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Modelling of Milk Kefir Fermentation for its Optimised Physicochemical and Microbiological Properties

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ABSTRACT

Milk kefir is a tangy, probiotic-rich fermented milk drink that is underexplored, partly due to the lack of broad awareness and complexity in standardisation of processing. This research studied the milk kefir fermentation process using response surface methodology (RSM) and artificial neural network (ANN). The RSM approach has optimised milk kefir quality at pH 4.4, viscosity at 1200 mPa.s and lactic acid at 0.8% at fermentation temperature of 35.8°C for 8.8 hours. The ANN model presented predictive capabilities with a higher coefficient of determination, R^2 values and comparably lower root mean square error (RSME) and lower average absolute deviation (AAD) as compared to RSM, suggesting that the ANN model is more effective in capturing nonlinear data for predicting quality responses of milk kefir. Bacterial and fungal composition of milk kefir was measured using metagenomics via next-generation sequencing. *Firmicutes* was found as the most dominant bacterial phylum, while *Ascomycota,* the fungi phylum in both optimised and commercial milk kefir. At the genus level, *Lactobacillus* was an abundant bacterium in optimised milk kefir, while *Streptococcus* in commercial milk kefir. Both *Lactobacillus* and *Streptococcus* are recognised as probiotics that promote improvement in gut health and support immunity. For fungi, the genus *Pichia* was detected in high abundance percentage in optimised milk kefir, while *Debaryomyces* in commercial milk kefir.

Keywords: Artificial neural network, fermentation, milk kefir, optimisation, response surface methodology

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INTRODUCTION

The increasing global demand for sustainability has underlined the necessity of promoting resilient food production, which contributes to minimal waste and long-term food security to ensure that future generations have access to sufficient, safe, and nutritious food. Fermentation is one of the most traditional practices that has been adopted to prevent losses in highly perishable food products. Milk is known to be the third largest category of food wastage after meat and vegetable products due to its perishable nature which contributed 17% of total value loss worth $165.6 billion in the United States (Buzby et al., 2014). Although fermentation process of milk has produced products such as cheese, whey, and yoghurt (Martin et al., 2021), milk kefir is less widely known as it is primarily limited to household levels from ancient times (Alves et al., 2021). Milk kefir was discovered in the Caucasian Mountain, between Russia and Georgia, and has been passed down from one generation to another for centuries (Gentry et al., 2023). Milk kefir is a fermented beverage that is obtained from fermentation of milk commonly sourced from cow, goat, or ewe with inoculated milk kefir grains consisting of microbial communities mainly lactic acid bacteria, yeast, and acetic acid bacteria (Leite et al., 2013). The dominant genera found in the milk kefir is lactic acid bacteria, including *Lactobacillus* sp.*, Leuconostoc* sp., *Pediococcus* sp. and *Lactococcus* sp. which serve as probiotics that regulate gut microbiota and prevent dysbiosis (Fiorda et al., 2017; Garofalo et al., 2015). For yeast, *Saccharomyces cerevisiae, Candida* kefyr, and *Kluyveromyces marixanus ssp. Marxianus* are found abundantly that facilitate in acid and alcohol fermentation (Fiorda et al., 2017). However, elucidation of the beneficial microbial composition of milk kefir has remained controversial, stemming from types of substrates, fermentation time, and temperature (Gul et al., 2015). Hence, optimising processing is essential for desired and consistent milk kefir quality to establish a favourable condition that support growth of beneficial microbes.

Fermentation of milk results in the change of physicochemical properties including pH, viscosity, microbial composition, and formation of various metabolites including organic acids, ethanol, carbon dioxide, and micronutrients (Rosa et al., 2017). Fermenting milk imparts health benefits such as probiotics (Bellikci-Koyu et al., 2019), antioxidants (Yilmaz-Ersan et al., 2018), and antidiabetics (Kahraman et al., 2021). The challenges in up-scaling production and quality control of milk kefir often arise from the complexity of fermentation performance, which is influenced by the interactions of factors such as time (Hecer et al., 2019), temperature (Hecer et al., 2019), and origin of kefir grain (de Sainz et al., 2020). The complex interactions between processing factors and milk kefir properties, coupled with a general lack of fundamental knowledge of fermentation process have aggravated the quality prediction of milk kefir using a single or general mathematical models.

The Response Surface Methodology (RSM) is a practical design of experiment and mathematical tool that has been used in research and manufacturing industries to

optimise processing factors in the production of yogurt (Akhi et al., 2024) and sausage (Florence Adeola et al., 2024). Artificial Neural Network (ANN) is a powerful machine learning system which involves computational and mathematical techniques that are designed to simulate learning algorithm of the human brain (Antil et al., 2020). Within the food sector, ANN has been applied to model and predict rheological properties of dough (Razmi-Rad et al., 2007), monitoring of winemaking process (Sipos, 2020), and more recently in the spray drying of coconut milk (Ming et al., 2021). This research aims to model and optimise fermentation conditions on pH, viscosity, and lactic acid percentage using the RSM and ANN methods. The prediction capabilities of both RSM and ANN were compared by root mean square error (RMSE), absolute average deviation (AAD), and coefficient of determination (R^2). The integration between both the conventional and machine learning predictive modelling provides a more robust modelling framework for process optimisation, specifically for milk kefir which has not been implemented. This research also identified the microbial composition of milk kefir using amplicon-based sequencing via the Next Generation Sequencing to account its diverse microbial communities, hence connecting optimisation and high-resolution microbial profiling that offers a more comprehensive understanding of milk kefir dynamics beyond physicochemical properties.

MATERIALS AND METHODS

Raw Materials and Reagents

Cow's milk (Farm Fresh Milk Sdn. Bhd. Malaysia), Tibetan kefir grains purchased from Nature Brands (Nature's Recipe, Selangor, Malaysia), and commercial milk kefir (BrightCow, Malaysia).

