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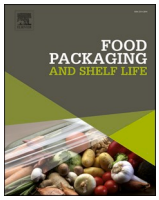

A salicylate-functionalized PET packaging to counteract blue discoloration on mozzarella cheese under cold storage

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ABSTRACT

In this work we tested a new technological solution to extend the shelf-life of Mozzarella cheese using a salicylate-functionalized PET packaging. To this aim, we inoculated ready-to-sell Mozzarella cheeses with *Pseudomonas lactis* ITEM 17298 strain, responsible for blue discoloration; mozzarella cheeses packed in treated or untreated trays were stored at 4 °C and monitored up to blue discoloration occurred. Results from microbiological analyses showed inhibitory effect against the pigmenting strain up to 8 days of cold storage in Mozzarella cheese packed in treated trays compared to the control samples. Likewise, CIELab values of b* and hue did not differ from those found on the uninoculated Mozzarella cheeses, delaying the appearance of bluish spots by approximately 2 days. Furthermore, the use of treated trays unpaired the formation of the biofilm by significantly reducing the amount deposited inside the treated trays compared to the control ones. For the first time, therefore, the use of an antimicrobial packaging with high technology readiness level has delayed the blue mozzarella occurrence specially contaminated samples. Further trials will have to be undertaken to optimize the salicylate concentration released in the tray in order to abate the risk of this fresh dairy product withdrawal from the market.

1. Introduction

High-moisture Mozzarella, the most-exported Italian cheeses worldwide, is a soft, fresh cheese characterized by a short shelf-life; the global Mozzarella Cheese market is valued at 10100 million US$ in 2018 and will reach 15100 million US$ by the end of 2025, growing at a compound annual growth rate (CAGR) of 5.2% during 2019–2025 (Absolute Reports, 2019). However, mozzarella shelf life is highly affected by spoilage microorganisms, responsible for off-flavor, texture deterioration and anomalous discolorations (Baruzzi, Lagonigro, Quintieri, Morea, & Caputo, 2012; Caputo et al., 2015).

Over the past decade, several widespread occurrences of anomalous blue coloration of Mozzarella cheese have been recorded in the United States and some European countries; blue discoloration events caused product withdrawn, cheese waste increase across the value chain with severe environmental impacts and economic losses by dairy industries, also as a consequence of damage to the company image (del Olmo, Calzada, & Nuñez, 2018). Although several studies linked the blue discoloration to high concentrations of *Pseudomonas fluorescens* belonging to called "blue branch" phylogenetic group (Nogarol, et al., 2013; Andreani et al., 2015), the constant updating of genomic data repositories allowed to reclassify them as *Pseudomonas lactis* (Quintieri, Caputo, De Angelis, & Fanelli, 2020a). In addition, recent evidences have highlighted that psychrotrophic pseudomonads (including *P. lactis* and *P. fluorescens*) showed the presence of antibiotic resistant genes (β-lactams and carbapenems), that can be acquired and transmitted by horizontal genetic transfer by increasing the risk associated with their persistence in food and the need to be controlled and deeper investigated (Quintieri, Fanelli, & Caputo, 2019a). It has been reported that *Pseudomonas* spp. contamination in dairy plants in mozzarella cheese derives from water used during the processing (Martin, Murphy, Ralyea, Wiedmann, & Boor, 2011); on pipe surfaces and dairy processing equipment *Pseudomonas* spp. persistence is promoted by their growth in biofilm state (Cherif-Antar *et al.* 2016). Biofilm, positively affected by

Abbreviations: LDHs, layered double hydroxides; PET, poly(ethylene terephthalate); SA, salicylic acid; OD, optical density; ANOVA, analysis of variance; CFU, colony forming unit; TBC, total mesophilic aerobic counts; CIE, Commission Internationale de l'Eclairage; CV, crystal violet.

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the low temperatures (Quintieri et al., 2019b; Rossi et al., 2018), also plays a role in spoilage by promoting enzyme production and color development (Teh *et al.* 2014; Quintieri et al., 2019b). Although the research of novel bio-preservatives targeting biofilm by spoilage bacteria are ongoing (Machado, Silva, Giaouris, Melo, & Simões, 2020), their application is still limited by the high production cost; *e.g.* in the case of blue discoloration, the control strategy based on the antibiofilm activity of lactoferrin derived peptides and was limited by the high cost of the purified protein to be hydrolyzed (Caputo et -al., 2015; Quintieri et al., 2019b). Promising results in the control of this anomalous discoloration have been also obtained by the application of acidified brine delaying the growth of the pigmenting strains (Faccia, Gambacorta, Natrella, & Caponio, 2019). A part from these two above mentioned applications, no additional strategies have been developed to counteract anomalous discoloration of Mozzarella cheese negatively emphasized by newspapers resulting in an image damage for the dairy manufacturers; accidentally events, indeed, occur due to a gap in the legislation related to non-pathogenic pseudomonads limits.

