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DETECTION AND PATHOGENICITY LEVEL SCREENING OF HEMOLYTIC BACTERIA ISOLATED FROM UNPACKED CHICKEN NUGGET

Ika Alfiyani
Diploma of Health Analyst Study Program, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang
ikaalfiani8@gmail.com

Ana Hidayati Mukaromah
Magister of Clinical Laboratory Science, Postgraduate Program, Uiversitas Muhammadiyah Semarang
ana.hidayati@unimus.ac.id

Muhammad Ardi Afriansyah
Diploma of Health Analyst Study Program, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang
afriansyah@unimus.ac.id

Stalis Norma Ethica
Magister of Clinical Laboratory Science, Postgraduate Program, Uiversitas Muhammadiyah Semarang
norma@unimus.ac.id

Abstract

Food poisoning is a severe threat that can lead to death. According to the WHO, cases of food poisoning have been on the rise in recent years. Pathogenic bacteria are responsible for food poisoning, often originating from contaminated food. Chicken nuggets, a popular ready-to-eat food, are susceptible to bacterial growth due to the risk of microbial contamination during storage. This study aims to assess the pathogenicity levels of bacteria in chicken nuggets when stored at room temperature for seven days. Samples from chicken nuggets left at room temperature were diluted and cultured on Nutrient Agar (NA) media. Subsequently, bacterial pathogenicity screening was conducted using MacConkey Agar (MC), Blood Agar Plate (BAP), and Chocolate Agar Plate (CAP). The results revealed six bacterial isolates labeled FMCN-1 to FMCN-6 (FMCN denoting Fatmawati Market chicken nugget). Most isolates exhibited a moderate level of pathogenicity (FMCN-2, FMCN-3, FMCN-4, FMCN-5, and FMCN-6), with only one isolate demonstrating low pathogenicity (FMCN-1). In conclusion, storing chicken nuggets at room temperature for seven days can lead to bacterial contamination with mostly moderate pathogenicity. Further molecular analysis is required to identify the potentially harmful bacteria.

Keywords

Bacterial Pathogenicity, Chicken Nugget, Hemolytic Bacteria, Pathogenicity Scoring

1. Introduction

Food contamination and poisoning a serious health problems that can lead to death (Mohammad et al., 2018). According to data from the World Health Organization (WHO), more than two million people die yearly due to food poisoning. In Indonesia, there are various types of food poisoning cases every year, so poisoning cases are categorized into topics of Extraordinary Events (Mabruroh & Ciptaningtyas, 2017).

The quality of foodstuffs plays a vital role in consumer health. Food quality is determined by chemicals such as nutrition, physical form, and the presence or absence of toxic microorganisms, in eating food ~~that has been~~ exposed to microorganisms resulting in *foodborne disease. Foodborne* disease is transmitted through food when the body digests (Nagaraj, 2021; Samoggia and Riedel, 2020). Food products often the course of *foodborne disease* bacteria are processed products from meat. One of the most famous processed meat products is chicken *nuggets*. *Chicken nuggets* are

ready-to-eat foods that have a high nutritional content. *Chicken nuggets* are also preferred and easy to get in traditional and mini markets (Barbut &Leishman, 2022).

Pathogenic bacteria are bacteria capable of causing human disease (Pakbin *et al.,* 2021). Because there is still a high level of microorganism contamination in food organizers' food. Hemolytic bacteria cause several cases of bacterial contamination of food. Hemolytic bacteria can lyse red blood cells ~~as a whole~~, which have pathogenic properties. Examples of pathogenic bacteria are *Pseudomonas rugosa, Escherichia coli, Staphylococcus aureus, and Salmonella* sp., *known to be pathogenic bacteria that cause many deaths* (Hwang & Park, 2015; El Ftouhy et al., 2022; Liu et al., 2023).

