Evaluating the production of freeze-dried Kefir yogurt supplements with *Cordyceps militaris*

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ABSTRACT

The research aimed to produce freeze-dried Kefir yogurt supplemented with *Cordyceps militaris*, diversifying fermented foods, while offering convenient storage, consumption options and health benefits. To enhance the structure and flavor of the product, ingredients such as maltodextrin, skimmed milk powder and gelatin were incorporated. Experiments also included the addition of sucrose to further enhance these attributes. The optimal formulation for the freeze-dried Kefir yogurt supplemented with *C. militaris* was determined as containing Kefir with 5% *C. militaris* powder, while maintaining a lactic acid bacteria (LAB) count of 7.4 $\times 10^7$ CFU/g. This formulation also received the highest sensory scores and positive feedback on its structure, flavor, and moisture content, which remained below 5%. The study revealed that the addition of *C. militaris* powder to Kefir yogurt did not significantly impact the LAB count. In conclusion, the research successfully developed a freeze-dried Kefir yogurt enriched with *C. militaris* which contains bioactive compounds such as Cordycepin (1712 mg/kg) and Adenosine (89.9 mg/kg), contributing to yogurt's potential as a promising healthy snack.

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1. Introduction

In Vietnam, digestive-related diseases affect up to 10% of the population, and the incidence is rising. These disorders, such as constipation, diarrhea, and gastroesophageal reflux disease, can significantly impact patients' health and daily life if left untreated. The digestive system, particularly the intestines, plays a crucial role in the immune system by protecting against disease-causing agents in food. Kefir, a fermented milk drink, contains more than 30 beneficial microflora offering various health benefits including improved digestion, antibacterial effects, and antioxidant activity (Rosa et al., 2017). Despite these advantages, Kefir remains less popular in Vietnam due to its unique taste and limited awareness. Additionally, its short shelf life and temperature-sensitive storage requirements present challenges (Ho et al., 2021).

Cordyceps militaris (CM) is known for its bioactive compounds with numerous health benefits, ranging from anti-inflammatory to neuroprotective properties (Das et al., 2010). It's composed of various bioactive compounds, including adenine, polysaccharide, cordycepin, adenosine, and cordyheptapeptide (Wu et al., 2019). The CM has been shown to enhance the NK cell activity and lymphocyte proliferation and partially increased Th1 cytokine secretion. Therefore, CM is safe and effectively increased cell-mediated immunity of healthy male adults (Kang et al., 2015). However, despite the availability of various *C. militaris*, their bitter taste and aroma limit their popularity among consumers. To address these issues, combining *C. militaris* with sweet and sour Kefir yogurt, following by freeze-drying could be optimal method to improve the sensory, biological, and nutritional characteristics of the product, and making it suitable for long-term storage and easy transportation (Ho et al., 2021). Therefore, this research aimed to diversify product options and make Kefir as well as *C. militaris* more accessible to Vietnamese consumers enhancing both taste and health benefits.

2. Materials and Methods

2.1. Kefir milk

Kefir yogurt was supplied by Tracy Kefir, Viet Nam. The Kefir fermentation process used the commercial unsweetened pasteurized cow's milk (Vietnam Dairy Products Joint Stock Company, Vietnam) as the medium. Kefir grains were added to the milk at a concentration of 7% (w/v) and the mixture was thoroughly mixed to ensure even distribution of the Kefir grains and the milk. The fermentation process occurred at 22°C for 22 - 24 h. Then, the Kefir yogurt product was refrigerated at 1 - 4°C to preserve its structure and flavor.

2.2. *Cordyceps militaris* powder

Cordyceps militaris dried powder with 6 ± 0.25% moisture content, supplied by the Research Institute of Biotechnology and Environment (RIBE) at Nong Lam University, Ho Chi Minh City, Vietnam. The powder was added directly to the Kefir yogurt at concentrations of 5%, 6%, or 7% and mixed thoroughly. The particle size of dried CM powder is around 1 mm that helps it to dissolve quickly in the fermented Kefir without forming lumps. These varying concentrations were tested to evaluate potential differences in sensory quality and bacterial counts resulting from the incremental incorporation of *C. militaris* into the Kefir yogurt.

