Immune and metabolic beneficial effects of Beta 1,3-1,6 glucans produced by two novel strains of Aureobasidium pullulans in healthy middle-aged Japanese men: An exploratory study

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doi: https://doi.org/10.1101/2021.08.05.21261640

Abstract

Background Imbalances in glucose and lipid metabolism in the background of a declining immune system, along with aging, make one prone to glucolipotoxicity-related diseases such as hepatic steatosis and to high risk of infection-related mortality, as with COVID-19, warranting a safe prophylactic measure to help regulate both metabolism and the immune system. Based on the beneficial effects of the AFO-202 strain of black yeast *Aureobasidium pullulans*-produced beta 1,3-1,6 glucan in balancing of blood glucose and immune enhancement, and that of the N-163 strain of the same species in lipid metabolism and immune modulation, in this pilot study, we have evaluated their specific benefits in healthy human subjects.

per day), IA for 35 days and IB for 21 days; Group II consumed a combination of AFO-202 beta glucan (2 sachets of 1 g each) and N-163 beta glucan (1 sachet of 15 g gel each per day), IIA for 35 days and IIB for 21 days. Investigations for immune stimulation, anti-glycaemic, and anti-cholesterolemia biomarkers were undertaken in all four groups.

Results In terms of metabolic control of glucose, the decrease in HbA1C and glycated albumin (GA) was significantly better in Group I compared with the other groups. Immune enhancement in terms of a significant increase of eosinophils and monocytes and marginal decrease in D-dimer levels, decrease in neutrophil-to-lymphocyte ratio (NLR), with an increase in the lymphocyte-to-CRP ratio (LCR) and leukocyte-to-CRP ratio (LeCR) was observed in Group I. Regulation of lipids by decrease in total and LDL cholesterol was better in Group II, and immunomodulation of coagulation-associated and anti-inflammatory markers by a decrease of CD11b, serum ferritin, galectin-3, fibrinogen was profound in Group II.

Conclusion *A. pullulans,* a polythermotolerant black yeast - produced AFO-202 beta glucan has balanced blood glucose with marginal immune enhancement in healthy individuals, which when combined with N-163 beta glucan, balanced the lipid profile and immunomodulation. This outcome warrants larger clinical trials to understand the mechanisms and explore the potentials of these safe food supplements in prevention and prophylaxis of diseases due to dysregulated glucose and lipid metabolism, such as fatty liver disease, and infections such as COVID-19 in which a balanced immune activation and immunomodulation are of utmost importance, besides their administration as an adjunct to existing therapeutic approaches of both communicable and non-communicable diseases.

Introduction

Metabolism imbalance is a gradually occurring condition leading to diabetes, heart disease, stroke, etc., and the risk varies between populations based on their genetic predisposition, diet, lifestyle, and environmental influences [**1**]. By the time an individual is diagnosed with any lifestyle illness requiring medication, further prevention and deceleration of the pathogenesis is an uphill task. To address this, exercise, dietary modifications such as intake of foods with low glycaemic index or fats, and medications are advised which are temporary and not definitive solutions [**1**]. The disease onset starts with elevated glucose levels leading to glucotoxicity in predisposed individuals and in high-fat diet populations; lipotoxicity occurs, and they both synergistically derail the metabolic homeostasis skewed towards dysregulation, paving the way for gradual onset of a nidus for diseases in many organs [**2**]. The liver, being the metabolic centre of the body, often

of reactive oxygen species (ROS) and the ability of the endogenous antioxidant system (AOS), in turn causing aging and various age-related chronic pathologies such as inflammation, neurodegenerative diseases, atherosclerosis, and vascular complications of diabetes mellitus [**3**]. Additionally, the immune reserves may be depleted in handling, and the circulating high levels of advanced glycation end products (AGEs) and lipids affects the functional capability of the immune cells, leading to high risk of disease severity [**3**] when COVID-19-like infections occur [**4**]. The fibrosis that ensues after a chronic inflammation-metabolic-immune dysregulation can lead to pulmonary or liver fibrosis, such as non-alcoholic steatohepatitis (NASH) [**2**], which could eventually culminate in carcinogenesis [**5**]. Glucotoxicity and lipotoxicity also cause gut dysbiosis [**6**], which is now increasingly considered the key factor influencing progression of infections, inflammations, and fibrosis, creating a viscous cycle.

