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2 Study on Synbiotic Beverages Based on Local Seeds and Oyster Mushrooms with the Addition of *Lactobacillus casei*

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1 ABSTRACT

Beverages are one of the most accessible liquid foods, can provide nutrients such as vitamins, minerals, antioxidants, organic acids and other bioactive substances for the body. To meet this need, the development of synbiotic drinks made from local grains is urgently needed. Synbiotic drink which is a combination of grain-based probiotics and prebiotics also has advantages for consumption for people with lactose intolerance as a substitute for dairy milk. This study was aimed to evaluate the effect of *Lactobacillus casei* with various concentration on synbiotic drinks based on local grains and cereals and oyster mushroom. Another aim from this study was to find out the best treatment for the characteristics according to SNI 7552:2018. In this study, 6 levels formulation of *L. casei* concentration (0%, 2%, 3%, 4%, 5%, 6%) were used. The experiment was non-factorial and arranged in Completely Randomized Block Design with 4 replications. The data homogeneity was tested using the Bartlett's test and data additivity was tested with the Tukey test. The data were then analyzed for variance and further processed using Least Significant Difference test (LSD) at 5% level. The results showed synbiotic drink with 4% of *L. casei* found to be the best treatment which had a total LAB of 9.83 log CFU/mL, 0.76% lactic acid, 3.52 pH with a color score of 3.53 (rather like), a aroma score of 3.32 (neutral), a taste score of 2.34 (neutral) and an overall acceptance score of 2.83 (neutral).

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1. INTRODUCTION

As food science and technology develops, it provides awareness to the public of the importance of changing towards a healthy lifestyle by adopting a good diet. Consumption of unsafe food is usually caused by the presence of harmful ingredients such as preservatives, colorant, and artificial sweeteners which may result in various health problems, especially digestive tract diseases. Gastrointestinal tract disease is a major contributor that affects many people, especially children, caused by a lack of fiber

consumption from both vegetables and fruit. A good food consumption pattern can be done by adopting a healthy lifestyle and consuming plant-based functional foods such as whole grains, legumes, cereals, fruit and vegetables (Sebastiani *et al.*, 2019). This has led to the development of plant-based synbiotic beverage innovations.

Synbiotic is a product of combining probiotics and prebiotics which has better benefits for the body, but grain-based synbiotic products have not been widely developed. Probiotic drinks are popular functional drinks with microbial content that is beneficial to health when consumed in sufficient quantities (Hill *et al.*, 2014). The benefits of probiotic drinks are obtained from the content of lactic acid bacteria (LAB) strains that are often used including *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* (Fijan, 2014). The use of probiotics as a fermented drink is widely known in the food industries, but this probiotic drink has a weakness, namely the viability problem of microbial survival in the digestive tract. The number of probiotic living cells in the digestive tract will decrease due to several factors such as increased acid production resulting in a decrease of pH and decreased LAB viability due to nutrient depletion by insufficient substrate available (Nakkarach & Ulaiwan, 2018). One way to overcome the adequacy of nutrition for these microbes is to add ingredients containing prebiotics.

Prebiotics are food ingredients that can be found in grains, fruits, and vegetables in which there are components that cannot be digested, but can stimulate the growth of probiotic microorganisms in the digestive tract (Gourbeyre *et al.*, 2011). Weaknesses of prebiotic drinks include unbalanced nutritional content for health due to lack of protein content, low bioavailability of vitamins and minerals due to several anti-nutritional substances in the food ingredients used (Aydar *et al.*, 2020). The deficiencies found in probiotic and prebiotic drinks can be overcome by making synbiotic drinks as a solution to improve and increase nutritional value. This can be done by fermentation using the addition of LAB to improve the bioavailability of vitamins and minerals in grain milk and increase protein digestibility by microbes.

