



## Manufacturing Kefir Starter Culture from Sumbawa Horse Milk Microbes for Potentially Probiotic Drinks

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### ABSTRAK

Susu kuda Sumbawa merupakan produk susu fermentasi lokal yang mengandung bakteri asam laktat dan yeast. Mikroba tersebut berpotensi sebagai starter kultur kefir (minuman probiotik) yang bermanfaat bagi penderita diabetes melitus tipe 2. Penelitian ini bertujuan untuk mendapatkan kultur starter kefir dari mikroba susu kuda Sumbawa yang berpotensi untuk dijadikan sebagai minuman probiotik. Mikroba yang digunakan sebagai kultur adalah dua isolat bakteri asam laktat (SKP K.3 : *Weissella confusa* dan SKP K.5 : *Lactococcus garvieae*) dan yeast YGC SKL K.3 (*Kazachstania humilis*). Kultur starter yang digunakan adalah bakteri asam laktat  $10^7$  CFU/ml dan khamir  $10^6$  CFU/ml (OD 600 : 0,3). Bakteri asam laktat dan yeast diinokulasikan ke dalam MRS-glukosa dengan perbandingan 1:1 dan 3:1. Hasil uji antagonis menunjukkan bahwa koloni bakteri asam laktat dan yeast dapat tumbuh baik pada media MRS (glukosa 5%) tanpa ada yang saling mendominasi. Hal ini menunjukkan bahwa yeast (SKL K.3) dapat dikombinasikan dengan bakteri asam laktat (SKP K.3 dan SKP K.5) sebagai kultur starter untuk kefir. Kultur starter kefir diinokulasi pada 0,5% volume susu sapi untuk menghasilkan kefir. Penelitian ini memberikan informasi awal yang dapat digunakan untuk penelitian selanjutnya tentang potensi mikroba dalam susu kuda Sumbawa.

### ABSTRACT

Sumbawa horse milk is local fermented milk products containing lactic acid bacteria and yeast. The microbes have potential to serve as a culture starter for kefir (probiotic drinks) that have benefit patients with type 2 diabetes mellitus. The study aims to obtain kefir starter culture from Sumbawa horse milk microbes for potentially probiotic drinks. The microbes used as cultures were two isolates of lactic acid bacteria (SKP K.3: *Weissella confusa* and SKP K.5: *Lactococcus garvieae*) and yeast YGC SKL K.3 (*Kazachstania humilis*). The starter culture used was lactic acid bacteria  $10^7$  CFU/ml and yeast  $10^6$  CFU/ml (OD 600: 0.3). Lactic acid bacteria and yeast were inoculated into MRS-glucose with a ratio 1:1 and 3:1. The results of the antagonist test showed that lactic acid bacteria and yeast colonies could grow well on MRS media (5% glucose) without any dominating each other. This indicates that yeast (SKL K.3) can be combined with lactic acid bacteria (SKP K.3 and SKP K.5) as a starter culture for kefir. Kefir starters were inoculated at 0.5% volume of the cow's milk to produce kefir. This

research provides initial information that can be used for future studies on the potential microbes in Sumbawa horse milk.

## 1. Introduction

Sumbawa horse milk is a local fermented milk product containing lactic acid bacteria (LAB) and yeast. These microbes can be used as a culture starter for kefir (probiotic drink) that have benefit patients with type 2 diabetes mellitus (DM) (Galiccia-Garcia et al., 2020). Type 2 DM is a metabolic disorder caused by a combination of defective insulin secretion by pancreatic  $\beta$ -cells and the inability of insulin-sensitive tissues to respond to insulin. According to the International Diabetes Federation (IDF), DM affects 1.9% of the global population and is ranked as the seventh leading cause of death worldwide. The IDF also notes that the prevalence of diabetes has been steadily increasing, with projections indicating a rise to over 300 million people by 2025.

