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NEW GROWTH MEDIUM FOR CULTURING LACTIC ACID BACTERIA AS PROBIOTIC CONSORTIUM ISOLATED FROM FERMENTED FISH (BUDU)

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ABSTRACT: This study aimed to obtain the best ratio of inoculums and types of alternative media in increasing the growth of the probiotic consortium with the observed variables consisting of viability, cell biomass and decrease in pH. Completely randomized design (CRD) factorial consisting of 2 factors with 3 replications, factor A were the probiotic consortium (A1: *Lactobacillus parabuchneri*: *L. buchneri*: *L. harbinensis*, *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner)* with ratio 1:1:1:1:1; A2: same consortium with ratio 1:1:1:1:2; A3: same consortium with ratio 1:1:1:2:1; A4: same consortium with ratio 1:1:2:1:1; A5: same consortium with ratio 1:2:1:1:1; A6: same consortium with ratio 2:1:1:1:1 and factor B were the type of alternative media (B1=control; B2=coconut water (90%) + cassava flour (5%) + fish waste flour (5%); B3=tofu liquid waste (90%) + flour onggok (5%) + fish waste meal (5%); B4= tofu whey (90%) + onggok flour (5%) + fish waste meal (5%). The results showed that there was an interaction between factor A and factor B which was highly significant ($P<0.01$) on viability, cell biomass and decrease in medium pH. In conclusion, the best ratio of probiotic consortium was 1:1:1:2:1, with growth medium coconut water (90%) + cassava flour (5%) + fish waste flour (5%) which resulted in a viability value of: 3, 02, cell biomass: 22.47 mg/ml and a decrease in the pH of the medium by 2.84.

Keywords: Cell biomass, Fermentation, Medium pH, Probiotic consortium, Viability.

INTRODUCTION

The safe criteria for probiotics are non-toxic and non-pathogenic, have clear taxonomic identification, can live in the target species, can survive, colonize and metabolize actively in the target indicated by resistance to digestive juices and bile salts, persistence in the digestive tract, and competition, with the host microflora, produce antimicrobial compounds, antagonist against pathogens, can change the immune response, does not change and is stable during storage and field processes (Gaggia et al., 2010). The lactic acid bacteria need a source of carbon and nitrogen as well as minerals and vitamins (Mıdık et al., 2020). The MRS (de Man Ragosa and Sharpe), is a selective medium for the growth of lactic acid bacteria, but it is expensive to use commercially and on a large scale (Champagne et al. 1991).

This research was to look a good growth characteristics of the consortium probiotics which have the potential to be made on an industrial scale with a special purpose for achieve high cell concentrations and medium prices that are cheap and easy to obtain by utilizing local resources such as various agricultural and industrial wastes. The similar study was reported by Champagne et al. (1991) which the production of probiotics in large quantities and used on an industrial scale is constrained by limited costs, cell production efficiency and difficulty in harvesting. Coghetto et al. (2016) added that probiotic cells can be produced using alternative carbon and nitrogen sources, such as agro-industrial residues, such as tapioca factory waste, fish offal meal, shrimp shell meal and other waste materials.

The waste or agro-industrial residues must contain a number of food substances that can support the growth of probiotic microbes such as carbon, protein and mineral sources such as tofu liquid waste, fish waste flour, coconut water and cassava flour, and shrimp waste flour. Marlida et al. (2022) found that liquid coconut waste, tapioca waste and shrimp shell waste, the best medium for good growth and high cell concentration of mixes probiotics of *Lactobacillus plantarrum* and *Saccharomyces cereviceae*. Heenan et al. (2002) reported that growth medium for culturing probiotic bacteria for applications in vegetarian food products such as media containing 25 g/L soy peptone, yeast extract and glucose monohydrate suitable for *Lactobacillus acidophilus*, *L. paracasei* ssp. *paracasei* and *Bifidobacterium lactis* better than commonly used media in laboratory MRS and RCM. Anggraini et al. (2019) added that the best natural growing medium for lactic acid bacteria in the *Pedioccocus acidilacti* group is tofu and palm sugar liquid waste, where the best concentration is 100% tofu liquid waste and 15% palm sugar resulting in the production of gamma-aminobutyric acid

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(GABA) up to 311,485 mg/L. It has been found that 5 isolates of lactic acid bacteria have probiotic abilities, where the 5 isolates are able to live at low pH, in 0.3% bile fluid, are able to stick to the walls of the small intestine and are able to kill pathogenic bacteria such as *E.coli, S. aureus* and *Salmonella enteridis* (Susalam et al., 2022). In the future, the five isolates will be made into a probiotic consortium that is ready to be applied and commercialized. To achieve this goal, it is necessary to find an alternative medium so that the five isolates can grow together with the right balance. The purpose of this study was to find alternative medium and a ratio of the five isolates of lactic acid bacteria isolated from fish fermented (budu) a traditional fermented food from West Sumatera, Indonesia that they play an optimal role as a probiotic consortium.

