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# Identification of Total Coliform Bacteria in Processed Enbal Food in Several Traditional Markets in Ambon City

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**Abstract**

*Enbal* is a traditional food from Maluku, originally from the Kei Islands, made from bitter cassava (*Manihot esculenta*). This processed food product holds significant historical and cultural value. However, *enbal* can become a source of illness if not prepared properly, leading to contamination and an increased risk of foodborne diseases. This study aimed to determine the microbiological quality through total plate count (TPC) analysis and bacterial identification of *enbal* at several traditional markets in Ambon City. It was a descriptive observational study with a true experimental laboratory approach. The TPC results for *enbal* food samples from Mardika market ranged from $14.5 \times 10^2$ CFU/g to $4.85 \times 10^4$ CFU/g, Waiheru market ranged from $8.3 \times 10^2$ CFU/g to $3.2 \times 10^5$ CFU/g, Batu Merah market ranged from $11.0 \times 10^4$ to $3.0 \times 10^5$ CFU/g, and Passo market ranged from $2.4 \times 10^2$ CFU/g to $3.75 \times 10^5$ CFU/g. The results of bacterial species identification from the four *enbal* food samples revealed *Klebsiella pneumoniae* and *Staphylococcus haemolyticus*. Thus, it can be concluded that the four *enbal* samples examined are safe for consumption as they do not exceed the Indonesian National Standard (SNI) 01-2997-1996, which is $1 \times 10^6$ CFU/g**.**

**Keywords:** Bacterial contamination; bitter cassava; CFU/g; SNI.

**Abbreviations**: FAO: Food and agriculture organization; CFU: colony-forming unit; TPC: total plate count; PCA: plate count agar; TNTC: too numerous to count; TFTC: too few to count; SNI: Indonesian national standard; CoNS: coagulase-negative *Staphylococci*; UTI: urinary tract infection.

## INTRODUCTION

Food is essential to human needs as one of the primary and basic physiological needs that support human life (Trivedi et al., 2019; Casman et al., 2022). However, food can also become a source of illness if it is not handled or prepared properly and hygienically (O'Shea et al., 2019). Food hygiene ensures the control of potential hazards, which can be identified using a hazard assessment approach, as well as the likelihood and severity of adverse impacts, determined using a risk assessment approach (Hassauer & Roosen, 2020). Traditional food markets, which are a source of livelihood for millions of people, can pose public health risks if proper food safety measures are not followed. Traditional markets themselves are places that sell various types of traditional products, including crafts, souvenirs, and traditional foods. In Maluku, one food product found in traditional markets is *enbal*, a processed food product.

*Enbal* is a food product made from bitter cassava (*Manihot esculenta*), processed in a way passed down through generations using simple methods (Leasa et al., 2018; Riry et al., 2013). Traditional processed products like *enbal* have significant historical and cultural value and are an essential to Indonesia's food diversity (Polnaya et al., 2016). *Enbal* is often consumed by the people of Maluku, especially in Southeast Maluku, and comes in several variants such as fried *enbal*, chocolate-cheese *enbal*, peanut *enbal*, and plain *enbal* (Kementerian Pendidikan dan Kebudayaan Indonesa, 2017). However, traditional food products also carry risks, such as health and food safety risks due to contamination by microorganisms, especially since most traditional food products are sold in traditional markets with low sanitation levels (Olowokure et al., 2023; Zamzammi, 2015).

Contamination in food can lead to an increase the number of foodborne diseases. Foodborne diseases are any illnesses caused by consuming contaminated food, contaminated by pathogenic bacteria, viruses, or

parasites. Globally, foodborne diseases are still not under control, and outbreaks can cause significant health and economic losses. The causes are unhygienic practices in food production, harvesting, and preparation (Adley & Ryan, 2016). Foodborne diseases are caused by food contamination and occur at every stage of the food production, distribution, and consumption chain. This can result from various forms of environmental contamination, including water, soil, or air pollution, as well as unsafe food storage and processing (World Health Organisation, 2010).

