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Research Article
Microorganism Test on Biscuits Combined With Red Algae Extract (*Eucheuma denticulatum*) and Tempeh (*Glycine max*)

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Abstract

Background and Objective: Biscuits are snacks that are widely circulated in the market but do not meet Indonesian National standards so they are harmful to consumer health. This study aims to determine the total plate count (TPC) value of bacteria and mold/yeast and determine the presence or absence of bacterial contamination of *Staphylococcus aureus* and *Escherichia coli* in biscuit products. **Materials and Methods:** This study is descriptive in nature using three different sample types. Total plate count (TPC) value testing was carried out using the pour plate method. Meanwhile, to determine the presence or absence of *Staphylococcus aureus* bacteria using MSA (mannitol salt agar) media with the spread plate technique. The *Escherichia coli* test uses EMBA (eosin methylene blue agar) media with a streak plate technique. **Results:** Three samples of biscuit formula obtained ALT of bacteria in sample A) 2.2×10^7 colonies/g, sample B) 1.9×10^7 colonies/g and sample C) 4.1×10^7 colonies/g. Mold/khamir obtained in sample A) 7.7×10^5 colonies/g, sample B) 5.1×10^6 colonies/g and sample C) 1.1×10^6 colonies/g. In the *Staphylococcus aureus* bacteria test, the results were not overgrown with *Staphylococcus aureus* bacteria and in the *Escherichia coli* bacteria test, the results were easily purplish red in color. **Conclusion:** It can be concluded that only formula C samples meet the requirements of the SNI quality standards. In the pathogenic microbial test, there was no growth of *Staphylococcus aureus* and *Escherichia coli* microbes in the three biscuit formula samples.

Key words: *Eucheuma denticulatum*, *Glycine max*, biscuits, microorganism, contamination, product

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biscuits are snacks or snacks that have low water content, made by baking dough made from wheat flour with or without substitutes, oil or fat, with or without the addition of other food ingredients and permitted food additives[1]. Biscuits until now are much favored by the public so that snacks or snacks from various economic groups and age groups. Saksono with his research stated that biscuit consumption increased by 5-8% along with the increase in domestic consumption[2].

Currently, there are many biscuits on the market in various shapes and flavors. However, not all biscuits on the market meet the Indonesian National Standards (SNI) so that they are harmful to consumer health. This can occur because the biscuits have been contaminated by physical, chemical and microbial contamination. Microorganism contamination in unqualified food can cause diseases when consumed by humans such as diarrhea, poisoning and more danger if the food is contaminated by pathogenic microorganisms which can be fatal to the person who consumes it. Microbes can contaminate food through water, dust, air, soil and processing equipment (during the production or preparation process)[3].

Food storage factors must also be considered because they will determine food safety and microbiological quality. Food storage from raw materials to products needs to be considered. Shelf life will also affect the proliferation of microbes in the product. Gut microbiota is known to affect host health at several different levels: By competing with pathogens, by interacting with the immune system and by producing beneficial and detrimental metabolites[4].

The quality of the raw materials determines the food consumption. Good raw materials that are not overgrown with microbes can be predicted to have a longer shelf life. Food that is stored for too long and has been contaminated with mold will change color, shape and texture. This is caused by the activity of the braid that continues to grow. The longer the incubation time, the more the number of mold colonies increases because it is supported by sufficient nutritional factors from food. Molds that contaminate food can interfere with human health, because some species of molds are capable of producing mycotoxins[5].

Pathogenic and non-pathogenic microbes when present in large quantities will be very harmful to the body. The microbiological quality of food and beverages is an important issue and needs great attention, because it can cause poisoning[6].

Several cases of poisoning were caused by the consumption of food and beverages, including in 2007 there was a snack poisoning in one of the hotels in Padang and based on the results of laboratory clinical tests, it was found that the food samples were positive for *Staphylococcus aureus* and *Bacillus cereus*[7].

The MUI's fatwa on microbes and microbial products says microbes are basically halal as long as they do no harm and are not exposed to uncleanliness. The microbial content found in food and beverages produced with the help of microbes is called microbial products[8]. Microbial products are halal as long as the ingredients, from preparation to production, are not haram and unclean[9]. In Islam, Muslims are required to consume halal food, because every food that Muslims consume will be ingrained in the body and become an important source of energy for life[10].

This study was conducted to test the contamination of *Staphylococcus aureus*, *Escherichia coli* and mold microorganisms in biscuits from a combination of red algae extract (*Eucheuma denticulatum*) and tempeh powder (*Glycine max*) based on product shelf life.

MATERIALS AND METHODS

Study area: The research was conducted at the Laboratory of Pharmaceutical Microbiology, Faculty of Medicine and Health Sciences, State Islamic University of Alauddin Makassar, from February to May 2023.

