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Research Paper

Effects of Fermentation Temperature, Drying Temperature, Caliber Size, Starter Culture, and Sodium Lactate on *Listeria monocytogenes* Inactivation During Salami Production

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ABSTRACT

The effect of fermentation and drying temperatures, caliber, and sodium lactate on *Listeria monocytogenes* inactivation was studied in salami, produced in a pilot scale, inoculated with 10^7 CFU/g *of Listeria innocua* ATCC® 33090 as a surrogate microorganism for *L. monocytogenes*. Fermentation temperature varied between 24 and 30°C, drying temperature between 14 and 20°C, caliber between 5.1 and 13.2 cm, and sodium lactate initial concentrations in salamis were 0 and 2%. *L. innocua* counts, pH and water activity were determined in salamis over time. Sodium lactate (2%) decreased pH drop and *Listeria* inactivation during fermentation. Baranyi & Roberts equation was used to fit the experimental data and to estimate, for each test condition, inactivation rate (k), initial (Y_0), and final counts of *L. innocua* (Y_{END}). Total inactivation was calculated as Y_0 minus Y_{END} (Y_0-Y_{END}). Then, using a Box Benkhen experimental design, a quadratic model for k and a two-factor interaction model (2FI) for $Y_0 - Y_{END}$ were obtained as functions of fermentation temperature, drying temperature, and caliber size. The models predicted that maximum k and $Y_0 - Y_{END}$, -2.62 ± 0.14 $\log_{10}$ CFU/g/day and 4.5 ± 0.1 $\log_{10}$ CFU/g, respectively, would be obtained fermenting at 30°C and drying at 20°C regardless of caliber. Drying at 14°C allowed *Listeria* growth until a water activity (a_w) of 0.92 was reached. Therefore, if initial *Listeria* contamination is high (3 $\log_{10}$ CFU/g), drying at low temperatures will compromise product safety.

Listeria monocytogenes is a human pathogenic bacterium that may cause listeriosis, a foodborne infection with a low morbidity and a high mortality rate (20–30%) (Buchanan et al., 2017; Lomonaco et al., 2015; Rees et al., 2017). *Listeria monocytogenes* is commonly found in meat and meat products such as salami (Braga et al., 2017; Doménech et al., 2015; EFSA & ECDC, 2021; Meloni, 2015; Mor-Mur & Yuste, 2010; Nightingale et al., 2006; Skandamis & Gounadaki, 2006).

In salami production, lactic acid fermentation (Ordóñez et al., 1999) is used as a preservation method through pH reduction and competitive exclusion in a medium containing between 2% and 3% of sodium chloride (Giello et al., 2018; Rust, 2015). Once a formulation has been established, the fermentation and drying conditions will determine *L. monocytogenes* growth, conditioning the decrease in pH and water activity (a_w) (Leistner, 2000) until reaching the restrictive values (pH ≤ 4.4 or a_w ≤ 0.92 or pH ≤ 5.0 and a_w ≤ 0.94) for *Listeria* growth (International Commission on Microbiological Specifications for Foods (ICMSF), 2005). Reaching these conditions will depend mainly on temperatures and relative moisture used during fermentation and drying (Casaburi et al., 2008), starter culture characteristics (Drosinos et al., 2006; Martín et al., 2022), sugars available for the fermentation, and the use of pH regulators (Girard, 1991; Pellicer et al., 2011) such as sodium lactate (Degenhardt & Anna, 2007; Pellicer et al., 2011). The addition of sodium lactate to salami delays bacterial growth by reducing the intracellular pH. This is because lactic acid, a weak acid, can diffuse across the cell membrane in its undissociated form, dissociate inside the cell, and acidify the cytoplasm (Shelef, 1994). Several commercial salami brands produced in the region con-

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tain sodium lactate, and the amount typically added is 2% (Degenhardt & Anna, 2007).

Starter cultures are selected microorganisms that beneficially influence the fermentation or maturation process. They usually include *Staphylococcus carnosus* and *Stapphylococcus xylosus* to impart the desired sensory characteristics to the final product (Casaburi et al., 2007; Stekelenburg & Kant-Muermans, 2001) and lactic acid bacteria (LAB) that produce bacteriocins with listericidal activity (Castellano et al., 2008; Martín et al., 2022; Nielsen et al., 1990; Olaoye et al., 2011). The LAB species most used to control *L. monocytogenes* growth in fermented sausages are *Pediococcus acidilactici*, *Lactobacillus sakei,* and *Lactobacillus curvatus* (Drosinos et al., 2006), the last two were renamed as *Latilactobacillus sakei* and *Latilactobacillus curvatus* by Zheng (Zheng et al., 2020). LAB listericidal activity varies according to the starter culture composition and the environmental conditions (Cosansu et al., 2010; de Souza Barbosa et al., 2015; Leroy & De Vuyst, 1999). Sodium lactate has been reported to retard the growth of many microorganisms, including *L. monocytogenes* (Houtsma et al., 1993; Shelef, 1994; Weaver & Shelef, 1993) and LAB (Deumier & Collignan, 2003; Lin & Lin, 2002; Stekelenburg & Kant-Muermans, 2001; Wang, 2000).

Salami fermentation temperature directly affects LAB growth rate, lactic acid production rate, and consequently pH drop rate. Some authors report that at higher fermentation temperatures, *Listeria* growth probability is greater (Brusa et al., 2021).

In Uruguay, fermentation temperatures range between 24 and 29°C and relative air humidity between 80% and 95% (Repiso, 2018). In this stage, since temperatures exceed 60°F (15.5°C), a maximum time in hours is established to reduce pH to 5.3 in order to limit *E. coli* O157:H7 and *S. aureus* growth (American Meat Institute Foundation (AMI), 1997). Therefore, a rapid pH reduction during fermentation is important not only for the control of *L. monocytogenes* but also for other pathogens.

After fermentation, the a_w decreases during the drying stage. In order to inhibit *Listeria* growth in the final product, the a_w must be less than 0.92. The time to reach this final value will depend on several factors, including the temperature and speed of the air in the drying chamber and on the salami caliber size. To reach the same a_w value, larger caliber sizes require longer drying periods (De Souza et al., 2018). Longer drying times result in greater *L. monocytogenes* reduction in salami (Hussein et al., 2022).

National *L. monocytogenes* regulation for salami establishes that the pathogen must be absent in 25 g sample (Decreto 588/008, 2008). However, the presence of *L. monocytogenes* in salami has been reported (Braga et al., 2017; Intendencia Municipal de Montevideo (IMM), 2023), suggesting that on occasions, the fermentation and drying processes were not able to eliminate this pathogen. Therefore, it is necessary that the salami production process be optimized. USDA-FSIS regulatory guidance indicates that validation studies for salami processing conditions must demonstrate that the process is capable of achieving a reduction of *L. monocytogenes* greater than 3 log (Hussein et al., 2022).

The strategy of obtaining experimental data using an experimental design followed by modeling approaches using the Response Surface Methodology (RSM) is presented as a useful tool to study and understand the behavior of *Listeria monocytogene*s and other pathogens during food processing (Brugnini et al., 2021; Dogan et al., 2022; Mataragas et al., 2006; Rosario et al., 2021). In turn, obtaining experimental data contributes to improving the available predictive models (Brusa et al., 2021; Polese et al., 2017).

Therefore, the aim of the present work was to study and model the effect of fermentation and drying temperature, caliber size, starter cultures, and the use of acidity regulators on the reduction of *L. monocytogenes* during the typical production of Uruguayan salami. For this purpose, a challenge study will be conducted using pilot-scale salami production inoculated with *Listeria innocua* as a surrogate microorganism for *L. monocytogenes* (Friedly et al., 2008; Tirloni et al., 2019). The study aims to establish the process conditions that will produce a reduction of *Listeria* greater than 3 $\log_{10}$ CFU/g.

Materials and Methods

Materials. Frozen and vacuum-packed pork meat and fat (boneless shoulder pulp and back fat, respectively) were acquired at La Bordona (Montevideo, Uruguay). Sodium nitrite (INS 250), sodium chloride, ascorbic acid (INS 300), polyphosphate (INS 452i), and sodium lactate (INS 325) were provided by ERESUR S.A. (Montevideo, Uruguay). Spices (peppercorn, ground nutmeg, coriander, and dehydrated garlic) were purchased at La Molienda S.A. (Montevideo, Uruguay). Artificial casing FIBROUS® 11/2, 5N, and D9 (Viskase® Companies, Inc.) were acquired at Prinzi S.A. (Montevideo, Uruguay). The starter cultures used were S1 (Texel® SA 306, DANISCO) provided by Liderfran S.A. (Montevideo, Uruguay) composed by *L. sakei, S. carnosus,* and *S. xylosus*; S2 (Bactoferm® F-1, Chr. Hansen) composed by *S. xylosus* and *P. pentosaceus*; and S3 (FLC, Chr. Hansen) composed by *S. xylosus, L. curvatus,* and *P. acidilactici.* Hansen starter cultures were acquired at Nortesur S.A. (Montevideo, Uruguay).

***Listeria innocua* culture preparation.** *Listeria innocua* strain ATCC® 33090 (Friedly et al., 2008; Tirloni et al., 2019) was maintained at − 80°C in a mixture of 20% glycerol (AppliChem) and 80% tryptic soy broth (TSB, Oxoid Ltd., Hampshire, UK) until use. The culture for salami inoculation was prepared in two steps. First, strain ATCC® 33090 was grown overnight aerobically at 35°C in 10 mL of TSB (Oxoid Ltd., Hampshire, UK) supplemented with 0.6% yeast extract (Oxoid Ltd., Hampshire, UK) in order to reach the stationary phase. Second, the overnight culture was subcultured in 90 mL of TSB and incubated for 12–16 h aerobically at 35°C. Then, cells were harvested by centrifugation at 4,000*g* for 10 min at 4°C and resuspended with phosphate−buffered saline (PBS; pH 7.4, Sigma) to 8–9 log CFU/mL. The inoculum was kept at 4°C and used within the same day.

Starter culture selection. A total of 500 g of minced pork meat containing 25% fat were placed in Cryovac T7335B bags. Starter cultures (S1, S2, or S3), and sugars were added according to the starter manufacturer's instructions. Samples were inoculated with 8 log CFU/g of *L. innocua* ATCC® 33090 and manually homogenized for 1 min. Then, the bags were sealed and incubated at 25°C for 48 h. Two sets of samples were prepared by duplicate and analyzed at 0, 24, and 48 h for *Listeria* content and pH. For *Listeria* quantification, 10 g of sample were taken, placed in sterile stomacher bags, and homogenized with 90 ml of Butterfield´s buffer at 230 rpm for 2 min. Appropriate dilutions were plated on Palcam Agar Base (Oxoid Ltd., Hampshire, UK) and incubated at 37°C for 48 h. Counts were log-transformed and compared using ANOVA followed by Fisher's LSD in order to select the starter culture that generated the greatest *L. innocua* reduction. For pH determination, the second set of samples was used. pH was measured at six different sites of the sample at times 0, 24, and 48 h. Mean values were analyzed by ANOVA followed by Fisher's LSD.

Salami preparation and inoculation with *Listeria innocua* ATCC® 33090. Salami samples were prepared in 25 kg batches within a pilot plant. The meat and fat mixture (75:25) was ground to a fine grain using between 15 and 17 turns of a cutter (LASKA KR 60–2 MV, Linz, Austria) with the blade and plate speed set to 1. Subsequently, the minced meat and fat mixture were transferred to the blender (LASKA T WU5 Vac/P, Linz, Austria) and 150 ppm sodium nitrite, 2.6% sodium chloride, 0.1% ascorbic acid, 0.40% polyphosphate, 0.03% peppercorns, 0.10% ground nutmeg, 0.30% coriander, 0.10% dehydrated garlic, 1% dextrose, starter culture, and sodium lactate were added according to the experimental design. This mixture was homogenized for 1 min (30 s clockwise and 30 s counterclockwise). Then, the mixture was inoculated with 10^7 CFU/g of *L. innocua*

ATCC® 33090 and mixed for an additional minute. After mixing, the salami were stuffed (with different artificial casing depending on the salami caliber size to be produced: FIBROUS® 11/2 for 5.1 cm salami, 5N for 9.15 cm and D9 for 13.2 cm) using a HANDTMANN VF 12 – 100 sausage stuffer (Servo, Poland). Salami were placed for fermentation in an ALFA LAVAL LR-6 chamber (Surrey, United Kingdom) at relative humidity (RH) of 90 ± 5% for 48 h, and then, dried for 26 days in an ALFA LAVAL kkt 21021 chamber (Surrey, United Kingdom) at RH of 78 ± 5%.

***Listeria innocua* counts in salami.** Two samples of 10 g of each salami were aseptically transferred to individual sterile bags with filter and homogenized with 90 mL of Butterfield´s buffer in a Stomacher Seward 400C Lab Blenders, at 230 rpm for 2 min. Dilutions were made in Butterfield buffer, and 100 μL of the appropriate dilutions of the homogenate were plated in duplicate on Palcam agar plates with the addition of the selective supplement for Palcam (SR0150 Thermo). Plates were incubated at 37°C, and *Listeria* colonies were counted at 48 h. Counts of *L. innocua* were expressed as $\log_{10}$ CFU/g of salami, and mean values were used for statistical analysis.

pH and water activity measurements in salami. The pH was measured with a Hanna® model 9025c pH meter (Nusfalau, Romania) with a spike electrode, penetrating to a depth similar to half the thickness of the salami (Novelli et al., 2017). The a_w measurements were carried out according to the ISO 21807: 2004 standard with a Rotronic HP23-AW-A-set-40 portable equipment (Bassersdorf, Switzerland). Calibration of the device was done with LiCl (Lithium chloride) standard humidity solutions provided by Rotronic AG (95% RH at 20°C and 80% RH at 20°C). pH and water activity measurements were performed at three different spots of each salami, and mean values were used for statistical analysis.

Sodium lactate effect during fermentation. Salami of 9.15 cm caliber were prepared with and without 2% sodium lactate and inoculated with *L. innocua* ATCC® 33090 according to the process described in 2.4 using starter S3. Then, salami were incubated for 48 h in the fermentation chamber (90% HR) at 24, 27, and 30°C. *L. innocua* counts were performed at 0 and 48 h, and pH was measured at 0, 24, and 48 h. For each experimental condition, *L. innocua* reduction (Red. Lis_{48h}) was calculated as the difference between the $\log_{10}$ CFU/g of *L. innocua* determined at the beginning and at the end of fermentation (48 h).

Experimental design to evaluate production conditions. A Box-Behnken experimental design was applied using the Design Expert 13.0 program. Independent variables were fermentation temperature (24, 27, and 30°C), product diameter (5.1, 9.15, and 13.2 cm), and drying temperature (14, 17, and 20°C). The experimental design included sixteen experimental runs with four replicates for the central point (Table 1). Salami were prepared without sodium lactate.

For each experimental run, *L. innocua* counts and pH of salami were determined at 0, 1, 2, 7, 14, 21, and 28 days. Water activity was measured at 0, 2, 7, 14, 21, and 28 days.

Baranyi & Roberts equation (Baranyi & Roberts, 1994) using the software DMFit version 3.5 Excel® available at the ComBase portal (https://browser.combase.cc/DMFit_Excel.aspx) was used to adjust the experimental *L. innocua* counts for each test condition over time. The Baranyi & Roberts equation used is presented in Eq. (1). *L. innocua* inactivation rate (k) expressed in $\log_{10}$ CFU/g/day, the initial counts of *L. innocua* at the beginning of the fermentation stage (Y_0), and the final counts of *L. innocua* reached at the end of the drying stage (Y_{END}) expressed in $\log_{10}$ CFU/g were estimated for each test condition. *L. innocua* reduction ($Y_0 - Y_{END}$) in $\log_{10}$ CFU/g was calculated for each test condition.

k ($\log_{10}$ CFU/g/day) and $Y_0 - Y_{END}$ ($\log_{10}$ CFU/g) were the responses analyzed in the Box-Behnken design. Responses were fitted to first−order, second−order, and quadratic models. For each response, the model with the best fit was selected by the Sequential Sum of Square Method and assessed based on statistically significant coefficients and R^2 values using ANOVA, with a significance level of $\alpha = 0.05$.

Table 1
Box-Behnken experimental design matrix with the experimental runs

Runs	Tferm (°C)[a]	Caliber size (cm)	Tdry (°C)[b]
1	30	13.2	17
2	30	9.15	20
3	30	9.15	14
4	30	5.10	17
5	27	13.2	20
6	27	13.2	14
7	27	9.15	17
8	27	9.15	17
9	27	9.15	17
10	27	9.15	17
11	27	5.10	20
12	27	5.10	14
13	24	13.2	17
14	24	9.15	20
15	24	9.15	14
16	24	5.10	17

[a] Tferm = Temperature in the fermentation chamber.
[b] Tdry = Temperature in the drying chamber.

$$Yt = Y0 + k * A(t) + \frac{1}{m} * In(1 + \frac{e^{-mk \cdot A(t)} - 1}{e^{m(Y0 - YEND)}}) \quad (1)$$

Y_t = *L. innocua* counts at time t ($\log_{10}$ CFU/g).
Y_0 = initial *L. innocua* counts ($\log_{10}$ CFU/g)).
k = *L. innocua* inactivation rate ($\log_{10}$ CFU/g/day).
Y_{END} = final *L. innocua* counts ($\log_{10}$ CFU/g).
t = time (day).
$A(t)$ = t (when no lag phase is considered).
m = curvature parameter (m = 10).

In addition, the change in *Listeria* counts during the drying stage was calculated as the difference between Y_{END} and the *Listeria* counts at the end of the fermentation stage (Y_{48h}), using the predicted value for Y_{END} and the experimental value for Y_{48h}, both expressed in $\log_{10}$ CFU/g. $Y_{END} - Y_{48h}$ above 0.5 indicated an increase in *Listeria* population and below −0.5 indicated a reduction. When $Y_{END} - Y_{48h}$ was between −0.5 and 0.5, it was considered that *Listeria* population remained constant during that period (Novelli et al., 2017). $Y_{END} - Y_{48h}$ values were analyzed by ANOVA followed by Fischer's LSD analysis.

Optimization and model validation. The optimal process conditions (Tferm, Tdry, and caliber size) were obtained by applying the following constraints on the response factors: i) maximize *L. innocua* inactivation rate (k) and ii) maximize *L. innocua* reduction between the initial and the final time of the process ($Y_0 - Y_{END}$).

To validate the proposed model, three experiments were carried out using the optimal conditions as the checkpoint. Experimental responses (k and $Y_0 - Y_{END}$) of the checkpoint were compared to the predicted results from the fitted models to evaluate the precision of the polynomial equations (value within IC95 predicted by model).

Results

Starter Culture selection. The three starter cultures tested caused a significant reduction ($P < 0.05$) in the pH of the mixtures during fermentation, reaching values below 5 at 48 h (4.8 for S1, 4.7 for S2, and 4.8 for S3) (Table 2). S1 and S3 significantly ($P < 0.05$) reduced *L. innocua* content in meat, while S2 had a negligible capacity to reduce *Listeria* under the trial conditions (Table 2). S1 produced a significant *Listeria* reduction of 1.9 ± 0.3 $\log_{10}$ CFU/g, and S3 reduced 3.7 ± 0.1 $\log_{10}$ CFU/g in 48 h. S3 was selected for the subsequent assays.

Table 2
Ph values measured at time zero,48 h and. *L. innocua* ATCC® 33090 reduction obtained in minced meat samples using different starter cultures at 25°C

Starter culture	Initial pH	pH at 48 h	*L. innocua* red. at 48 h (log_{10} CFU/g)[d]
S1[a]	5.5 ± 0.0Aa[e]	4.8 ± 0.0Ba	1.9 ± 0.3a
S2[b]	5.4 ± 0.0Ab	4.7 ± 0.0Bb	0.6 ± 0.0b
S3[c]	5.6 ± 0.0Ac	4.8 ± 0.0Ba	3.7 ± 0.1c

[a] S1 = *L. sakei* + *S. carnosus* + *S. xylosus*.
[b] S2 = *S. xylosus* + *P. pentosaceus*.
[c] S3 = *S. xylosus* + *L. curvatus* + *P. acidilactici*.
[d] *L. innocua* initial inoculum $1x10^8$ CFU/g.
[e] Values followed by different uppercase letters within rows are significantly different ($P < 0.05$). Values followed by different lowercase letters within columns are significantly different ($P < 0.05$).

Sodium lactate effect on *Listeria* reduction and pH during fermentation. The addition of 2% sodium lactate to the salami formula affected *Listeria* and pH reduction during fermentation. After 48 h of fermentation, *L. innocua* reduction, regardless of the fermentation temperature, was always significantly lower ($P < 0.05$) when 2% sodium lactate was included in the salami formulation (Table 3) and varied between 1.7 ± 0.2 and 3.2 ± 0.0 log_{10} CFU/g. In the absence of sodium lactate in the temperature range tested, no variation was observed in *Listeria* reduction with temperature change (Table 3). The reduction of *Listeria* in salami with 2% sodium lactate was higher at 30°C (3.2 ± 0.0 log_{10} CFU/g) than at 27°C and 24°C (1.7 ± 0.2 and 2.0 ± 0.2 log_{10} CFU/g, respectively) (Table 3). Regarding pH, irrespective of fermentation temperature, the addition of sodium lactate decreased the pH drop rate during fermentation. pH values were always higher in salami with lactate than in salami without lactate, except for a fermentation temperature of 27°C at 48 h (Table 3).

Effect of caliber size, fermentation temperature, and drying temperature on *Listeria* inactivation rate (k), and on the difference in *Listeria* population in salami without sodium lactate at the beginning (Y_0) and at the end (Y_{END}) of the process according to the Baranyi & Roberts equation. For all the experimental runs, *Listeria* log counts over time were satisfactorily modeled according to Baranyi & Roberts equation without considering a latency phase (Fig. A1 in Supplementary material). R^2 of the fit varied between 0.73 and 0.94, and the standard errors for k and Y_{END} varied between 0.26 and 0.72, and between 0.1 and 0.3 respectively indicating an adequate fit for experimental microbiological models (Table 4) (Gan et al., 2007; Mataragas et al., 2011). The *Listeria* inactivation rate varied between −2.04 and −3.17 log_{10} CFU/g/day and Y_{END} varied between 2.7 and 3.9 log_{10} CFU/g (Table 4).

Inactivation rate analysis (k, log_{10} CFU/g/day). A quadratic model was used to fit the inactivation rate k ($P < 0.0001$), which allowed to explain 89% of the variance ($R^2 = 0.89$). Fermentation temperature, fermentation temperature^2, and interaction between fermentation temperature*caliber (Tferm*caliber) were the significant terms ($P < 0.05$) in the model, while drying temperature (Tdry) was not significant (Eq. (2)). The model did not show lack of fit ($F = 2.42$, $p = 0.2516$), the adjusted R^2, predicted R^2 parameters were satisfactory (0.85 and 0.68 respectively) and the term "Adequate precision" was 15.3, this conditions allow to navigate in the design space. The model equation for the inactivation rate is presented in Eq. (2).

$$\begin{aligned} Y_1 &= k(log_{10}CFU/g/day) \\ &= -14.02861 + 1.09097 * Tferm - 0.337037 * Caliber \\ &\quad + 0.012963 * Tferm * Caliber - 0.024444 * Tferm^2 \end{aligned} \quad (2)$$

The 3D response surface plots allow to visualize the response in the design space (Fig. 1). The inactivation rate increased as Tferm increased. Changes in k were more pronounced between 27 and 30°C than between 24 and 27°C (Fig. 1). Although the salami caliber size was not a significant variable, the interaction between caliber and Tferm was significant. This effect was negligible at temperatures below 26°C, becoming more relevant at higher temperatures (Fig. 1). As Tferm increased k tended to increase as caliber size decreased (Fig. 1).

Reduction level analysis ($Y_0 - Y_{END}$, log_{10} CFU/g). The reduction level ($Y_0 - Y_{END}$) was fitted ($P < 0.0001$) with a reduced two-factor interaction model (2FI model) with an R^2 of 0.94 and a similarity between predicted R^2 and adjusted R^2 (0.75 and 0.89). The lack of fit was not significant ($F = 1.26$, $p = 0.4607$) and the "Adequate Precision" term was 17.4, allowing to navigate in the design space. Tferm, caliber, Tdry, and the interaction between Tferm*caliber and Tferm*Tdry were significant terms of the model ($P < 0.05$), and the model equation for ($Y_0 - Y_{END}$) is presented in Eq. (3).

Table 3
Listeria reduction and pH value at 24 and 48 h of fermentation for 9.15 cm caliber salami at different fermentation temperatures and in the presence/absence of sodium lactate

Tferm (C)[a]	Red.Lis_{48h} (log_{10} CFU/g)[b]		pH at 24 h		pH at 48 h	
	0% SL[c]	2% SL[d]	0% SL	2% SL	0% SL	2% SL
30	4.5 ± 0.1Aa[e]	3.2 ± 0.0 Ba	5.4 ± 0.1 Aa	5.7 ± 0.1 Ba	4.9 ± 0.0 Aa	5.1 ± 0.0 Ba
27	4.3 ± 0.1Aa	1.7 ± 0.2 Bb	5.6 ± 0.1 Aa	6.1 ± 0.0 Bb	5.0 ± 0.0 Ab	5.1 ± 0.1 Aa
24	4.5 ± 0.2Aa	2.0 ± 0.2 Bb	5.9 ± 0.1 Ab	6.1 ± 0.0 Bb	5.2 ± 0.0 Ac	5.4 ± 0.0 Bb

[a] Tferm = fermentation chamber temperature in °C. Tferm (mean value ± standard deviation) for 30 °C (29.6 ± 0.2°C), 27°C (26.6 ± 0.2°C), and 24°C (23.7 ± 0.2°C).
[b] Red.Lis_{48hs} = *L. innocua* reduction after 48 h of fermentation.
[c] 0% SL = 0% Sodium lactate (w/w).
[d] 2% SL = 2% Sodium lactate (w/w).
[e] Means of two values per samples ± standard deviation. Mean values followed by different uppercase letters within rows are significantly different ($P < 0.05$). Mean values followed by different lowercase letters within columns are significantly different ($P < 0.05$).

Table 4
Parameters estimated according to the Baranyi & Roberts equation using DMFit software

Tferm[a] (°C)	Caliber (cm)	Tdry[b] (°C)	k[c] (log_{10} CFU/g/day)	R^2 [d]	Y_{END}[e] (log_{10} CFU/g)	Y_0[f] (log_{10} CFU/g)
30	13.2	17	−2.64 ± 0.47	0.92	2.6 ± 0.2	7.1
30	9.15	20	−2.68 ± 0.23	0.98	2.7 ± 0.1	7.3
30	9.15	14	−2.81 ± 0.41	0.93	3.5 ± 0.1	7.4
30	5.1	17	−3.17 ± 0.26	0.97	2.7 ± 0.1	7.1
27	13.2	20	−2.21 ± 0.41	0.89	3.2 ± 0.2	7.7
27	13.2	14	−2.23 ± 0.61	0.81	3.6 ± 0.3	7.9
27	9.15	17	−2.29 ± 0.34	0.94	3.2 ± 0.1	7.4
27	9.15	17	−2.42 ± 0.36	0.94	3.2 ± 0.1	7.3
27	9.15	17	−2.37 ± 0.38	0.94	3.3 ± 0.1	7.5
27	9.15	17	−2.24 ± 0.45	0.93	3.2 ± 0.2	7.5
27	5.1	20	−2.30 ± 0.42	0.90	3.2 ± 0.2	7.5
27	5.1	14	−2.13 ± 0.52	0.86	3.8 ± 0.2	7.6
24	13.2	17	−2.14 ± 0.69	0.73	3.6 ± 0.3	7.8
24	9.15	20	−2.29 ± 0.59	0.79	3.4 ± 0.3	7.5
24	9.15	14	−2.18 ± 0.36	0.93	3.6 ± 0.2	7.7
24	5.1	17	−2.04 ± 0.72	0.73	3.9 ± 0.3	7.7

[a] Tferm = fermentation chamber temperature in °C. Tferm (mean value ± standard deviation) for 30 °C (29.6 ± 0.2°C), 27°C (26.6 ± 0.2°C), and 24°C (23.7 ± 0.2°C).
[b] Tdry = drying chamber temperature in °C. Tdry (mean value ± standard deviation) for 20°C (19.8 ± 1.0°C), 17°C (17.4 ± 0.4°C) and 14°C (14.8 ± 0.4°C).
[c] k = *Listeria* inactivation rate predicted value ± root mean squared error (RMSE).
[d] R^2 = R^2 of the fit.
[e] Y_{END} = *L. innocua* predicted at the end of the process (2 days of fermentation + 26 days of drying) ± RMSE.
[f] Y_0 = *L. innocua* at the beginning of the process.

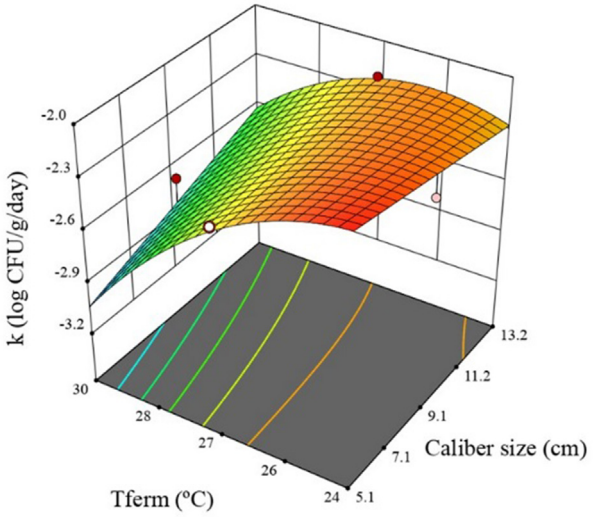

Figure 1. 3D response surface plots generated from the Box-Behnken design showing the effect of fermentation temperature (Tferm) and salami caliber size on *L. innocua* inactivation rate k (log_{10} CFU/g/day) for a drying temperature of 20°C.

$$Y_2 = (Y_0 - Y_{END})\,(\log_{10}\mathrm{CFU/g}) = 7.46179 - 0.204028 * \mathrm{Tferm} + 0.341152 * \mathrm{Caliber} - 0.392701 * \mathrm{Tdry} - 0.007407 * \mathrm{Tferm} * \mathrm{Caliber} + 0.018611 * \mathrm{Tferm} * \mathrm{Tdry} - 0.005761\,\mathrm{Calibre} * \mathrm{Tdry} \quad (3)$$

Figure 2A, 2B, and 2C show the effect of the interaction between Tferm and Tdry in $Y_0 - Y_{END}$. It is observed that $Y_0 - Y_{END}$ increases (hence also the total reduction of *Listeria* in the process) with the simultaneous increase of Tferm and Tdry. This effect is observed for the three salami calibers evaluated. Figure 2D, 2E, and 2F show the interaction between Tferm and salami caliber size. It is observed that $Y_0 - Y_{END}$ tends to decrease when Tferm and caliber decrease, and this decrease is more pronounced as Tdry increases.

Optimization and model validation. Based on the models obtained, the process conditions that maximize *L. innocua* inactivation rate (k) and total *L. innocua* reduction ($Y_0 - Y_{END}$) with a desirability factor close to 1 were: Tferm of 30°C and Tdry of 20°C for the three calibers sizes. Using these conditions, the model predicted a k of −3.04 ± 0.16 log_{10} CFU/g/day and a ($Y_0 - Y_{END}$) of 4.6 ± 0.1 log_{10} CFU/g (caliber size 5.1 cm), −2.83 ± 0.16 log_{10} CFU/g/day and 4.7 ± 0.1 log_{10} CFU/g (caliber size 9.15 cm) and −2.62 ± 0.1 6 log_{10} CFU/g/day and 4.8 ± 0.1 log_{10} CFU/g (caliber size 13.2 cm).

The experimental responses at Tferm of 30°C and Tdry of 20°C for salami caliber size 9.15 cm were compared to the predicted model results to evaluate the precision of the polynomial equations. The experimental values for k and $Y_0 - Y_{END}$ were −2.62 ± 0.14 log_{10} CFU/g/day and 4.5 ± 0.1 log_{10} CFU/g, respectively, both experimental values were within the 95% CI of the predicted outcome by the models.

***Listeria innocua* behavior during drying.** Despite having achieved a *Listeria* reduction greater than 3 log_{10} CFU/g at the end of the process (28 days), in some experimental runs, *Listeria* population at 28 days (Y_{END}) was higher than the *Listeria* population at the end of the fermentation step (Y_{48h}). The change in *Listeria* counts between the end of the drying stage and the end of fermentation ($Y_{END} - Y_{48h}$) (Table B1 in Supplementary material) was calculated. Analysis of variance (ANOVA) of $Y_{END} - Y_{48h}$ indicated that caliber size was not a significant variable ($P > 0.05$). A new ANOVA test was performed grouping $Y_{END} - Y_{48h}$ by drying temperature for different fermentation temperatures (Fig. 3). This analysis showed that for all conditions tested with Tdry of 14°C, regardless of the fermentation temperature, $Y_{END} - Y_{48h}$ was ≥ 0.5 log_{10} CFU/g, indicating that *Listeria* growth occurred. At a Tdry of 17°C, only the condition with fermentation at 24°C allowed the growth of *Listeria*. When drying was at 20°C for all the fermentation temperatures tested, $Y_{END} - Y_{48h}$ was between −0.5 and 0.5 log_{10} CFU/g, indicating that *Listeria* counts did not change in this period (Fig. 3).

Y_{END} = *Listeria* at the end of the process (28 days); Y_{48h} = *Listeria* at the beginning of drying stage. Error bars indicate the standard deviations (n = 3). Different uppercase letters within a drying temperature indicate significant differences ($P < 0.05$). Under the conditions that allowed the growth of *Listeria* during drying, the salamis required

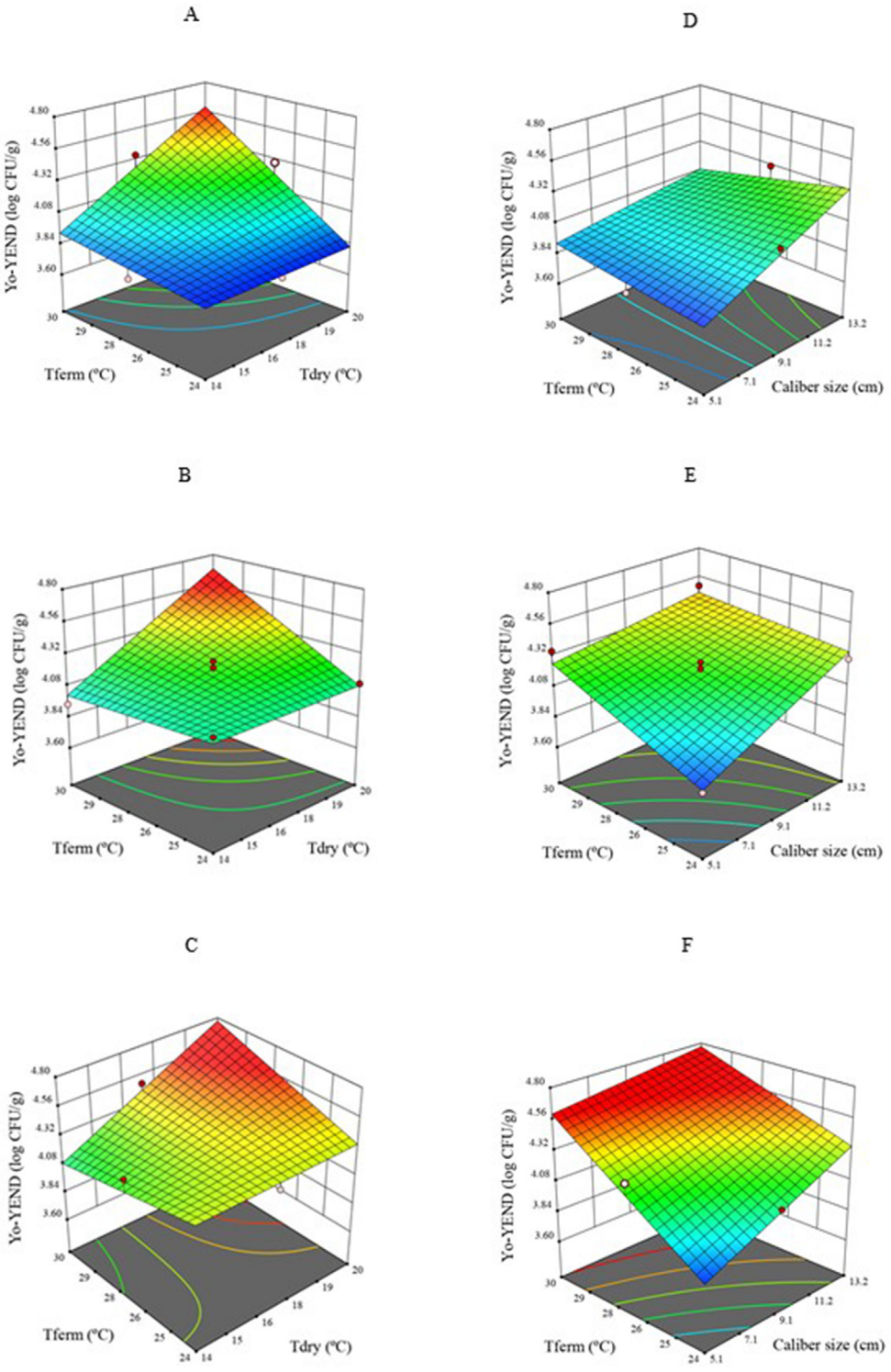

Figure 2. 3D response surface plots generated from the Box-Behnken design showing: the effect of fermentation temperature (Tferm) and drying temperature (Tdry) on *L. innocua* reduction (log_{10} CFU/g) for a 5.1 cm salami (A) 9.15 cm salami (B) and 13.2 cm salami (C). Effect of fermentation temperature (Tferm) and salami caliber size on *L. innocua* reduction $Y_0 - Y_{END}$ (log_{10} CFU/g) for a drying temperature of 14°C (D) 17°C (E) and 20°C (F).

more time to reach pH and a_w restrictive for *Listeria* growth (pH ≤ 5.0 and $a_w \leq 0.94$ or $a_w \leq 0.92$) (Table B2 in Supplementary material), once restrictive a_w was reached, *Listeria* counts remain constant up to day 28.

Discussion

In the present work, the fate of *Listeria* during salami production was studied at a pilot scale using *L. innocua* strain ATCC® 33090 as

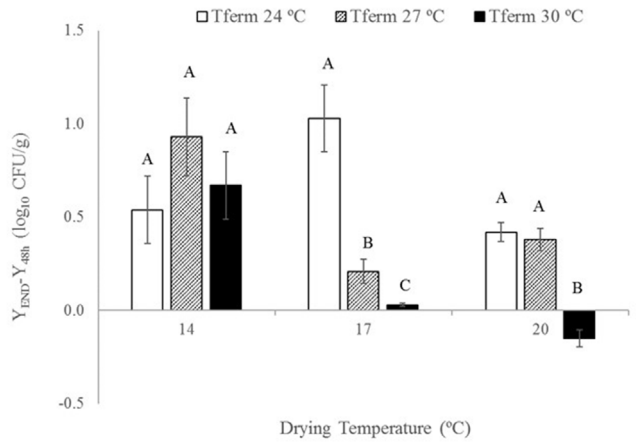

Figure 3. Variation in *Listeria* counts during the drying stage expressed as $Y_{END} - Y_{48h}$.

a surrogate microorganism. First, the incidence of different starter cultures and sodium lactate on *Listeria* reduction and pH drop during fermentation were analyzed. Then, the effect of fermentation and drying temperatures and salami caliber size on the survival of *Listeria* was analyzed using the RSM approach.

The three starter cultures tested caused a significant ($P < 0.05$) pH drop, below 5, in the mixture, indicating that the fermentation process was adequate (Ordóñez et al., 1999). However, the ability to reduce *Listeria* population was different for each starter culture (Table 2). This difference could be explained by the bacterial composition of the starter cultures. S1, S2, and S3 contain potentially bacteriocin − producing LAB species as *L. sakei*, *L. curvatus*, *P. acidilactici,* and *P. pentosaceus* (Castellano et al., 2008; Martín et al., 2022; Olaoye et al., 2011); the greater reduction of *L. innocua* produced by S3 may be due to the joint bactericidal action of *P. acidilactici* and *L. curvatus* while *S1 and S2* contain only one bactericidal specie. In this regard, Nielsen et al. (1990) reported that *P. acidilactici* produces a bacteriocin with an inhibitory and bactericidal effect against *L. monocytogenes* associated with fresh meat and Casaburi et al. (2016) reported that the *Lactobacillus curvatus* 54M16 strain produces an active bacteriocin against *L. monocytogenes* in culture media. Giello et al. (2018) confirmed that the *Lactobacillus curvatus* 54M16 strain was capable of inhibiting *L. monocytogenes* in fermented sausages during the course of fermentation. S2 contains *P. pentosaceus* which, despite being a potentially bacteriocin-producing species (Olaoye et al., 2011), in this work, no significant listericidal effect was observed. This can be explained by the dependence of bacteriocin production on the *P. pentosaceus* strain used and its interaction with the food matrix (Cosansu et al., 2010; de Souza Barbosa et al., 2015).

The addition of 2% sodium lactate during fermentation interfered with pH drop and *Listeria* reduction (Table 3). Although sodium lactate has bacteriostatic action against several pathogens such as *L. monocytogenes*, *Salmonella* spp., *Staphylococcus aureus,* and *Clostridium* spp. (Houtsma et al., 1993; Shelef, 1994; Weaver & Shelef, 1993), in this study, it was not observed an extra *Listeria* inhibition due to the addition of sodium lactate. The main role of sodium lactate in salami production is to regulate the rate of acidification (Ordóñez et al., 1999) by inhibiting LAB growth extending their lag phase (Deumier & Collignan, 2003; Lin & Lin, 2002; Stekelenburg & Kant-Muermans, 2001; Wang, 2000). Therefore, the addition of sodium lactate may decrease lactic acid production and possibly bacteriocin production by *L. curvatus* and *P. acidilactici* contained in the starter used. In this study, the use of sodium lactate under working conditions might compromise product safety by not allowing more than a 3 log cfu/g *L. innocua* reduction after 48 h of fermentation. Furthermore, the use of sodium lactate in the tested conditions extended the time while salami are exposed to pH and temperature conditions favorable for the growth of *Listeria* (pH > 5.0 and $a_w > 0.94$ or $a_w > 0.92$) and for other pathogens, such as *E. coli* and *Staphylococcus aureus* (pH > 5.3 and temperature > 15.5°C) (American Meat Institute Foundation (AMI), 1997). The addition of lower concentrations of sodium lactate should be studied to fully define its impact on the process.

