Diversity and Enumeration of Lactic Acid Bacteria in Traditional Fermented Fish Product *Bakasang* from Bacan and Sanana, North Maluku

Nurmaya Papuangan[1)*], Srimaya Maswara[1)], Hasna Ahmad[1)], Nurhasanah[1)]

[1)]Biology Education Program, Khairun University
[*]Corresponding E-mail: nurmaya.papuangan@unkhair.ac.id;

Abstrak

Bakasang adalah salah satu produk fermentasi tradisional yang berbahan dasar isi perut (jeroan) ikan tuna (*Thunnus* spp.) dan ikan cakalang (*Katsuwonus pelamis*) dengan penambahan garam dalam jumlah yang tinggi sekitar 20% atau lebih. Proses fermentasi bakasang berlangsunng secara alami dengan bantuan aktivitas mikroorganisme indigenous, terutama kelompok bakteri asam laktat (BAL). BAL adalah kelompok bakteri yang dapat memproduksi asam laktat yang berperan penting dalam pengawetan, pembentuk cita rasa, serta memiliki potensi fungsional probiotik. Penelitian ini bertujuan mengevaluasi keanekaragaman dan jumlah BAL dalam produk bakasang lokal dari daerah Bacan dan Sanana, sebagai dasar dalam pengembangan pangan fungsional berbasis sumber daya lokal. Enumerasi dan isolasi BAL dilakukan menggunakan metode pour plate pada media *de Man, Rogosa, and Sharpe* (MRS) agar, dilanjutkan dengan karakterisasi morfologi koloni, pewarnaan Gramm dan karakterisasi fisiologis. Hasil penelitian menunjukan bahwa jumlah BAL dalam produk bakasang dari kedua daerah berkisar antara 10^5 hingga 10^8 CFU/g. Jumlah BAL dari daerah Bacan sebesar 2,4 x 10^8 CFU/g lebih tinggi dibandingkan dengan bakasang dari daerah Sanana yang hanya sebesar 4,6 x 10^5 CFU/g. Hasil karakterisasi makroskopis, mikroskopis, dan fisiologis mengindikasikan kemiripan dengan genus *Lactobacillus* dan *Lactococcus*. Keanekaragaman BAL ini menunjukkan adanya mikrobiota lokal yang berpotensi dikembangkan sebagai agen fermentasi dan probiotik dalam industri pangan fungsional.

Kata kunci: Fermenasi, Pangan Lokal, Probiotik, Lactobacillus, Lactococcus

Abstract

Bakasang is a fermented fish product traditionally made using the viscera (internal organs) of tuna (*Thunnus* spp.) and skipjack tuna (*Katsuwonus pelamis*), with the addition of a high concentration of salt, typically around 20% or more. The fermentation process occurs naturally and spontaneously through the activity of indigenous microorganisms, particularly those belonging to the lactic acid bacteria (LAB) group. LAB are Gram-positive, catalase-negative bacteria capable of producing lactic acid, which plays a crucial role in preservation and flavor development, and also possesses potential probiotic functionality. This study aims to evaluate the diversity and population of LAB in locally produced *bakasang* from the Bacan and Sanana regions, as a basis for the development of functional foods utilizing local microbial resources. Enumeration and isolation of LAB were conducted using the pour plate method on de Man, Rogosa, and Sharpe (MRS) agar, followed by morphological colony characterization, Gram staining, and physiological profiling. The results showed that LAB populations in *bakasang* samples from both regions ranged from 10^5 to 10^8 CFU/g. The LAB count in *bakasang* from Bacan (2.4 × 10^8 CFU/g) was higher compared to that from Sanana (4.6 × 10^5 CFU/g). Macroscopic, microscopic, and physiological characterization of the isolates indicated similarities to the genera *Lactobacillus* and *Lactococcus*. The diversity of LAB observed in this study reflects local microbiota with promising potential to be developed as fermentation agents and probiotics for the functional food industry.

