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Optimisation of the inhibitory effect of *Lactiplantibacillus plantarum*, nisin, and lysozyme to prevent the late blowing defect in a cheese model

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Abstract: The present study employed response surface methodology (RSM) to optimise the prevention of late blowing defects in cheese during storage. The aim was to enhance the inhibition of *Clostridium sporogenes* in cheese by manipulating three independent variables: *Lactiplantibacillus plantarum* (utilising two different strains, labelled as A and B, the total cheese inoculation rate was 2% overall and the A : B ratio ranging from 25% to 75%), and lysozyme (ranging from 0 to 0.2 mg·L^{-1}). The response variables considered in this model cheese study included the *Clostridium* count, pH, and titratable acidity. The results showed that the optimal conditions for inhibiting *C. sporogenes* and preventing late blowing defects in cheese were achieved with an *L. plantarum* A : B ratio of 49.54 : 50.46%, nisin at a concentration of 1.762 mg·L^{-1}, and lysozyme at 0.2 mg·L^{-1}. These results demonstrated not only effective inhibition of *C. sporogenes*, a pivotal contributor to late blowing defects in cheese but also indicated favourable outcomes in terms of acidity parameters, which are crucial quality criteria for cheese production. The application of Response Surface Methodology revealed that late blowing defects can be prevented using relatively lower concentrations of antimicrobial agents, along with a judicious selection of appropriate cultures. This research highlights the potential for more efficient and cost-effective strategies to maintain cheese quality by minimising the risk of late blowing defects.

Keywords: *Clostridium sporogenes*; food preservation; antimicrobials; probiotic culture; endospore

Late blowing defect (LBD) is a significant fermentation-related issue associated with the presence of *Clostridium* spp. in producing semi-hard and hard cheeses. This defect manifests as the production of butyric acid, acetic acid, CO_2, and H_2 during the cheese ripening process due to lactate fermentation. The resulting butyric acid fermentation within the cheese matrix leads to the formation of irregular cracks, an undesirable taste, and odour. Consequently, such products are unappealing to consumers and result in substantial losses in the dairy industry (Le Bourhis et al. 2007; Martínez Cuesta et al. 2010; D'Incecco et al. 2018; Silvetti et al. 2018; Morandi et al. 2021).

The late blowing defect is primarily attributed to obligate anaerobes, spore-forming *Clostridium* species capable of fermenting lactate. These microorganisms are responsible for the defect's occurrence in cheeses, as *Clostridium* spores are prevalent and can withstand pasteurisation during milk and cheese production. Consequently, the defect develops during cheese

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storage (Storari et al. 2016; Rodi et al. 2020). Notably, Clostridia can induce LBD even when present in milk at concentrations as low as 50–1 000 spores per litre (Gómez-Torres et al. 2014; Oliveira et al. 2016). While *C. tyrobutyricum* is recognised as the primary culprit for LBD in cheese, other *Clostridium* species such as *C. butyricum*, *C. sporogenes*, and *C. beijerinckii* also significantly contribute to butyric acid fermentation in cheese (Le Bourhis et al. 2007; Martínez Cuesta et al. 2010; Garde et al. 2011; Gómez-Torres et al. 2014).

In cheese production, various methods are employed to prevent LBD, including antimicrobial agents such as nisin, lysozyme, nitrate, bactofugation, and microfiltration. Nisin, a polypeptide consisting of 34 amino acids produced by *Lactococcus lactis* spp. lactis is particularly effective against Gram-positive and spore-forming microorganisms. It exhibits activity against vegetative cells and spores in spore-forming bacteria (García et al. 2010; Gharsallaoui et al. 2016). Lysozyme, a small enzyme that catalyses the hydrolysis of specific polysaccharides in bacterial cell walls, is widely distributed and found in various human and animal secretions, body fluids, and tissues. Commercially, it is obtained from chicken egg albumin. It is known for its bactericidal effect against Gram-positive bacteria, but it is ineffective against Gram-negative bacteria due to differences in cell wall and lipopolysaccharide structures (Lucera et al. 2012).

Lactic acid bacteria (LAB) are recognised as effective bioprotective agents (Holzapfel et al. 1995). Their antimicrobial properties derive from the organic acids they produce, such as lactic acid and acetic acid, as well as from antimicrobial compounds known as bacteriocins, which can inactivate pathogenic microorganisms (Meghrous et al. 1999; Martínez Cuesta et al. 2010; Ávila et al. 2020). Lactic acid bacteria are crucial in imparting distinctive characteristics to dairy products. Among these, *L. plantarum* is noteworthy, found in various foods, including pickles, spices, sourdough, and dairy products. Due to its ability to thrive in low-pH environments, ferment different sugars, and produce various metabolites, *L. plantarum* is a potential probiotic microorganism (Jabbari et al. 2017). In cheese production, *L. plantarum* is often employed as a co-culture (Duan et al. 2019).

