International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

The Effect of *Acetobacter aceti* Addition at Different Concentrations on the Ethanol Content of Kefir

Khusnul Khothimah*[1], Lilik Eka Radiati[2], Herly Evanuarini[2]
[1]Post Graduate Prog, Faculty of Animal Science, Universitas Brawijaya, Malang, Indonesia 65145.
[2]Department of Animal Products Technology, Faculty of Animal Science, Universitas Brawijaya, Malang, Indonesia 65145.
*Corresponding Author: khusnul240196(at)gmail.com

Abstract**—** *The objective of this research was to determine the best concentration of Acetobacter aceti isolate added to kefir for reducing ethanol accumulation based on probiotic viability, total plate count, total yeast, pH value, and ethanol content. The materials used in this research were kefir made from milk, kefir grains and Acetobacter aceti isolate. The method used in this research was a completely randomized design with 4 treatments (0.25×8.67 log cfu/ml, 0.5×8.67 log cfu/ml, 0.75×8.67 log cfu/ml and 1×8.67 log cfu/ml) and 4 replications. The results showed that the effect of adding Acetobacter aceti isolate was not significant on total yeast but highly significant (P<0.01) on total lactic acid bacteria, total plate count, pH value, and ethanol content in kefir. Adding 1×8.67 log cfu/ml of Acetobacter aceti isolate to the kefir resulted in the best properties, in which the total lactic acid bacteria was 7.62 ± 0.52 log cfu/ml, total plate count was 8.53 ± 0.09 log cfu/ml, total yeast was 5.06±0.08 log cfu/ml, pH value was 5.16 ± 0.02 and ethanol content was 0.02 ± 0.01%. Therefore, the addition of Acetobacter aceti to kefir at 1 mL×8.67 log cfu/ml is recommended for producing kefir with low ethanol content*.

Keywords**—** *Total Lactic Acid Bacteria; Total Plate Count; Total Yeast; pH Value; Ethanol Percentage*.

I. INTRODUCTION

Kefir is a fermented product that has many benefits for human health and has been used for a long time. Some of the nutrients contained in kefir include minerals, vitamins, essential amino acids, and several other compounds such as calcium, phosphorus, magnesium, potassium, sodium, chloride, vitamins A, B2, B6, B12, C, D, E, carotene, thiamin, folic acid, niacin, and others [1]. Kefir has different characteristics from other fermented drinks because its ethanol content. The ethanol is formed by lactose hydrolyzing-bacteria and yeast. When yeast hydrolyzes lactose, it will produce CO_2 and ethanol in kefir. Ethanol as a secondary metabolite of the lactose fermentation process by yeast is undesired by some consumers. Excessive ethanol intake has negative pharmacological effects on the body, such as cardiovascular and central nervous system problems [2]. Ethanol is also an antimicrobial agent, which can affect the resistance and stability of microorganisms in kefir. The level of ethanol must be minimized so that the kefir products produced have better health benefits for consumers. Previous research has been conducted to reveal that it is possible to reduce the ethanol content in food. Reducing ethanol level can be done by converting ethanol compounds into other compounds, such as vinegar making process using ingredients containing sugar such as wine. The process involves two stages of fermentation. The first one is ethanol fermentation by converting glucose to ethanol by *Saccharomyces cerevisiae*. The second stage is acetic acid fermentation which oxidizes ethanol to acetic acid by *Acetobacter aceti* [3]. The use of *Acetobacter aceti* in the ethanol oxidation process is safe because it occurs through the natural fermentation process. Moreover, acetic acid produced from biological processes has a better flavor [4]. The aim of this study was to determine the effect of adding *Acetobacter aceti* isolates at different concentrations on the ethanol content produced in kefir based on the microbiological and chemical qualities of kefir.

II. MATERIALS AND METHODS

2.1 Kefir Preparation

CV Natural Probiotic Indonesia kindly provided kefir grains and fresh milk. *Acetobacter aceti* isolate was provided by Agrotekno Lab in Yogyakarta and tested for the number of cells/mL at the Faculty of Food Technology, Universitas Gadjah Mada, Yogyakarta. The media (milk) were replaced with the fresh one three or four times a week. Activated grains were used for respective assay. Cow's milk was incubated with 5% grains and was given additional *Acetobacter aceti* isolate (8.67 log cfu/ml) according to the following groups; 0.25 ml, 0.5 ml, 0.75 ml, and 1 ml, at 27°C for 24 h. After fermentation, the grains were removed by filtration with a sieve of 1-mm^2 mesh size, and the supernatant was designated as the kefir beverage.