Data from the Food and Agriculture Organization (FAO) estimates that around 600 million people, nearly 1 in 10 people worldwide, fall ill after consuming contaminated food, and 420,000 people die each year (Davidson & Emerita, 2017). In Europe, it is reported that 44 people per minute, an average of 23 million people per year, fall ill due to food poisoning, and it is estimated that 4,700 people die each year (Negru & Brickman, 2019). In Indonesia, the highest number of reported food poisoning outbreaks occurred between 2008 and 2011, with 54.8% of cases reported. The highest incidence of food poisoning occurred in West Java province, with 163 incidents out of a total population of 10,962. Food poisoning cases in Indonesia from 2000 to 2015 totaled 61,119 cases out of a population of 715,579 at risk (Arisanti et al., 2018).

A study conducted by Condro and Santoso (2017) on sweet potato chips in Jayapura City showed that samples obtained from four household industries were positive for *Escherichia coli* contamination. Another Syafira et al. (2021) study on cassava getuk sold in traditional markets in Cimahi City found that 4 out of 5 samples exceeded the safe food limit, with the highest total plate count of 1.4 x $10^6$ CFU/gram. Another study by Tallo and Pani (2023) on gaplek cassava samples from Belu and Ende Regencies showed that the samples tested had the highest yeast and mold count in both regencies of 8.7 x $10^5$ CFU/gram, which exceeded the threshold set by the Food and Drug Supervisory Agency of Indonesia Regulation number 13 of 2019, with a maximum colony number of $10^5$ CFU/gram. Many studies have been conducted on total plate count analysis in various food products, yet there is no research on total plate count analysis and bacterial identification of *enbal* products. Therefore, this study aimed to analyze the microbiological quality of *enbal* at several traditional markets in Ambon city through total plate count (TPC) analysis and bacterial identification.

## MATERIALS AND METHODS

### Study area

This study used a quantitative descriptive research design with a true experimental laboratory approach. The research was conducted at the Microbiology Laboratory of the Faculty of Medicine, Universitas Pattimura, and the Office for Health Laboratory and Medical Device Calibration of Maluku Province, Ambon, Indonesia, from June to July 2024.

### Procedures

*Tools and Materials*

Materials used in this study were 100 gr of processed plain enbal food obtained from each of the 4 markets in Ambon City, 0.85% NaCl solution (Merck), Plate Count Agar (PCA) (Merck), Nutrient Agar (Merck), sterile distilled water (WaterOne). The tools used were cottons, 20 ml and 100 ml measuring cylinders (Iwaki), 250 ml and 500 ml erlenmeyer flasks (iwaki), 100 ml beaker glass (Iwaki), test tubes (pyrex), micropipette (Socorex), glass spreader (Iwaki), autoclave (All American), vortex mixer (VM-300 Gemmy), inoculating needle, aluminium foil (Klinpak), plastic wrap (Klinpak), bunsen burner, mortar and pestle, petri dishes (OneMed), tweezers, inoculation loop, label paper, cotton, microscope (Olympus), glass slides (OneMed), and VITEK 2 Compact (bioMérieux).

*Sample Preparation*

The samples used in this study were processed *enbal* food obtained from several traditional markets in Ambon, Indonesia. After conducting a survey, it was found that plain processed *enbal* was only available in 4 out of 8 actively operating traditional markets in Ambon City: Mardika Market, Passo, Waiheru, and Batu Merah. From these four markets, a simple random sampling method was applied using a lottery system to select processed plain *enbal* from vendors in each market.

*Sterilization of Equipment*

The glassware was wrapped in parchment paper and sterilized using an autoclave at 121°C for 15 minutes. After sterilization, the tools were placed in an oven at 150°C for 1 hour (Ma'at, 2009).

*Preparation of Plate Count Agar (PCA) Medium*

A total of 22.5 gr of plate count agar powder was mixed with sterile distilled water until the solution volume reached 1 liter. The medium was then boiled on a heater until fully dissolved, and subsequently sterilized in an autoclave at 121°C and 760 mmHg for 15 minutes (Mariani et al., 2020).

*Serial Dilution and Bacterial Isolation*

One gram of the grounded sample is placed into a test tube and mixed with 9 ml of sterile distilled water, then homogenized using a vortex mixer with the tube closed to achieve a $10^{-1}$ dilution. To prepare a $10^{-2}$ dilution, 1 ml of the initial dilution suspension is transferred to a new test tube containing 9 ml of sterile distilled water and homogenized. This process was repeated to create $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions in the same manner. The isolates

from these dilutions were then inoculated onto PCA medium using the spread plate technique, repeated twice and incubated at 37°C for 24-72 hours (Jamilatun, 2022).

