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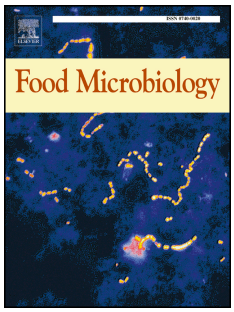

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Pepsin-digested bovine lactoferrin prevents Mozzarella cheese blue discoloration caused by *Pseudomonas fluorescens*

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Abstract

The aim of this work was to check the efficacy of bovine lactoferrin hydrolyzed by pepsin (LFH) to prevent blue discoloration of Mozzarella cheese delaying the growth of the related spoilage bacteria. Among 64 *Pseudomonas fluorescens* strains, isolated from 105 Mozzarella samples, only ten developed blue discoloration in cold-stored Mozzarella cheese slices. When Mozzarella cheese samples from dairy were treated with LFH and inoculated with a selected *P. fluorescens* strain, no pigmentation and changes in casein profiles were found up to 14 days of cold storage. In addition, starting from day 5, the count of *P. fluorescens* spoiling strain was steadily ca. one log cycle lower than that of LFH-free samples. ESI-Orbitrap-based mass spectrometry analyses allowed to reveal the pigment leucoindigoidine only in the blue LFH-free cheese samples indicating that this compound could be considered a chemical marker of this alteration. For the first time, an innovative mild approach, based on the antimicrobial activity of milk protein hydrolysates, for counteracting blue Mozzarella event and controlling psychrotrophic pigmenting pseudomonads, is here reported.

Keywords: cheese spoilage; blue Mozzarella; indigoidine; ESI-Orbitrap-MS; antimicrobial peptides; cheese shelf life.

1. Introduction

Italian traditional Mozzarella is a fresh table pasta filata cheese with a high moisture (HM) content (50-60%), usually dipped into a governing liquid (GL), mainly made up of tap water, brine and whey that

preserve the soft-springy texture and high amounts of expressible serum throughout 10-12 days of cold storage.

A combination of longer storage times and refrigeration temperatures causes an advantage particularly to psychrotrophic pseudomonads that can become the dominant non lactic bacteria population in milk and in fresh cheeses such as Mozzarella (Cantoni et al., 2003; De Jonghe et al., 2011; Franciosi et al., 2011; Martin et al., 2011; Morales et al., 2005).

Recently, the occurrence of very high loads of non lactic acid bacteria populations, mainly composed of *Pseudomonas*, *Acinetobacter* and *Rhanella* strains, was found to be responsible for casein hydrolysis and exfoliation of the outer surface of Mozzarella (Baruzzi et al., 2012). In addition, several cases of anomalous discoloration were reported in HM Mozzarella cheese and referred to the contamination by *Pseudomonas putida* (reddish discoloration; Soncini et al. 1998), *Pseudomonas fluorescens* biovar IV and *Pseudomonas libanensis* (bluish discoloration; Cantoni et al., 2003), *Pseudomonas gessardii* (yellow-purple spots; Cantoni et al., 2006) and *P. fluorescens* (greenish and fluorescent discoloration; Franzetti and Scarpellini, 2007) thanks to the production of different pigments (pyoverdin, pyocianin, pyorubin and pyomelanin; Palleroni, 2005).

In June 2010, the Rapid Alert System for Food and Feed (RASFF) reported many cases referred to as "blue Mozzarella cheese". At first, it was developed on high moisture (HM) Mozzarella cheese manufactured in Germany, and latter in other European countries. These cheeses, properly kept in cold storage conditions, became blue after opening the packs. German authorities demonstrated that tap water, containing *Pseudomonas* spp., was the source of cheese contamination (RASFF, 2010).

Many approaches have been undertaken to control the microbiota responsible for HM Mozzarella cheese spoilage such as the use of lysozyme and Na_2-EDTA (Sinigaglia et al., 2008), essential oil (Gammariello et al., 2008) or the use of silver nanoparticles in bio-based nanocomposite coatings (Gammariello et al., 2011). The replacement of the GL with a natural polysaccharide-based gel allowed

to stabilize Mozzarella microflora and cheese texture up to 15 days (Laurienzo et al., 2006). Recently, Quintieri et al. (2012) provided a direct evidence of the ability of bovine lactoferrin hydrolyzed by pepsin (LFH), containing the antimicrobial peptide lactoferricin B (LfcinB), to delay the growth of pseudomonads and coliforms contaminating commercial HM Mozzarella cheese samples under cold storage condition. Furthermore, antimicrobial activity of LfcinB was registered on plasma coating functionalized surfaces useful to obtain an active packaging for controlling the growth of pseudomonads causing cheese spoilage (Quintieri et al., 2013a).

Recently, Nogarol et al. (2013) isolated 132 pulsotypes of *P. fluorescens* from dairy products, without giving information about their ability to develop cheese pigmentation.

In order to fill this gap, in the present work, we selected, among the aforementioned *P. fluorescens* pulsotypes, those developing Mozzarella cheese blue discoloration and checked the efficacy of LFH, added in the GL, in controlling the growth of these spoiler bacteria and preventing their off-color spoilage.

2. Materials and Methods

2.1. Bacterial strains, growth media and culture conditions

Sixty-four strains of *P. fluorescens* were isolated from 105 samples of HM Mozzarella cheese by the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZS, Turin, Italy); the molecular characterization of 181 *P. fluorescens* strains, including the 64 strains analysed in this work, was previously reported by Nogarol et al. (2013).

All strains, if not otherwise mentioned, were grown overnight at 30 °C (150 rpm) in Plate Count Broth (PCB Difco™, Becton Dickinson, Milan, Italy). Fresh cultures were transferred in 200 µL of Nutrient Broth (BioLife Italiana, Milan, Italy) containing 20% glycerol and stored at -80 °C.

All experimental activities, described in the following paragraphs, have been summarized by a graphical scheme shown in the Fig. 1.

2.2. Screening of pigment production

An overnight culture of each selected *P. fluorescens* strain was spotted (2 µL) in triplicate onto Petri dishes of King A and King B agar media (Sigma Aldrich, Milan, Italy) and incubated at 25 °C for allowing the development of pyocyanin (blue colonies) and pyoverdin (green colonies), respectively (King et al., 1954), whereas the yellow-green fluorescence of the same colonies was daily observed under UV light using a Wood's lamp (λ = 300-400 nm). In addition, the strains were spotted on potato dextrose agar (PDA; Oxoid S.p.A., Rodano, Milan, Italy; Palleroni, 1984), in order to detect the eventual appearance of black colonies after 4 days of incubation at 15 °C (Martin et al. 2011, Palleroni, 2005). Colonies without any pigmentation were also registered (Fig 1). The pigmentation patterns displayed by each strain on the three different media were registered and compared with the NTSYSpc software (release 2.0; Applied Biostatistics Inc., Setauket, New York, USA) by using the band-based Dice similarity coefficient (SD), according to the following formula reported by Nei and Li (1979):

$$SD = \frac{2n_{XY}}{n_X + n_Y}$$

where n_{XY} is the number of the same color colony shared by two different strains and $n_X + n_Y$ is the sum of types of colors registered. The clustering of all fingerprints (dendrogram) was performed applying the unweighted pair group method by using average (UPGMA) linkages (Sneath and Sokal, 1973) with the NTSYSpc software (Applied Biostatistics Inc.).

All strains developing black colonies on PDA and at least one strain representative of each pigmentation group, obtained by cluster analysis, were assayed for color development on HM Mozzarella cheese-disks (Fig 1). Briefly, disks (20 x 5 mm; cut with a cork borer) of fresh Mozzarella

cheeses, purchased from a local dairy farm and produced by microbial acidification, were obtained under sterile condition and transferred to 12-well plates. Mozzarella cheese-disks were covered with the fresh bacterial cell suspension (3 ml) of each strain diluted with sterile saline solution at the final concentration of 3 log cfu/ml. Cheese controls were covered by the same volume of uninoculated sterile saline solution. All samples were prepared in triplicate and incubated at 4 °C for 5 days.

2.3. Antimicrobial effect of lactoferrin hydrolysate in vitro

Bovine lactoferrin (BLF; NZMP lactoferrin 7100, Fonterra, Boulogne-Billancourt, France) was hydrolyzed by pepsin (LFH), as previously reported (Quintieri et al., 2012).

LFH was tested *in vitro* in 96 well microplates only against the *P. fluorescens* strains developing black colonies and blue discoloration on Mozzarella cheese disks (Fig 1). A fresh culture (16 h) of each selected *P. fluorescens* strain was inoculated (final concentration of ca. 3 log cfu/ml) in 0.2 ml of PCB, containing 10, 25, 50 or 100 mg/ml of LFH. Cultures were incubated at 30 °C for 48 h, reading their optical density (OD) at 600_{nm} with the Microplate Reader Versamax (Molecular Devices; New York, USA) every 12 h and up to 48 h. At the end of the incubation time, in order to calculate the minimal lethal concentration (MLC) of LFH, 1% of the 48 hour-old treated *Pseudomonas* cultures, without any apparent microbial growth, was inoculated in LFH-free PCB medium and incubated at 30 °C for 48 h.

2.4. Effect of LFH on blue discoloration of Mozzarella cheese disks

At first, the efficacy of the MLC of LFH was evaluated on Mozzarella cheese disks, prepared as reported above and covered with 3 ml of a filter-sterilized (0.22-μm-pore size, Millipore, SpA, Milan, Italy) LFH solution or 0.95% NaCl solution, containing 3 log cfu/ml of each strain developing black colonies on PDA (Fig 1). Cheese-disks controls with the same volume of uninoculated sterile saline

136 solution were included in the assay. All samples were prepared in triplicate and incubated at 4 °C for 5
137 days.
138
139 *2.5. Validation of LFH effect on the commercial ball-shaped Mozzarella cheese*
140 Finally, commercial chemically acidified ball-shaped HM Mozzarella cheeses were used to confirm
141 LFH efficacy, chosen at its MLC, against cheese blue discoloration caused by a selected pigmenting
142 strain. Briefly, ball-shaped samples (13-15 g per piece), freshly manufactured by a local dairy farm,
143 was dipped in 150 ml of ice-cold GL composed of sterile tap water (trial A), water amended with of
144 LFH (trial B), water inoculated with the selected strain (trial C), or water inoculated with the same
145 strain and amended with LFH (trial D). Cheese packs (in triplicate) were incubated at 4 °C for 14 days.
146 At days 0, 1, 3, 5, 7, 10 and 14, microbiological and chemical analyses were performed on GL, whereas
147 drained cheese samples were analyzed for microbial content and CIELab changes and then they were
148 aerobically kept at 4 °C for 12 h (Fig. 1).
149
150 *2.5.1. Color determination*
151 In order to analyze the color appearance on Mozzarella cheese samples throughout their storage period,
152 colorimetric CIE (*Commission Internationale de l'Eclairage*) coordinates L* (lightness), a* (redness)
153 and b*(yellowness) were recovered on 3 random points of cheese using the ChromaMeter CR-400
154 (Konica Minolta, Osaka, Japan) equipped with a D65 illuminant (6504 K), following the
155 manufacturer's instructions. The visible color differences (ΔE) of the treated Mozzarella samples were
156 calculated as the appearance of the untreated control cheese samples, applying the following equation:
157 $$\Delta \mathbf{E} = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2} \ .$$

158 Hue (h°) and chroma (C*) of cheese samples, corresponding to the basic tint and the saturation of
159 color, respectively, were calculated as follows: $\mathbf{h}^{\circ} = \tan^{-1}\left(b^{*}/a^{*}\right)$; $\mathbf{C}^{*} = \sqrt{\left(a^{*}\right)^{2} + \left(b^{*}\right)^{2}}$ (CIE, 2004).
160 Furthermore, color determination was also carried out in order to evidence eventual differences in CIE
161 Lab values between chemically and microbially acidified ball-shaped Mozzarella cheese samples
162 inoculated with *P. fluorescens* 84095 and stored for 5 days, as reported above.
163
164 *2.5.2. Microbiological analyses*
165 At days 0, 1, 3, 5, 7, 10 and 14, aliquots of the GL (5 ml for each replicate) were collected and stored at
166 -20 °C for further studies. One hundred microlitres from the GL of each trial (A, B, C and D) were
167 plated on PCA in order to ascertain total mesophilic aerobic counts and on *Pseudomonas* agar base
168 (PSA; containing *Pseudomonas* CFC selective supplement; BioLife Italia) to enumerate *Pseudomonas*
169 spp.. Plates were aerobically incubated at 30 °C for 24 h. The same aliquots were also plated on potato
170 dextrose agar (PDA, Oxoid S.p.A.; supplemented with cycloheximide 100 mg/ml to inhibit yeast and
171 mould growth) to detect the selected *P. fluorescens* strains developing black colonies on this medium at
172 15 °C after 4 days (Martin et al., 2011).
173 The same microbiological analyses were performed on Mozzarella cheese samples (withdrawn from
174 GL and aerobically incubated at 4 °C for 12 h), after homogenizing one part of cheese with nine parts
175 of sterile sodium citrate solution (20 g/l).
176
177 *2.5.3. Molecular analyses*
178 Black colonies (ca. 60), isolated from PDA Petri dishes with the highest decimal dilutions of GL and
179 cheese samples, respectively, were analyzed by the two-step RAPD-PCR protocol, as previously

reported by Baruzzi et al. (2012). Fingerprints of each isolate were compared with those of the selected strain inoculated in Mozzarella cheese samples.

The taxonomic identification of the typed isolates, at each time sampling, was confirmed by amplifying and sequencing the 16S rRNA gene with EUP F (50- GAAGAGTTTGATCATGGCTC-30) and EUP R (50-AGGAGGTGATCCAGCCGCA-30) primers (Wiesburg et al., 1991) and comparing rDNA sequences with those present in the Basic BLAST Search (Altschul et al., 1997) and against nucleotide collection (nr/nt). Besides, typed isolates were further identified by amplifying and sequencing the *rpo*B gene (1247 bp), as described by Ait Tayeb et al. (2005).

2.5.4. Evaluation of LfcinB concentration in governing liquid

The residual concentration of antimicrobial LfcinB, present in LFH peptide mixture added to the GL of trials B and D was monitored at different days of cold storage as previously reported by Quintieri et al. (2012).

2.5.5. Evaluation of Mozzarella cheese spoilage

The concentration of small peptides and amino acids(expressed as glycine content) from GL and the casein Urea-PAGE pattern of the outer part of Mozzarella cheese were evaluated in accordance with Baruzzi et al. (2012). The experiments were carried out in triplicate.

2.5.6. Extraction and analysis of blue pigment

The extraction of blue pigment from HM Mozzarella cheese samples (trials A, B, C and D) was performed as reported by Kuhn et al. (1965) for the broth culture of *Pseudomonas indigofera*, with some modifications. Briefly, 1.3 g of each HM Mozzarella sample was homogenized in 4 ml of MQ water (Millipore Company, Bedford, Mass., USA) shaken in a 10 ml stainless steel jar with a grinding