Processed products from red beans, black glutinous rice, black sesame and oyster mushrooms are potential vegetable ingredients when processed into prebiotic milk and synbiotic drinks. The use of red beans, black glutinous rice, black sesame and oyster mushrooms which contain carbohydrates, protein, vitamins, minerals, and bioactive components can act as nutrient-rich ingredients for probiotic bacteria during fermentation. The use of oyster mushrooms besides aiming to increase the nutritional value, especially amino acids, is also expected to increase the added value of mushrooms which have a short shelf life. Aufkha & Purbasari (2021) reported that the texture of oyster mushrooms packaged in PE plastic without holes was no longer able to maintain the texture on the fourth day of storage. One of the LAB that can be used to make synbiotic drinks is the bacterium *Lactobacillus casei*. *Lactobacillus casei* is a homofermentative type of lactic acid bacteria that has a higher fermentation ability compared to other types of bacteria to break down glucose into lactic acid in ingredients up to 90% (Afzal *et al.*, 2011). However, according to Umam *et al.* (2012) the use of the right concentration of LAB for a drink so that it can be categorized as a synbiotic is when it reaches a minimum value of probiotic strains that can reach the digestive tract of 10⁶-10⁷ CFU/mL. Therefore this study aims to develop a functional drink from a mixture of red beans, black glutinous rice, black sesame, and oyster mushrooms as a blend to provide quality synbiotic drinks with the right starter concentration and be accepted by consumers.

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2. MATERIALS AND METHODS

The materials used in this study were red beans, black glutinous rice, oyster mushrooms, inulin, *Lactobacillus casei* (v/v) culture, skim milk, glucose, MRSB media, MRSB, distilled water, PP, NaCl, and 70% alcohol. The tools used in this study were analytical balances, soymilk maker, refrigerator, test tubes, petri dishes, incubators (Heraeus), autoclaves, bunsen and staves, pipette tips, knives, pH meters, micropipette, tissue, filter cloth, hot plate, glass bottle, stopwatch, colony counter, vortex mixer VM-300, measuring cup, beaker glass and Erlenmeyer.

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2.1. Experimental Design

The single factor study was designed using a Completely Randomized Block Design arranged with 4 repetitions. The factor used was the concentration of *L. casei* (L) starter, namely L1 (0%), L2 (2%), L3 (3%), L4 (4%), L5 (5%), and L6 (6%) in (v/v). The data obtained were analyzed for homogeneity with Bartlett's test and the additivity was tested with Tukey's test, then the data were analyzed for variance to find out the estimators of variance of error, and to find out the effect of the treatments. The data were analyzed further with the least significant difference test (LSD) at the level of significance $\alpha = 5\%$. The observations included microbiological properties, namely total LAB, chemical properties including pH, total lactic acid, and organoleptic tests. The best treatment was further analyzed by determining the proximate test (water content, protein, fat, carbohydrates and ash) as well as testing the content of dietary fiber.

2.2. Starter Preparation

One ose of pure *Lactobacillus casei* culture was inoculated into a test tube containing sterile MRS Broth media and incubated for 48 h at 37 °C for culture rejuvenation. Bacterial culture was inoculated with 4% (v/v) to be grown into 5% (w/v) pasteurized skim milk medium at 121 °C for 15 min, and incubated for 48 h at 37 °C. The resulting culture is called the main culture, which then was inoculated into 5% (w/v) sterile skim milk medium and 100 mL of grain extract with the addition of 3% (w/v) glucose, then incubated for 48 h at temperature 37 °C to obtain a working culture. The working cultures used in the manufacture of synbiotic drinks were based on (Rizal et al., 2016) with slight modifications, namely 0%, 2%, 3%, 4%, 5%, and 6% (v/v).

2.3. Preparation of Grain and Oyster Mushroom Extract

The milk extraction process from red bean, black glutinous rice, black sesame grains, and oyster mushrooms was based on previous research (Yustiana et al., 2022). Red beans (60 g) and black glutinous rice (30 g) were washed with clean water to remove dirt and soaked in water for 6 h, then drained. Oyster mushroom (30 g) was sorted and washed to be mixed into the soymilk maker with 30 g of black sesame, then it was made up to 1000 mL with water and cooked for 30 min. The results of cooking with the soymilk maker were filtered with a filter cloth. The filtrate was added with 30 g of inulin (Yustiana et al., 2022).