Screening the pathogenicity level of bacteria in food can be done relatively by using hemolysis tests using *MacConkey* (MC) and *Blood Agar Plate* (BAP) (Jabeen Fathima et al., 2019; Ethica et al., 2019). MC media is selective because it contains bile salts, *crystal violets,* and lactose. BAP media it has 5% blood, so it can isolate and grow various difficult bacteria to cultivate (Bonnet et al., 2020).

There are three types of hemolysis: α-, β-, and γ-hemolysis. The β-hemolysis bacteria can destroy red blood cells and hemoglobin until the colony produces a clear zone. The α-hemolysis bacteria can partially lyse the red blood cells until around the colony has a green color. The γ-hemolysis bacteria cannot destroy red blood cells, so they cannot change color around the colony (Ogunshe & Falode, 2021; Afriansyah & Ethica, 2023. The level of pathogenicity in bacteria can be measured using a combination of observations on MC, BAP, and *Chocolate Agar Plate* (CAP) media (Darmawati *et al.,* 2021). This study aimed to determine the presence of hemolytic bacteria in chicken nuggets stored at room temperature for seven days and screen their pathogenicity level using MC media, BAP, and CAP.

2. Materials and Method

2.1 Materials

Unpacked chicken nuggets purchased at the Fatmawati Pedurungan Semarang, allowed to stand at room temperature for seven days, were used as a sample to isolate hemolytic bacteria.

2.2. Method

2.2.1 Media preparation

This research was conducted at the Integrated Laboratory of Universitas Muhammadiyah Semarang in December 2021-January 2022. All tools to be used are washed clean, then dried and sterilized. The device is sterilized using an autoclave at a temperature of 121 ° C with a pressure of 1 atm for 15 mins. All activities were carried out aseptically. The solid media Nutrient Agar (NA), MacConkey Agar (MC). Blood Agar Plate (BAP) and Chocolate Agar Plate (CAP) were prepared by weighing the media material and then dissolved in 1000 mL of *aquadest*. The mixture was heated to dissolve; then, each was put into an Erlenmeyer covered using a cotton swab. And sterilized in an autoclave at 121°C and 1 atm for 15 mins; the obtained solution was poured into Petri dishes of 20 ml each and then left to cool (Darmawati et al., 2021; Abebe et al., 2023).

2.2.2 Bacterial isolation

As much as 1 g of chicken nugget sample was mashed. Five test tubes were sterilized and filled with physiological NaCl of 9 ml in each tube and then ready for serial dilution by 10^{-1}, 10^{-2}, 10^{-3}, 10-4, and 10^{-5}. The late sample was weighed 1 g and then put into a 10^{-1} dilution tube and homogenized using a vortex. From the 10-1 dilution tube, a sample of 1 mL was taken and then inserted into the 10^{-2} tube. These steps were continued to lower concentration to reach 10^{-5} dilution. The dilution results were then cultured on NA media by taking 1 ml and inoculated on NA media using spread plate techniques. Each unique colony observed after 24 h was marked and ready for subculture and purification (Pamungkas et al., 2018; Fuad et al., 2021)

2.2.3 Bacterial purification

The bacterial purification procedure was done by taking each unique bacterial colony grown from different dilution sources, inserting them into physiological NaCl, and then homogenizing them. A loopful of bacteria from the inoculum was then sub-cultured on NA media and incubated for 1 x 24 h at 37 ºC. The growing colonies were morphologically observed (Ethica et al., 2018).

2.2.4 Bacterial morphology identification

Then, Gram-staining was carried out by preparing object glass, smeared by a loopful of inoculum from each unique bacterial colony obtained. The object glasses were allowed to dry, then fixated by passing each over the fire. The next step was to drip each object glass with Gram A as the leading dye and allow it to stand for 3 mins. The drinks were washed with running water, followed by dripping Gram B solution, let to stand for 1 min, and then rinsed under running water. Solution of Gram C was dripped on the rinsed object glass, then let to stand for 1 min. Then again, each of the

glasses was washed under running water before it dripped again with Gram D and allowed to stand for 1 min. Each prepared object glass was observed using a microscope (Miller & Miller, 2017).

