

# Development of yogurt fortified with four varieties of common bean (*Phaseolus vulgaris*) whey by using response surface methodology: a preliminary study

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**Abstract** In recent years, there has been a growing interest in developing novel foods with improved health and nutritional characteristics, particularly through the supplementation and development of dairy products with plant-based ingredients. In this study, the response surface methodology (RSM) was employed to optimize the ingredient formulation and processing parameters of common bean whey-fortified yogurt (CBWFY) production containing *Lactobacillus bulgaricus*, and common bean whey (CBW) with a high probiotic count, superior physicochemical and textural properties, and desirable sensory attributes. The experiments were planned using the “box-Behnken design” (BBD) with three independent variables: fermentation time (0–10 h), common bean ratio (25–100%), and the amount of starter culture (1–5%). To assess the physicochemical properties of the yogurt, such as pH, titratable acidity, viable cell count, and syneresis of the CBWFY, they were determined and optimized. In all the common bean whey samples, the

optimum conditions were obtained by supplementing cow milk with 25% common bean whey (CBW), an inoculation ratio of 1–4%, and fermentation for 5.54 h. Fermentation time and CBW concentration significantly affected the viability of *L. bulgaricus* and the physicochemical attributes of yogurt. This study demonstrated that the addition of cow milk with 25% CBW from the white bean variety produced probiotic yogurt with the highest *L. bulgaricus* population (up to 8.55 log CFU/mL) compared to the other varieties and an enhancement in the yogurt’s pH and acidity, while a decrease in yogurt syneresis occurred. In general, the results of the current study showed that adding up to 25% white common bean whey to probiotic yogurt can be an option for producing yogurt with potential functional and sensory quality.

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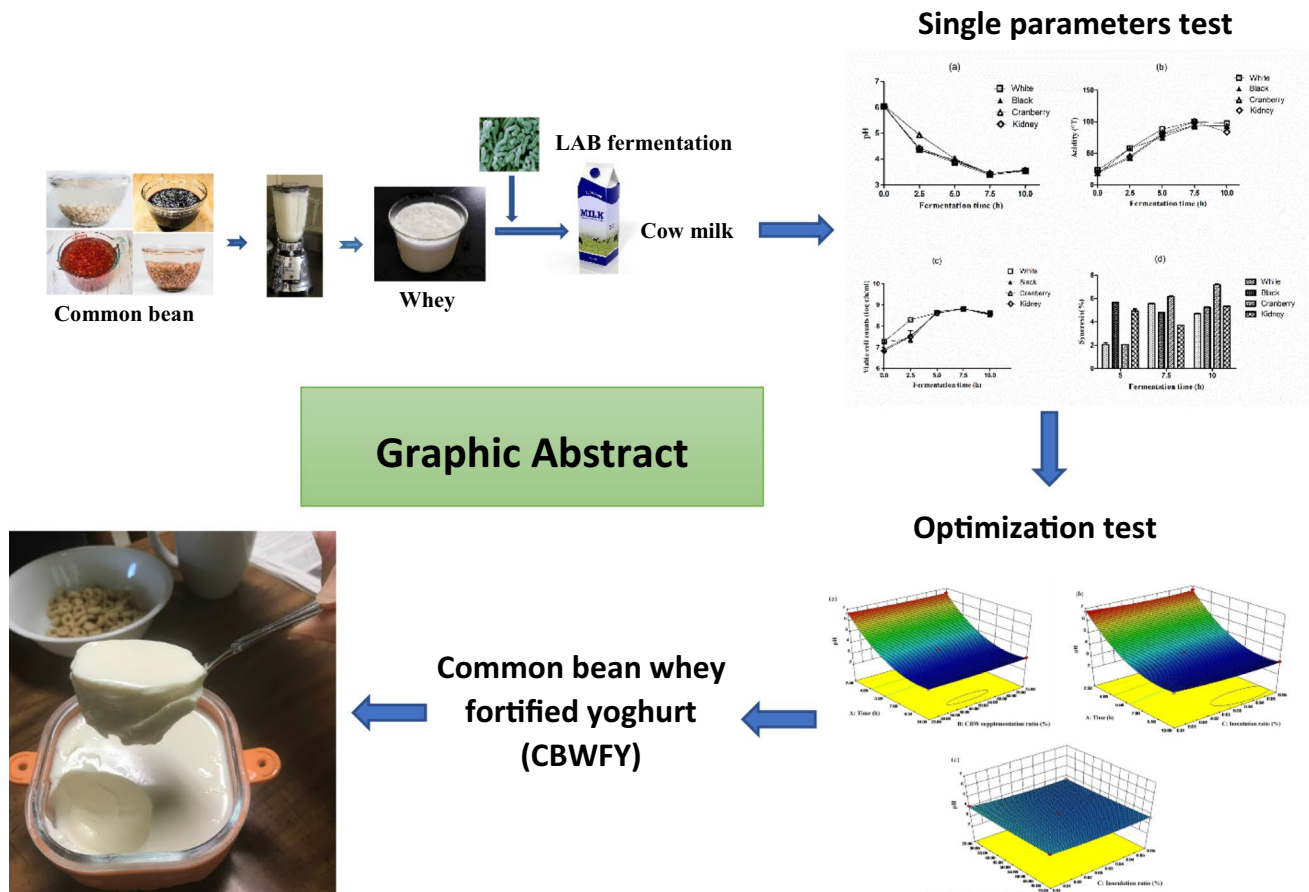
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## Graphical abstract



**Keywords** Yogurt · Common bean wheys · *L. bulgaricus* · Syneresis · RSM

### Abbreviations

3D	Three-dimensional
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
BBD	Box–Behnken design
BCA	Bicinchoninic acid
CBW	Common bean whey
CBWFY	Common bean whey fortified yogurt
CFU/mL	Colony forming unit/milliliter
CICC	The China Center of Industrial Culture Collection
CM	Cow milk
GRAS	Generally recognized as safe
LAB	Lactic acid bacteria
MBM	Mung bean milk
mg/mL	Milligrams per milliliter
MRS	De Man, Rogosa and Sharpe
PAPD	Priority academic program development
pH	Power of hydrogen

RSM	Response surface methodology
SBM	Soyabean milk
SHYs	Soyabean and hemp protein fermented yogurts
TA	Titrateable acidity
USA	United States of America
W/V	Weight/volume

### Introduction

Over the past few decades, there has been a growing interest in creating innovative foods with enhanced health and nutritional benefits. Concurrently, the global flavored milk market has been experiencing rapid expansion and is projected to reach a substantial value of USD 68.8 billion by 2024 (Bakr 2013; Mishra and Mishra 2013; Sawale et al. 2020). The growing interest in this subject has been significantly amplified by the supplementation and enhancement of dairy products with plant-based components, including

legumes, seeds, grains, nuts, vegetables, and fruits. This trend is driven by various factors, such as the pursuit of a healthier lifestyle, concerns for animal welfare, health issues such as lactose intolerance, and heightened environmental awareness (Aydar et al. 2021; Moyib et al. 2015). Nevertheless, the primary concern is food security, with an immediate need to address protein malnutrition (Pradeepkiran 2019). At the same time, food producers are increasingly interested in plant-based proteins. Hence, pulses could be exploited as sustainable, climate change-resistant, and high-quality protein sources (Bessada et al. 2019). Fortunately, owing to their low cost and high protein content (18.5–32%), pulses are considered (the meat of the poor) and are a good source of plant proteins for many of the world's population, particularly in developing countries (Zahir et al. 2020). Moreover, due to their nutritional, environmental, and economic benefits, these proteins have a promising future in the food industry (Emkani et al. 2022).

In yogurt production, significant attention is given to using plant-derived milk, such as those obtained from peas, peanuts, lentils, almonds, corn, and soy, as the primary ingredients. As a result, food manufacturing has been actively developing plant-based milk alternatives in recent years (Emkani et al. 2022). For instance, soy milk, as a nondairy substitute for cow's milk, includes high-quality protein, dietary fiber, and isoflavone. However, to produce a functional food, the incorporation of soy milk with lactic acid bacteria (LAB) is a suitable method that meets the demands of consumers (Atik et al. 2021). Yogurt, a nutrient-dense dairy product, is typically produced through the lactic acid fermentation of bovine milk or milk-derived ingredients. This process involves carefully controlled temperatures and environmental conditions, achieved by employing a symbiotic culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Yankey et al. 2023). Milk acid production and the synthesis of aromatic compounds are attributed to the function of these starter cultures. Using these starter cultures has enhanced our understanding of LAB's probiotic role and their 'generally recognized as safe' (GRAS) status in developing functional foods (Amakromo et al. 2012; Rui et al. 2017). Therefore, due to its sensory properties, it is broadly consumed as a nutritious and healthy food (Innocente et al. 2016; Li et al. 2019). Based on its structure and composition, yogurt is categorized as plain or flavored. Plain yogurt comprises dairy constituents without additives, while flavored yogurt contains additives (Chandan 2017).