Keywords: Fermentation, Local Food, Probiotics, Lactobacillus, Lactococcus

INTRODUCTION

Fermentation is the oldest food processing and preservation method that has long been utilized by coastal communities in North Maluku, especially in the utilization of local resources such as fish. Bakasang is one of the traditional fermented products made from fish as the main ingredient, especially the internal organs of tuna, skipjack, anchovy, and sardines. The fermentation process occurs naturally with the addition of a high amount of salt, around 20% or more, and is incubated for several days to weeks. The product is processed based on customs and experiences passed down through generations using very simple technology and equipment. Bakasang is used as a side dish, flavor enhancer, or seasoning that has a texture similar to a yellow-brown sauce and contains amino acids. (Yanti & Dali, 2014)

The fermentation process of bakasang occurs naturally and spontaneously with the help of indigenous microorganisms that develop and contribute to the formation of flavor, aroma, and stability of the product. One of the main groups of microorganisms in bakasang fermentation is Lactic Acid Bacteria (LAB) (Li et al., 2024; Yanti & Dali, 2014). Lactic acid bacteria (LAB) have a significant contribution to the development of flavor, enhancement of product shelf life, and have the potential as natural probiotics (J. P. Tamang, Watanabe, et al., 2016a). LAB are Gram-positive facultative anaerobic bacteria that can survive in various habitats, such as the gastrointestinal tract of animals and humans, canned foods, dairy products, fermented foods, as well as various tropical fruits and vegetables. LAB is widely utilized in food fermentation processes as a preservative agent, fermentation culture, and probiotic food component due to its ability to produce lactic acid from sugar fermentation. The production of lactic acid lowers the environmental pH, thus inhibiting the growth of pathogenic microorganisms and spoilage-causing microbes (Aritonang et al., 2019; Ray & Joshi, 2015). LAB also produces various other bioactive compounds such as bacteriocins, enzymes, and vitamins (Klaenhammer et al., 2002). The main Genus of LAB such as Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus, dan *Micrococcus* serve as natural starters in various food fermentations, including dairy products, vegetables, and traditional fermented fish products such as bakasang. (Surono, 2016; J. P. Tamang, Shin, et al., 2016; Yanti & Dali, 2014).

Although bakasang is a traditional fermented product with local wisdom from the coastal communities in North Maluku and has high functional potential, research that specifically examines the microbiota composition, especially the characterization of Lactic Acid Bacteria (LAB), is still very limited. LAB plays an important role in fermentation through its contribution to flavor development, extending shelf life, and potential as probiotics. The lack of data on the quantity, diversity, and physiological properties of LAB in bakasang from various regions of North Maluku creates a knowledge gap regarding the utilization of local microbiota as a natural starter and probiotic candidate. Therefore, studies that quantitatively and qualitatively map the LAB profile are crucial as a basis for the development of functional foods based on traditional fermentation.

METHOD

Tools and Materials

The materials used are 70% and 95% alcohol, aquades, 0.85% NaCl, selective De Man, Rogosa and Sharpe (MRS) Agar with the composition: 1% bacteriological peptone, 1% beef extract, 0.5% yeast extract, 2% glucose, 0.5% CaCO3, MRS broth, H2O2, Gram staining kit, and sterilized aquades.

Sampling of Bakasang

The sample of bakasang was obtained from local producers in the Bacan and Sanana areas, North Maluku. The sampling of bakasang was conducted using a purposive sampling approach, by identifying local producers who routinely produce traditional bakasang in both areas with a fermentation time of 2 weeks. The selection of locations was based on the availability of representative products and the sustainability of traditional fermentation practices. All samples that were two weeks old were collected aseptically, placed in sterile containers, and stored at a cold temperature during the transportation process to the laboratory for analysis.