*Analysis of Total Plate count (TPC)*
The analysis of total plate count was performed by counting bacterial colonies per petri dish. Colony counts were carried out to determine the level of bacterial contamination in the samples, and were only performed on petri dishes with colony counts between 30 and 300. The observed number of colonies were then used to calculate the total plate count using the formula (Yunita et al., 2022; Yunita et al., 2016):

$$\text{TPC} = \text{Numbers of Colonies} \times \frac{1}{\text{Dilution Factors}}$$

*Purification of Selected Bacteria*
Bacteria that grow and have different morphologies are selected considering colony color and shape differences across all petri dishes. The five selected colonies were purified on PCA medium using the spread plate technique with the quadrant streak method and incubated for 24 hours (Sa'adah, 2020).

*Macroscopic Characterization*
Macroscopic characterization was carried out by directly observing the isolates grown on agar media, including colony size, colony shape, colony elevation, colony margin, surface texture, as well as colony color and turbidity (Bergey & Hendricks, 1994; Tille, 2022).

*Microscopic Characterization*
A purified bacterial colony was taken and smeared onto a glass slide pre-treated with 0.96% NaCl. The smear was spread using an inoculating loop and dried by fixation over a flame. Crystal violet (purple) stain was applied to the smear and left on the slide for 1 minute before being rinsed with distilled water. Lugol's iodine solution was then added to enhance the affinity of the crystal violet stain, left for 1 minute, and rinsed again. Next, a decolorizer (96% alcohol) was applied for 1 minute, followed by another rinse with distilled water. The final step of the Gram staining process involved counterstaining with safranin for 30 seconds to color the bacterial cells. The slide was then rinsed, dried, and observed under a microscope at 100x magnification using immersion oil (Jawetz et al., 2019; Tille, 2022).

*Bacterial Identification*
Aseptically transfer 3.0 mL of sterile NaCl solution (0.45% to 0.50% NaCl, pH 4.5 to 7.0) into a clear plastic test tube (polystyrene, 12 mm x 75 mm). Using a sterile swab or inoculation loop, transfer a purified bacterial colony into the test tube. Prepare a homogeneous bacterial suspension with a density of 0.50 to 0.63 McFarland standard using a calibrated Vitek-2-Compact system (Astuty et al., 2023).

**Data analysis**
The data analysis in this study was conducted using Microsoft Excel and the obtained data was compared with SNI 01-2997-1996.

## RESULTS AND DISCUSSION

**Total plate count (TPC) analysis**
The results showed that the Total Plate Count (TPC) of *enbal*, as presented in Table 1, remained below the maximum bacterial contamination limit set by the Indonesian National Standard (SNI) 01-2997-1996, which is $1.0 \times 10^6$ CFU/g (Sagen, 2023). Based on the calculations in Table 1, all *enbal* samples analyzed were found to have microbial contamination levels within the acceptable range. The comparison between the total plate count and the SNI is illustrated in Figure 1, confirming that the bacterial count in *enbal* remains within safe consumption limits. These findings indicate that the *enbal* food studied is still safe for daily consumption.

**Table 1.** Results of total plate count (tpc) analysis for enbal.

| Dilution | Markets (Mean ± SD) | | | | SNI (CFU/gr) |
|---|---|---|---|---|---|
| | Mardika | Waiheru | Batu Merah | Passo | |
| $10^{-1}$ | $14.5 \times 10^2$ | $8.3 \times 10^2$ | TNTC[a] | $2.4 \times 10^2 \pm 21.0$ | $1 \times 10^6$ |
| $10^{-2}$ | $10.5 \times 10^3 \pm 6.5$ | $39.5 \times 10^2 \pm 3.5$ | TNTC[a] | $6.05 \times 10^3 \pm 23.5$ | |
| $10^{-3}$ | $4.85 \times 10^4 \pm 11.5$ | $33.5 \times 10^3 \pm 1.5$ | $11.0 \times 10^4$ | $5.5 \times 10^4 \pm 21.0$ | |
| $10^{-4}$ | TFTC[b] | $3.2 \times 10^5 \pm 1.0$ | $3.0 \times 10^5$ | $3.75 \times 10^5 \pm 5.5$ | |