Numerous studies have reported that supplementation of pulses into humans' daily diet may reduce the risks of chronic illnesses such as cardiovascular disease, certain forms of cancer, diabetes, constipation, obesity, hypertension, and osteoporosis (López-Barrios et al. 2016). Additionally, animal investigations revealed that the orderly feeding

of common beans reduces colon cancer incidence (Campos-Vega et al. 2009). For these reasons, several interests have been boosted in developing novel pulse-supplemented products. For example, a bean curd formed by many pulses of protein wheys, including mung beans, lentils, smooth peas, winged beans, and peas, was investigated (Cai et al. 2001; Campos-Vega et al. 2009; Kleintop et al. 2013).

The potential health benefits and tastes of novel products are associated with increasing demands and consumer acceptance. To increase traditional yogurt's nutritional quality and flavor, supplementing plant ingredients such as soya and pea protein, lentils, chickpeas, beans, soya flour, soy whey, and pea fiber may be beneficial (Azi et al. 2021). A previous investigation revealed that milk supplementation with pea ingredients, particularly pea fiber, has provided minor advantages for growing yogurt starter culture (Zare et al. 2012). Significantly higher viable cell counts and acid production were reported by adding 1–3% lentil flour. Compared to nonsupplemented control yogurt, 3% lentil flour supplementation minimally altered the physical and rheological properties. This suggests that the incorporation of plant ingredients supports viable cell growth and acid production. Moreover, the minimum whey separation (syneresis) was found with 3% lentil flour, whereas a significantly higher level was reported with 1–2% supplementation (Zare et al. 2011). This indicates that various ratios of pulse ingredients have different impacts on the final product's texture and viable cell count. Due to the LAB strain acidification rate improvement, a novel product has been developed individually with pulse whey, such as pea protein and fiber. Fortifying yogurt with chickpea water whey resulted in higher *Streptococcus thermophilus* counts compared to the control sample (Zahir et al. 2020).

To the best of our knowledge, there is limited information regarding the effect of yogurt supplementation with common bean whey (CBW) on the pH, acidity, growth of yogurt cultures, and syneresis. With its high protein and fiber content, adding CBW to yogurt may alter the physical and sensory properties of the final supplemented yogurt formula, thus necessitating further investigation. Response surface methodology (RSM) is a great application for detecting optimal conditions during food processing (CEYLAN and ÖZER 2020). Hence, RSM was used to optimize the process parameters and obtain ideas for the latter study and research to provide a theoretical basis. In this study, a filtered supernatant portion of common bean slurry as starch whey residue named common bean whey (CBW) was used for supplementation of cow milk to produce novel common bean whey-fortified yogurt (CBWFY), and the effect of yogurt supplementation with CBW on acid production, viable cell count, pH, and syneresis varied depending on fermentation time, supplementation ratio, and inoculation ratio by using response surface methodology (RSM).

## Materials and methods

### Raw materials

#### Common bean

Four varieties of common bean (*Phaseolus vulgaris*), namely, black, white, kidney, and cranberry, were purchased from a local supermarket in Nanjing, P. R. China, and stored at 4 °C until use.

#### Milk

The packed cow milk (CM) was obtained from a local supermarket in Nanjing, P. R. China, and stored at 4 °C until use.

#### Starter culture

The active starter culture for preparing yogurt, i.e., *Lactobacillus bulgaricus*, was obtained from the China Center of Industrial Culture Collection (CICC), Beijing, China.

### Preparation of common bean whey

The selected common bean seeds were rinsed and then soaked in distilled water for approximately 12 h at room temperature. The soaked beans were drained and dispersed in sixfold distilled water to make a slurry, which was homogenized using a homogenizer (BE601AB, Midea, China). To eliminate the insoluble part, a 200-mesh screen cloth was used to filter the slurry, which was then stored at 4 °C for approximately 12 h to allow any precipitated starch to settle. The precipitated starch was subsequently removed from the system. The supernatant, known as common bean whey (CBW), was carefully decanted and collected. To prepare CBW for inoculation, it was sterilized by boiling for 5 min. To make a CBW-CM mixture with CBW content ranging from 0 to 100%, a different portion of CM was added to the CBWs. Fermentation was commenced by inoculating *L. bulgaricus* at 1–5% ratios and carried out at 37 °C temperatures for various durations (i.e., 0, 2.5, 5, 7.5, and 10 h).

### Proximate composition

To determine the proximate analysis of samples (whey), the Association of Official Analytical Chemists—AOAC (2005) methods were used (Shimelis and Rakshit 2005). The BCA method with a BCA kit (P0010, Beyotime, P. R. China) was used to determine protein content using serum albumin as a standard. Lipid analysis was carried out following the alkaline hydrolysis method described by the Association of Official Analytical Chemists.

### Enumeration of *L. bulgaricus* in the yogurt samples

Viable cell counts were determined using the plate enumeration method. To prepare dilutions, 1 g of each sample was diluted and vortexed aseptically with 9 mL of sterile physiological saline (0.85%, w/v). *L. bulgaricus* was counted on MRS agar (pH 6.2 ± 0.2, Oxoid-CM0361, Unipath, Basingstoke, UK). Confirmed colonies were counted under anaerobic incubation at 37 °C for 48 h. Cell counts were expressed as log CFU/mL.

### Measurement of pH and titratable acidity

A pH meter was used to measure the pH values (IS 128, Yimai Technology Co, Shanghai, P. R. China). A titration method was used to determine the acidity of the samples. Accurately, 10 g of sample was carefully weighed and mixed with 20 mL of distilled water. The samples were titrated with 0.1 M sodium hydroxide with phenolphthalein as an indicator. Acidity (°T) was calculated using the following equation:

$$\text{Acidity (°T)} = \frac{C \times V \times 100}{M \times 0.1}$$

where C is the concentration of sodium hydroxide, V is the volume of sodium hydroxide consumed, and M is the weight of the sample.

### Syneresis measurements

To determine the spontaneous amount of whey separation from yogurt, syneresis of samples were performed according to the method described by (Lucey et al. 1998). Briefly, 40 g of each sample was weighed in a petri dish and then sealed with Glad® wrap (polyethylene film) to prevent evaporation during fermentation. After fermentation, surface whey was collected and weighed consciously. Five replicates were done for each sample. Syneresis was expressed as the percentage of whey separated from the gel over initial weight of the gel.

### Experimental design

#### The analysis

Response surface methodology (RSM) was employed to optimize CM-supplemented yogurt production, considering the influence of multiple factors and their interactions on viable LAB cell counts. The three factors were selected as fermentation time (A), CBW-CM ratio (B), and inoculum ratio (C) at three different levels: low (coded as -1), medium (0), and high (+1). Preliminary experiments were conducted to select these three levels, which indicated that

a fermentation time of 0–10 h, common bean whey concentrations of 25–100%, and inoculation ratio of 1–5% yield acceptable novel yogurt products. To minimize the impact of unaccounted factors on the observed response, the experiments were randomized. The study encompassed a total of 17 experiments, with three factors, each evaluated at four different levels.

**Optimization of CBW yogurt formulation**

To determine the optimal conditions for CM supplementation with CBW probiotic yogurt production, the study calculated the maximum value for the response (*L. bulgaricus* count) and range levels for the independent variables (fermentation time, common bean whey ratio, and inoculation ratio). In contrast, the variables of syneresis were considered at the minimum level, and the variable acidity was considered a target level.

**Optimization and verification procedure**

Box–Behnken was used to statistically optimize the processing conditions (including three variables) to obtain the CBW and inoculation ratio with a short fermentation time. However, in the range of experiments, by fixing and changing the two variables and center point, three-dimensional (3D) surface plots were produced to describe the interaction

influence of the independent variables on dependent variables. Furthermore, a comparative analysis was conducted between the experimental data obtained from various verification experiments and the expected values derived from the response regression, aiming to assess the adequacy of the response surface equation.