Enumeration and Isolation of LAB

Enumeration and isolation of LAB were performed using the pour plate method, starting with a tiered serial dilution. LAB isolation from the sample was carried out using selective media for Lactobacillus, namely MRS agar enriched with 0.5% $CaCO_3$ and MRSA broth. A total of 1 gram of sample from each area was dissolved in 9 mL of sterile 0.85% NaCl and homogenized, resulting in a dilution of 10^{-1}. Next, 1 mL of suspension from the 10^{-1} dilution was mixed again with 9 mL of sterile 0.85% NaCl to obtain a dilution of 10^{-2}. This dilution process continued stepwise until reaching a dilution of 10^{-7}. A total of 1 mL of culture from the 10^{-3} to 10^{-7} dilutions was pipetted and placed into sterile petri dishes, followed by pouring about 20 mL of MRSA media that had been supplemented with 0.5% $CaCO_3$. Each dilution was performed in duplicate and the mixture was shaken evenly with a motion resembling the number eight. After the media solidified, the petri dish was incubated upside down at 30 °C for 48 hours. After incubation, the growing LAB colonies were counted to determine the number of CFUs, followed by Gram staining and catalase testing. The CFU calculation of LAB was performed by the guidelines of the TPC method, which is the number of colonies in the range of 25–250 colonies per petri dish (referring to SNI 01-2332.3-2006) and expressed in cfu/g using the following formulation:

$$N = \frac{\sum C}{[(1 \times n1)+(0,1 \times n2)] x\, d} \ldots\ldots\ldots\ldots\ldots (1)$$

Ket:
ΣC = total number of colonies from all counted plates
N = number of colonies per ml/gram
n1 = the number of cups in the first dilution
n2 = the number of cups in the second dilution
d = the dilution level obtained from the first Petri dish is calculated

Characterization of LAB Isolate

Characterization of LAB isolates was conducted both macroscopically and microscopically. The macroscopically grown LAB colonies were observed for shape, color, edge, and surface of the colonies. To determine cell morphology and Gram characteristics, Gram staining and microscopic observation were performed.

Catalase Test and Fermentation Type

The catalase test is used to confirm the character of LAB, which is catalase negative. The catalase test is performed by adding two drops of hydrogen peroxide (H_2O_2) solution to a 24-hour-old bacterial colony placed on a glass slide. A positive reaction is indicated by the appearance of air bubbles as a result of the decomposition of H_2O_2 by the catalase enzyme into water (H_2O) and oxygen (O_2), which shows the bacteria's ability to produce that enzyme. To test the type of fermentation, LAB isolates are grown in MRS broth media in a test tube containing a Durham tube. The culture is then incubated for 48 hours at 37 °C. LAB has a heterofermentative type of fermentation if gas is present in the Durham tube, and if no gas is present in the Durham tube, it is homofermentative.

RESULTS AND DISCUSSION

Characterization of Organoleptic Bakasang

The organoleptic characterization of batik samples from Bacan and Sanana shows differences that reflect variations in microbiological activity during the fermentation process. The same brown color in both samples indicates protein degradation and a mild Maillard reaction commonly occurring in fish-based fermentation (Li et al., 2024). (Purwaningsih et al., 2011) states that the brown color that appears is suspected to originate from the hydrolysis process and microbial activity during fermentation and boiling of the offal. Meanwhile, the dull brown color observed during storage is related to the original color of the raw materials, which tends to be reddish-brown. Additionally, the formation of the brown color can also be caused by the browning reactions that occur due to the cooking of protein- and amino acid-rich offal.

The difference in consistency between the thick, homogeneous, and slightly soft Bacan base and the more liquid, less homogeneous, and somewhat coarse Sanana is likely due to the composition of the raw materials and the rate of substrate degradation by proteolytic enzymes produced by microbes, primarily lactic acid bacteria (LAB) and other microbes (J. P. Tamang, Watanabe, et al., 2016a). The decrease in texture is related to microbial activity during storage, which breaks down the main macromolecules, namely proteins, into derivative compounds such as peptides and amino acids, accompanied by the release of water (H_2O). (Purwaningsih et al., 2011).