Reactivation of Kefir Grain and Milk Kefir Production

The reactivation and preparation of kefir grain was modified from the method by Hecer et al. (2019). Kefir grains were reactivated in a container of milk for 12 hours at 25°C for three incubation cycles. In each incubation cycle, the kefir grains were separated using a sieve and transferred into a new batch of fresh milk. For milk kefir production, the reactivated kefir grains were inoculated in fresh cow milk with a ratio of 1:30 (w/v) as recommended by Gentry et al. (2023). The mixture was stirred gently before being placed in the incubator with a programmable temperature and humidity chamber (Model TMJ-9712, T Machine Technology Co., Ltd., China) for fermentation. The humidity was fixed at 60% RH throughout all experiments. Upon incubation, milk kefir grains were separated and the milk kefir obtained was left to set at room temperature for 10 minutes prior to the analysis.

pH Measurement

The pH value of milk kefir was measured using a pH meter (Milwaukee MW 100 Pro, United States). The pH meter was calibrated with pH 4 and pH 7 buffer solutions. The tip of the pH meter was immersed in 3 mL of milk kefir. The pH value was recorded once the reading remained stable. The pH value of milk kefir was obtained in triplicate.

Viscosity Measurement

Viscosity was measured using a viscometer (Model BDV-8S, Biobase, China). The spindle used was No. 2 and the speed was set at 30 rpm. A total amount of 120 mL of milk kefir was poured into a beaker, and the spindle was lowered into the milk kefir until it touched the detection level. Measurement of viscosity was performed in triplicate at a constant temperature of 25°C.

Lactic Acid Percentage Measurement

The determination of titratable acidity was performed following Triwibowo et al. (2020). Several studies have demonstrated that milk kefir is composed mainly of lactic acid, hence, measuring titratable acidity of milk has been perceived as a common quantitative method of the lactic acid percentage (Putri et al., 2020; Triwibowo et al., 2020). Based on Codex Alimentarius, the minimum titratable acidity for milk kefir is 0.6% (Food & Agriculture Organisation, 2022). A total of 5 mL of milk kefir was pipetted and transferred into the Erlenmeyer flask, followed by the addition of 2-3 drops of phenolphthalein solution. The mixture was titrated with 0.1N sodium hydroxide solution until a vibrant pink was observed. The lactic acid percentage was calculated following Eq. 1:

$$\text{Lactic acid percentage (\%)} = \frac{\text{Amount of NaOH added (ml)} * 0.009}{\text{Amount of milk kefir (ml)}} * 100\% \quad [1]$$

Response Surface Methodology (RSM) Experimental Design and Modelling

A two-factor Central Composite Design with five levels was generated using statistical software (Minitab Release 14, Minitab Inc., US). The processing factors, temperature and time are presented in Table 1, with a total of 39 runs varying temperature (X_1) from 18°C to 38°C and time (X_2) ranging from 5 hours to 9 hours. The fermentation time was set based on a preliminary experiment where milk kefir was fermented within this range of time and started to over-ferment after 9 hours based on the occurrence of whey separation. A temperature of 28°C and 7 hours were set as the centre points. To minimise variances, the experimental run was performed in triplicate. The central point was repeated five times to validate the reproducibility of the method. The responses measured included milk kefir's

pH (Y_1), viscosity (Y_2) and lactic acid percentage (Y_3). For modelling, an overall quadratic equation of the response variables as Eq. 2 was generated based on regression analysis from statistical tool (Minitab Release 14, Minitab Inc., US).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_{12} \quad [2]$$

where Y is the response variable, X_1 and X_2 are fermentation temperature and time respectively, β_0 is a constant coefficient, β_1, β_2 are linear regression coefficient, β_{11}, β_{22} are quadratic regression coefficient, β_{12} is interactive regression coefficient.

RSM Optimisation and Validation

The optimum incubation temperature and time were determined setting targets of pH at 4.4 based on Lengkey and Balia (2014), viscosity at 1200 mPa.s based on Setyawardani et al. (2019) and lactic acid percentage at 0.8% as recommended by Yilmaz et al. (2022). The optimised temperature and time were then used to predict values of response variables using a quadratic model (Eq. 2). A validation experiment was performed by using the optimum incubation temperature and time in the incubator to obtain responses which were then compared with those predicted using the model. The response variables' outcomes were compared with the predicted values using Eq. 3 to determine the adequacy of the mathematical model.

$$\text{Residual standard error, RSE } (\%) = \frac{\text{Experimental value} - \text{Predicted value}}{\text{Experimental value}} \times 100\% \quad [3]$$

Artificial Neural Network (ANN) Modelling

ANN modelling was performed and trained using MATLAB 2024a software (Mathworks, Natick, USA) along with the neural net fitting tool (nftool) from neuron network model to build the architecture graph. The same data collected for RSM was used in ANN modelling for similar response variable prediction. Before ANN training, data were normalised based on Eq. 4 to maintain a uniform attention during training process:

$$X_{normalised} = \frac{X_{d.v} - X_{mean}}{s} \quad [4]$$

where $X_{d.v.}$ is data value, X_{mean} is mean of dataset and s is standard deviation of dataset.

To preserve flexibility and to account for complexity in approximating nonlinear function of any desired level of accuracy, the number of neurons within a hidden layer was adjusted and a two-layer feedforward multilayer perceptron with a sigmoid transfer function was chosen as the nonlinear analysis method. The number of neurons within the

input and output layers was set based on the number of inputs and outputs respectively. The neural network topologies were built by performing adjustments on the neurons in hidden layers varying from 1 to 10. Levenberg-Marquardt backpropagation learning algorithm was employed as default to train the neural network. The dataset was segregated into 70% for training, 15% for validation and 15% for testing according following method proposed by Kantono et al. (2022). The performance of the ANN was evaluated based on mean squared error (MSE). A lower MSE was preferable when training model to obtain a minimal error in the model which was suggested by Kantono et al. (2022). The exact ANN architecture of present study consisted of one input layer with two neurons known for input factors, namely fermentation temperature and time, one hidden layer with five neurons and an output layer consisting of three neurons representing pH, viscosity and lactic acid percentage. Once the network was trained iteratively with a different number of neurons and the lowest MSE was achieved, the predicted response variables were generated and recorded. Every neuron consists of multiple inputs, multiplied by corresponding weights, total up, added extra bias and inserted into activation function to generate a single output through Eq. 5.

$$z = f(\sum_{i-1}^{n} w_i\, x_i + d) \quad [5]$$

where z is the output from neuron, x_i is the input value, w_i is the connection weight, d is the bias value and f is the activation function.