*Statistical analysis*

Design Expert software, V7.0.0 (StaEase Corp., Minneapolis, MN, USA), was used to handle the modeling, statistical analyses, and “box-Behnken design” (BBD) matrix (Table 1). In this study, RSM was employed for data analysis using the commercial statistical package, Design-Expert version 7.0.0 (Statease Inc., Minneapolis, USA). All experiments were carried out in triplicate. Multiple regression analysis was performed to fit the best model for each response. The significance of the regression equation models was determined using the correlation coefficient and the lack-of-fit test. The analysis of variance (ANOVA), regression coefficients of individual linear or quadratic models, and the optimization of the polynomials were performed at  $p < 0.01$  and  $p < 0.05$ . The full quadratic equation for each yogurt variable was derived using the RSM and is presented as follows:

$$Y = b_0 + b_1A + b_2B + b_{11}C + b_{22}X_{22} + b_{12}ABC$$

**Table 1** Box–Behnken design for the coded and actual values of independent variables

Independent variables				Dependent variables (response obtained from the experiment)			
Run	Time (hr)	CBW (%)	Inoculation ratio (%)	pH	Acidity (°T)	LAB count (log CFU/mL)	Syneresis (%)
1	2.50	25	3	6.66	33	8.13	0.85
2	2.50	50	1	6.75	18	7.66	1.90
3	10.00	25	3	3.10	97	10.98	0.72
4	6.25	50	3	3.63	90	9.16	11.83
5	6.25	25	5	3.66	112	9.30	0.27
6	6.25	75	5	3.64	70	9.18	22.20
7	2.50	75	3	6.58	32	8.98	23.80
8	6.25	50	3	3.63	90	9.16	11.83
9	6.25	25	1	4.11	79	9.05	0.41
10	10.00	50	5	3.12	122	11.04	9.87
11	6.25	50	3	3.63	90	9.16	11.83
12	6.25	75	1	3.90	59	9.31	16.36
13	2.50	50	5	6.68	39.67	8.87	14.50
14	6.25	50	3	3.63	90	9.16	11.83
15	10.00	50	1	3.29	98	10.93	14.13
16	6.25	50	3	3.63	90	9.16	11.83
17	10.00	75	3	3.18	96.33	11.01	28.62



**Table 2** Proximate composition of CM and four varieties of CBW

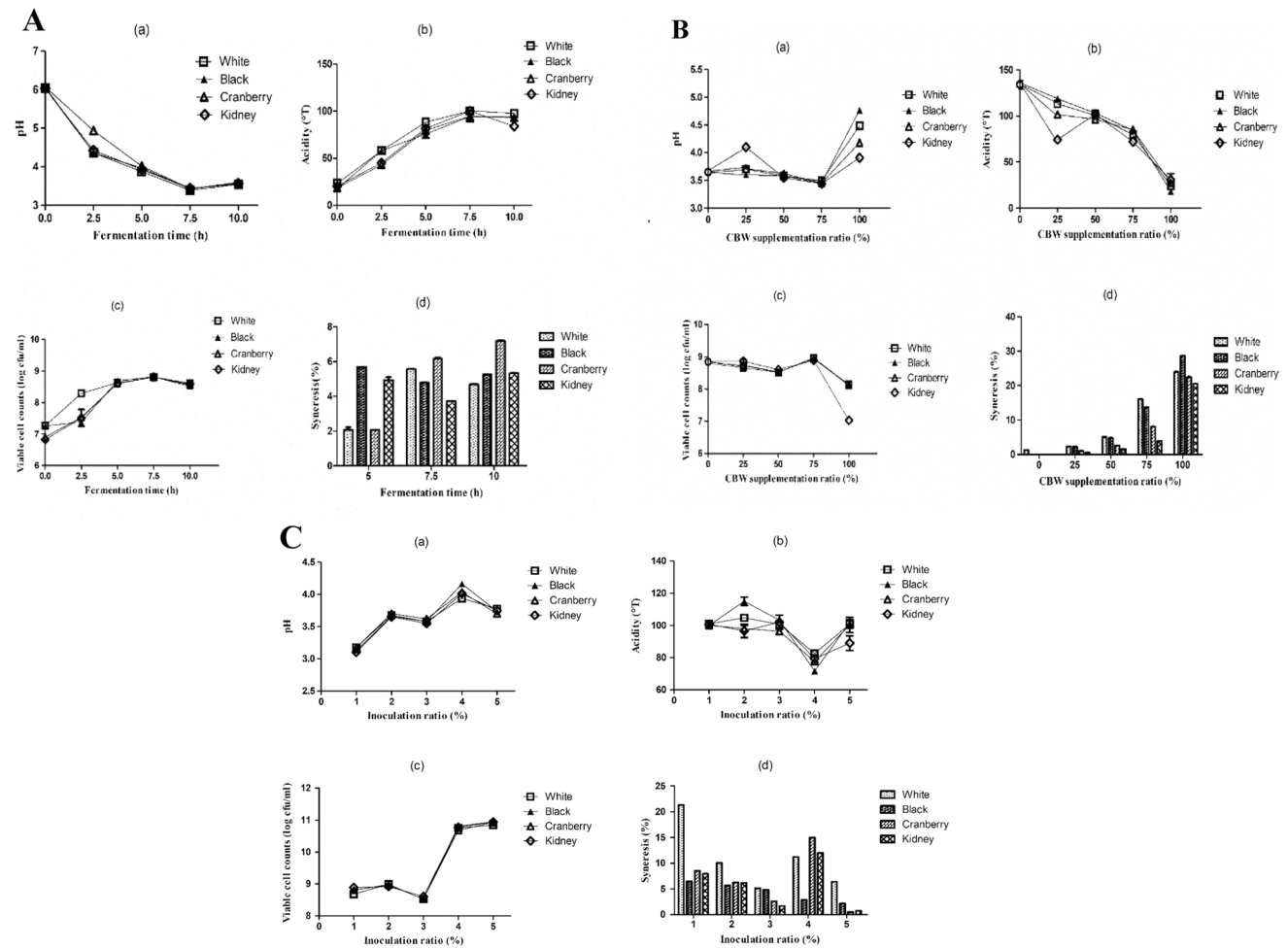
Proximate components	Cow	White	Black	Kidney	Cranberry
Protein (%)	3.20	2.66	2.65	2.62	2.65
Lipid content (%)	3.50	0.26	0.27	0.29	0.28
Ash content (%)	0.77	0.66	0.96	0.92	0.87
Moisture (%)	86.78	91.86	92.20	90.75	92.31

where Y is the response (pH, acidity rate, LAB count, and syneresis),  $b_0$  is a constant,  $b_1$  and  $b_2$  are the linear coefficients,  $b_{11}$  and  $b_{22}$  are the quadratic coefficients, and  $b_{12}$  is the interaction coefficient. A, B, and C represent fermentation time, CBW concentration, and inoculation

concentration, respectively. The initial single-parameter test data were analyzed using ANOVA (SAS 9.1, SAS Institute INC. NC, USA). Multiple comparison tests were carried out to determine the significant differences between means ( $p < 0.05$ ) using IBM SPSS Statistics.

## Results and discussion

In the present study, we investigated the effect of CM supplementation with CBW on pH, acidity, LAB counts, and syneresis by performing single-parameter tests and using RSM.



**Fig. 1** A Single parameter test showing the effect of different fermentation times on pH, acidity, log CFU/mL, and syneresis; white (□), black (▲) cranberry(Δ), kidney (◇) (a) pH vs fermentation time; b) acidity vs fermentation time; c) log CFU/mL vs fermentation time, and d) syneresis vs fermentation time. B Single parameter test showing the effect of different % of CBW supplementation on pH, acidity, log CFU/mL, and syneresis; white (□), black (▲),

cranberry(Δ), kidney (◇) a) pH vs CBW ratio; b) Acidity vs CBW ratio; c) log CFU/mL vs CBW, and d) Syneresis vs CBW ratio. (C) Single parameters test showing the effect of different inoculation ratios on pH, acidity, log CFU/mL, and syneresis; white (□), black (▲), cranberry(Δ), kidney (◇) a) pH vs inoculation ratio; b) acidity vs inoculation ratio; c) log CFU/mL vs inoculation ratio, and d) syneresis vs inoculation ratio

## Proximate composition

The protein content of the four varieties of CBW ranged from 2.62 to 2.66%, the lipid content from 0.27 to 0.32%, the ash content from 0.66 to 0.96%, and the moisture content from 90.75 to 92.31%. The protein content of CBW differed significantly from that of cow milk ( $p < 0.05$ ), whereas the lipid content was lower. This suggests that CBW may be a suitable supplementary pulse for developing novel foods. The moisture content of CBW was significantly ( $p < 0.05$ ) higher than that of CM, as previously reported by (Elechi et al. 2023). No significant difference was observed in ash content among all varieties of CBW when compared to CM, except for the low value obtained with white bean whey (0.66 mg/mL) (Table 2).

## Single parameter tests

*The effect of fermentation time on pH, acidity, bacterial count, and syneresis.*

To evaluate the effect of fermentation time on the pH, acidity, viable cell counts, and syneresis of the yogurt, different fermentation times (0–10 h) were applied to CM supplemented with 50% of different CBWs (Fig. 1A). Fermentation of CM-CBW with *L. bulgaricus* demonstrated a progressive decline in pH value for all investigated CBWs (Fig. 1Aa). The initial pH value was ranged from 6.04 to 6.07, then dropped to 3.40–3.43 at 7.5 h of fermentation time and subsequently increased slightly to 3.55–3.57 at 10 h. This rapid decrease ( $\Delta\text{pH} = 2.64$ ) is characteristic of the LAB strain. A similar observation was reported by (Huang et al. 2022). Gan et al. (2017) found that after approximately 6 h of fermentation as the fermentation time increased, the pH values of fermented mung bean milk (MBM) and soya bean milk (SBM) steadily decreased, which was more pronounced during the first 9 h of fermentation and then stabilized. This decline mainly results from the accumulation of organic acids such as lactic acid produced by LAB (Gan et al. 2017). However, the pH of soybean and hemp protein-fermented yogurts (SHYs) did not significantly differ during the first 2 h of fermentation but notably decreased from 2 to 6 h. The authors also noted that during fermentation, this reduction is primarily due to the microorganism's utilizing carbohydrates as a carbon source to produce acids that lower the pH of the yogurt, such as lactic acid and short-chain fatty acids (Xu et al. 2022).