Description: TNTC: Too Numerous To Count; TFTC: Too Few To Count

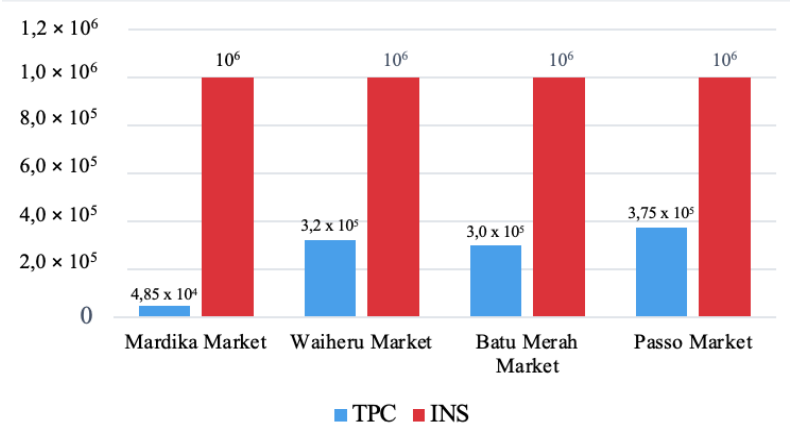


**Figure 1.** Comparison of total plate count for each market with Indonesian national standard.

## Bacterial Identification

Different macroscopically bacterial colonies were identified based on their characteristics, including size, shape, margin, elevation, and color (Figure 2).

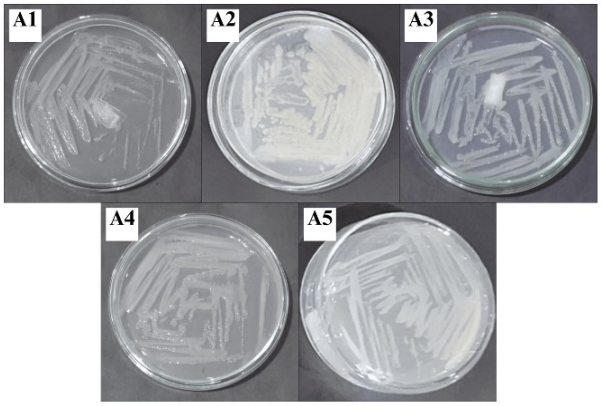


**Figure 1.** Purification results of five selected bacterial isolates (A1, A2, A3, A4, and A5) after 48 hours of incubation.

The purified bacterial colonies were regrown and analyzed macroscopically, with the observation results documented in Table 2, which shows similarities and differences in the macroscopic characteristics of each selected isolate.

**Table 2.** Macroscopic characterization of bacteria from enbal food processing samples.

| Isolates | Morphology characteristics of the colonies | | | | |
|---|---|---|---|---|---|
| | **Colony Size** | **Colony Shape** | **Colony Elevation** | **Colony Margin** | **Color and Turbidity** |
| A1 | Medium | Irregular | Umbonate | Undulate | Milky White |
| A2 | Medium | Irregular | Flat | Undulate | Milky White |
| A3 | Medium | Irregular | Umbonate | Undulate | Milky White |
| A4 | Medium | Irregular | Raised | Undulate | Milky White |
| A5 | Medium | Irregular | Flat | Undulate | Milky White |

The bacterial isolates obtained from *Enbal* food products were subsequently characterized based on their size, morphology, pigmentation, and Gram reaction under microscopic examination. The results of the microscopic characterization (Figure 3), where isolates A1 and A4 exhibit similar cellular structures and belong to the same Gram classification. These isolates form *Staphyl* aggregates with a coccoid morphology and appear purple upon Gram staining, indicating that they are Gram-positive *Staphylococcus*. Conversely, isolates A2, P3, and A5 display analogous cellular morphology and Gram staining properties, presenting as rod-shaped bacilli with a red coloration. This observation confirms that these three isolates are classified as Gram-negative bacteria.