Materials: The materials used in this study 5 g of biscuits were used, 70% alcohol, sterile distilled water, nutrient agar (NA) medium, potato dextrose agar (PDA) medium, mannitol salt agar (MSA) medium, lactose broth (LB) medium and eosin methylene blue agar (EMBA) medium, measuring cup, vortex, dropper pipette, volume pipette, spoit, test tube, beaker glass, stirring glass, petri dish, bunsen, lighter, hotplate, erlenmeyer, test tube rack, ose needle, spatula, fat cotton, aluminum foil, label paper and colony counter.

Methods

Sample preparation: Prepare for food samples composition in the form of biscuits as shown in the Table 1 and biscuit formula 1-3 shown in the Fig. 1. Then, a dilution bottle containing 9 mL of sterile distilled water was prepared[11]. After that, 1 g of biscuits was weighed and then crushed until smooth with a mortar[12]. The crushed sample is then put into a dilution bottle and shaken until homogeneous (dilution 10^{-1}). From dilution 10^{-1}, then taken 1 mL with a spoit and put into a dilution bottle and shaken until homogeneous (dilution 10^{-2}). From dilution 10^{-2}, take 1 mL with a spoit and put into a dilution bottle, shaken until homogeneous (dilution 10^{-3}) do the same for dilutions 10^{-4}, 10^{-5} and 10^{-6}.

Fig. 1(a-c): Biscuit formula 1, 2 and 3

Table 1: Biscuit composition of combination read algae extract and tempeh

Material	Usability
Red algae extract	Main ingredients
Tempeh powder	Main ingredients
Sago flour	Fastener
Stevia sugar	Sweetener
Vanilla powder	Fragrance
Water	Solvent

Microbiological test of biscuit preparations

Bacterial ALT test: In the bacterial ALT test, three dilutions of biscuit food were taken, namely dilutions 10^{-4}, 10^{-5} and 10^{-6} then pipetted as much as 1 mL and then put into a sterile petri dish. After that, natrium agar medium was added until the entire bottom of the cup was covered, then homogenized by rotating 7 times to the left and 7 times to the right allowing it to solidify[13]. Then, incubated for 1×24 hrs at 37°C and counted the number of bacterial colonies present[14].

Mold ALT test: In the mold ALT test, three dilutions of biscuit food were taken, namely dilutions 10^{-4},10^{-5} and 10^{-6} were pipetted as much as 1 mL and then placed in a sterile petri dish. After that, PDA medium was added until the entire bottom of the cup was covered, homogenized and allowed to solidify. Then, incubated for 3×24 hrs at room temperature, counted the number of existing mold colonies[15].

***Staphylococcus aureus* bacteria test:** In the *Staphylococcus aureus* bacterial test, three dilutions of biscuit food were taken, namely dilutions of 10^{-4}, 10^{-5} and 10^{-6} then each dilution was taken as much as 1 mL and then put into a petri dish containing MSA media that had solidified using the scatter cup method[11]. Then the petri dish containing the inoculant was incubated using an incubator at 37°C for 24 hrs. Observations were then made of the colony characteristics (color and shape of colonies) formed on the test media and compared with the characteristics of colonies that generally grow on MSA media[16]. *Staphylococcus aureus* colonies will show round-shaped, yellow-colored colonies surrounded by a yellow zone around the colony[17].

***Escherichia coli* bacteria test:** Prepared nine test tubes each dilution consists of 3 tubes in which there is a durham tube in an inverted state. From dilution 10^{-1}, 1 mL was pipetted into the first series test tube containing 9 mL lactose broth (LB) medium. From dilution 10^{-2}, 1 mL was pipetted into the second series test tube containing 9 mL lactose broth (LB) medium, the same was done for dilution 10^{-3}. All tubes were homogenized and incubated for 1×24 hrs at 37°C[18]. A positive reaction with air bubbles in the durham tube and or a color change from yellow to cloudy in the test solution[19].

From the tubes that gave positive results, 1 ose was taken and then inoculated in a scratch on eosin methylene blue agar (EMBA) medium. Furthermore, it was incubated at 37°C for 24 hrs. A positive reaction is characterized by the formation of metallic green colonies which are *colonies* of *Escherichia coli* bacteria[20].

Data analysis: Data obtained from the calculation of the number of bacterial colonies, mold/khamir in the total plate count (TPC) test and identification of *Staphylococcus aureus* and *Escherichia coli* bacteria in biscuits were analyzed descriptively in tabular form accompanied by narration.

RESULTS AND DISCUSSION

Bacterial TPC test results on biscuits: Total plate count test of bacteria in biscuit samples obtained results at a shelf life of 1 month were shown in the Table 2.