2.2.5 Screening of Hemolytic Bacteria

Bacterial isolates that have been obtained were tested for hemolytic activity using BAP media. Bacterial samples on pure NA media were then isolated on BAP media and incubated for 24 h at 37°C. The presence of hemolytic zones around the media indicates the presence of bacteria that can lyse red blood cells. The hemolytic activity test result can be inferred from the hemolytic index formed in the media (Afriansyah & Ethica, 2023).

2.2.6 Screening by Pathogenicity Scoring

Screening by pathogenicity scoring was conducted by plate-based pathogenicity assessment system first reported by Darmawati et al., 2021. The plate-based method used the observations on MAP, BAP, and CAP. Non-fermenting bacteria that cannot show a violet medium color on the MAP would be labeled "-". Meanwhile, bacteria that indicate fermenters would be labeled "+". Colonies on BAP media with complete hemolysis were marked with the label "β", while colonies with incomplete hemolysis and no hemolysis at all were marked with the labels "α and γ". The level of bacterial pathogenicity will be considered *very high* if it shows signs of colonies characteristic of bacteria that are difficult to grow, which generally grow on CAP, MAP, and BAP media (Darmawati et al., 2021).

3. Results and Discussion

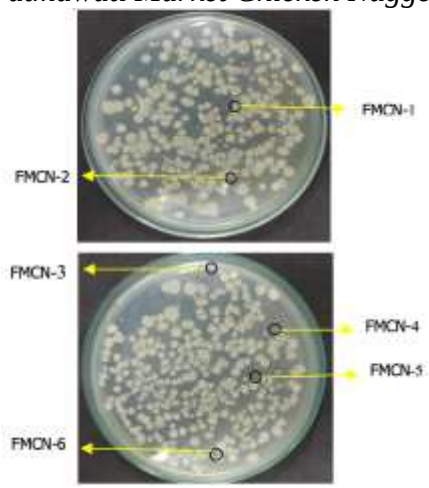
This study aimed to isolate hemolytic bacteria in chicken nugget samples stored at room temperature for seven days and screen for their pathogenicity level based on the pathogenicity scoring system (Darmawati et al., 2021). After seven days, the nugget sample changed its shape to drier, with a dark brownish color and unpleasant odor as signs of decay (Figure 1).

Figure 1: *Sample of Unpacked Chicken Nuggets Bought From Fatmawati Market of Semarang City after Seven Days of Storage at Room Temperature*

(Source: Self/Authors' Illustration).

Chicken nugget samples stored at room temperature for seven days were weighed at 0.5 g. Then, the serial dilution was carried out using physiological NaCl to minimize or reduce the number or density of microbes suspended in the liquid so that their colonies could be well-separated. The method used for bacterial isolation is the spread plate method, which is suitable to provide better visibility on colony shapes of microorganisms grown on agar media (Masi et al., 2023). The results on plates from the 10^{-5} graded dilution showed the best visibility of bacterial colonies where each colony formed was fully separated. From the related bacterial suspension (with 10^{-5} graded dilution), as much as 20 µL was taken using a micropipette, distributed into solid NA media evenly, and then incubated 1 x 24 h at 37 ºC. The results of the bacterial cultivation process on NA media can be seen in Figure 2.