The predicted responses obtained from both RSM and ANN models were then compared in terms of prediction capabilities using RMSE, AAD and R^2 given in the Eqs. 6-8.

$$\text{Root Mean Squared Error (RMSE)} = \sqrt{\frac{1}{n}\sum_{i=1} |Y_{i,exp} - Y_{i,pred})^2} \quad [6]$$

$$\text{Absolute Average Deviation (AAD)} = \frac{100}{n}\left[\sum_{i=1}^{n}\left(\frac{|Y_{i,exp} - Y_{i,pred}|}{Y_{i,pred}}\right)\right] \quad [7]$$

$$\text{Coefficient of Determination } (R^2) = 1 - \frac{\sum_{i=1}^{n}(Y_{i,exp} - Y_{i,pred})^2}{\sum_{i=1}^{n}(Y_{i,exp} - Y_{mean})^2} \quad [8]$$

Amplicon-based Sequencing

Microbiological composition of optimised and commercial milk kefir was identified and compared by performing amplicon sequencing which includes 16S sequencing for bacterial diversity and ITS sequencing for fungal diversity. Prior to sequencing, microbial DNA of milk kefir samples was isolated using DNeasy Powerfood Microbial Kit (Qiagen, Hilden,

Germany) in accordance with protocol stated in Qiagen's handbook. Extracted DNA was processed for clean-up utilising AMPure XP beads (Beckman Coulter®, Brea, CA, USA) to purify the product. Then, extracted DNA was amplified specifically targeting the 16S V3-V4 regions and the ITS 2-region. Successful amplification was determined by presence of desired band in positive control and absence band in negative control. DNA amplicons were read and sequenced using Illumina Miseq System (Illumina, San Diego, California) at 2x250bp configuration, which generates approximately 100,000 raw reads per sample. Raw sequencing data collected were pre-processed using DADA2 in Rstudio (Version 2024.12.0-467) for bioinformatics analysis. Steps of pre-processing raw data involve checking sequencing quality to identify and trim low-quality regions, learning error rate, merging the contigs, defining taxonomy, collating number of sequences at different stages and creating phyloseq object. Bar plot featuring microbial composition of optimised and commercial milk kefir were constructed and their abundance percentage of dominant taxa was compared.

RESULTS AND DISCUSSION

Regression Modelling Analysis using RSM

Table 1 shows the experimental results of CCD with experimental responses including pH, viscosity and lactic acid percentage. Table 2 lists the regression coefficients, β and respective p-value from regression modelling using Eq. 2 yielding prediction models for each response (Eqs. 9-11) obtained from Minitab. It was found that all linear terms of temperature (X_1) and time (X_2) and the interaction term of temperature and time (X_{12}) were giving significant impacts to the responses. The quadratic term of temperature (X_1^2) was not significant towards pH and viscosity ($P > 0.05$) while for quadratic term of time (X_2^2) was not significant for the pH and lactic acid percentage.

Table 1
Experimental runs generated from CCD for collected milk kefir responses. Responses with subscript RSM and ANN are both predicted values from modelling using Eqs. 2 and 5

Run	X_1	X_2	Y_1			Y_2			Y_3		
			Y_{exp}	Y_{rsm}	Y_{ann}	Y_{exp}	Y_{rsm}	Y_{ann}	Y_{exp}	Y_{rsm}	Y_{ann}
1	28	7	5.55	5.47	5.52	1448.6	1536.3	1438.6	0.43	0.43	0.43
2	28	8	5.31	5.36	5.38	1395.3	1367.1	1424.6	0.45	0.48	0.47
3	28	7	5.53	5.47	5.52	1477.8	1536.3	1438.6	0.43	0.43	0.43
4	28	7	5.53	5.47	5.52	1528.3	1536.3	1438.6	0.41	0.43	0.43
5	28	7	5.46	5.47	5.52	1489.1	1536.3	1438.6	0.47	0.43	0.43
6	18	9	6.23	6.25	6.22	567.4	571.2	567.6	0.36	0.35	0.36
7	28	8	5.31	5.36	5.38	1375.0	1367.1	1424.6	0.43	0.48	0.47

Table 1 *(continued)*

Run	X_1	X_2	Y_1			Y_2			Y_3		
			Y_{exp}	Y_{rsm}	Y_{ann}	Y_{exp}	Y_{rsm}	Y_{ann}	Y_{exp}	Y_{rsm}	Y_{ann}
8	18	9	6.24	6.25	6.22	568.9	571.2	567.6	0.36	0.35	0.36
9	28	7	5.45	5.47	5.52	1485.0	1536.3	1438.6	0.47	0.43	0.43
10	23	7	5.72	5.75	5.72	1623.2	1469.6	1629.1	0.34	0.36	0.32
11	28	7	5.45	5.47	5.52	1477.8	1536.3	1438.6	0.47	0.43	0.43
12	23	7	5.74	5.75	5.72	1634.0	1469.6	1629.1	0.32	0.36	0.32
13	23	7	5.74	5.75	5.72	1647.1	1469.6	1629.1	0.34	0.36	0.32
14	28	6	5.51	5.63	5.56	1416.3	1206.0	1465.1	0.40	0.37	0.41
15	18	5	5.94	5.96	5.97	360.2	413.1	361.1	0.25	0.29	0.28
16	18	5	5.97	5.96	5.97	361.0	413.1	361.1	0.31	0.29	0.28
17	38	9	4.22	4.21	4.19	1209.2	1178.8	1184.6	0.92	0.91	0.90
18	33	7	4.97	5.14	5.01	1746.9	1691.3	1740.5	0.58	0.56	0.59
19	28	7	5.40	5.47	5.52	1427.3	1536.3	1438.6	0.43	0.43	0.43
20	33	7	4.98	5.14	5.01	1746.6	1691.3	1740.5	0.56	0.56	0.59
21	38	5	5.62	5.58	5.62	700.3	692.3	669.6	0.50	0.52	0.51
22	28	7	5.44	5.47	5.52	1421.1	1536.3	1438.6	0.43	0.43	0.43
23	28	7	5.40	5.47	5.52	1413.7	1536.3	1438.6	0.43	0.43	0.43
24	28	7	5.38	5.47	5.52	1412.8	1536.3	1438.6	0.54	0.43	0.43
25	38	5	5.62	5.58	5.62	654.2	692.3	669.6	0.50	0.52	0.51
26	28	7	5.38	5.47	5.52	1445.5	1536.3	1438.6	0.47	0.43	0.43
27	38	5	5.61	5.58	5.62	651.1	692.3	669.6	0.50	0.52	0.51
28	28	6	5.51	5.63	5.56	1408.7	1206.0	1465.1	0.41	0.37	0.41
29	33	7	4.98	5.14	5.01	1727.3	1691.3	1740.5	0.59	0.56	0.59
30	38	9	4.23	4.21	4.19	1197.3	1178.8	1184.6	0.92	0.91	0.90
31	28	7	5.37	5.47	5.52	1412.3	1536.3	1438.6	0.47	0.43	0.43
32	18	5	5.98	5.96	5.97	359.6	413.1	361.1	0.31	0.29	0.28
33	28	7	5.88	5.47	5.52	1498.9	1536.3	1438.6	0.38	0.43	0.43
34	18	9	6.22	6.25	6.22	568.4	571.2	567.6	0.36	0.35	0.36
35	28	7	5.87	5.47	5.52	1493.1	1536.3	1438.6	0.36	0.43	0.43
36	38	9	4.23	4.21	4.19	1208.3	1178.8	1184.6	0.92	0.91	0.90
37	28	8	5.31	5.36	5.38	1353.0	1367.1	1424.6	0.45	0.48	0.47
38	28	6	5.51	5.63	5.56	1413.0	1206.0	1465.1	0.40	0.37	0.41
39	28	7	5.86	5.47	5.52	1490.0	1536.3	1438.6	0.34	0.43	0.43

