The effect of kefir grain concentration and fermentation duration on characteristics of cow milk-based kefir

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Abstract Cow's milk is very easily damaged because of the content of decomposing bacteria that occurs very quickly, so that the milk becomes inappropriate for consumption. Current technological developments in food sector provide an alternative diversification of milk processing products. One such technology is fermentation of cow's milk into kefir. The method used in this study is a Completely Randomized Design method (CRD) without any repetition. The raw materials used are cow's milk, kefir grains with various concentrations, namely 3%, 5% and 7% per 1L kefir, with different fermentation lenght namely 18 hours, 21 hours and 24 hours. Furthermore, the characteristics of lactic acid levels were tested using alkalimetry method, protein content test using the formol method, and the Lactic Acid Bacteria (LAB) test using *Man Rogosa Sharpe* (MRS) on growing medium. Characteristic test results show that lactic acid levels are optimal at 5% kefir grain concentration as well as optimal protein levels at 5% kefir grain concentration, while total LAB obtained are 1.80 × 107 cells / mL at 3% kefir grain concentration.

1. Introduction

Milk is one food that contains several important substances needed by humans, namely carbohydrates (lactose), protein, fat, vitamins, and minerals. However, fresh milk is very easily damaged due to contamination by decomposing bacteria that can occur very quickly, so that the milk becomes damaged and not suitable for consumption. The effort to extend the usability, shelf life, and economic value of milk is done through processing techniques. One of the most supportive milk processing efforts is fermentation [1]. Current fermented milk products are yogurt and kefir [2].

Kefir is a heterofermentative fermentation product that has a taste, color and texture that resembles yogurt and has a distinctive aroma like tape [3]. The basic ingredients used in making kefir are usually sourced from animal milk such as goats [4], cattle and buffaloes [5]. In addition, it can also be sourced from vegetable milk from beans such as soybeans, green beans, tolo beans, and red beans and peanuts [6].

The purpose of this research was to determine the most optimal conditions of fermentation kefir with three effect factors, specifically: fermentation duration, concentration and addition of almond milk. The selection of fermentation duration is expected to know the optimal time of fermentation in order to save on production costs later but still obtain kefir products according to CODEX standards. The selection of kefir grain concentration is expected to obtain optimal results of lactic acid bacteria, when consumed by the body. The addition of almond milk is expected to increase the nutritional value in kefir and increase flavor variability.

The advantages of kefir are the presence of probiotic bacteria that are proven to improve the digestive process by providing the microflora needed and can inhibit the growth of pathogenic bacteria in the digestive tract. In addition, kefir provides natural resistance to infections in the intestine, prevents constipation, produces vitamin B and antimicrobial compounds [7].

The fermentation process of cattle's milk becomes kefir using Lactic Acid Bacteria (BAL) and yeast that work in symbiosis. Lactic Acid Bacteria produce lactic acid and the breakdown of lactose which stimulates the growth of microorganisms, while yeast produces alcohol in the form of ethanol and carbon dioxide from lactose [8]. The growth of microorganisms from kefir will produce lactic acid, alcohol, carbon dioxide, acetaldehyde, acetoin and diacetyl which will create a unique kefir flavor [9]. Lactic acid is produced from lactose degradation, while diacetyl and acetoin are formed from citric and lactose acids. While acetaldehyde is produced from protein degradation which is used as a source of growth for microorganisms [10].

The more concentration of kefir grain is added, the higher the ability of microbes to break down glucose to produce primary metabolites (lactic acid and alcohol) and secondary metabolites (antibacterial activity and polyphenols) [2]. Kefir storage at low temperature (4^{o}C) must be done with the aim to produce a kefir texture that is suitable and useful to inhibit BAL activity so that kefir acidity is relatively stable. The addition of kefir grain is at least 3 grams per liter of milk and the fermentation time is 24 hours at room temperature to produce kefir with quality in accordance with the standard of fermented milk [11].

According to Astawan [12] "the duration of fermentation affects the antibacterial activity, because the longer the bacterial fermentation becomes more active, the more the amount, so the ability to break down the substrate is greater. To achieve kefir with the best quality in addition to the length of fermentation, the concentration of kefir grain also affects the quality of kefir. According to Nihayah, [13] "that the best kefir quality of cow's milk with the addition of 5% dtarter concentration produces the highest quality kefir in accordance with SNI". The duration of fermentation can affect the product produced, because during fermentation there is a breakdown of milk nutrition. According to Kunaepah [14], this is because the longer the time for microbial fermentation to multiply so that the number increases and the ability to break down the substrate is greater.

