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Fermentation of Germinated Native Black Rice Milk Mixture by Probiotic Lactic Acid Bacteria

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Abstract**—**This research aimed to demonstrate probiotic germinated native black rice juice fermentation by lactic acid bacteria (*Lactobacillus casei* TISTR 390). Germinated native black rice juice was inoculated with a 24-h old lactic culture and incubated at 30 °C for 72 hours. Changes in pH, acidity, total soluble solid, and viable cell counts during fermentation under controlled conditions at 0-h, 24-h, 48-h, and 72-h fermentations were evaluated. The study found out that the change in pH and total soluble solid of probiotic germinated black rice juice significantly ($p \leq 0.05$) decreased at 72-h fermentation (5.67±0.12 to 2.86±0.04 and 7.00±0.00 to 6.40±0.00 ºbrix at 0-h and 72-h fermentations, respectively). On the other hand, the amount of titratable acidity expressed as lactic acid and the viable cell count significantly ($p \leq 0.05$) increased at 72-h fermentation (0.11±0.06 to 0.43±0.06 (% lactic acid) and 3.60 x 10^6 to 2.75 x 10^8 CFU/ml at 0-h and 72-h fermentations, respectively). Interestingly, the amount of γ-Amino Butyric Acid (GABA) had a significant difference ($p \leq 0.05$) twice as high as that of the control group (0.25±0.01 and 0.13±0.01 mg/100g, respectively). In addition, the free radical scavenging capacity assayed by DPPH method also showed that the IC_{50} values were significantly ($p \leq 0.05$) higher than the control (147.71±0.96 and 202.55±1.24 mg/ml, respectively). After 4 weeks of cold storage at 4 °C, the viable cell counts of lactic acid bacteria reduced to 1.37 x 10^6 CFU/ml. In conclusion, fermented germinated native black rice juice could be served as a healthy beverage for vegans and people who are allergic to cow milk products.

Keywords**—**Germinated native black rice, probiotic, lactic acid bacteria, *Lactobacillus casei*.

I. Introduction

PROBIOTICS have several beneficial effects on human health. Lactic acid bacteria have been proven to exert health-promoting activities such as adjustment of the immune response to a desired level, and enhancement of resistance against pathogens and reduction of blood cholesterol levels [1]. Nowadays, available probiotic products on the market are usually dairy-based and cannot be consumed by individuals who suffer lactose intolerance and allergies due to milk proteins [2]. In addition, with an increase in vegan consumers throughout developed countries, there is also a demand for vegan probiotic products. Moreover, foods such as fruits, vegetables, and grain are reported to contain a wide variety of antioxidant components, including phytochemicals. Phytochemicals, such as phenolic compounds and anthocyanin, are considered beneficial for human health decreasing the risk of degenerative diseases by the reduction of the oxidative stress and inhibition of macromolecular oxidation [1].

Rice (*Oryza sativa* L.) is the principal staple food for more than half of the world's population including Thailand [3]. In fact, Thai farmers grow rice in many areas of Thailand, including the province of Chanthaburi. Native black rice (Khao hawm mae paya tong dum) is a traditional rice variety in the district of Kao Kitchakut in Chanthaburi province. The said rice has a black pigment. In rice pigmentation, anthocyanin is reported to possess a free radical scavenging activity [4]. In addition, black rice contains more nutritional components, (i.e. dietary fibers, phytic acid, and vitamins E and B) than the ordinary milled rice [5]. Moreover, Sangkitikomon et al. reported that anthocyanin from black rice has higher antioxidant activity than red rice and rice berry [6]. For this reason, black rice is a popular ingredient among snacks and desserts [7]. Germinated black rice offers more considerable benefit especially it has an increasing GABA, dietary fiber, inositols, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc, γ-oryzanol and prolylendopeptidase inhibitor. Additionally, the germination of black rice releases its bound minerals, which makes the rice more absorbable by the body and tender and tastier [9].

GABA is a neurotransmitter in the brain and the spinal cord of mammals. This substance can lower hypertension, promote sleepiness and has benefits for human health [8]. On the other hand, there was little information reported in producing non-diary probiotic from germinated native black rice. Therefore, the purpose of this study was to investigate the suitability of germinated native black rice for production of probiotic juice by lactic acid bacteria (*L. casei* TISTR 390). Finally, the amount of antioxidant activity, GABA content, and sensory evaluation were compared between fermented and unfermented germinated native black rice.

II. Materials and Methods

A. Materials

Native black rice (Khao hawm mae paya tong dum) was purchased from a local farmer in the district of Kao Kitchakut in Chanthaburi province and was then transported to the laboratory.

B. Strain and Culture

Probiotic lactic acid bacteria (*L. casei* TISTR 390) were obtained from the Microbiological Resources Center at the Thailand Institute of Scientific and Technological Research in Pathum Thani, Thailand. The culture was grown at 37 °C for

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24 hours in a MRS (de Man Rogosa and Sharpe) broth (dextrose 20.0 g/l, meat peptone 10.0 g/l, beef extract 10.0 g/l, yeast extract 5.0 g/l, sodium acetate 5.0 g/l, disodium phosphate 2.0 g/l, ammonium citrate 2.0 g/l, tween 80 1.0 g/l, magnesium sulphate 0.1 g/l, manganese sulphate 0.05 g/l) and used as inoculum.