MATERIALS AND METHODS

Samples

Lactobacillus parabuchneri, *L. buchneri*, *L. harbinensis*, *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner* were used as starter cultures. They were obtained from the Laboratory of Feed and Technology, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia. The cultures were stored in a 10% skim milk mixture and 1% sucrose under -20 °C. Alternative materials such as whey tofu, coconut water, tofu liquid waste, fish waste flour and cassava flour were purchased from the local market.

Chemicals

Chemicals used in this study were MRS Broth medium (de Man Rogosa and Sharpe Broth), PDA (Potatoes Dextrose Agar), and PDB (Potatoes Dextrose Broth). All media used were also purchased from Merk, Germany.

Animals and biological material

Biological materials involved in this study were *L. parabuchneri*, *L. buchneri*, *L. harbinensis*, *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner* of our own collection isolated from the previous study.

Experimental design

The completely randomized design (CRD) factorial consisting of 2 factors with 3 replications, factor A were the probiotic consortium (A1: *L. parabuchneri*: *L. buchneri*: *L. harbinensis*, *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner* with ratio 1:1:1:1:1; A2: same consortium with ratio 1:1:1:1:2; A3: same consortium with ratio 1:1:1:2:1; A4: same consortium with ratio 1:1:2:1:1; A5: same consortium with ratio 1:2:1:1:1; A6 same consortium with ratio 2:1:1:1:1 and factor B were the type of alternative media (B1=control; B2=coconut water (90%) + cassava flour (5%) + fish waste flour (5%); B3=tofu liquid waste (90%) + flour onggok (5%) + fish waste meal (5%); B4= tofu whey (90%) + onggok flour (5%) + fish waste meal (5%). The observed variables were viability, cell biomass and decrease in pH.

The measured parameters

Cell viability and cell biomass assay were measured according to Pires et al (2013). pH was measured for each natural medium (Matouskova et al., 2021). The all founded data were analyzed using analysis of variance for a Completely Randomized Design. The significance of differences between treatments were tested with Duncan's Multiple Range Test (DMRT), with a significant value at P>0.05 (Steel and Torrie, 1995).

Sample preparation

There were three alternative media: 1) the media based on coconut water consisted of coconut water, cassava flour and fish waste flour; 2) the media based on tofu liquid waste consisted of tofu liquid waste, cassava flour, and fish waste flour; 3) the media based on whey tofu consisted of whey tofu, cassava flour and fish waste flour. The combination of *Lactobacillus Parabuchneri*, *Lactobacillus Buchneri*, *Lactobacillus Harbinensis*, *Schieferilactobacillus Harbinensis* and *Lentilactobacillus Parabuchner* (probiotic consortium) were based on the TPC (Total Plate Count) results and were divided into six ratios, namely 1:1:1:1:1; 1:1:1:1:2; 1:1:1:2:1; 1:1:2:1:1; 1:2:1:1:1 and 2:1:1:1:1, cultured on MRS-B and incubated at 37 °C for 24 hours. The experiment was triplicated.

RESULTS AND DISCUSSUON

Based on research that has been done regarding the effect of different media in probiotics consortium ratio on viability, it can be seen in Table 1. Statistical analysis showed that there was an interaction between factor A (probiotic consortium ratio) and factor B (type of media) significantly different (P<0.01) on viability. In the A3B2 treatment, the viability was higher than all treatments, namely 3.02. This value was not significantly different (P>0.05) with treatment A3B1, but significantly different (P<0.01) with A3B3 and A3B4. The stability of the viability value of the probiotic consortium is thought to be influenced by the nutritional value content in B2 media, which has an abundant carbon source due to a combination of coconut water waste, cassava flour, fish waste flour. The ratio of probiotic consortium ini A3 was dominated by *Schieferilactobacillus Harbinensis*. Colares et al. (2021) reported that *S. harbinensis* Ca12 is one of the

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probiotics that has passed resistance tests at low pH, bile salts, different candida tests, aggregation, coaggregation, has high adhesion to HT-29 cells, and inhibits HT-29 cell *Samonella* growth. Colares et al. (2021) added *S. harbinensis* Ca12 to grow optimally, supplemented with whey was marked by an increase in cell biomass of around 600%.