Note. where Y_{exp} is experimental value, Y_{rsm} is value predicted by RSM model, Y_{ann} is value predicted by ANN model

Table 2
Regression coefficients, β *and their p-value for responses, pH (Y_1), viscosity (Y_2) and lactic acid percentage (Y_3) for processing factors temperature (X_1) and time (X_2)*

Term	Y_1		Y_2		Y_3	
	β	p-value	β	p-value	β	p-value
Constant	4.848	0.000	-9698	0.000	0.874	0.000
X_1	0.1374	0.000	-105.5	0.000	-0.0706	0.000
X_2	0.006	0.000	3462	0.000	0.020	0.000
X_1^2	-0.00095	0.576*	1.77	0.144*	0.001081	0.023
X_2^2	0.0313	0.461*	-249.8	0.000	-0.0060	0.600*
X_{12}	-0.02071	0.000	4.11	0.008	0.004275	0.000

Note. $*P > 0.05$

$$Y_1 = 4.848 + 0.1374\,X_1 + 0.006\,X_2 - 0.00095{X_1}^2 + 0.0313\,{X_2}^2 - 0.02071\,X_{12} \quad [9]$$

$$Y_2 = -9698 - 105.5\,\mathrm{X}_1 + 3462\,\mathrm{X}_2 + 1.77\,{X_1}^2 - 249.8\,{X_2}^2 + 4.11\,\mathrm{X}_{12} \quad [10]$$

$$Y_3 = 0.874 - 0.0706\,\mathrm{X}_1 + 0.020\,\mathrm{X}_2 + 0.001081\,{X_1}^2 - 0.0060\,{X_2}^2 + 0.004275\,\mathrm{X}_{12} \quad [11]$$

The goodness of fit of the models was determined by the coefficient of determination, R^2. The R^2 values of pH, viscosity and lactic acid percentage were 92.19%, 95.03%and 94.51%, respectively. The results are much higher than the R^2 obtained by a parallel study by Abadl et al. (2023) on optimising coconut milk kefir, with R^2 value of 75%. Ghasemlou et al. (2012) explained that a high value of R^2 above 90% shows a close agreement between the experimental and predicted values proposed by the modelling equations, suggesting that the models are considered valid and highly reliable.

Response Surface Analysis using RSM

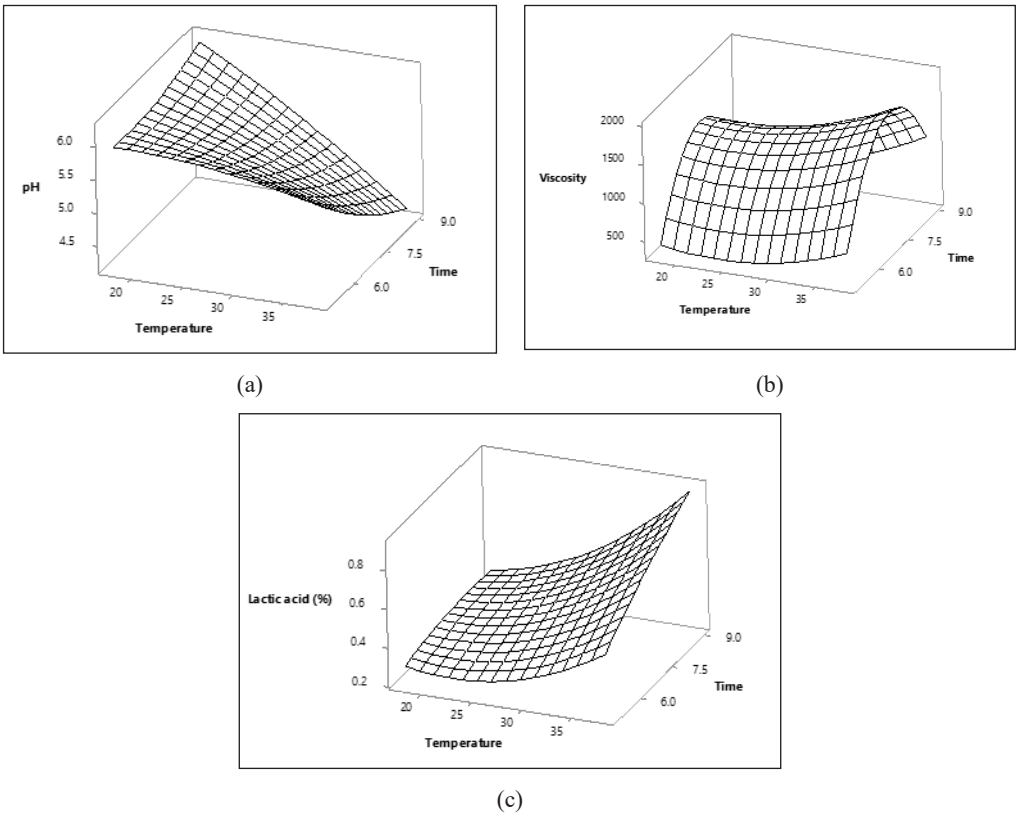
Figure 1a shows a sharp decrease in milk kefir pH with an increase in both fermentation temperature and time. Milk kefir pH incubated for 9 hours at a higher temperature of 38°C resulted in a lower pH of 4.23 compared to a pH of 5.96 when incubated at a lower temperature of 18°C for 9 hours. Milk kefir fermented at 38°C for 9 hours had the lowest pH which was sufficient to inhibit growth of undesirable spoilage microorganisms and preserve shelf life of the product. Alegbeleye et al. (2022) explained that maintaining a pH range between 4.2 and 4.6 was important in maintaining safety quality of food product.

