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Physico-chemical Properties and Microbiological Composition of Optimised *Tempeh* and Milk Kefir

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The fermentation process of two selected fermented foods, *tempeh* and milk kefir are commonly performed at small scale in homes as part of healthy diet following local culture practise. Due to much variations in every fermentation process, the changes in physico-chemical and microbiological properties were studied and compared with commercial products. The fermentation parameters including temperature, time and humidity were optimised for desired product qualities using Response Surface Methodology (RSM) following the three factors Box Behnken design (BBD) for *tempeh* and two factors central composite design (CCD) for milk kefir. The optimised processing conditions for *tempeh* was 33.1 °C for 34.1 hours at 60 % relative humidity while for milk kefir was 35.8 °C for 8.8 hours. It was found that both optimised *tempeh* and milk kefir had significantly greater counts of lactic acid bacteria, aerobic plate counts and mold or yeast. Amplicon sequencing results have identified beneficial microbes that are probiotic including high abundance of *Bacillus* and *Weisella* in optimised *tempeh* while milk kefir contained mostly *Lactobacillus* and *Streptococcus*.

1. 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. Common fermented foods like kimchi and sauerkraut are examples of preservation of cabbage while yogurt and cheese are good ways to extend shelf life of milk. *Tempeh* and kefir are other two rising fermented food because of their reputative health benefits which promote gut health thus evaluating both products offer comparative insights of their fermentation process, impact of optimisation on their physico-chemical and microbiological properties.

Tempeh is a mold-fermented soybean product originated from Indonesia and is consumed widely amongst Malaysians. Tempeh serves as an alternative nutrient-dense source of protein, dietary fiber, vitamins and minerals at an affordable price hence has become a staple food for the locals (Astuti et al., 2000). In the fermentation process, soybeans are reduced in carbohydrate content, protease inhibitors, phenols and phytic acid concentrations. Fermented soybeans develop texture, flavours, antioxidant components and isoflavones which make tempeh an excellent source of nutrition (Mani and Ming 2017). There is also significant biochemical changes through the action of microorganisms or enzymes (Teoh et al. 2024).The predominantly small-scale or household production of tempeh has driven research to help transition from traditional methods to more standardised processing leading to improved product uniformity (Rizal et al., 2022).

Milk kefir is a fermented beverage from 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). Fermenting milk is highly advantageous because it does not only preserve the milk but also imparts health benefits in the form of probiotics (Bellikci-Koyu et al., 2019) and anti-inflammatory effects (Rodrigues et al., 2016). The challenges in up-scaling production and quality control of milk kefir often arise from the complexity of fermentation performance, which is strongly influenced by the interactions of various factors such as time, temperature (Hecer et al., 2019) and origin of kefir grains (de Sainz et al., 2020).

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This research aimed to model and optimise fermentation conditions using response surface methodology (RSM), a statistical and mathematical tool that is used to model and optimise processes which evaluates the relationships between multiple input variables and their impact on response variables to achieve the best outcomes. The Box Behnken design (BBD) was chosen for *tempeh* optimisation involving three processing parameters of fermentation time, temperature and humidity as it more suitable and employs a more economical design that requires fewer experimental runs compared to the central composite design (CCD) (Calfee and Piontkowski, 2016). The CCD was used in the milk kefir optimisation involving two processing parameters of temperature and time.

2. Materials and Methods

Organic soybean for *tempeh* making was purchased locally (Radiant Whole Food, Selangor, Malaysia) and commercial inoculum Ragi *Tempeh* (Raprima, Yogyakarta, Indonesia) from mold *Rhizopus* spp. was used as *tempeh* starter. Commercial *tempeh* was from UMMI Family Enterprise, milk kefir was made using cow's milk (Farm Fresh Milk Sdn Bhd, Malaysia) and Tibetan kefir grains was from Nature's Recipe, Selangor, Malaysia. Commercial kefir was obtained from Brightcow Malaysia Sdn. Bhd.