Table 1- Average consortium probiotic viability on different media and probiotic ratio

Probiotics* \ Different growth medium	B1: (Oxoid (CM 359)	B2: (Coconut water (90%) + cassava flour (5%) + fish waste flour (5%)	B3: (Tofu liquid waste (90%) + cassava flour (5%) + fish waste flour (5%)	B4: (Whey tofu (90%) + cassava flour (5%) + fish waste flour (5%)	Average
A1: consortium probiotic* with ration 1:1:1:1:1	2.71^{ab}	2.79^{a}	1.40^{de}	1.58^{de}	2.16^{a}
A2: consortium probiotic* with ration 1:1:1:1:2	2.91^{a}	2.68^{ab}	1.38^{e}	1.30^{e}	2.07^{a}
A3: consortium probiotic* with ration 1:1:1:2:1	2.58^{ab}	3.02^{a}	1.28^{e}	1.16^{e}	2.01^{ab}
A4: consortium probiotic* with ration 1:1:2:1:1	1.68^{d}	2.76^{a}	1.20^{e}	1.27^{e}	1.73^{b}
A5: consortium probiotic* with ration 1:2:1:1:1	1.95^{cd}	2.76^{a}	1.32^{e}	1.19^{e}	1.80^{ab}
A6: consortium probiotic* with ration 2:1:1:1:1	2.00^{c}	2.76^{a}	1.32^{e}	1.20^{e}	1.82^{ab}
Average	2.31^{b}	2.80^{a}	1.34^{c}	1.28^{d}	

Note: different superscripts show highly significant different effects (P<0.01); *Consortium probiotic: *L. parabuchneri*: *L. buchneri*: *L. harbinensis*: *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner*

The results of observations on the parameter value of cell biomass can be seen in Table 2. Average probiotic consortium cell biomass. Based on the DMRT follow-up test, it showed that the A3B2 treatment was higher significantly different (P<0.01) compared to all treatments on cell biomass values. Leroy and de Vuyst (2001) found that the availability of carbon and nitrogen elements as well as the adequacy of minerals and vitamins in the media at the beginning of growth has the potential to form the final biomass. Pato et al. (2021) added, the basic material of carbon sources is the largest component in the medium and the product is in the form of cell biomass.

In the medium B2, containing coconut water, 100 grams of coconut water contains electrolytes including potassium (250 mg), phosphorus (20 mg), iron (0.29 mg), zinc (0.1 mg) and water (94.99 mg). g), as well as carbohydrates (3.71 g), sugar (2.61 g), protein (0.72 g), vitamin C (2.4 mg), Vitamin B6 (0.032 mg), Pantothenic Acid (0.043 mg), Folate (3 µg), Thiamine/vitamin B1 (0.03 mg) (USDA, 2016). In our research mineral and vitamin in the coconut water can be supported the growth of probiotic consortium faster than whey and tofu liquid, because mineral and vitamin signifcant increase in biomass. The same finding also reported by a group of researchers, whereas minerals and vitamins are very important in a microbial growth medium (Pandiyan et al., 2012; Santos et al., 2014; Chiang et al., 2015).

The pH greatly affects the growth of the probiotic consortium. As seen in Table 3, the initial pH of the medium was made the same for each growth medium, but after being inoculated with various probiotic consortium ratios, the reduction pH of the medium was very different. We can see in Tables 1 and 2, the biomass and viability also low if decreased pH lower. The results of the analysis of variance showed that there was an interaction between factor A (probiotic ratio) and factor B (type of media) which had a highly significant (P<0.01) effect on the decreased pH of the medium.

One of the uniqueness of lactic acid bacteria is the formation of acid at the end of fermentation, so that an effective method in determining the parameters of technological application is the formation of acid and the concentration of biomass. The decrease in pH is thought to be due to the active work of the probiotic consortium in carrying out the fermentation so that a lot of organic acids are produced, especially lactic acid. A group of researchers has been reported that increase acidity making increase taste and pH the medium become lower (Maslami et al., 2018: Anggraini et al., 2019; Harnentis et al., 2020; Marlida et al., 2022).