Against the given background, as a remedy, what we require ideally should:

I. **Regarding glucotoxicity and lipotoxicity:**

1. ***At an early stage or before onset of disease:***

• Balance the blood glucose levels, especially the post-prandial spike

• Balance the blood cholesterol level without side effects

2. ***Post-disease onset stage:*** During and after onset of the glucotoxicity and/or lipotoxicity

• Control glucose and cholesterol with no interaction with other drugs prescribed

• Balance the blood cholesterol level without side effects; control LDL and VLDL without affecting HDL

• Be able to control inflammation and the accumulation of free fatty acids (FFA)

3. ***After progression of disease with chronic sequalae stage*****:** Post-onset of pathogenesis causing organ and/or systemic inflammation

• Control organ inflammatory reaction to avoid fibrosis

• Balance micro-inflammation of the gut

II. Regarding systemic wellness and immune balance:

1. All through the above stages:

• Support the immune system, especially during aging, by enhancing it to prevent illnesses from disease-

- Reverse gut dysbiosis

Although a single such prophylactic measure or component is almost impossible, we selected two products of strains from the black yeast *A. pullulans* which have a track record of safety [**7**–**9**].

AFO-202 benefits

The AFO-202 strain-produced beta glucan has been shown to normalize Hba1c and fasting, post-prandial blood glucose levels in patients with type II diabetes [**7**]. It has been shown to decrease elevated LDL and VLDL cholesterol and triglycerides in clinical studies of metabolic syndrome [**8**]. Enhancement of immune cells such as natural killer (NK) cells and macrophages, apart from suppression of pro-inflammatory cytokines such as IL-1beta, IL-2, IL-6, IL-12 (p70+40), interferon-gamma (IFN-gamma), tumour necrosis factor-alpha (TNF-alpha), or soluble Fas ligand (sFasL) while enhancing beneficial cytokines such as interleukin-8 (IL-8) or soluble Fas (sFas) and antibodies has been reported [**9**]. Apart from these beneficial immune and metabolic modulations, a decrease in the neutrophil-to-lymphocyte ratio (NLR) and increase in lymphocyte-to-C-reactive protein (CRP) ratio (LCR) and leukocyte-to-CRP ratio (LeCR) are particularly significant in COVID-19 [**10**], as the dysregulation of these parameters has been correlated with progression of the disease and higher odds of mortality [**11**]. The potential of the AFO-202 beta glucan as an immune adjuvant in the prophylaxis of COVID-19, along with beneficial anti-coagulopathy benefits, has been described [**12**–**15**].

N-163 benefits

While AFO-202 is relevant to both metabolic and immune regulation, the anti-inflammatory, anti-fibrotic potential of N-163 has been reported with significance in a NASH animal model [**16**], along with a decrease in inflammation-associated lipid parameters such as non-esterified free fatty acids (NEFAs) [**17**]. Thus, N-163 is more relevant in the stages of progressed disease status.

Before addressing specific disease targets, we sought to study the effects of AFO-202 and N-163-produced beta glucans in the middle-aged, healthy subjects, as they have been the most vulnerable population for metabolic diseases [**18**] and severe COVID-19.

Methods

The study was conducted in compliance with the ethical principles based on the Declaration of Helsinki and the Ethical Guidelines for Medical Research Involving Human Subjects (notified by the Ministry of Education,

Network-Clinical Trial Registry (UMIN-CTR) of Japan [**19**]. The study was conducted at the Chiyoda Paramedical Care Clinic, Tokyo, Japan.

Study Subjects

The study was designed as an exploratory study in healthy Japanese male volunteers aged 40 to 60 years with four intervention conditions: two test food groups and two durations of intake in each test food group. As the minimum number of participants required for statistical comparisons within and between intervention conditions is four per intervention condition, a total of 16 target study participants was determined.

The person in charge of the allocation, as specified in the study protocol, allocated the study subjects to the four groups as evenly as possible, giving first priority to pre-test BMI, second priority to weight, and third priority to height.

Subjects who met the selection criteria in **Table 1** and did not fall under any of the exclusion criteria were eligible for the study.

Table 1

View inline

: **Inclusion and exclusion criteria**

Intervention

The duration of the study food intake and the schedule of visits for each group are shown in **Table 2**.

Table 2

View inline

: **Interventions and Study groups**

Evaluations

The following tests were carried out after written consent was obtained from the study subjects.