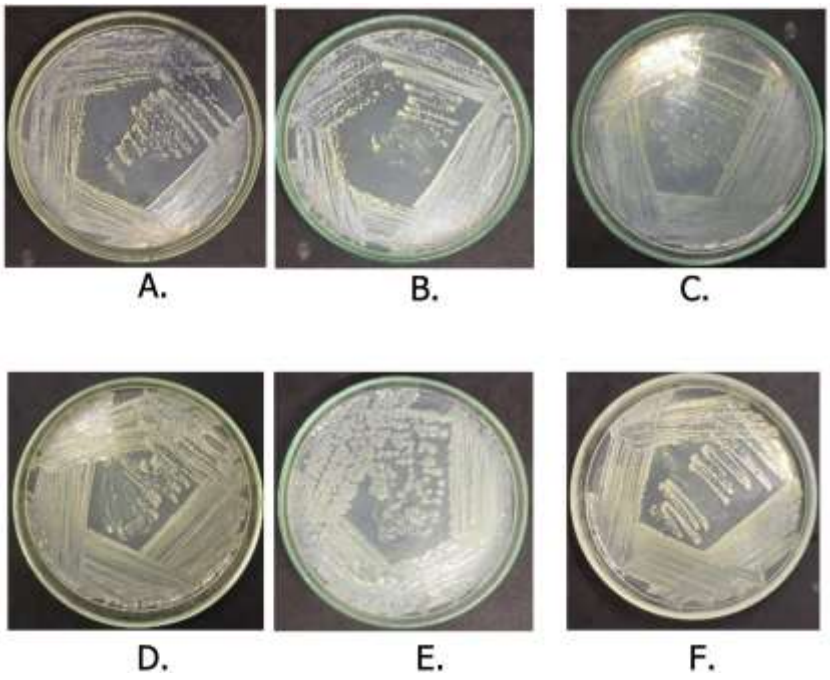
Figure 2: *Six Bacterial Unique Colonies Cultivated on Nutrient Agar Media from 10^{-5} Dilutions of Bacterial Suspension Stock. Each Strain was Labeled Where FMCN Refers to Fatmawati Market Chicken Nugget*



(Source: Self/Authors' Illustration).

The results of culturing bacteria from 10^{-5} dilution stock out on NA media using the spread plate method showed growth, as seen in Figure 2. As seen in Figure 2, there were six different bacterial colonies with visually distinct characteristics. Each of the six bacterial colonies was then subjected to the purification stage as they were considered other bacterial species. The method used at the purification step is the streak plate method. This stage isolates microorganisms from the mixture (Rajapaksha et al., 2019). This method was used to isolate microbial colonies on agar media to obtain separate colonies and pure cultures. The purification results of pure bacterial isolates were then observed morphologically, and the results are presented in Figure 3.

Figure 3: *Colony Purification Results Of Each Of 6 Isolated Bacteria With Unique Colony Isolated From Unpacked Chicken Nugget After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). A. Strain FMCN-1, B. FMCN-2, C. FMCN-3, D. FMCN-4, E. FMCN-5 Dan F. FMCN-6 Where FMCN Refers To Fatmawati Market Chicken Nugget*



(Source: Self/Authors' Illustration).

This identification includes shape, elevation, margin, size, consistency, color, and properties. Microscopic observations include cell morphology and color. Gram-positive bacteria will be purplish-blue, while Gram-negative bacteria will be red (Miller & Miller, 2017). The morphological characteristics of bacterial cells can be seen in Table 1.

Based on the results of bacterial purification on Natrium Agar media, different morphological characteristics of the colonies were observed (Table 1). The FMCN-1 FMCN-2,

FMCN-3, FMCN-4, FMCN-5, and FMCN-6 isolates have the same round shape colonies. All colonies are small except the FMCN-5 colony, which had a moderate size. The edges of all colonies are the same, namely entire, as is the elevation; all colonies have the same elevation, namely Convex (curved). The consistency of FMCN-1, FMCN-2, FMCN-3, FMCN-4, FMCN-5 colonies were smooth unless FMCN-6 had a mucoid consistency.