The trend was in agreement with Abadl et al. (2023) who reported a gradual decrease in pH when increasing temperature of coconut milk kefir fermentation from 25°C to 35°C. In terms of fermentation time, Kivanc & Yapici (2019) observed a pH drop from 6 to 4.5 after 24 hours of milk kefir fermentation. The pH reduction is due to increased lactic acid bacteria (LAB) and its metabolism activity (Ribeiro et al., 2020). Higher fermentation temperature and longer fermentation time encourage the microorganism to proliferate rapidly and favourably, thereby increasing the LAB population which metabolises lactose into lactic acids aided by β-galactosidase enzyme. The presence of lactic acids contributes to a higher concentration of H^+ ions which drastically decreases milk kefir pH drastically (Divayanti et al., 2023).

Figure 1b shows that milk kefir viscosity gradually increased with fermentation temperature from 18°C to 38°C. With fermentation time, milk kefir viscosity also increased rapidly, reaching a peak at 7 hours, beyond which there was a gradual decline. A maximum viscosity of 1746.9 m.Pas was achieved when milk kefir was fermented at 33°C for 7 hours. Milk kefir viscosity of 1200-1600 m.Pas has been recommended by Putri et al. (2020) and it has been set as a reference for quality control as it dictates the organoleptic properties including texture, mouthfeel and cohesiveness. The increasing trend of milk kefir viscosity was consistent with Putri et al. (2020), who found that milk kefir fermented at 37°C resulted in a higher viscosity than at room temperature of 25°C. The increase of viscosity when raising fermentation temperature is attributed to the increase of kinetic energy in the molecules of milk kefir, encouraging casein coagulation and intermolecular interaction between the carbohydrate and proteins, which leads to the formation of a well-structured and stronger gel matrix (Khubber et al., 2021; Putri et al., 2020). The non-linear increase of viscosity with fermentation time was similar to that of Kivanc & Yapici (2019), who conducted a prolonged fermentation time at 22°C, where milk kefir viscosity reached its peak at 48 hours before decreasing as they focused on the formation of gel network between carbohydrate-protein network and how the gel network led to changes in viscosity during fermentation. The increase in viscosity over fermentation time is due to more and stronger molecular interactions and cross-linkages between molecules such as polysaccharides and protein (Akhi et al., 2024). The decrease in viscosity after the peak is due to whey separation when the protein network starts to denature and destabilise as a result of the growth of an extremely acidic environment from the continuous fermentation (Rekha et al., 2012).

Figure 1c shows that the lactic acid percentage increased with fermentation temperature and time due to favourable conditions for the growth and multiplication of lactic acid bacteria (Triwibowo et al., 2020). Milk kefir fermented at 38°C for 9 hours at 0.918% was the highest in terms of lactic acid percentage, and it was three times of that fermented at 18°C for 5 hours at 0.316%. The Codex Alimentarius regulated by Food and Agriculture

Organisation (2022) requires milk kefir to have at least 0.6% of lactic acid. The increase of lactic acid percentage with fermentation temperature and time is consistent with the work by Ribeiro et al. (2020), who reported that a significant increase in lactic acid percentage by a 12.17-fold higher in titratable acidity rate when kefir incubation temperature was increased from 17°C to 32°C. Kivanc & Yapici (2019) showed a gradual increase in lactic acid percentage from 0.14% to 1.0% in an extended fermentation of milk kefir from 2 to 24 hours. Hecer et al. (2019) showed that milk kefir incubated at 30°C consisted of higher *Lactobacillus* spp. count at 6.37 $\log_{10}$ CFU/mL compared to milk kefir incubated at 20°C at 6.04 $\log_{10}$ CFU/mL. The advantage of high lactic acid percentage is that it inhibits spoilage microorganisms as the acidification process drives a low pH and enhances food safety. The inverse relationship between lactic acid percentage and pH is reported in various studies (Moonga et al., 2019).



(a) (b)

(c)

Figure 1. Response surface plot illustrating interaction effect of fermentation temperature and time on (a) pH, (b) viscosity, and (c) lactic acid percentage of milk kefir

Optimisation using RSM

The optimisation of milk kefir at a targeted pH 4.4, viscosity 1200 m.Pas and lactic acid percentage 0.8% yielded fermentation temperature at 35.78°C and fermentation time at 8.84 hours. The composite desirability obtained was 0.9717, indicating that the setting optimally achieved a favourable outcome for all responses as a whole as the result is close to one with individual desirability of pH, viscosity and lactic acid percentage at 0.9464, 0.9742 and 0.9950, respectively (Table S1). Composite and individual desirability are essential for evaluating how well the combination of processing variables satisfies the targeted response values. The validation experiment performed at the suggested optimum conditions was compared with the predicted. The residual standard error (RSE) shows that all errors were lower than 10%, where predicted and actual values are in agreement (McLaughlin & Magee, 1998), which also suggests that predictions made from models (Eqs. 9-11) are accurate and valid. Figure 2 also shows the behaviour of actual responses generated from the optimised factors demonstrated that as fermentation temperature and time increased, the lactic acid percentage and viscosity increased while the pH decreased. For viscosity, it increased with temperature, but over time, it trends up before down at around 7 hours, as seen in the response surface plot, Figure 1b.