2.2 Total Lactic Acid Bacteria

Total lactic acid bacteria value was tested using the spread plate method [5]. The first step was to fill 1 mL of bacterial stock into tube 1 and homogenize it with a vortex. Then 1 ml of bacterial suspension was taken from tube 1, transferred to tube 2, and homogenized. The 1 ml of bacterial suspension from tube 2 was taken, transferred again to tube 3, and homogenized again, and so on until tube 6. After dilution, the dilution results were cultured into a plate containing MRS agar by using a spread plate method using bent glass (scatter plate). The dilution of the cultures on the plate containing MRS agar media was started from a 10^{-4} dilution to a 10^{-6} dilution. For each dilution, 0.1 ml was taken using a micropipette and poured onto a plate containing MRS agar. Furthermore, the Petri dishes were incubated inside an incubator at 37^0C for 24 hours in anaerobic condition. The growing colonies were then observed based on colony morphology (shape, edge, elevation, and color), and the number of colonies on the plate was counted (cfu/ml).

Khusnul Khothimah, Lilik Eka Radiati, and Herly Evanuarini, "The Effect of *Acetobacter aceti* Addition at Different Concentrations on the Ethanol Content of Kefir," *International Research Journal of Advanced Engineering and Science*, Volume 7, Issue 2, pp. 18-21, 2022.

International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

2.3 Total Plate Count

Total plate count was tested using the pour plate method [5]. The first step was to fill 1 ml of bacterial stock into tube 1 and homogenize with a vortex. Then 1 ml of bacterial suspension from tube 1 was taken, transferred to tube 2 then homogenize. The 1 ml of bacterial suspension from tube 2 was transferred to tube 3 and homogenized, and so on until tube 6. After dilution, the dilution results were cultured into a dish and poured PCA media using the pour plate method. The dilution that was cultured on a plate containing PCA media was started from a 10^{-4} dilution to a 10^{-6} dilution. Each dilution was taken (1 ml) using a micropipette and poured into Petri dishes. Furthermore, the Petri dishes were incubated inside an incubator at 37^0C for 24 hours in anaerobic condition. The growing colonies were then observed based on colony morphology (shape, edge, elevation, and color), and the number of colonies on the plate was counted (cfu/ml).

2.4 Total Yeast

The total yeast was tested using the spread plate method [5]. The first step was to fill 1 ml of bacterial stock into tube 1 and homogenize it with a vortex, then take 1 ml of bacterial suspension from tube 1 then transferred to tube 2 then homogenized again and taken 1 ml of bacterial suspension from tube 2 and transferred again to tube 3 and homogenized again. After dilution, the dilution results were cultured into a plate containing PDA media by using a spread plate method using a bent glass (scatter plate). The dilution of the cultures on the plate containing PDA media was started from a 10^{-1} dilution to a 10^{-3} dilution. For each dilution, 0.1 mL was taken using a micropipette and poured onto a plate containing PDA media. Furthermore, the Petri dishes were incubated in an incubator at 25^0C for 48 hours in anaerobic condition. The growing colonies were then observed based on colony morphology (shape, edge, elevation, and color), and the number of colonies on the plate was counted (cfu/ml).

2.5 pH Value

Kefir pH value was measured by using a pH meter [6]. The pH meter was calibrated by inserting the electrode into a buffer with pH 7, then rinsing the electrode with distilled water until the electrode was clean and wiped with suction paper, then calibrated again by inserting the pH meter electrode into a buffer with pH 4. Sample pH was determined by dipping the electrode into the sample solution until the number on display was stable.

2.6 Ethanol

Ethanol content was analyzed using a gas chromatography (GC) [7] Agilent 6890 N with 20M carbowax column, FID detector, 250^0C injector temperature, 60^0C initial temperature and 140^0C final temperature with increasing temperature of 3^0C per minute.