In the results of the bacterial ALT test, it was found that three test sample formulas did not meet the requirements with the results of contamination in formula 1 which amounted to 2.2×10^7 colonies/g as shown in the Fig. 2. Formula 2 which was 1.9×10^7 colonies/g as shown in Fig. 3. And formula 3 which was 4.1×10^7 colonies/g as shown in Fig. 4. Determination of total plate count (ALT) of bacteria with the standard plate count (SPC) method using nutrient agar (NA) medium. From the observation results of the three biscuit formulas, the results showed that they did

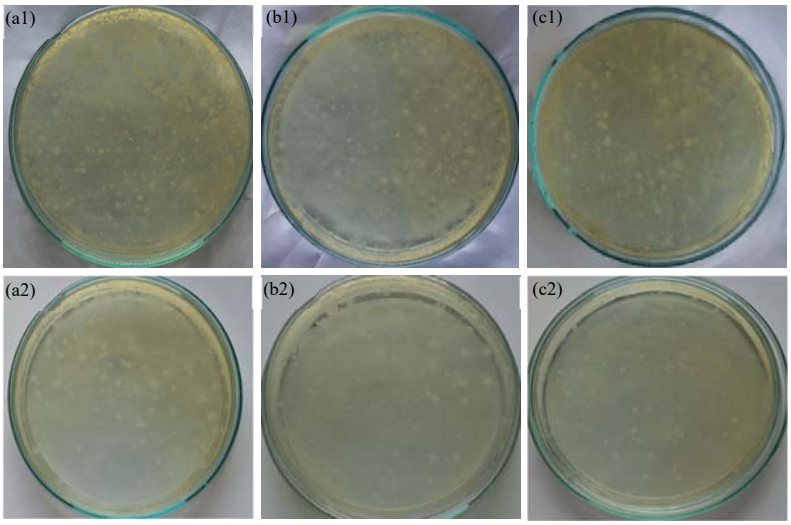

Fig. 2(a1-c2): ALT result of formula 1 bacteria, (a1) Sample formula 1 dilution 10^{-4} (cup 1), (a2) Sample formula 1 dilution 10^{-4} (cup 2), (b1) Sample formula 1 dilution 10^{-5} (cup 1), (b2) Sample of formula 1 dilution 10^{-5} (cup 2), (c1) Sample of formula 1 dilution 10^{-6} (cup 1) and (c2) Sample of formula 1 dilution 10^{-6} (cup 2)

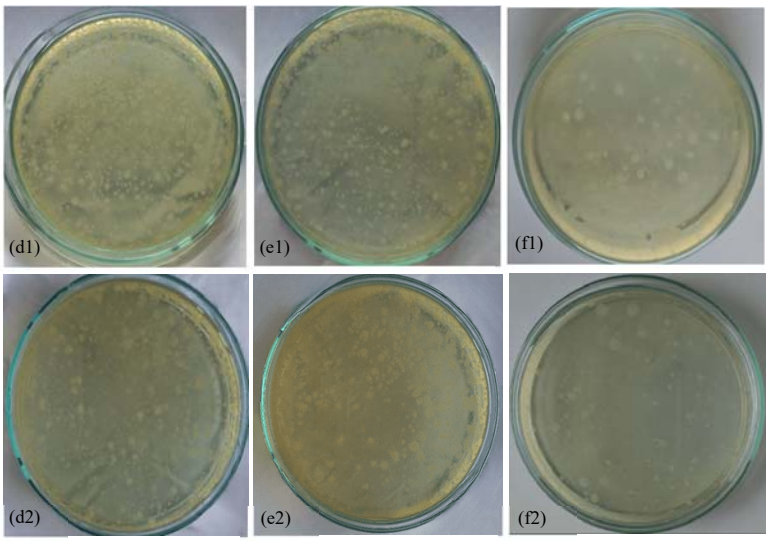

Fig. 3(d1-f2): ALT results of Formula 2 bacteria, (d1) Sample formula 2 dilution 10^{-4} (cup 1), (d2) Sample formula 2 dilution 10^{-4} (cup 2), (e1) Sample formula 2 dilution 10^{-5} (cup 1), (e2) Sample formula 2 dilution 10^{-5} (cup 2), (f1) Sample formula 2 dilution 10^{-6} (cup 1) and (f2) Sample formula 2 dilution 10^{-6} (cup 2)

Table 2: Results of bacterial total plate count test on biscuits

		Number of colonies					
Sample name	Dilution	Cup 1	Cup 2	Average	Alt (colonies/g)	Contamination standard	Ket
Biscuit 1	10^{-4}	205	67	136	2.2×10^{7} colonies/g	1×10^{4} colonies/g	TMS
	10^{-5}	94	51	72.5			
	10^{-6}	79	39	59			
Biscuit 2	10^{-4}	239	158	198.5	1.9×10^{7} colonies/g		TMS
	10^{-5}	216	98	157			
	10^{-6}	49	33	41			
Biscuit 3	10^{-4}	∞	∞	∞	4.1×10^{7} colonies/g		TMS
	10^{-5}	221	109	165			
	10^{-6}	87	45	66			

