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Microbiology quality and shelf life analysis of enteral formulas based on tempeh flour and yam flour

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ABSTRACT

Background: *Critically ill patients have an increased risk of developing infection. Enteral formula that given to patients must meet food safety which includes microbiology quality. In powder form, powder formula is a solution to suppress microbial growth, although it is still susceptible to oxidation. Shelf life is useful to determine the oxidation status.*
Objectives: *This study aimed to analyze the value of TPC, Salmonella, E. coli and shelf life of enteral formula.*
Methods: *This study was a completely randomized experimental design of one factor, namely the length of storage for values of TPC, Salmonella and E. coli with variations in storage for 0, 1, 2, and 3 hours at room temperature. Data on the TPC test was analyzed using Kruskal-Wallis. The temperature used for shelf life with TBA based-Arrhenius equation is 25^{0}C, 35^{0}C, and 45^{0}C for 28 days.*
Results: *There was a difference in the length of storage of 0, 1, 2, and 3 hours on the value of TPC. The TPC value at 0 and 1 hour did not exceed the normal limit. The value of Salmonella was negative/25 g and < 3/g for E. coli. The shelf life of enteral formulas was respectively 25^{0}C, 35^{0}C and 45^{0}C for 44.89, 28.26 and 18.32 days.*
Conclusion: *The longer the length of storage, the higher the TPC value. In accordance with the Indonesian standard (SNI), there is no contamination of Salmonella and E. coli in the enteral formula. The longest shelf life is at 25^{0}C.*

Keywords *: enteral formula; microbiology quality; shelf life*

INTRODUCTION

Patients with critically ill conditions develop metabolic changes, which lead to an increase in protein catabolism, resulting in a significant loss of lean body mass.[1] Not only muscle mass but also its energy stores are depleted, and nutrients are used at high levels. This catabolic state results in weight loss, sarcopenia, and malnutrition.[2] In critical conditions, the patients could also have swallowing disorder, decreased awareness and appetite which cause them to have difficulty in meeting their nutritional needs. The presence of this condition leads to prolong intensive care, increase infections, increase complications, and increase mortality.[3] The main goal of nutritional support is to prevent malnutrition and its complications by modulating the patient's stress response.[2]

Nutritional support in critically ill patients can be performed by the administration of enteral formulas if the digestive system is functioning.[4] Enteral formulas are generally available in two types, namely Commercial Enteral Formula (FEK) in powder form and Homemade Enteral Formula (FERS) in liquid form made from a variety of fresh ingredients.[5] FEK is considered to have nutritional content that is more easily adjusted and more hygiene guaranteed but tends to be expensive for patients who do not receive full medical assistance.[6,7] FERS is more economical but more likely bearing a high risk of cross-contamination.[6] Recently, many FERS products have been developed, but there is still a big issue of hygiene and shelf life. Hospitalized-patients are a vulnerable group exposed to infections. There are food safety criteria that must be met by FERS in line with the patient's condition. The Food and Drug Administration (FDA) has recommended that Total Plate Count (TPC) level in enteral formulas is strictly below 1x10^4 CFU/g.[8] Moreover, standar in Indonesia for enteral formula according to Indonesia National Standard (SNI) is by looking at bacteria which determine the safety aspect of the food: *Salmonella* is negative/25 g and <3/g *for Escherichia coli*.[9]

Microbial growth can be influenced by storage time and environmental conditions such as temperature, nutrition, and supporting water activity. Storage time is the time between foodstuffs produced until the material is still suitable for consumption. Food products have a time limit to be safely consumed.[10] Brewed enteral formulas can only be stored for four hours at room temperature and showing exponentially grown microbial growth for more hours.[11]

To reduce the pathogenic microbial growth in food, the production chain must be shortened by making enteral formula in powder form. Powder enteral formula requires a brewing process only before being administered to the patients, so it has a lower risk of contamination than liquid formula.[5] This study uses flour-based ingredients, which are tempeh flour and

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yam flour, to maintain the quality and shelf life of the product.[12] In addition skim milk, maltodextrin, soybean oil, and granulated sugar is added to meet the nutritional content recommended by European Society of Parenteral and Enteral Nutrition (ESPEN).[13]