Recently, nanoscience and nanotechnology gave an interesting contribution towards the development of food packaging to be applied downstream of the manufacturing process (Dasarahally-Huligowda, Goyal, & Suleria, 2019). Among nano-fillers that can modify the polymers' properties and functionalities Layered Double Hydroxides (LDHs) are very attractive and versatile (Costantino, Nocchetti, Gorrasi, & Tammaro, 2011; Forano, Costantino, Prévot, & Gueho, 2013; Rives, del Arco, & Martín, 2014). They are biocompatible and can be produced with simple procedures and high level of purity, and have been widely proposed as carriers of antimicrobials for active packaging development (Costantino, et al. 2009). In particular, its functionalization with salicylate anions (listed in EC-Directive 10/2011/EC of 14 January 2011) was effective to extend shelf life of fresh or processed food under cold storage conditions (Bugatti, Vertuccio, Zuppardi, Vittoria, & Gorrasi, 2019; Bugatti, Viscusi, & Gorrasi, 2020; Gorrasi, et al., 2020).

In light of these considerations, the aim of this work was the application of active salicylate-LDH coated PET trays to counteract the blue discoloration of Mozzarella cheese and extend shelf-life of this fresh cheese under purchase conditions. In this way the Mozzarella cheese, also if accidentally contaminated by pigmenting pseudomonads, would gain a greater commercial advantage compared to the use of traditional trays.

2. Material and methods

2.1. Preparation of active film

Poly(ethylene terephthalate) (PET) trays (length: 18 cm, width: 14 cm, height: 7 cm), have been thermoformed by Selepack spa (Italy) from laminae 350 μm thick (trade name: R.PET TRA S2 F740 S350, 2157400350). The closure of the PET trays is made of the same material as the tray. The active filler is constituted of a LDH in which the nitrate anion has been substituted with the salicylate anion (listed in EC-Directive 10/2011), the modification was conducted accordingly to a previously reported procedure (Frunza, Lisa, Popa, Miron, & Nistor, 2008) and kindly provided from Nice Filler Startup. A water based paint food grade, whose constituents are in accordance with the EC-Directive 2002/72 including amendments, was purchased from Inx srl (Lodi, Italy). The trade name is Inx 1–7801–7000, with a solid content 42 ± 2%, viscosity 20 s at 20 °C. The resin was mixed to the active filler at 7 wt% (4.2% of active molecule) through a high energy ball milling at ambient temperature, for 30 min at 450 rpm. The PET laminae were coated with the active coating (Treated sample) by using an automatic coater. The composite filler weight was 12 ± 0.5 g/m^2 on dry resin. The laminae, after the coating, were thermoformed to obtain the active trays; trays used as control, thermoforming the uncoated PET laminae, were also produced.

2.2. Bacterial culture conditions

Pseudomonas lactis ITEM 17298, widely studied for its spoilage traits including blue mozzarella cheese discoloration (Caputo et al., 2015; Quintieri et al., 2019b; Quintieri, Caputo, Brasca, & Fanelli, 2021), was maintained at − 80 °C as pure stock cultures in Nutrient Broth (NB; Oxoid S.p.A., Rodano, Milan, Italy) supplemented with glycerol 30% (vol/vol). The strain was routinely refreshed (30 °C, 24 h) by streaking onto Luria Bertani (LB; Sigma Aldrich, Milan, Italy) agar; then it was cultivated for 16 h at 30 °C and 150 strokes/min into 25 mL of LB broth to reach the optical density (OD) at 600 nm of *ca.* 0.25 (corresponding to 7.00 log CFU/mL), used in the subsequent experiments as initial inoculum.

2.3. Effect of salicylate on blue discoloration of Mozzarella cheese disks

At first, the efficacy of increasing concentration of salicylic acid (SA; 0.005, 0.5, 5.0, 50 mg/mL) to counteract blue cheese discoloration by the selected strain was evaluated on Mozzarella cheese disks. Briefly, disks (20 × 5 mm; cut with a cork borer) of fresh Mozzarella cheeses were obtained under sterile condition and transferred to 12-well plates; then, they were covered with sterile saline solution (3 mL) inoculated with the fresh bacterial cell suspension at the final concentration of *ca.* 4 log CFU/mL and containing or not salicylate at the above mentioned concentrations. Cheese controls were covered by the same volume of uninoculated sterile saline solutions supplemented or not with the antimicrobial compound. All samples were prepared in triplicate and incubated at 4 °C until color appearance. ITEM 17298 growth was monitored by plating serial dilution of inoculated or un-inoculated solutions on PDA potato dextrose agar (PDA) supplemented with 100 mg/L of chloramphenicol. ITEM 17298 dark-blue colonies were enumerated after incubation at 15 °C for 4–5 days (Caputo et al., 2015).

2.4. Salicylate release kinetic

The release kinetic of salicylate from the active trays were evaluated at ambient temperature using a Shimadzu (Japan) UV-2401 PC spectrometer. The tests were performed using 4 cm^2 specimens placed into 25 mL of physiological solution and stirred at 100 rpm in an orbital shaker (VDRL MOD. 711 +, Asal S.r.l., Italy). The release medium was withdrawn at fixed time intervals and replenished with fresh medium. The considered band was 230 nm.