Pre-test and before intake

Blood sampling volume: 34 mL

Background survey: gender, date of birth, age, smoking habits, drinking habits, eating habits, current medical history, medication, treatment, previous history, allergies (to drugs and food), regular use of food for specified

At *pre-test and before intake,* Day 4 of intake, Day 8 of intake, Day 22 of intake, Day 36 of intake, and Day 15 of post-observation

➢ Medical history and physical measurements: medical history, height, weight, BMI, temperature

➢ Physiological examination: systolic blood pressure, diastolic blood pressure, pulse rate

➢ Haematology 1: White blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb), haematocrit (Ht), MCV, MCH, MCHC, platelet (PLT) count

➢ Haematology 2: Basophil, Eosinophil, Neutrophil, Lymphocyte, Monocyte counts

➢ Haematology 3: D-dimer, prothrombin time (PT), Ferritin, Fibrinogen

➢ Blood biochemistry: T-Cholesterol, LDL-Cholesterol, HDL-Cholesterol, triglycerides (TG), HbA1c (NGSP), glycated albumin (GA)

➢ Immunological test : CRP, blood IgG, blood IgM, blood IgA

➢ Cellular immunity test: CD11b in monocyte fraction

➢ IL-2, IL-6, IL-7, IL-8, IFN-γ, sFas ligand

➢ Galectin-3

Daily diary

Daily diary: Participants kept a diary from the day of the start of test food consumption until the day before the 36th day of consumption and the 15th post-observation day.

The following items were recorded in the diary: intake of test foods, body temperature; intake of food for specified health uses, functional foods, and health foods; intake of restricted foods; subjective symptoms; visits to medical institutions; treatment; and use of medicines.

Examples of restricted foods

The following are examples of restricted foods.

Supplements rich in beta-glucan: supplements containing beta-glucan extracted and concentrated from yeast, barley, mushrooms, and seaweed.

Foods claimed to stimulate the immune system: yoghurt, lactobacillus beverages, bifidobacteria powder,

2. WBC, RBC, Hb, Ht, PLT, MCV, MCH, MCHC
3. Basophils, eosinophils, neutrophils, lymphocytes, monocyte counts
4. CRP, IgG in blood, IgM in blood, IgA in blood
5. IL-2, IL-6, IL-7, IL-8, IFN-γ, sFas ligand

Secondary endpoints

1. Coagulopathy related markers
   i. Ferritin, D-dimer, PT, Fib, CD11b in monocyte fraction, galectin-3
2. Blood glucose level
3. HbA1c, GA
4. Cholesterol level
   i. TG, T-Cho, HDL-Cho, LDL-Cho

Safety evaluation items

Incidence of adverse effects

Data analysis

Target population for analysis

After all data had been obtained, a decision was made on the handling of cases and the acceptance or rejection of data for all cases. Intention to treat (ITT) was defined as the group of all study subjects excluding those who did not consume the study food. The Full Analysis Set (FAS) was defined as the population from which the ITT excluded subjects who withdrew or dropped out of the study. The FAS excluding subjects who met the following exclusion criteria for PPS analysis was defined as the Per Protocol Set (PPS).

Exclusion criteria for PPS analysis

All study subjects who consumed the test food were included in the analysis. However, if a study subject met any of the following exclusion criteria, the study investigator and sponsor discussed and decided how to handle the test results.

3) Repeated failure to take the indicated precautions

4) Failure to comply with the fasting and smoking cessation rules, or any other significant behaviour that undermined the reliability of the test results

5) A breach of the exclusion criteria determined after completion of the test

6) Other obvious reasons for omission

Data-handling criteria

For each test item in the relevant test, if the measured value could not be obtained because it was below the lower or above the upper limit of measurement, the lower or upper limit of measurement was substituted. Values exceeding the range of three times the standard deviation from the mean value for each test item were considered outliers and were not used in the analysis. Missing values were not complemented, but there were no missing values in this study.

Statistical analysis

The statistical significance level was set at 5%, two-sided. SPSS26.0 (IBM Japan, Ltd.) and Microsoft Excel (Microsoft Corporation) were used as analysis software. An unpaired t-test, Fisher's exact test (Bonferroni correction), Dunnett certification, and a correspondence t-test were performed.

Results

Sixteen patients (ITT) who fulfilled all the selection criteria and none of the exclusion criteria were selected to start the study. Because one study subject (No. 4) with leukocyte abnormalities (suspected leukaemia) discontinued or dropped out of the study, 15 study subjects who participated throughout the entire study period were considered FAS. In addition, two study subjects (Nos. 11 and 16) were excluded as a result of deliberation at the case review meeting because they fell under "6) Other obvious reasons for omission" in the "*Exclusion criteria for PPS* analysis" section. The reason for the exclusion of study subject No. 11 was that there were more outliers (mean ± 3SD) for this subject than for other study subjects, and it was judged that the exclusion might affect the study results. Thus, subjects Nos. 11 and 16 were excluded from the analysis. After excluding these two subjects from the FAS, 13 subjects were included in the PPS. The CONSORT diagram of the trial is presented as **figure 1**. There were no significant differences between the test food groups (four groups) in terms of BMI, height, and weight, which were the test subject allocation factors.