2.7 Experimental Design

The research method used was an experimental laboratory method with a completely randomized design with four replications. Different addition concentrations of *Acetobacter aceti* (8.67 log cfu/ml) were 0.25 ml (T1); 0.5 ml (T2); 0.75 ml (T3), and 1 ml (T4).

2.8 Statistical Analysis

Obtained data was analyzed statistically using analysis of variance (ANOVA). If there was any significant effect or very significant effect analysis will be continued using Duncan Multiple Range Test.

III. Results and Discussion

3.1 Kefir grains

Table 1 shows the total lactic acid bacteria and total yeast of the kefir grain were 1.67×10^6 cfu/g (6.22 log cfu/g) and 1.41×10^5 cfu/g (5.15 log cfu/g), respectively. The total number of kefir grain microbes with lactic acid bacteria as the majority microorganisms in kefir grains was 10^7 cfu/g while yeast was around 10^4 cfu/g [8]. It is in line with the results in the study and the numbers met the standards, which had a total LAB (lactic acid bacteria) value of 1.67×10^6 cfu/g and a total yeast value of 1.41×10^5 cfu/g.

3.2 Acetobacter aceti

Table 1 shows the data of *Acetobacter aceti* used in this research analyzed on total number of cells/ml of isolates, the results obtained were 4.75×10^8 cfu/ml (8.67 log cfu/ml). *Acetobacter aceti* is acetic acid bacteria found in kefir grains, although it is not found in some grain compositions. Acetic acid bacteria are not the majority of bacteria in kefir grains, but their presence causes various microorganisms in kefir. The number of *Acetobacter aceti* cells used in this study is higher than in literature studies as in general, the number of acetic acid bacteria in kefir grains does not exceed 20% of the total population of kefir grains [9,10].

TABLE 1. Data on total lactic acid bacteria test results, total yeast, and total cell of *Acetobacter aceti* isolate

	Total Lactic Acid Bacteria log cfu/g	**Total yeast log cfu/g**	**Total cell/ml log cfu/ml**
Kefir grain	6.22	5.15	
Acetobacter aceti	-	-	8.67

TABLE 2. Average value of total lactic acid bacteria test results, total plate count, total yeast, pH value, and ethanol percentage of samples

	Total Lactic Acid Bacteria log cfu/ml	**Total Plate Count log cfu/ml**	**Total Yeast log cfu/ml**	**pH Value**	**Ethanol %**
T1	5.82 ± 0.47^a	6.94 ± 0.21^a	5.35 ± 0.02	5.24 ± 0.03^a	0.07 ± 0.02^a
T2	6.76 ± 0.26^b	7.03 ± 0.23^a	5.32 ± 0.17	5.32 ± 0.02^b	0.06 ± 0.01^{ab}
T3	7.32 ± 0.29^c	8.14 ± 0.29^b	5.21 ± 0.21	5.27 ± 0.02^c	0.05 ± 0.01^b
T4	7.62 ± 0.52^d	8.53 ± 0.09^b	5.06 ± 0.08	5.16 ± 0.02^d	0.02 ± 0.01^b

^{a,b,c,d} Different superscripts in the same column showed significant differences ($P < 0.01$).

Khusnul Khothimah, Lilik Eka Radiati, and Herly Evanuarini, "The Effect of *Acetobacter aceti* Addition at Different Concentrations on the Ethanol Content of Kefir," *International Research Journal of Advanced Engineering and Science*, Volume 7, Issue 2, pp. 18-21, 2022.

