LACTOSE OXIDASE AS AN INHIBITORY AGENT EFFECTIVE AGAINST BLUE DISCOLORATION IN TWO TYPES OF MOZZARELLA CHEESE

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ABSTRACT

Blue discoloration in fresh mozzarella cheese (FMC) is a defect caused specifically by *Pseudomonas fluorescens*. In the current study, lactose oxidase (LO) was demonstrated to be an effective hurdle to prevent the formation of blue color defects in FMC. In cheese produced through direct acidification (DA) by addition of citric acid, surface application of LO at a concentration of 1.2 g/L resulted in retention of conventional color characteristics; a concentration of 0.12 g/L also performed favorably, depending on variables such as product composition or initial contamination level. A LO concentration of 0.012 g/L did not show an inhibitory effect. Quantification of LO's effects on blue discoloration in fresh mozzarella produced by starter fermentation (SF) revealed less consistency than that seen with DA FMC. However, based on the researchers' visual assessments, the 1.2 g/L concentration performed the best out of those investigated, with obvious blue coloration occurring in only 1 out of 6 samples. This study demonstrates a promising clean-label approach to prevent blue color defects in FMC; further studies need to be conducted to fine-tune this process depending on the method used for acidification of cheese, potential initial levels of contamination, and packaging applied.

BIOGRAPHICAL SKETCH

The writer, Pablo Fernando Torres Frenzel, was born in Valdivia, Chile in 1986. He cursed his bachelor's degree of Food Engineering in the Universidad Austral from Chile between 2016- 2013. In 2014 he moved to the city of La Union, Chile, where he started his professional career at the Cooperativa Agricola y Lechera de La Union. In 2020, he went to Cornell University, pursuing the Master of Professional Study in Food Science and Technology with Dr. Wiedmann.

In May 2021, Pablo Fernando Torres Frenzel, will finish his MPS degree at Cornell, with the finished work of the summary report of Lactose Oxidase as an inhibitory agent effective against blue discoloration in two types of Mozzarella cheese

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LIST OF ABBREVIATIONS

ΔE: total color change

BSC: bio-safety cabinet

CIE: Commission Internationale de l'Eclairage

DA: direct acidification

FMC: fresh mozzarella cheese

LO: lactose oxidase

NS: “negative control” sample

P0: sample inoculated with *P. fluorescens,* but that did not receive a LO treatment

P0.012: 0.012 g lactose oxidase/liter

P0.12: 0.12 g lactose oxidase/liter

P1.2: 1.2 g lactose oxidase/liter

PPC: post-pasteurization contamination

SF: starter fermentation.

1. INTRODUCTION

Food waste is one of the major concerns faced by the food industry nowadays. In the specific case of the dairy industry, it is estimated that in the U.S., the total value of food lost due to waste at the retail level is $165.6 billion annually, predominantly comprising fluid milk, cheese, and cultured products, all susceptible to contamination with spoilage organisms during the manufacturing process (Martin et al., 2021). Microbial contamination is one of the largest factors adversely affecting the profitability of the dairy industry, as it shortens shelf lives and increases perishability of some fresh products. One of the products included in this category is fresh mozzarella cheese (FMC). Mozzarella is a cheese produced using the pasta-filata technique, which is characterized by a processing step in which a high temperature is essential. In this step, by the application of hot water or steam, the curd is mechanically worked (stretched) to obtain a semi-flowable plastic mass marked by a striking rearrangement of the curd structure, with unique textural and melting characteristics (Kindstedt, 2002). The manufacturing process requires the acidification of the curd, which is achieved by one of two methods: starter fermentation (SF), involving the addition of defined or undefined thermophilic starter strains; or direct acidification (DA), generally involving addition of citric acid. The drop in pH causes casein demineralization and makes the curd pliable in hot water (Natrella et al., 2020; Ricciardi et al., 2015). This pliability is the key attribute that makes mozzarella the most desirable cheese for pizza makers around the world, as well as being a popular table cheese for at-home consumption (McMachon & Oberg, 1993). In the case of FMC, the product has a final moisture content between 60 and 65%, with a very soft body and milky flavor (Faccia et al., 2019).

It is well known that any strategies for delaying microbial deterioration of FMC must primarily address the inhibition of psychrotolerant bacteria, among which *Pseudomonas* spp. play a primary role (Faccia et al., 2019). The *Pseudomonas* genus includes several species of rod-shaped, Gram-negative, aerobic, non-fermentative, mesophilic and/or psychrotolerant bacteria (De Jonghe et al., 2011). *Pseudomonas* spp. are characterized as having simple nutritional requirements and are capable of adapting to a wide variety of food matrices, due to their production of enzymes such as proteases, lipases, pectinases, and lecithinases (Caldera et al., 2015). Because of their resistance characteristics, strains of the *Pseudomonas* genus are difficult to eradicate once introduced into the production environment. The ability of these microorganisms to adapt to different environments is also likely accounted for by the propensity of this genus to form biofilms, providing these bacterial populations with a high level of resistance (C. Rossi et al., 2018). Contamination following heat-treatment, referred to as post-pasteurization contamination (PPC), is one of the most common ways in which *Pseudomonas* is introduced into FMC (Baruzzi et al., 2012). These bacteria can grow at refrigeration temperatures, and their presence is directly correlated to anomalous blue coloration in food (Circella et al., 2020). In fresh cheeses, once the package is opened and the product is exposed to air, a blue or purplish-blue discoloration develops on the surface of the cheese (del Olmo et al., 2018). Instances of this issue have surged over the last decade worldwide, with events identified in countries like the U.S., Italy, Germany, England, and Spain (del Olmo et al., 2018). Studies have been conducted to investigate this problem in FMC (Cenci-Goga et al., 2014; del Olmo et al., 2018), rabbit carcasses (Circella et al., 2020), and queso fresco (N. H. Martin et al., 2011). The news media highlighted this defect in 2010, when a batch of 70,000 mozzarella cheese balls, originating from a German plant, turned blue after shipment to Italy (RASFF, 2010), and, partly due to the breaking of this story, new interest has been spurred

among researchers in finding new methods for detecting and preventing this issue (Caputo et al., 2015; Cenci-Goga et al., 2014; Faccia et al., 2019; N. H. Martin et al., 2011).