In addition, the addition of Almond milk to get an acceptable taste of kefir. Almond milk has antioxidant content in the form of α-tocopherol and is rich in Monounsatured Fatty Acid. Besides almonds also contain vitamin E, biotin, manganese, copper, fiber, protein, phosphorus, selenium, iron, riboflavin, potassium, tryptophan, magnesium, vitamin D, and calcium [15]. According to Maguire [16] In addition, almonds also compare 0.3 grams of unsaturated fat per gram and also a mixture of phenols and polyphenols which are included in flavonoids [17].

2. Materials and Methods

2.1. Materials

The material used in the study was pure cattle's milk from the Pangudi Mulyo farm Gunungpati, and kefir grain. The materials used in the analysis are aquades, NaOH solution, phenolphthalein solution (PP indicator), saturated potassium oxalate solution, and *Man Rogoso and Sharpe* (MRS).

2.2. Method

The research method consists of preliminary research, main research, and characteristics test. The experimental design used in this study was a Completely Randomized Design (CRD) without repetition. The treatment design consisted of two factors. The first factor is the concentration of kefir grain with concentrations of 3%, 5%, and 7%. The second factor is the duration of fermentation of 18 hours, 21 hours and 24 hours. The chemical design carried out in this study was a characteristic test consisting of analysis of lactic acid levels, analysis of protein levels and analysis of total LAB.

2.3. Preliminary Research

Preliminary research is used to find the exact concentration of kefir grain and fermentation time. The kefir grain's variables of concentration used were 3%, 5%, and 7%. The research raw materials used were 1L pure cow's milk for each kefir grain concentration variable and each length of fermentation time, which then fermented with different time variables namely 18 hours, 21 hours and 24 hours.

1 L pure cow's milk was pasteurized at 90 C for 15 minutes, then the cooling process was carried out in a sterile container. Milk that has been cold was filtered and then be treated with two different treatments. For the first treatment, the milk was inoculated with kefir grains of different concentrations namely 3%, 5% and 7% with 24-hour fermentation in the incubator. The second treatment of the milk was inoculated with kefir grains with 5% concentration which then was fermented in an incubator at different times of 18 hours, 21 hours and 24 hours. The third treatment of the milk was inoculated with kefir grains with 5% concentration which then was fermented in an incubator at 24 hours and factor is the addition of almond milk with concentrations of 10%, 20%, and 30%. When the fermentation process was complete, the kefir was filtered and then the most exact concentration and fermentation time were decided by doing the characteristic test which includes lactic acid test, protein test, and total BAL test. Kefir grain concentration and fermentation time were selected based on the results of the three characteristic tests that approached kefir standard quality according to the 2003 CODEX standard and the 2009 SNI (Indonesian National Standard).

The procedure of the research on producing kefir includes several stages, namely:

1. Preparation

   Equipment used for the research is sterilized first by washing and cleaning the tools that are going to be used.
2. Pasteurization

   Pure cow's milk was pasteurized at 90^{o}C for 15 minutes using the stove while the milk is stirred. Then the milk that has been heated is put into a sterile container for cooling process to a temperature of 20 – 25^{o}C.
3. Inoculation

   The milk that has been cooled is then filtered to separate the milk from the polluter, then kefir grains is added with various concentrations of 3%, 5%, and 7%.
4. Fermentation

   Milk that has been inoculated, then fermented at 37^{o}C for 18 hours, 21 hours and 24 hours in the incubator. In the fermentation process, two layers will be formed, namely the curd layer and the clear layer (whey).
5. Filtering

   The fermentation process is complete, the milk is stirred and filtered to separate between kefir optima (mixed whey and curd) and kefir prime (curd), then a cooling process is conducted at 4 ^{o}C.
6. Chemical Analysis

   The kefir curd product that is produced is then carried out the chemical analysis, namely analysis of lactic acid levels, analysis of protein levels and analysis of total LAB.

2.4. Characteristics Test

a. Lactic acid level test

   Lactic acid levels in kefir can be known by conducting a sample test using the Alkalimetry method. Alkalimetry testing was carried out by standardizing NaOH solution first, then kefir curd in Erlenmeyer was added with 1% PP solution and titrated with NaOH solution until it turns into pink.

b. Protein level test

   Protein levels in kefir can be discovered by conducting a sample test using the formol method. Formol method can be done by making potassium oxalate solution first, then kefir curd in Erlenmeyer is added with aquades, 1% PP indicator solution, potassium oxalate solution and titrated with NaOH solution until pink discoloration occurs.

c. Total LAB test

   The total LAB in kefir can be done using the cup count method using *Man Rogoso and Sharpe* (MRS) growing media. The plating is done by taking 1 mL of kefir sample which has been diluted into a cup, carried out at dilution 10^{6} – 10^{8}.