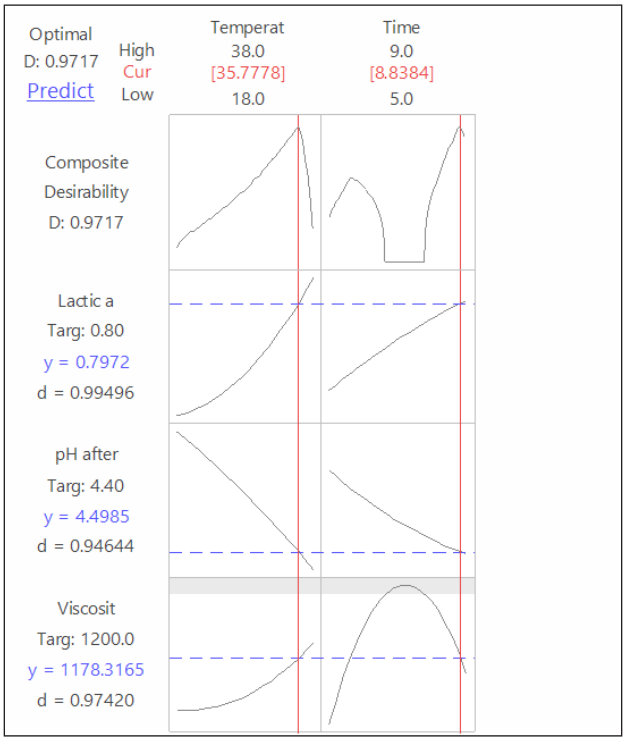


Figure 2. Response behaviour of lactic acid percentage, pH and viscosity predicted from the processing conditions

Comparison of the Predictive Capability between ANN and RSM Models

The neural network model (ANN) was established through training, validation, and testing. In constructing ANN modelling, the feedforward multilayer perceptron and 70% data were used as the training set, while the remaining 30% data was used for validation and testing set, each at 15%. One hidden layer with five neurons was chosen to construct ANN model where fermentation temperature and fermentation time were input parameters, while pH, viscosity and lactic acid percentage were the output parameters. During the training of neurons, it was found that the accuracy of the model declined after five neurons within the hidden layer. The increase of neurons beyond 5 caused the model to become overly complex and overfit the training data, leading to a high predictive error. The response values predicted by ANN model using the same dataset of RSM are presented in Table 1 as Y_{ann}.

RSM and AAN models' predictive capability were compared using statistical parameters including RMSE, AAD and R^2 which were calculated following Eqs. 6-8 as shown in Table 3. The RMSE and AAD values for all predicted responses of pH, viscosity and lactic acid percentage from the ANN model were lower when compared to the RSM model, suggesting the ANN model was more accurate in terms of smaller predictive errors and smaller absolute average deviations. For the coefficient of determination, R^2, the ANN model also presented a higher R^2 value than the RSM model by 1.92%, 4.26% and 0.70% for pH, viscosity and lactic acid percentage respectively. Achieving a low RMSE, low AAD and high R^2 are the criteria that are generally sought to define a highly accurate model as lower RMSE implies a minimal average prediction error, a lower AAD reflects consistency and minimal average deviation from actual values while a high R^2 indicates the goodness of fit of the model (Kantono et al., 2022). Prior research also agreed that ANN model provided a better predictive and generalisation ability with lower percentage error as compared to RSM in the prediction of solubility, colour change, antioxidant activity and total anthocyanin content of spray-dried pomegranate juice by Youssefi et al. (2009). The superiority of the predictive accuracy of ANN which yielded lower RMSE, lower AAD and higher R^2 values across all predicted response values can be explained by its universal capability in approximating nonlinearity of the system while the RSM is limited to second-order polynomial (Youssefi et al., 2009). The RSM, however, can provide

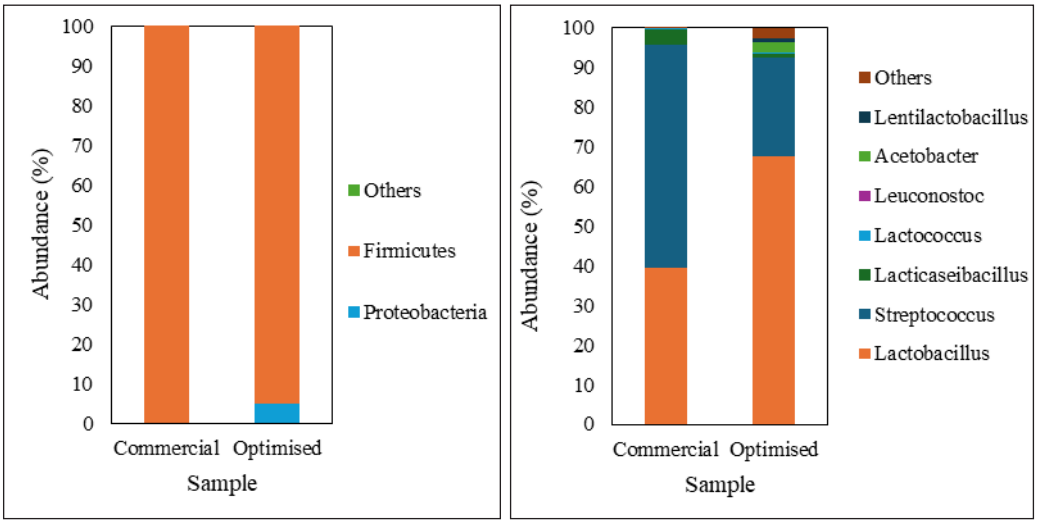
Table 3
Comparison of predictive capabilities of RSM and ANN modelling on responses

	pH			Viscosity			Lactic Acid Percentage		
Parameters	**RSM**	**ANN**	**Difference**	**RSM**	**ANN**	**Difference**	**RSM**	**ANN**	**Difference**
RMSE	0.046	0.035	- 0.011	0.073	0.026	- 0.47	0.00261	0.00258	- 0.00003
AAD (%)	0.116	0.056	- 0.06	1.121	0.089	- 1.032	0.692	0.008	- 0.684
R^2 (%)	92.19	94.11	+ 1.92	95.03	99.29	+ 4.26	94.51	95.21	+ 0.70

information such as individual and interaction effects of processing variables. Modelling milk kefir fermentation using both RSM and ANN methods has helped to bridge traditional fermentation practices to a more systematic and scientific approach of producing consistent and quality milk kefir product.