Aroma or smell is a specific attribute in determining the deliciousness of a food product. The aroma in fermented products also indicates the contribution of microbial metabolite compounds. The fish aroma and fragrance in Bacan fermented products are suspected to originate from the production of volatile compounds such as diacetyl and acetoin, which are commonly produced by the genera Lactobacillus and Lactococcus, while the fish aroma and acidity in Sanana fermented products indicate fermentation activity that produces organic acids, such as lactic acid and acetic acid (Ray & Joshi, 2015). The volatile compounds involved are alcohol carbonyl, sulfur, hydrocarbons, and bromophenol, while the non-volatile compounds involved are free amino acids, peptides, nucleotides, and organic bases from food products (Zhang et al., 2020). Meanwhile, the fishy smell is caused by the presence of ammonia and glutamic acid (Ohta, 1995).

pH is one of the physicochemical factors that play an important role in determining the shelf life of food materials. Generally, a pH ≤ 4.5 in fermented foods is considered safe for consumption because it can inhibit the growth of pathogens such as *Clostridium botulinum* and other spoilage bacteria (Ray & Joshi, 2015). Traditional fermented products such as kimchi and fermented fish usually show a final pH ranging from 3.5 to 4.5, depending on the type of substrate and microbes involved during the fermentation (Li et al., 2024). A pH value of 6 in the Bacan and Sanana fish sauce samples indicates that fermentation has taken place but has not yet reached the peak acidity point. This is relevant considering that the fermentation age is still 14 days, during which LAB typically shows maximum activity between the 10th and 21st days (Surono, 2016). In addition, the fermentation process in both types of waste occurs under conditions with high salt concentrations and without any additional carbohydrates as a source of microbial energy. Carbohydrates serve as the main substrate for the metabolism of lactic acid bacteria (LAB), which produce lactic acid as the primary product of fermentation. The production of this acid lowers the pH, creates anaerobic conditions, and inhibits the growth of pathogenic microorganisms (J. P. Tamang, Shin, et al., 2016). The decrease in pH value during the fermentation process in the substrate indicates the activity of lactic acid bacteria (LAB) that grow and develop (Desniar et al., 2023) states that BAL metabolizes carbohydrates, especially glucose from dry rice, into simple organic acid compounds that contribute to the increase of total titratable acidity (TTA), and subsequently lowers the pH value to become more acidic. According to (Haryo et al., 2016) *Lactobacillus plantarum* is known to be able to synthesize amylase and β-glucosidase enzymes, which play a role in the hydrolysis of polysaccharides into monosaccharides.

According to (Pratomo et al., 2020), the decrease in pH during fermentation is a result of the activity of lactic acid bacteria (LAB) that produce acid compounds during the fermentation process. The decrease in pH by LAB is primarily due to the production of organic acids, especially lactic acid. In addition, LAB are also capable of synthesizing other compounds such as acetic acid, ethanol, volatile aromatic compounds, bacteriocins, exopolysaccharides, and various enzymes, which synergistically contribute to extending shelf life, improving texture and aroma, and the stability of probiotic microbes in the product, as well as forming the sensory characteristics of the final product that are preferred by consumers. (J. P. Tamang, Watanabe, et al., 2016a).

Overall, these differences in physical and chemical characteristics indicate variations in the activity of local microbial communities that not only affect sensory quality but also the functional potential of fermented products as sources of natural starter cultures and probiotic candidates in the development of functional foods based on traditional fermentation.