The present study aimed to control *C. sporogenes*, a common cause of late blowing defects in cheese production, and to fill the research gap caused by the lack of studies on it. To mitigate this defect, we have investigated and optimised a combination of *L. plantarum* strains, nisin, and lysozyme-known for their antimicrobial properties. Our goal is to prevent *C. sporogenes* spores from affecting the cheese production process. Additionally, we have explored the effect of these factors on acidity values (pH and acidity %), which are critical quality indicators. This study contributes to the limited literature on the simultaneous assessment of these factors within the cheese matrix, aiming to prevent LBD without significantly altering the cheese's quality properties.

MATERIAL AND METHODS

Development, storage and spore preparation of *Clostridium sporogenes* strains. *C. sporogenes* 73 and *C. sporogenes* 97, isolated from a cheese with LBD and raw milk, respectively, in the Department of Food Engineering at Süleyman Demirel University, were used (Ertürkmen and Öner 2023). *Clostridium* strains were maintained at –40 °C with 5% glycerol, and they were activated in Reinforced Clostridial Medium (RCM; Merck, Germany) under anaerobic conditions at 37 °C for 48 h before sporulation.

For spore preparation, *C. sporogenes* cultures were incubated in Bryant Burkey Broth (BBB; Merck, Germany) for one week at 37 °C. The obtained culture was centrifuged (5 000 × g, 15 min, 20 °C) and washed twice with sterile distilled water. The spore suspension was stored at –40 °C for cheese making. Spore suspensions were heat-treated at 80 °C for 20 min before being used in cheese making (Gómez Torres et al. 2015).

***Lactiplantibacillus plantarum* strains development and storage.** Two different strains of *L. plantarum* were employed in the cheese-making process. The probiotic properties of these strains had been previously determined in distinct studies, and the cultures were identified as *L. plantarum* AB 6-25 and *L. plantarum* Lb 9 (Kılıç et al. 2013; Yalçınkaya et al. 2022). For clarity in this study, they were denoted as *L. plantarum* A and *L. plantarum* B, respectively. To prepare the cultures for cheese production, they were retrieved from the –40 °C freezer and activated in de Man, Rogosa, Sharpe (MRS) medium (Merck, Germany) at 37 °C for 24 h. Subsequently, the activated cultures were inoculated into 10% skim milk (Sigma Aldrich, Germany).

Model cheese production. All model white cheeses were prepared in duplicate on separate days in a laboratory setting. Raw cow's milk was sourced from a farm in the Acıpayam region of Denizli province. The raw milk underwent pasteurisation at 72 °C for 15 s and was cooled to 35 °C. At this temperature, 0.02 $g \cdot L^{-1}$ of CaCl was added. The pasteurised milk was then evenly distributed into vats, each containing 5 litres of milk, to produce 17 different cheese varieties (as listed in Table 1). Approximately 4 log $MPN \cdot mL^{-1}$ (MPN – most

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Table 1. Chemical and microbiological analysis results of 17 different model cheeses after 45 days of storage

Cheeses	Independent variables			Results							LBD symptoms
	X_1 (mg·L^{-1})	X_2 (mg·L^{-1})	X_3 (mg·L^{-1})	pH	lactic acid (%)	dry matter (%)	*Clostridium sporogenes* (log MPN·g^{-1})	*Lactobacillus* (log CFU·g^{-1})	*Streptococci* (log CFU·g^{-1})	TMAB (log CFU·g^{-1})	
1	50 (0)	12 (1)	0.2 (1)	5.50 ± 0.01	0.68 ± 0.01	51.69 ± 0.07	2.18 ± 0.06	8.82 ± 0.06	8.80 ± 0.14	9.02 ± 0.05	yes
2	50 (0)	12 (1)	0.0 (–1)	5.54 ± 0.04	0.66 ± 0.01	51.70 ± 0.40	2.24 ± 0.09	8.51 ± 0.08	8.41 ± 0.10	8.63 ± 0.10	yes
3	50 (0)	0 (–1)	0.0 (–1)	4.36 ± 0.02	2.19 ± 0.06	46.70 ± 0.34	3.36 ± 0.02	8.26 ± 0.04	8.49 ± 0.16	8.55 ± 0.12	no
4	50 (0)	0 (–1)	0.2 (1)	4.71 ± 0.01	1.93 ± 0.01	48.36 ± 0.36	2.93 ± 0.03	8.21 ± 0.07	8.54 ± 0.08	8.48 ± 0.13	no
5	50 (0)	6 (0)	0.1 (0)	5.46 ± 0.03	0.80 ± 0.02	50.41 ± 0.08	2.84 ± 0.00	8.42 ± 0.18	8.47 ± 0.06	8.49 ± 0.08	yes
6	25 (–1)	12 (1)	0.1 (0)	5.55 ± 0.01	0.71 ± 0.03	52.80 ± 0.32	2.34 ± 0.00	8.62 ± 0.23	8.52 ± 0.06	8.43 ± 0.04	yes
7	25 (–1)	6 (0)	0.2 (1)	5.48 ± 0.02	0.60 ± 0.02	53.55 ± 0.04	2.93 ± 0.03	8.76 ± 0.11	8.46 ± 0.12	8.64 ± 0.08	yes
8	25 (–1)	0 (–1)	0.1 (0)	4.56 ± 0.01	2.06 ± 0.06	51.77 ± 0.15	3.71 ± 0.02	8.86 ± 0.16	8.45 ± 0.04	8.91 ± 0.11	no
9	25 (–1)	6 (0)	0.0 (–1)	5.49 ± 0.02	1.24 ± 0.01	50.66 ± 0.00	3.53 ± 0.01	8.89 ± 0.14	8.45 ± 0.04	8.86 ± 0.23	yes
10	75 (1)	12 (1)	0.1 (0)	5.39 ± 0.02	0.77 ± 0.01	49.77 ± 1.06	2.19 ± 0.05	8.63 ± 0.02	8.53 ± 0.17	8.69 ± 0.22	yes
11	75 (1)	6 (0)	0.2 (1)	5.31 ± 0.01	0.92 ± 0.04	52.79 ± 0.65	3.16 ± 0.05	8.50 ± 0.03	8.75 ± 0.32	8.56 ± 0.14	no
12	75 (1)	6 (0)	0.0 (–1)	5.36 ± 0.04	0.71 ± 0.01	51.49 ± 0.16	3.53 ± 0.01	8,84 ± 0.01	8.73 ± 0.04	8.63 ± 0.07	yes
13	75 (1)	0 (–1)	0.1 (0)	5.09 ± 0.49	2.06 ± 0.03	51.87 ± 0.06	4.00 ± 0.04	8.51 ± 0.08	8.73 ± 0.09	8.48 ± 0.13	no
14	50 (0)	6 (0)	0.1 (0)	5.43 ± 0.03	0.82 ± 0.02	50.83 ± 0.49	2.54 ± 0.00	8.48 ± 0.16	8.49 ± 0.16	8.60 ± 0.14	yes
15	50 (0)	6 (0)	0.1 (0)	5.49 ± 0.01	0.81 ± 0.00	50.91 ± 0.08	2.69 ± 0.04	8.63 ± 0.16	8.45 ± 0.04	8.59 ± 0.08	yes
16	50 (0)	6 (0)	0.1 (0)	5.48 ± 0.01	0.82 ± 0.02	49.84 ± 0.49	2.84 ± 0.00	8.67 ± 0.02	8.56 ± 0.12	8.50 ± 0.14	yes
17	50 (0)	6 (0)	0.1 (0)	5.44 ± 0.01	0.81 ± 0.01	50.25 ± 0.75	2.73 ± 0.00	8.48 ± 0.14	8.56 ± 0.06	8.46 ± 0.09	yes

X_1 – *Lactiplantibacillus plantarum* A : B ratio; X_2 – nisin; X_3 – lysozyme; MPN – most probable number; CFU – colony forming unit; TMAB – total mesophilic aerobic bacteria; LBD – late blowing defect

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probable number) of *C. sporogenes* strains, mixed at a 1:1 ratio, were inoculated into the vats. *L. plantarum* strains were added to the milk used for cheese production at a rate of 2%, with A:B ratios ranging from 25–75, 50–50, and 75–25%. Additionally, a 0.5% yogurt culture (Chr Hansen YC350) was incorporated into all samples as a starter culture to enhance acidity. At this stage, nisin (min. 1 000 IU·mg^{-1}; Maysa A.Ş., Türkiye; IU – international unit) and lysozyme (Liquid Lysozyme Chloride; ≥ 9 × 10^6 U·mg^{-1}; Maysa A.Ş, Türkiye; U – unit) were introduced into the milk at concentrations of 0, 6, and 12 mg·L^{-1} for nisin, and 0, 0.1, and 0.2 mg·L^{-1} for lysozyme, respectively. Rennet (Marzyme® 55 800 IMCU; Danisco, Denmark) was added to induce clot formation within 90 min. After breaking the clot (approximately 1 cm^3) and separating the whey, the cheeses were pressed. Following pressing, the cheeses were immersed in a 12% brine solution at 25 °C for 12 h. Each vat yielded approximately 500 g of cheese (dimensions: 7 × 7 × 10 cm^3). The cheese samples were stored at 4 °C for 45 days, because white cheeses in Türkiye are stored at this temperature and LBD can often be observed.

Microbiological analyses. Total mesophilic aerobic bacteria (TMAB), lactic acid bacteria, and *Clostridium* strains were enumerated in cheese samples. For the microbiological analysis of all cheeses, 10 g of cheese samples were homogenised in Ringer's solution. Tenfold serial dilutions were then prepared, and the counts were determined from the appropriate dilutions. Samples for counting lactic acid bacteria were spread-plated on M17 (Merck, Germany) and MRS agar (Merck, Germany). The plates were incubated at 37 °C for 48 to 72 h. Plate count skim milk agar (Merck, Germany) was used for the enumeration of TMAB at 35 °C for 24 to 48 h (De Man et al. 1960; Terzaghi and Sandine 1975). *Clostridium* spores were quantified using the MPN method in BBB (Merck, Germany) medium, as modified by Garde et al. (2011). To inactivate vegetative cells, the prepared serial dilutions were heated at 80 °C for 20 min. After transferring 1 mL of these serial dilutions into tubes containing 10 mL of BBB, 2 mL of sterile melted paraffin was added. The samples were then incubated at 37 °C for up to 1 week, and the tubes were assessed for gas formation. The results were expressed in log MPN·g^{-1}.

Chemical analyses. Titratable acidity, pH, and dry matter analyses were conducted on the cheese samples. Each analysis was performed at least twice for accuracy. pH measurements for the cheese samples were obtained by immersing a pH meter electrode (WTW, Germany) directly into the cheese. The titratable acidity of the model cheeses was determined through titration using 0.1 mol·L^{-1} NaOH (Merck, Germany), as previously outlined Jooyandeh et al. (2015). Titratable acidity values for the cheeses are expressed as a percentage of lactic acid. Additionally, dry matter analysis was carried out according to established procedures (Jooyandeh et al. 2015).

Detection of late blowing defect. At the end of the storage of cheeses, LBD, which occurs as a result of butyric acid fermentation by *Clostridium* spp., was detected by sensory evaluation. For this purpose, cheeses were subjected to odour and visual controls. LBD was detected by deterioration of cheese packaging, cracks/splits in the cheese matrix and bad odour due to butyric acid. For the verification cheese produced at the optimum points mentioned in the 'Experimental design, verification production and statistical analysis' section, sensory evaluation was also carried out to determine butyric acid content. Butyric acid contents, the volatile components of the cheese samples were analysed using gas chromatography-mass spectrometry (Model; QP 2010 ultraseries; Shimadzu, Japan). Analysis parameters are as follows; The oven was kept at 40 °C for 2 min and increased to 250 °C with an increase of 4 °C per min. It was kept at this temperature for 5 min. The electron ionisation was set at –70 eV and the ion source temperature at 250 °C. Chromatographic separation was carried out in a column (Restek Rx-5 Sil MS 30 m × 0.25 mm, 0.25 μm). The carrier gas was helium and the flow rate was 1.61 mL per min.

Experimental design, verification production, and statistical analysis. The Response Surface Method (RSM) was employed to conduct an optimisation study aimed at preventing the occurrence of the late blowing defect in cheese. The Box-Behnken design was selected as the experimental framework, with Design Expert (version 12.0) as the statistical analysis tool. The independent variables for the optimisation study were defined as follows: *L. plantarum* A:B ratio (X_1), nisin concentration (X_2), and lysozyme concentration (X_3). Three distinct levels were established for each independent variable and coded as –1, 0, and +1. These variables were set as follows: X_1 with ratios of 25:75, 50:50, and 75:25%; X_2 with concentrations of 0, 6, and 12 mg·L^{-1}; and X_3 with concentrations of 0, 0.1, and 0.2 mg·L^{-1}.

The Box-Behnken 2^3 factorial design was implemented, encompassing five replications at the central point, resulting in 17 experimental trials. In this study, the response variables chosen were *Clostridium* count (Y_1),

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pH (Y_2), and acidity percentage (Y_3). The data obtained from these experimental trials were input into the Design-Expert software package, and the significance of the potential regression models and the coefficients within the model were determined through an analysis of variance (ANOVA).

Upon conducting the ANOVA for each independent variable, a quadratic model was the most appropriate. The model equations can be formulated using the coefficients of the independent variables based on the derived quadratic model. As a result, estimated values can be computed from these equations, as represented by Equation 1 below:

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k}\beta_{ii} X_i^2 + \sum_{i=1}^{k-1}\sum_{j=i+1}^{k}\beta_{ij} X_i X_j \quad (1)$$

where: Y – dependant variable; X_i, X_j – independent variables; k – number of independent variables; β_0, β_i, β_{ii}, and β_{ij} – constant coefficient, linear, quadratic, and the interaction coefficient of the two factors.

Verification production was carried out using the method mentioned in the 'model cheese production' section at the optimum points obtained with RSM. Differences between *Lactobacillus* count, *Streptococci* count, TMAB count and dry matter values of the samples were determined using one-way analysis of variance (ANOVA) and Tukey test ($P < 0.05$).

RESULTS AND DISCUSSION

Microbiological and chemical analysis results of model cheeses. The pH, % lactic acid, dry matter, and microbiological analyses were carried out in trial model cheeses after 45 days of storage. The pH values of the cheeses ranged from 4.35 to 5.55. and the titration acidity between 0.6% and 2.19%. Dry matter (%) values of the cheeses were determined in the range of 46.70–52.80%.

Microbiological results were determined as *Lactobacillus* number in the range from 8.21 ± 0.07 log CFU·g^{-1} to 8.89 ± 0.14 log CFU·g^{-1} (CFU – colony forming unit), M17 number in the range from 8.41 ± 0.10 log CFU·g^{-1} to 8.8 ± 0.14 log CFU·g^{-1}, and total mesophile aerobic count in the range from 8.43 ± 0.04 log CFU·g^{-1} to 9.02 ± 0.05 log CFU·g^{-1}. The number of *Clostridium* spores was determined in the range from 2.18 ± 0.06 log MPN·g^{-1} to 4.00 ± 0.04 log MPN·g^{-1}. Microbiological and chemical analysis results of cheeses were given in Table 1.

No statistically significant differences were observed in the counts of TMAB (except for samples 1 and 6), *Lactobacillus* (except for samples 3, 4, and 9), and *Streptococci* in the cheeses ($P > 0.05$). It was determined that the antimicrobial substances (nisin and lysozyme) did not have a significant effect on the populations of cultures typically used in cheese production.

A notable distinction was observed in the pH values of the cheeses. The pH was higher in cheeses with a higher nisin concentration (Table 1). This elevated pH (pH > 5.39) was found to encourage the growth of *C. sporogenes*, leading to the development of cracks and crevices in these cheeses, which are indicative of LBD. While the use of elevated nisin concentrations in cheeses effectively inhibited spore growth, it was also observed to affect the sensory characteristics of the cheeses. Consequently, our objective was to produce the most suitable cheese by optimising the independent variables used in our study to prevent LBD in cheeses without compromising their overall quality.

Effects of independent variables on responses in model cheeses. The data regarding the *Clostridium* counts obtained from the experimental model cheeses were input into the Design-Expert software, and a quadratic model was selected as the most appropriate model. Statistically insignificant variables in the determined model were simplified through the backward elimination method. The analysis of variance conducted on the quadratic model, which illustrates the relationship between the independent variables and the count of *C. sporogenes*, is presented in Table 2.

As indicated in Table 2, nisin (X_2) and lysozyme concentrations (X_3) had a statistically linear effect on *C. sporogenes* inhibition, while the *L. plantarum* A : B ratio (X_1^2) had a quadratic effect ($P < 0.05$). On the other hand, the linear effects of *L. plantarum* A : B ratio (X_1), *L. plantarum* A : B ratio × nisin interaction (X_1X_2), *L. plantarum* A : B ratio × lysozyme interaction (X_1X_3), nisin × lysozyme interaction (X_2X_3) and quadratic effects of nisin (X_2^2) and lysozyme (X_3^2) were statistically insignificant ($P > 0.05$).

Data on *C. sporogenes* counts in the model cheese are presented in Table 1. The lowest count of *C. sporogenes* was determined to be 2.18 log MPN·L^{-1} in cheese No. 1, containing 50% *L. plantarum* A, 12 mg·L^{-1} nisin, and 0.2 mg·L^{-1} lysozyme. The highest value was recorded in cheese No. 13, which contained 75% *L. plantarum* A, 0 mg·L^{-1} nisin, and 0.1 mg·L^{-1} lysozyme. According to the results, an increase in nisin concentration corresponded to a decrease in *C. sporogenes*. This reduc-

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Table 2. Quadratic model analysis for *Clostridium sporogenes* counts response in cheeses

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value
Model	4.3400	4	1.0900	27.19	< 0.0001*
X_1	0.8809	1	0.8809	22.05	0.0005*
X_2	3.1000	1	3.1000	77.61	< 0.0001*
X_3	0.2592	1	0.2592	6.49	0.0256*
X_1^2	0.9623	1	0.9623	24.09	0.0004*
Residual	0.4793	12	0.0399	–	–
Lack of fit	0.4174	8	0.0522	3.37	0.1277
Pure error	0.0619	4	0.0155	–	–
Corrected total	4.8200	16	–	–	–

*Significant at $P < 0.05$; $R^2 = 0.9006$; adjusted $R^2 = 0.8675$; predicted $R^2 = 0.7665$; X_1 – *Lactiplantibacillus plantarum* A : B ratio; X_2 – nisin; X_3 – lysozyme; X_1^2 – quadratic effect of *L. plantarum* A : B ratio; *df* – degrees of freedom; R^2 – coefficient of determination

tion in *C. sporogenes* count due to the increase in nisin concentration is illustrated in Figures 1A and 1C. It has been demonstrated in numerous prior studies that nisin has an antimicrobial effect on *Clostridium* spp. spores (Meghrous et al. 1999; Hofstetter et al. 2013; Ávila et al. 2014). Němečková et al. (2010) reported that 4 mg per kg nisin in cheese slurry reduced the *C. tyrobutyricum* count from 1.5 ± 0.3 log MPN·g^{-1} initially to < 0.5 log MPN·g^{-1} after 2 weeks. In a study examining the anticlostridial effect of *Lactococcal* and *Enterococcal* cultures in Dutch-type boiled cheese, nisin (20 mg·L^{-1}), used as a control, resulted in the *C. tyrobutyricum* count remaining at the initial level of 1 log MPN·g^{-1} at the end of the first 30 days. However, no LBD symptoms were observed in the cheese (Havlíková et al. 2018).

It was observed that the *C. sporogenes* counts decreased with increasing lysozyme concentration (Figure 1B). However, it was determined that this decrease was relatively less than the decrease caused by nisin (Figure 1A). Martínez-Cuesta et al. (2010) reported that adding 25 mg·L^{-1} lysozyme to milk can prevent LBD caused by *C. tyrobutyricum* in semi-hard cheese. The effect of *L. plantarum* cultures on *C. sporogenes* is evident in Table 2 and Figure 1B, with the optimal effect observed when *L. plantarum* is used in an A : B ratio of approximately 50 : 50. As seen in Figure 1B and Table 2, it was determined that the varying concentrations of *L. plantarum* (A and B cultures) did not affect the *C. sporogenes* counts.

For the pH values of model cheeses, the most suitable model was found to be the quadratic in the Design Expert program. Table 3 shows the relationship between independent variables and pH values obtained as a result of the analysis of variance performed on the quadratic model.

As shown in Table 3, linear (X_2) and quadratic (X_2^2) effects of nisin, as well as the interaction of *L. plantarum* A : B ratio and nisin interaction (X_1X_2), were determined to be statistically significant on pH ($P < 0.05$). However, the linear effects of *L. plantarum* A : B ratio (X_1), lysozyme (X_3), and the interaction of *L. plantarum* A : B ratio and lysozyme (X_1X_3), as well as the quadratic effects of nisin and lysozyme (X_2X_3), *L. plantarum* A : B ratio (X_1^2) and lysozyme (X_3^2) on pH were found to be statistically insignificant ($P > 0.05$).

The pH value exhibited changes based on the concentration of *L. plantarum* A and nisin (Figure 1D). Consequently, lower concentrations of nisin should be employed to attain lower pH values in cheeses. Diverse findings have been reported regarding the effect of nisin on cheese pH. Samelis et al. (2003) noted that the pH values of cheeses to which nisin was added (at concentrations of 100–500 IU·g^{-1}) during the production of Anthotyros cheese (a type of cheese made from whey in Greece) were higher than those of control samples without nisin, after 45 days of storage. Kallinteri et al. (2013) reported that using nisin in Galotyri cheese had no significant effect on pH during storage. Furthermore, Ávila et al. (2020) employed a nisin-producing *Lactococcal* starter in cheese production to prevent LBDs. They observed that the pH remained stable during storage in control cheeses without *C. tyrobutyricum*, but the pH rose from the 14th day in a cheese containing *C. tyrobutyricum*. This phenomenon was attributed to the deacidification caused by the metabolism of *Clostridium* cells in previous studies (Le Bourhis et al. 2007; Matijašić

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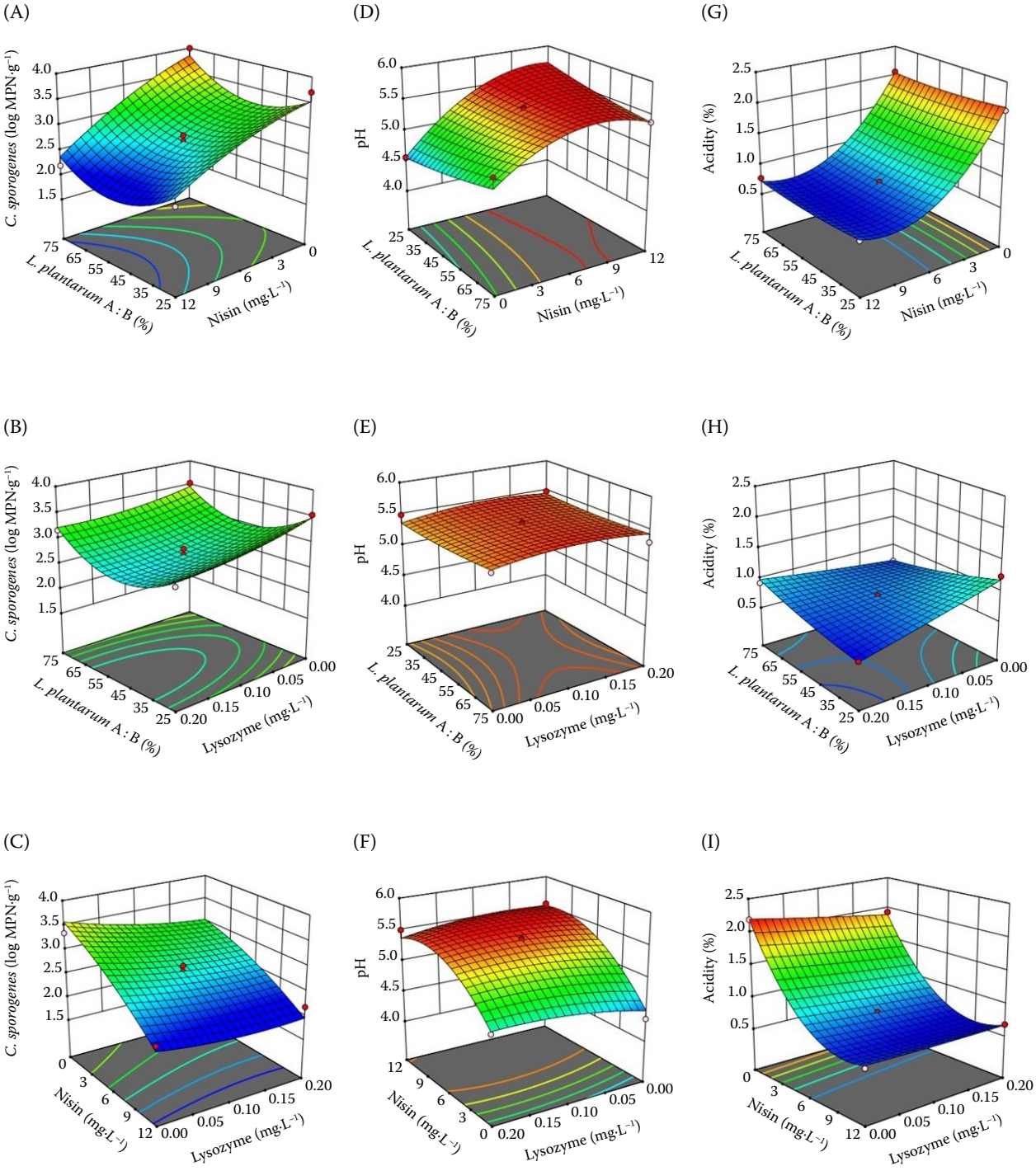

Figure 1. The effects of independent variables on the *Clostridium sporogenes* counts, pH, and acidity (%): Effect of (A) *Lactiplantibacillus plantarum* A : B ratio and nisin; (B) *L. plantarum* A : B ratio and lysozyme; (C) nisin and lysozyme; (D) *L. plantarum* A : B ratio and nisin; (E) *L. plantarum* A : B ratio and lysozyme; (F) nisin and lysozyme; (G) *L. plantarum* A : B ratio and nisin; (H) *L. plantarum* A : B ratio and lysozyme; and (I) nisin and lysozyme

MPN – most probable number

et al. 2007; Gómez-Torres et al. 2014; Gómez-Torres et al. 2015). On the other hand, lysozyme had no significant effect on pH.

The most suitable model for acidity percentage values of the model cheeses was determined to be quadratic using the Design Expert program. An analysis of vari-

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Table 3. Quadratic model analysis for pH response in cheeses

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value
Model	2.5000	5	0.5003	117.97	< 0.0001*
X_1	0.0210	1	0.0210	4.95	0.0479*
X_2	1.7500	1	1.7500	412.28	< 0.0001*
X_3	0.0078	1	0.0078	1.84	0.2019
X_2X_3	0.0380	1	0.0380	8.97	0.0122*
X_2^2	0.6861	1	0.6861	161.79	< 0.0001*
Residual	0.0467	11	0.0042	–	–
Lack of fit	0.0409	7	0.0058	4.09	0.0958
Pure error	0.0570	4	0.0014	–	–
Corrected total	2.5500	16	–	–	–

*Significant at $P < 0.05$; $R^2 = 0.9817$; adjusted $R^2 = 0.9734$; predicted $R^2 = 0.9375$; X_1 – *Lactiplantibacillus plantarum* A : B ratio; X_2 – nisin; X_3 – lysozyme; X_2X_3 – nisin × lysozyme interaction; X_2^2 – quadratic effects of nisin; *df* – degrees of freedom; R^2 – coefficient of determination

ance was conducted on the quadratic model, which illustrates the relationship between the independent variables and the acidity percentage values. The results of this analysis are presented in Table 4.

As shown in Table 4, nisin (X_2) and lysozyme (X_3), *L. plantarum* A : B ratio × lysozyme interaction (X_1X_3), nisin × lysozyme (X_2X_3) interaction and nisin quadratic (X_2^2) have statistically significant effects on acidity % ($P < 0.05$). However, it was determined that the effects of *L. plantarum* A : B ratio (X_1) linear, *L. plantarum* A : B ratio × nisin interaction (X_1X_2), *L. plantarum* A : B ratio (X_1^2) quadratic and lysozyme ($X_3{}^2$) quadratic effects on acidity % were statistically insignificant.