Powder-based and milk-based products undergone a decrease in quality in respect of fat oxidation, odor changes, browning reactions and changes in organoleptic elements due to oxygen mass, moisture content, microorganisms, and toxic chemicals.[14] Soybean oil are rich in long chain unsaturated fatty acids thus have lower storage stability because it is more sensitive to oxidation reaction. The shelf life of powder formula could be determined by the value of the malondialdehyde (MDA) level which is useful in evaluating the oxidation status of food in the initial phase of autoxidation.[15,16] Based on these problems, the aim of this study was to analyze the microbiology quality and shelf life analysis of enteral formulas with various storage times.

MATERIALS AND METHODS

Study Design

This study is part of a study entitled "GLITEROS Enteral Formula for Patients with Hyperglycemia Based on Tempeh Flour and Yam Flour" within the field of Food Technology and Food Microbiology sciences. This research was a completely randomized, one-factor randomized design. The study was conducted in March-July 2019, which consisted of preliminary and main research.

Preliminary Research

Preliminary research conducted at the CV Chem-Mix Pratama Yogyakarta Analysis Laboratory included proximate tests (carbohydrates, fats, proteins, fiber, and water), food fiber, viscosity, osmolarity, and protein digestibility. Before conducting the main research, the study was to determine the level of treatment by estimating the calculation of the material used based on the requirements of the enteral formula for patients with critically ill. The composition formulations can be seen in Table 1.

Table 1. Composition of Enteral Formula Based on Tempeh Flour and Yam Flour

Composition	A1 Formula	A2 Formula	A3 Formula
Tempeh Flour (g)	60	70	60
Yam Flour (g)	60	42	90
Skimmed Milk(g)	50	50	50
Soybean Oil (g)	15	15	15
Maltodextrin (g)	50	50	50
Sugar (g)	13	13	13
Total (g)	268	240	278

Looking at the three enteral formulas with a ratio of the amount of Tempeh and Yam flour that is 1: 1 (A1), 5: 3 (A2) and 2: 3 (A3), the best result was the one with a ratio of Tempeh and Yam flour 1: 1 (A1). The A1 formula selected has met the requirements of an enteral formula for critically ill with hyperglycemia patients, both in terms of nutrient composition and energy density.[17] The selected formula will be used for microbiological and shelf life test sample.

Main Research

The formulas were prepared at the formula kitchen, National Diponegoro Hospital (RSND). Microbiological tests including TPC were carried out at Laboratorium Terpadu Universitas Diponegoro, while *Salmonella* and *E. coli* at Balai Laboratorium Kesehatan Semarang. Morever, the shelf life test based on Tio Barbituric Acid (TBA) numbers was carried out at the Unika Soegijapranata Food Technology Laboratory.

Microbiological tests were carried out with 4 variations of treatment, namely analyzing the amount of TPC, *Salmonella,* and *E. coli* in liquid formula and steeping with a storage time of 1 hour, 2 hours and 3 hours at closed room temperature. The test was carried out with three repetitions in each treatment so that 12 samples were analyzed for microbiology. TPC analysis used the Nutrient Agar (NA) medium by planting one gram of the sample which has been diluted into a petri dish, then incubated. TPC count results in the form of CFU/ml colonies. As for the *Salmonella* bacteria, the analysis used the *Salmonella* identification method. *Salmonella* detection testing uses *Buffered Pepton Water* (BPW) as a non-selective liquid media, *Tetrationat Broth* (TB) and *Bismuth Sulfith Agar* (BSA) as a selective medium to isolate *Salmonella*. Analysis of *E.coli* bacteria using the MPN (Most Probable Number) method with *Lactose Broth* media in presumptive tests and *Brilliant Green Lactose Bile Broth* media in confirmation tests.[18]

The shelf life test was using the *Arrhenius* model accelerated shelf-life testing (ASLT) method based on TBA values using 3 variations of storage temperature 25°C, 35°C and 45°C once every seven days for 28 days. The selection of storage temperature was based on guidelines for determining the temperature of shelf-life testing on dry food.[19] Enteral formula products were packaged in aluminum foil sachets by milk powder packaging provisions.[16] The tests were carried out with two repetitions so that there are 30 samples to be analyzed. Data obtained from TBA were plotted against time and three product storage temperatures to produce a linear regression equation $y = bx + a$.