Based on the pH value results, no significant differences were observed among the different varieties, except for a higher pH obtained in CM fortified with cranberry bean whey at 2.5 h of fermentation. As previously investigated by other studies, the pH value is mainly influenced by LAB growth performance and the accumulation of acids, which

are also linked to the metabolic activity and growth requirements of LAB (Xiao et al. 2015). Our result aligns with those of previous studies, which reported that the pH reduction from 6 to 4.6 after 6 h of fermentation was attributed to the increased population of lactic acid bacteria (LAB) and the subsequent breakdown of lactose into lactic acid (Yankey et al. 2023). During fermentation, a decrease in pH value has been shown to increase the formation of nonvolatile acids, such as pyruvic and lactic acids, which can impact the organoleptic properties of the final product.

The acidity of yogurt prepared from all CBWs increased steadily from 17.67–23.33 to 100–100.33 °T as the fermentation time extended from 0 to 7.5 h, followed by a slight decrease was observed when fermentation was prolonged to 10 h (Fig. 1A. b). This trend was consistent with the pH changes during fermentation. A similar observation was reported by (Mir et al. 2021); who noted the highest acidity value in yogurt with an incubation time of 6.5 h. The rapid growth of LAB led to the production of organic acids. However, the final pH for each yogurt remained approximately at pH 3.57. This could be attributed to CBW's higher carbohydrate content, especially lactose, which is converted into glucose and galactose during the fermentation process by lactic acid cultures. Glucose is subsequently transformed into lactic acid, giving CBW yogurt a robust buffer capacity that requires more acid to lower the pH. This aligns with previously published results in kefir-fermented beverages (Paredes et al. 2022). The decreasing pH and increasing acidity observed in the yogurt may be attributed to the specific active ingredients derived from CBW that play a prebiotic role and promote the growth of LAB.

For LAB, the rate of pH decline indicates their growth performance. Consequently, the viable cell number was also monitored during fermentation. In this test, CM-CBW yogurt was inoculated with *L. bulgaricus* at a cell density of log 6.82–7.26 CFU/mL. Microbial growth reached log 8.81–8.82 CFU/mL when the fermentation time was extended from 0 to 7.5 h. A slight reduction was observed when fermentation was prolonged to 10 h (Fig. 1A.c). This indicates that accompanied by the decline in pH value, the viable cell number of *L. bulgaricus* gradually increased at 7.5 h of fermentation and then slightly decreased at the later stage (10 h) of fermentation (Fig. 1A.c). A similar finding was reported by Xiao et al. (2015), who found that in soy whey, the highest viable cell counts were obtained at 10 h of fermentation (Xiao et al. 2015). Tu et al. (2019) also reported that soy whey has enough nutritional composition (e.g., glucose and fructose) to support LAB growth and metabolism (Tu et al. 2019). The reduction in microbial growth might be due to reaching the microbial population at the stationary phase in the CBW. A previous study also demonstrated that lactose depletion, oligosaccharide richness, and limited sulfur-containing amino acids might contribute

to the growth retardation of LAB strains in yogurt fortified with pulse ingredients (Rui et al. 2015). It is generally required that yogurt or fermented milk possesses at least  $10^6$  log CFU/mL, representing approximately one million viable cells per gram at the time of consumption. To maintain these numbers, it is important to monitor viability during the fermentation time (Huang et al. 2022).

Syneresis of the yogurt samples was measured as “whey-off,” which separates the liquid phase from the gel, an undesirable phenomenon in yogurt. It is preparing a symptom of nonhomogeneities in the gel system of the yogurt; hence, higher whey separation is relevant to gel instability which arises in conditions such as the high temperature of incubation, extreme heating pretreatment of milk, disturbances while the gel is still weak, production of lactic acid in low amounts and low total solids content with the temperature of incubation which is also related to the pH of the yogurt system. Measuring it becomes essential because whey separation in large amounts is considered a quality defect in set-type yogurts (Mir et al. 2021; Zare et al. 2012). The syneresis of CM-CBW yogurt is affected by fermentation time as well as bean variety (Fig. 1A. d). Yogurts supplemented with white and cranberry bean whey demonstrated the least syneresis at 5 h of fermentation, whereas black and kidney bean whey showed the least syneresis at 7.5 h. In terms of acidification, no significant differences were observed among different CBWs when the fermentation time was prolonged to 7.5 h. In contrast, kidney bean whey showed a slightly lower value than the other varieties at 10 h. Syneresis was positively related to fermentation time from 7.5 to 10 h, indicating that longer fermentation times induced nonhomogeneities in the gel. It appears that compared to white and cranberry bean wheys, black and kidney bean wheys delayed the formation of the gel, resulting in higher whey separation. In comparison to CM yogurt, all CM-CBW yogurt exhibited high syneresis amounts, except CM supplemented with white and cranberry, which showed approximately levels of syneresis at 5 h of fermentation time. A fermentation time of 7.5 h was suggested to maintain the appropriate pH, acidity, viable cell counts, and syneresis of CM-CBW yogurt; thus, it was selected for the following analysis.

#### *The effect of the CBW supplementation ratio on pH, acidity, bacterial count, and syneresis.*

To evaluate the effect of the CBW supplementation ratio on the yogurt fermentation process, various CBW percentages (25–100%) were added to CM, and the fermentation was terminated at 7.5 h. The results of pH, acidity, viable cell counts, and syneresis are presented in Fig. 1B. All investigated CM-CBW yogurts showed no significant pH differences when the supplementation ranged from 25

to 75%. However, a higher pH was observed when kidney bean whey was applied at 25% supplementation (Fig. 1B. a). Conversely, 100% of additional CBW supplementation resulted in a pronounced pH increase, which was significantly ( $p < 0.05$ ) higher and ranged from 3.90 to 4.77. This observation aligns with the acidity and viable cell count results (Fig. 1B. b, c). This effect may be due to the higher content of CBW and the consequent reduction in lactose availability as a crucial carbon source for LAB growth. As a result, fewer viable cells were able to convert lactose into lactic acid, leading to the retention of high acidity. A similar finding was reported by Huang et al. (2022). However, it contrasts with studies that found an increase in guava juice content resulted in a lower pH in soy-based desserts (Granato et al. 2010). Sulmiyati et al. (2019) also reported that the highest pH value was associated with goat-milk kefir produced by adding 2% kefir grain, while the lowest pH was found in goat-milk kefir produced with a 6% kefir grain supplementation (Sulmiyati et al. 2019).

The acidity trends in different varieties of CBW-fermented yogurt are illustrated in (Fig. 1B. b). Among all varieties of common bean, there was no significant changes in acid production except a significantly ( $p < 0.05$ ) lower acidity obtained for CM fortified with 25% kidney bean whey, as indicated by the pH results. A more pronounced reduction in acidity was observed ( $p < 0.05$ ) in the 50%, 75%, and 100% CBW-supplemented samples. The lower acidity levels suggest that a high level of CBW might not be an ideal substrate for *L. bulgaricus*. A similar result was reported by Huang et al. (2022), who found that quinoa supplementation led to low acidity levels (Huang et al. 2022). This difference was attributed to the chemical composition of the substrate. Higher CBW content is associated with higher carbohydrate content but lower protein, fat, and ash content. In addition, the starch in CBW may absorb water, and the LAB may lack water for their physiological activities, resulting lower LAB count.

Viable LAB cell counts showed minimal variation as the CBW supplementation ratio increased from 0 to 75%. However, a 100% CBW supplementation ratio led to a significant reduction in viable LAB cell counts for all tested yogurts (Fig. 1B. c). The decline in viable LAB cell counts may be attributed to the depletion of lactose in the culture, which serves as a vital carbon source for LAB growth. It may have also contributed to the higher pH and lower acidity observed in these samples, as the slow growth of lactic acid bacteria resulted in reduced lactic acid production. Previous studies have also shown that cow milk possesses a greater buffering capacity compared to pulse ingredients (Grasso et al. 2020).