Comparison of Bacterial Composition

Figure 3a shows abundance percentage of phyla across optimised and commercial milk kefir. Among phyla, *Firmicutes* were found as the most dominant phylum in both optimised and commercial milk kefir. Commercial milk kefir exhibited a higher abundance percentage of *Firmicutes* than optimised milk kefir, where both accounted for 99.99% and 94.86%, respectively. *Proteobacteria* was also found in optimised milk kefir at abundance percentage of 5.09% while only a small amount was detected in commercial milk kefir which was 0.002%. A similar finding was reported recently by Sumarmono et al. (2023) who indicated *Firmicutes* and *Proteobacteria* were the dominant phyla that were found in milk kefir. *Actinobacteria* and *Bacteroidetes* were other phyla that were identified in optimised milk kefir despite in small percentages, which contributed for less than 0.001% of abundance (Table S2). *Firmicutes* as the dominant phylum serves a significant role in regulating dietary fibre uptake and colonise intestinal mucosa which leads to homeostasis that promote health promoting function for gut (Sun et al., 2023). *Firmicutes* is also known as short-chain fatty acid-producing bacteria which assist in regulating glucose metabolism and insulin sensitivity, relieving inflammatory conditions among diabetic patients (Cifuentes



(a) (b)

Figure 3. Abundance percentage of bacterial composition in commercial and optimised milk kefir. (a) Phylum level and (b) Genus level

et al., 2024). *Proteobacteria* as the second dominant phylum in optimised milk kefir are mainly responsible for serving carbohydrate digestion in human body and trigger immune response (Hou et al., 2022).

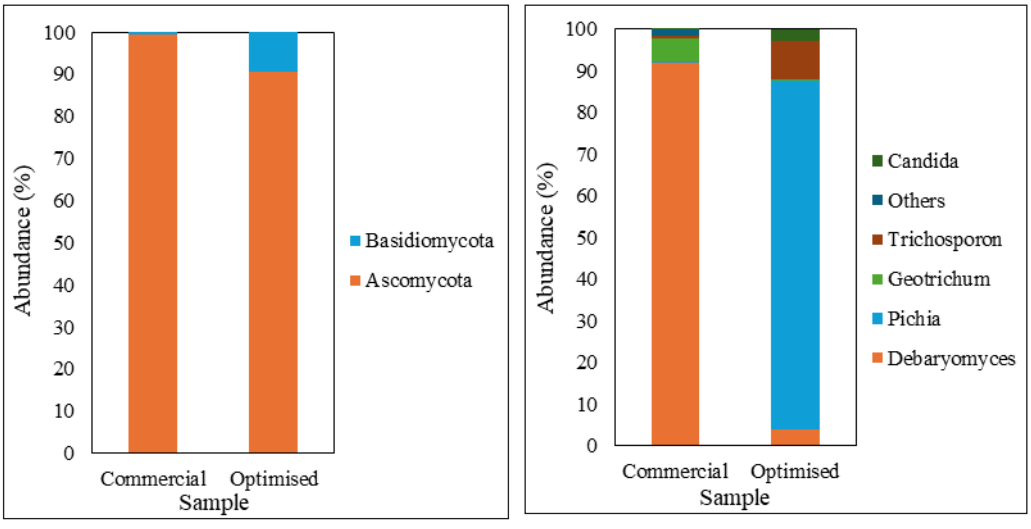
Figure 3b shows abundance percentage of genera composition in commercial and optimised milk kefir. At genus level, optimised and commercial milk kefir consisted of a total of up to 14 and 6 genera respectively (Table S2), suggesting that optimised milk kefir exhibited a higher diversity of genera in microbial community, providing a wider range of beneficial genera with health potential. *Streptococcus*, *Lactobacillus*, *Lacticaseibacillus*, *Lactococcus* and *Leuconostoc* were the top five dominant genera found in commercial milk kefir. Optimised milk kefir comprised five dominant genera namely, *Lactobacillus*, *Streptococcus*, *Acetobacter*, *Lentilactobacillus* and *Lacticaseibacillus*. A similar finding was established in a study by Sumarmono et al. (2023), who have noted that *Lactobacillus* were predominantly detected in cow and goat milk kefir at high abundance percentage. The abundance percentage of *Lactobacillus* and *Lentilactobacillus* were observed to be higher in optimised milk kefir as compared to commercial milk kefir by a difference of 28.12% and 0.97% while abundance percentage of *Streptococcus*, *Lacticaseibacillus*, *Lactococcus,* and *Leuconostoc* were found higher in commercial milk kefir as compared to optimised milk kefir, by a difference by 31.11%, 2.95%, 0.01% and 0.1% respectively. *Lactobacillus, Streptococcus*, *Lentilactobacillus, Lacticaseibacillus, Leuconostoc,* and *Lactococcus* are recognised as probiotics as they are mainly associated with maintaining gut health and preventing pathogen invasion (Ajibola et al., 2023; Garcia et al., 2020). In fermentation, they are also a lactic-acid producing bacteria which facilitate fermentation process by lowering the pH during fermentation process that aim to preserve and extend shelf life (Ajibola et al., 2023). 16S amplicon sequences have effectively revealed the presence of other genera in optimised milk kefir that was accounted in relatively small abundance percentages. Of these, *Pseudomonas, Klebsiella, Acinetobacter,* and *Bacillus* were detected in optimised milk kefir and are typically associated with gut microbiota (Karaliute et al., 2022; Lee et al., 2019). Optimised milk kefir can serve as a potential candidate as a health-boosting product due to its high diversity in potential genera and consisting of relatively high abundance percentage of *Lactobacillus* which is often associated with mounting clinical evidences in supporting health benefits such as strengthening immunity (Rastogi et al., 2022), enhancing gut health (Huynh & Zastrow, 2023) and controlling blood glucose level (Kumari et al., 2023)

Comparison of Fungal Composition

Figure 4a shows bar plot featuring abundance percentage of fungal phyla across optimised and commercial milk kefir. Two phyla namely *Ascomycota* and *Basidiomycota* were detected in both milk kefir. *Ascomycota* was the dominant phylum in both milk kefir,

accounting for more than 90%. This was consistent with the results of both Sumarmono et al. (2023) and Marsh et al. (2013) who found that *Ascomycota* was detected abundantly in milk kefir originating from Ireland and the United Kingdom. In the present study, commercial milk kefir exhibited a higher relative abundance percentage of *Ascomycota* as compared to optimised milk kefir by a difference of 8.77%. Meanwhile, *Basidiomycota*, another phylum was found to be more abundant in optimised milk kefir as compared to commercial milk kefir by a difference of 8.76%. *Ascomycota* serves a vital role in fermented beverages in fermenting sugars and breaking down anti-nutritive factors (Cason et al., 2020). In medical field, members of *Ascomycota* also exhibit anti-diabetic properties, regulate lipid levels and serve as alpha-amylase inhibitors (Jiang et al., 2025). *Basidiomycota* also exhibit multiple roles, from decomposers in soil to enzyme development and bioremediation. *Basidiomycota* has been extensively reviewed recently due to its bioactive compounds that contribute to antimicrobial, anticancer and anti-inflammatory activities (Oliveira et al., 2024).