Enumeration and Isolation of Lactic Acid Bacteria

The enumeration results show that the highest number of LAB was found in the Bacan product at 2.4 x 10^8 CFU/g, while in Sanana it was only 4.6 x 10^5 CFU/g. This difference can be attributed to variations in salt concentration, pH, environmental conditions, substrate types, and the availability of carbohydrates during fermentation. High salt levels in fish fermentation can restrict the types of microbes that grow and lead to the dominance of halophilic LAB (Kim et al., 2022). The higher the concentration of salt, the higher the number of lactic acid bacteria and halophilic bacteria (Udomsil et al., 2011a). In the fermentation of bakasang, the presence of carbohydrates from both additives and leftover substrates supports the growth of Lactic Acid Bacteria (LAB), especially heterofermentative types such as Leuconostoc and Pediococcus, which also produce secondary flavor compounds (Ray & Joshi, 2015; Vinderola et al., 2019). The main metabolic activity that has been the focus in the use of LAB as a fermentation agent, which has long been used in the food, pharmaceutical, and agricultural industries, is carbohydrate metabolism, utilizing carbon molecules as its primary energy source to ferment lactic acid as its fermentation product (Hayek et al., 2013). Certain pH levels are among the aspects that can affect the growth rate of microbes in the environment (Antoni, 2016). LAB shows optimal growth at a pH of 5.5 - 6.5, although it can still survive at a pH of 4.0 - 7.0 with a decrease in metabolic activity outside this optimal range (J. Tamang et al., 2015).

Morphological and Physiological Characterization of LAB

The isolation results yielded five LAB isolates, namely isolates BKSB-32, BKSB-52, BKSB-61, BKSB-62 obtained from Bacan fermentation and isolate BKSS-41 from Sanana fermentation. The phenotypic characterization results of the five LAB isolates from the fermentation products showed variation in colony morphological traits, cell morphology, and physiological capabilities (Table 1). Colony colors varied between cream and white, with the dominant colony shape being irregular, except for isolates BKSB-61 and BKSS-41, which were round and had convex elevation. All isolates had smooth colony margins with cocci-shaped cells (BKSB-32, BKSB-52, BKSB-61, BKSS-41) and bacilli (BKSB-62). The Gram reaction of all five isolates was categorized as Gram-positive bacteria and showed a negative catalase reaction, which is characteristic of the isolation results yielded five BAL isolates, namely isolates BKSB-32, BKSB-52, BKSB-61, BKSB-62 obtained from Bacan fermentation and isolate BKSS-41 from Sanana fermentation. The phenotypic characterization results of the five LAB isolates from the fermentation products showed variation in colony morphological traits, cell morphology, and physiological capabilities (Table 1). Colony colors varied between cream and white, with the dominant colony shape being irregular, except for isolates BKSB-61 and BKSS-41 which were round and had convex elevation. All isolates had smooth colony margins with cocci-shaped cells (BKSB-32, BKSB-52, BKSB-61, BKSS-41) and bacilli (BKSB-62). The Gram reaction of all five isolates was categorized as Gram-positive bacteria and showed a negative catalase reaction, which is characteristic of the LAB group. (Axelsson, 2004).

Table 1. Morphological and Physiological Characterization of Five BAL Isolates from Bacan and Sanana.

Test Parameter	LAB Isolate				
	BKSB-32	**BKSB-52**	**BKSB-61**	**BKSB-62**	**BKSS-41**
Macroscopic					
Colony color	Cream	White	Cream	White	Cream
Colony form	Irregular	Irregular	Round	Irregular	Round
The Edge of the Colony	Entire	Entire	Entire	Entire	Entire
Colony Elevation	Flat	Flat	Convex	Flat	Convexss
Microscopic					
Colony Cell	Coccus	Coccus	Coccus	Basil	Coccus
Cell Arrangement	Clustered	Clustered	Clustered	Diplobasil	Clustered
Gram Reaction	Positive	Positive	Positive	Positive	Positive
Spores	-	-	-	-	-
Physiological					

Fermentation Type	Heterofermentative	Heterofermentative	Heterofermentative	Heterofermentative	Heterofermentative
TSIA	A/A	A/A	A/A	A/A	A/A
Indol	-	-	-	-	-
Motility	-	-	-	-	-
MR	-	-	-	-	-
VP	-	-	-	-	-
Citric acid	-	-	-	-	-
Gelatin Hydrolysis	+	+	+	+	+
Catalase	-	-	-	-	-