3. Results and Discussion

3.1. Preliminary Research

The preliminary study that had been conducted was to determine the two selected variables, namely in the form of starter concentration (kefir seedlings) and fermentation length used as a reference in the main study. The test results from various starter concentration variables are shown in table 1.

Table 1. The results of kefir characteristic test towards kefir grain

Test	Concentration (gram)		
	3	5	7
Lactic Acid	1.575	1.890	2.175
Protein	3.717	4.480	6.280
Total of Lactic Acid Bacteria	1.80×10^7	9.80×10^6	6.50×10^6

Meanwhile, the test results with the variable of the fermentation length are shown in table 2.

Table 2. The results of kefir characteristic test towards fermentation length

Test	Fermentation (hours)		
	18	21	24
Asam Laktat	1.305	1.425	1.890
Protein	3.512	3.691	4.480
Total of Lactic Acid Bacteria	7.45×10^6	9.45×10^6	9.80×10^6

Table 3. The test results for adding almond milk to the characteristics

Test	Almond Milk Concentration (%)		
	10	20	30
Asam Laktat	1.42	1.55	3.58
Protein	19.104	15.102	16.264
Total of Lactic Acid Bacteria	1.67×10^7	13.37×10^7	18.27×10^7

The three tables of the content test above show that the average result obtained increases with the increasing concentration of kefir grain and fermentation length and different results with the addition of almond milk. According to Ema Rosiana [18] states that the more the number of bacteria inoculated into cow's milk, the greater the chemical changes that occur in it. Based on the obtained data, the highest value in the use of kefir seedling concentration of 7% and with a 24-hour fermentation period.

3.2. Main Research

3.2.1. Lactic Acid. The following two tables show that the obtained lactic acid content is higher along with the increase in the contribution of kefir seedlings or the length of fermentation. This is caused by the acidic effect of fermented milk, so there is a change in lactose to lactic acid. This happens because there is an activity of enzymes produced by lactic acid bacteria and also the contained compound in milk, such as albumin, citrate and phosphate casein. Besides, this also affects nutritional factors and fermentation length which will affect microbes that work to form lactic acid. According to the CODEX Standard, fermented milk has lactic acid levels contained in kefir which is a

minimum of 0.6%. Meanwhile, according to the Indonesian National Standard, the kefir lactic acid level is 0.5 - 2.0%, so that all samples of the research conducted have fulfilled the standards.

3.2.2. Protein . Those two tables indicate that the obtained protein content is also getting higher. This is because microbes in kefir have the ability to break down proteins from complex compounds into simpler compounds by the help of protease enzymes as energy supply and the growth. The protease enzyme causes the protein to be hydrolyzed into amino acids [19]. This is in accordance with the research of Irfatun Nihayah [13] which states that as the starter concentration is getting high, the level of cow's milk protein is also high. Besides, the results are in accordance with the CODEX and SNI Standards for fermented milk, the level of protein contained in kefir is minimum 2.7%, so the protein contained in cow's milk kefir fulfills the standards.

3.2.3. The Total of Lactic Acid Bacteria. The results of the study show that there is an effect of kefir grain concentration and duration of fermentation on the total LAB obtained. The total BAL obtained is in the range of 1.8 × 107 - 9.80 × 106. The results are in accordance with the SNI standard and CODEX for the total BAL of cow's milk kefir which is at least 107 cfu / gram. The calculation of total LAB will be higher if more lactose content is found in cow's milk. Lactose becomes one of the factors that influence the growth of Lactic Acid bacteria. According to Septiani [20], the more good microorganisms present in kefir, the better the quality of kefir. However, if the microorganism lacks nutrients (carbon, nitrogen, vitamins and minerals) on the substrate in conducting cell multiplication, the total BAL value will decrease [21].

4. Conclusions

The results of the study are as follows:
The concentration of kefir grain influences the characteristics of kefir, where the highest levels of lactic acid, protein and total LAB in a sequence are 2.175%, 6.28% and 1.8 × 107 cfu / gram. However, the optimal addition of the kefir grain and standard closeness to all three kefirs is in a concentration of 3%. And the fermentation length affects the characteristics of kefir, where the highest lactic acid, protein and total lactic acid bacteria are obtained from the 24 hour fermentation time, which are 1.575%, 3.717% and 1.80 × 107 cfu / gram. And the effect of adding almond milk to the characteristics of cow's milk kefir is incrise a protein content of 15.102% -19.104%, lactic acid content is 1.42% -3.58%, and total lactic acid bacteria content is (11.67 – 18.27 x 108 CFU / mL)