Information :
y = Characteristic value of product
x = Storage time (days)
a = Initial characteristic value of product
b = Rate of characteristic change

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The value of quality degradation constant (k) was obtained from the linear regression equation, then ln k was plotted with 1 / T to result the intercept and slope value of the linear regression equation ln k = ln k0 - (Ea / R) (1 / T). After obtained the activation energy characteristics and the value of k0, the Arrhenius equation was calculated by the formula k = k0. e-E / RT.

Information :
k = Constant decrease in quality
k0 = Constant (not temperature dependent)
E = Activation energy
T = Absolute temperature (K)
R = Gas constant (1,986 cal / mol K)

The k value obtained was calculated into the equation of the reaction sequence t = (A0 - At) / k.

Information :
A0 = Initial value of shelf life
At = Final value of shelf life
t = Shelf life (days)
k = Constant decrease in quality

Those formulas resulted in the shelf life of enteral formulas for each specified temperature.[20]

Statistical Analysis

The independent variables in this study were the storage time and storage temperature in the enteral formula. The dependent variables of this study included the value of TPC, *Salmonella*, and *E.coli* and the shelf life. The TPC test was analyzed using Kruskall Wallis statistical test with a degree of confidence of 95%, while the shelf life test of the data was analyzed using Microsoft Excel.

RESULTS

***Total Plate Count* (TPC)**

The results of the TPC test analysis showed that there was a significant difference between the storage time and the TPC value ($p < 0.05$). Based on table 2, the lowest TPC value was in the storage time of 1 hour, 0.2 x 10^4 CFU/ml, while the highest value is in the storage time of 3 hours with the value of 1.5 x 10^4 CFU/ml. The storage time of 2 and 3 hours showed that the TPC value of enteral formula samples was failed to meet the requirement as the TPC value was more than 1 x 10^4 CFU/ml.[8] Further tests showed that there were significant differences between storage times of 0 and 3 hours, 1 and 3 hours and 2 and 3 hours with the same p-value is 0.046 ($p < 0.05$).

Salmonella* Identification and *Most Probably Number* (MPN) of *E. coli

Salmonella identification test results showed that at storage time 0 hours (powder), 1 hour, 2 hours and 3 hours no *Salmonella* was detected and so also the MPN value of *E. coli* was <3/g as depicted in table 3. This is in accordance with SNI in formulas for medical purposes.[9]

Table 2. TPC Test Results with Various Storage Times

Variable	Total Plate Count Value (TPC)		p
	Median (Min-Max) x10^4	Mean ± SD	
0 hours / Powder	0,45(0,3-0,95)[a]	0,6 x 10^4 ± 0,3 x 10^4	0,023*
1 hours	0,25(0,03-0,36)[a]	0,2 x 10^4 ± 0,2 x 10^4	
2 hours	1,1(0,8-1,4)[a]	1,1 x 10^4 ± 0,3 x 10^4	
3 hours	1,5(1,5-1,6)[b]	1,5 x 10^4 ± 0,1 x 10^4	

*significance < 0,05

Table 3. *Salmonella* Identification and MPN *E.coli* Test Results

Storage Time	*Salmonella* Identification Test Results (-/+)	MPN value of *E.coli*
0 hour/ powder	Negative/25 g	< 3/g
1 hour	Negative/25 ml	<3/ml
2 hour	Negative/25 ml	< 3/ml
3 hour	Negative/25 ml	< 3/ml

Table 2. Results of TBA Analysis

Days-	TBA Values		
	25°C	35°C	45°C
0	0.341	0.341	0.341
7	0.302	0.187	0.274
14	0.277	0.274	0.281
21	0.272	0.302	0.439
28	0.287	0.397	0.431

Table 3. Linear Regression Equation of TBA Parameters

Temperature	Regression Equation		R^2	
	Ordo 0	Ordo 1	Ordo 0	Ordo 1
25°C	y=0.0020x + 0.3234	y=0.0064x – 1.132	0.6186	0.6148
35°C	y=0.0032x + 0.2548	y=0.0112x – 1.390	0.2100	0.1905
45°C	y=0.0049x + 0.2842	y=0.0134x – 1.249	0.4752	0.4354

Shelf Life Analysis

The longer the storage time and the higher storage temperature gave impact in TBA value change, which were depicted in table 4. Results of TBA analysis then were plotted in order to obtain the regression equation.