2.5. Mozzarella cheese trials

Cow milk high moisture (HM) Mozzarella cheese ("nodini", weighing *ca.* 30 g/sample) were used to confirm Active packaging efficacy against cheese blue discoloration caused by the selected pigmenting strain *P. lactis* ITEM 17298. Briefly, cheese samples, manufactured and purchased from a Puglia dairy farm on the same day, were cold stored for 3 h before draining their governing liquid and transferred in triplicate into coated and uncoated plastic trays (three samples/tray). Each tray has been filled with 190 mL of inoculated or un-inoculated cold autoclaved tap water (*ca.* 4 log CFU/g; following the same ratio product/governing liquid of commercial mozzarella cheese). Then, the trays were incubated at 4 and 15 °C until color development occurred. At different times of cold storage (0, 3, 5, 10 days) cheese samples were withdrawn and subjected to pH determination with the Φ 340 pH/Temp Meter system Beckman Coulter, Fullerton, CA, USA) and microbiological analyses.

2.6. Microbiological analyses

For microbiological analyses 10 g of Mozzarella cheese sample were aseptically homogenized in 90 mL of sterile 0.9% NaCl solution in a stomacher (Lab-Blender 400, PBI International, Milano, Italy), and decimally diluted in 0.1% (w/v) sterile peptone saline solution (0.9%

NaCl) before plating on the selective media. In particular, *P. lactis* ITEM 17298 was detected on potato dextrose agar (PDA) incubated at 25 °C for 3–5 days (Caputo et al., 2015); pseudomonads were grown on *Pseudomonas* agar base (PSA, amended with *Pseudomonas* CFC selective supplement, Oxoid) at 30 °C for 24 h (ISO/TS 11059:2009; IDF/RM 225:2009); total mesophilic aerobic counts (TBC) were enumerated on Plate Count Agar (PCA) supplemented with 100 mg/L of cycloheximide after incubation at 30 °C for 24 h (ISO 4833, 2003).

2.7. Color determination

Color appearance of Mozzarella cheese samples stored at 4 °C in PET trays was determined at 0, 3, 8 and 10 days measuring colorimetric CIE (Commission Internationale de l'Eclairage) coordinates L* (lightness), a* (redness) and b* (yellowness) on 3 random points of cheese surface with ChromaMeter CR-400 (Konica Minolta, Osaka, Japan; illuminant: D65; observer: 2°) and Color Data Software SpectraMagic NX (Konica Minolta), as previously reported (Caputo et al., 2015). Hue (h°) and chroma (C*) of cheese samples (corresponding to the basic tint and the saturation of color, respectively) as well as color differences (ΔE*ab) between treated and untreated samples regardless of ITEM 17298 inoculation were calculated as described for a soft cheese by Loi et al. (2020).

2.8. Biofilm determination

After mozzarella cheese sampling, PET and Active trays from inoculated samples were recovered and washed three times with 200 mL of water in order to remove debris and planktonic cells. Then, biofilm cells adhering to the tray surface were stained with 200 mL crystal violet (CV; 0.1%, w/v). After a second washing step, CV incorporated by biofilm was solubilised with 30% acetic acid (v/v) and its absorbance (OD) was measured at 570 nm.

2.9. Evaluation of shelf life

The Gompertz equation was applied to estimate the shelf life (S.L.) of blue mozzarella cheese for active and untreated trays by fitting the equation (1) (Corbo, Del Nobile, & Sinigaglia, 2006):

$$\log(\mathrm{CFU/g}) = \mathrm{K} + \mathrm{A} * \exp\left\{-\exp\left\{\left[(\mu_{max} * 2.7182) * \frac{\lambda - t}{\mathrm{A}}\right] + 1\right\}\right\} \quad (1)$$

where μ_{max} is the maximum growth rate, λ is the lag phase (days), K (log (CFU/g)) represents the initial level of bacterial count, A represents the maximum bacteria growth achieved at the stationary phase while t is the time (days). The estimation of Gompertz's equation parameters allowed to evaluate the shelf life by using the equation (2):

$$\mathrm{S.L.} = \lambda - \frac{\mathrm{A} * \left\{\ln\left[-\ln\left(\frac{\mathrm{A.L.}-\mathrm{K}}{\mathrm{A}}\right)\right] - 1\right\}}{\mu_{max} * 2.7182} \quad (2)$$

Where A.L. is the acceptability limit for milk and milk-derived products (6 log(CFU/g)). All analyses were carried out in triplicate and the media and standard deviations were evaluated.

2.10. Statistical analyses

Data analysis was carried out using IBM SPSS Statistics release 20 (IBM, Armonk, NY, USA). All datasets were checked for homogeneity of variance by Levene's test ($P < 0.05$) before variance analyses. ITEM 17298 counts in Mozzarella cheese plugs, colorimetric coordinates of cheese in trays and biofilm biomass amounts were analyzed using Independent Student t-test ($P < 0.05$) comparing treated and untreated samples inoculated or not with target strain. One-way ANOVA was applied for comparing ΔE values over storage time of Mozzarella cheese using *post hoc* Tukey's HSD test. Two-way ANOVA was used for analyzing the effects of storage time and treatment tray on counts of different microbial groups. If homogeneity of variance missed, the analysis was conducted with non-parametric tests such as Mann Whitney test and Kruskal Wallis test continued with a Dunn's *post hoc* test ($P < 0.05$).