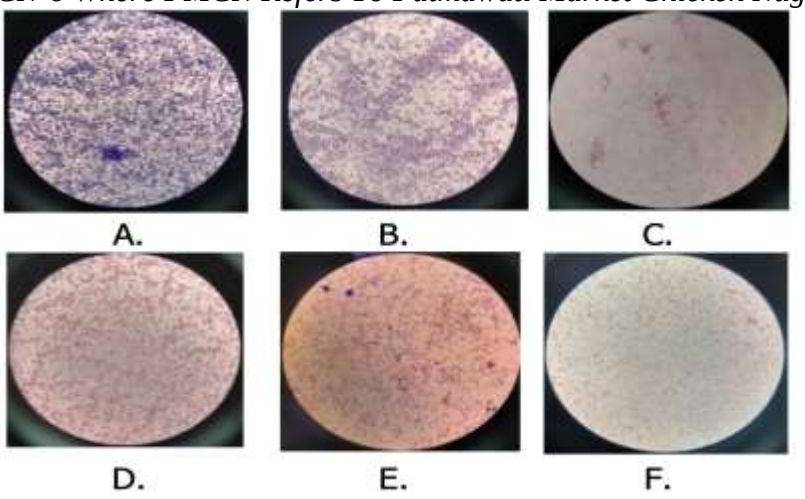
Table 1. *Morphology Characteristics Of Unpacked Bacterial Colonies Isolated From Unpacked Chicken Nuggets After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). FMCN Refers To Fatmawati Market Chicken Nugget*

Sample Code	Shape	Color	Size (mm)	Consistency	Elevation	Edge
FMCN-1	*round*	Cream	*small*	*smooth*	*convex*	*entire*
FMCN-2	*round*	Opaque white	*small*	*smooth*	*convex*	*entire*
FMCN-3	*round*	Clear	*small*	*smooth*	*convex*	*entire*
FMCN-4	*round*	Yellow	*small*	*smooth*	*convex*	*entire*
FMCN-5	*round*	Opaque white	*moderate*	*mucoid*	*convex*	*entire*
FMCN-6	*round*	Opaque white	*small*	*smooth*	*convex*	*entire*

(Source: Self/Authors' Own Illustration).

The following process was Gram staining on obtained six strains to determine the various shapes and uniformity of cells (Prayekti & Sumarsono, 2012). Uniform cells indicate that the isolate is pure without contaminants (See Figure 4).

Figure 4: *Colony Purification Of Each Of 6 Isolated Bacteria With Unique Colony Isolated From Unpacked Chicken Nugget After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). A. Strain FMCN-1, B. FMCN-2, C. FMCN-3, D. FMCN-4, E. FMCN-5 Dan F. FMCN-6 Where FMCN Refers To Fatmawati Market Chicken Nugget*



(Source: Self/Authors' Own Illustration).

Table 2. *Morphology Characteristics Of Bacterial Cells Isolated From Unpacked Chicken Nugget After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). FMCN Refers To Fatmawati Market Chicken Nugget*

No	Isolate code	Shape	Arrangement	Gram Staining
1	FMCN-1	*coccus*	clustered	positive
2	FMCN-2	*coccus*	clustered	positive
3	FMCN-3	*bacillus*	solitary	negative
4	FMCN-4	*coccus*	*In row*	positive
5	FMCN-5	*spored bacillus*	Solitary	positive
6	FMCN-6	*coccus*	*In row*	positive

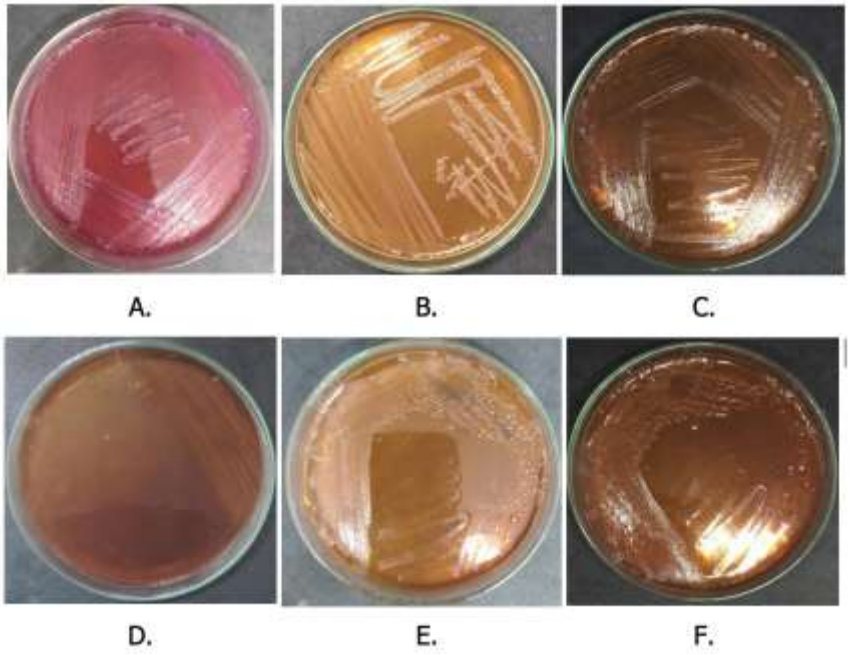
(Source: Self/Authors' Own Illustration).