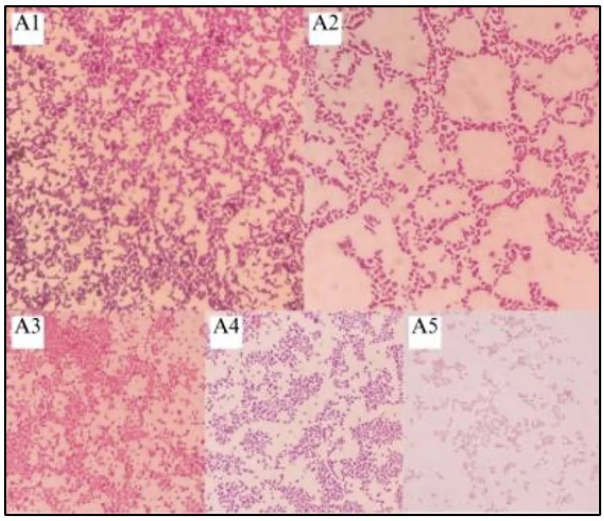


**Figure 2.** Microscopic characterization results of bacterial isolates.

Identification of the five selected bacterial isolates using the Vitek-2-Compact system revealed two bacterial species. Isolates A2 and A4 were identified as *Staphylococcus haemolyticus*, while isolates A2, A3, and A5 were identified as *Klebsiella pneumoniae*. The biochemical identification results, presented in Table 3, demonstrate the bacterial species of the selected isolates with varying similarity levels for each isolate.

**Table 3.** Results of Bacterial Identification Using the Vitek-2-Compact System.

| Isolates | Bacterial Species | Significance | Bacterial Habitate |
|---|---|---|---|
| A1 | *Staphylococcus haemolyticus* | 91% | Human skin |
| A2 | *Klebsiella pneumoniae* | 97% | Human oral cavity, skin, gastrointestines |
| A3 | *Klebsiella pneumoniae* | 97% | Human oral cavity, skin, gastrointestines |
| A4 | *Staphylococcus haemolyticus* | 85% | Human skin |
| A5 | *Klebsiella pneumoniae* | 89% | Human oral cavity, skin, gastrointestines |

## Discussion

### Total plate count analysis

Based on research findings, the total plate count of bacteria from the four *enbal* processed food samples examined met the safety standards set by the Indonesian National Standard. This may be attributed to several factors, such as proper and clean packaging. Food packaging is essential as a barrier and protection against spoilage and additional contamination. Safirin et al. (2023) stated that appropriate packaging helps maintain product quality and increases consumer acceptance. Similarly, Warto & Samsuri (2020) explained that packaging is vital in enhancing the safety and durability of processed food products.

Another factor that can help suppress bacterial growth is the processing method of *enbal* itself, which involves high-temperature heating. Sutiko et al. (2020) in their study on the effect of heating duration on non-vacuum-packaged wet spring rolls concerning total plate count (TPC), found that heating processed foods reduced bacterial count. Heating extends shelf life and eliminates organisms such as bacteria, molds, and yeasts that may contaminate processed food products. Pratama et al. (2016) revealed that heating significantly kills microbes and pathogens responsible for spoilage. Additionally, heating is an essential process for achieving the desired organoleptic characteristics of food.

One crucial factor contributing to suppressing bacterial growth in *enbal* processed food is its composition. *Enbal* is a dry processed food with low moisture content, which inhibits bacterial growth. Microorganisms require a specific level of water or moisture to thrive in food products. Preetha & Narayanan (2020) explained that foods with high water content are more susceptible to spoilage due to the rapid growth of spoilage and pathogenic microorganisms. In contrast, foods with low water content are less likely to be damaged by microorganisms. Microbial growth ceases when moisture levels drop below the minimum threshold, but microbes do not immediately die and may remain inactive in food for a certain period (Talakua et al., 2024).

Several factors may contribute to the high total plate count in one of the sampling locations, such as the quality of raw materials, storage errors, or cross-contamination during production until the product reaches consumers. Cross-contamination can occur through environmental transmission to food due to inadequate sanitation practices. According to Condro & Santoso (2017), contamination in processed foods may originate from the production process, from raw material reception to the final product, as well as poor hygiene in marketing facilities. Differences in total plate count across the four markets may be due to variations in distribution distances, leading to longer storage times and allowing bacteria to incubate for extended periods.

Hammond et al. (2015) emphasized that food distribution and storage are closely related, as longer distribution distances require additional precautions since bacterial growth increases over time. Astutiningsih et al. (2022) also found that the total microbial count rises with prolonged storage duration. The location of vendors may also contribute to the relatively higher total plate count of bacteria in Passo market than other markets. *Enbal* vendors in Passo market are situated at an intersection, a high-traffic area with many vehicles and pedestrians passing through. This factor may contribute to the higher total plate count in Passo market. Rostina & Mutiana (2018) stated that sales locations with high vehicle traffic can increase the spread of dust and bacteria, leading to greater contamination of food products and an increase in bacterial counts.