3.3 Total Lactic Acid Bacteria

Different addition levels of *Acetobacter aceti* gave a very significant difference (P<0.01) to the total lactic acid bacteria. The number of LAB in kefir tended to increase along with the addition of the concentration of *Acetobacter aceti*. The highest LAB count was found in T4 (7.62 ± 0.52 log cfu/ml) with the addition concentration of *Acetobacter aceti* isolate of 1 ml×8.67 log cfu/ml, and the lowest total LAB count was found at T1 which was 5.82 ± 0.47 log cfu/ml with the addition of the concentration of *Acetobacter aceti* as much as 0.25 ml×8.67 log cfu/ml. The increase in the total number of LAB in kefir was because *Acetobacter aceti* is an acetic acid bacterium capable of producing vitamin B12 which can be used to support the growth and physiological functions of other microorganisms, resulting in a mutualism symbiosis between acetic acid bacteria and lactic acid bacteria [11]. Therefore, higher addition level of *Acetobacter aceti* resulted in a higher number of LAB in kefir. Other factors also can cause the increasing number of total LAB in kefir. Even though LAB are mesophilic bacteria that have optimum growth at 30^0C-40^0C, they can still grow optimally in fermentation at room temperature (27^0C) with the availability of nutrients produced by acetic acid bacteria with an optimum growth temperature of 25^0C-30^0C. In the optimum temperature range, the growth of LAB will increase along with the increase in environmental temperature, which causes an acceleration of the growth rate so that the population also increases [12].

3.4 Total Plate Count

Different addition concentrations of *Acetobacter aceti* gave a very significant difference (P <0.01) on the total plate count (TPC) of kefir. Table 2 shows that the TPC of kefir tended to increase along with the increasing concentration of *Acetobacter aceti*. The highest TPC was found in T4, which was 8.53 ± 0.09 log cfu/ml with the addition concentration of *Acetobacter aceti* of 1 ml×8.67 log cfu/ml and the lowest TPC value was found in T1, which was 6.94 ± 0.21 log cfu/ml, with the addition concentration of 0.25 ml×8.67 log cfu/ml. The increase in the TPC value of kefir was due to the addition of *Acetobacter aceti* concentration, which caused an increase value in the total number of cells in the media. Kefir fermentation temperature at room temperature (27^0C) is the optimal temperature for the growth of *Acetobacter aceti* bacteria. The optimum temperature for the growth of *Acetobacter aceti* is around 25^0C-30^0C [13]. The increase in total microbes in kefir also implies an increase in the number of lactic acid bacteria because the optimum environment supports the growth of lactic acid bacteria. The lactic acid bacteria starts to grow up to a maximum of 4 hours [14] so that in the 24 hour fermentation period, lactic acid bacteria have experienced their growth phase.

3.5 Total Yeast

Different addition concentrations of *Acetobacter aceti* did not give significant difference from the total yeast average value in kefir. The mean value in Table 2 of the results of the study can be seen that the total mean value of yeast tends to decrease along with the addition of the concentration of *Acetobacter aceti*. The highest average total yeast value was found at T1 namely, 5.35 ± 0.02 log cfu/ml with the addition of the concentration of *Acetobacter aceti* as much as 0.25 ml×8.67 log cfu/ml, and the lowest average total yeast value was found at T4 which was 5.06 ± 0.08 log cfu/ml with the addition of 1 ml×8.67 log cfu/ml of *Acetobacter aceti* concentration. The decrease in the total mean value of kefir yeast was due to nutrient competition between microbes in kefir. Lactic acid bacteria undergo a growth phase earlier than yeast so that the available nutrients for yeast decreased. The lactic acid bacteria and yeast use the same substrate, namely glucose, which causes competition to get nutrients [15]. Another thing that can cause a decrease in total yeast value is because its growth can support *Acetobacter aceti* as if it grows on GYC media containing yeast extract. *Acetobacter aceti* can become a predator for yeast so that the amount of yeast in kefir decreases as in research on the development of growth of *Acetobacter spp*, GYC media is used to support growth and maintenance [16].

3.6 pH Value

Different addition concentrations of *Acetobacter aceti* gave a very significant difference (P <0.01) to the average pH value of kefir. The average value in Table 2 of the study results can be seen that the average pH value of kefir tends to decrease with the addition of the concentration of *Acetobacter aceti*, but has an increase in T2. The highest average pH value is found in T2, which is 5.32 ± 0.02 with the addition of the concentration of *Acetobacter aceti* as much as 0.5 ml×8.67 log cfu/ml, and the lowest average pH value is found at T4, namely 5.16 ± 0.02 with the addition of concentration. *Acetobacter aceti* as much as 1 ml×8.67 log cfu/ml. The decrease in the average pH value in kefir is because *Acetobacter aceti* can convert ethanol to acetic acid, so that along with the addition of the concentration of *Acetobacter aceti* isolates given, the higher the ethanol is converted to acetic acid. Thus the pH value decreases. The *Acetobacter aceti* bacteria become a bioreactor for the transformation of alcohol into acetic acid with very limited production of other metabolites [17]. Another factor that can cause a decrease in the pH value of kefir is the optimum growth of lactic acid bacteria that can convert lactose in milk into lactic acid more optimally so that it impacts decreasing the pH value of kefir. The pH range value in lactic acid bacteria was 4-6 [18].