The Gram staining mechanism is based on the compositional structure of the bacterial cell wall. Gram-negative bacteria contain higher levels of lipids or fat-like substances than those contained in Gram-positive bacteria. The cell walls of Gram-negative bacteria cause the retraction of lipids, thus increasing the permeability of the cell walls of Gram-negative bacteria. Thus, the crystal violet complex that has entered the cell wall during the initial step in the staining process can be extracted. Because of this, Gram-negative bacteria lose this color (Sastry & Bhat, 2018).

Based on the results of Gram staining of bacterial isolates, it was found that FMCN-1 and FMCN-2 isolates had the form of clustered coccus with Gram positive characteristics marked by purple cells, while the FMCN-3 isolate had a bacillus form with a solitary arrangement with Gram negative characteristics marked by cells. the pink one. The FMCN-4 isolate has a coccus shape with a row arrangement with Gram-positive properties indicated by the color purple.

The FMCN-5 isolate has the form of a gram-positive spore bacillus with a solitary arrangement, while the FMCN-6 isolate has a coccus form with a row arrangement and has Gram-positive characteristics which are marked with a purple color.

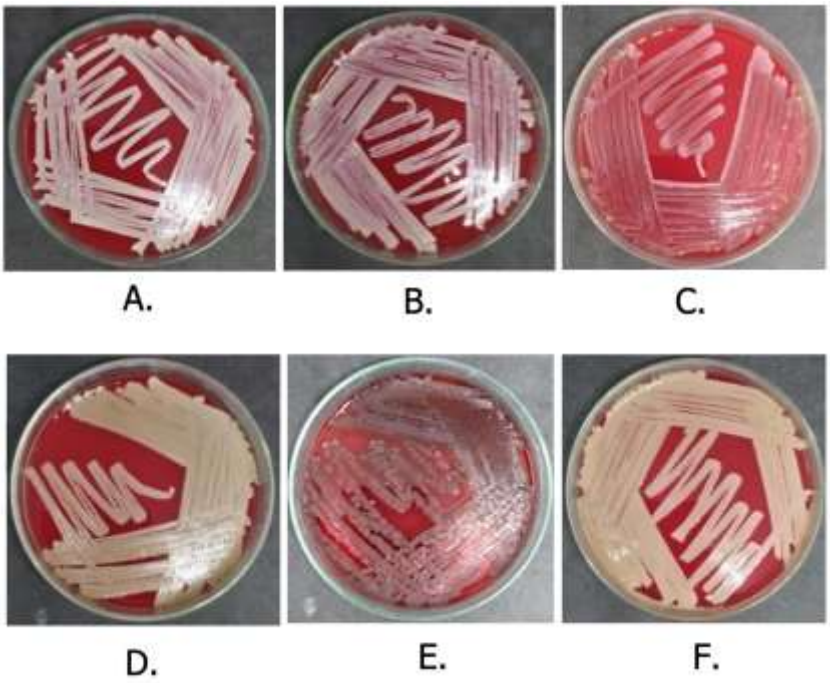
Figure 5: *Colony Purification Of Each Of 6 Isolated Bacteria With Unique Colony Isolated From Chicken Nugget After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). A. Strain FMCN-1, B. FMCN-2, C. FMCN-3, D. FMCN-4, E. FMCN-5 Dan F. FMCN-6 Where FMCN Refers To Fatmawati Market Chicken Nugget*



(Source: Self/Authors' Illustration).