MS: Qualified and TMS: Not eligible

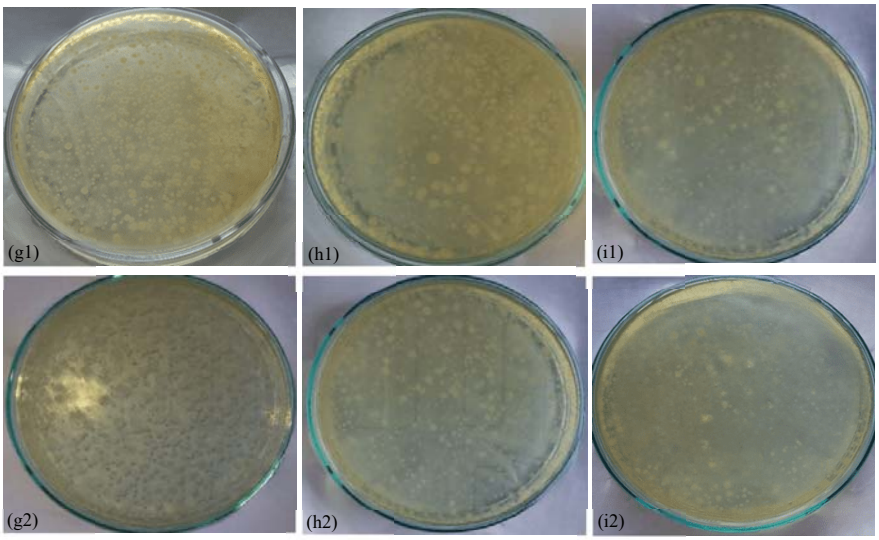

Fig. 4(g1-i2): ALT results of Formula 3 bacteria, (g1) Sample formula 3 dilution 10^{-4} (cup 1), (g2) Sample formula 3 dilution 10^{-4} (cup 2), (h1) Sample formula 3 dilution 10^{-5} (cup 1), (h2) Sample formula 3 dilution 10^{-5} (cup 2), (i1) Sample of formula 3 dilution 10^{-6} (cup 1) and (i2) Sample of formula 3 dilution 10^{-6} (cup 2)

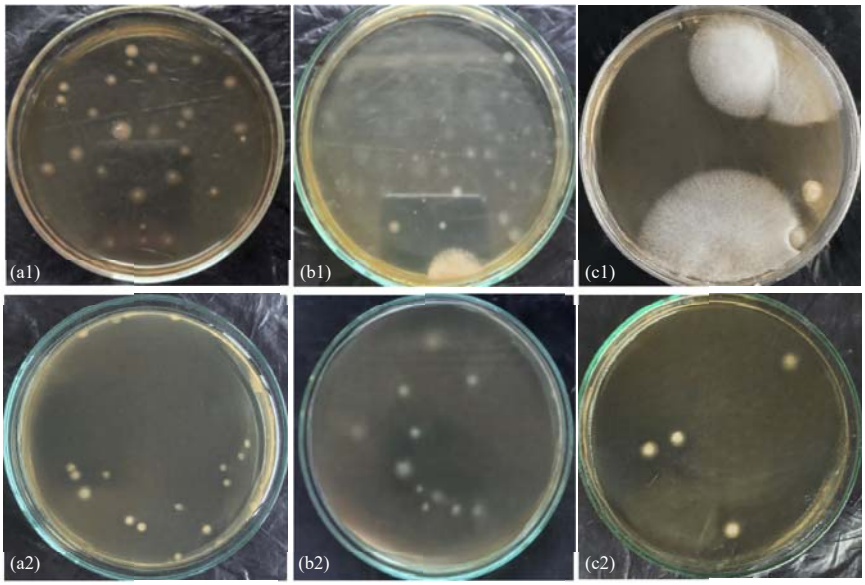

Fig. 5(a1-c2): ALT results of mold/yeast of Formula 1, (a1) Sample formula 1 dilution 10^{-4} (cup 1), (a2) Sample formula 1 dilution 10^{-4} (cup 2), (b1) Sample of formula 1 dilution 10^{-5} (cup 1), (b2) Sample formula 1 dilution 10^{-5} (cup 2), (c1) Sample of formula 1 dilution 10^{-6} (cup 1) and (c2) Sample formula 1 dilution 10^{-6} (cup 2)

not meet the requirements because the number of bacterial colonies exceeded the limit according to the National Standardization Agency, 2011. Microbial contamination biscuit quality testing parameters: Total plate number max., 1×10^4 colonies/g. Based on these provisions, the three biscuit formulas do not meet the requirements[14].