C. Germinated Native Black Rice Juice Preparation

Germinated native black rice was prepared by modifying the method of [10]. Briefly, the rice seed was selected and soaked in water on a tray at the ratio of 1:10 (rice:water) at 40 °C for 6 hours. The water was then drained and incubated for 24 hours. The germination was discontinued by drying in a hot air oven at 55 °C for 4.5 hours. The obtained germinated native black rice was cooked in a rice cooker with a ratio of 1:2 (rice:water). The cooked rice was blended by using a blender with a ratio of 1:10 (rice:water) and then the obtained juice was filtrated by using some whites. The germinated native black rice juice was then added with 3.5% (w/v) sugar and 0.35% (w/v) salt and pasteurized for 10 minutes. The obtained juice was transferred in an Elenmeryer flask (250 ml). Finally, the germinated native black rice juice was sterilized in an autoclave at 121 °C for 15 minutes.

D. Fermentation of Probiotic Native Black Rice Medium

Fermentation experiments were preceded in a 250-ml Erlenmeyer flask, each containing 100 ml of the sterilized juice. All samples were inoculated with a 24-h old culture (> 10^6 CFU/ml) and incubated at 30 °C for 72 hours.

E. Effect of Cold Storage on Cell Viability in the Probiotic Native Black Rice Medium

After 72 hours of fermentation at 30 °C, the fermented samples (100 ml) were stored at 4 °C for 4 weeks. Samples were taken at weekly intervals, and the viability of probiotic cultures in probiotic native black rice medium was determined and expressed as colony forming unit (CFU).

F. Chemical and Microbiological Analyses

Samples were taken at 24-h intervals for chemical and microbiological analyses. The pH was measured with a pH meter (Subutex, Taiwan). Total acidity, expressed as percent lactic acid, was determined by titrating with 0.02 N NaOH to pH of 8.2. Total soluble solid was analyzed by hand refractometer (Atago, Japan). Viable cell counts (CFU/ml) were evaluated by the standard plate method with lactobacilli MRS medium after a 48-h inoculation at 30 °C.

G. DPPH Radical Scavenging Activity and GABA Content Determination

The free radical scavenging activity was determined by the method of [11] by Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI) in Bangkok, Thailand. Briefly, one gram of sample was extracted with 10 ml ethanol. The solution was separated by centrifugation at 6,000 rpm. The obtained supernatant was tested by mixing with ethanol at the various concentrations of 10, 20, 30, 40, and 50 µg/ml. The sample (1 ml) was mixed with 0.1 mM DPPH (2.2-diphenyl-1-picrylhydrazyl) solution in 95% ethanol (1 ml) and incubated in dark for 30 minutes. The absorbance was determined by using a spectrophotometer at 517 nm. Vitamin C (L-ascorbic acid), Vitamin E (Tocopherol), and BHT (butylated hydroxytoluene) were used as the reference standard compounds. The percentage of radical scavenging activity was calculated with:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_1)/ A_0] \times 100$$

A_0 = the absorbance of control reaction

A_1 = the absorbance of test compound

The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against the sample concentration. GABA content was sent to be analyzed by the Institute of Food Research and Product Development (IFRPD) at Kasetsart University in Bangkok, Thailand.

H. Sensory Evaluation

The non-diary probiotic germinated native black rice for 3-day fermentation at 30 °C were sensory evaluation and compared with unfermented juice by 50 untrained panelists (staff and students) from the Department of Product Development and Management Technology at Rajamangala University of Technology Tawan-ok in Chanthaburi Campus,. The panelists evaluated the sample using a nine-point hedonic scales ranging from 1 (extremely dislike) to 9 (extremely like) [12]. Each panelist evaluated the samples for color, flavor, taste, texture, and overall acceptability.

I. Data Analysis

Properties analysis was carried out in three replicates. The data were subjected to Analysis of Variance (ANOVA) ($p \leq 0.05$) [13]. Mean with significant differences were separated by Duncan's Multiple Range Test (DMRT) using computer software. For the sensory evaluation, mean with significant differences were compared by T-test.

III. Results and Discussion

Change in pH, acidity, total-soluble solid, and cell viability of *L. casei* TISTR 390 were presented in Table I. *L. casei* TISTR 390, reduced the pH level significantly ($p \leq 0.05$) from an initial value of 5.67±0.12 to 3.37±0.03, 3.05 ±0.06 and 2.86 ±0.04 after 24, 48 and 72 hours fermentation, respectively. Lactic acid bacteria increased the acidity from an initial value of 0.11±0.06% titrable acidity expressed as lactic acid (v/v) to 0.13±0.00, 0.22±0.06 and 0.43±0.06 (%v/v) after 24, 48 and 72 hours fermentation, respectively. For the total soluble solid content, *L. casei* TISTR 390 slowly utilized sugar from 7.00±0.00 to 6.40±0.00 °Brix after 24 hours of fermentation and remained the same at the end of fermentation (72 hours). For the cell viability, lactic acid cultures grew rapidly in the germinated native black rice juice and reached a viable cell population of greater than 2.75×10^8/ml after 72 hours of fermentation at 30 °C.