5. References

[1] Ketut M 2018 *Keanekaragaman Bakteri Asam Laktat dan Khamir pada Kefir Susu Sapi dan Kefir Susu Kedelai* Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Lampung.
[2] Safitri M F 2013 *JATP* **2 (2)** 87-92.
[3] Haryadi, Nurliana dan Sugito 2013 *Nilai pH dan Jumlah Bakteri Asam Laktat Kefir Susu Kambing setelah Difermentasi dengan Penambahan Gula dengan Lama Inkubasi yang Berbeda* Program Studi Pendidikan Dokter Hewan Fakultas Kedokteran Hewan Universitas Syiah Kuala Banda Aceh.
[4] Verruck, Silvani, Adriana Dantasb and Elane Schwinden Prudencioa 2018 *Functionality of The Components from Goat's Milk, Recent Advances for Functional Dairy Products Development and Its Implications on Human Health* Department of Food Science and Technology Federal University of Santa Catarina Brazil.
[5] Gul O M, Mortas I, Atalar M, Dervisoglu and T Kahyaoglu 2015 *Manufacture and Characterization of Kefir Made from Cow and Buffalo Milk, Using Kefir Grain and Starter Culture* Program of Food Technology Ondokuz Mayiz University, Turkey.
[6] Fratiwi, Yulneriwarni dan Noverita 2008 *Fermentasi Kefir dari Susu Kacang–Kacangan* Fakultas Biologi Universitas Nasional Jakarta.
[7] Sari N K, 2007 *Tren dan Potensi Susu Sapi* Food Review Maret 2007 32-36 PT Media Pangan Indonesia.
[8] Dertli, Enes and ahmet Hilmi Çon 2007 *Microbial Diversity of Tradisional Kefir Grains and Their Role on Kefir Aroma* department of Food Engineering Faculty of engineering Bayburt

university Turkey.
[9] Oh N S, H A Lee, J H Myung, J Y Lee, J Y Joung, Y K Shin, and S C Baick 2013 *Kor. J. Food. Sci. An.* **33** 25-330.
[10] Diana I 2018 *Isolasi dan Karakterisasi Bakteri Asam Laktat Proteolitik dari Kefir* Fakultas Matematika Dan Ilmu Pengetahuan Alam Universitas Lampung.
[11] Sawitri M E 2011 *J. Tapro* **12** 15-21.
[12] Made A 2008 *tetap Sehat Dengan Produk Makanan Olahan* Solo: tiga serangkai.
[13] Nihayah I 2015 *Pengaruh Konsentrasi Starter Terhadap Kualitas Kefir Susu Sapi dan Pemanfatannya sebagai Penurun Kadar Kolesterol Darah Mencit (Mus musculus) Jurusan biologi* fakultas sains dan teknologi UIN maulana malik Ibrahim Malang.
[14] Kunaepah U 2008 *Tesis Pengaruh Lama Fermentasi dan Konsentrasi Glukosa Terhadap Aktivitas Antibakteri Polifenol Total dan Mutu Kimia Kefir Susu Kacang Merah* Universitas Diponegoro Semarang.
[15] Chen CY, Lapsley K, and Blumberg J 2006 *J. Sci. Food. Agric.* **86** 2245-2250
[16] Maguire LS, O'Sullivian SM, Galvin K, O'Connor TP, and O'Brien NM 2004 *Int. J. Food. Sci. Nutr.* **55** 171-178.
[17] Rossi E F, Hamzah dan Ferbriyani 2016 *Perbandingan Susu Kambing dan Susu Kedelai dalam Pembuatan Kefir.* Fakultas Pertanian Universitas Riau, Pekanbaru
[18] Rosiana, Ema, Nurliana dan T. Armansyah TR 2013 *Kadar Asam Laktat dan Derajat Asam Kefir Susu Kambing yang di Fermentasi dengan Penambahan Gula dan Lama Inkubasi yang Berbeda* Program Studi Pendidikan Dokter Hewan Fakultas Kedokteran Hewan Universitas Syiah Kuala Banda Aceh.
[19] Julianto, Budi, Rossi E dan Yusmarini 2016 *Karakteristik Kimiawi dan Mikrobiologi Kefir Susu Sapi dengan Penambahan Susu Kedelai* Jurusan Teknologi Pertanian Fakultas Pertanian Universitas Riau.
[20] Septiani A H,Kusrahayu K, dan Legowo A M 2013 *J. Anim Sci* **2(1)** 225 – 231.
[21] Rossi E, F Hamzah dan Febriyani 2016 *Perbandingan Susu Kambing dan Susu Kedelai dalam Pembuatan Kefir* Fakultas Pertanian Universitas Riau Pekanbaru.