2.4. Synbiotic Beverage Manufacturing Process

As much as 100 mL of extract of oyster mushroom and grains was pasteurized at 70 °C for 15 min and cooled to near room temperature. Next, the working cultures of *Lactobacillus casei* were inoculated with concentrations of 0, 2, 3, 4, 5, 6% (v/v) and incubated at 37 °C for 48 h (Rizal et al., 2016, with modifications).

2.5. Analysis of Total LAB

The synbiotic drink sample was diluted in stepwise. As much as 1 mL of the synbiotic drink was put into 9 mL of sterile physiological saline, then homogenized and 1 mL of the solution was taken from the first tube and then put into a second test tube containing 9 mL of sterile physiological saline to obtain a 10⁻¹ dilution. And so on until it reaches the desired dilution (10⁻⁸ to 10⁻¹⁰). A sample of the solution from the desired dilution is taken 1 mL using a pipette and then put into a sterile petri dish then added 10-15 mL of sterile MRS Agar medium. The cup that already contains the media and sample is leveled by moving it vertically to form a figure 8 and left to freeze. Petri dishes were incubated at 37 °C for 48 h in an inverted position to prevent microbes from being exposed to moisture during the incubation process, then the number of colonies was counted (Rahayu & Nurwitri, 2012).

2.6. Analysis of Total Lactic Acid

As much as 1 mL of sample was put into Erlenmeyer and diluted with 10 mL of distilled water and added 2 drops of phenolphthalein indicator (PP). The mixture is then titrated with 0.1 N NaOH solution, and the end point of the titration is determined after a constant pink color is formed (AOAC, 2012).

2.7. Degree of Acidity (pH)

Testing the pH of synbiotic drinks from grain extracts and oyster mushrooms was carried out using a pH meter (AOAC, 2012).

2.8. Sensory Test

The sensory assessment of synbiotic drinks from the extract of grains and oyster mushroom was carried out by hedonic test (level of preference) covering color, aroma, taste and overall acceptance. The sensory test was carried out after 48 hours of fermentation of the synbiotic drink. Tests were carried out by 25 semi-trained panelists, 19 of whom were female and 6 male. The age of the panelists is between 18-19 years (students who have taken the Sensory Test course). Samples were coded with 3 numbers and served randomly in plastic cups of uniform size (Setyaningsih et al., 2010).

2.9. Proximate Analysis

Proximate analysis in this study was carried out on the results of the synbiotic drink from the best treatment, namely with a starter concentration of *Lactobacillus casei* 4%. The proximate test was carried out at the Saraswanti Indo Genetech Laboratory (Bogor) with parameters and standard methods as shown in Table 1.

Table 1. Proximate test parameters and methods adopted

Proximate parameter	Standard method
Water content	SNI 01-2891-1992 point 5.1.
Protein content	18-8-31/MU/SMM-SIG (Kjeltech)
Fat content	18-8-5/MU/SMM-SIG point 3.3.3 (Weibul)
Carbohydrate content	FAO 2003 Food Energy methods of analysis and conversion factors
Ash content	SNI 01-2891-1992 point 6.1.

2.10. Dietary Fiber Test

Analysis to determine the levels of dietary fiber was also carried out only on the best results, namely synbiotic drinks resulted from treatment with a starter concentration of 4% *Lactobacillus casei*. The analysis was also conducted at the Saraswanti Indogenetech Laboratory (Bogor) used the standard method 18-8-6-2/MU/SMM-SIG.

3. RESULTS AND DISCUSSION

3.1. Chemical Properties

Table 2 presents chemical characteristic of symbiotic drinks resulted from different treatment of concentrations of *Lactobacillus casei* starter. The properties to be analyzed including Total Lactic Acid Bacteria (LAB), Total Lactic Acid, and pH.