AKK test results on biscuits: Mold total plate count test on biscuit samples obtained results at a shelf life of 1 month were shown in the Table 3.

In the AKK test results, the contamination results in formula 1 were 7.7×10^5 colonies/g as shown in Fig. 5. Formula 2 was 5.1×10^6 colonies/g as shown in Fig. 6. and formula 3 was 1.1×10^6 colonies/g as shown in Fig. 7.

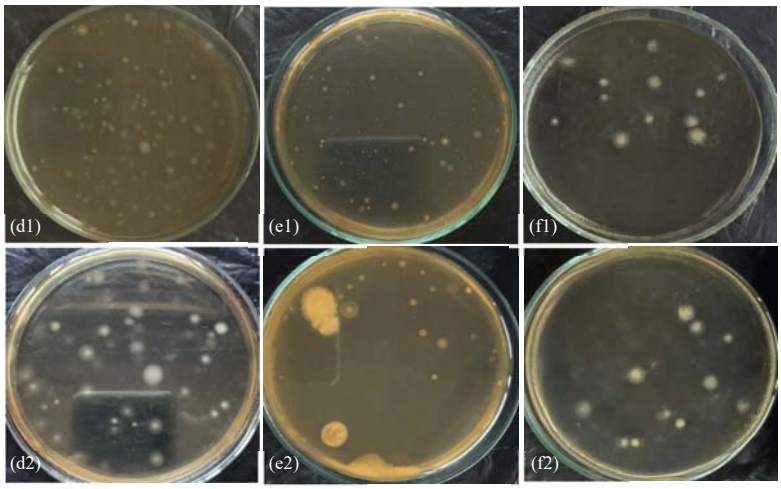

Fig. 6(d1-f2): ALT results of mold/yeast of formula 2, (d1) Sample formula 2 dilution 10^{-4} (cup 1), (d2) Sample formula 1 dilution 10^{-4} (cup 2), (e1) Sample of formula 2 dilution 10^{-5} (cup 1), (e2) Sample formula 2 dilution 10^{-5} (cup 2), (f1) Sample of formula 2 dilution 10^{-6} (cup 1) and (f2) Sample formula 2 dilution 10^{-6} (cup 2)

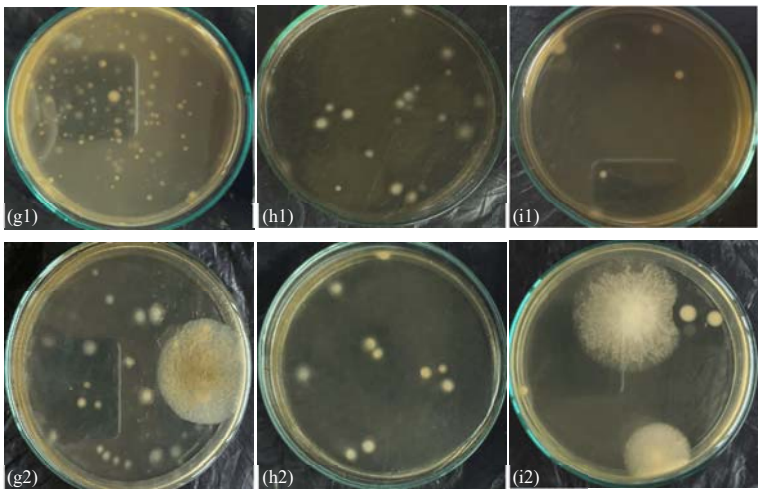

Fig. 7(g1-i2): ALT results of mold/yeast of formula 3, (g1) Sample formula 3 dilution 10^{-4} (cup 1), (g2) Sample formula 3 dilution 10^{-4} (cup 2), (h1) Sample of formula 3 dilution 10^{-5} (cup 1), (h2) Sample formula 3 dilution 10^{-5} (cup 2), (i1) Sample of formula 3 dilution 10^{-6} (cup 1) and (i2) Sample formula 3 dilution 10^{-6} (cup 2)

Table 3: AKK results on biscuits combination read algae extract and tempeh

		Number of colonies					
Sample name	Dilution	Cup 1	Cup 2	Average	Alt (colonies/g)	Contamination standard	Ket
Biscuit 1	10^{-4}	27	20	23.5	7.7×10^{5} colonies/g	2×10^{2} colonies/g	TMS
	10^{-5}	15	11	13			
	10^{-6}	5	4	4.5			
Biscuit 2	10^{-4}	130	44	87	5.1×10^{6} colonies/g		TMS
	10^{-5}	40	18	29			
	10^{-6}	12	11	11.5			
Biscuit 3	10^{-4}	73	24	48.5	1.1×10^{6} colonies/g		MS
	10^{-5}	23	10	16.5			
	10^{-6}	6	5	5.5			

MS: Qualified and TMS: Not eligible

Determination of the total plate number AKK value with the standard plate count (SPC) method using potato dextrose agar (PDA) medium. From the observation of the three biscuit formulas, the results showed that formula 1 and formula 2 did not meet the requirements because the number of AKK colonies exceeded the limit. Microbial contamination biscuit quality testing parameters: In mold/chamir max., 2×10^2 colonies/g[14]. Based on these provisions, of the three biscuit formulas, only formula 3 meets the requirements and formula 1 and formula 2 do not meet the requirements.

