involved in inducing this effect. In general, EPS have been reported to contribute to the rheology of fermented foods and provide health-promoting properties to food [15]. *L. plantarum* has been attracting considerable attention, since it is one of the most prominent EPS-producing lactic acid bacteria [16]. In fact, EPS produced by *L. plantarum* strains isolated from tarhana have been shown to promote the growth of probiotic strains in the fermentation medium [17]. Therefore, it is reasonable to consider that the EPS produced by *L. plantarum* K-3 and contained in FP-AMV had a prebiotic effect on the growth of eight lactic acid bacteria and five bifidobacteria.

Interestingly, FP-AMV showed different optimal concentrations for the growth of *L. gaserri* and *B. longum* (Figures 2b and 3). It was suggested that both the bacteria had different sensitivities to the same bacterial growth factor derived from *L. plantarum* K-3, or that different bacterial growth factors acted on both the bacteria. Therefore, although the bacterial growth factors contained in FP-AMV have been not yet identified, it is necessary to determine the optimal concentration for balancing gut microbiota when developing a new beverage based on FP-AMV to improve the gut environment.

Table 1. *The influence of FP-AMV on the growth of Lactobacillus, Bifidobacterium, and Clostridium species.*

Bacteria	Relative turbidity (%)
Lactobacillus acidophilus	$106.96 \pm 0.64^{**}$
Lactobacillus casei	102.88 ± 0.49
Lactobacillus delbrueckii subsp. *bulgaricus*	$110.86 \pm 0.12^{*}$
Lactobacillus gasseri	$247.50 \pm 0.50^{**}$
Lactobacillus helveticus	$134.98 \pm 0.09^{**}$
Lactobacillus plantarum	$115.26 \pm 1.20^{*}$
Lactobacillus rhamnosus	$118.05 \pm 0.43^{*}$
Lactobacillus salivarius	105.05 ± 0.59
Lactococcus lactis subsp. *lactis*	$116.35 \pm 0.49^{**}$
Pediococcus pentosaceus	$109.81 \pm 0.23^{**}$
Bifidobacterium adolescentis	$160.07 \pm 1.99^{*}$
Bifidobacterium bifidum	$146.53 \pm 0.59^{**}$
Bifidobacterium breve	$151.55 \pm 0.53^{**}$
Bifidobacterium longum	$168.98 \pm 0.59^{**}$
Bifidobacterium longum subsp. *infantis*	$153.10 \pm 0.49^{**}$
Clostridium perfringens	$22.62 \pm 9.30^{*}$

The turbidity of the suspension incubated without samples was taken as 100% (control). Experimental data are presented as mean ± standard error, and significant differences (using two-tailed Student's *t* -tests) from the values in control are indicated by $*p < 0.05$,.$**p < 0.01$.

3.3. The Suppressing Effect of FP-AMV on the Growth of C. Perfringens

The *in vitro* prebiotic assay showed that the growth of *C. perfringens* was potently suppressed by FP-AMV (Table 1). Since FP-AMV had the minimum effect on the pH of the solvent in this assay, it was suggested that antibacterial substances were included in FP-AMV. The raw material for FP-AMV contains polyphenols such as vanillic acid (4-hydroxy-3-methoxybenzoic acid) and its derivatives, which have been reported to have antibacterial effects [18]. Moreover, it is possible that *L. plantarum* K-3 produces antibacterial substances similar to other *L. plantarum* strains [19, 20].

3.4. The Prebiotic Effect of FP-AMV

The *in vitro* prebiotic assay demonstrated the growth-promoting effect of FP-AMV on typical lactic acid bacteria and bifidobacteria. Therefore, an animal experiment was conducted to investigate the influence of FP-AMV ingestion on the gut microbiota. In Group I mice, weight gain and some parameters indicating the status of lipid metabolism, such as TG, ALT, and AST, were high by the introduction of a high-fat diet (Table 2). In contrast, the effect of a high-fat diet on the mice of Group II was alleviated by the ingestion of FP-AMV. Gut microbiota analysis of fecal samples collected from the mice in Groups I and II showed that the ingestion of FP-AMV increased the ratios of *Lactobacillus* and *Bacteroides* in the gut microbiota (Figures 4a and 4b). In addition to contributing to the improvement of the gut microbiota in obese mice, it was suggested that probiotic species increased by FP-AMV may have influenced the improvement of lipid metabolism.