Figure 4b shows bar plot featuring abundance percentage of fungal genera for optimised and commercial milk kefir. The most abundant genus in commercial milk kefir was *Debaryomyces* (91.81%) but only a small amount was found in optimised milk kefir (3.84%). In optimised milk kefir, *Pichia* (83.66%) was found dominantly while commercial milk kefir consisted of less than 1% of *Pichia*. A similar finding was found in a study by Rahmani et al. (2022) who detected *Pichia* abundantly in milk kefir. In contrast, research by Sumarmono et al. (2023) reported *Kazachstania* and *Kluyveromyces* as the dominant genus in cow milk kefir. *Pichia* serves an important role in fermentation where it acts as a biofilm



(a) (b)

Figure 4. Abundance percentage of fungal composition in commercial and optimised milk kefir. (a) Phylum level and (b) Genus level

producer that adheres to kefir grain surface and establishes a symbiotic relationship with *Lactobacillus* where they utilise bioproducts as energy and microbial growth factors, which also have the role of contributing to lactic and alcohol fermentation (Hamet et al., 2013; Wang et al., 2012). Referring to Table S3, *Geotrichum* was found in a higher percentage in commercial milk kefir compared to optimised milk kefir by a difference of 5.23%. Meanwhile, optimised milk kefir showed a higher percentage of *Trichosporon* and *Candida* by 8.79% and 2.65% respectively when compared to commercial milk kefir. Other genera with abundance percentage less than 1% in commercial milk kefir include *Cyberlindnera*, *Gyoerffyella*, *Aspergillus*, *Dipodacus, Wickerhamiella,* and *Cutaneotrichosporon* while *Mycoaciella* and *Clasdosporium* were found in small percentages in optimised milk kefir. This showed that the optimum processing parameters have also helped provide favourable conditions that encouraged the growth of beneficial bacteria such as *Pichia* as the dominant fungal species which facilitates microbial growth for successive lactic and alcohol fermentation.

CONCLUSION

Modelling milk kefir fermentation using both RSM and ANN methods has helped bridge traditional fermentation practices to a more systematic and scientific approach to producing consistent and quality milk kefir product. By optimising processing parameters using RSM, target physicochemical qualities such as pH, viscosity and lactic acid percentage can be achieved. It also allows determination of combinations of processing parameters that provide a critical impact on changes of qualities. The recommended fermentation temperature of 35.78°C and fermentation time of 8.84 hours according to the definition of desired milk kefir quality with targeted values of 4.4 pH, 0.8% lactic acid and 1200 mPa.s. While the ANN gave a better modelling predictive capability, the RSM has provided more information, including interaction effects of processing factors and the optimal conditions to produce targeted milk kefir quality. The optimisation approach has not only helped in determining optimal processing parameters for desired quality of milk kefir but also created favourable conditions that promote the enhanced growth of beneficial bacteria including probiotics *Lactobacillus. Pichia* was found to be the dominant fungal which serves as an important bioproduct and microbial growth factor in successive lactic and alcohol fermentation. This validated modelling framework ultimately enables manufacturers to adapt for industrial manufacturing with targeted physicochemical quality in a more efficient and low-cost manner. Scaling-up to a pilot-scale level and more research on product shelf life and stability integrated with consumer sensory studies will be helpful in commercialisation validation of milk kefir.

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SUPPLEMENTARY DATA

Table S1
Response optimisation and desirability

Responses	Goal	Target	Predicted Result	Experimental Result	RSE (%)	Desirability
Y_1	Target	4.4	4.4985	4.51	0.25	0.9464
Y_2	Target	1200	1178.32	1296.6	9.12	0.9742
Y_3	Target	0.8	0.7972	0.792	0.66	0.9950

Table S2
Bacterial composition of optimised and commercial milk kefir

Sample	Commercial	Optimised_R1	Optimised_R2	Optimised_R3
Phylum				
Firmicutes	99.99754	96.45881	93.05435	95.1441
Proteobacteria	0.002457	3.518859	6.918533	4.830824
Spirochaetes	0	0	0	0
Bacteroidetes	0	0.00072	0	0
Candidatus_Saccharibacteria	0	0	0	0
Actinobacteria	0	0	0.001731	0
Fusobacteria	0	0	0	0
Genus				
Streptococcus	56.06486	24.95623	23.06716	26.81051
Lactobacillus	39.57859	69.22007	67.80139	66.08603
Lacticaseibacillus	3.952579	1.262293	0.768405	0.980652
Lactococcus	0.224704	0.151302	0.263635	0.233323
Leuconostoc	0.135068	0.034583	0.047304	0.043182
Pseudomonas	0.002456	0.016571	0.029421	0.022288
Acetobacter	0	1.464030	3.376484	2.505955
Lentilactobacillus	0	0.831442	1.101843	0.990402
Klebsiella	0	0.744984	1.192990	0.697879
Moraxella	0	0.010087	0.017306	0.019502
Acinetobacter	0	0.006484	0.008076	0.009054
Bacillus	0	0.002882	0.007499	0.004615
Brachybacterium	0	0	0.001731	0
Massilia	0	0	0.001731	0

Table S3
Fungal composition of optimised and commercial milk kefir

Sample	Commercial	Optimised_R1	Optimised_R2	Optimised_R3
Phylum				
Ascomycota	99.3535	99.7347	92.7599	79.2698
Basidiomycota	0.64647	0.26529	7.24009	20.7302
Genus				
Debaryomyces	91.8081	3.76292	4.12425	3.65451
Geotrichum	5.52877	0.80983	0.08234	0
Cyberlindnera	0.77788	0.04887	0	0
Trichosporon	0.62359	0.23736	7.24551	20.7512
Gyoerffyella	0.41948	0	0	0
Pichia	0.34394	92.5929	85.9132	72.4812
Aspergillus	0.17679	0	0	0
Dipodascus	0.13822	0	0	0
Candida	0.10447	2.52025	2.63473	3.09196
Wickerhamiella	0.04982	0	0	0
Cutaneotrichosporon	0.02893	0	0	0
Mycoaciella	0	0.02793	0	0
Cladosporium	0	0	0	0.02115