The utilization of enzyme-based preservation technologies has been shown to be a novel and interesting method for preserving dairy foods, specifically as pertains to the clean label approach. For consumers of dairy products such as cheeses, it is not uncommon to read labels that contain enzymes in their ingredient lists, as microbially-produced enzymes such as rennet have been used in the manufacture of cheeses for decades (Ben Amira et al., 2017). Milk's lactoperoxidase system is an endogenous antimicrobial system that helps preserve raw milk, and is made up of three components: lactoperoxidase, thiocyanate, and hydrogen peroxide (H_2O_2) (Rivera Flores et al., 2020). Here, the role of lactoperoxidase is to catalyze the oxidation of thiocyanate by H_2O_2, resulting in the generation of hypothiocyanite ions, which have antimicrobial properties (de Wit and van Hooydonk, 1996). The performance of the lactoperoxidase system depends on the concentrations of thiocyanate and H_2O_2. Milk's endogenous thiocyanate can be present at a close-to-optimal concentration, depending on the breed of the producing animal, udder health, and type of feed (Kussendrager & Van Hooijdonk, 2000), but H_2O_2 is only present in trace amounts in raw milk, and its concentration must be increased if the lactoperoxidase system's effects are to be optimized (Rivera Flores et al., 2020). Lactose oxidase (LO, EC 1.1.99.18) is a commercially available food-grade enzyme from the cellobiose oxidase class (Lund et al., 2019); LO exhibits a high specificity toward lactose, oxidizing this disaccharide into lactobionic acid, with the simultaneous release of H_2O_2 (Ahmad et al., 2004). Like hypothiocyanite ions, H_2O_2 has been shown to perform as an effective antimicrobial agent in milk (Martin et al., 2014) and queso fresco (Kozak et al., 2018). Lactobionic acid has also been demonstrated to possess antimicrobial properties, specifically with regard to *P. fluorescens* (Kang et al., 2020). Lara-Aguilar & Alcaine

studied the effects of LO in raw milk, demonstrating its positive effects when used at refrigerated temperatures, as well as in situations where cold-chain access is limited (Lara-Aguilar & Alcaine, 2019a). Specifically, they studied the inhibitory effects of LO on four foodborne pathogens (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus),* one spoilage bacterium (*Pseudomonas fragi*), and one mold (*Penicillium fragi*); LO effectively inhibited all organisms under investigation, particularly *Pseudomonas fragi*, most likely due to the sensitivity of this genus to H_2O_2 (Lara-Aguilar & Alcaine, 2019b). LO has also been tested in ultra-high temperature (UHT) milk as a means of preventing "age gelation" caused by *Pseudomonas* spp., and has demonstrated good performance in UHT milk stored over a 6 mo period, when a concentration of 0.24 g/L was used (Rivera Flores et al., 2020).

Despite the potential for LO to inhibit blue defects as well as the presence of *Pseudomonas* spp. in FMC, no definitive studies have been conducted investigating the application of LO to dairy matrices of variable lactose concentration (Lara-Aguilar & Alcaine, 2019b). Residual lactose concentrations of 6.0 ± 0.3 m*M* and 3.0 ± 0.3 m*M* have been detected in FMC (Fior di latte) made from cow's milk using DA and SF, respectively (Minervini et al., 2012). With all of this in mind, the following hypothesis has been formulated: The enzyme LO can be used to inhibit the development of blue discoloration defects produced by *P. fluorescens* in FMC.

Here, the hypothesis is tested with different concentrations of LO in cheese elaborated by two different processes: DA and SF.

2. MATERIALS AND METHODS

2.1 Preparation of cheese samples, surface treatment with LO

All of the cheese blocks used in this study were purchased at a local market. Samples of vacuum-packed fresh mozzarella, each representing either the DA or the SF process of elaboration, were selected from two different brands. Working under sterile conditions and using a sterile knife, the cheeses were cut into rectangles approximately 4 x 1 x 2.5 cm in size. Each piece was placed into a separate sterile 90 mm-diameter polystyrene Petri dish and stored for approximately 1 hour at 6 ºC, after which the appropriate treatment was applied, as described below.

First, a LO (Lacto Yield, Chr. Hansen, Milwaukee, WI) stock solution was prepared containing 24 g LO per liter of Milli-Q water (Milli-Q Advantage A10 system, MilliporeSigma, Burlington, MA); this solution was sterilized using a 0.45 um vacuum filter. The stock was then combined with sterile Milli-Q water to produce three diluted solutions with concentrations of 2.4, 0.24 and 0.024 g/L, respectively. Finally, these dilutions were each mixed with an equal volume of sterile Milli-Q water to produce working solutions with the respective concentrations of 1.2 (P1.2), 0.12 (P0.12) and 0.012 (P0.012) g/L. Each of the previously prepared pieces of cheese was treated with 100 uL of one of the three LO working solutions; in this way, whereas each piece of cheese only received one of the three LO treatments, ultimately each type of cheese was treated with each of the working solutions. A sterile L-shaped cell spreader was used to distribute the appropriate solution over the surface of each piece of cheese. The cheese samples were then held at 6 ºC for 1 h, so that the applied LO solution could absorb into its respective surface. For each cheese product tested, a sample (P0) was prepared that would later be inoculated with *P. fluorescens* (as described in section 2.2), but that did not receive a LO treatment; an additional “negative control” sample

(NS) was also prepared that received neither a LO treatment nor a *P. fluorescens* inoculum. In place of a LO solution, these samples were treated with sterile Milli-Q water.