Based on table 5, reaction ordo kinetics were chosed by comparing the correlation coefficient (R^2) for each linear regression equation. A reaction ordo with a greater R^2 value is the reaction used, thus in the estimation of shelf life based on the TBA follows the zero ordo reaction. This data showed that changes in TBA numbers during storage followed linear kinetics or a constant rate of increase in TBA.

Table 4. Parameters of Arrhenius-Equation of TBA Values During Storage

Temperature (K)	1/T (x)	k	Ln k (y)
298	0.003356	0.0020	-6.2146
308	0.003247	0.0032	-5.7446
318	0.003145	0.0049	-5.3185

Based on table 6, the value of quality decrease (k) are greater when the storage temperature are higher. The value of k states the rate of reaction changes in TBA value. The larger the value of k, the bigger the rate of reaction change in TBA. The values of 1/T and ln k were plotted and a linear regression equation was obtained $y = -4246.6x + 8.0381$ with $R^2 = 0.9999$. The correlation coefficient was near to 1 or R equal 1, meaning that the temperature was extremely influencing the reaction of changes in TBA numbers. The activation energy (Ea) of the change in TBA number was 8433.76 cal/mol. These calory contributed in starting the change of TBA numbers. [14]

The shelf life of enteral formulas was calculated using the linear regression equation of TBA numbers. From each equation, the k value was obtained and further used to calculate the shelf life of the product, as shown in table 7.

Table 5. Results of Store Life for Enteral Formulas at Various Temperatures

Temperature		k Value	Shelf-life (Days)
K	^{0}C		
298	25	0.002005	44.89
308	35	0.003184	28.26
318	45	0.004912	18.32
328	55	0.007381	12.19

DISCUSSION

Total Plate Count (TPC)

Total Plate Count is a quantitative method used to find out all the total microorganisms both molds, yeasts and bacterial colonies (pathogens and non-pathogens) that grow on food. The higher the TPC value and exceeds the standard, the lower the quality of food.[21] The results of TPC test on brewed enteral formula based on tempeh flour and yam flour showed that the duration of storage (0 hours, 1 hour, 2 hours and 3 hours) differed significantly to the value of TPC with a value of $p = 0.023$. Based on further tests the results obtained were significant differences in storage time of 0 and 3 hours, 1 and 3 hours and 2 and 3 hours.

There was a significant difference in storage time 0, 1 and 2 hours with 3 hours due to increased bacterial activity. Bacteria need time to divide, which is called generation time. Bacterial generation time varies greatly depending on species and growth conditions. The more complex the cell's characteristics are, the longer it will take. Bacteria divide faster than yeast and mold. Bacteria could divide and grow optimally in about 20 minutes, while yeast around 90 minutes and mold 180 minutes.[22] Other results showed that enteral formula powder samples have a higher TPC value than steeping samples with storage duration of 1 hour. Both samples were still suitable for consumption and researchers performed the procedures in accordance with standards ranging from storing materials, making formulas, and testing processes. [28]

Temperature is one of the environmental factors that influence microbial growth. Each microbe has a certain temperature range and optimum temperature for its growth. Most food-destroying microbes are mesophile microbes that grow well at a temperature of 20-45°C.[23] Enteral formulas was brewed at 70°C, after being stored for 1 hour, 2 hours and 3 hours resulting a decrease in temperature which are 32°C, 29°C and 27°C respectively. The longer the storage, the higher the TPC value caused by decreased in temperature.