2.3. Food additives

The production of freeze-dried Kefir involves the incorporation of several food additives to maintain the overall quality of the kefir as well as the density of beneficial bacteria in the final product. Maltodextrin (MD), used as a stabilizer, is sourced from Tereos FKS in Indonesia. Skimmed milk powder (SMP), an essential component for enhancing the nutritional profile and texture is imported from New Zealand, and gelatin (G), which contributes to the structural integrity and mouthfeel of the final product, is obtained from Ewald-Germany. To determine the optimal formulation, each additive MD, SMP, and G was incorporated at three different concentrations (5%, 10%, and 15%). A control sample containing only Kefir and *Cordyceps militaris* (CM) without any additives was also prepared. All samples were then subjected to freeze-drying and subsequently evaluated based on lactic acid bacteria counts and sensory attributes.

2.4. Preparation of the freeze-dried kefir yogurt supplemented with CM

The freeze-dried yogurt was prepared followed by Doan et al (2022). Briefly, the kefir milk after fermentation (24 h) was mixed with CM powder and each additive MD, SMP, and G, respectively as described above. Finally, 10% sucrose was added to the mixture to reduce the bitterness of CM. Then, the mixture was poured into silicone molds (21 x 4 x 16 cm), placed on stainless steel trays and were placed in the freeze dryer (Harvestright, UT, USA) operating at - 30°C, and dried at 45°C, with a total drying time of 30 h (Yamaguchi et al., 2019; Ismail et al., 2020; Pham, 2022).

2.5. Lactic acid bacteria count

The lactic acid bacteria (LAB) were isolated from Kefir yogurt products using a serial dilution method. Briefly, the sample was serially diluted to 10^{-5} and 100 µL aliquots from dilution factors of 10^{-3} to 10^{-5} were spread onto MRS medium. The MRS medium consisted of 0.4% yeast extract, 0.8% beef extract, 1% peptone, 2% D-glucose, 0.2% K_2HPO_4, 0.02% $MgSO_4$, 0.004% $MnSO_4$, 0.1% Tween 80, and 0.5% sodium acetate ($C_2H_3NaO_2$), with 2% agar powder added, following the formulation by de Man et al. (1960). Uniform colonies were collected and subcultured on MRS agar at 37°C for 48 h. Colony morphology, gram staining, catalase, oxidase, urease, motility, and endospore staining were performed for preliminary identification. The LAB count is calculated using the following formula:

$$A = \frac{N}{n_1 \times V \times f_1 + ... + n_i \times V \times f_1}$$

Where:

A: The number of bacteria in 1 gram of the sample (CFU/mL).

N: The total number of colonies counted on the selected plates.

V: The volume of the sample solution inoculated onto each plate (mL).

n_i: The number of plates inoculated at the i-th dilution level.

f_i: The corresponding dilution factor.

2.6. Sensory evaluation

The sensory attributes of Kefir yogurt and freeze-dried snacks were evaluated using a 9-point hedonic scale, as described by Noel et al. (2011). Thirty consenting volunteers rated their liking or disliking of various attributes, such as appearance, aroma, taste, texture, and overall acceptability, on a scale ranging from 1 means "Dislike Extremely" to 9 means "Like Extremely". The results of the sensory tests provided valuable insights into consumer acceptance and preferences for the product.

2.7. Moisture content

The moisture content of the dried yogurt tablets was measured by drying the samples at 105°C in a forced-air drying oven (Ahn et al., 2014), until a constant weight was obtained. The moisture content was then calculated according to the Vietnamese standard TCVN 10788:2015.

$$W = \frac{M1 - M2}{M1} \times 100$$

Where:

W: Moisture content of the yogurt tablets (%);

M1: Initial weight of the yogurt tablets (g);

M2: Weight of the yogurt tablets after drying (g).

2.8. Data analysis

The data was analyzed using one-way ANOVA with Minitab 16 software to determine statistically significant differences among treatments (α = 0.05).

3. Results and Discussion

3.1. Bacterial count in Kefir yogurt

The colonies isolated from Kefir yogurt appeared round, smooth, and milky white in color. The bacteria were Gram-positive and tested positive for catalase activity, while negative for oxidase. The isolates demonstrated the ability to utilize calcium carbonate ($CaCO_3$) but were non-motile and did not form endospores. Based on these biochemical tests, the isolated bacteria were characterized as lactic acid bacteria (LAB). After 24 h of fermentation, the LAB count in Kefir yogurt was 5.4 x 10^8 CFU/mL. Previous studies have also reported the presence of beneficial probiotic microorganisms in Kefir yogurt. For instance, Rosa et al. (2017) reported that the probiotic microorganisms in the Kefir yogurt are in range of 10^4 - 10^7 CFU/mL. In the present study, the Kefir yogurt product contained a higher number of beneficial bacteria, which is expected to promote potential positive health effects (Mijačević et al., 2001; Castellone et al., 2021).

3.2. The effect of CM powder on the quantity of LAB after mixing with Kefir yogurt

Liquid Kefir yogurt, supplemented with varying concentrations of CM, was utilized to assess the density of lactic acid bacteria (LAB). This experiment was performed prior to the freeze-drying process to investigate the impact of CM powder addition on the population of beneficial bacteria in Kefir yogurt. The results of this analysis are summarized in Table 1.

Table 1. Lactic acid bacteria (LAB) count of liquid Kefir yogurt after *Cordyceps militaris* (CM) supplementation

CM supplementation ratio in Kefir (%)	The average LAB count (Log CFU/mL)
0	$8.73^a \pm 0.01$
5	$8.72^a \pm 0.008$
6	$8.73^a \pm 0.008$
7	$8.74^a \pm 0.008$

The results are presented as the mean ± SD. Different letters in the same column indicated that values were significantly different (P < 0.05).

All four samples had a LAB count of 8 log CFU/mL, indicating potential probiotic benefits (Table 1). There was no statistically significant difference in the number of LAB observed after the addition of CM, suggesting that CM did not influence LAB density in Kefir yogurt. According to the study of Ghasempour et al. (2014), consuming 100 mL of Kefir daily offers beneficial probiotics, enzymes, and nutrients. Besides, a review by Truong et al. (2023) has shown that CM contains nucleosides, sterols, polysaccharides, and phenolic compounds that can support immunity and provide antioxidant supplementation to the body. Additionally, *C. militaris* is a valuable source of natural carotenoids, which are secondary metabolites in the fruiting body and mycelium that may contribute to the color of the freeze-dried yogurt (Figure 1) (Lee et al., 2020). It has been shown that carotenoids possess strong antioxidant properties, which help mitigate oxidative stress and protect retinal cells from damage (Chen et al., 2022). Therefore, the evaluation of bioactive activities of these compounds in the freeze-dried Kefir yogurt is necessary in future research.

Another research by Huynh et al. (2022) found that using 0.25 to 0.5 g of CM powder did not result in any adverse effects on mice and had a liver-protective impact through the ability to reduce AST-ALT liver enzyme activity in plasma, reduce Malondialdehyde content, and inhibited hepatocellular lipid peroxidation. Based on these findings, adding 5%, 6%, and 7% CM powder to 100 mL of Kefir yogurt may provide health and digestive benefits without any adverse effects on the human body. Due to insignificant effects on density of LAB when adding CM powder to Kefir, the optimal concentration of CM powder was selected based on sensory evaluation results below.

3.3. Sensory evaluation

To determine the optimal concentration of CM to be added to Kefir yogurt before freeze-drying, a sensory evaluation was conducted. Initial samples displayed a bitter taste, attributed to the inherent bitterness of CM. Therefore, the formular of the was modified following the approach outlined by Doan et al. (2022) with 10% sucrose was incorporated. Sensory evaluation results indicated that Kefir yogurt supplemented with 5% CM powder achieved the highest sensory scores, regardless of the presence of sucrose (Table 2). Consequently, the 5% CM powder concentration was selected as the optimal ratio for Kefir yogurt production.

Table 2. Sensory evaluation (n = 30) of fresh Kefir yogurt with *Cordyceps militaris* (CM) supplement

Samples	Average score	
	Set 1: without sucrose	Set 2: add 10% sucrose
100% Kefir (Control)	$3.38^b \pm 0.57$	$8.04^a \pm 0.28$
Kefir + 5% CM	$3.98^b \pm 0.43$	$8.55^a \pm 0.22$
Kefir + 6% CM	$2.68^b \pm 0.37$	$7.11^a \pm 0.31$
Kefir + 7% CM	$2.48^b \pm 0.29$	$6.79^a \pm 0.30$

The results are presented as the mean ± SD. Different letters in the same row indicated that values were significantly different (P < 0.05).

3.4. Identification of optimal additive(s) for the freeze-dried Kefir yogurt supplemented with CM

The food additives were incorporated into kefir yogurt to maintain the overall quality of the kefir as well as the density of beneficial bacteria in the final product after freeze-drying. Samples containing 5% MD and 5% SMP exhibited structural damage, while those with 10% and 15% MD, SMP retained their shape (Figure 1). The yogurt sample with gelatin was shrunk and cannot maintain the shape well (G sample, Figure 1). Therefore, yogurt samples with MD and SMP supplementation were used to investigate the viability of LAB after freeze-drying.

The results showed significant differences between the control and the samples with additives, as well as variations in the effect of different additive ratios (Table 3). After supplementing with MD and SMP, there was a significant increase in the LAB quantity at all ratios. Among the three ratios of additives, the products with 15% additives showed the highest LAB count, followed by 10%, and 5%. This trend was consistent for both type of additives, MD and SMP. These results were consistent with previous studies (Reddy et al., 2009; Ismail et al., 2020), in which LAB survival was higher in Kefir spray-dried with MD compared to non-fat samples. The inclusion of 10% sugar as lyoprotective agents markedly enhanced the survival rates of both lactic acid bacteria and yeasts was also shown in the study of Chen et al. (2006). These findings also align with research by Pham (2022) on the positive impact of increased additive ratios in enhancing the survival of LAB after freeze drying Kefir yogurt.

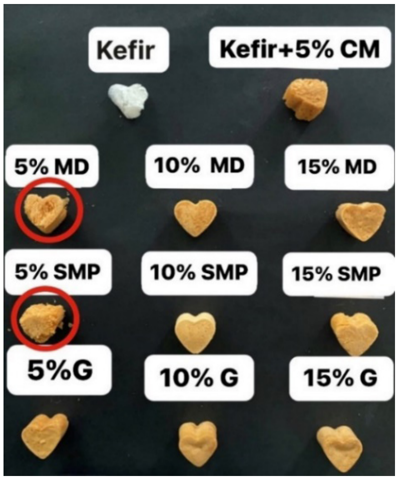

Figure 1. Freeze-dried CM-supplemented Kefir yogurt with additives.

The structural integrity of all four samples remained intact, and the LAB count was within an acceptable range as a beneficial bacterial count of at least 6 Log CFU/g is linked to positive health effects (Castellone et al., 2021). Therefore, the 10% and 15% of MD and SMP samples were selected for further testing in the next content.

Table 3. Variations in lactic acid bacteria (LAB) counts among the Kefir samples after freeze-drying

Sample	Average LAB count (Log CFU/g)
Kefir + 5% CM	$6.74^{d} \pm 0.02$
Kefir + 5% CM + 5% MD	$7.74^{c} \pm 0.01$
Kefir + 5% CM + 10% MD	$7.80^{b} \pm 0.01$
Kefir + 5% CM + 15% MD	$7.87^{a} \pm 0.01$
Kefir + 5% CM + 5% SMP	$7.75^{c} \pm 0.03$
Kefir + 5% CM + 10% SMP	$7.81^{b} \pm 0.01$
Kefir + 5% CM + 15% SMP	$7.87^{a} \pm 0.02$

The results are presented as the mean ±SD. Different letters in the same column indicated that values were significantly different (P < 0.05).

3.5. Improving the sensory taste of the freeze-dried Kefir yogurt formula supplemented with CM

After selecting the optimal ratio and type of additives to preserve structural integrity and ensure a stable LAB count in the kefir snacks, further improvement in the product's taste was identified as necessary. While the sourness of the kefir and bitterness of the CM were moderated, the product's overall flavor remained unpalatable due to the strong taste of CM powder. Following the approach used in the previous research on dried yogurt products by Doan et al. (2022), 10% sucrose was added to improve the sensory properties of freeze-dried Kefir. The moisture content and LAB viability of the sugar-added product were presented in Tables 4 and 5.

Table 4. The result average moisture content (%) of the freeze- dried Kefir

5% CM Kefir yogurt	Average moisture (%)	TCVN 7729: 2007
MD 10% + 10% Sucrose	$1.16^{d} \pm 0.02$	Pass (< 5%)
MD 15% + 10% Sucrose	$2.84^{b} \pm 0.02$	Pass (< 5%)
SMP 10% + 10% Sucrose	$2.03^{c} \pm 0.03$	Pass (< 5%)
SMP 15% + 10% Sucrose	$3.07^{a} \pm 0.02$	Pass (< 5%)

The results are presented as the mean values (± SD). Different letters in the same column indicated that values were significantly different (P < 0.05). MD: Maltodextrin; SMP: Skimmed milk powder.

3.6. LAB count after freeze-drying

Table 5. Lac tic acid bacteria (LAB) density of four freeze-dried Kefir yogurt samples

5% CM Kefir yogurt	Average LAB count (Log CFU/g)
MD 10% + 10% Sucrose	$7.80^{b} \pm 0.01$
MD 15% + 10% Sucrose	$7.87^{a} \pm 0.01$
SMP 10% + 10% Sucrose	$7.81^{b} \pm 0.01$
SMP 15% + 10% Sucrose	$7.87^{a} \pm 0.01$

The results are presented as the mean values (± SD). Different letters in the same column indicated that values were significantly different (P < 0.05). MD: Maltodextrin; SMP: Skimmed milk powder.

All four samples meet the moisture content requirements of the Vietnamese standard for heat-treated milk products TCVN 7729:2007 (Table 4). This demonstrates good preservation efficiency of freeze-dried yogurt. In addition, the results in Table 5 showed that the samples with 15% added maltodextrin and skimmed milk powder have higher LAB count than those added at 10%. However, all four samples complied with the CODEX STAN 243 - 2003 requirement for a count of at least 7 log CFU/g of beneficial bacteria (CAC, 2003). Therefore, the optimal formula for freeze-dried kefir was decided by sensory evaluation score. The results of Table 6 showed that the 15% MD with 10% sucrose freeze-dried Kefir achieved the highest sensory evaluation score of 8.62 while maintaining structural integrity.

Table 6. Sensory evaluation scores (n = 30) of freezes- dried Kefirs supplemented with *Cordyceps militaris* (CM)

Sample	Average score
MD 10% + 10% Sucrose	$7.80^{b} \pm 0.40$
MD 15% + 10% Sucrose	$8.62^{a} \pm 0.26$
SMP 10% + 10% Surcose	$7.02^{c} \pm 0.39$
SMP 15% + 10% Surcose	$6.44^{d} \pm 0.31$

The results are presented as the mean values (± SD). Different letters in the same column indicated that values were significantly different (P < 0.05). MD: Maltodextrin; SMP: Skimmed milk powder.

Through the evaluation of structural integrity and sensory scores, the 15% maltodextrin added 10% sucrose sample stood out with the best results, maintaining its structure after drying and density of beneficial LAB. Additionally, the product quality was evaluated based on cordycepin and adenosine content, yeast and mold counts. The product met the requirements of the Vietnamese Standard TCVN 7030:2002 for heat-processed dairy products, with no presence of yeast or mold detected, thereby confirming the product's safety for consumption.

The final Kefir products were sent for quantification to the Center for Analytical Services and Experimentation (CASE), concluding that the freeze-dried Kefir contained Cordycepin of 1712 mg/kg and Adenosine of 89.9 mg/kg. The results show that the inclusion of 5% CM powder in the product, using approximately 15 g, equivalent to 30 dried Kefir yogurt tablets supplemented with CM, would be sufficient to meet the body's immunity and digestive support needs based on the study Sun et al. (2014). Furthermore, the incorporation of *C. militaris* powder into Kefir yogurt could potentially enhance some beneficial effects. For example, the protective effects of *C. militaris* polysaccharide-supplemented probiotic yogurt have been demonstrated in mice, where daily consumption for 28 days led to notable improvements in liver health. Research by Han et al. (2020) showed that this supplementation significantly reduced liver damage in mice with alcoholic liver injury. The effects included a marked decrease in liver index, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, as well as reductions in total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) levels in the serum. In addition, the cordycepin in *C. militaris*, which structurally resembles adenosine, plays a critical role in DNA and RNA synthesis. As demonstrated by Thuy et al. (2021), cordycepin can integrate into the RNA and DNA structures of bacteria and viruses, interfering with nucleic acid biosynthesis and modification. This activity limits the growth of harmful microorganisms, adding another layer of health benefits to the product. Given these findings, further research is needed to explore the full bioactive potential of the developed freeze-dried Kefir yogurt supplemented with *C. militaris*, particularly in relation to its effects on liver health, microbial growth, and overall nutritional value.

4. Conclusions

The findings of this study indicate that freeze-dried Kefir yogurt could serve as a promising market alternative for the dairy industry given its high beneficial lactic acid bacteria count. The addition of *C. militaris* powder to the Kefir yogurt did not significantly impact the LAB density, indicating compatibility between CM and Kefir yogurt. However, the structural integrity of dried Kefir yogurt was notably influenced by the choice of additives used in the formulation. Among the three tested additives, maltodextrin demonstrated superior preservation of LAB viability while also maintaining the structural integrity of freeze-dried Kefir yogurt.

Conflict of interest

All authors declare that they have no conflict of interest.

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