3. Results

3.1. Antimicrobial activity of salicylate

Before PET trays functionalization, the antimicrobial activity of salicylic (SA) against ITEM 17298 was previously checked by dipping inoculated Mozzarella cheese disks in saline solutions containing different SA concentrations. Results showed that blue discoloration appeared on inoculated and untreated cheese disks starting from the 5th day of incubation at 4 °C; only the highest SA concentration (50 mg/mL) inhibited color development throughout cold storage (Fig. S1). This effect was consistent with a significant concentration-dependent reduction in microbial load of inoculated ITEM 17298 in the Mozzarella plugs stored at 4 °C (Fig. 1). The reduction by *ca.* 3 log CFU/mL was registered in presence of SA 5 mg/mL at day 5 of storage in comparison with the untreated sample; however, this inhibitory effect was not enough to inhibit color development. By contrast, no viable count was detected at 5 and 8 days of cold storage under the exposure of SA at 50 mg/mL (Fig. 1).

3.2. Active filler and salicylate release

Fig. S2 reports the release of salicylate (mg) as function of the time (days), from the active tray (4 cm^2). It is evident a first fast release in one day that could be attributed to the active molecule on the surface of the coating and/or a part of not intercalated salicylate. In the saline solution (190 mL) covering mozzarella cheese, total average concentrations of salicylate released by tray surface was *ca.* 2.1 mg/mL. The second slow step, up to 30 days, is due to the progressive de-intercalation of the active molecule from the LDH layers for the exchange of salicylate with the chlorine ions in the release medium.

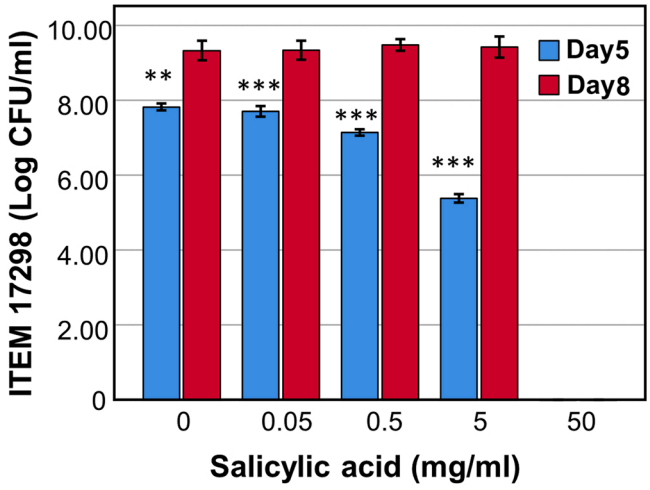

Fig. 1. *Pseudomonas lactis* ITEM 17298 loads on Mozzarella cheese plugs with different concentrations of salicylic acid added to the governing liquid and incubated at 4 °C for 5 and 8 days. Values represent mean ± standard deviation (N = 3). Significant differences: * *, $P < 0.01$; * **, $P < 0.001$ (Independent Student *t*-test).

3.3. Evaluation of mozzarella cheeses packed in functionalized trays during cold storage

Laboratory scale trials were carried out to assess the efficacy of the treated trays to counteract blue discoloration of Mozzarella cheese caused under low temperature by *P. lactis* ITEM 17298 at the purchase conditions of this cheese.

Fig. 2 shows microbial loads registered in mozzarella cheeses packed in the untreated-PET trays or treated-trays throughout cold storage. Presumptive pseudomonads and total bacterial mesophiles (TBC) loads from Mozzarella cheese samples were slightly affected by addition of the selected blue pigmenting strain ITEM 17298 regardless of the tray treatment and storage temperature (Fig. 2); indeed, at the beginning of the incubation, microbial counts in the inoculated samples registered *ca.* 6 log CFU/g, whilst they were equal to *ca.* 4 CFU/g, on average in each un-inoculated sample. Overall, except for ITEM 17298 determined on PDA as the selective medium, no significant differences were registered in TBC and total pseudomonads between treated and untreated samples (Fig. 2, panel A and B). By contrast salicylate caused an inhibitory effect against ITEM 17298 over 8 days of cold storage; then, after additional 2 days of incubation no difference in blue pigmenting strain load was registered between samples (Fig. 2, panel C).

Mozzarella cheese samples did not show apparent differences except for the bluish spots that were registered as early as day 8 only in the inoculated samples stored in untreated trays (Fig. 3). Conversely, in treated trays the inoculated samples displayed a color appearance similar to the controls without the addition of the ITEM 17298 strain. This difference in color disappeared after 2 days of storage resulting in a similar faint blue nuance for both inoculated samples regardless of treatment (Fig. 3). As shown in Fig. S3, the barely noticeable blue discoloration of Mozzarella cheese was mostly due to high average lightness (L*) and low relative saturation (C*, data not shown) values that were statistically similar among all analyzed samples during storage. Nevertheless, the blue discoloration of the inoculated samples kept in treated trays was also confirmed by b* (yellow-redness) and hue (h°) values that were significantly different than those untreated only up to day 8 (Fig. 3). These results are more evident considering CIE distance metric ΔE differences between treated and untreated samples in relation to inoculation and storage time (Fig. 4). PET trays, functionalized or not, and containing inoculated samples was also evaluated for the quantification of biofilm biomass formed on their surface (Fig. 5). Interestingly, at days 8 and 10 of cold storage, significant decreases in biofilm biomass (by *ca.* 50%) were registered in treated samples in comparison with untreated ones.