2.2 Preparation and application of Pseudomonas fluorescens inocula

In order to reproduce the blue discoloration defect seen on FMC, an isolate with the designation "FSL W5-0203" was obtained from Cornell University's Food Safety Lab; this isolate represents the strain *P. fluorescens* biovar IV. This specific isolate has been demonstrated in a previous study (Reichler et al., 2019) to produce blue color defects in mozzarella.

To resuscitate, the cryo-preserved culture of *P. fluorescens* was streaked onto brain heart infusion (BHI) agar (BD Diagnostics, Franklin Lakes, NJ) and incubated for 24 h at 30 ℃. A single colony from the plate was then used to inoculate a tube of 5 mL of BHI broth (BD Diagnostics); this was done in duplicate. The tubes were incubated for 18 h at 25 ℃ with shaking at 185 rpm. Following incubation, the culture tubes were vortexed to homogenize, and representative samples of each tube were measured at 600 nm using a Genesys 6 spectrophotometer (Thermo Scientific, USA) to determine their optical densities (ODs), so as to provide an estimate regarding a volume of culture that would contain a sufficient number of CFU for subsequent steps. Eppendorf tubes containing the appropriate volumes of these inocula were centrifuged at 12,000 x *g* for 2 min, the supernatants were removed, and each pellet was resuspended with 1 mL of sterile 1x phosphate-buffered saline (PBS). Serial dilutions of these cultures were plated in order to precisely determine the cell concentrations of the respective undiluted cell resuspensions from which they were prepared; these plates were counted using a Chemopharm® Color QCount model 530 (Advanced Instruments, Inc., Norwood, MA). Using this method, the undiluted cell resuspensions were determined to contain ~ 7.37 log CFU/ml. Once these concentrations were obtained, the ~ 7.37 log CFU/ml

resuspensions were again subjected to serial dilution using 1x PBS, to generate suspensions with concentrations of ~5.55 log CFU/ml, as determined by the same process of serial dilution, plating, and enumeration via QCount. 50 uL aliquots of these ~5.55 log CFU/ml suspensions were spot-inoculated over the surfaces of each of the samples P0, P1.2, P0.12, and P0.012. This process resulted in each cheese sample receiving on its 10 cm2 surface an inoculum that averaged ~ 4.25 log CFU for three biological replicates, with a standard deviation of 0.34 log CFU. The cheese samples were stored at 6 ºC for 16 days, to simulate slightly abusive refrigeration temperatures.

2.3 Quantification of color change

To quantify the efficacy of LO in inhibiting the development of blue color, on days 4, 8, 12, and 16, photographs of each sample were taken with a Canon Power Shot SX530 HS camera, configured with the following parameters: aperture (f): 5.6; shutter speed: 1/60 s; ISO-800; focal length: 50 mm. To limit exposure to ambient light, pictures were taken in a bio-safety cabinet (BSC) (Nuaire NU-425-400, Plymouth, USA) in a darkened room; the fluorescent lamp inside the BSC provided the light for the pictures. For quantification of each cheese sample's color, the Commission Internationale de l'Eclairage (CIE) colorimetric standards were used to determine the applicable coordinates (Caputo et al., 2015). For each cheese's surface upon which inoculum and/or treatment was applied, the ImageJ program (ImageJ, v 1.52, Wayne Rasband, National Institutes of Health, USA) was used to determine the values of L^*, a^*, and b^*, which represent, respectively, lightness (an L^* value of 0 represents pure black; an L^* value of 100 is a diffuse white); the position on the green-red spectrum (negative a^* values are green; positive values are red); and the position on the blue-yellow spectrum (negative b^* values are blue; positive values are yellow).

For each cheese sample, the total color change (ΔE) was calculated and used as the primary means of evaluation of overall difference between each respective treatment sample and the appropriate NS from the same time point, based on equation (1);

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

The NS samples provided a baseline against which the ΔE values of the other treatments were measured. On each successive sampling day, each treatment sample was compared with its respective NS, and a ΔE value for each treatment was calculated for that time point.

2.4 Evolution of pH in cheese samples

For each of the SF and DA cheeses under investigation, samples were prepared and treated with 400 uL of one of the three LO working solutions: P0.012, P0.12, or P1.2. For each cheese product, a control sample was also prepared, to which was applied sterile Milli-Q water in place of LO. A pH meter with a surface electrode (HI14140, Hanna Instruments) was used to take a pH measurement of the surface of each cheese sample on days 0, 4, 8, 12, and 16. Samples were stored at 6 °C between measurements.

2.5 Statistical analyses

For the purposes of statistical analyses, 3 biological replicates were performed, each of them with two technical replicates. Inferential statistical tests were performed with JMP Pro 15 software (SAS Institute, Cary, NC). Significance level was set to 0.05. ANOVA and Tukey's honestly significant difference test were used to compare differences among the means of the treatments for the same time point for the values of the color coordinates (L^*, a^*, and b^*) and for ΔE. The “each pair Student’s t-test” was used to compare the differences between time points for the means of

each treatment. Pictures representing the changes in color seen in all treatments of DA and SF samples on day 16, as well as pictures representing the evolution of color seen in NS samples over the course of the 16 days of the trial, are presented for descriptive purposes.

3. RESULTS AND DISCUSSION

3.1 Changes in color and pH in DA mozzarella cheese treated with LO

In the case of fresh mozzarella made by DA, significant differences between treatments were not detected on either day 4 (P = 0.762) or day 8 (P = 0.866), and mean values for Δ*E* were in the range of 1.5 – 2.5 (Figure 1).

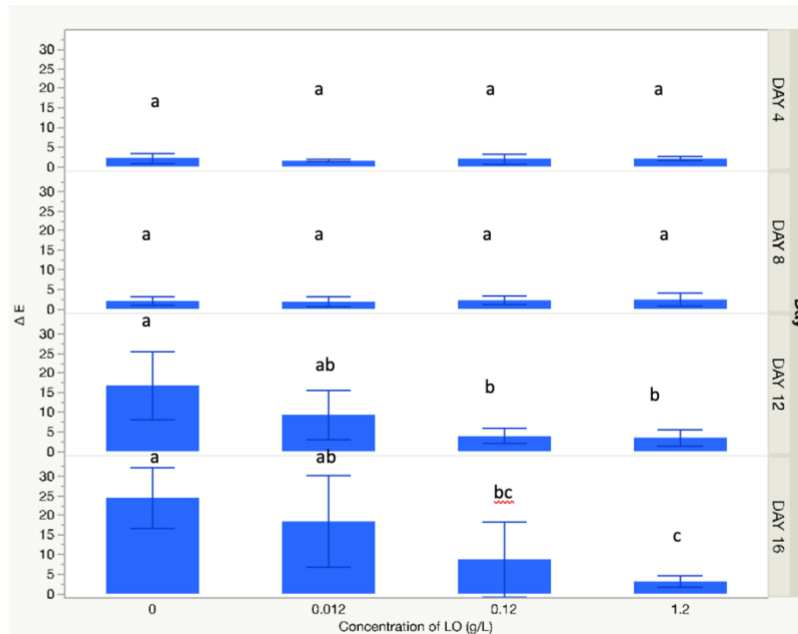

Fig. 1 Overall color change (Δ*E*) in mozzarella cheeses, made by direct acidification subsequently inoculated with *Pseudomonas fluorescens* and surface-treated with solutions with selected concentrations of lactose oxidase (LO), during 16 days of storage at 6°C

[a-c] Within each row, treatments that do not share a common letter are significantly different

The measures on day 12 showed significant differences between treatments (P = 0.002), reaching a maximum mean value of 16.8 for the sample P0, in which *P. fluorescens* was not exposed to any LO, followed by the P0.012 sample, to which the lowest concentration of LO was applied (Figure 1). On day 16, the differences between treatments increased further (P = 0.001), and the P0 sample reached the highest mean value of 24.4 (Figure 1). In a previous study, utilizing the same method of direct inoculation on FMC made by DA, the change in color was detected after 2 d, but this

involved an inoculation level of approximately 6.7 log CFU (Reichler et al., 2019). However, in our study, with an initial inoculation level of ~4.25 log CFU, the color change was not clearly evident until some point between days 8 and 12. This study's initial concentration of *Pseudomonas*, were it to occur in the context of PPC, would be considered high enough to cause defects in products, as was reported in a study from the UK, in which the majority of instances of PPC, originating in the filling step, resulted from relatively low levels of between 1-50 psychrotolerant gram-negative bacteria per 100 mL of product (Schroder, 1984).

Figure 2 displays images of all cheese samples, comprising the three biological replicates, as of each sample's respective day 16. This figure gives a visual representation of the fact that across biological replicates, the highest ΔE value occurred in the P0 samples (Figure 1). It also gives an indication that for each replicate, the mean ΔE value of the duplicate P0 samples directly correlates with the total number of samples in that replicate that underwent a color change over the majority of their surfaces (Figure 2).

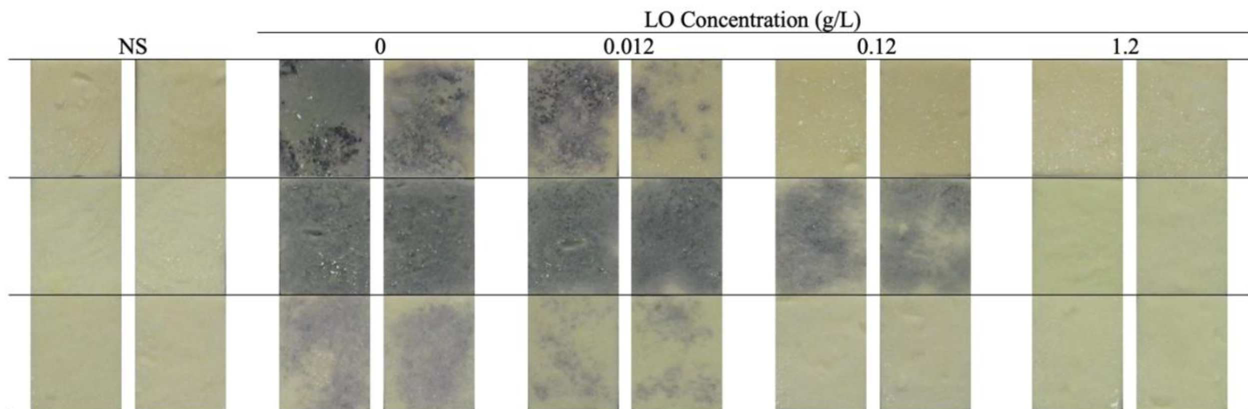

Fig. 2 Photographs of fresh mozzarella cheeses, made by direct acidification (DA), on the 16th day following inoculation with *Pseudomonas fluorescens* and surface-treatment with solutions with selected concentrations of lactose oxidase (LO); also pictured are "negative control" samples (NS), which received neither an inoculum nor a LO treatment.

As of day 12, P0's values for L^*, a^*, and b^* started to show significant differences compared to

the respective values of the NS. These differences increased by day 16, reaching mean values, in the case of P0, of 51.5 for *L** (reflecting a decrease in brightness as darker colors developed), 4.0 for *b** (indicating a tendency to transition from a yellow to a blue color) and -2.0 for *a** (denoting a tendency to shift from a green to a red color) (Table 1).

Table 1. CIE colorimetric coordinates for direct acidification (DA) fresh mozzarella cheese, inoculated with *Pseudomonas fluorescens* and surface-treated with lactose oxidase (LO).

Day	Colorimetric coordinate	NS[1]	LO concentration (g/L)			
			0	*0.012*	*0.12*	*1.2*
4	L*	72.9[a]	72.1[a]	72.6[a]	71.5[a]	71.6[a]
	a*	-5.7[a]	-5.2[a]	-5.6[a]	-5.8[a]	-5.3[a]
	b*	15.8[a]	15.3[a]	15.2[a]	16.0[a]	15.6[a]
8	L*	73.1[a]	72.4[a]	72.6[a]	71.6[a]	71.9[a]
	a*	-5.4[a]	-5.3[a]	-6.1[a]	-6.2[a]	-5.9[a]
	b*	15.4[a]	15.2[a]	16.4[a]	16.3[a]	16.2[a]
12	L*	71.8[a]	59.3[b]	65.0[ab]	69.0[a]	72.5[a]
	a*	-6.1[b]	-2.8[a]	-4.7[ab]	-5.1[ab]	-6.1[b]
	b*	16.8[a]	6.7[b]	11.4[ab]	16.0[a]	15.0[a]
16	L*	71.4[a]	51.5[c]	55.3[bc]	64.6[ab]	71.9[a]
	a*	-5.3[cd]	-2.0[a]	-3.3[ab]	-4.5[bc]	-6.9[d]
	b*	17.4[a]	4.0[c]	8.6[bc]	14.4[ab]	19.0[a]

[a-d] Within each row, each single letter indicates a discrete group the values of which are significantly different from those of all other single letters; coupled letters indicate groups the values of which are intermediate between (not significantly different from) the two groups represented by the coupled letters' two individual letters

[1] NS: "Negative control" sample, which received neither a *P. fluorescens* inoculum nor a LO treatment

These CIE coordinates reflect the final blue-grayish colors developed due to the actions of *P. fluorescens* (Figure 2). This strain tends to produce a gray color when the pH of the sample is close

to the natural pH of fluid milk (pH 6.70), whereas exposure of this strain to a lower pH tends to result in a bluer discoloration (Reichler et al., 2019). At the other extreme, sample P1.2 produced both the lowest mean ΔE value (3.1) and the smallest standard deviation for day 16, displaying consistently minimal color changes by this final day (Figure 1). In fact, none of the P1.2 samples generated any blue color spots by day 16 (Figure 2), and the L^*, a^*, and b^* values of these samples did not show significant differences from those of NS over the same time period (Table 1). Although it fluctuated over time, P1.2's mean pH value ultimately underwent a decrease during the 16 days of the trial, whereas those of samples P0, P0.012, and P0.12 increased over the same time period (Figure 3). P1.2’s decrease in pH is a phenomenon not generally seen during the process of rind formation; rind formation is usually associated with an increase in pH, accompanied by a reduction in water activity, as seen in cheeses that are partially or completely exposed to air for purposes of ripening (Chatelard-chauvin et al., 2015).

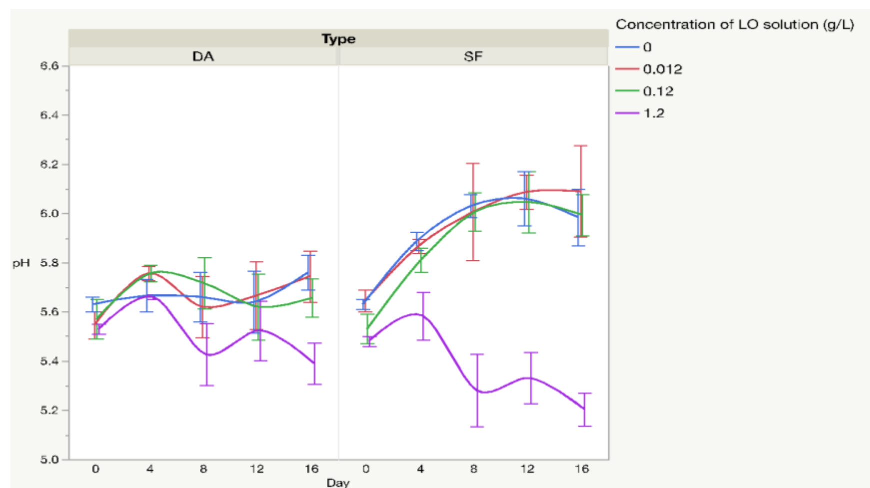

Fig. 3 Evolution of pH of mozzarella cheese, made by direct acidification (DA) or starter fermentation (SF), during 16 d of storage at 6ºC following surface treatment with solutions of lactose oxidase (LO) at concentrations of 0, 0.012, 0.12 or 1.2 g/L.

P1.2’s decrease in pH is likely attributable to the oxidation of lactose to lactobionic acid, with accompanying increase in H_2O_2 concentration, the overall combination of which presumably

caused the inhibition of *P. fluorescens* and any related blue discoloration (Figure 3). The present results indicate the efficacy of LO at a concentration of 1.2 g/L in inhibiting degradation of color characteristics of FMC, made by DA, during instances of contamination by *P. fluorescens* at a concentration of up to 4.25 log CFU per 10 cm^2. Applicable to this level of contamination, this study's methodology suggests a potential tool to extend the shelf life of the product, conceivably effective in scenarios both of PPC at the processing site (Martin et al., 2011), and of contamination during the retail consumer's removal of packaging and subsequent refrigerated storage of the product at home (Cenci-Goga et al., 2014).

In the case of the P0.12 samples, a mean ΔE value of 8.7 was measured as of day 16; this value was not significantly different from the lower ΔE value of P1.2, but was significantly different from the higher mean ΔE value of the P0 samples (Figure 1). At this LO concentration of 0.12 g/L, 4 of the 6 samples underwent no significant changes in color (Figure 2); this is despite the fact that the 2 samples that did develop blue discoloration initially received an inoculum of fewer CFUs (3.87 log CFU) than those of the 4 samples that did not undergo discoloration (average of 4.45 log CFU). These last results and associated inoculation concentrations demonstrate that there is some inhibitory effect with a LO surface application of 0.12 g/L; in the manufacturing setting, however, this inhibitory effect may be partially or completely counteracted, depending on the variability inherent to the process of batch manufacturing of mozzarella. Such process variability, although generally considered both inevitable and acceptable, can result in variability in terms of the product's pH, moisture content, and/or concentration of colloidal calcium, any of which may in turn adversely affect the results of the application of the proposed methodology. Results by Lara-Aguilar & Alcaine (2019b) demonstrated that at a LO concentration of 0.12g/L, the level of lactose present in the environment plays a crucial role. Whereas LO concentrations of 1.2 and 12 g/L

required no lactose for their inhibitory effects, inhibition was only seen with 0.12 g/L when there was concomitant exposure of the organism to lactose (Lara-Aguilar & Alcaine, 2019b). In DA mozzarella cheese, as indicated in section 1, lactose is generally present at a concentration of 6.0 ± 0.3 m*M* in the final product (Minervini et al., 2012); this combination of lactose concentration and LO surface application of 0.12 g/L appears to be insufficient to interact in a way that would consistently inhibit blue discoloration. In another study, the authors hypothesized that citric acid, used to acidify milk during the manufacture of cheese, may also exert an inhibitory effect on the development of blue pigment; the authors base this hypothesis on the fact that citric acid is known to inhibit enzymatic browning in fruits and vegetables, and to chelate metal ions that could either act as cofactors for enzymes that produce pigments, or that could themselves directly catalyze oxidative reactions (Reichler et al., 2019). The initial level of contamination is also a factor that has some effect on blue coloration. For example, a study examining the development of blue color under different storage conditions revealed that the initial concentration of contaminants, storage time, storage conditions, and number of times that packaging was opened all correlate with the development of blue color in some, but not all, of the samples tested (Cenci-Goga et al., 2014). Another point to consider in the performance of LO at the concentration of 0.12 g/L is the method of surface application. The current study utilized a surface treatment representative of those used in industry in steps leading up to vacuum packaging. In the industrial manufacture of cheese, shower technology or dipping are common and practical ways to apply surface treatments, as in the case of natamycin utilization (Ollé Resa et al., 2014). Most previous studies that have focused on investigating or preventing blue discoloration by *P. fluorescens* in FMC have focused on cheeses packaged in liquid (preserving brine) (Cenci-Goga et al., 2014; Caputo et al., 2015; Faccia et al., 2019); future research could elucidate the performance of LO at a concentration of 0.12 g/L

in this context.

All samples treated with the lowest concentration of 0.012 g of LO per liter developed blue coloration by day 16 (Figure 2). On Day 16, P0.012's CIE colorimetric coordinates and *ΔE* showed no significant differences compared to those of sample P0; the P0.012 treatment did not demonstrate any inhibitory effect on blue coloration under the conditions outlined in this study (Figure 1; Table 1). This may be related to the fact that at this lowest LO concentration, inhibitory properties may require the presence of lactose in the milieu, as discussed previously; mozzarella cheese does not provide the minimum concentration of lactose necessary for this concentration of LO to suppress the generation of blue color (Lara-Aguilar & Alcaine, 2019b; Minervini et al., 2012). Based on the data captured in this study, we propose a minimum inhibitory concentration of between 0.12 g and 1.2 g of LO per liter to act as a hurdle against the development of blue color defects by *P. fluorescens*, when present at a concentration of ~4.25 log CFU/10 cm2 or less in DA mozzarella cheese. A concentration of 0.012 – 0.12 g/L could be effective, depending on the initial counts of the contamination and the standardization of the manufacturing process, which largely determines the subsequent level of variability with respect to composition and characteristics. The methodology proposed in this paper, in addition to strict adherence to and validation of good manufacturing practices, will help ensure that risks of PPC, and associated consequences, consistently remain as minimal as possible.

3.2 Changes in color and pH in SF mozzarella cheese treated with LO

The results regarding color change over a 16-day period for SF cheese inoculated with *P. fluorescens* and treated with selected concentrations of LO showed that comparisons between the various treatments' *ΔE* values within each day for days 4, 8, 12, and 16 did not reveal significant

differences (Figure 4).

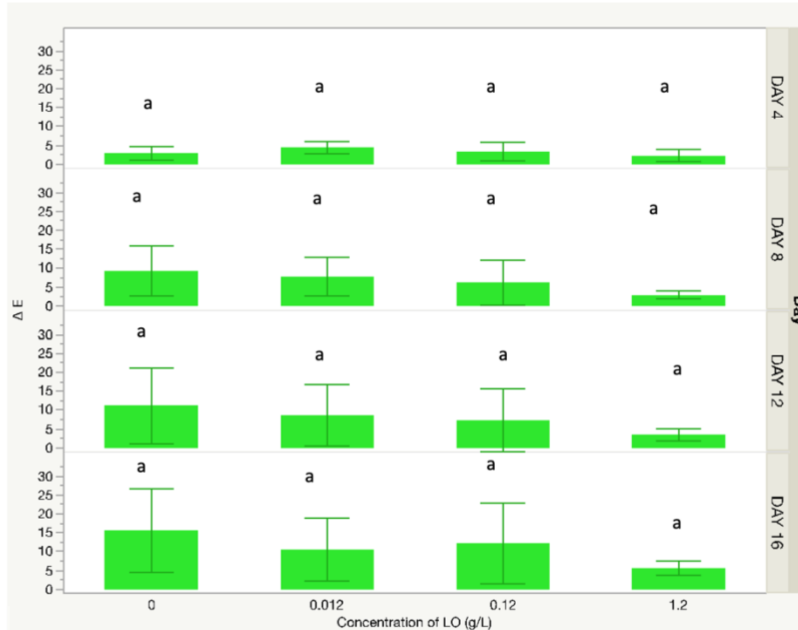

Fig. 4 Overall color change (ΔE) in mozzarella cheeses, made by starter fermentation, subsequently inoculated with *Pseudomonas fluorescens* and surface-treated with solutions with selected concentrations of lactose oxidase (LO), during 16 days of storage at 6°C

[a-c] Within each row, treatments that do not share a common letter are significantly different

In these trials, SF mozzarella cheese behaved fundamentally different from DA cheese with respect to coloration, as the SF NS consistently exhibited a color change in the absence of *P. fluorescens* inoculation; this illustrates the fact that, in addition to applied treatments, means of acidification of the cheese is itself a variable that may influence color change (Figure 5). The samples' color parameter values are displayed in Table 2, wherein in the case of the NS samples, a significant difference is seen between the $b*$ values of day 4 and day 16 ($P < 0.0001$), based on the results of an "each pair Student's t" test. NS's $b*$ value on day 16 corresponds to an intense yellow-green color, as seen in Figure 5.

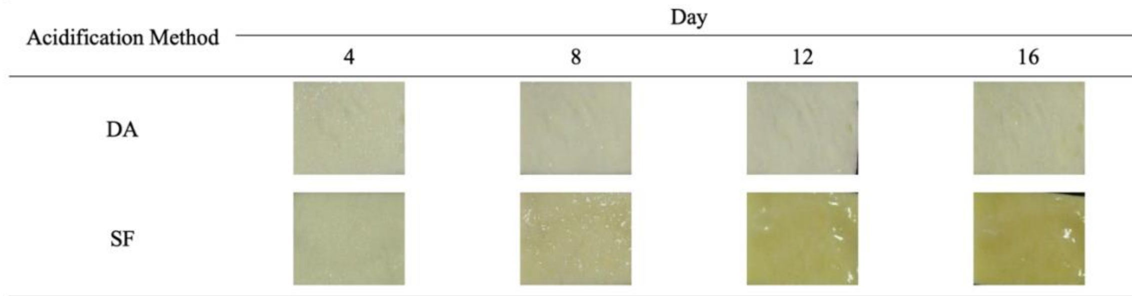

Fig. 5 Evolution of color of uninoculated/untreated fresh mozzarella cheese samples (NS), made by direct acidification (DA) or starter fermentation (SF), during 16 days of storage at 6°C

Table 2. CIE colorimetric coordinates for starter-fermented (SF) fresh mozzarella cheese, inoculated with *Pseudomonas fluorescens* and surface-treated with lactose oxidase (LO).

Day	Colorimetric coordinate	NS[1]	LO concentration (g/L)			
			0	*0.012*	*0.12*	*1.2*
4	L*	70.5[a]	70.4[a]	67.1[b]	68.7[ab]	69.5[a]
	a*	-6.5[a]	-6.3[a]	-6.2[a]	-6.7[a]	-6.1[a]
	b*	19.5[a]	19.3[a]	19.3[a]	19.7[a]	18.0[a]
8	L*	68.8[a]	64.3[a]	65.8[a]	67.2[a]	68.9[a]
	a*	-6.0[a]	-4.4[a]	-4.9[a]	-4.8[a]	-5.6[a]
	b*	20.3[a]	15.9[a]	16.9[a]	16.7[a]	17.6[a]
12	L*	70.0[a]	62.6[a]	65.1[a]	65.7[a]	69.1[a]
	a*	-7.8[a]	-6.6[a]	-6.6[a]	-6.8[a]	-7.3[a]
	b*	26.4[a]	19.7[a]	20.9[a]	20.3[a]	21.9[a]
16	L*	67.5[a]	59.6[a]	61.9[a]	61.6[a]	69.2[a]
	a*	-9.1[a]	-5.3[a]	-7.7[a]	-7.3[a]	-7.3[a]
	b*	33.00[a]	24.2[a]	26.0[a]	24.0[a]	24.1[a]

[a-b] Within each row, each single letter indicates a discrete group the values of which are significantly different from those of all other single letters; coupled letters indicate groups the values of which are intermediate between (not significantly different from) the two groups represented by the coupled letters' two individual letters

[1] NS: "Negative control" sample, which received neither a *P. fluorescens* inoculum nor a LO treatment

In comparison, when stored under the same conditions, there was no significant difference (P =

0.315) between the DA cheese NS's mean b^* coordinates from day 4 and day 16 (Table 1). A previous study that examined microbial counts and physico-chemical parameters in high-moisture mozzarella cheese produced results similar to ours with regard to both processes of acidification; that study demonstrated an increase in the b^* value for SF control samples, but not for DA control samples, after 5 days of refrigerated storage (Ricciardi et al., 2015).

With regard to the present study's P0 samples, the scenario was nearly the opposite of that of the NS samples. With DA, all of the P0 samples developed a blue-gray color over the majorities of their surfaces by day 16 (Figure 2). In contrast, with SF, of the six P0 samples (representing three biological replicates each with duplicate samples), only the surfaces of two samples underwent a definitive change in color by day 16, while the other four developed only small, dispersed patches of blue color during the same time period (Figure 6).