3.7 Ethanol

Different addition concentrations of *Acetobacter aceti* gave a very significant difference (P <0.01) to the average value of ethanol kefir levels. The average value in Table 2 of the study results can be seen that the total average value of kefir acid tends to decrease with the addition of the concentration of *Acetobacter aceti.* The highest average ethanol content is in T1, which is 0.07 ± 0.02% with the addition of the *Acetobacter aceti* concentration of 0.25 ml×8.67 log cfu/ml. The lowest average ethanol content is in T4, which is 0.02 ± 0, 01% with the addition of the concentration of *Acetobacter aceti* as much as 1 ml×8.67 log cfu/ml. The ethanol content of kefir varies. It is reported that the ethanol content of kefir varies from 0.01% to 1% [19]. The decrease in ethanol content in kefir is closely related to the yeast population in kefir. The smaller the yeast population, the smaller the ethanol value produced. In addition, the availability of nutrients also greatly determines the amount

Khusnul Khothimah, Lilik Eka Radiati, and Herly Evanuarini, "The Effect of *Acetobacter aceti* Addition at Different Concentrations on the Ethanol Content of Kefir," *International Research Journal of Advanced Engineering and Science*, Volume 7, Issue 2, pp. 18-21, 2022.

of ethanol produced as the yeast makes use of glucose which is then converted into ethanol. Yeast grown on the media uses glucose for glycolysis [20]. The conditions of anaerobic fermentation, causing pyruvic acid, will not undergo oxidative decarboxylation but is converted into CO_2 and acetaldehyde, which are then broken down into ethanol and energy in smaller amounts [21]. Another factor that can lead to a decrease in the average value of ethanol in kefir is the addition of *Acetobacter aceti* isolates known to convert ethanol to acetic acid and other metabolites. The alcohol fermentation by acetic acid bacteria is carried out in two sequential reactions catalyzed by the enzymes pyrroquinoline quinone (PQQ) - dependent alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The reaction lies on the cytoplasmic membrane and transfers electrons to ubiquinone Q10. PQQ-ADH is a cinochemoprotein-cytochrome complex and catalyzes periplasm. The first step is ethanol oxidation by transferring electrons to Q10 and producing acetaldehyde, which is normally a substrate for other enzymes (ALDH) and converted to acetic acid during the second step of ethanol fermentation [22]. Therefore, as the concentration of *Acetobacter aceti* isolates increases, the ethanol content in kefir gets lower.

IV. Conclusions

The present results refer to the possibility of producing milk kefir contains very limited ethanol content with good microbial properties as well as high acceptance rates. The addition of *Acetobacter aceti* to kefir at 1 ml×8.67 log cfu/ml is recommended for producing kefir with low ethanol content and high probiotic that beneficial to human health.

Acknowledgements

The Researchers would like to thank all those who contributed to the writing of this scientific paper.