Table 2. Total lactic acid bacteria at various concentrations of *Lactobacillus casei* starter

Treatment	LAB (log CFU/mL)	Total Lactic Acid (%)	pH
<i>Lactobacillus casei</i> 6%	10.07 a	0.99 a	3.24 e
<i>Lactobacillus casei</i> 5%	10.03 a	0.85 b	3.38 d
<i>Lactobacillus casei</i> 4%	9.83 b	0.76 c	3.52 c
<i>Lactobacillus casei</i> 3%	9.73 bc	0.69 d	3.55 c
<i>Lactobacillus casei</i> 2%	9.61 c	0.57 e	3.55 c
<i>Lactobacillus casei</i> 0%	9.36 d	0.31 f	3.77 a
BNT ($\alpha = 0.05$)	0.19	0.04	0.03

Note: The average value followed by the same letter in the same column is not significantly different on the LSD test with $\alpha = 0.05$.

3.1.1. Total Lactic Acid Bacteria (LAB)

The total LAB of the synbiotic drink from mixed-grain and oyster mushroom extracts increased due to the addition of starter concentration during the fermentation process (Table 1). This is because the number of bacteria is proportional to the concentration: the higher the concentration of the starter added, the higher the number of bacteria that will grow so that the total number of LAB also increases. *Lactobacillus casei* is a lactic acid bacterium that requires carbohydrates as a carbon source in the formation of metabolites during its growth. According to Ranadheera et al. (2017) Oyster mushrooms and grains are a type of prebiotic which contain various carbohydrates for the growth of probiotic bacteria such as fructooligosaccharides, galactooligosaccharides, raffinose, inulin and several types of non-digestible peptides.

According to Pranayanti & Sutrisno (2015) with the availability of sufficient amounts of nutrients derived from the prebiotic ingredients used can trigger the growth of lactic acid bacteria. Prebiotic ingredients from red beans, black glutinous rice, black sesame, and oyster mushrooms in this study were able to enhance the probiotic function of *L. casei* bacteria by increasing the viability of the bacteria to grow and their vitality in the digestive tract in the human body (Ramchandra & Shah 2010). The total LAB value of the synbiotic drinks in this study ranged from 9.36 log CFU/mL (2.33×10^9 CFU/mL) to 10.07 log CFU/ml (11.73×10^9 CFU/mL). Based on the results of this study, the total LAB value of all treatments with different concentrations of starter complied with the

stipulated SNI for flavored lactic fermented drinks, namely a minimum of 1×10^6 CFU/mL (SNI 7552: 2018).

3.1.2. Total Lactic Acid

The total lactic acid value in the synbiotic drink in this study ranged from 0.31-0.99% (Table 2) with a tendency for total lactic acid to increase as the number of starters was increased. The high or low levels of lactic acid obtained depend on the amount of starter and the ability of the starter used to form lactic acid during the 48 h fermentation process.

Lactic acid bacteria have different abilities to produce acid, depending on the starter species, the conditions and composition of the media and the environment. *Lactobacillus casei* used as a starter in this study is a group of bacteria that produce large amounts of lactic acid as the end result of sugar or carbohydrate metabolism (Suharyono *et al.*, 2012). According to Rachman *et al.* (2018) starter lactic acid bacteria such as *Lactobacillus casei* will carry out the process of breaking down carbohydrates and simple sugars through continued metabolism so that they can produce lactic acid and other organic acids which have an impact on the total acid value. Based on the results of this study, it was shown that the total lactic acid value with the number of different concentrations of starter in all samples of synbiotic drinks from extract of grains and oyster mushrooms complied with the stipulated SNI for fermented drinks, namely a minimum of 0.2-0.9% (SNI 7552: 2018). The higher the concentration of the LAB starter, the higher the total lactic acid due to the activity of the bacteria. This is supported by the lower pH value along with increasing levels of lactic acid. The starter concentration added in the manufacture of this synbiotic drink is still within normal limits for using nutrients from grains and oyster mushrooms and does not exceed the nutritional requirements, so that optimization in the formation of lactic acid does not decrease and meets quality requirements.