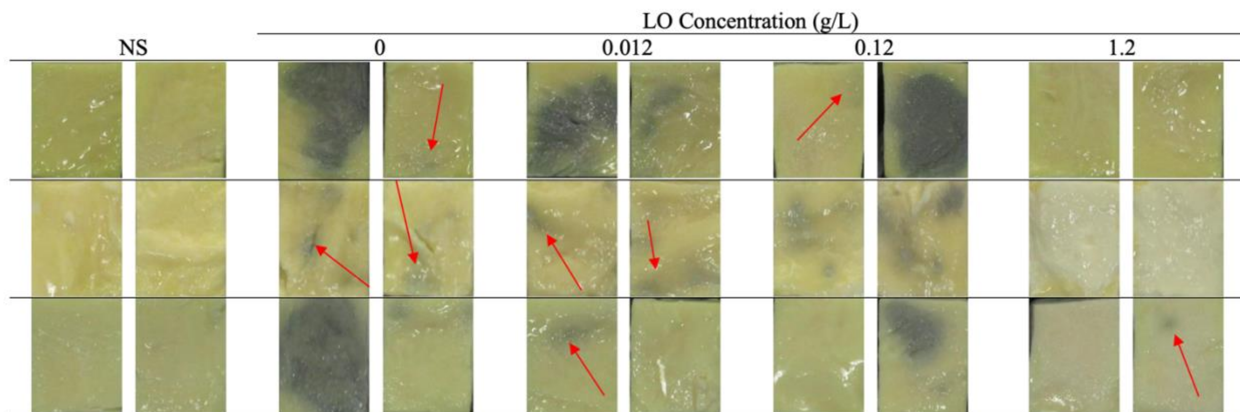

Fig. 6 Photographs of fresh mozzarella cheeses, made by starter fermentation (SF), on the 16th day following inoculation with *Pseudomonas fluorescens* and surface-treatment with solutions with selected concentrations of lactose oxidase (LO); also pictured are "negative control" samples (NS), which received neither an inoculum nor a LO treatment. Red arrows indicate the presence of blue color in instances in which it is not easily discerned.

One hypothesis for this is that production of SF FMC often involves the common starter culture *Streptococcus thermophilus*, which produces bacteriocins that may act as a hurdle against spoilage by the *P. fluorescens* used in this study (Guidone et al., 2016; F. Rossi et al., 2013).

The SF P1.2 samples produced the lowest mean *ΔE* value (5.7) on day 16, while the SF P0 samples achieved the highest mean *ΔE* (15.7), a pattern similar to that of DA (Figure 1). As previously stated, however, unlike the ΔE values for DA, for SF day 16 measurements, a larger standard deviation for P0, combined with a smaller range between minimum and maximum mean values than that of DA, due to differences intrinsic to SF, translated into a lack of significant difference across treatments. The spotty quality of the color changes in the SF P0 day 16 samples no doubt contributed to the large standard deviation (Figure 4, Figure 6). For SF, the P1.2 treatment performed the best, as only one out of these 6 samples developed blue color (Figure 6). P0.12 and P0.012 both developed blue color in five of their respective six samples (Figure 6). These results demonstrate that application of LO as a 1.2 g/L solution to the cheese surface can fairly effectively inhibit *P. fluorescens*, although the effectiveness of this application appears more powerful when utilized with DA cheese, as in this study, at this concentration, all analyzed samples were free from blue color (Figure 2). One explanation for this difference could be related to the amount of residual lactose in cheese produced by each respective acidification method, with a higher average concentration for DA cheese, as compared to SF cheese (Minervini et al., 2012). This variation in residual lactose concentration, in the presence of LO, would theoretically result in a higher level of production of H_2O_2 in DA cheese as compared to that of SF cheese. This last explanation is brought into question by examining the pH data in Figure 3. Here, the SF P1.2 displayed a larger overall decline in pH over 16 days, as compared to the DA P1.2 (Figure 3). This is in spite of the fact that the DA cheese most likely had a higher concentration of residual lactose that could serve as a substrate for the production of lactobionic acid and H_2O_2, the presumed drivers of the observed reduction in pH for both types of cheese. This downward pH trend for P1.2 cheeses of both acidification methods is an uncommon phenomenon, as FMC surface pH generally rises during

the period of rind formation (Chatelard-chauvin et al., 2015).

Further studies need to be conducted with these products to determine the optimum minimum concentration that provides the desired effect. Sensory analyses also need to be conducted to investigate effects on flavor. Additionally, future research must investigate LO's effects on the functional characteristics of FMC, such as on browning defects resulting from residual lactose and galactose, as seen particularly in SF cheese (Oberg et al., 1991).

In the quest to inhibit blue discoloration by *P. fluorescens* in SF mozzarella cheese, we have collected no data that would definitively determine the optimum LO concentration to be applied. Nevertheless, surface-application of a solution of 1.2 g LO per liter demonstrated the best performance, all things considered; further studies need to be executed to verify the validity of this concentration, and ultimately to reach the goal of preventing the spoilage of mozzarella cheese due to blue color defects by *P. fluorescens*.

4. CONCLUSIONS

Our findings support the utilization of LO, via surface application, as a viable method to prevent blue color defects in FMC as caused by *P. fluorescens*, when present due to a contamination event that resulted in an initial inoculation level of 4.25 log CFU/10 cm^2 or less. This LO treatment provides better results in FMC made by DA, but still shows promise in SF FMC. Manufacturers will need to consider several parameters in order to determine the most suitable LO concentration for their products; these parameters include the type of acidification method used for production of cheese, final concentration of residual lactose in the product, storage temperature, potential initial contamination levels, and type of packaging. Future studies should compare sensory and functional properties of FMC following application of LO.

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