References

[1] Otes S and O Cagindi 2003 Kefir : A Probiotic Dairy-Composition, Nutritional and Therapeutic Aspects *Pakistan Journal of Nutrition* 2 (2) pp 54-59
[2] Tritama T K 2015 Konsumsi Alkohol dan Pengaruhnya terhadap Kesehatan *Majority* 4 (8) pp 7-10
[3] Hardoyo, A E Tjahjono, D Primarini, Hartono and Musa 2007 Kondisi Optimum Fermentasi Asam Asetat Menggunakan *Acetobacter Aceti* B166 *J. Sains MIPA* 13 (1) pp 17-20
[4] Palimbong S 2017 Pengaruh Konsentrasi *Acetobacter aceti* dan Lama Fermentasi Terhadap Total Asam Cairan Fermentasi Pepaya Burung (*Carica papaya*, L.) *J. Sains dan Teknologi Pangan* 2 (2) pp 478-485
[5] Sanders E R 2012 Aseptic Laboratory Techniques: Plating Methods *Journal of Visualized Experiments* 63 pp 1-18
[6] AOAC 2005 Official Methods of Analysis of the Association of Official Analytical Chemists by the Association of Official Analytical Chemist (Maryland)
[7] Winarni I and T B Bardant 2017 Pembuatan Bioetanol dari Limbah Kayu Sengon (*Falcataria moluccana* (Miq.) Barneby & J.W. Grimes) dengan Metode Substrat Konsentrasi Tinggi *Jurnal Penelitian Hasil Hutan* 35 (4) pp 231-242
[8] Codex Alimentarius Commision 2003 Codex Standard for Fermented Milk: Codex STAN 243 FAO/WHO Food Standards
[9] Garrote G L, A G Abraham and G L De Antoni 2001 Chemical and Microbiological Characterisation Of Kefir Grains *Journal of Dairy Research* 68 (4) pp 639-652
[10] Miguel M G C P, P G Cardoso, L A Lago and R F Schwan 2010 Diversity of Bacteria Present in Milk Kefir Grains Using Culture-Dependent and Culture-Independent Methods. Food Research International 43 pp 1523-1528
[11] Schwan R F, K T M Guedes and D R Dias 2015 Kefir - Grains And Beverages: A Review *Scientia Agraria Paranaensis* 14 (1) pp 1-9
[12] Suriasih K, W R Aryanta, G Mahardika and N M Astawa 2012 Microbiological and Chemical Properties of Kefir Made of Bali Cattle Milk *Food Science and Quality Management 6* pp 12-22
[13] Samuel O, J Lina and O Ifeanyi 2016 Production of Vinegar from Oil-palm Wine Using Acetobacter Aceti Isolated from Rotten Banana Fruits *Universal Journal of Biomedical Engineering* 4 (1) pp 1-5
[14] Mardalena 2016 Fase Pertumbuhan Isolat Bakteri Asam Laktat (BAL) Tempoyak Asal Jambi yang Disimpan Pada Suhu Kamar *Jurnal Sain Peternakan Indonesia* 11 (1) pp 58-66
[15] Sinurat R L, C N Ekowati, Sumardi and S Farisi 2018 Karakteristik Kefir Susu Sapi dengan Inokulum Ragi Tape *Jurnal Ilmiah Peternakan Terpadu* 6 (2) pp 111-116
[16] Kowser J, M G Aziz and M B Uddin 2015 Isolation and Characterization of *Acetobacter Aceti* From Rotten Papaya *J. Bangladesh. Agril. Univ*. 13 (2) pp 299-306
[17] Mas A, M J Torija, M D C G Parrilla and A M Troncoso 2014 Acetic Acid Bacteria and the Production and Quality of Wine Vinegar *Scientific World Journal* pp 1-6
[18] Adamberg K, S Kask, T M Laht and T Paalme 2003 The Effect Of Temperature and pH On The Growth Of Lactic Acid Bacteria: A pH-Auxostat Study *International Journal Of Food Microbiology* 85 pp 171 – 183
[19] Adams M R and M O Moss 2008 Food Microbiology: Third Edition Royal Society of Chemistry (Cambridge)
[20] Kim B H and G M Gadd 2008 Bacterial Physiology and Metabolism. Cambridge University Press (Cambridge)
[21] Draphco C M, N P Nhuan and T H Walker 2008 Biofuels Engineering Process Technology The McGrawHill Companies, Inc (USA)
[22] Manzo S G, J E Escamilla, A G Valdez, G L Velazquez, A V Carlo, J M Quino, I D L M D L Mora, I G Torrez, S E Flores, M L C Zentella, R A Espinosa, P M H Kroneck and M E S Torres 2015 The Oxidative Fermentation of Ethanol in *Gluconacetobacter diazotrophicus* Is a Two-Step Pathway Catalyzed by a Single Enzyme: Alcohol-Aldehyde Dehydrogenase (ADHa) *Int. J. Mol. Sci.* 16 pp 1293-1311