TABLE I
CHANGE IN PH, ACIDITY, TOTAL-SOLUBLE SOLID AND CELL VIABILITY OF *L. CASEI* TISTR 390 CULTURES IN GERMINATED NATIVE BLACK RICE JUICE

Time (h)	0	24	48	72
pH	5.67±0.12^{a}	3.37±0.03^{b}	3.05±0.06^{c}	2.86±0.04^{d}
Acidity (% lactic acid v/v)	0.11±0.06^{d}	0.13±0.00^{c}	0.22±0.06^{b}	0.43±0.06^{a}
Total soluble solid (Brix)	7.00±0.00^{a}	6.40±0.00^{b}	6.40±0.00^{b}	6.40±0.00^{b}
Viable cell count (CFU /ml)	3.60 x 10^{6b}	2.33 x 10^{7b}	2.79 x $10^{7\,b}$	2.75 x $10^{8\,a}$

Mean with different letters are statistically different (p≤ 0.05) according to DMRT.

Bars represent standard deviation from triplicate determination.

Table II shows the effect of cold storage on the cell viability of *L. casei* TISTR 390 in fermented germinated native black rice juice. The viable cell counts of lactic acid bacteria were also higher than 10^6/ml even after 4 weeks of cold storage at 4 °C. The viable cell counts of lactic acid bacteria sharply decreased significantly from 2.57 x 10^{10}/ml to 3.55 x 10^7, 2.43x10^7, and 1.37 x10^6/ml after 2, 3, and 4 weeks, respectively. In addition, antioxidant activity and GABA content were compared between probiotic germinated native black rice juice and unfermented juice. The result found out that fermented sample had significantly (p ≤ 0.05) higher free radical scavenging capacity than the unfermented sample: IC_{50} values had 147.71±0.96 and 202.55±1.24 mg/ml, respectively. Remarkably, the amount of GABA had a significant difference (p≤0.05) two-fold as high as that of the control group (0.25±0.01 and 0.13±0.01 mg/100g, respectively) (Table III). However, for sensory evaluation, fermented native black rice juice exhibited lower scores in colour, germinated aroma, taste, and acceptability than the unfermented germinated native black rice (Table IV). This could be due to the age factor of most consumer panelists, the students, who normally do not like fermented rice juice. Therefore, the acceptability scores were lower.

TABLE II
EFFECT OF COLD STORAGE ON THE CELL VIABILITY OF *L. CASEI* TISTR 390 CULTURES IN GERMINATED NATIVE BLACK RICE JUICE

Time (week)	Survival (CFU/ml)
0	2.75 x $10^{8\,b}$
1	2.57 x $10^{10\,a}$
2	3.55 x $10^{7\,b}$
3	2.43 x $10^{7\,b}$
4	1.37 x $10^{6\,c}$

Mean with different letters are statistically different (p≤ 0.05) according to DMRT.

TABLE III
ANTIOXIDANT ACTIVITY AND GABA CONTENT OF NON-DIARY PROBIOTIC GERMINATED NATIVE BLACK RICE JUICE

Treatments	DPPH assay IC50 (mg/ml)	GABA content (mg/100g)
control	202.55 ±1.24^{b}	0.13 ±0.01^{a}
Probiotic germinated black rice juice	147.71±0.96^{a}	0.25± 0.01^{b}

Mean with different letters are statistically different (p≤ 0.05) according to T-test.

Each data represents mean of three replications with standard error.

TABLE IV
MEAN SENSORY SCORES OF NON-DIARY PROBIOTIC GERMINATED NATIVE BLACK RICE JUICE

Time (h)	Colour	Aroma	Taste	Texture	Acceptability
control	6.49^{a}	7.00^{a}	6.51^{a}	6.55^{a}	6.96^{a}
Probiotic germinated black rice juice	6.24^{b}	5.29^{b}	4.59^{b}	5.49^{b}	5.33^{b}

Mean with different letters are statistically different (p≤ 0.05) according to T-test.

IV. CONCLUSION

The results showed that lactic acid bacteria (*L. casei* TISTR 390) was found out to be capable of rapidly utilizing germinated native black rice juice for the cell synthesis and lactic acid production. It reduced the pH to as low as 2.86 and increased the acidity to as high as 0.43%, and the viable cell counts (CFU) reached 10^8/ml after fermentation of 72 hours at 30 °C. *L. casei* TISTR 390 survived under the low pH and high acidity conditions during 4 weeks of cold storage at 4 °C. Interestingly, the antioxidant activity (DPPH assay) and the amount of GABA of fermented germinated native black rice juice had significantly (p ≤ 0.05) higher than that the control group. In conclusion, fermented germinated native black rice juice could be used as a good raw material for lactic acid fermentation. The product could also serve as a healthy beverage for vegans and milk allergic consumers. However, the taste could be improved to suit other consumers' preference.

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