Probiotics are an alternative used to treat various diseases in developing countries such as type 2 DM (Ostadrahimi et al., 2015). Probiotics are drinks resulting from the fermentation of microorganisms in the form of bacteria and yeast which provide health benefits (Chen et al., 2014). The bacteria used are LA B from the *Lactobacillus* and *Bifidobacterium* species (FAO, 2021). The main species of LAB that have characteristics of probiotic bacteria are *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifidobacteria* (Fijan, 2014). Several studies have examined the effect of consuming probiotics in reducing glucose levels and glucose tolerance of LAB and yeast microorganisms. Gulnaz et al. (2021) shows that probiotics (*Lactobacillus sakei* Probio65 and *Lactobacillus plantarum* Probio-093) and their metabolites as natural anti-diabetic therapy through the modulation of gut microbiota and inhibit diabetes-causing enzymes. Sari et al (2017), states that giving *L. casei* fermented milk as a therapy to mice can reduce blood glucose levels. Liu et al. (2022), that study showed results *L. plantarum* Y15 may serve as a potential probiotic for developing food products to ameliorate T2D. Sangwan and Singh (2014) explains that consumption of probiotic fermented milk LGG and *L. casei* NCDC 19 significantly suppresses various risk factors for type 2 DM. Numerous studies have identified various yeast genera that have the potential to act as probiotics. These include *Kluyveromyces* sp, *Saccharomyces* sp, *Torula* sp, *Kazachstania humilis*, *Wickerhamomyces anomalus*, and *Pichia kudriavzevii* Schwan et al., 2015; Punyauppath et al., 2022). According to research by Abid et al. (2022), *Saccharomyces cerevisiae*

var. *boulardii* is one of the most commonly used biotherapeutic agents that possess a range of beneficial properties such as antibacterial, antiviral, anticarcinogenic, antioxidant, anti-inflammatory, and immunomodulatory properties. Furthermore, research conducted by Punyaappa-path et al (2022), highlights that *Kazachstania bulderi* KKKS4-1 is the most effective probiotic in terms of its hemolytic activity, antimicrobial activity against pathogenic bacteria, tolerance to low pH and bile salts, and hydrophobicity.

Kefir is probiotic drink derived from fermented milk using kefir grains (LAB and yeast). The microbes found in kefir are classified as probiotics which are beneficial for human health (John & Deeseenthum, 2015). The LAB content contained in kefir is *L. brevis*, *L. cellobiosus*, *L. acidophilus*, *L. casei*, *Lc. Lactis*, *Streptococcus salivarius* ssp. *thermophilus*, *Leuconostoc mesenteroides*, and *L. cremoris* (John & Deeseenthum, 2015; Abdelfatah & Mahboub, 2018). The yeast content of kefir is *Kazachstania*, *Kluyveromyces*, *Candida*, *Torulopsis*, and *Saccharomyces* sp (John & Deeseenthum, 2015). Research by Ostadrahimi et al. (2015) states that kefir can be useful as a complementary or adjuvant therapy in the treatment of diabetes.

Previous research has successfully identified LAB microorganisms using 16S rRNA primers and yeast using ITS/5.8S rRNA primers from Sumbawa horse milk. The LAB isolate species that were identified were *Enterococcus faecium* (SK 1.5), *Weissella confusa* (SKP K.3), *Lactococcus garvieae* (SKP K.5), *Enterococcus thailandicus* (SKP K.9), *Lactobacillus fermentum* (SKL K.4), *Enterococcus faecalis* (M.SKL K.1), and *Lactococcus petauri* (SKP K.4) (Kusdianawati et al., 2020). From the LAB isolates obtained, three types of bacteria closely related to *Enterococcus* sp., *Lactococcus garvieae*, and *Lactococcus petauri* were identified as new bacteria in Sumbawa horse milk compared to previous research reported by Mulyawati et al. (2019), Sujaya et al. (2008) and Widiada et al. (2006). The yeast isolate that was identified from Sumbawa horse milk was *Kazachstania humilis* (Kusdianawati & Isworo, 2019). Based on this research, the results showed that LAB and yeast contained in Sumbawa horse milk have the potential to be used as a starter culture for kefir. Therefore, this research aims to *obtain kefir starter culture from Sumbawa horse milk microbes for potentially probiotic drinks*.

## 2. Materials dan Methods

### 2.1. Materials

Samples Sumbawa horse milk was collected at horse farms in Penyaring Village, LAB isolates SKP K.3 (*Weissella confusa*) and SKP K.5 (*Lactococcus garvieae*), yeast YGC SKL K.3 (*Kazachstania humilis*), YGC medium, MRS medium, cow's milk, and glucose.

### 2.2. Preparation of LAB and Yeast Isolates

The LAB isolates SKP K.3 (*Weissella confusa*) and SKP K.5 (*Lactococcus garvieae*), which were isolated from the milk of Sumbawa wild horses from Penyaring Village. These isolates had been successfully isolated in previous research (Kusdianawati et al., 2020). The LAB isolate SKP K.3 and LAB isolate SKP K.5 were cultured in MRS medium and then incubated at 28 °C for 24 in an anaerobic condition. The yeast isolate YGC SKL K.3 (*Kazachstania humilis*), is the yeast isolate that is used to make kefir seed starter. It was isolated from the milk of Sumbawa wild horses that are kept in Lenangguar Village (Kusdianawati & Isworo, 2019). Isolate YGC SKL K.3 was cultured in YGC medium and then incubated for 20 hours at 28 °C using an incubator shaker.

### 2.3. Antagonistic Testing Procedure and Preparation of Kefir Starter Cultures

Preparation of starter for making kefir starter culture using the modified method of Thongheam et al. (2008). The LAB isolate SKP K.3 (*Weissella confusa*) and LAB isolate SKP K.5 (*Lactococcus garvieae*) were incubated at 28 °C for 18 hours. The yeast YGC SKL K.3 (*Kazachstania humilis*) yeast isolate was incubated at 28 °C for 20 hours. Starter culture can be used when it reaches  $10^6$ – $10^8$  CFU/mL ( $10^7$  CFU/ml for LAB and  $10^6$  CFU/ml for yeast). The antagonist test is used to prepare the kefir starter before it is cultivated in a growth media. The antagonistic tests between LAB and yeast can be performed to measure the inhibitory capacity of the organisms against other microorganisms and make sure these microbes were not dominating each other.

The antagonist test involves using MRS media mixed with glucose. A spread plate method was used with a ratio of LAB to yeast of either 1:1 or 3:1. Based on the results of the antagonist test, a starter culture for kefir was created using the modified Simova et al. (2002) method. The starter culture was made up of LAB  $10^7$  CFU/ml and

yeast  $10^6$  CFU/ml. Once the LAB and yeast culture reached a ratio of OD 600: 0.3, it was inoculated into 150 ml of MRS-Glucose media. The ratio of LAB SKP K.3 (*Weissella confusa*) to yeast YGC SKL K.3 (*Kazachstania humilis*) was 1:1 or 3:1, and the ratio of LAB SKP K.5 (*Lactococcus garvieae*) to yeast YGC SKL K.3 (*Kazachstania humilis*) was also 1:1 or 3:1. The mixture was then incubated at 28 °C in an incubator shaker for 30 hours.

#### 2.4. Kefir Production

Kefir was prepared using a modified method by Simova et al. (2002) and (Safitri & Swarastuti, 2011). The process involved preparing the kefir seed starter, which included LAB, yeast, and cow's milk. The milk was first subjected to pasteurization at 92 °C for 15 minutes. Next, the kefir starter culture was added to the milk at a ratio of 0.5% of the milk volume. The mixture was then incubated at room temperature for 24 hours.

### 3. Result and Discussion

#### 3.1. Preparation of LAB and yeast isolates

The isolates LAB SKP K.3 (*Weissella confusa*) and LAB SKP K.5 (*Lactococcus garvieae*) were successfully isolated from the milk of horse milk in Penyaring Village, Sumbawa Regency (Kusdianawati et al., 2020), where these LAB species have the potential to be used as probiotics. Based on the results of previous research by (Kusdianawati et al., 2023), metabolites extracted from LAB SKP K.3 have activity against *S. aureus* ATCC 6538 (2.33 cm), *S. typhimurium* ATCC (2.4 cm), *L. monocytogenes* BTCC B693 (2.26 CM), and EPEC K.1.1 (2.56 cm). The isolate LAB SKP K.3 also has inhibitory activity against the  $\alpha$ -glucosidase enzyme at 53.57% (Kusdianawati et al., 2023). This is supported by (Sharma et al., 2018) that *Weissella confusa* (KR780676) isolated from idli batter (Indian traditional fermented food) produces galactan exopolysaccharide (EPS) which can reduce cholesterol,  $\beta$ -galactosidase and has antioxidant activity that is beneficial for health. Based on research by Lakra et al. (2020), *Weissella confusa* MD1 and *Weissella cibaria* MD2 isolated from a fermented batter have probiotic potential. Both strains (MD1 and MD2) have been found to exhibit antimicrobial activity against foodborne pathogens such as *E. coli*, *S. enterica*, *L. monocytogenes*, *S. typhi*, and *S. aureus*. Additionally, they displayed high bile

salt hydrolase activity in the sodium taurocholate and bile salt mixture. Strains MD1 and MD2 have both shown a significant reduction in cholesterol levels by 67.11% and 78% (Lakra et al., 2020). The LAB strain-coded PP29 (*Weissella confusa*) was isolated from a Romanian commercial yogurt. *W. confusa* strains fermentations in media with different anthocyanin concentrations from *Hibiscus sabdariffa* L., to increase the exopolysaccharide (EPS) yield, leading to augmented probiotic and prebiotic properties (Dimofte et al., 2022).

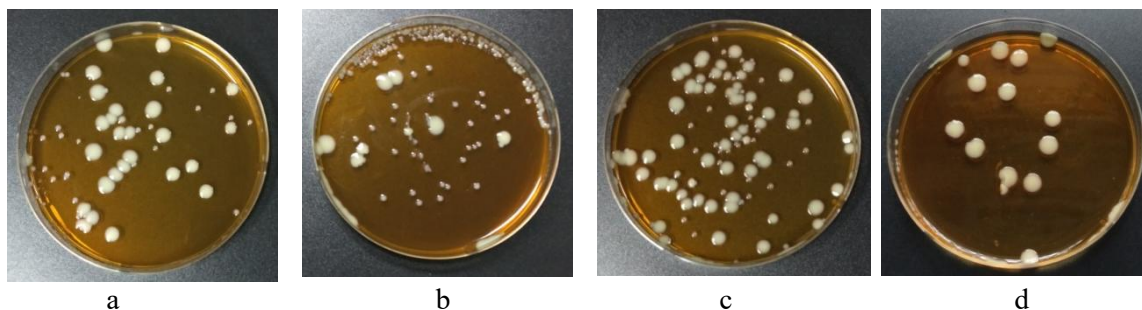
The isolate LAB SKP K.5 (*Lactococcus garvieae*) has metabolite extract activity on the bacteria *S. aureus* ATCC 6538 (2.3 cm), *S. typhimurium* ATCC (2.4 cm), *L. monocytogenes* BTCC B693 (2.4 CM), and EPEC K.1.1 (2.43 cm). In addition, the isolate LAB SKP K.5 also has inhibitory activity against the  $\alpha$ -glucosidase enzyme 6.25% (Kusdianawati et al., 2023). Based on the research by Abdelfatah & Mahboub (2018), that *L. garvieae* of dairy origin can produce an inhibitory substance that can control pathogenic *S. aureus* during cheese manufacture. furthermore, (Abdelfatah & Mahboub, 2018)] that *Lactococcus garvieae* isolated from fresh cow's milk has the potential as a probiotic to control the pathogenic bacteria *Staphylococcus aureus*.

The yeast isolate YGC SKL K.3 (*Kazachstania humilis*) was successfully isolated from the milk of horse milk in Lenangguar Village, Sumbawa Regency (Kusdianawati & Isworo, 2019). *Kazachstania humilis* is one of the yeast species found in kefir seeds and has the potential as a probiotic (Yang et al., 2014; Azhar & Abdul Munaim, 2019) Kaur & Dua, 2022). Based on the research of (Punyauppa-path et al., 2022), the yeast genera *Kazakhstan* (33.0%) isolated from Thai fermented food products is one of the yeast genera that have the potential to be used as a probiotic. Based on probiotic characteristic analysis of ten selected yeast strains, *Kazachstania bulderi* KKKS4-1 showed the strongest probiotic characteristics in terms of hemolytic activity, antimicrobial activity against pathogenic bacteria, tolerance to low pH and bile salt, and hydrophobicity. Probiotics using bacteria and yeast in type II DM sufferers showed a significant reduction in total cholesterol, triglycerides, CRP, HbA1c, fasting plasma glucose, insulin, and blood pressure. This research shows that probiotics can be used as additional therapy to improve metabolic control in type II DM sufferers (Kocsis et al., 2020).

### 3.2. Antagonistic Testing Procedure and Preparation of Kefir Starter Cultures

The results of the antagonist test indicated that both LAB and yeast colonies could grow well without dominating each other, indicating compatibility for kefir production (**Figure 1**). Several studies showed that LAB and yeast can interact well in milk content. According to Kusdianawati & Isworo (2019) and (Kusdianawati et al., 2020), Sumbawa horse milk contains LAB and yeast which interact with each other to produce distinctive aroma. The interaction of LAB and yeast, it can be used as a starter culture to produce kefir.

Yeast can produce highly concentrated ethanol and flavor components making it essential for fermented milk. In particular, LAB is an important part of fermentation as it produces organic acids, aromatic compounds, and small peptides that inhibit the growth of undesirable organisms. Interaction of symbiotic organisms resulting from the fermentation process has been characterized by some properties, including the production of carbon dioxide and other metabolites (among others vitamins, co-enzymes, and growth factors) and the activity of substances with the proteolytic and lipolytic activity of yeast fermentation (Ganzorig et al., 2016).

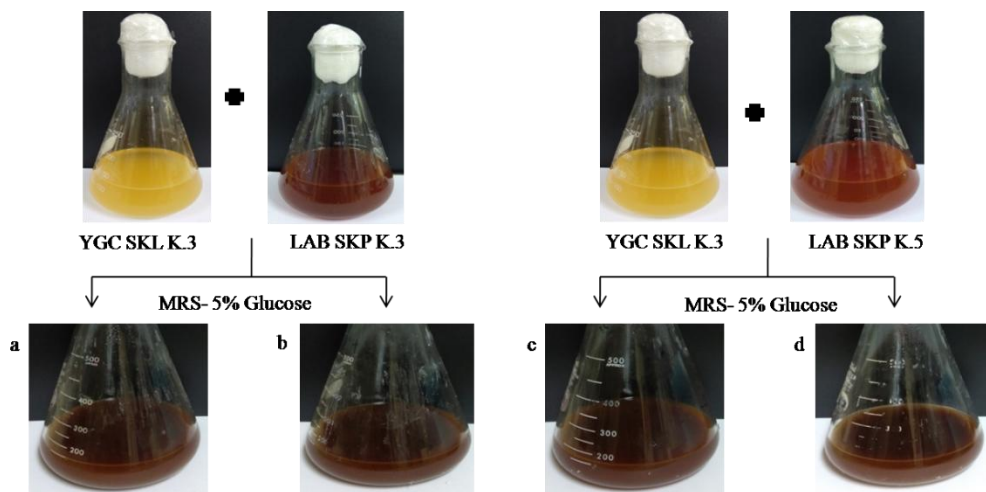


**Figure 1.** Antagonist test results for LAB and yeast colonies with a comparison of 1: 1 and 3: 1. a.) LAB SKP K.3 (1): Yeast YGC SKL K.3 (1); b.) BAL SKP K.3 (3): Yeast YGC SKL K.3 (1); c.) BAL SKP K.5 (1): Yeast YGC SKL K.3 (1); d.) BAL SKP K.5 (3): Yeast YGC SKL K.3 (1)

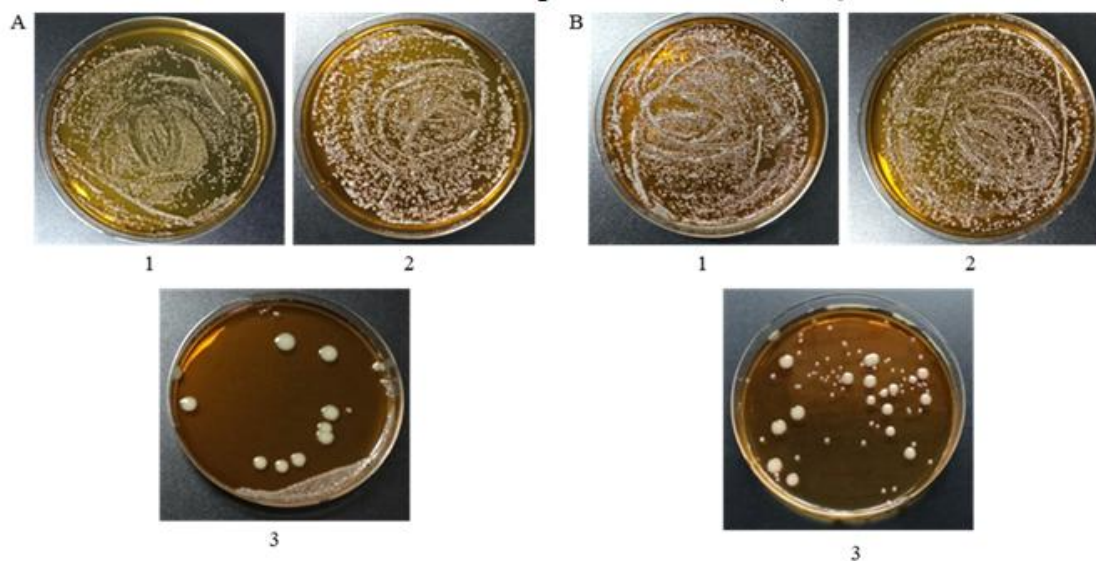
*Saccharomyces unisporus* (*Kazakhstania unispora*) from Kazakhstani qymyz is a kind of yeast that can ferment galactose (Kondybayev et al., 2023). In non-lactose fermentation, the LAB in milk contributes to the dominance of yeast. This indicates that the fermentation process for Kazakhstani qymyz involves two steps: first, the lactose is hydrolyzed by the LAB, which metabolizes the glucose and releases the leftover galactose into the environment, where the yeast consumes it. Since Sumbawa horse milk has a high

lactose content (6.2%), this can also occur with that kind of milk. According to Widiada et al. (2006), horse milk fermentation (pH 3.1) results in an elevated lactose content due to the overabundance of organic acids from milk and yeast. Sumbawa horse milk's sour taste has been linked to both yeast and LAB counts, which are dependent on storage time fluctuations.

Making kefir starter culture from LAB and yeast must reach  $10^6$ - $10^8$  CFU/ml. The starter culture used LAB  $10^7$  CFU/ml and yeast  $10^6$  CFU/ml. LAB and yeast (OD 600: 0.3) were inoculated into MRS-Glucose (150 ml) with a ratio of LAB to yeast (1:1 and 3:1) (Thongheam et al., 2008) (**Figure 2**).



**Figure 2.** Starter culture for kefir in a comparison of (1) LAB : (1) yeast and (3) LAB: (1) yeast. a.) LAB SKP K.3 (1): Yeast YGC SKL K.3 (1); b.) LAB SKP K.3 (3): Yeast YGC SKL K.3 (1); c.) LAB SKP K.5 (1): Yeast YGC SKL K.3 (1) ; d.) LAB SKP K.5 (3): Yeast YGC SKL K.3 (1)



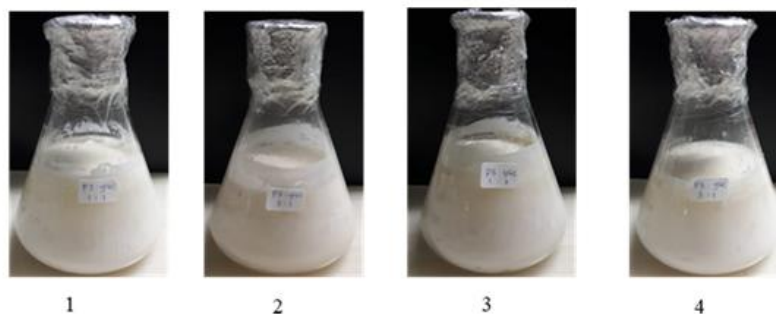
**Figure 3.** Results of spread of LAB and yeast isolates from starter culture. A.  $10^3$  dilution starter culture; B.  $10^6$  dilution starter culture