Brewed enteral formulas could only be stored for four hours at room temperature. If more than four hours, the microbes grow exponentially.[11] According to the Food and Drug Administration related special formulas for health, including enteral formulas, TPC levels are not allowed more than $1x10^4$ CFU/g.[8] Enteral formula steeping with 2 and 3 hours storage time were not suitable for consumption because TPC values of more than $1x10^4$ CFU/ml are obtained. Powder and steeping samples with 1 hour of storage showed that enteral formula samples were still suitable for consumption.

***Salmonella* Identification**

Salmonella identification test shows that there is no *Salmonella* contamination in the powder sample and enteral formula steeping with a storage time of 1 hour, 2 hours and 3 hours.[24] This research uses the ingredients of yam flour, tempeh flour, skim milk, soybean oil, granulated sugar, and maltodextrin. The use of these ingredients was one of the factors causing the absence of *Salmonella* contamination. Another possibility for

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the absence of *Salmonella* contamination in enteral formulas was the cleanliness factors such as the condition of the room and equipment in accordance with the requirements of the Ministry of Health, and the condition of the handlers who use Personal Protective Equipment during the process of making enteral formulas. Transmission of *Salmonella* bacteria via fecal-oral were not developed as long as the environment including the handlers maintain cleanliness.[25,26]

The presence of *Salmonella* in food is considered harmful to health. The presence of *Salmonella* could cause disease in the human body called *salmonellosis*. *Salmonellosis* is caused by food contaminated by *Salmonella*. *Salmonellosis* is characterized by symptoms that arise acutely, abdominal pain, diarrhea, nausea and sometimes vomiting. *Salmonella* is transmitted to humans normally when humans consume food contaminated with the bacteria.[26] The latest study estimates that there are 80.3 million annual cases of *Salmonella*-related diseases worldwide. About 5% of all hospital patients experience septicemia.[27]

Salmonella identification test is a qualitative analysis that aims to determine the presence of Salmonella in food. *Salmonella* is pathogenic, the presence of these bacteria in food can cause foodborne diseases such as diarrhea.[26,28] Indonesian Standards (SNI) guidelines state the safe limit of *Salmonella* values for milk-based liquid foods is negative/25 grams, meaning that there should be no *Salmonella* in 25 grams of food samples.[9]

Most Probably Number (MPN) of *E.coli*

The value of MPN *E. coli* sample of enteral formula based on tempeh flour and yam either in powder or steeping with a storage duration of 1 hour, 2 hours and 3 hours still met the Indonesian standrad (SNI) requirements which state the MPN *E. coli* limit on milk products is <3 per gram or per ml.[9] *Escherichia coli* are part of *Enterobacteriaceae*, gram-negative bacteria, rod-shaped, facultative and non-spore anaerobic. *Escherichia coli* can live on a variety of substrates. The presence of E. coli in food is usually through polluted water source media.[24] The World and Health Organization has recommended brewing the formula at a temperature of 70-76°C to avoid the presence of coliform bacteria. One of the causative factors MPN *E. coli* values according to the standard that is in this study brewing enteral formula samples carried out at 70°C.

Escherichia coli become a pathogen if the number of these bacteria in the digestive tract increases. *Escherichia coli* which produce enterotoxins are found as a cause of diarrhea throughout the world. *Escherichia coli* in food causes poisoning that affects stomach pain, diarrhea and fever. The field of food microbiology states that *Escherichia coli* is known as an indicator of sanitation bacteria so that the presence of these bacteria in food shows that in one or more stages of food processing is contaminated and shows conditions inadequate sanitation.[29]

The *Escherichia coli* test uses the MPN method which estimates the closest amount of *E.coli*. The advantage of this method is better sensitivity to microorganism concentrations that are less than the plate count. MPN is suitable for samples with low concentrations of microorganisms, especially from the type of water, milk or food samples, especially those that have dissolved particles in it. The MPN method output is the MPN value which is interpreted as an estimate of the number of individual bacteria. The smaller the MPN value, the higher the quality of the food, and the more suitable for consumption.[30]