The plate-based pathogenicity level test included differentiation of bacteria that are lactose fermenters and non-lactose fermenters using MacConkey Agar Plate. The selective nature of the medium is attributed to Crystal violet and bile salts inhibiting most gram-positive bacteria. Neutral Red is a pH indicator that becomes red at a pH below 6.8 and colorless at a pH greater than 6.8 (Aryal, 2018). Results of the lactose fermentation and non-lactose fermenter tests for each bacterial colony isolate are shown in Figure 5. Based on Figure 5, it can be seen that the bacterial isolates are non-lactose fermenters as indicated by the growth of colorless colonies in isolates FMCN-2, FMCN-3, FMCN-4, FMCN-5, and FMCN-6. In contrast, in isolates FMCN-1 is a lactose fermenter which is characterized by the growth of pink bacteria. Most bacteria capable of fermenting lactose are non-pathogenic (Pamungkas et al., 2018).

Figure 6: *Colony Purification Of Each Of 6 Isolated Bacteria With Unique Colony Isolated From Chicken Nugget After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). A. Strain FMCN-1, B. FMCN-2, C. FMCN-3, D. FMCN-4, E. FMCN-5 Dan F. FMCN-6. Where FMCN Refers To Fatmawati Market Chicken Nugget*

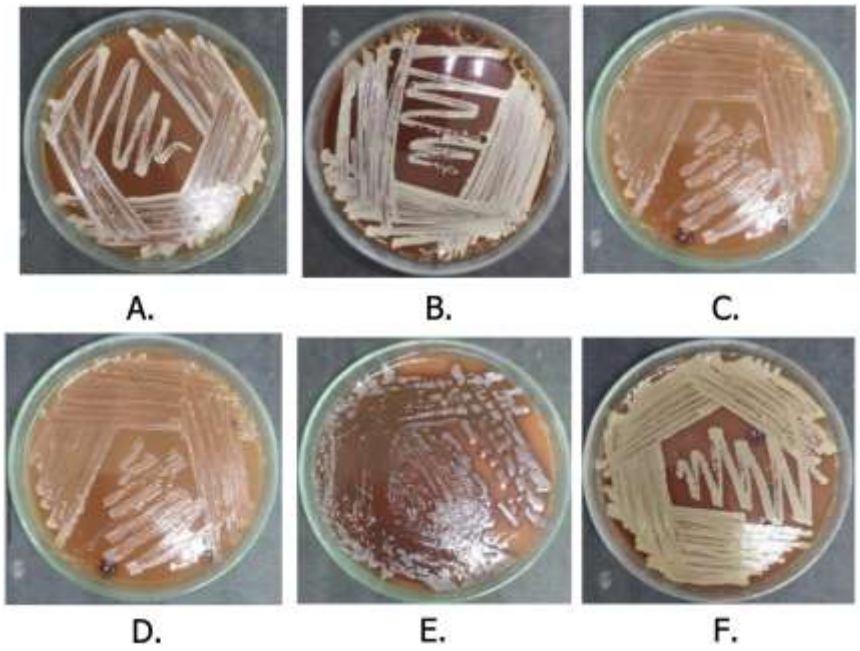


(Source: Self/Authors' Illustration).

The BAP test results for each bacterial colony isolate are shown in Figure 6. BAP media is an enriched and differential medium. It is called an enriched medium because it contains 5% blood, so it can enhance the growth of various types of bacteria that are difficult to cultivate (Tidjani Alou, 2020). Results in Figure 6 show that the six bacterial isolates are unable to hemolyze red blood cells (unable to change the color of the medium around the colony) or γ-hemolyze (non-hemolytic), so they are classified as bacteria with a low level of pathogenicity.