Additionally, the microbial growth sustained during the addition of different percentages of CBW and the number of viable microorganisms is an important factor in the final product regarding acidification and nutritional health



benefits attributed to yogurt starters as probiotics. In comparison to the CM, viable counts of *L. bulgaricus* in CBW-supplemented yogurt varied from log 8.94 to 8.97 CFU/mL after the addition of 75% CBW, and reductions occurred from log 8.14 to 7.03 CFU/mL in all samples of CBW with 100% addition. These data showed that the nutrients carried by the CBW affect the growth of the yogurt cultures differently than those carried by CM. Remarkably, adding 50% CBW enhanced the acidification rate, increasing the log CFU/mL values simultaneously, but exhibiting a reduction rate of 100% addition in the fermented products compared to CM. Similar to previous reports, the starch in quinoa absorbs much water, and the LAB lack water for life activities, so the number of LAB is low (Pachekrepapal et al. 2021); however, it is in contrast with studies reporting that *L. bulgaricus* counts of the product increased with sea buckthorn fruit syrup (Selvamuthukumaran and Khanum 2015), and Silva et al. (2018), stated that samples with higher soymilk kefir percentages exhibited higher *lactobacilli* cell counts (SILVA et al. 2018).

Syneresis of yogurt supplemented with 25, 50, 75, and 100% of different CBWs is shown in (Fig. 1B. d). The lowest syneresis was occurred at 25% CBW supplementation regardless of the bean variety. Yogurt supplemented with 25% kidney and cranberry bean whey showed values of 0.55 and 1.03%, respectively, which were even lower than the syneresis of CM yogurt, indicating maximum homogeneity. This contrasts with Xu et al. (2022), who reported that the addition of a small amount of hemp protein (5%) to yogurt increased syneresis, resulting in more whey precipitation (Xu et al. 2022). A significantly ( $p < 0.05$ ) higher value was observed for the 50% CBW-supplemented samples, whereas the 100% common bean-supplemented samples exhibited the highest degree of whey separation. In terms of whey separation, supplementation with 50% or more with CBW significantly ( $p < 0.05$ ) promoted gel instability, leading to increased whey separation. The elevated syneresis was mainly attributed to the fragile three-dimensional gel network structure of the fermented yogurt. The substantial presence of CBW likely contributed to a looser three-dimensional network and a weaker gel system. This unstable gel system had a reduced ability to retain water molecules, and the gel structure was more prone to contraction, leading to whey precipitation (Pérez et al. 2021).

Different bean varieties had varying effects on syneresis when the supplementation exceeded 50%. It appears that lower syneresis levels were observed in cranberry and kidney bean varieties, especially at 50% and 75% supplementation ratios, in comparison to white and black bean supplementation, indicating the formation of a stronger gel by the former two varieties. This resulted in the lowest syneresis, attributed to the 25% CBW-supplemented yogurt. A similar finding was reported by Nehaa et al. (2022), who found that

a formulation containing 7.13 mL of peanut milk, 10 mL of oat milk, and 7.86 mL of coconut milk showed a low syneresis rate (Nehaa et al. 2022). Youssef et al. (2016) reported that increased pea concentration leads to products with higher syneresis (Youssef et al. 2016). Overall, the addition of a certain amount of CBW to plant-based yogurt can reduce syneresis or whey separation, effectively addressing one of the most common defects in fermented dairy products.

#### *The effect of inoculation ratio on pH, acidity, bacterial count, and syneresis*

To evaluate the effect of the inoculation ratio on the fermentation process, varied inoculation ratios (1–5%) were applied to CM supplemented with 50% of different CBWs, and the fermentation was terminated at 7.5 h. The results of pH, acidity, viable cell counts, and syneresis are shown in (Fig. 1C).

The results showed that increasing the inoculation ratio from 1 to 4% led to an increase in yogurt pH values ranging from 3.10–3.18 to 3.94–4.16, whereas an inoculation ratio of 5% resulted a decrease in the yogurt pH (Fig. 1C. a). The effect of inoculation concentration on the acidity of CBWs is shown in (Fig. 1C. b). Among different bean varieties, the acidity trend showed significant differences ( $p < 0.05$ ) when comparing inoculation ratios from 1 to 5%. Inoculating CBW by 1–3% showed no significant changes, except high acidity observed in CM supplemented with black (100–115 °T) and white bean whey (101–104.66 °T) at a 2% inoculation ratio. In contrast, a more pronounced reduction (82.67–71.66 °T) was observed at 4% inoculation and subsequently increased at a 5% inoculum concentration. Based on the inoculation ratio result, a high pH value and a low acidity rate were observed at a 4% inoculation ratio. Conversely, high microbial growth was achieved with 5% inoculation due to the rapid expansion of the microbial population, resulting in more acid production by the conversion of lactose into lactic acid. Thus, inoculum concentration strongly influenced microbial growth but did not necessarily correspond to acidity as the highest values of log 10.85–10.95 CFU/mL were obtained. This suggests that a 3% inoculation concentration with a fermentation time of 7.5 h was the optimal inoculation ratio for the growth of *L. bulgaricus* in CBW. However, this did not align with the acidity results, as 2% inoculum concentration provided the highest acidity. A similar finding was reported by (Shu et al. 2014).

The increases in viable cell counts for all varieties of CBW were significantly different ( $p < 0.05$ ) at the inoculation ratios of 1–5% (Fig. 1C. c). Inoculation of CBW at 1–3% showed no significant differences ( $p < 0.05$ ) in microbial growth except for slight changes at 2 and 3% inoculation ratios, while substantial microbial growth was observed at a

4% inoculation concentration. The microbial counts reached their highest values at a 5% inoculation ratio. Changes in syneresis were observed in all CM-CBW yogurts with increasing inoculum concentrations (Fig. 1C. d). Syneresis decreased for CM-CBW yogurt inoculated with *L. bulgaricus* at 1%, 2%, and 3% inoculation ratios. However, a significantly higher syneresis value was obtained for white bean whey fortification at a 1% inoculation ratio. Conversely, significantly higher syneresis was observed for the samples inoculated with 4%, indicating that at a 1% inoculation ratio, initial whey separation occurred due to low microbial activity, resulting in higher whey separation. Additionally, a 5% inoculation ratio exhibited maximum homogeneity (low whey separation) in comparison to 1%, 2%, and 4%, suggesting that at 5%, there was more microbial growth. When considering the effect of the inoculum ratio on syneresis, inoculation with 4% resulted in higher syneresis, whereas a 5% inoculum achieved maximum homogeneity (low whey separation). This contradicts the finding that increased syneresis was observed with an increasing inoculum concentration, as reported by (Bansal et al. 2015). This phenomenon could arise from both the initial removal of whey

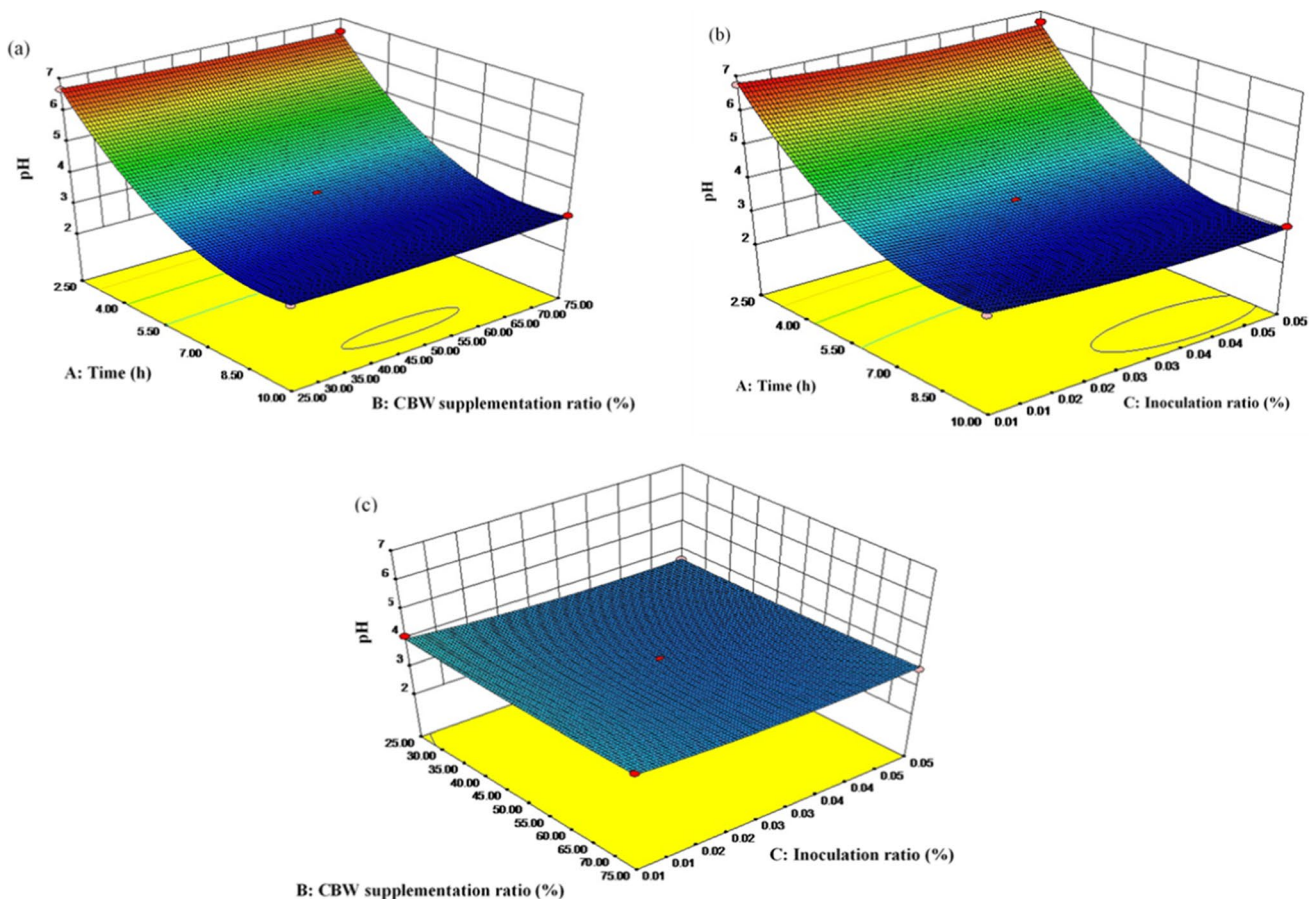
and the presence of starch, which can be further linked to microbial activities. Consistent with earlier research conducted by Zare et al. (2013), intensified acidification leads to increased separation of initial water from the gel matrix (Zare et al. 2013).