3.3.1. Evaluation of shelf life

The results concerning *P.. lactis* ITEM 17298 growth in untreated and active trays stored at 4 °C were reported in Fig. 6**.** The evaluated parameters from equations 1 and 2 are reported in Table 1. Means having different superscript lowercase letters for a parameter are significantly different ($P < 0.05$) through the Tukey test. The statistical analysis showed significant differences ($P < 0.05$). Table 1 clearly evidences the differences between active and untreated trays. Moreover, a noticeable increase in lag time needs to be highlighted, which almost doubled for

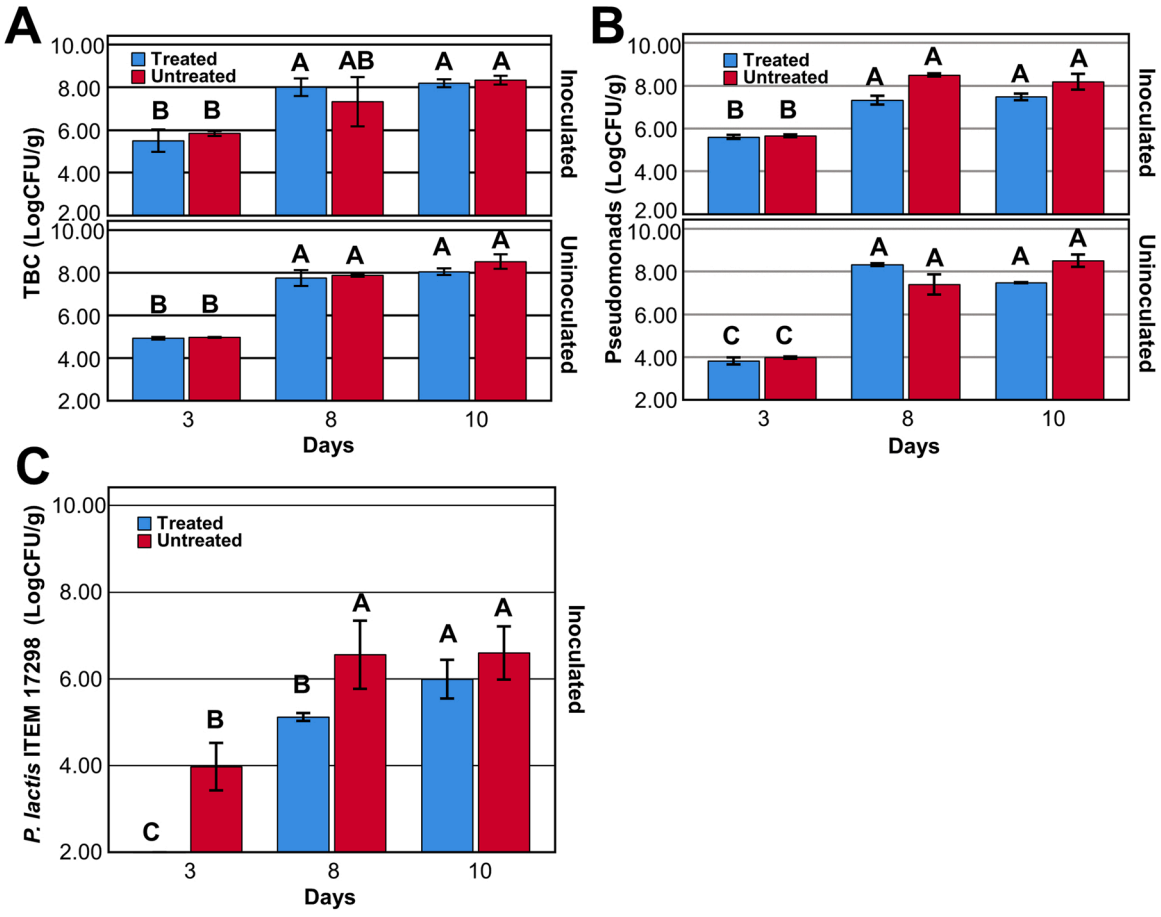

Fig. 2. Counts of different microbial groups (A: total mesophilic aerobic counts, TBC; B: *Pseudomonas* spp.; C: *Pseudomonas lactis* ITEM 17298) from Mozzarella cheese samples dipped in autoclaved governing liquid with or without inoculating ITEM 17298 and packed in the coated and uncoated PET packages during incubation at 4 °C for 10 days. Bars represent mean ± standard deviation (N = 3). Different letters represent significant differences within inoculated or uninoculated cheese samples according to the Kruskall-Wallis test ($\chi^2(2) = 32.76–15.23$, $p < 0.009$) and Dunn's *post hoc* test ($P < 0.05$).