Shelf Life Analysis

Shelf life is a period for products that are sensory and nutritional content still acceptable and safe for consumption. Shelf-life studies are very important for fast and perishable food products. The shelf life of food products could be suspected by two methods, Extended Storage Studies (ESS) and Accelerated Shelf Life Test (ASLT). ESS is called a conventional method by storing a product in normal conditions, changes in quality and shelf life are observed. This method requires a very long time, so it is recommended to use the ASLT method by accelerating changes in quality on critical parameters. This method uses environmental conditions that can accelerate the reaction of a decrease in the quality of food products. Food products are stored at extreme temperature conditions where damage to food products occurs faster so that the critical parameters decrease in quality due to the influence of heat. The higher the storage temperature, the reaction rates of various chemical compounds will be increasingly fast.[31]

Powdered milk formula obtained from modified cow's milk and added with polyunsaturated fatty acid (PUFA) has low chemical stability, thus it could not be stored in a longer period. Compared to other types of fat, PUFA is more susceptible to oxidation. In this study, the source of fat for enteral formulas was obtained from soybean oil, which has a high content of Polyunsaturated Fatty Acids (PUFA) which is less stable to oxidation. Food damage starts from the formation of peroxides which cause the product to be unstable and reactive, resulting in carcinogenic compounds and loss of nutritional value of food.[16] It is very important to make enteral formulas with appropriate packaging and storage temperatures to protect products from oxidative damage.

Powdered enteral formula requires oxidation parameters during storage under different conditions. Parameters that could be used to monitor the autoxidation process are the detection of

malondialdehyde (MDA). Malondialdehyde is the most important autoxidation product and is used as an indicator of the fat peroxidation process. Malondialdehyde could be evaluated through the thiobarbituric acid (TBA) test, which is the simplest, quickest and most sensitive method because it could determine food oxidation in the initial phase. On the other hand, the analysis of TBA has the disadvantage that the product has been sensitively damaged, but the TBA number is still low.[16] The results of this study indicated that $R^2 = 0.9999$, the correlation coefficient was close to 1 or $R \approx 1$, meaning that temperature was very influential to the reaction of changing TBA values. The activation energy (Ea) of the change in TBA value was 8433.76 cal/mol, which meant to start the TBA value, those were the amount of energy needed.

Considering the shortage of the TBA number method, when estimating shelf life the organoleptic observations were also made which included the aroma, taste, and color compared to the control, which was stored at temperatures around 10-14°C. On the 7th day, there has been a darker color change and rancid aroma. Furthermore, on the 14th day until the 28th day the sample aroma was grassy and fatty odour and had the darkest color and bitter taste. The color change that occurs was called the browning reaction caused by high temperatures. Storage at high temperatures even in the short term can cause lactose crystallization which can accelerate non-enzymatic browning reactions. Under these conditions, browning reactions occur more quickly than fat oxidation.[32] There was a change in aroma caused by the formation of hexanal and heptanal compounds from PUFA oxidation. Hexanal and the paralysis cause the product to have a rancid and piercing aroma.[33] The results of this study indicated that storage with a temperature of 25^0C had a physical characteristic that was not much different from the control sample both of aroma, taste, and color.

Determination of shelf-life of enteral formula products based on tempeh flour and yam using a calculation of shelf life of zero ordo, because the value of R^2 in the Arrhenius equation is greater than ordo 1. The equation used to determine shelf life is $y = -4246.6x + 8.0381$. The equation can be used for the desired temperature analog. Dried food products such as milk powder can be stored at room temperature (25°C) or refrigerator temperature (4°C).[37] Through the calculation of shelf life with the above equation, the shelf life of enteral formula products with aluminum foil packaging is stored at 4^0C it has a shelf life of 132.24 days, 25°C has a shelf life of 44.89 days, 35°C has a shelf life of 28.26 days and 45°C has a shelf life of 18.32 days. The results of the calculation of shelf life in accordance with the theory that the more the temperature rises the greater the damage that occurs and the shelf life of the product becomes shorter.[34]

CONCLUSION

Enteral formula based on tempeh flour and yam flour could be applied in hospitals because it has appropriate microbiological quality for medical purposes and long shelf life compared to liquid FERS. The shelf life of enteral formula based on tempeh flour and yam flour were obtained that the temperature of 25^0C is the ideal storage temperature because it has a longer shelf life of 44.89 days and the physical properties are not much different from the control.

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