3.1.3. pH value

The results of the analysis showed that the pH of the synbiotic drink treated with various concentrations of *Lactobacillus casei* ranged from 3.24 to 3.77 (Table 2). The pH value obtained is still within the normal threshold from the results of lactic acid bacteria activity, which is around 3-6 (Rasbawati *et al.*, 2019). The pH value tends to decrease with increasing concentration of the starter given to each treatment of the synbiotic drink from grains and oyster mushroom extracts. This is due to the activity of lactic acid bacteria by *L. casei* which continuously produces lactic acid and other organic acids so that the product becomes acidic and the pH tends to decrease.

Lactobacillus casei is a group of facultative homofermentative lactic acid bacteria where almost all of the glucose in the material is converted to lactic acid through the Embden-Meyerhof metabolism in producing lactic acid (Rachman *et al.*, 2018). The lactic acid bacteria will be excreted out of the cell and produce lactic acid which accumulates in the fermentation medium by producing a sour taste through the release of H^+ protons which causes a decrease in the pH value (Sintsari *et al.*, 2014).

3.2. Sensory Test

Table 3 reveals hedonic score of symbiotic drinks resulted from different treatment of concentrations of *Lactobacillus casei* starter. Sensory parameters included color, aroma, taste, and overall acceptance.

Table 3. Hedonic score for synbiotic drink produced from various concentrations of *Lactobacillus casei* starter

Treatment	Color	Aroma	Taste	Overall Acceptance
<i>Lactobacillus casei</i> 6%	3.26 c	2.96 c	2.29 cd	2.67 c
<i>Lactobacillus casei</i> 5%	2.87 d	2.98 bc	2.43 b	2.99 a
<i>Lactobacillus casei</i> 4%	3.53 a	3.32 a	2.34 bc	2.83 b
<i>Lactobacillus casei</i> 3%	3.57 a	3.05 b	2.20 d	2.66 c
<i>Lactobacillus casei</i> 2%	3.33 b	3.33 a	2.75 a	2.95 a
<i>Lactobacillus casei</i> 0%	2.53 e	1.94 d	2.08 e	1.94 d
BNT ($\alpha = 0.05$)	0.05	0.07	0.09	0.11

Note: The average value followed by the same letter in the same column is not significantly different on the LSD test with $\alpha = 0.05$.

3.2.1. Color

The color score of the synbiotic drink from grains and oyster mushroom extracts ranged from 2.53 to 3.56 (Table 3). The color of the synbiotic drink produced in each treatment tends to vary from black to brownish black caused by the basic ingredients and composition in the product manufacturing process. The average rating of the color given by the panelists ranged from 2.53 (dislike) to 3.57 (rather like). Red bean with the most composition compared to other ingredients causes the color of the drinks tend to be dark red or brownish. Meanwhile, the black color in the synbiotic drink is caused by the presence of anthocyanin color pigments in black glutinous rice which are metabolized by the *Lactobacillus casei* bacteria so that anthocyanins are degraded due to changes in pH, temperature, and the presence of oxygen and light during incubation (Hardoko et al., 2010).

3.2.2. Aroma

The score for the aroma of the synbiotic drink from grains and oyster mushroom extracts was in between 1.94-3.33 (Table 3). Increasing the concentration of starter in the manufacture of synbiotic drinks causes a decrease in the level of preference for aroma. This is presumably due to the aroma of lactic acid which is formed due to the activity of lactic acid bacteria. According to Karti et al. (2018), lactic acid bacteria play a role in the formation of the distinctive sour aroma of yogurt produced by the presence of lactic acid, acetaldehyde, diacetyl, acetic acid, and volatile compounds obtained from the bacterial fermentation process. Based on the organoleptic test, the aroma of the synbiotic drink with increasing concentration of starter was concluded in general by the panelists, namely that it smelled sour which was quite pungent and unpleasant.