The CAP test results relied on specific morphology and hemolysis to identify colonies corresponding to the pathogen species. Observation on the media was carried out after 24 h incubation, and the results are shown in Figure 7. As seen in number 7, all isolated strains in this study did not establish similar colonies with certain highly pathogenic bacteria commonly growing in CAP, such as *Streptococcus pneumoniae*, *Neisseria meningitides*, *N. gonorrhoeae*, and *Haemophilus influenza* (Darmawati, et al., 2021).

Figure 7: *Colony Purification Of Each Of 6 Isolated Bacteria With Unique Colony Isolated From Chicken Nugget After 7-Day Incubation At Room Temperature On Nutrient Agar Medium (SMA). A. Strain FMCN-1, B. FMCN-2, C. FMCN-3, D. FMCN-4, E. FMCN-5 Dan F. FMCN-6 Where FMCN Refers To Fatmawati Market Chicken Nugget*



(Source: Self/Authors' Illustration).

Table 2. *Screening Results Based On Plate-Based Pathogenicity Scoring System by Darmawati Et Al. (2021) Of 6 Bacterial Strains Isolated From Unpacked Chicken Nugget after 7-Day Incubation at Room Temperature Where Fmcn Refers To Fatmawati Market Chicken Nugget*

Isolate Code	MacConkey Agar Plate (MAP) Result	Blood Agar Plate (BAP) Result	Chocolate Agar Plate (CAP) Result	Pathogenicity Score
FMCN-1	+	γ	-	Low
FMCN-2	-	γ	-	Medium
FMCN-3	-	γ	-	Medium
FMCN-4	-	γ	-	Medium
FMCN-5	-	γ	-	Medium
FMCN-6	-	γ	-	Medium

(Source: Self/Authors' Own Illustration).

All observation results from Gram-staining, MC, BAP, and CP were summarized in Table 2. The summarized data will be used for the initial pathogenicity scoring for each of the 6 bacterial strains isolated from unpacked chicken nugget samples.

The plate-based pathogenicity level assessment system established in this study is based on observation techniques previously reported on MAP, BAP, and CAP (Darmawati et al., 2021). In principle, non-fermenting bacteria that cannot show violet media color on MP are labeled "-", while bacteria that lead fermentation are labeled "+". Bacterial colonies on BAP with complete hemolysis are labeled “β”, while those with incomplete hemolysis and no lysis at all are labeled “α and γ”.

Bacterial pathogenicity levels are considered very high if the bacteria can show signs of colonies of characteristic bacteria that are difficult to grow, which generally grow on Chocolate Agar media, regardless of the distinct colonies on MAP and BAP. Pathogenic bacterial species that are difficult to grow but can grow on Chocolate Agar media with distinctive colony shapes are *Streptococcus pneumoniae*, *Neisseria meningitides*, *N. gonorrhoeae*, and *Haemophilus influenza* bacteria (Darmawati et al., 2021).

Tri et al. (2018) reported detecting Enterobacteriaceae contamination in bulk-packaged chicken nuggets and Bandar Lampung. The study found Enterobacteriaceae in bulk chicken nuggets and packaged chicken nuggets, namely *Shigella sonnei* and *Proteus mirabilis*, known as emerging pathogenic bacteria. In one sample, Gram-positive cocci were found, which is in line with the results from this study.

Overall, it can be concluded that unpacked chicken nuggets purchased at Fatmawati Market, Pedurungan, Semarang, which were left for seven days at room temperature, contained several bacterial isolates with low to moderate levels of pathogenicity based on test results on MC, BAP, and CAP media. Consuming chicken nuggets that have bacteria with an average level of pathogenicity will undoubtedly carry the risk of causing food poisoning. Further investigation is needed based on our screening to identify species of hemolytic bacteria that may contaminate chicken nuggets to determine their exact pathogenicity.

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