***Staphylococcus aureus* bacteria contamination test results:** Test results for *Staphylococcus aureus* bacteria in biscuit samples on MSA media were shown in the Table 4.

In the results of testing *Staphylococcus aureus* bacteria using mannitol salt agar (MSA) medium as a selective medium by scattering, the results obtained were negative because there were no colonies that grew on mannitol salt agar (MSA) media. The MSA media is a medium commonly used to detect the growth of *Staphylococcus aureus* in a test material. On MSA media, *Staphylococcus aureus* colonies will show round, yellow colonies surrounded by a yellow zone around the colony. The yellow zone is formed due to the ability of the colony to ferment mannitol which is one of the components contained in MSA media. Colonies that appear yellow are caused by *Staphylococcus aureus* colonies producing lipochrome pigments that are golden yellow and orange yellow. This pigment appears during the 24 hrs incubation process at 37°C. *Staphylococcus aureus* test results formula 1 as shown in Fig. 8. *Staphylococcus aureus* test results formula 2 as shown in Fig. 9. *Staphylococcus aureus* test results formula 3 as shown in Fig. 10.

***Escherichia coli* bacteria contamination test results:** Test results for *E. coli* bacteria on biscuit samples test results for *Staphylococcus aureus* bacteria in biscuit samples on MSA media were shown in the Table 5.

The first *Escherichia coli* bacteria test carried out is a presumptive test using LB (lactose broth) media as shown in Fig. 11. The fermentation results are positive if the *E. coli* bacteria have air bubbles in the durham tube and or experience a color change from yellow to cloudy in the test solution. *Escherichia coli* bacteria test results, a1-a3 sample formula 1, b1-b3 sample formula 2 and c1-c3 sample formula 3 as shown in Fig. 12. The result obtained on LB media is turbidity in the sample. Then the positive results were continued on the selective medium, namely EMBA media by scratching and the results obtained were negative, namely the absence of a metallic green color but a purplish pink color which indicated that all samples incubated on EMBA media did not grow *E. coli* as shown in Fig. 13, but indicated the presence of coliform.

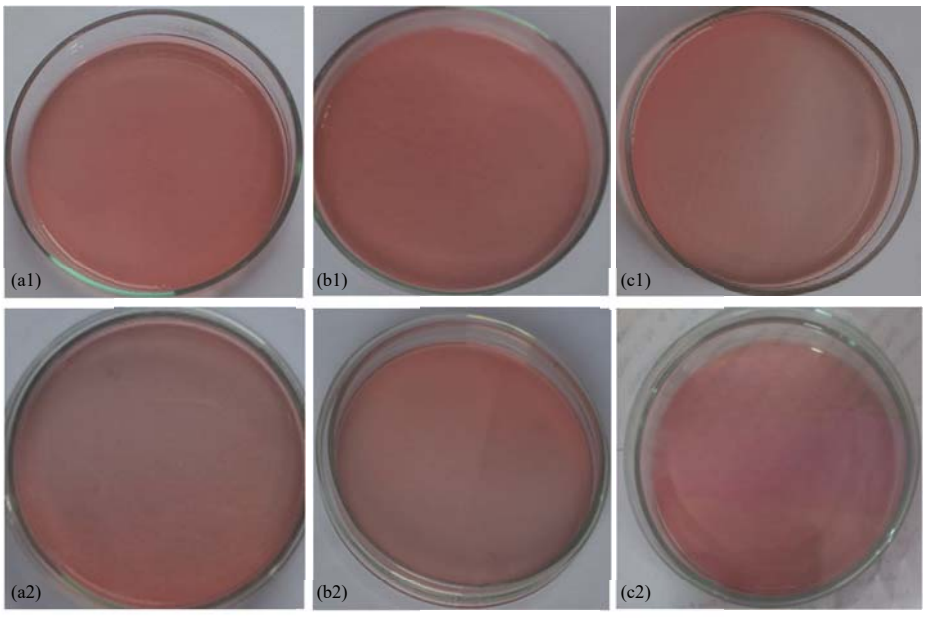

Fig. 8(a1-c2): *Staphylococcus aureus* test results formula 1, (a1) Sample formula 1 dilution 10^{-1} (cup 1), (a2) Sample formula 1 dilution 10^{-1} (cup 2), (b1) Sample of formula 1 dilution 10^{-2} (cup 1), (b2) Sample formula 2 dilution 10^{-2} (cup 2), (c1) Sample of formula 1 dilution 10^{-3} (cup 1) and (c2) Sample formula 2 dilution 10^{-3} (cup 2)