### Optimization of fermentation time, inoculation ratio, and common bean whey ratio by RSM.

Response surface methodology (RSM) can provide valuable data for analyzing the responses of multiple factors and their interactions. It has been employed in numerous studies to optimize fermentation conditions. Since the test results for single parameters yielded similar outcomes across all common bean varieties, including white, black, kidney, and cranberry, we have selected white beans for further discussion as follows.

#### RSM model for pH

The purpose of pH determination is to assess the quality of yogurt which pH and acidity are being most critical factors.



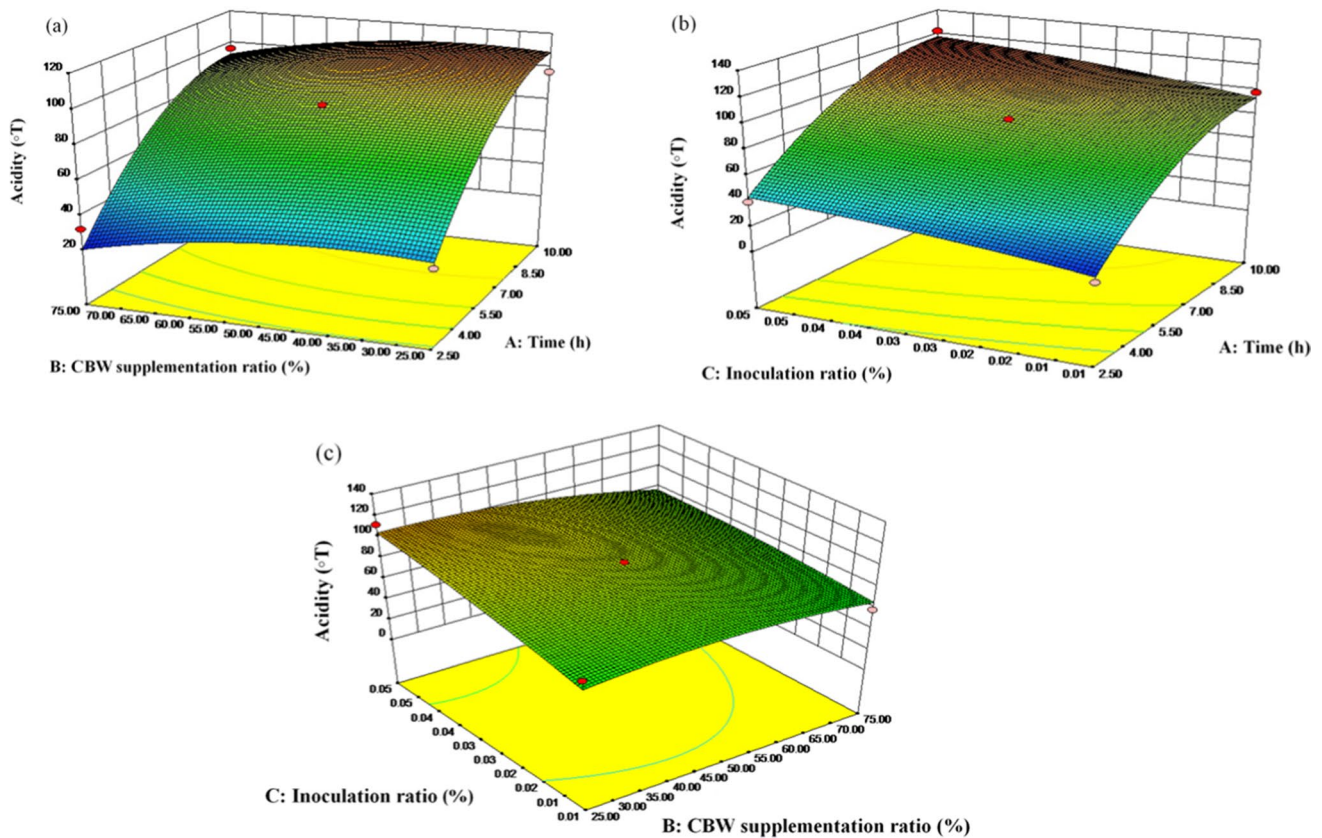
**Fig. 2** Response surface plot, showing the pH as affected by fermentation time, CBW ratio, and inoculum concentration

The quadratic model for pH was statistically significant ( $p < 0.0001$ ), signifying the model’s significance. The  $R^2$  coefficient,  $R^2$ Adj coefficient, and the F and P values of the lack of fit confirm that this model can effectively stimulate CBW fermentation progress. (Refer to supplementary tables 1, 5, 9, and 13). Figure 2 illustrates the pH results influenced by fermentation time, inoculation ratio, and CBW ratio.

Among the three factors, the pH of common bean whey was significantly ( $p < 0.05$ ) affected by fermentation time (A) ( $p < 0.0001$ ), followed by inoculation ratio (C) and CBW ratio (B). However, the interactions between the CBW ratio and fermentation time (AB), inoculation ratio and fermentation time (AC), and CBW ratio (BC) and inoculation ratio, were not significant ( $p > 0.05$ ) (Fig. 2). The highest pH value (6.75) was observed in yogurt supplemented with CBW at a 2.5 h fermentation time, 50% CBW, and 1% inoculation ratio, implying that an increase in CBW results in higher pH. This indicates that during the fermentation process, an increase in the CBW ratio, a shorter fermentation time, and a lower inoculation ratio did not allow LAB to fully convert lactose into lactic acid (partially hydrolyzed CBW in the yogurt), leading to an elevated pH value. Mir et al. (2021)

reported a significant increase in pH (5.15) yogurt noted with increasing gum concentration (Mir et al. 2021).

The lowest pH value (3.10) was observed in the sample containing 25% CBW, a 3% inoculation ratio after 10 h of fermentation time. This can be attributed to the improved growth and survival of probiotic bacteria, which converted more lactose into lactic acid. This finding is consistent with the result obtained from the addition of 75% almond milk (Aydar et al. 2021). At the linear level, fermentation time had a significant but negative effect on the pH of the final product and a positive and significant effect at the quadratic level. This indicates that an increase in fermentation time at the linear level leads to a reduction in pH. Increasing fermentation time and inoculum ratio resulted in a reduction in the pH value. Bansal et al. (2015) reported that high inoculum concentration significantly affected pH reduction (Bansal et al. 2015). Adding 25% CBW and 10 h of fermentation period decreased the pH (Fig. 2a–c). This demonstrates the significant influence of the inoculum ratio, CBW, and fermentation time on pH reduction during fermentation. For example, a fermentation time of 10 h consistently produced lower pH values than a fermentation time of 2.5 h. This was anticipated because extensive fermentation processes result in a prolonged acidification process, converting more lactose



**Fig. 3** Response surface plot, showing the acidity as affected by fermentation time, CBW ratio, and inoculum concentration