2.1 *Tempeh* and milk kefir production

The traditional procedure for making *tempeh* was followed with steps of soaking, draining and rinsing with fresh water before manual dehulling. 100 g of soybeans were soaked in clean water overnight for 8 h at room temperature, then dehulled manually before boiling for 25 minutes at soybean to water ratio of 1:3 (w/v) then drained. The weight of soaked and cooked soybeans increased to about 200 g were then left on sieves for excess water removal and cooled down to below 35 °C. *Tempeh* starter was added at 2 g/kg (Hasbullah and Silvy, 2020), mixed thoroughly and filled into two polyethylene bags at about 100 g each, pressed and flattened slightly to make block size of 6 cm x 9 cm. The packed blocks of *tempeh* were placed in an incubator (TMJ-9712B, T-Machine Technology Co., Lt, Taiwan) following settings generated by the BBD design.
Milk kefir production followed the methods of Hecer et al. (2019) with modifications of fermentation temperature to suit the current experiment. Kefir grains were priorly activated by inoculating in pasteurised cow milk at ratio of 1:30 (w/v) for three incubation cycles of 12 h each at 25 °C (Gentry et al., 2023). The mixture was stirred gently and placed in the incubator for fermentation following the CCD design at constant humidity of 60 % RH following Standard MS1525:2014. At the end of fermentation, kefir grains were separated from the mixture and collected milk kefir was cooled down for 10 minutes prior to the analysis.

2.2 RSM optimisation

The RSM designs using BBD and CCD respectively for *tempeh* and milk kefir optimisation were generated using a statistical software (Minitab Release 14, Minitab Inc., US). The BBD requires fewer runs and efficiently estimates quadratic effects making it ideal for exploring interactions among three factors for *tempeh* fermentation while CCD's flexibility and efficiency in exploring non-linear behaviour and establishing a quadratic model suited milk kefir fermentation optimising two factors. A total of 15 runs for the three-factor BBD *tempeh* optimisation (Daji et al., 2022) were determined at setting targets of maximum values for textural attributes (Erkan et al. 2020), colour parameters (maximum L*, minimum a* and b*) (Muzdalifah et al., 2017) and pH at 7.0 (Handoyo and Morita, 2006). A total of 39 runs for the two-factor CCD for milk kefir optimisation was determined by setting targets of pH at 4.4 as recommended by Lengkey and Balia (2014), viscosity at 1200 mPa.s based on Setyawardani et al. (2019) and lactic acid percentage at 0.8 % following recommendations of Yilmaz et al. (2022).
For *tempeh* analysis, Texture Profile Analysis was used to assess textural attributes using a texture analyser (TA-XTplus, Stable Micro Systems, Surrey, U.K.). *Tempeh* samples sized 2 cm x 2 cm were placed at the center of the platform where two compression cycles at 5 s interval were made by a 75 mm stainless steel cylinder compression probe at 50 % strain of sample height (Erkan et al., 2020). Colour analysis was conducted using a colour spectrophotometer (Hunter lab UltraScan VIS, US) in quadruplicates. pH was measured using a pH meter (Milwaukee MW 100 Pro, United States) in triplicates. 10 g of *tempeh* was blended with 100 mL of distilled water in blender for 30 seconds to make slurry while for milk kefir, 3 mL was used. The tip of pH meter probe was immersed into the tempeh slurry or milk kefir.
Viscosity was measured using a viscometer (Model BDV-8S, Biobase, China). Spindle No. 2 was used at speed set at 30 rpm. 120 mL of milk kefir was poured into a beaker and the spindle was lowered until it touches the detection level. Measurement of viscosity was performed in triplicates at 25 °C. The determination of titratable acidity was performed to quantify lactic acid percentage (Putri et.al. 2020). 5 mL of milk kefir was pipetted into the Erlenmeyer flask, followed by adding 3 drops of phenolphthalein solution. The mixture was titrated with 0.1 N

sodium hydroxide solution until a vibrant pink was observed. The lactic acid percentage was calculated using equation below.

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

2.3 Microbiological Plate Count

25 g sample was mixed with 225 mL of 0.1 % peptone water (Oxoid, UK) then homogenised using a stomacher for 30 seconds. Subsequently, a series of dilutions from 10^{-1} up to 10^{-8} were prepared. From each dilution, 1 mL was extracted and pipetted onto 3M petrifilm plates. Aerobic Plate Count was determined using 3M Petrifilm AC Plate following AOAC Method 990.12, while 3M Petrifilm Rapid Yeast and Mold Count (RYM) Plate was used for the yeast and mold analysis following AOAC Method 2014.05. The plated samples were then incubated at 35 °C for 48 h for AC plates while for RYM plates were incubated at 25 °C for 48 h. The cultivation of lactic acid bacteria (LAB) was performed using MRS agar (M641, HiMedia, India) supplemented with 1 % $CaCO_3$ and incubated in an anaerobic gas-jar at 30 °C for 48 h following GB 4789.35-2016. Number of colonies (CFU/g) on plates were manually counted.