Note : (-) is negative, (+) is posistive and A/A is Acid

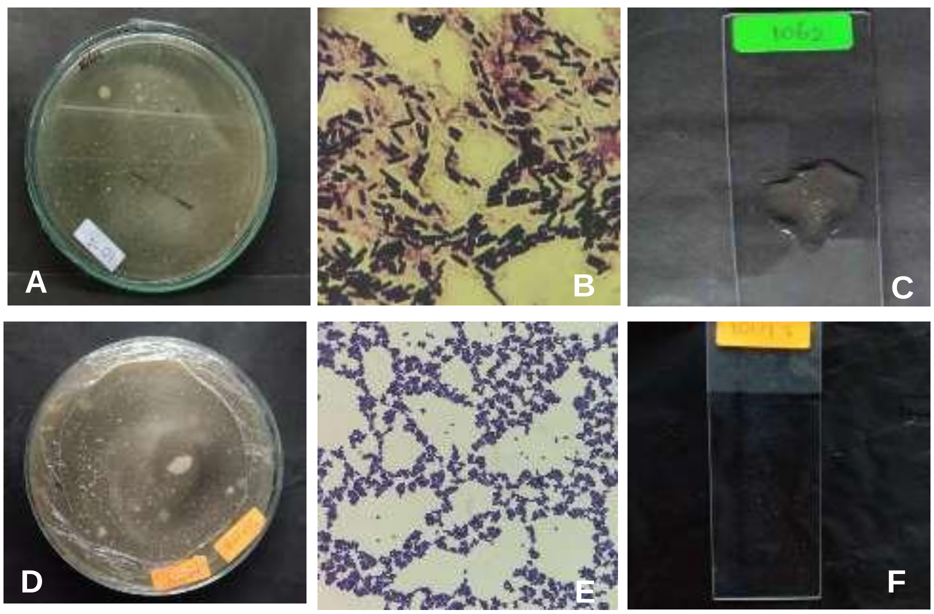

Figure 1. Growth of Isolate BKSB-62 on MRSA Media (A); Shape and Arrangement of Isolate BKSB-62 Cells (Gram-positive Bacilli) (B); Catalase Negative Isolate BKSB-62 (C); Growth of Isolate BKSS-41 on MRSA Media (D); Shape and Arrangement of Isolate BKSS-41 Cells (Gram-positive Coccus) (E); Catalase Negative Isolate BKSS-41 (F)

LAB generally shows a negative reaction to the catalase test, which is one of its characteristics. *Lactobacillus plantarum* and *Lactobacillus acidophilus* do not show catalase activity. This activity supports LAB in producing lactic acid as a main metabolite, which plays a role in the natural preservation of fermented products such as yogurt and bakasang (Pratiwi et al., 2017; Yanti & Dali, 2014). The catalase test results showed no gas bubbles when the isolate was treated with hydrogen peroxide solution, indicating that the LAB isolate from both samples showed a negative reaction (Surono, 2016; Zang et al., 2020).

The fermentation pattern of all isolates is classified as heterofermentative, characterized by gas production in MRS broth media with Durham tubes, supporting the ability of LAB to produce various metabolites during the fermentation process (J. P. Tamang, Watanabe, et al., 2016). LAB is classified into two groups based on its fermentation type, namely homofermentative and heterofermentative lactic acid bacteria (Yanti & Dali, 2014). The homofermentative fermentation process produces only one type of component, namely lactic acid, whereas heterofermentative fermentation produces various other compounds or components, such as ethanol, acetate, lactic acid, and carbon dioxide. According to (Surono, 2016), homofermentative bacteria are unable to produce CO_2 gas, because they do not have the enzyme pyruvate oxidase that can convert pyruvate into CO_2 and acetyl phosphate, followed by the formation of H_2O_2. Lactic acid bacteria produce secondary metabolites such as diacetyl, H_2O_2, reuterin, and bacteriocins that serve to maintain the health of the digestive tract for those who consume them, as they have antimicrobial properties against pathogenic microbes. (Afriani, 2010; Arena et al., 2016; Desniar et al., 2013; Lewus & Montville, 1991).