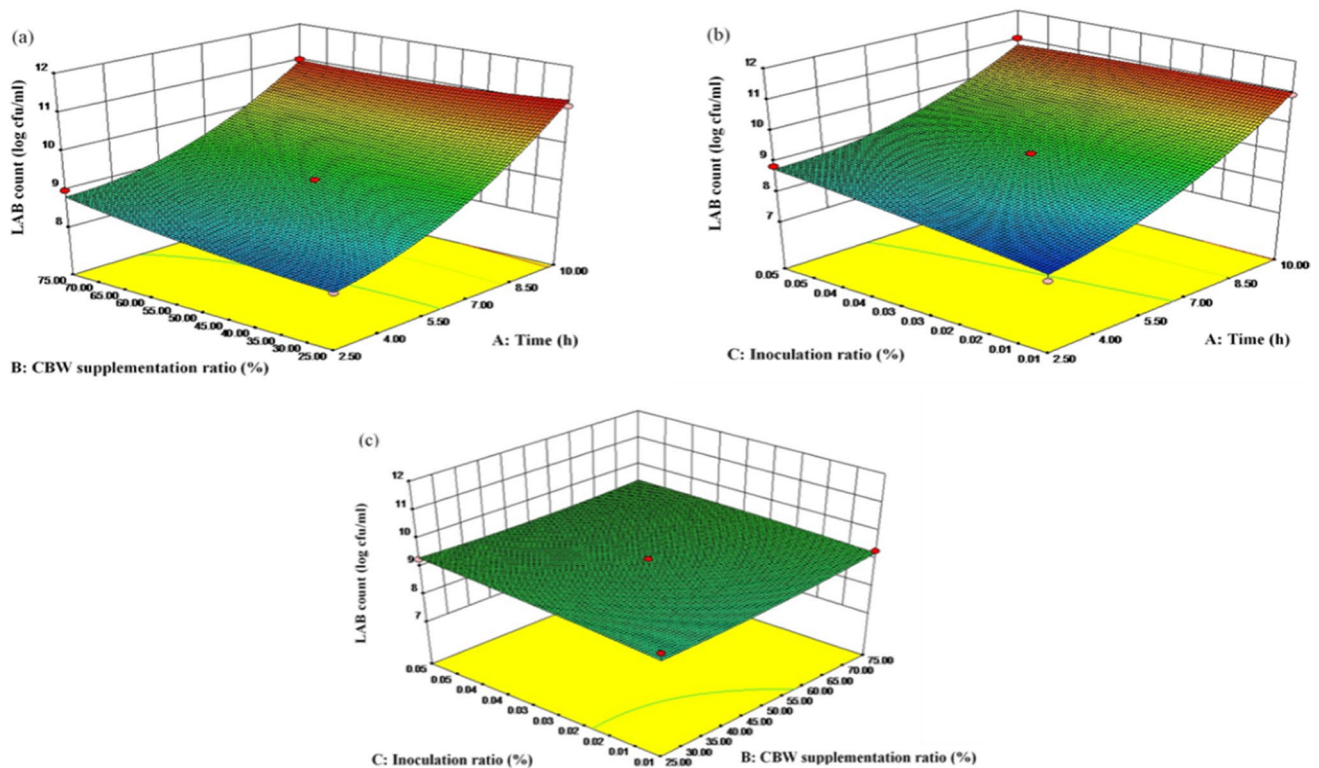


compounds into lactic acid and other organic acids (acetic acid, citric acid, formic acid, butyric acid). The accumulation of these acids results in a lower pH, as indicated by the regression coefficients (Yankey et al. 2023).

#### RSM model for acidity

During fermentation, the equivalent percentage (%) of lactic acid present in the yogurt was measured by titratable acidity (TA). Lactose is the main carbohydrate converted into glucose and galactose during the fermentation process by lactic acid cultures. Glucose is further reduced to lactic acid at the end of the metabolic chain. The accumulation of lactic acid and other organic acids leads to the continuous breakdown of lactose, reducing the yogurt's initial pH from 6.5 or 6.3 at the beginning of the fermentation to 4.5–4.3 at the end of the process (Miele et al. 2017). The quadratic model for acidity was statistically significant ( $p < 0.0005$ ), confirming the model's significance. The  $R^2$  coefficient  $R^2$ Adj coefficient, and F and P values for lack of fit confirmed that this model can effectively stimulate CBW fermentation progress. (Refer to supplementary tables 2, 6, 10, and 14). Figure 3 shows the acidification trend in CBW-supplemented yogurt affected by fermentation time, inoculation ratio, and CBW ratio. It's worth noting that other factors, which may not have been considered, could also play a role in the total titratable

acidity in the yogurt matrix. Regarding acid production, the CBW was significantly affected by fermentation time ( $p < 0.0005$ ), except for the interaction between the CBW ratio and inoculation ratio (BC), where the inoculation ratio had a more pronounced influence than the CBW ratio. This suggests that fermentation time allowed the LAB strain to convert more lactose into lactic acid and had a significant and positive effect on the acidity of the yogurt (Fig. 3). This result suggests that an increase in fermentation time leads to an increase in acidity (Fig. 3). This trend is shown in Fig. 3, where the highest (122 °T) acidity was related to yogurt supplemented with CBW at a 10 h fermentation time, 50% CBW and 5% inoculation ratio, while the lowest acidity (32 °T) was found in the sample containing 75% CBW and a 3% inoculation ratio at a 2.5 h fermentation time. A similar finding was reported by Bansal et al. (2015), who found that incubation time had a significant positive effect on acidity and that the product acidity increased with increasing inoculum concentration (Bansal et al. 2015). This implies that the inoculum concentration and fermentation time affect the acidification process, resulting in a lower pH and increased acidity. As a result, the increasing fermentation time and inoculum ratio result in higher acidity. In contrast, when adding 75% CBW and using a 2.5-h fermentation time, LAB activity ceased, leading to reduced acidity. Therefore, an extended fermentation time promotes the conversion of



**Fig. 4** Response surface plot, showing the LAB count as affected by fermentation time, CBW ratio, and inoculum concentration

lactose in the yogurt milk into lactic acid. Lactic acid plays a crucial role in developing some of the distinctive characteristics of yogurt, including its flavor, tartness, and thickening (Tomovska et al. 2016).

*RSM model for viable cell counts*

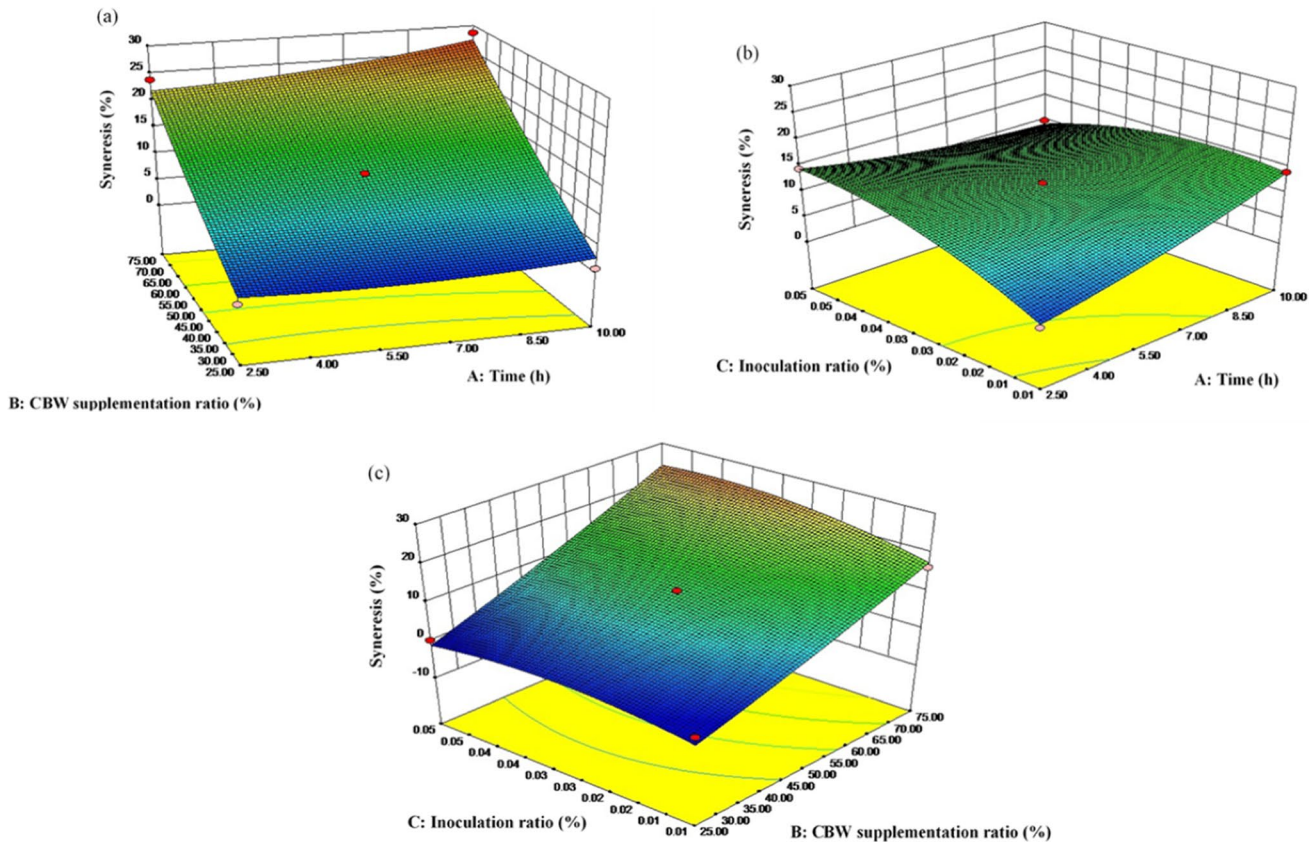
In addition to the acidification rate, the number of *Lactobacilli* in the fermented yogurt is also important. The quadratic model for viable cell counts was statistically significant ( $p < 0.0001$ ), confirming the model’s significance. The  $R^2$  coefficient,  $R^2$ Adj coefficient, and F and P values for lack of fit verified that this model can effectively stimulate CBW fermentation progress. (Refer to supplementary tables 3, 7, 11, and 15). The LAB count of common bean whey was significantly ( $p < 0.05$ ) affected by fermentation time (A) ( $p < 0.0001$ ), followed by the CBW ratio (B) and the inoculation ratio (C). However, the interactions between the CBW ratio added and fermentation time (AB), inoculation ratio and fermentation time (AC), and CBW ratio (BC) and inoculation ratio, were not significant ( $p > 0.05$ ) (Fig. 4). The highest (11.04 log CFU/mL) LAB counts were observed in yogurt-supplemented with CBW at a 10 h fermentation time, 50% CBW, and 5% inoculation ratio, while the lowest