2.4 Amplicon Sequencing for Microbiological Composition

Bacterial composition of optimised and commercial *tempeh* and milk kefir samples were identified by performing amplicon 16S sequencing. Microbial DNA of samples were isolated using DNeasy Powerfood Microbial Kit (Qiagen, Hilden, Germany) in accordance with Qiagen's handbook. Extracted DNA was proceeded for clean-up using AMPure XP beads (Beckman Coulter®, Brea, CA, USA) and amplified at 16S V3-V4 regions. Successful amplification was determined by presence of desired band in positive control and absence band in negative control. DNA amplicons was read and sequenced using Illumina Miseq System (Illumina, San Diego, California) at 2 x 250 bp configuration which generates approximately 100,000 raw reads per samples. 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, learning error rate, merging the contigs, defining taxonomy, collating number of sequences at different stages and creating phyloseq object. Bar plot featuring abundance percentage of dominant taxa was compared.

3. Results and Discussion

3.1 Optimisation using RSM

The optimisation results of *tempeh* at optimum texture attributes, colour and pH 7 yielded fermentation temperature at 33.1 °C, fermentation time of 34.1 h and humidity of 60.5 % RH (Relative Humidity). Table 1 shows that the overall composite desirability obtained was acceptable at 0.8756 with most responses exhibiting high desirability scores. High errors of more than 20 % occurred in springiness and chewiness due to products of two attributes while colours a* and b* were due to variations of pigmentation degradation during fermentation. The pH value in *tempeh* fermentation has exhibited high consistency with target value with negligible error of 0.14 % and a strong desirability of 0.9160. Optimisation of milk kefir targeted at pH 4.4, viscosity 1200 m.Pas and lactic acid percentage 0.8 % yielded fermentation temperature at 35.8 °C and fermentation time of 8.8 h. The high composite desirability of 0.9717 suggests favourable outcomes for all responses.

3.2 Microbiological plate count

Table 2 shows that aerobic plate count, lactic acid bacteria and mold increased progressively during fermentation process for a duration of 48 h for *tempeh* and 11 h for milk kefir. This increase reflected the effective proliferation of microorganisms during fermentation process. Based on the optimised fermentation duration of 34.1 h found for *tempeh*, the measured aerobic plate counts was 5.0 x 10^8 CFU/g and lactic acid bacteria was 6.1 x 10^7 CFU/g, both of which are higher than commercial *tempeh.* The commercial *tempeh* unexpectedly showed higher mold counts of 1.1 x 10^5 CFU/g which could have controlled the proliferation of microbes during *tempeh* fermentation. Optimised milk kefir surpassing 8 h's fermentation also showed higher aerobic plate count, lactic acid bacteria count and yeast than the commercial sample. While yeast was not found in *tempeh*, mold was not detected in milk kefir.

Table 1: Response optimisation and desirability of tempeh and milk kefir

Response	Goal	Target	Results		Error (%)	Desirability
			Predicted	Experimental		
Tempeh						
Hardness (g)	Maximum	-	1426.93	1296.57	9.14	0.9426
Springiness (mm)	Maximum	-	0.390	0.506	29.74	0.9475
Cohesiveness	Maximum	-	0.225	0.222	1.33	0.5429
Chewiness (g.mm)	Maximum	-	118.09	145.38	23.11	0.9517
Gumminess (g)	Maximum	-	316.79	287.38	9.28	0.8369
L*	Maximum	-	79.62	80.76	1.43	0.9648
a*	Minimum	-	1.80	1.33	26.11	0.9043
b*	Minimum	-	12.03	9.43	21.61	0.9799
pH	Target	7.0	6.95	6.96	0.14	0.9160
Milk kefir						
pH	Target	4.4	4.4985	4.51	0.25	0.9464
Viscosity (m.Pas)	Target	1200	1178.32	1296.6	9.12	0.9742
Lactic Acid Percentage (%)	Target	0.8	0.7972	0.792	0.66	0.9950

Table 2: Microbiological Plate Count for commercial and optimised tempeh and milk kefir

Tempeh	Commercial	Fermentation Time (h) for Optimised				
		2	16	32	48	
Aerobic Plate Counts	6.2×10^6	2.3×10^4	7.3×10^7	5.0×10^8	1.6×10^9	
Lactic Acid Bacteria	2.4×10^7	1.5×10^4	4.3×10^7	6.1×10^7	6.3×10^7	
Mold	1.1×10^5	NG(<10)	3.3×10^2	4.6×10^2	7.2×10^3	
Yeast	NG(<10)	NG(<10)	NG(<10)	NG(<10)	NG(<10)	
Milk Kefir	Commercial	Fermentation Time (h) for Optimised				
		0	2	5	8	11
Aerobic Plate Counts	2.92×10^5	1.6×10^4	1.7×10^5	7.7×10^5	5.4×10^6	5.5×10^6
Lactic Acid Bacteria	4.8×10^6	8.1×10^3	4.9×10^4	9.2×10^5	7.8×10^6	2.8×10^7
Yeast	2.7×10^4	10	5.9×10^3	2.4×10^4	4.6×10^4	6.0×10^6
Mold	NG(<10)	NG(<10)	NG(<10)	NG(<10)	NG(<10)	NG(<10)