Table 4: *Staphylococcus aureus* bacteria results

		Staphylococcus aureus		
Sample	Dilution	C1	C2	Colonies on MSA media
Formula 1	10^{-1}	-	-	Not overgrown with *S. aureus* bacteria
	10^{-2}	-	-	Not overgrown with *S. aureus* bacteria
	10^{-3}	-	-	Not overgrown with *S. aureus* bacteria
Formula 2	10^{-1}	-	-	Not overgrown with *S. aureus* bacteria
	10^{-2}	-	-	Not overgrown with *S. aureus* bacteria
	10^{-3}	-	-	Not overgrown with *S. aureus* bacteria
Formula 3	10^{-1}	-	-	Not overgrown with *S. aureus* bacteria
	10^{-2}	-	-	Not overgrown with *S. aureus* bacteria
	10^{-3}	-	-	Not overgrown with *S. aureus* bacteria

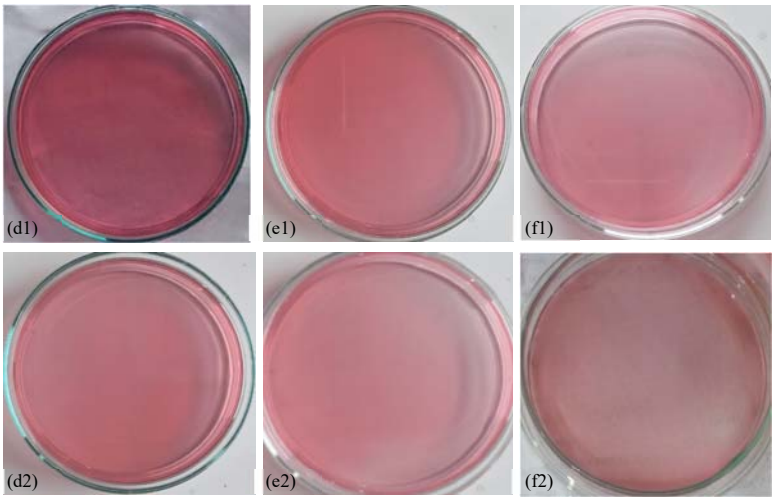

Fig. 9(d1-f2): *Staphylococcus aureus* test results formula 2, (d1) Sample formula 2 dilution 10^{-1} (cup 1), (d2) Sample formula 1 dilution 10^{-1} (cup 2), (e1) Sample of formula 2 dilution 10^{-2} (cup 1), (e2) Sample formula 2 dilution 10^{-2} (cup 2), (f1) Sample of formula 2 dilution 10^{-3} (cup 1) and (f2) Sample formula 2 dilution 10^{-3} (cup 2)

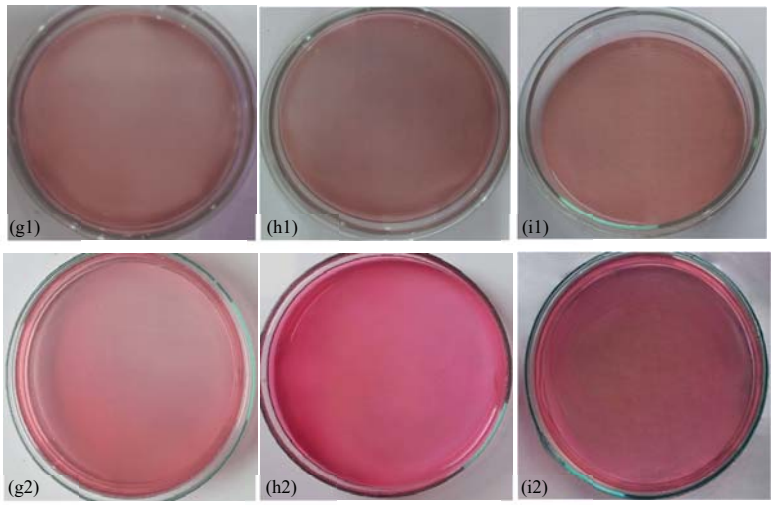

Fig. 10(g1-i2): *Staphylococcus aureus* test results formula 3, (g1) Sample formula 3 dilution 10^{-1} (cup 1), (g2) Sample formula 3 dilution 10^{-1} (cup 2), (h1) Sample of formula 3 dilution 10^{-2} (cup 1), (h2) Sample formula 3 dilution 10^{-2} (cup 2), (i1) Sample of formula 3 dilution 10^{-3} (cup 1) and (i2) Sample formula 3 dilution 10^{-3} (cup 2)