LAB counts (7.66 log CFU/mL) were found in the sample containing 50% CBW, and a 1% inoculation ratio at a 2.5 h fermentation time (Fig. 4a–c).

This observation aligns with the acidity results, where a 50% addition of CBW resulted in the highest acidity of 122 °T. A similar finding was reported by those who stated that a 3% sucrose addition into mung bean milk (MBM) yielded the highest acidity (Wu et al. 2015). Tabasco et al. (2014), also reported that *L. bulgaricus* had a higher preference toward lactose than glucose as a substrate, suggesting that 50% of CM provides lactose for LAB growth in yogurt, leading to the highest LAB counts were obtained (Tabasco et al. 2014). In conclusion, increasing fermentation time and inoculum ratio resulted in higher LAB counts. By adding 50% CBW and extending the fermentation time to 10 h, more LAB viability occurred, leading to increased viable cell counts.

*RSM model for syneresis*

Syneresis provides an indication of nonhomogeneities in the fermented milk system. This parameter is undesirable in yogurt and negatively affects the consumer preferences. When it occurs, customers may believe that there



**Fig. 5** Response surface plot, showing syneresis as affected by fermentation time, CBW ratio, and inoculum concentration



is something microbiologically wrong with the product, impacting their purchasing decisions. The quadratic model for syneresis was statistically significant ( $p < 0.0001$ ), indicating that the model was significant. Moreover, the lack of fit was insignificant, demonstrating that the models correlated well with the measured data. The  $R^2$  coefficient,  $R^2$  Adj coefficient, and F and P values of lack of fit confirmed that this model can effectively stimulate CBW fermentation progress. (Refer to supplementary tables 4, 8, 12, and 16). Syneresis of CBW was significantly ( $p < 0.05$ ) affected by fermentation time (A) ( $p < 0.0001$ ), followed by CBW ratio (B) and inoculation ratio (C), suggesting that an increase in fermentation time resulted in decrease syneresis. This is consistent with the pH result for a 10 h fermentation time, which resulted in a low pH (3.10). Mir et al. (2021) reported that the almond gum concentration and fermentation time significantly affected yogurt syneresis. They observed a significant reduction in syneresis with an increase in the concentration of almond gum. According to the authors, the reduction in syneresis of the yogurts could be attributed to the increased viscosity and strong interaction of almond gum polysaccharides with milk proteins. However, the presence of  $Ca^{+2}$  ions in yogurt milk may have assisted the interaction of gum polysaccharides with milk proteins (Mir et al. 2021). Furthermore, the interaction between the CBW ratio added and fermentation time (AB), as well as inoculation ratio and fermentation time (AC) and finally, CBW ratio (BC) and inoculation ratio, were significant ( $p < 0.05$ ) (Fig. 5). This demonstrates the significant contribution of CBW, fermentation time and inoculation ratio in controlling syneresis in yogurt production. This observed tendency was expected as increasing CM increases yogurt milk's total solids content, particularly protein.

The syneresis results for all 3 treatments are shown in Fig. 5. The highest (28.62%) syneresis was related to yogurt supplemented with CBW at a 10 h fermentation time, 75% CBW, and a 3% inoculation ratio. This indicated that the highest levels of syneresis might be due to samples that contained lower levels of CM. Our result is consistent with those of Aydar et al. (2021), who found that the syneresis rate increased with an increased almond milk (AM) content in yogurt samples (Aydar et al. 2021). In contrast, the lowest syneresis (0.27%) was found in the sample containing 25% CBW and a 5% inoculation ratio at 6.25 h of fermentation time. The lower syneresis levels in these samples can be ascribed to the higher levels of CM. Lower CM results in a weaker three-dimensional yogurt gel, increasing whey expulsion from the gel (Yankey et al. 2023). The increasing fermentation time and CBW ratio both contributed to increased syneresis. Adding 25% CBW and extending the

fermentation time to 6.25 h resulted in reduced syneresis. Our study showed that increasing the fermentation time significantly reduced the yogurt syneresis, with no significant impact from the CBW ratio.

The optimization results indicate that the reduction in pH can be attributed to the increased production of lactic acid by *L. bulgaricus*. This finding aligns with previous research that demonstrated how a higher availability of nutrients, particularly essential amino acids and lactose, leads to faster acid production by lactic acid bacteria (Grasso et al. 2020; Zare et al. 2013). The reduction in acidity may be attributed to the retardation of microbial growth (Fig. 3a–c). This result is consistent with earlier studies that found adding 1–2% lentil flour led to faster acidification but only became significant after 2.5 h of incubation (Zare et al. 2011). The enhancement in LAB count is likely due to the increased availability of nutrients, resulting in a higher number of viable cell counts.

As shown in previous studies, the addition of pulse ingredients improved the growth rate of LAB counts (Mousavi et al. 2019). The reduction in syneresis can be related to the absorption of free water by CBWs (Fig. 5a–c). These results are similar to those reported by Zare et al. (2013) where syneresis decreased in samples with 1–3% pea fiber added (Zare et al. 2013).

The optimum acidity, LAB count, and syneresis were achieved in all common bean wheys by adding 25% CBW supplementation, an inoculation ratio of 1–4%, and a fermentation period of 5.54 h. The recommended acidity range was 70–70.003 °T, with a LAB count ranging from 8.54 to 9.69 log CFU/mL and a syneresis rate of 0.57–3.14%. Confirmatory experiments conducted under the optimized conditions—namely, 25% CBW supplementation, a 1–4% inoculation ratio, and a fermentation duration of 5.54 h—yielded results indicating acidity within the range of 70–70.005 °T, a LAB count between 8.55 and 9.75 log CFU/mL, and syneresis levels of 0.58–3.15%. Since these outcomes align with previous estimates, these conditions were applied for further investigations. The above results indicate that the CBW ratio, inoculum ratio, and fermentation time were the most critical factors influencing pH, acidity, LAB count, and syneresis. Therefore, to determine the optimum ranges, and to produce the ideal CBWFY, it is important to establish the ranges of these factors. Generally, a good yogurt should have a reasonable pH, maximum viable cells, and low syneresis.

## Conclusions

Currently, attention to developing novel plant-based products, particularly pulse ingredients, is increasing. For the first time, the present investigation revealed that supplementation of

CM with 25% CBW resulted in the production of yogurt with significantly lower pH and higher viable counts of *L. bulgaricus* compared to the control samples. A stable gel structure and lower syneresis were obtained with 25% CBW supplementation. Due to the higher protein and fiber content and based on the microbial and physical attributes explored, the findings suggest that CBW could potentially be considered a source of prebiotic and physical improvement ingredients for novel yogurt production. As product storage might affect the physicochemical properties of fermented novel products, further studies focusing on physicochemical testing will be very valuable.

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**Author contributions** All co-authors contributed substantially to this research article. AZ conceived the idea, designed the experimental methodology, and reviewed the manuscript; AZ implemented the experimental work analyzed the results, and prepared the manuscript; MH and EN organized the raw material supplies.

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**Data availability** The data supporting this study's findings are available from the corresponding author upon request.

#### Declarations

**Conflict of interest** There are no conflict of interest to declare.

**Ethical approval** In this study, no animals were involved; hence, no ethical clearance or approval was needed.

**Consent to publication** All authors have given their full consent for the submission of this research article in this journal.

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