### Identification of bacterial isolates from *enbal* processed food samples

Purification and re-culturing of five selected xisolates were conducted based on their most prominent macroscopic characteristics. These characteristics included colony size, colony shape, colony elevation, colony edges, and colony color, which were assessed following *Bergey's Manual of Determinative Bacteriology*. After macroscopic characterization, Gram staining was performed for microscopic identification, revealing that two isolates were Gram-positive bacteria and three were Gram-negative bacteria. Gram staining was also used to determine the appropriate placement of isolates on the Vitek 2 GP card or Vitek 2 GN card for physiological and biochemical identification using the Vitek-2-Compact system.

Subsequently, bacterial identification was carried out at the Health Laboratory and Medical Equipment Calibration Center of Maluku Province using the VITEK-2-Compact system. The results identified two bacterial species: *Staphylococcus haemolyticus*, which was found in *enbal* processed food samples from

Mardika Market (A1) and Waiheru Market (isolate A4), and *Klebsiella pneumoniae*, which was identified in samples from Waiheru Market (isolate A2) and Passo Market (isolates A3 and A5).

*Staphylococcus haemolyticus* belongs to the coagulase-negative staphylococci (CoNS) group and is a commensal bacterium on human skin. However, it can cause severe infections in various body systems, such as meningitis in the nervous system, endocarditis in the cardiovascular system, prosthetic joints within the musculoskeletal system, and systemic infections like bacteremia, which commonly occur in hospital environments and among healthcare workers. *S. haemolyticus* has also been associated with septicemia, peritonitis, otitis media, and diabetic ulcers (Eltwisy et al., 2022a; 2022b).

*Staphylococcus haemolyticus* in food is suspected to result from contamination from human skin to the food. If consumed in high amounts, it may lead to foodborne illnesses and other systemic diseases. This finding aligns with the study by (Regecová et al., 2021), who successfully identified several *Staphylococcus* species, including *S. haemolyticus*, from 193 isolates of various food types, such as Alaska pollock (*Theragra chalcogramma*), Atlantic mackerel (*Scomber scombrus*), Atlantic herring (*Clupea harengus*), bryndza cheese (Regecová et al., 2021). Besides *S. haemolitycus*, other *Staphylococcus* species such as *S. sciuri, S. gallinarum,* and *S. pseudintermedius* were also found in smoked yellowfin tuna (Haurissa et al., 2024).

*Klebsiella pneumoniae* is a coliform bacterium belonging to the Gram-negative, rod-shaped, non-motile group. It ferments glucose and naturally exists as part of the normal human flora, found in the mouth, skin, intestines, and feces. *Klebsiella pneumoniae* is often associated with bacteremia, pneumonia, and urinary tract infections (UTIs), particularly in individuals with compromised immune systems or those exposed to nosocomial infections. Additionally, *K. pneumoniae* is a predisposing factor for liver abscesses in several Asian countries (Saif & Sami, 2020).

Contamination of food with *K. pneumoniae* can occur through transmitting its normal flora, such as from human lips or skin, onto food, making its presence detectable during bacterial identification tests. This finding aligns with the study conducted by (Hartantyo et al., 2020), which found that 147 out of 698 food samples—including vegetables, spices, and raw meat tested positive for *Klebsiella pneumoniae* through PCR analysis.

The findings of this study indicate that although the total plate count remains within safe limits, the possibility of bacterial contamination in processed food still exists. Excessive contamination can lead to an increased incidence of foodborne illnesses such as diarrhea or typhoid fever (Todd, 2014).

## CONCLUSIONS

*Enbal* products obtained from four traditional markets in Ambon City: Mardika Market, Waiheru Market, Batu Merah Market, and Passo Market are considered safe for consumption from a microbiological perspective based on SNI 01-2997-1996. The bacterial identification of processed *enbal* food exhibits the presence of both Gram positive and Gram-negative bacteria, namely *Staphylococcus haemolyticus* and *Klebsiella pneumoniae*.

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***Competing Interests:*** The authors declare that there is a conflict of interest.

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