The process of decreasing the pH value in natural growth media is also caused by the release of hydrogen ions (H+) due to the breakdown of glucose which results in lactic acid which increases acidity. Febriningrum (2013) reported the pH value in a solution shows the amount of H+ concentration. The higher the pH value in a certain solution, the fewer H+ ions contained in the solution, conversely the lower the pH value, the greater the number of H+ ions.

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Table 2 - Average consortium probiotic biomass on different media and probiotic ratio

Probiotics* \ Different growth medium	B1: (Oxoid (CM 359)	B2: (Coconut water (90%) + cassava flour (5%) + fish waste flour (5%)	B3: (Tofu liquid waste (90%) + cassava flour (5%) + fish waste flour (5%)	B4: (Whey tofu (90%) + cassava flour (5%) + fish waste flour (5%)	Average
A1: consortium probiotic* with ration 1:1:1:1:1	20.60^{gh}	20.62^{de}	20.82^{efgh}	20.72^{efgh}	20.69
A2: consortium probiotic* with Ration 1:1:1:1:2	20.71^{efgh}	20.30^{b}	20.87^{ef}	20.70^{fgh}	20.90
A3: consortium probiotic* with ration 1:1:1:2:1	20.67^{fgh}	22.47^{a}	20.75^{efgh}	20.68^{fgh}	21.14
A4: consortium probiotic* with ration 1:1:2:1:1	20.70^{fgh}	21.15^{cd}	20.92^{ef}	20.80^{efgh}	20.89
A5: consortium probiotic* with ration 1:2:1:1:1	20.59^{gh}	21.03^{de}	20.88^{ef}	20.88^{ef}	20.85
A6: consortium probiotic* with ration 2:1:1:1:1	20.55^{h}	21.27^{bc}	20.87^{ef}	20.83^{efg}	20.88
Average	20.64^{b}	21.31^{a}	20.85^{b}	20.77^{b}	

Note: different superscripts show highly significant different effects (P<0.01); *Consortium probiotic: *L. parabuchneri*: *L. buchneri*: *L. harbinensis*: *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner*

Table 3 - Average consortium probiotic pH decrease on different media and probiotic ratio

Probiotics* \ Different growth medium	B1: (Oxoid (CM 359)	B2: (Coconut water (90%) + cassava flour (5%) + fish waste flour (5%)	B3: (Tofu liquid waste (90%) + cassava flour (5%) + fish waste flour (5%)	B4: (Whey tofu (90%) + cassava flour (5%) + fish waste flour (5%)	Average
A1: consortium probiotic* with ration 1:1:1:1:1	0.92^{g}	2.76^{b}	1.14^{f}	1.14^{f}	1.49
A2: consortium probiotic* with ration 1:1:1:1:2	0.93^{g}	2.81^{a}	1.15^{f}	1.75^{c}	1.66
A3: consortium probiotic* with ration 1:1:1:2:1	0.90^{gh}	2.84^{a}	1.17^{f}	1.62^{d}	1.63
A4: consortium probiotic* with ration 1:1:2:1:1	0.87^{h}	2.84^{a}	1.17^{f}	1.65^{d}	1.63
A5: consortium probiotic* with ration 1:2:1:1:1	0.91^{g}	2.82^{a}	1.17^{f}	1.63^{d}	1.63
A6: consortium probiotic* with ration 2:1:1:1:1	0.90^{gh}	2.81^{a}	1.16^{f}	1.56^{e}	1.60
Average	0.91^{bc}	2.81^{c}	1.16^{b}	1.56^{a}	

Note: different superscripts show highly significant different effects (P<0.01); *Consortium probiotic: *L. parabuchneri*: *L. buchneri*: *L. harbinensis*: *Schieferilactobacillus harbinensis* and *Lentilactobacillus parabuchner*

CONCLUSION

The conclusion of this study were the ratio of the best probiotic consortium is 1:1:1:2:1, with growth medium coconut water (90%) + cassava flour (5%) + fish waste flour (5%) which results in a viability value of: 3, 02, cell biomass: 22.47 mg/ml and a decrease in the pH of the medium by 2.84.

DECLARATIONS

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Ethical approval

This article does not contain any studies that would require an ethical statement.

Authors' contribution

M. Kudus Susalam and Y. Marlida planned all stages of the research, Harnentis assisted with research in the laboratory and Jamsari assisted in checking the final draft of the publication

Conflict of interests

All researchers involved in the research stated that there were no conflicts of interest either

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