The more starter that is added, the stronger the fermented characteristic sour aroma which causes a decrease in the panelist's preference level. Panelists tend to prefer fermented drinks with a sour aroma that is not too strong. This is in line with Rizal et al. (2016) who stated that the characteristic aroma of synbiotic products is obtained from lactic acid as a result of LAB metabolism in providing sharpness of taste and aroma that occurs due to the presence of volatile components in the product's basic ingredients. The unpleasant aroma wafting from the synbiotic drink is a distinctive odor from grains caused by the activity of the lipoxygenase enzyme found in red beans and the volatile content in black glutinous rice, black sesame and oyster mushrooms during the fermentation process by *Lactobacillus casei* bacteria.

3.2.3. Taste

The score for the taste of the synbiotic drinks produced in this study ranged from 2.08 to 2.75 (Table 3). Sayuti *et al.* (2013) stated that the sour taste in the fermented products obtained comes from lactic acid as a result of the cellular metabolism of lactic acid bacteria. The increase in the amount of lactic acid that is formed occurs due to the high number of bacteria that work so that it affects the decrease in the pH value which causes the product to taste more sour. This is because the taste of the synbiotic drink is closely related to the total lactic acid and the pH value where the higher the total lactic acid obtained, the lower the product pH value and the more sour the taste of the synbiotic drink.

3.2.4. Overall Acceptance

Overall acceptance is the assessment given by the panelists. The test results showed that the panelist's level of overall preference for synbiotic beverage products ranged from 1.94-2.99 (dislike to slightly like). The results of the preference test can be seen in Table 3. The level of overall preference with the right starter concentration is rather favorable with the treatment of the starter concentration used not too high or low because the panelists were still able to accept it in terms of color, aroma, and taste. The difference in the level of panelist overall acceptance of the synbiotic drink was due to the difference of the concentration of the addition of *Lactobacillus casei*.

3.5. Best Treatment

Selection for the best treatment in this study was carried out using the Hierarchy Process Analysis method which was carried out by determining targets or criteria in the decision component variables through iteration processes I and II to determine the Eigen value of how much influence a variable has. The results of the analysis showed that the synbiotic drink made from grains and oyster mushroom extracts treated with the addition of *Lactobacillus casei* starter at a concentration (L4) of 4% was the best.

3.6. Proximate content

Proximate testing of synbiotic grain and oyster mushroom drinks with the best treatment, namely a concentration of 4% *Lactobacillus casei* was carried out to determine the chemical components of the beverage product. The proximate analysis results obtained in the form of water, protein, fat, carbohydrates and ash content are presented in Table 4.

Table 4. Proximate content of synbiotic drinks from grains and oyster mushroom extracts with the best treatment (4% starter *Lactobacillus casei*)

Parameter	Value	SNI
Water (%)	86,94	-
Protein (%)	1,65	Min 1 %
Fat (%)	1,15	0,6-2,9 %
Carbohydrate (%)	9,96	-
Ash (%)	0,30	Max 1 %

3.6.1. Dietary Fiber

The test results for dietary fiber content in the best treatment of synbiotic drinks from grains and oyster mushroom extracts were 2.62%. The decrease in dietary fiber content in the synbiotic drink was influenced by the addition of lactic acid bacteria which indicated that LAB uses dietary fiber in the product as a nutrient for its growth. The dietary fiber content of this synbiotic drink is higher when compared to other grains-based drinks. Vanga & Raghavan (2018) reported that the dietary fiber contents of almond milk, soy milk, and coconut milk were 0.64, 0.96, 0.25%, respectively.

4. CONCLUSION

The conclusion from this study was that the concentration of starter *Lactobacillus casei* significantly affected the total lactic acid bacteria, total lactic acid and pH in the synbiotic drink made from grains and oyster mushroom extracts. The best synbiotic drink was obtained at a starter concentration of *Lactobacillus casei* 4% with a color score of 3.53 (rather like), aroma score 3.32 (rather like), taste score 2.34 (rather like), overall acceptance score 2.83 (kinda like). Furthermore, the best synbiotic drink from grains and oyster mushroom extracts has a water content of 86.94%, protein 1.65%, fat 1.15%, carbohydrates 9.96%, ash content 0.30%, and fiber 2.62%. All parameters comply with SNI so that this drink has the potential to be developed and promoted as a health drink.

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