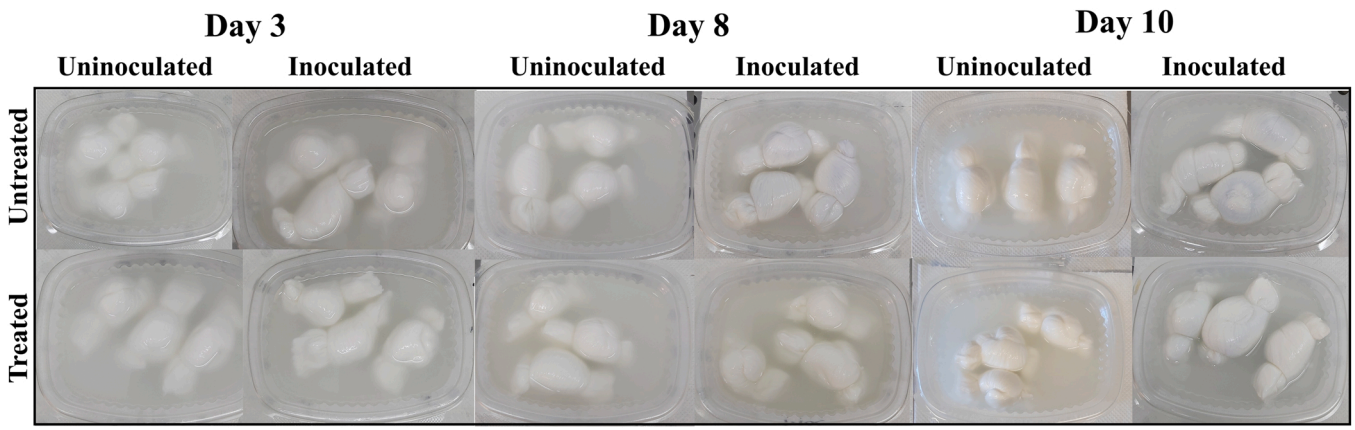

Fig. 3. Appearance of Mozzarella cheese samples inoculated or not stored at 4 °C in the coated and uncoated PET trays with the governing liquid for 10 days.

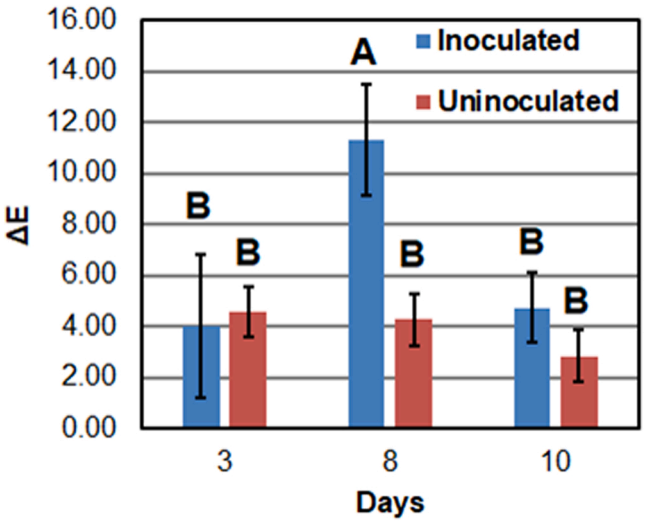

Fig. 4. CIE distance metric ΔE differences between Mozzarella cheese samples (inoculated or not with *Pseudomonas lactis* ITEM 17298) stored in treated and untreated trays at 4 °C for 10 days. Bars represent mean ± standard deviation (N = 3). Different letters represent significant differences according to the Tukey test ($F(5,12) = 5.989$, $p = 0.005$).

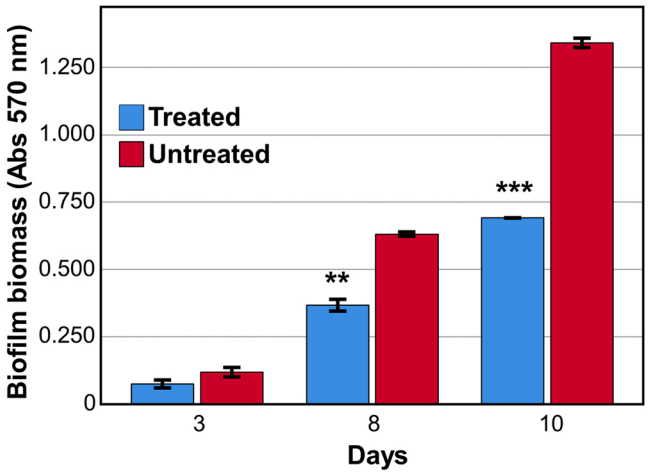

Fig. 5. Biofilm biomass produced on the inner surface of treated and untreated trays containing Mozzarella cheese samples inoculated with *Pseudomonas lactis* ITEM 17298 and stored at 4 °C for 10 days. Bars represent mean ± standard deviation (N = 3). Significant differences: * *, $P < 0.01$; * **, $P < 0.001$ (Independent Student t-test).

the active tray (from 4 days to 7 days). As reported in Table 1, the active tray allowed to extend the shelf life of blue mozzarella cheese up to about 9 days noticeably prolonged in comparison with its shelf life in untreated tray (about 7 days).

4. Discussion

The shelf-life of high moisture Mozzarella cheese is directly related to its storage method. Indeed, this fresh cheese is stored at a low temperature in a governing liquid limiting moisture and quality loss in a very short period (Jana & Mandal, 2011); under these conditions Mozzarella cheese undergoes critical physico-chemical and microbiological changes characterized by an increase in psychrotrophic pseudomonads more quickly and huger than that of other thermotolerant microbial groups (Baruzzi et al., 2012). In spite of these changes, Mozzarella cheese is still a saleable food displaying approximately 12 days of shelf life (Faccia et al., 2019; Guidone et al., 2016). During cold storage and the shelf-life period, the appearance of anomalous discolorations produced by very high concentrations of *P. fluorescens* (> 7–8 log CFU/g) causes the immediate withdrawal from the market in accordance with legislation (Reg. CE n. 178/2002). Psychrotrophic pigmenting pseudomonads enter the Mozzarella cheese production line

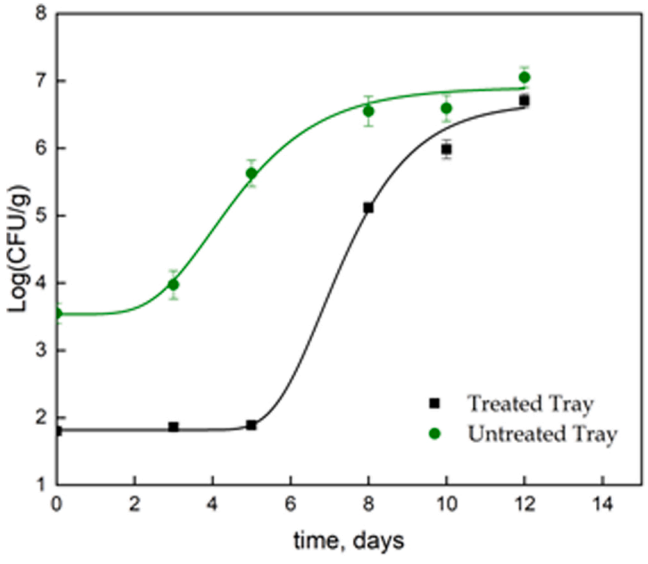

Fig. 6. Evolution of *Pseudomonas lactis* ITEM 17298 bacteria during storage time (scatters) and fitting curves (solid lines) obtained from Eq. 1.

Table 1
Gompertz's equation parameters evaluated by using Eqs. (1) and (2).

	Treated tray	Untreated tray
K	1.82 ± 0.04^{a}	3.53 ± 0.19^{a}
A	1.78 ± 0.08^{a}	1.24 ± 0.09^{b}
μ_{max} ($days^{-1}$)	0.52 ± 0.04^{a}	0.29 ± 0.07^{b}
λ (days)	6.82 ± 0.17^{a}	3.98 ± 0.35^{b}
Shelf life (days)	8.58 ± 1.01^{a}	7.03 ± 0.95^{b}
R^2	0.995	0.989

by tap water, usually used for governing liquid or dairy equipment washing (Carminati *et al.* 2019). Although several works have been reported on this topic in the last decade (Cenci-Goga *et al.* 2014; Andreani et al., 2015; Caputo et al., 2015 and Caputo, Quintieri, Cavalluzzi, Lentini, & Habtemariam,; 2018; del Olmo *et al.* 2018; Rossi et al., 2018; Faccia et al., 2019), a few control strategies have been developed (Caputo et al., 2015; Faccia et al., 2019) and, to the best of our knowledge, none of them have so far been transferred to the production line at industrial scale.

In this paper, for the first time, we presented a practical, readily transferable solution based on trays functionalized with salicylic acid to pack mozzarella cheeses inoculated with the blue pigmenting *P. lactis* ITEM 17298 (Caputo et al., 2015; Quintieri et al., 2020a).

A preliminary evaluation of antimicrobial effect of SA used as additive in the governing liquid of inoculated cheese disks confirmed its effectiveness to delay pseudomonads growth. In particular, a significant ITEM 17298 load reduction was registered starting from 5 mg/mL. SA is, indeed, a natural antimicrobial used in a wide range of pharmaceutical formulations and also listed among molecules authorized to come into contact with foods (EC-Directive 10/2011/EC of 14 January 2011); the efficacy of a low SA concentration (30 mM) was also demonstrated against pathogenic pseudomonas by altering the membrane proteome (Bandara, Sankaridurg, Zhu, Hume, & Willcox, 2016). A concentration of SA ten-fold higher than 5 mg/mL exhibited a bactericidal effect and completely inhibited the formation of the characteristic blue spots on the cheese discs.