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Figure 1:

CONSORT Flow diagram of the trial

The intake rates (number of days and number of packets) for FAS and PPS were 100.00% for both test food groups (I and II).

Comparisons between the test food groups using the change from pre-consumption values showed statistically significant differences in the parameters outlined in **Table 3**.

I. **AFO-202 beta glucan:**

1. ***Glucose metabolism:***

HbA1C:

In Group I, the decrease was greater by -0.23 ± 0.06% after 35 days of intake compared with Group II (-0.08 ± 0.05%), which showed a statistically significant higher value ($p < 0.05$) (**Figure 2A**).

Glycated albumin (GA):

RBC

After 21 days of consumption, the RBC was statistically significantly higher ($p < 0.05$) in Group I (4.0 ± 5.3 x 104/µL) compared with test Group II (-8.8 ±5.6 x 104/µL).

Hb

After 21 days of consumption, the value in Group I (0.13 ± 0.12 g/dL) ($p < 0.01$) was statistically significantly higher compared with that of Group II (-0.38 ± 0.15 g/dL).

Haematocrit (Ht)

After 21 days of intake, Group I (-0.03 ± 0.40%) showed statistically significant higher Ht values than did Group II (-1.50 ± 0.29%) ($p < 0.01$).

Eosinophils

A statistically significant difference was found between the test food groups in terms of the change from pre-treatment to post-treatment ($p < 0.05$). Eosinophil count (0.50 ± 0.54%) was higher in Group I compared with Group II (-0.36 ± 0.61%) (**Figure 3A**).

Monocytes

After 7 days of consumption, Group I (6.63 ± 0.51%) showed a statistically significantly higher monocyte value than did Group II (5.00 ± 0.82%) ($p < 0.05$). After 21 days of consumption, Group I (1.93 ± 0.47%) also showed a statistically significant increase compared with Group 2 (0.87 ± 0.21%) ($p < 0.05$) (**Figure 3B**).

CRP

At 21 days, the decrease in CRP was greater in Group I (level= 0.0517 mg/dl) compared with Group II (0.1329 mg/dl), which was statistically significant ($p < 0.05$) (**Figure 3C**).

IL-7

After 7 days of consumption, the IL-7 level was statistically significantly higher ($p < 0.05$) in Group I (4.33 ±0.87 pg/mL) compared with Group II (2.67 ±0.55 pg/mL).

IL-8

Group I's IL-8 values (7.003 ±0.929 pg/mL) were statistically significantly higher than those of Group II (5.230 ±0.469 pg/mL) after 7 days of intake ($p < 0.05$).

D-dimer

After 35 days of intake, the D-dimer decrease in Group I (-0.30 ±0.10 µg/mL) was statistically significantly

In terms of LCR and LeCR, at day 35, the increase from baseline value was greater in Group I compared with Group II (**Figure 4A-C**). The results however were not statistically significant.

II. **N-163:**

1. **Regulation of lipid parameters:**

Total cholesterol (T-Cho):

After 21 days of intake, the T-Cho decrease in Group II (-12.8 ± 4.0 mg/dL) was statistically significantly higher than that of the test food Group I (9.0 ± 12.3 mg/dL) ($p < 0.05$) (**Figure 5A**).

LDL cholesterol (LDL-Cho)

There was a statistically significant decrease in LDL-Cho in Group II, at 124.0 ±25.3 mg/dL, after 21 days of consumption, compared with 134.0 ±25.2 mg/dL before consumption ($p < 0.01$) (**Figure 5B**).

2. **Immuno-modulation and anti-inflammatory effects:**

IL-2

The increase to 0.3743 ± 0.1165 pg/mL after 14 days of post-observation in Group II was statistically significant higher ($p < 0.05$) than the 0.1220 ± 0.0635 pg/mL value in Group 1.

Blood IgA

After 21 days of intake, Group II (340.3 ±64.9 mg/dL) had a statistically significantly higher blood IgA value than did Group I (175.0 ±9.5 mg/dL) ($p < 0.01$).

MCHC

After 7 days of consumption, the MCHC was statistically significantly higher ($p < 0.05$) in Group II (32.56 ± 0.55%) compared with Group I (31.85 ± 0.55%).

Serum galectin, ferritin, and fibrinogen

The decrease in serum fibrinogen, ferritin and galectin-3 was greater in Group II compared with Group I, but the difference was not significant (**Figure 6A-C**).

CD11b

An increase in CD11b in the monocyte fraction was observed in Group II after 21 days of ingestion compared with Group I but it was not statistically significant (**Figure 6D**).

Other parameters: