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The Antilisterial Effect of *Latilactobacillus sakei* CTC494 in Relation to Dry Fermented Sausage Ingredients and Temperature in Meat Simulation Media

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Abstract: *Listeria monocytogenes*, the causative agent of listeriosis, is a relevant pathogen in dry fermented sausages (DFSs), and the application of antilisterial starter cultures is an effective intervention strategy to control the pathogen during DFS production. The effect of factors in relation to DFS formulation and production, NaCl (0–40 g/L), Mn (0.08–0.32 g/L), glucose (0–40 g/L) and temperature (3–37 °C), on the behaviour of *L. monocytogenes* when cocultured with *Latilactobacillus sakei* 23K (non-bacteriocinogenic) and CTC494 (bacteriocinogenic) strains was studied through a central composite design in meat simulation media. *L. sakei* and *L. monocytogenes* counts, pH, lactic acid production and bacteriocin activity were determined in mono and coculture. The pH decrease and lactic acid production were highly influenced by glucose, while production of sakacin K by *L. sakei* CTC494 was observed at moderate (10 and 20 °C), but not at the lowest (3 °C) and highest (37 °C), temperatures. Coculture growth had no effect on the acidification and bacteriocin production but inhibited and inactivated *L. monocytogenes* when *L. sakei* 23K entered the early stationary phase and when *L. sakei* CTC494 produced sakacin K. Optimal conditions for achieving a 5-log units reduction of *L. monocytogenes* were at 20 °C, 20 g/L of NaCl, 0.20 g/L of Mn and 40 g/L of glucose, those highlighting the importance of considering product formulation and fermentation conditions for bioprotective starter cultures application.

Keywords: *Listeria monocytogenes*; starter culture; bioprotection; bacteriocin; meat fermentation

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1. Introduction

Listeria monocytogenes is a facultative anaerobe foodborne pathogen found in a wide range of environments, including water, soil, faeces, food processing environments, and food [1]. This pathogen, when ingested, is the causative agent for listeriosis, a very severe foodborne illness with high mortality rate between 20 and 30%, making *L. monocytogenes* one of the most significant pathogens encountered in food [1,2]. Ready-to-eat (RTE) products (e.g., deli meats, soft cheeses, and seafood) are the main source of listeriosis outbreaks. Dry fermented sausages (DFSs) are not generally linked to food poisoning [1,3], due to the low probability of growth of the pathogen in these foods thanks to the effect of hurdles combination, with pH and water activity (a_w) reductions achieved during fermentation and drying processes [4]. However, DFSs (especially low-acid type) are occasionally involved in withdrawals and recalls if the raw meat is contaminated with *L. monocytogenes* and the lethality of the fermentation process is not able to decrease to compliance levels (i.e., <2 log CFU/g) [5]. In the last 4 years, the European Rapid Alert System for Food and Feed portal (RASFF) [6] has recorded 13 notifications of *L. monocytogenes* in DFSs. The ubiquitous nature

of *L. monocytogenes* allows it to be found at all stages of the pork processing chain, including raw pork. Specifically, pork can be contaminated in industrial environments when in contact with equipment and surfaces, where the pathogen can adapt and persist for extended periods, for instance in biofilm formations (i.e., aggregation ability of microorganisms on wet surfaces and grow in microcolonies, persisting in the environment) [7–9]. This increases the risk of cross-contamination during post-processing stages, which can pose a listeriosis risk to vulnerable consumer groups such as pregnant woman and individuals with weakened immune systems. Chilling temperatures during pork storage before DFS manufacturing can support *L. monocytogenes* growth [10], since it can multiply under relatively broad range of conditions of temperature (−1.5–45 °C), pH (4.5–9.0), a_w (0.92–0.99) and salt concentrations (<16%) [10–12]. Despite European Regulation (EC) 2073/2005 establishing a maximum of 100 CFU/g in ready-to-eat (RTE) foods like DFSs, which are unable to support the growth of *L. monocytogenes*, 1.24% of fermented sausage units tested positive, and 0.11% exceeded the maximum limit according to enumeration tests for the pathogen in EU official samplings conducted during the 2018–2022 period [3,13,14]. As shown by several authors [8,15], *L. monocytogenes* is highly present in the equipment, industrial surfaces, the meat batter used for DFS, and final products, which is of concern.

Mediterranean-style DFSs are produced with a mixture of lean pork and fat with ingredients and additives including salt, dextrose, black pepper or paprika, sodium ascorbate and nitrifying agents. The production process of DFSs consists of fermentation and drying steps that reduce the meat batter pH and a_w, turning DFSs into self-stable meat products not supporting bacterial growth [16]. Acidification and decrease in water activity transform nutritious fresh meat environments to harsh conditions for microorganism growth and development [10]. In the framework of the hurdle technology, both the formulation and production processes (i.e., decrease in pH and a_w) provide antimicrobial barriers that contribute to the food safety of the final product. In industrial production, lactic acid bacteria (LAB) are frequently used as starter cultures to promote the product acidification through fermentation. Some LAB strains (the so-called bioprotective LAB) produce small antimicrobial peptides (i.e., bacteriocins such as sakacin and enterocin) with strong antimicrobial activity against *L. monocytogenes* [17] that can be used as a strategy to control the pathogen [18,19]. In fermented meat products, the bioprotective culture *Latilactobacillus sakei* CTC494 (formerly *Lactobacillus sakei* CTC494), a producer of sakacin K, has been used as starter culture in different types of DFSs, enhancing food safety due to its antilisterial effect [20,21]. The sakacin K antilisterial effect of *L. sakei* CTC494 has been previously tested with *Listeria innocua* coculture in meat simulation media [22], but it is the first time that it is experimented in coculture with the pathogenic *L. monocytogenes*. Sakacin K is a class IIa bacteriocin composed of 40 amino acid residues and a molecular weight of 3802 Da. Its structural gene is located on a 60 Kbp plasmid harboured by the *L. sakei* CTC494 strain. The sakacin K mode of action is similar to other bacteriocins such as sakacin A and curvacin A. These bacteriocins specifically attach and form pores in the membrane of *Listeria*, disrupting the cell homeostasis and thereby reducing cell viability [21,23]. Among the DFS ingredients influencing the behaviour of LAB, glucose and black pepper have shown to promote the growth, while NaCl can reduce their growth. For instance, increasing the amount of salt, from 2 to 3.5% or up to 6%, was reported to interfere with the LAB growth, bacteriocin production or the binding of the bacteriocins to the target microbiota cell membrane receptors [24,25]. In contrast, glucose, as carbon source, assists bacteriocin biosynthesis and bacterial growth [26]. Similarly, black pepper, with a high manganese content (76 ppm), promotes the LAB growth and fermentation of the meat batter [27,28]. Environmental conditions during fermentation, including temperature and sausage ingredients and additives (e.g., salt, nitrite, magnesium, manganese, oxygen, and fat), were reported to influence the growth of *L. sakei* CTC494 and sakacin K production [29,30]. Accordingly, in the DFS production process, ingredients, additives, temperature, and initial microbial communities have cross-related effects on the fermentation; hence [31], that can have an impact on the bacterial growth and metabolism.

Response Surface Methodology (RSM) is a robust and cost-effective approach [32] that allows to study the influence of several factors on bacterial growth response and metabolite production behaviour through a multifactorial experiment [33]. Previous RSM studies have shown reliable inferences when testing the influence of ingredients and additives used in RTE food on the growth and inhibition of *Listeria* spp. [2,33,34]. The optimal application of factors promoting the production of antimicrobials (e.g., bacteriocins and organic acids) and inhibition of pathogens can be used as a tool to improve food safety.

The objective of the present study was to evaluate the effect of factors related with the DFS formulation and production (glucose, manganese, salt, and temperature) on the behaviour of a non-bacteriocinogenic and bacteriocinogenic *L. sakei* strains growing in mono and coculture with *L. monocytogenes*. An RSM approach was applied to identify those combinations enhancing the antilisterial potential of *L. sakei* used as starter culture in DFSs.

2. Materials and Methods

2.1. Experimental Design

The effect of NaCl, manganese (Mn) and glucose (Gluc) concentration and temperature (T) on the behaviour of *L. sakei* and *L. monocytogenes* in monoculture and coculture was evaluated through two complete factorial designs ($\alpha = 2^{3/4}$), using a circumscribed Central Composite Design (CCD) [35], with three independent factors each (Table 1). The central levels of the independent factors were selected regarding the typical concentrations and fermentation temperatures employed on industrial DFS production [20,31]. A total of 40 experimental runs were designed, of which 30 were conducted. Overlapping combinations between CCD1 and CCD2 (10 experiments) were performed only once (Table 2, in italics). For three factors, a second-degree polynomial equation was modelled for each CCD.

Table 1. Independent factors of the circumscribed Central Composite Designs (CCDs) 1 and 2, operating at five levels for each factor, used to evaluate the effect of ingredients and temperature of DFS on *L. sakei* and *L. monocytogenes* behaviour.

Levels [a]	CCD1 Factors			CCD2 Factors
	x1 [b]	x2 [c]	x3 [d]	x2 [c]
−1.6818	0.00	0.08	0.00	3.01
−1.0000	8.18	0.13	8.18	9.90
0.0000	20.18	0.20	20.18	20.00
+1.0000	32.18	0.27	32.18	30.11
+1.6818	40.36	0.32	40.36	37.00

[a] To maintain rotatability and orthogonality, the scaled value for α relative to the coded values ± 1 was 1.68 ($2^{3/4}$). [b] Factor 1 represents the concentration of NaCl (g/L) for CCD1. The corresponding a_w theoretical values regarding NaCl concentration were 1.00 (0.00 g/L), 1.00 (8.18 g/L), 0.99 (20.18 g/L), 0.98 (32.18 g/L) and 0.98 (40.36 g/L) [36]. [c] Factor 2 represents the concentration of Mn (g/L) and temperature (°C) for CCD1 and CCD2, respectively. [d] Factor 3 represents the concentration of glucose (g/L) for CCD1.

Table 2. Concentrations of the evaluated factors according to the CCD1 and CCD2 and observed and predicted values of *L. monocytogenes* reduction (log) and inactivation rate constant (k_d; h^{-1}) when in coculture with the bacteriocinogenic *L. sakei* CTC494.

Experiment	x1	x2	x3	NaCl (g/L)	Mn (g/L)	T (°C)	Gluc (g/L)	Observed Reduction (log) [1]	Predicted Reduction (log) [1]	Observed k_d (h^{-1}) [1]	Predicted k_d (h^{-1}) [1]
1	−1.00	−1.00	−1.00	8.18	0.13	NE [2]	8.18	−3.24	−2.81	−0.12	−0.06
2	−1.00	−1.00	1.00	8.18	0.13	NE	32.18	−4.14	−4.29	−0.17	−0.09
3	−1.00	1.00	−1.00	8.18	0.27	NE	8.18	−4.56	−2.81	−0.20	−0.06
4	−1.00	1.00	1.00	8.18	0.27	NE	32.18	−4.59	−4.29	−0.15	−0.09
5	1.00	−1.00	−1.00	32.18	0.13	NE	8.18	−2.59	−2.39	−0.07	−0.06
6	1.00	−1.00	1.00	32.18	0.13	NE	32.18	−2.37	−3.87	−0.06	−0.09
7	1.00	1.00	−1.00	32.18	0.27	NE	8.18	−3.34	−2.39	−0.08	−0.06
8	1.00	1.00	1.00	32.18	0.27	NE	32.18	−2.64	−3.87	−0.07	−0.09

Table 2. *Cont.*

Experiment	x1	x2	x3	NaCl (g/L)	Mn (g/L)	T (°C)	Gluc (g/L)	Observed Reduction (log) [1]	Predicted Reduction (log) [1]	Observed k_d (h^{-1}) [1]	Predicted k_d (h^{-1}) [1]
9	*0.00*	0.00	*1.68*	*20.18*	0.20	*NE*	*40.36*	*−5.59*	*−5.46*	*−0.13*	*−0.09*
10	*0.00*	0.00	*−1.68*	*20.18*	0.20	*NE*	*0.00*	*0.35*	*−2.96*	*−0.01*	*−0.03*
11	0.00	1.68	0.00	20.18	0.32	NE	20.18	−5.62	−4.21	−0.12	−0.13
12	0.00	−1.68	0.00	20.18	0.08	NE	20.18	−5.16	−4.21	−0.19	−0.13
13	*1.68*	0.00	*0.00*	*40.36*	0.20	*NE*	*20.18*	*−1.75*	*−1.40*	*−0.06*	*−0.04*
14	*−1.68*	0.00	*0.00*	*0.00*	0.20	*NE*	*20.18*	*−2.07*	*−2.11*	*−0.04*	*−0.04*
15	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−5.69*	*−4.21*	*−0.14*	*−0.13*
16	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−5.56*	*−4.21*	*−0.10*	*−0.13*
17	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−5.61*	*−4.21*	*−0.13*	*−0.13*
18	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−3.72*	*−4.21*	*−0.16*	*−0.13*
19	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−3.10*	*−4.21*	*−0.14*	*−0.13*
20	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−4.15*	*−4.21*	*−0.10*	*−0.13*
21	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−3.26	−2.48	−0.02	−0.01
22	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−5.29	−3.97	−0.04	−0.04
23	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.52	−0.89	−0.03	−0.02
24	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.53	−2.37	−0.03	−0.05
25	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−3.26	−2.06	−0.02	−0.01
26	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−2.81	−3.55	−0.02	−0.04
27	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.60	−0.47	−0.02	−0.02
28	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.60	−1.95	−0.02	−0.05
29	0.00	1.68	0.00	*20.18*	NE	37.00	*20.18*	−1.62	0.31	−0.05	−0.02
30	0.00	−1.68	0.00	*20.18*	NE	3.01	*20.18*	−0.02	−2.37	0.00	0.00

[1] *L. monocytogenes* in coculture with the bacteriocinogenic *L. sakei* CTC494. [2] NE: Factor Not Evaluated in this experiment. In italics: Overlapped combinations between CCD1 and CCD2.

2.2. Bacterial Strains and Inoculum Preparation

The bacteriocinogenic strain *L. sakei* CTC494, known for antilisterial activity [21] and sourced from the Institute of Agrifood Research and Technology (IRTA) Food Safety and Functionality Programme culture collection, and the non-bacteriocinogenic strain *L. sakei* 23K [37] were selected for this study. *L. monocytogenes* CTC1034 (serotype 4b), sourced from IRTA's collection, was used as the target pathogen. *L. sakei* and *L. monocytogenes* strains were isolated from DFS and stored at −80 °C in De Man Rogosa and Sharpe broth (MRS, Oxoid, UK) and Brain Heart Infusion broth (BHI, Beckon Dickinson, Sparks, MD, USA), respectively, both supplemented with 20% glycerol as a cryoprotectant.

Before the experiments, *L. sakei* and *L. monocytogenes* strains were cultured in MRS at 30 °C and BHI at 37 °C, respectively, for 8 h. Subsequently, the strains were pre-adapted in Meat Simulation Media (see Section 2.3), supplemented with NaCl (20.18 g/L), Mn (0.20 g/L), and glucose (20.18 g/L) (i.e., to concentrations at the central conditions of the CCD), and then incubated for 16 h at 20 °C.

2.3. Experiments with L. sakei Strains and L. monocytogens in Mono and Coculture in Meat Simulation Media

The experiments with *L. sakei* strains and *L. monocytogenes*, both in mono and coculture, were carried out in Meat Simulation Media (MSM) based on the formulation by Sánchez Mainar et al. [38]. The basic composition per litter included 11.0 g of Bacto proteose peptone No. 3 (Gibco™, Thermo Fisher Scientific, San Diego, CA, USA), 8.8 g of Beef extract (Difco™, Thermo Fisher Scientific, San Diego, CA, USA), 2.2 g of Yeast Extract (Liofilchem, Roseto degli Abruzzi, Italy), 0.038 g of $MnSO_4$:$4H_2O$ (Merck KGaA, Darmstadt, Germany), and 1 mL of Tween 80 (Merck KGaA, Darmstadt, Germany). The pH was adjusted to 5.80, simulating the pH of a DFS meat batter.

Following pre-adaptation, the strains were inoculated into tubes containing 10 mL of MSM with different initial concentration of *ca.* 5 log CFU/mL for *L. sakei* (Ls) strains, within the typical range of concentrations of *L. sakei* starter cultures [20,31], and *ca.* 3 log CFU/mL for *L. monocytogenes* (Lm), a level expected in pork [5], under mono and coculture conditions. For the coculture experiments, the inoculation of *L. sakei* (CTC494 or 23K):

L. monocytogenes resulted in an inoculation ratio of 5:3, corresponding to a logarithmic scale. The set of experiments in both mono and coculture were stored at 20 °C for CCD1 and at 3, 10, 20, 30 and 37 °C for CCD2 (Table 2) during a period from 2 to 28 days.

Samples for microbiological, pH, lactic acid and bacteriocin activity analysis were taken periodically (one tube per sampling time) with a temperature-dependent frequency: every hour for 30–37 °C cultures, every 3 h for 10–20 °C and every 24 h for 3 °C.

2.4. Microbiological Analyses

Microbiological determinations were performed by 10-fold serially diluting the cultures in physiological saline solution (1 g/L of peptone and 8.5 g/L of NaCl). *L. sakei* strains were enumerated in MRS agar plates (de Man, Rogosa and Sharpe; Merck, Darmstadt, Germany) and incubated at 30 °C for 72 h under anaerobic conditions using sealed jars with AnaeroGen sachets (Oxoid Ltd., Altrincham, UK). *L. monocytogenes* was enumerated in CHROMagar™ *Listeria* plates (CHROMagar, Paris, France) incubated at 37 °C for 48 h.

2.5. Physicochemical and Metabolite Determinations

pH values were measured using a puncture electrode model 5232 connected to a portable pHmeter PH25 (Crison Instruments S.A., Alella, Spain). The minimum pH (pH_{min}) was recorded, while the pH reduction was calculated as the difference between the initial pH (5.80) and the pH_{min}.

The determination of lactic acid concentration (LA; [g/L]) was indirectly measured by spectrophotometry directly from the culture supernatant, as by Borshchevskaya (2016) [39]. The coloured product resulting from the reaction of lactate ions with iron (III) chloride was measured at a wavelength of 390 nm.

Sakacin K activity of *L. sakei* CTC494 cultures was analysed through the spot-on lawn test [34,35]. In brief, culture supernatant was pasteurized (10 min at 80 °C), and proteins were precipitated with ammonium sulphate (0.3 g/mL) and stored overnight at 4 °C. The pelleted proteins were then resuspended in phosphate buffer and 2-fold serially diluted. Subsequently, 10 µL drops were placed onto the surface of a semisolid TSAYE overlay (Tryptone Soya agar with 0.6% yeast extract and 7.5 g/L of agar) inoculated with an overnight culture of *L. monocytogenes* CTC1034 in TSBYE (Tryptone Soya broth with 0.6% yeast extract). Plates were incubated overnight at 30 °C for 24 h. The results of sakacin K activity were expressed in arbitrary units (AU/mL), defined as the highest dilution causing a clear inhibition zone on the lawn of the target strain.

The maximum values of lactic acid concentration (LA_{max}) and bacteriocin activity (BAC_{max}) were determined. Yield of lactic acid (Y_{LA}; mg/log CFU·h) and bacteriocin (Y_{BAC}; AU/log CFU·h), expressing the metabolite production as a function of bacterial biomass, were estimated by fitting the modified Luedeking and Piret model [40].

2.6. Assessment of Growth and Inactivation Parameters

Plate counts for *L. sakei* CTC494 and 23 K, as well as *L. monocytogenes* in mono and coculture, were transformed into decimal logarithmic values. The Logistic growth model without delay ($\lambda = 0$) [41] was fitted to the growth curves obtained from the experimental datasets generated by CCD1 and 2, using the MS Excel 2016 Solver add-in (Microsoft, Redmond, WA, USA). This model was used to obtain the kinetic parameters of growth, including the maximum specific growth rate (μ_{max}; h^{-1}) and maximum population density (N_{max}; log CFU/mL). The goodness of fit of the Logistic model was assessed by root-mean-square error (RMSE) and coefficient of determination (R^2), respectively.

The growth potential (i.e., log increase) of *L. sakei* (CTC494 and 23K) and *L. monocytogenes* strains was calculated as the difference between the maximum and the initial bacterial concentrations (log CFU/mL) using concentrations estimated by the Logistic model. For coculture experiments, pathogen inactivation (i.e., log reduction) was calculated as the difference between the maximum and the minimum concentrations (log CFU/mL).

The inactivation rate constant (k_d; h^{-1}) of *L. monocytogenes* in coculture with bacteriocinogenic *L. sakei* CTC494 was estimated as the slope of the first-order equation fitted to the log counts of the pathogen between 12 and 36 h, the interval of time in which *L. monocytogenes* showed a linear decrease.

2.7. Response Surface Methodology and Data Analysis

The effects of the independent factors (i.e., NaCl, Mn, glucose, and temperature) on the dependent physicochemical (i.e., pH_{min}, LA_{max}, Y_{LA}, BAC_{max} and Y_{BAC}) and microbiological factors (i.e., μ_{max}, N_{max}, growth potential, log reduction and k_d) were assessed by the Response Surface Methodology. The JMP v16.0.0 software (SAS Institute Inc., Cary, NC, USA) was used to fit a polynomial model for each response applying a stepwise regression approach, with forward direction and combined rules of the Bayesian Information Criteria (BIC) to achieve a parsimonious model with only statistically significant factors (p-value < 0.05). This methodology allowed the evaluation of linear, quadratic, and interactive effects of independent factors on microbial behaviour (dependent factors). Statistic tests of lack of fit, summary of fit, and Analysis of Variance (ANOVA) were used to evaluate the goodness of fit of polynomial equations and test significant differences (p-value < 0.05). The significance of the regression model and estimated parameters were evaluated by the lack-of-fit test. Two- and three-dimensional surface plots were drawn to illustrate the effect of the independent factors on significant models with insignificant lack of fit. Differences between the mono and cocultures responses were calculated using the t-test within the replicates of the central level of CCD.

3. Results and Discussion

The present study evaluated the effect of NaCl (0, 8.18, 20.18, 32.18 and 46.36 g/L), Mn (0.08, 0.13, 0.20, 0.27 and 0.32 g/L), glucose (0, 8.18, 20.18, 32.18 and 40.36 g/L) and temperature (3, 9.9, 20, 30 and 37 °C) (Table 1) on the physicochemical parameters (pH_{min}, LA_{max}, BAC_{max}, Y_{LA} and Y_{BAC}) and microbiological parameters (μ_{max}, N_{max}, growth potential, log reduction and k_d) associated with the behaviour in mono and coculture of *L. sakei* (CTC494 and 23K) and/or *L. monocytogenes*. The factor's impact main results on mono and coculture are described in the following sections, while detailed results are reported in Supplementary Table S1. All polynomial equations and the results of statistic tests (i.e., lack of fit, summary of fit and ANOVA) for *L. sakei* and *L. monocytogenes* strains are described in Supplementary Table S2.

3.1. Assessment of the Effect of NaCl, Mn, Glucose, Temperature and Coculture in the Acidification and Bacteriocin Production in Meat Simulation Media

3.1.1. pH Decrease and Lactic Acid Formation

The decrease in pH values and the production of lactic acid depended on the bacterial species and were clearly influenced by the NaCl and glucose concentrations in the medium and the incubation temperature. Sodium chloride has been described to interfere with bacterial growth due to its role as an a_w-lowering agent [25], and glucose is the main ATP-energy source for LAB fermentation and is fully converted to lactate by homofermentative *L. sakei*. Temperature modulates cell growth and, consequently, the use of the nutrient resources available in the media [42].

The strongest acidification, representing a decrease of 2.0 pH units from an initial pH of 5.80, was observed for both *L. sakei* strains (CTC494 and 23K) in mono and coculture with *L. monocytogenes*, reducing the pH of the medium to pH_{min} values of *ca.* 3.80 at glucose concentrations $\geq$ 8 g/L (i.e., all experiments except for experiment 10) (Figure 1A). Specifically, the lowest pH_{min} was achieved when the glucose concentration was the highest (40 g/L) by the strains *L. sakei* 23K and CTC494 both in mono and coculture, with pH_{min} ranging from 3.6 to 4.0 (reduction of *ca.* 2.0 pH units). When glucose was not added (i.e., experiment 10), the highest pH_{min} was achieved by *L. sakei* 23K (pH of 5.1) and *L. sakei* CTC494 (pH of 5.4), showing significant differences with the lowest values (p < 0.05). The

same results were observed in coculture experiments (pH_{min} *ca.* 5.1), resulting in a reduction of only *ca.* 0.7 pH units and the lowest LA_{max} concentrations (*ca.* 0.55 g/L), as detailed in Supplementary Table S1. Conversely, in *L. sakei* monoculture experiments with higher NaCl concentration (32.18 g/L) at 20 °C (experiments 5, 6, 7 and 8), the pH_{min} values were slightly higher, *ca.* 4.00, representing a reduction of 1.8 pH units (Figure 1A). Barbieri et al. (2022) [43] showed that in fermentations of *L. sakei* in defined liquid medium (initial pH of 6.50), when glucose was not a limiting factor, the pH was reduced by 2.6 units, whereas when glucose was limiting, the reduction of pH was only 0.5 units.

In contrast, *L. monocytogenes* reached pH_{min} values in MSM of *ca.* 4.4, with the exception of experiment 10, where glucose was not added and the pH_{min}, with a value of 5.1, was the highest, corresponding to a reduction of 0.7 pH units (Figure 1A). Different pH reductions were due to the different availability of glucose, and when it was limiting (i.e., not added to the MSM), there was a lower production of lactic acid and, therefore, a less pronounced reduction in pH. On the contrary, when glucose was not a limiting factor (i.e., present in sufficient quantity, such as 40 g/L), the metabolic activity of LAB and the pathogen strains was accelerated, and a more significant reduction in the pH of the MSM was observed. Glucose is a fermentable sugar used by bacteria to generate lactic acid as a fermentation metabolite that promotes a pH drop. Specifically, for homofermentative bacteria (e.g., *L. sakei*), lactic acid is obtained as the sole product of glucose metabolization (i.e., 1 mole of glucose yields 2 moles of lactic acid and 2 moles of ATP, or 1 g of lactic acid per 1 g of substrate) [44].

As expected, higher LA_{max} values were registered when higher pH reductions occurred and lower pH_{min} were observed (Supplementary Table S1), as previously shown for *L. sakei* CTC494 [42]. On the contrary, and similar to the pH_{min} results, the lowest values of LA_{max} were achieved when glucose was not added (0.5, 0.7 and 0.6 g/L of LA_{max} for *L. sakei* 23K, CTC494 and *L. monocytogenes*, respectively). *L. sakei* strains produced more LA (>3 g/L) than *L. monocytogenes* (*ca.* 1.5 g/L) ($p < 0.001$) (Figure 1A). This fact could be related to metabolic differences between *L. sakei* and *L. monocytogenes*. *L. sakei* strains are homofermentative, performing glycolysis as the most efficient way to produce energy (i.e., ATP), and LA is the main fermentation product. In contrast, *L. monocytogenes* also produces ethanol and formate as products of its metabolism [45]. In the coculture experiments, the same observation (i.e., higher reductions in LA_{max} and pH in *L. sakei* monocultures) was made due to the acid produced by *L. sakei* strains with no acidification differences regarding the *L. sakei* strain in coculture ($p > 0.05$). However, coculture of the 23K strain and *L. monocytogenes* often showed higher values (e.g., 5.86 g/L of LA_{max} concentration in experiment 4) than the CTC494 coculture (e.g., 5.09 g/L of LA_{max} concentration in experiment 4) [45].

Overall, the highest LA_{max} and pH reductions registered by both coculture experiments were similar to those observed by monoculture (>3 g/L and a decrease of 2 pH units) and slightly higher than those of *L. monocytogenes* monoculture (*ca.* 1.5 g/L and 1.5 pH units) ($p < 0.01$) (Figure 1A). In the central point experiment runs, no coculture effect was observed, and similar pH_{min} (i.e., 3.72 ± 0.01 and 3.75 ± 0.03, for the 23Kco and CTC494co, respectively) and LA_{max} (i.e., 4.47 ± 0.12 g/L and 4.12 ± 0.32 g/L) were similar to those obtained in monoculture experiments for *L. sakei* strains ($p > 0.05$) (Figure 1A). These results indicated that simultaneous growth of *L. sakei* with *L. monocytogenes* had no relevant effect on acidification.

The RSM showed in experiments with *L. sakei* strains that glucose and NaCl had quadratic and linear effects, respectively, on LA_{max} values, whereas in coculture with *L. monocytogenes*, only glucose had a quadratic effect ($p < 0.05$) on the pH_{min} and LA_{max} concentrations (see polynomial equations coefficients in Supplementary Table S2). LA yield (Y_{LA}) was influenced by glucose with linear or quadratic effects for all strains. However, the polynomial models obtained showed poor goodness of fit to observed Y_{LA} data (Supplementary Table S2). In food fermentations, high LA production is desirable from a food safety perspective, and, in DFS, the antilisterial effect of LAB has been linked to the antimicrobial effect of LA (i.e., pH drop) [20].

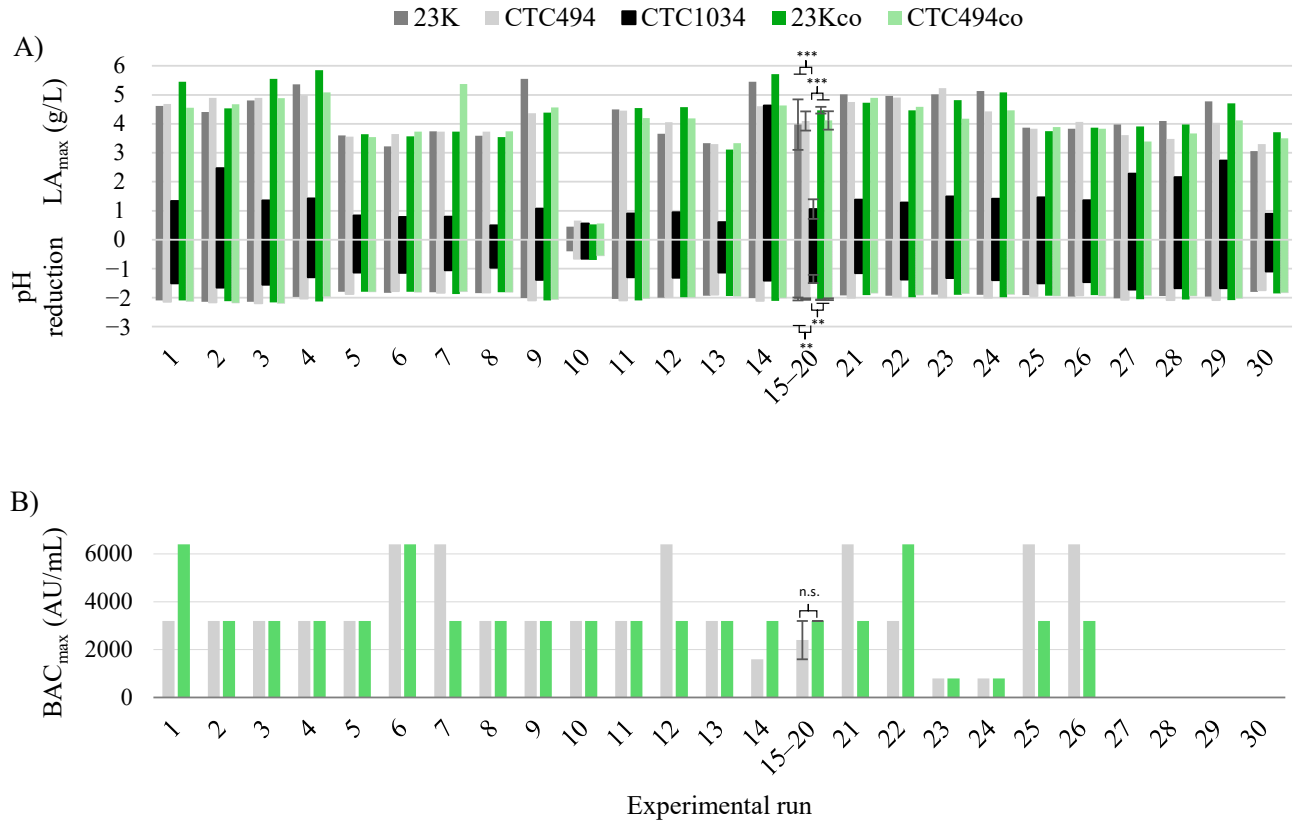

Figure 1. Maximum concentration of lactic acid (LA_{max}) and pH reduction of *L. sakei* 23K, CTC494 and *L. monocytogenes* CTC1034 in monoculture and in coculture with *L. sakei* 23K (23Kco) and *L. sakei* CTC494 (CTC494co) (**A**), and maximum bacteriocin activity (BAC_{max}) registered by *L. sakei* CTC494 (**B**). Central points (experiments 15–20) are expressed as mean and standard deviation of 6 replicates, and statistical significance is indicated: *p*-value $p < 0.01$ (**), $p < 0.001$ (***), $p > 0.05$ (non-significant, n.s.).

3.1.2. Sakacin K Activity

The main factors affecting bacteriocin production by *L. sakei* CTC494, measured as maximum activity recorded during the experiment (i.e., BAC_{max}), were temperature and Mn concentration (g/L). Leroy and de Vuyst [30] studied the effect of Mn limitation, which resulted in a considerable decrease in cell growth and lactic acid and bacteriocin production. The most favourable temperatures for bacteriocin activity in monoculture experiments were moderate (i.e., 10 and 20 °C), reaching a maximum activity of 6400 AU/mL in experiments 6, 7, 12, 25 and 26, whereas in coculture the higher BAC_{max} values were observed in experiments 1, 6 and 22 (Figure 1B), indicating that there were no consistently higher values in coculture. Consequently, sakacin K production by *L. sakei* CTC494 was not stimulated by the presence of the pathogen, although induction of bacteriocin production has been reported in *Lactiplantibacillus plantarum* strains. This trait may be associated with a quorum sensing-related mechanism involving cell-to-cell contact and is therefore specific to the strain and determined by the bacteriocin production regulatory operon [46].

BAC activity was detected at the late exponential phase (t = 15 h) and in general increased to reach its maximum (BAC_{max} of 3200 AU/mL) at the early stationary phase (see Figure 2 as an example). However, when experiments were conducted at higher temperatures (i.e., 30 and 37 °C), sakacin K activity was ≤800 AU/mL for experiments 23, 24, 27, 28 and 29, while at low temperature (i.e., 3 °C; experiment 30), no *BAC* activity was detected (Figure 1B). Previous studies with the CTC494 strain showed that at pH 6.5, the optimal temperature for *BAC* production was between 20 and 25 °C and was undetectable at 34 °C [29]. Furthermore, it was observed that at NaCl concentrations of 2%, *L. sakei* bacteriocin activity was decreased [25], and above 8% of NaCl, the sakacin

K activity was not detected [29]. For another *L. sakei* strain, CCUG 42687, the maximum sakacin P production was also reported at 20 °C, with a concentration seven times higher than at 30 °C [47]. Temperatures for some fast-fermented European-style DFS fermentation coincide with the highest values of bacteriocin activity, maximising the antilisterial effect of the bioprotective starter culture. At temperatures higher than 25 °C, although cells grow faster, bacteriocin activity drops significantly due to the combined effect of a lower specific productivity and a higher bacteriocin degradation rate, and cell yield decreases as the energy needed for maintenance becomes more important [42].

The concentrations of Mn and NaCl (CCD1) showed a significant effect ($p < 0.05$) on BAC_{max} and Y_{BAC}, which was also influenced by temperature (CCD2) (Supplementary Table S2). Y_{BAC} in coculture also showed similar yields to those obtained in monoculture, with values <300 AU/log CFU·h at extreme temperatures (3 °C ≤ and ≥ 30 °C), while at tested star concentrations of Mn (i.e., experiments 11 and 12), Y_{BAC} was >2000 AU/log CFU·h (Supplementary Table S1). Polynomial models for Y_{BAC} highlight temperature and Mn as the main factors influencing sakacin K production with quadratic effects (Supplementary Table S2). Leroy and De Vuyst [42] observed that the production of sakacin K by *L. sakei* CTC494 was highly influenced by pH and that the optimal temperature and pH for growth were different from those maximising bacteriocin production. Specifically, the optimum temperatures and pH values for *L. sakei* CTC494 growth were reported to be 33.5 °C and 6.15, while a pH of 5.0 and 23 °C were the most favourable conditions for bacteriocin production [30,42]. Enhancing the production of lactic acid and sakacin K can be used not only to maximise the antilisterial properties of *L. sakei* CTC494 when applied as a bioprotective culture in foods but also to increase the antimicrobial activity of the strain prepared as a postbiotic (i.e., microbial cells inactivated with/without metabolites) [48].

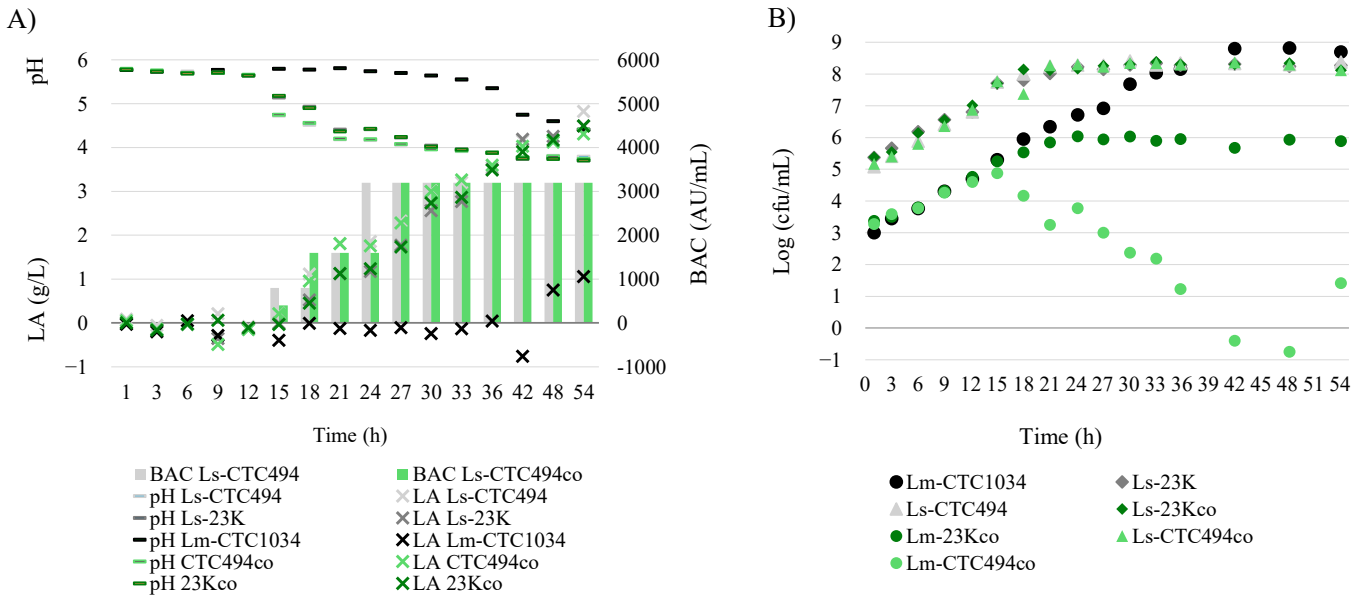

Figure 2. Physicochemical parameters (pH, lactic acid (*LA*) and bacteriocin activity (*BAC*)) (**A**) and microbial counts (**B**) of strains in mono- and coculture of experiment run 9 (star point of glucose, 20 g/L of NaCl, 0.20 g/L of Mn, 40 g/L of glucose at 20 °C). Ls: *L. sakei*, Lm: *L. monocytogenes*, co: coculture.

3.2. Impact of NaCl, Mn, Glucose, Temperature and Coculture in the Behaviour of L. sakei and L. monocytogenes

3.2.1. Bacterial Growth under DFS Fermentation Conditions in MSM

Microbiological parameters (μ_{max}, N_{max} and growth potential) of the strains were highly influenced by temperature. In both mono- and coculture, the highest and the lowest μ_{max} values were observed at 37 °C and at 3 °C for the two *L. sakei* and *L. monocytogenes* (around 1.0 h^{-1} at 37 °C and 0.025 h^{-1} at 3 °C) (Figure 3A in monoculture; Figure 3D,G,H in

coculture; Supplementary Table S1), which is in agreement with previous studies evaluating the effect of temperature on the growth of *L. sakei* CTC494 [42].

In monoculture experiments, the N_{max} ranged between 7.5 and 8.7 log CFU/mL for *L. sakei* strains and were slightly higher (8.4–9.1 log CFU/mL) for *L. monocytogenes* (Supplementary Table S1). Previous studies in cooked ham also showed higher N_{max} values for *L. monocytogenes* CTC1034 than *L. sakei* CTC494 [19]. NaCl, Mn, glucose and temperature had a microbiologically and statistically relevant effect on N_{max}, although they were only significant for the CTC494 CCD1 model (Figure 3B).

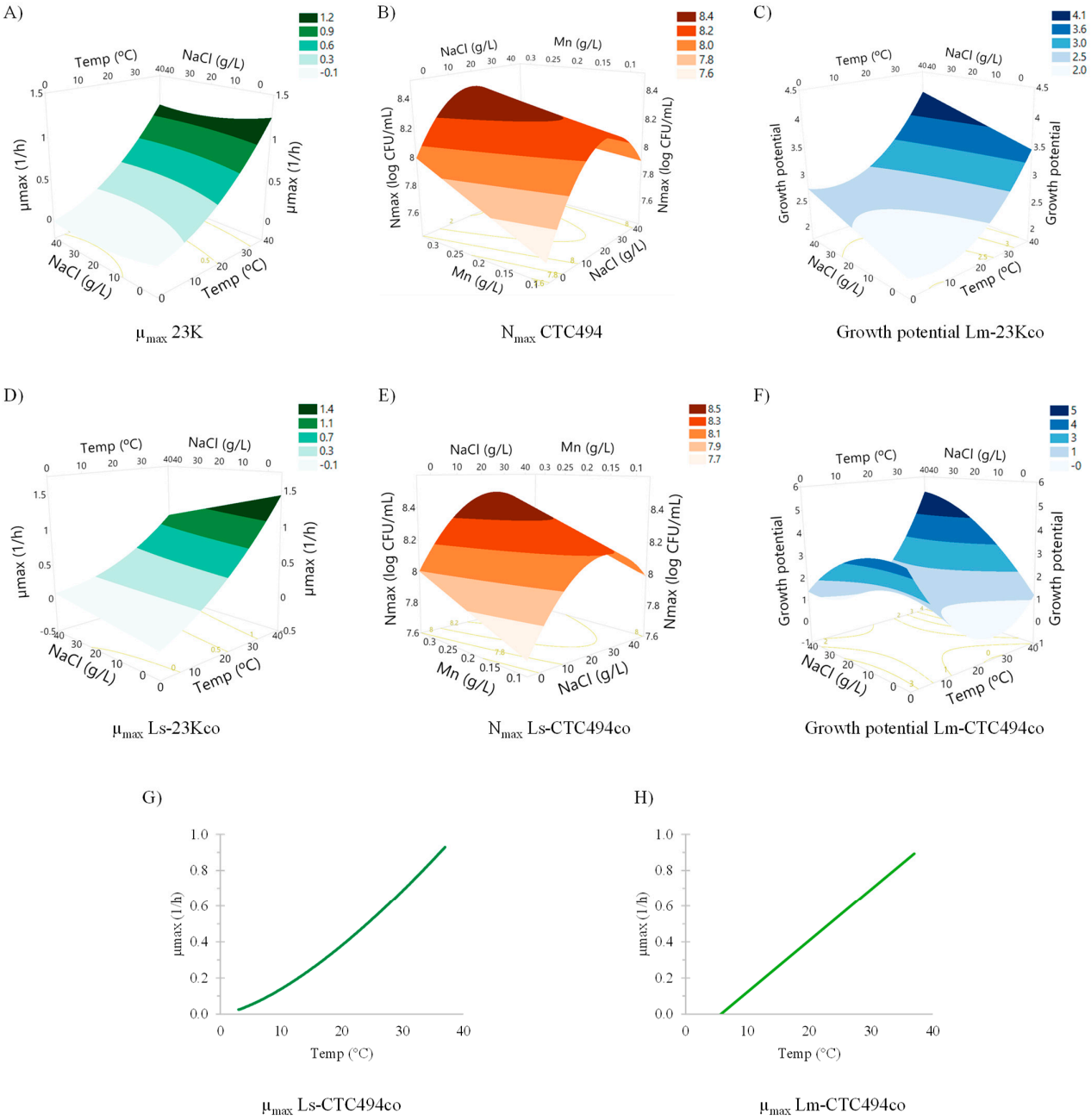

Figure 3. Microbiological parameters with significant effects response to independent factors. In monoculture, μ_{max} of *L. sakei* 23K (**A**) and N_{max} of *L. sakei* CTC494 (**B**). In coculture, growth potential of *L. monocytogenes* CTC1034 in coculture with *L. sakei* 23K (Lm-23Kco) (**C**), μ_{max} of *L. sakei* 23K in coculture (Ls-23Kco) (**D**), N_{max} of *L. sakei* CTC494 in coculture (Ls-CTC494co) (**E**), growth potential of *L. monocytogenes* CTC1034 in coculture with *L. sakei* CTC494 (Lm-CTC494co) (**F**), μ_{max} of Ls-CTC494co (**G**) and of Lm-CTC494co (**H**).

Overall, the growth potential of the strains was affected by the independent factors NaCl and glucose, with temperature having the most remarkable effect. In monoculture experiments with *L. sakei* strains, growth potential ranged from 2.4 to 5.1 for 23K and 2.3 to 3.8 for CTC494, with the highest values observed in experiments with low salt concentrations and intermediate temperatures (5.1 log for 23K in experiment 3 and 3.8 log for CTC494 strains in experiment 23). In contrast, in monoculture, *L. monocytogenes* exhibited higher growth potential, ranging from 4.8 to 6.0 log, attributed to its lower initial concentration and higher N_{max} compared to *L. sakei* (Figure 4). Coculture did not affect *L. sakei* N_{max}, as shown for CTC494 strain in Figure 3B,E. This is in agreement with other experiments with this strain in cooked ham [19] and the strain *L. sakei* 706 (sakacin A producer) in MRS broth [49].

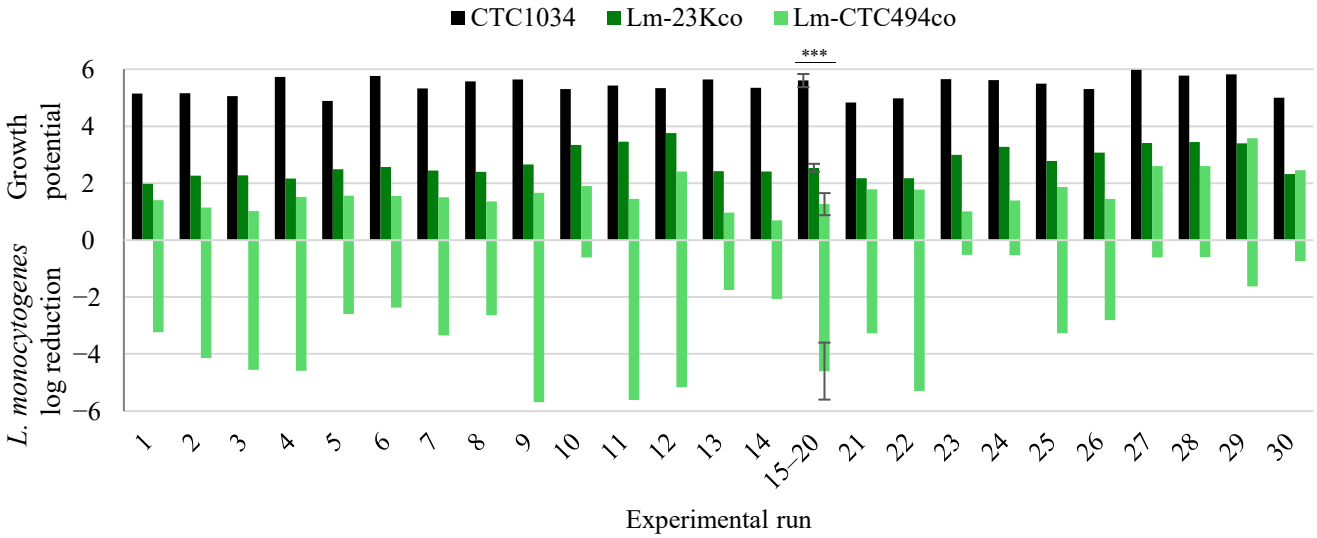

Figure 4. Growth potential of *L. monocytogenes* CTC1034 in monoculture and coculture with *L. sakei* 23K (Lm-23Kco) and CTC494 (Lm-CTC494co) and log reduction of *L. monocytogenes* in coculture with *L. sakei* CTC494 (Lm-CTC494co). Growth potential statistical significance ($p < 0.001$; ***) in central points (experiments 15–20) is indicated.

However, it is remarkable that coculture with *L. sakei* strains considerably reduced N_{max} of *L. monocytogenes* ($p < 0.001$), especially the bacteriocinogenic CTC494 strain (Figure 4). Specifically, *L. monocytogenes* reached N_{max} values ranging from 4 to 7 log CFU/mL in coculture with *L. sakei* strains, while in monoculture experiments, the pathogen always exhibited $N_{max} > 8.4$ log CFU/mL (Supplementary Table S1).

Multiple factors may be involved in interactions between bacterial populations in the same ecosystem, including competition for nutrients, production of metabolites (e.g., organic acids and bacteriocins), signalling molecules and cell-to-cell contact mechanisms [50]. The results in coculture experiments showed that inhibition of *L. monocytogenes* growth occurred when the dominant population, i.e., *L. sakei* 23 K, reached its N_{max}, leading to an early entry of the pathogen into the stationary phase. This phenomenon is known as the Jameson effect [51], which has been observed by several authors studying non-bacteriocinogenic competition between *L. sakei* and *L. monocytogenes* in broth media simulating DFS fermentation conditions [52–54]. On the other hand, the bacteriocinogenic strain CTC494 not only inhibited the growth of *L. monocytogenes* but also promoted its inactivation by more than 5 log units. Similar results were observed for the non-pathogenic *Listeria innocua* by Leroy et al. [47] in broth. The different behaviour of *L. monocytogenes* in coculture with 23K and CTC494 strains is shown in Figure 2B, which provides a representative example of the microbial kinetics together with the corresponding pH, *LA* and *BAC* production profiles (Figure 2A).

Considering the impact of the evaluated factors on the coculture experiments between *L. sakei* CTC494 and *L. monocytogenes*, the highest N_{max} (5.5 log CFU/mL) for the pathogen was observed at extreme temperatures of 3 and 37 °C, and at 30 °C with high NaCl concentration of 32.18 g/L (Supplementary Table S1). These conditions correspond to those combinations of factors that did not favour bacteriocin production (i.e., experiments 23, 24, 27, 28, 29 and 30) (Figure 1B). Accordingly, the growth potential of *L. sakei* and *L. monocytogenes* in coculture was also influenced by temperature and NaCl ($p < 0.05$), and in the case of *L. monocytogenes*, it was significantly reduced ($p < 0.001$) by the presence of the dominant population, i.e., *L. sakei*, especially in experiments where the CTC494 strain produced bacteriocin (Figure 3C,F). More specifically, *L. monocytogenes* growth potential in monoculture was above 5 log and decreased, depending on the experimental conditions, to 1.4–3.5 log in coculture with *L. sakei* 23K and to 0.9–3.9 log in coculture with *L. sakei* CTC494 (Figure 4). In contrast, for both *L. sakei* strains, experiments at the lowest temperature (3 °C), at the highest temperature (37 °C), and at high temperature and high NaCl (30 °C and 32 g/L), the lowest reductions in the growth potential of *L. monocytogenes* (<1 log) were observed (Figure 4).

3.2.2. *L. monocytogenes* Inactivation by the Sakacin K Producer *L. sakei* CTC494

L. monocytogenes inactivation was only observed in the presence of *L. sakei* CTC494, with reductions ranging from −0.6 to −5.7 log. The lowest reductions correlated with experiments with lower BAC (i.e., experiments 23, 24, 27 and 28) (Figures 4 and 1B, respectively). The promotion of *L. sakei* CTC494 by glucose or Mn at 20 or 10 °C (e.g., experiments 9 and 11) enhanced inactivation of the pathogen and reduced N_{max} the most (i.e., >5.2 log). The mechanism explaining this phenomenon would be related with the model of action of sakacin-type bacteriocins, which specifically targets *Listeria* spp. by interacting with bacterial cell surface and cell membrane and forming pores, leading to cell permeabilization and ultimately to cell death [17,55]. Specifically for experiment 9, the highest reduction was observed with the maximum glucose concentration, which increased *LA* and sakacin K production. Similarly, high inactivation of *L. monocytogenes* was also observed by Pleasants et al. [49] in coculture experiments with *L. sakei* 706, a sakacin A producer, under conditions of 20 °C and pH 7 in MRS broth.

The highest pathogen inactivation rate (k_d; h^{-1}) in coculture with CTC494 was $-0.20\ h^{-1}$, at 20 °C, with 8 g/L of NaCl and glucose and 0.27 g/L of Mn (experiment 3). Under these conditions, the k_d coincided with the highest reductions in pH, high LA production (*ca.* 5 g/L) (Figure 1A) and a reduction in the growth potential of *L. monocytogenes* by approximately 1 log compared to the pathogen in monoculture (e.g., 5 log) (Figure 4).

Experiments 9 and 3 reinforce that the combination of mild temperature (20 °C) and high glucose (≥32 g/L) determines the best strategy to inactivate the pathogen. As shown in Figure 5, the combined concentrations of NaCl (i.e., 10 to 20 g/L), glucose (i.e., 20 to 30 g/L) and temperature (i.e., 10 to 20 °C) had a statistically significant effect on pathogen inactivation in terms of inactivation rate and log reduction. Usual formulation of DFSs (g per kg of meat) consists of NaCl (20), maltodextrin (20), pepper (3) (i.e., Mn (0.228)) and ripening temperature of 13 °C [31], coinciding with central values of the studied factors. The obtained results show that low NaCl (i.e., 0.8% [*w*/*v*]) and abundant glucose and Mn availability in the media at mild temperature (i.e., 20 °C) enhance bacteriocin activity, therefore *L. monocytogenes* inactivation.

The polynomial equation indicated that the highest predicted pathogen reduction (−5.5 log) and k_d ($-0.13\ h^{-1}$) would be achieved at the optimal combinations of 40.36 and 20.18 g/L of glucose, respectively, together with 20.18 g/L of NaCl at 20 °C (Table 2). The analysis also showed two and three quadratic two-way interactions for NaCl ($p < 0.05$) and temperature ($p < 0.01$) for *L. monocytogenes* reduction and for all independent factors ($p < 0.01$) for k_d (Supplementary Table S2).

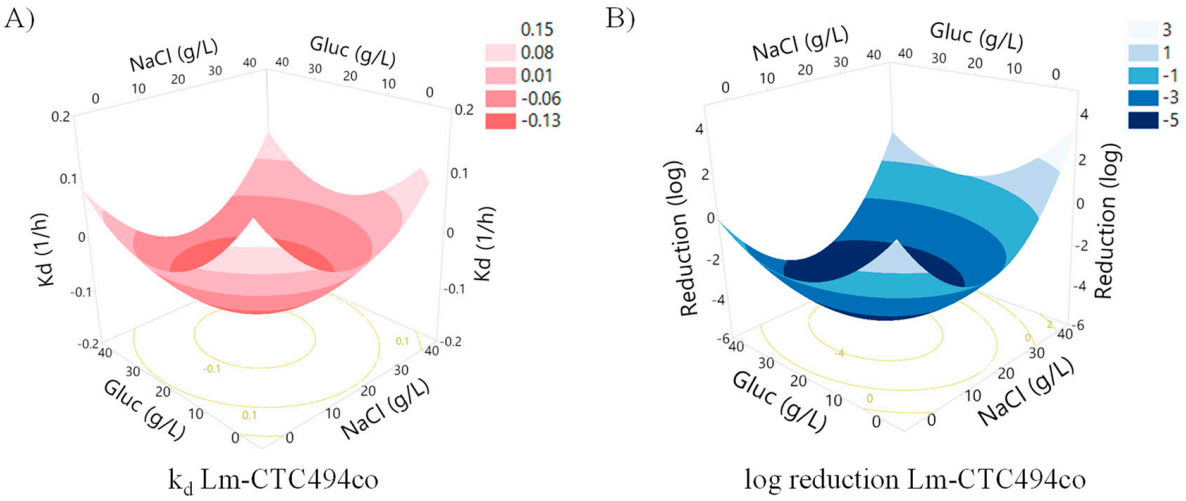

Figure 5. Inactivation parameters of *L. monocytogenes* in coculture with the bacteriocinogenic *L. sakei* CTC494 showing significant effects in response to independent factors. (**A**) Inactivation rate (k_d; h^{-1}). (**B**) log reduction.

The optimal conditions to increase pathogen inactivation mainly depended on glucose concentration, as shown by the growth potential. In this regard, Figure 4 shows that the lowest pathogen growth also resulted in higher inactivation rates, such as in experiment 9 (with a maximum glucose concentration of 40 g/L). However, the combination of NaCl and glucose have also shown an effect on sakacin K production and, consequently, on the pathogen inactivation. In agreement, Leroy and De Vuyst [30] showed that when high glucose was combined with 10–20 g/L of NaCl, increased *L. innocua* inactivation rates were observed. Additionally, as temperature also determined the inactivation rate, sakacin K amounts have been reported to be enhanced at 20 °C in comparison to higher temperatures of 30 °C. Temperatures $\leq$ 20 °C are recommended to promote the antilisterial potential of bacteriocinogenic strains in DFSs [30].

4. Conclusions

The inhibition of *L. monocytogenes* through the application of *L. sakei* represents a control measure to enhance the food safety of dry fermented sausages as this species reduces the growth potential of the pathogen through the reduction of N_{max}. However, only the application of the bacteriocinogenic *L. sakei* CTC494 has an antilisterial effect, providing an additional level of control by the reduction in the pathogen population. At mild temperatures around 20 °C and formulations promoting *L. sakei* growth (such as high glucose concentrations and Mn), together with lactic acid and sakacin K production, the inactivation of *L. monocytogenes* was enhanced, resulting in a reduction of more than 5-log units.

The safety of DFSs must be achieved through reduction of the levels of *L. monocytogenes* during the production process. The findings of this study underscore the critical role of starter cultures within the framework of the hurdle technology. Furthermore, their efficacy can be enhanced by application under optimal conditions for *L. sakei* growth and/or bacteriocin production.

The response surface methodology was a useful approach to identify the most appropriate temperature, NaCl and glucose conditions, taking into account the complex interactions between multiple variables to increase the inactivation of *L. monocytogenes* in meat simulation media in coculture with *L. sakei* CTC494. Further research needs to be performed on a real DFS food matrix to confirm the optimal conditions of application as a useful strategy for DFS manufacturers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060326/s1, Table S1: Experimental and predicted data for each experiment run of CCDs 1 and 2; Table S2: Polynomial equations and Response Surface statistical parameters.

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Conflicts of Interest: The authors declare no conflicts of interest.

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