These results have been partially confirmed on Mozzarella packed in active trays and stored under commercial conditions. In PET trays, mozzarella cheese discoloration appeared at the 8 day of cold storage; this delay in color development (8th day) in packed mozzarella cheeses in comparison to cheese disks (5th day) could be attributed to the microaerophilic condition which occurred in PET trays in comparison to multiwell plates. In spite of this, preserved mozzarella recorded a significant reduction of target inoculated strain load up to 8 days compared to the control samples and although the treatment did not completely inhibit the strain, the blue appearance became evident only at 10 days of storage. These results were in accordance with previous data since the release of the active substance from the packaging into the preserving liquid reached the concentration of 2.1 mg/mL after 3 days of incubation. Interestingly, the present work reported for the first time that the biomass biofilm deposited by the target strain on the inner surface of the tray during its storage was significantly lower than in the samples in control trays. Under environmental conditions such as cold storage, pseudomonads show the ability to grow as surface-attached communities also named as biofilm, embedded in a self-produced extracellular matrix composed of exopolysaccharides (EPS), DNA, and other components (Quintieri et al., 2019a,b). Biofilm is a important adaptation and survival mechanism commonly employed by bacteria to counteract stress conditions (*e.g.* such as heat, cold, salt, acid and preservatives). Our data were in accordance with other Authors that investigated SA mechanism of action on *P. areuginosa* and other *P. fluorescens*; in particular, SA interfered with quorum sensing (QS) system responsible for biofilm regulation (Gerner, Almqvist, Thomsen, Werthén, & Trobos, 2021; Lemos et al., 2014). This finding was also reported by other scholars who had observed that several genes including *rhlR* and *lasR*, encoding for virulence factors, proteases synthesis, EPS, and biofilm formation were down-regulated in the presence of SA (Da, Heroux, Pakzad, & Schiffmacher, 2010).

Recent studies reported that biofilm formation is strictly correlated with activation of pathways addressed to the microbial spoilage such as pigment release; this latter was classified as adaptive response to environmental stress such as oxidative stress by low temperatures (Chattopadhyay *et al.* 2011; Quintieri et al., 2020a; Quintieri et al., 2020b). In light of this consideration, it can be supposed that an antibiofilm effect could occur on the surface of the cheese under the pressure of released SA. Recently, the application of hydrolysates of lactoferrin and of the LFcin peptide against ITEM 17298 planktonic cells resulted in the inactivation of the biosynthetic pathways related with biofilm and leuco-indigoidine synthesis, of which the oxidized form indigoidine is responsible for blue discoloration on mozzarella cheese (Caputo et al., 2015; Quintieri et al., 2019b). Lactoferrin hydrolysate confirmed its efficacy on Mozzarella cheese trials. However, the high costs of the active hydrolysate made these technologies unattractive to the dairy environment and especially for Mozzarella cheese manufacturing. By contrast, the LDL-PET salicylate trays can be readily transferred at industrial scale and, as also confirmed by Gompertz modeling, favor an approximate two-day extension of the shelf-life.

In addition to economic advantages for the dairy industry, the application of eco-friendly solutions, such as the herein presented active LDH-PET, acquires added value also in relation to the current sustainable development goals (Wikström et al., 2019). Indeed, reducing food spoilage and improving food quality are some of the biggest challenges facing food insecurity and food waste, which represent two of the most current social issues of this century (Bajželj, Quested, Röös, & Swannell, 2020). In addition to the pressures on natural resources and the environment, the current COVID-19 pandemic has limited access to fresh foods and also increased consumption of long term stored foods (Roe, Bender, & Qi, 2020); the research of new technologies and innovative solutions to improve fresh food storage and processing facilities, post-harvest and cold food chain management has indeed undergone an acceleration. Although further experiments will have to be carried out to optimize the concentrations of released salicylate and its release kinetics, the herein presented results, moving in this direction, set the stage for the development of active packaging able to prevent anomalous cheese discoloration by extending product shelf life.

5. Conclusions

In this work the developed methodology was able to extend the shelf life of mozzarella cheese, intentionally inoculated with blu pigmenting *P. lactis,* by 2 days, delaying discoloration appearance. In addition to this, the results regarding the effects of SA on biofilm formation, previously demonstrated to be correlated with pigment release, suggest the role of this compound as antibiofilm agent and paves the way towards novel SA application also including the clinical setting. To this regards an *ad hoc* experimental design will be performed in the future to decipher SA mechanism of action in bacteria cells.

Moreover, it is noteworthy to highlight the versatility of such methodology producing active cheap, safe and easily industrially scalable packaging able to extend the shelf life of almost any food, by using a natural nano-carrier to which it is possible to bond any type of active molecule by simple ion exchange reaction; indeed, the appropriate ratio LDH/molecule allow both to bond inside the LDH layers the exact amount of compound responsible for the antimicrobial or antibiofilm activity and to apply it on different food packaging materials (polymers as well as aluminium) addressed to several type of foods.

Thus, in the case of mozzarella cheese the developed food grade active packaging could be ready to be exploited for the preservation of mozzarella cheese and protection from alterative bacteria without making any changes to its production process.

CRediT authorship contribution statement

Laura Quintieri: Conceptualization, Investigation, Formal analysis, Data curation, Validation, Writing – original draft. **Leonardo Caputo**: Methodology, Data curation, Formal analysis, Writing – review & editing. **Valeria Bugatti**: Methodology, Data curation, Formal analysis, Writing – review & editing. **Giuliana Gorrasi**: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2022.100850.

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