Fig. 11: Test on LB media sample formula 1, 2, 3

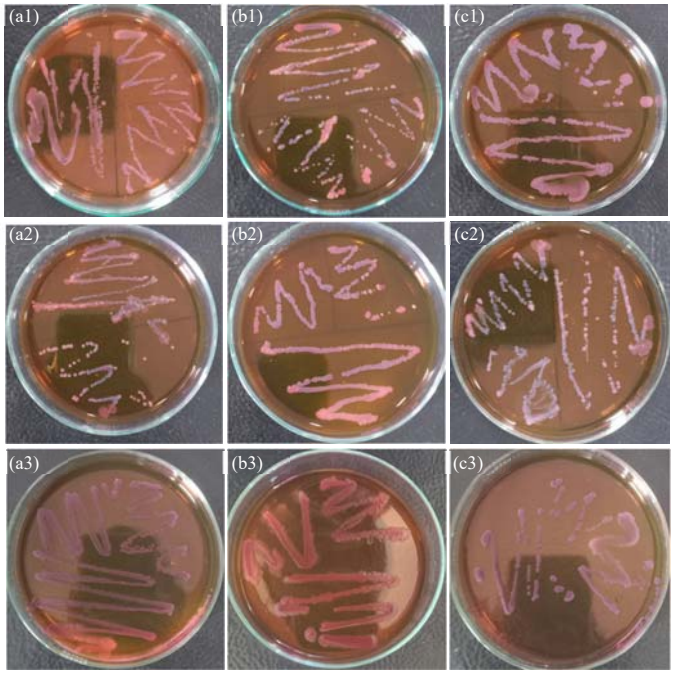

Fig. 12(a1-c3): *Escherichia coli* bacteria test results, (a1-a3) Sample formula 1, (b1-b3) Sample formula 2 and (c1-c3) Sample formula 3

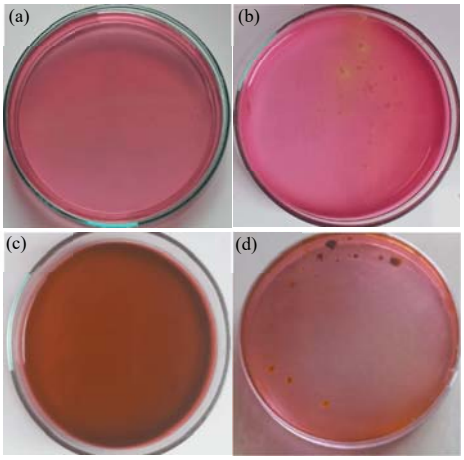

Fig. 13(a-d): MSA and EMBA control media, (a) Mannitol salt agar (MSA) media, (b) Mannitol salt agar (MSA) media+*Staphylococcus aureus* bacteria, (c) Eosin methylene blue agar (EMBA) media and (d) Eosin methylene blue agar (EMBA) media+*Escherichia coli* bacteria

Table 5: *Escherichia coli* bacteria results on biscuits combination red alga and tempeh

Sample	Colonies on LB media	Colonies on EMBA media	*Escherichia coli*
Formula 1	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
Formula 2	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
Formula 3	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative
	Turbidity occurs in the sample	Purplish pink (Coliform)	Negative

CONCLUSION

The conclusion of this study is that at a shelf life of 1 month, biscuit formulas 1, 2 and 3 for the ALT test obtained results do not meet the requirements based on Indonesian National Standards regarding the number of bacterial colonies contained in biscuit formulations. As for the AKK test, the results of the three biscuit formulas showed that only formula 3 met the requirements, formula 1 and formula 2 did not meet the requirements. From 3 samples of biscuit formulas that have been tested for bacterial ALT and mold/khamir, colonies with different ALT values are obtained, namely bacteria A with a value of 2.2×10^7 colonies/g, bacteria B with a value of 1.9×10^7 colonies/g and bacteria C with a value of 4.1×10^7 colonies/g. Mold/khamir A obtained a value of 7.7×10^5 colonies/g, mold/khamir B obtained a value of 5.1×10^6 colonies/g and mold/khamir C obtained a value of 1.1×10^6 colonies/g. And in the pathogenic microbial test (*Staphylococcus aureus* and *Escherichia coli*) no microbial growth was found.

SIGNIFICANCE STATEMENT

This study aims to determine the TPC value of bacteria and mold/yeast and determine the presence or absence of bacterial contamination in biscuit products. This study found that in the pathogenic microbial test *(Staphylococcus aureus* and *Escherichia coli)* there was no microbial growth in the biscuits used as samples. This study is a reference for further research related to the contamination of biscuits with microorganisms based on the shelf life of the product.

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