The acidity values of the cheeses ranged from 0.6% to 2.19%. The lowest acidity % value was observed in cheese 7, with an *L. plantarum* A ratio of 25%, a nisin concentration of 6 mg·L^{-1}, and a lysozyme concentration of 0.2 mg·L^{-1}. This cheese was followed by cheeses 1 and 2, with acidity % values of 0.68 and 0.66, respectively. The acidity value decreases with increasing nisin and lysozyme concentrations (Figure 1G–I).

Determination of optimum points and optimisation control. After entering the results obtained in our study into the Design Expert program, we calculated the optimal values of the independent variables. While determining these optimal values, we aimed to mini-

Table 4. Quadratic model analysis for acidity % response in cheeses

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value
Model	5.2100	7	0.7445	359.47	< 0.0001*
X_1	0.0025	1	0.0025	1.18	0.3050
X_2	3.6700	1	3.6700	1773.04	< 0.0001*
X_3	0.0545	1	0.0545	26.29	0.0006*
X_1X_3	0.1764	1	0.1764	85.17	< 0.0001*
X_2X_3	0.0196	1	0.0196	9.46	0.0132*
X_1^2	0.0092	1	0.0092	4.42	0.0648
X_2^2	1.2600	1	1.2600	609.05	< 0.0001*
Residual	0.0186	9	0.0021	–	–
Lack of fit	0.0164	5	0.0033	5.74	0.0576
Pure error	0.0023	4	0.0006	–	–
Corrected total	5.2300	16	–	–	–

*Significant at $P < 0.05$; $R^2 = 0.9964$; adjusted $R^2 = 0.9937$; predicted $R^2 = 0.9768$; X_1 – *Lactiplantibacillus plantarum* A : B ratio; X_2 – nisin; X_3 – lysozyme; X_1X_3 – *L. plantarum* A : B ratio × lysozyme interaction; X_2X_3 – nisin × lysozyme interaction; X_1^2 – *L. plantarum* A : B ratio quadratic effects; X_2^2 – quadratic effects of nisin; *df* – degrees of freedom; R^2 – coefficient of determination

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mise the *Clostridium* count, maintain the pH value between 4.35 and 5.00, and the acidity percentage within the range of 1.35 to 2.19. These limits were defined based on the significance of acidity development in cheese, as established by our study during the optimisation process. Because the development of acidity in cheese is an important parameter, and therefore, these limits were determined depending on our study while calculating the optimum conditions. The optimum conditions in the Design Expert program; *L. plantarum* A : B ratio was determined as 49.54 : 50.46%, 1.762 mg·L^{-1} nisin and 0.2 mg·L^{-1} lysozyme.

Verification production was carried out with the optimum values obtained from the Design Expert program. The number of *C. sporogenes*, pH value and acidity of cheeses produced at these optimum points were found to be 3.08 ± 0.16, 5.27 ± 0.02, and 1.32 ± 0.02 log MPN·g^{-1}, respectively. Control samples were also generated with and without *C. sporogenes*, along with the validation production. As a result of the optimisation study, the verification cheeses and control cheeses produced were analysed. On the first day of storage, it was determined as 3.93 ± 0.26 log MPN·g^{-1} in the verification cheese and 4.62 ± 0.11 log MPN·g^{-1} in the control cheese containing *Clostridium* strains. At the end of storage, experimental values were obtained as *Clostridium* number 2.96 log MPN·g^{-1}, pH 5.00, and acidity 1.43%. The obtained results and estimated values were compared and it was determined that the results were very close to each other. These data demonstrated the accuracy and viability of the model. Upon comparing the control sample containing *C. sporogenes* with the verification production cheese after the storage period, it was observed that the count of *C. sporogenes* spores in the verification production cheese was lower by 1.8 log CFU·g^{-1}. No spores were detected in the control sample that did not contain *C. sporogenes*. Also, the concentration of butyric acid in the validation cheese was approximately 2.32 times lower compared to the control cheese containing *C. sporogenes*.

CONCLUSION

The response surface methodology (RSM) has been effectively applied to cheese containing *L. plantarum*, nisin, and lysozyme to mitigate late blowing defect (LBD). The method's accuracy is supported by several key observations: a logarithmic decrease in the *C. sporogenes* counts, the emergence of gas eyes in the cheese (a standard indicator of late blowing), and the absence of bloating in the packaging. Furthermore, combining these different antimicrobial agents has demonstrated a notable synergistic effect. Additionally, favourable outcomes concerning pH and acidity percentage indicate the practicality of this model. Consequently, it is recommended that various antimicrobial agents be employed in modest quantities to combat LBD in the dairy industry. This approach should be coupled with selecting starter cultures with relatively lower susceptibility to antimicrobial agents.

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