3.3 Microbiological composition

Figure 1 shows the abundance percentage of bacterial composition in milk kefir and tempeh at genus level from bioinformatics analysis. Milk kefir displayed a smaller variability of microbial composition compared to tempeh. Milk kefir samples were dominated by Lactobacillus and Streptococcus quite equally. Lactobacillus (67.70 %) was found slightly more abundantly in optimised milk kefir while Streptococcus (56.06 %) in the commercial sample. Both the Lactobacillus and Streptococcus are generally recognised as probiotics with mounting clinical evidences that promote gut health, strengthening immunity (Ajibola et al., 2023) and controlling blood glucose level (Kumari et al. 2023). They are also lactic-acid producing bacteria which facilitates fermentation process by lowering down pH during fermentation process that aim to preserve and extend food shelf life (Ajibola et al., 2023).

Optimised tempeh was characterised by mainly the Bacillus (42.77 %) and Weissella (41.51 %) while the commercial tempeh presented higher variability in types of microbes besides of Bacillus (2.35 %) and Weissella (40.33 %), but also Streptococcus (11.72 %) Limosilactobacillus (10.86 %) and Acinetobacter (3.95 %). Bacillus species produce amylases and proteases that can break down complex carbohydrates and proteins (Li et al., 2023) and also synthesise extracellular polysaccharides and antimicrobial lipopeptides which inhibit spoilage and pathogenic microorganisms (Gopikrishna et al., 2021). Bacillus is recognised as probiotics and have demonstrated significant health benefits, including the support of digestion, improvement of nutrient absorption (Elshaghabee et al., 2017), gastric issues, cancer and hepatic disease (Gopikrishna et al., 2021). Weissella are lactic acid bacteria that contribute to distinctive flavours and aromas of fermented products and are increasingly recognised for their potential probiotic properties (Yulandi et al., 2020). Limosilactobacillus has been demonstrated to improve gut health by maintaining microbiota balance and producing antimicrobial compounds such as organic acids and caries prevention through its inhibitory activity in vitro against cariogenic bacteria (Félix-Sicairos et al., 2024). Some Acinetobacter strains have been explored for producing bioactive compounds, including antimicrobial peptides (Roy et al., 2022). Although Lactobacillus is often associated with mounting clinical evidences in supporting health benefits, Bacillus, spore-forming bacteria is said to have

advantages over the Lactobacillus species as it is heat stable and can be stored at room temperature without any deleterious effects on its viability and also being resistant to acidic gastric environment.

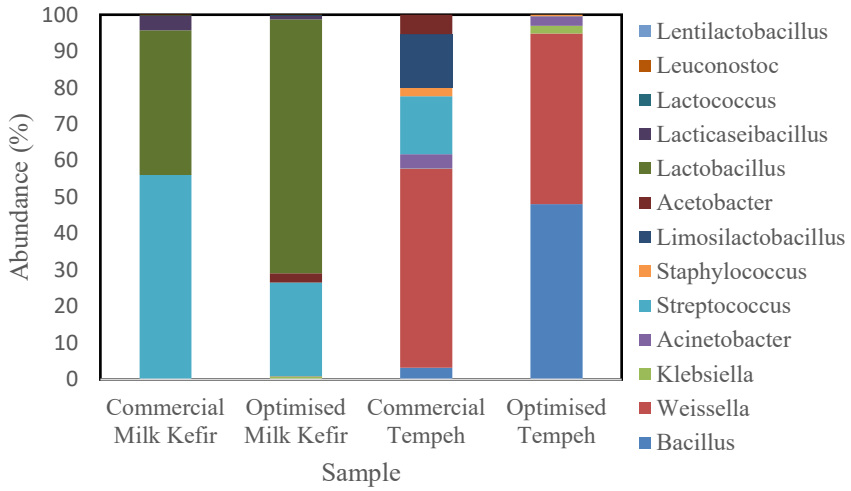

Figure 1: Abundance Percentage of Bacterial Composition of Milk Kefir and Tempeh

4. Conclusions

Optimising *tempeh* and milk kefir fermentation has helped bridge traditional fermentation practices to a more systematic and scientific approach of producing consistent and better quality of fermented products especially in enhanced lactic acid bacteria count which is associated with probiotics functions of maintaining good gut health and strengthening immune system. The optimisation process also seems to play a role in the composition of microbial variability and contents although the variations microbial community may be primarily influenced by sources of raw material.

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