Based on the data from **Figure 2 and 3**, the LAB and yeast isolates from the  $10^3$  dilution starter culture exhibited significant growth, making it impossible to determine the comparison of the number of colonies between yeast and LAB. However, when spreading LAB and yeast isolates from the starter culture dilution  $10^6$ , it was observed that yeast colonies were more dominant compared to LAB colonies. The results indicate that yeast (SKL K.3) can be effectively combined with LAB (SKP K.3 and SKP K.5) to create a suitable starter culture for kefir.

### 3.3. Kefir production

Kefir is a probiotic drink made from fermented milk using kefir grains, which contain microorganisms in the form of lactic acid bacteria and yeasts (John & Deeseenthum, 2015). The yeasts found in kefir include *Kazachstania*, *Kluyveromyces*, *Candida*, *Torulaspora*, *Pichia*, *Cryptococcus*, *Debaromyces*, *Lachancea*, *Torulaspora*, *Zygosaccharomyces*, *Trichosporon*, and *Saccharomyces* (Sangwan & Singh, 2014; Leite et al., 2015; Sari et al., 2017). Additionally, *Kazachstania humilis* (*Candida humilis*, *Candida milleri*), commonly found in sourdoughs worldwide, is one of the dominant species in type I sourdoughs (Carbonetto et al., 2020; García-Ortega et al., 2022; Wittwer et al., 2022).

Some yeast species are major constituent of the microbiota of dairy products including fermented milks (horse, camel, cow, yak and goat), yogurt and cheese (Ganzorig et al., 2016; Fröhlich-Wyder et al., 2019). In fermented milks, yeasts share some biochemical and physiological characteristics such as utilization of lactic acid, assimilation of lactose, stimulation of amino acid and vitamins, growth at low temperatures and influences of tastes and flavors (Fröhlich-Wyder et al., 2019). The LAB identified in the kefir grains used in this study were LAB SKP K.3 (*Weissella confusa*) and LAB SKP K.5 (*Lactococcus garvieae*). Additionally, the yeast content of the kefir grains in this study was found to be YGC SKL K.3 (*Kazachstania humilis*).



**Figure 4.** Kefir production (1) Starter culture combination LAB SKP K.3 (1): Yeast YGC SKL K.3 (1); (2) Starter culture combination LAB SKP K.3 (3): Yeast YGC SKL K.3 (1); (3) Starter culture combination LAB SKP K.5 (1): Yeast YGC SKL K.3 (1); (4) Starter culture combination LAB SKP K.5 (3): Yeast YGC SKL K.3 (1)

Starter cultures (YGC SKL K.3 + LAB SKP K.3 and YGC SKL K.3 + LAB SKP K.5) for producing kefir were successfully developed with a fermentation time of 36 hours using cow's milk (**Figure 4**). This research serves as a promising foundation for developing probiotic kefir using Sumbawa horse milk microbes, with further testing needed to establish clinical benefits for individuals with type 2 diabetes mellitus.

#### 4. Conclusions

The isolates LAB SKP K.3 (*Weissella confusa*) and LAB SKP K.5 (*Lactococcus garvieae*) were isolated from horse milk in Penyaring Village. Additionally, the yeast YGC SKL K.3 (*Kazachstania humilis*) was isolated from horse milk in Lenangguar Village, Sumbawa Regency. The results of the antagonist test indicated that both LAB and yeast colonies could grow well without dominating each other, indicating

compatibility for kefir production. Kefir from starter cultures (YGC SKL K.3 + LAB SKP K.3 and YGC SKL K.3 + LAB SKP K.5) were successfully produced with a fermentation time of 36 hours. This research serves as a promising foundation for developing probiotic kefir using Sumbawa horse milk microbes, with further testing needed to establish clinical benefits for individuals with type 2 diabetes mellitus.

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