Biochemical tests such as TSIA show an A/A reaction (acid/acid), indicating fermentation of glucose and lactose/sucrose. All isolates were negative for indole, motility, MR, VP, and citrate tests, and showed the ability to hydrolyze gelatin, indicating potential protein degradation during fermentation. *Lactobacillus plantarum* produces gelatinase that hydrolyzes peptide bonds in gelatin (Afriani, 2010; Arena et al., 2016; Wikandari et al., 2012). This activity is detected through the gelatin hydrolysis test, where gelatin melts due to protein degradation (Nuryanti et al., 2021; Raharja et al., 2023)

This phenotypic variation indicates that LAB isolates from the bakasang consist of different species or strains with varying ecological adaptations. The characteristics of the isolates suggest a possible affiliation with the genus Lactobacillus, Lactococcus, or Leuconostoc and show potential as natural starter cultures that can be developed in the production of functional foods based on traditional fermented fish. (Axelsson, 2004; Ray & Joshi, 2015; Surono, 2016).

Correlation of pH, Salt Concentration, and Comparison with Asian Fish Fermentation

pH conditions and salt concentration are two key environmental factors that influence the dynamics of microorganism populations during fish fermentation. In this study, the products of fermentation from Bacan and Sanana showed a final pH of around 6, which is considered more neutral compared to other traditional fish fermentation products in Asia such as *pla-ra* (Thailand), *shiokara* (Japan), or *jeotgal* (Korea), which generally have final pH values ranging from 4.5 to 5.0 (J. P. Tamang, Watanabe, et al., 2016a; Vinderola et al., 2019). A lower pH tends to indicate the dominance of LAB and the success of fermentation in producing organic acids.

The salt concentration in traditional ferments ranges from 20% or more, which serves as a natural selective agent against microbiota, allowing only the growth of halotolerant microorganisms such as LAB. This high salt content is in line with common fish fermentation practices in tropical regions, where salt is used to suppress the growth of pathogens and enhance product safety (Ray & Joshi, 2015). However, excessively high salt levels can also inhibit the metabolic activity of LAB and slow down the decline in pH, as is suspected to occur in the samples from Sanana.

In a similar study, pla-ra from Thailand contained 15–20% salt and showed an final pH of around 5.2–5.4, with dominance of *Tetragenococcus halophilus* and *Lactobacillus* spp. (Udomsil et al., 2011b). Meanwhile, *jeotgal* from Korea, although also using high salt, has a broader final pH variation depending on the raw materials and fermentation time, yet it still shows the dominance of lactic acid bacteria and other halophilic bacteria (J. P. Tamang, Watanabe, et al., 2016a).

The difference in pH value and LAB density in bakasang indicates that local practices and fermentation conditions (temperature, moisture content, carbohydrate availability, and duration of fermentation) significantly determine the direction of fermentation and the resulting microbiota profile. Therefore, optimizing traditional fermentation such as bakasang requires a deep understanding of the correlation between these parameters to improve the quality and safety of the product as well as its functional potential as a probiotic food.

CONCLUSION

The diversity and number of lactic acid bacteria (LAB) in the bakasang products from Bacan and Sanana indicate the potential of endogenous microbes as fermentation agents and probiotics. The highest LAB concentration was recorded in Bacan bakasang (2.4×10^8 CFU/g), which is greater than Sanana (4.6×10^5 CFU/g). The characterization results showed a similarity of the isolates with the genera Lactobacillus and Lactococcus. This data supports the prospect of utilizing local LAB in the formulation of functional foods based on traditional fermentation.

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