The strategy of fitting *Listeria* log colony number over time for each test condition with Baranyi & Roberts equation and then modeling k and $Y_0 - Y_{END}$ as a function of Tferm, Tdry, and caliber size was adequate to explain *Listeria* behavior. In the present study, the Baranyi & Roberts model fit was adequate (R^2 0.73–0.97) (Gan et al., 2007; Mataragas et al., 2011). A greater difference was observed between the predicted and the experimental *Listeria* counts in the area of the curve corresponding to the first days of the drying stage (Fig. A1 in Supplementary Materials), nevertheless, in all cases, k and Y_{END} were predicted with an adequate standard error (Table 4). The RSM generated models for k and $Y_0 - Y_{END}$ with an adequate fit (0.89 and 0.94, respectively), which allows to navigate in the design space (Gan et al., 2007) and to predict these responses as a function of Tferm, Tdry, and salami caliber size.

Tferm was the main factor in the quadratic model for k. According to the model, an increase in Tferm produced an increase in k (Fig. 1). *Listeria* inactivation during fermentation is mainly due to the action of lactic acid and bacteriocin, products of the metabolism of the lactic acid bacteria present in the starter culture (de Souza Barbosa et al., 2015; Leroy & De Vuyst, 1999; Martín et al., 2022). In this study, the pH drop after 24 h of fermentation at Tferm 30°C was 0.79 while at Tferm 24°C was only 0.09 (Table B3 in Supplementary material) suggesting a higher salami content of lactic acid at 30°C. Several authors have reported that temperatures above 25°C favor lactic acid bacteria growth and bacteriocin production (Mataragas et al., 2003; Yang et al., 2018).

In the *Listeria* total reduction level ($Y_0 - Y_{END}$) model, the three factors Tferm, Tdry, and caliber size were significant ($P < 0.05$). An increase in Tferm and Tdry contributed to increase $Y_0 - Y_{END}$. The conditions that maximized both k and $Y_0 - Y_{END}$ values were, for all calibers, a fermentation temperature of 30°C and a drying temperature of 20°C. There are reports showing that the probability of *Listeria* growth increases as fermentation temperature increases (Brusa et al., 2021; Polese et al., 2017). These reports are based on a growth/non-growth predictive model developed by Polese et al. (2017) for Italian artisanal salamis. The preprocessing conditions of artisanal Italian salamis differ from those used in this study; therefore, there may be discrepancies.

In this study, a *Listeria* reduction greater than 3 $\log_{10}$ CFU/g at the end of the process (28 days) was observed for all the experimental conditions, but in the experimental runs with Tdry 14°C and in those with Tferm 24°C and Tdry 17°C *Listeria* counts at 28 days (Y_{END}) were higher than at the end of the 48 h fermentation step (Y_{48h}), suggesting that these conditions allowed *L. monocytogenes* to grow during the first days of the drying stage ($Y_{END} - Y_{48h} \geq 0.5$ $\log_{10}$ CFU/g). This could be explained because when salami were placed at temperatures below 20°C during drying lactic acid bacteria metabolism (Mataragas et al., 2003) was slowed down causing a reduction in lactic acid and bacteriocin production (Mataragas et al., 2003; Yang et al., 2018), and therefore, *Listeria* grew until restrictive a_w was reached. The salami produced under these conditions reached the restrictive conditions for *Listeria* growth in food (pH ≤ 5.0 and $a_w \leq 0.94$ or $a_w \leq 0.92$) at longer times compared to salami that did not show growth during drying (Tables B2 and B4 in Supplementary material). In addition, once the restrictive condition of $a_w < 0.92$ was reached, *Listeria* counts remained unchanged until day 28. Hussein et al. (2022) reported that in *L. monocytogenes,* inoculated salami extending drying from 30 to 60 days at 21°C increases *Listeria* reduction by approximately 2 $\log_{10}$

CFU/g. This suggests that prolonging the drying time would be beneficial in further reducing *Listeria* counts.

In conclusion, this is the first study that reports the modeling of *Listeria* behavior under the process conditions (Tferm, Tdry, and caliber size) used in South America. The experimental data were obtained from salami produced in a pilot scale and inoculated with *Listeria innocua* ATCC® 33090 as a surrogate microorganism for *L. monocytogenes*.

According to our results, the use of 2% sodium lactate is not recommended since it compromises product safety. Another important observation is that different starter cultures produced different levels of *Listeria* reduction with a similar pH drop during the first 48 h of fermentation. Data also show that even though water activity has reached a value less than 0.92, *Listeria* in salami will survive for a time that can extend for several weeks. An extension of the drying time may benefit product safety allowing *Listeria* counts to decay before the product is released to the market.

Therefore, although pH drop during fermentation and water activity decrease during drying are parameters that must be controlled during salami production, to guarantee the safety of the processes, dry fermented sausage manufacturers should validate their formulation (starter culture used, ingredients, and amounts added) and processing conditions (fermentation temperature, drying temperature and caliber) to demonstrate that *Listeria* inactivation target (greater than 3 log CFU/g) and other pathogens inactivation are achieved. This validation should be carried out at the setting of the production process and after any change in salami formulation or processing conditions. Once the process's ability to reduce the level of *Listeria* by more than 3 log CFU/g has been validated, then pH and a_w values during the process will become true safety indicators.

CRediT authorship contribution statement

Giannina Brugnini: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jesica Rodríguez:** Writing – review & editing, Investigation, Formal analysis. **Soledad Rodríguez:** Investigation, Formal analysis. **Inés Martínez:** Writing – review & editing, Conceptualization. **Ronny Pelaggio:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Caterina Rufo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfp.2024.100286.

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