Table 2. *The influence of FP-AMV on weight gain and serum biochemical parameters of mice.*

	Group I	Group II
Weight gain (g)	19.7 ± 0.5	$18.1 \pm 0.8^{*}$
Serum TG (mg/dL)	99.7 ± 11.1	$64.2 \pm 5.1^{*}$
Serum ALT (U/L)	10.6 ± 0.6	$7.8 \pm 0.8^{*}$
Serum AST (U/L)	45.0 ± 5.5	$27.8 \pm 3.9^{*}$

Experimental data are presented as mean ± standard error, and significant differences (using two-tailed Student's *t*-tests) from the values in Group I are indicated by $*p < 0.05$.

Although the usefulness of *Lactobacillus* in the gut environment and gut immune system has been proved [21], it has also been reported that the production of immunoglobulin A (IgA) in Peyer's patches is induced effectively by *Bacteroides,* and bacterial products, such as polysaccharide A, derived from *Bacteroides* can modulate the T cell-dependent immune reaction [22]. Thus, *Lactobacillus* and *Bacteroides* species can be utilized to promote individual health. As these improvements are closely related to the activation of gut immunity, further studies should investigate the influences of FP-AMP on IgA production in Peyer's patches and the T cell-dependent immune reaction.

In contrast, it was found that the mice in Group II had a lower prevalence of *Clostridium* than that in Group I (Figure 4c). Some species of *Clostridium* produce short-chain fatty acids such as acetic acid and butyric acid; however, this genus

also contains pathogenic bacteria. Therefore, to utilize FP-AMV as a prebiotic food, it is imperative to prove that FP-AMV can reduce the prevalence of harmful species in *Clostridium*.

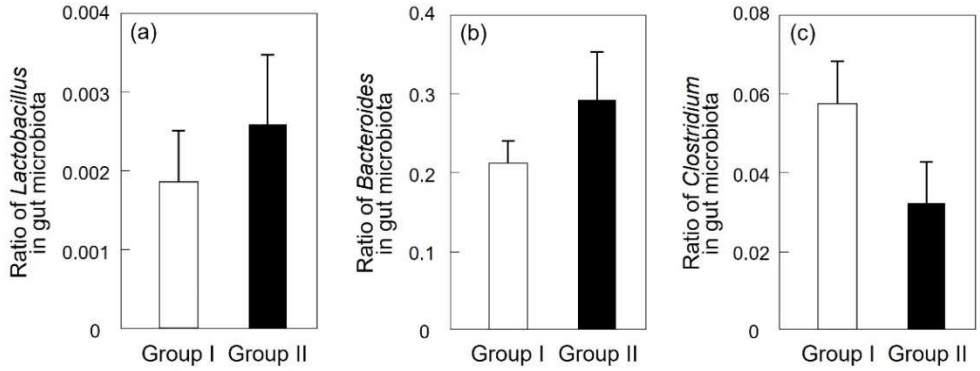

Figure 4. *The influence of FP-AMV on the ratios of Lactobacillus (a), Bacteroides (b), and Clostridium (c) in the gut microbiota. The schedule of the ingestion of FP-AMV in the animal experiment is described in the text and Figure 1. The fecal samples collected from all C57BL/6JJcl mice were used for gut microbiota analysis.*

4. Conclusion

In this study, it was suggested that FP-AMV could contribute to improvements in the gut microbiota and gut environment, based on *in vitro* prebiotic assays and animal ingestion experiments. Gut microbiota is involved in the regulation of lipid metabolism and immunity. Therefore, in the future, we would like to conduct an ingestion test of FP-AMV for human subjects to demonstrate its possibility as a prebiotic food that also contributes to the improvement of lipid metabolism and immunity.

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Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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