ball (Ø 10 mm) for 5 min at 15 hertz by using a Mixer Mill MM 301 (Retsch Technology GmbH, Düsseldorf, Germany). Then, Mozzarella cheese extracts were processed for UV/visible light absorbance measurements using the spectrophotometer Ultraspec 3100pro (Amersham Pharmacia Biotech Italia). In particular, the pH of all samples was lowered to 4.6 with 6 M HCl (50 µL) in order to precipitate caseins. After centrifugation (10,000 × *g* for 30 min), supernatants (1 ml) were collected and their absorbance values were registered at 582 nm. The same supernatants were adjusted to pH 9.0 with 50 µL of 10 M NaOH in order to cause the related shift of absorbance maximum to 425 nm. Moreover, Mozzarella cheese extracts were subjected to LC-High Resolution-MS analysis for pigment characterization. Three milliliters for each sample were loaded on 3-kDa ultrafiltration tubes (Amicon Y3 filters, Millipore SpA, Milan, Italy). Filtrates were then analysed by a HPLC and High Resolution Mass Spectrometry system consisting in a (U)HPLC Accela™ Pump (Thermo Fischer Scientific, San Josè, USA) coupled through the ESI interface to an Exactive™ Orbitrap-based Mass Spectrometer (Thermo Fisher Scientific). Chromatographic separation was accomplished on a C_{18} Kinetex column (100 x 2.1 mm x 2.6 µm, 100 Å; Phenomenex, Torrance, CA, USA) applying the following elution gradient: from 10% to 50% of A (A=acetonitrile+1% acetic acid; B=water +1% acetic acid) in 20 min, from 50% to 70% in the following 10 min, then up to 90% for other 10 min and isocratic for 5 min before reconditioning for 15 min. MS analyses were performed in positive polarity and the system was operated at the resolution as high as 50,000 amu in full scan mode. MS instrumental settings: scan range 100-1000 m/z; Microscan, 1 Hz; AGC, balanced 1 x 10^6; injection time, 100 ms; sheath gas, 15; auxiliary gas, 5; capillary temperature, 250 °C; capillary voltage, 32.50 V; tube lens voltage, 130 V; skimmer voltage, 30 V; heater, 30 °C.

2.6. Statistical analyses

A randomized complete block design was used to study the effect of treatments, storage time and their interaction and block (triplicate trials) on microbial counts, CIELab coordinates and ΔE coefficients. Statistical analysis was carried out using the SPSS statistical package release 8.0 (SPSS Inc., Hong Kong, China). Raw data of color difference were normalized using an arcsine-root transformation before the analysis. The results from all variables were standardized transforming them to a z-value distribution with the mean value zero and standard deviation of 1. Then, data were analyzed using the General Linear Models Statistical Procedure to check the individual effects of the factors studied (time and treatment) as well as the interaction between them.

Multiple comparisons among individual means were made by the Fisher's least significant difference (LSD) post hoc test after rejecting the homogeneity of their variances using the Levene's test with an α level of $P < 0.05$.

3. Results and Discussion

3.1. Characterization of P. fluorescens strains for pigment production

Even though several *P. fluorescens* strains have already been isolated from HM Mozzarella cheese affected by blue discoloration (Nogarol et al., 2013), the correlation between *P. fluorescens* strains and off-color of Mozzarella has not been found yet. Thus, in the present work, 64 *P. fluorescens* strains coming from the 181 pulsotypes analyzed by Nogarol et al. (2013) were further screened for their ability to produce pigmented colonies on three different media.

All the assayed strains were clustered in seven groups with Dice similarity coefficients ranging from 0 to 0.80 (Fig. 2). In particular, ten strains developing black colonies on PDA and fluorescence on both King's media, grouped in the clusters I, II and III. The strains displaying green and/or fluorescent

250 colonies were clustered in the groups IV, V and VI, and 14 strains that did not show any pigmentation
251 grouped in the cluster VII (Fig. 2).
252 Interestingly, all the ten strains displaying black colonies on PDA developed blue discoloration on
253 Mozzarella cheese-disks, as previously reported by Cantoni et al. (2003) for other *P. fluorescens* biovar
254 IV strains that produced a blue, non diffusible pigment on HM Mozzarella cheese and by Martin et al.
255 (2011) for a fresh Latin-style cheese. Under the experimental conditions of the present work, no
256 discoloration was observed on cheese-disks inoculated with strains developing fluorescence on King A
257 and/or King B media (Fig. 1S; supplementary data). This result could depend on the specific
258 physiological needs of the assayed *P. fluorescens* strains.
259
260 *3.2. Antimicrobial effect of lactoferrin hydrolysate in vitro*
261 Eight out of the ten *P. fluorescens* strains, developing black colonies on PDA, were inhibited in PCB
262 amended with 10 mg/ml of LFH; however, the remaining two strains 84095 and 15620 did not grow
263 when inoculated in PCB with 50 mg/ml of LFH that was established being the MLC. These results are
264 in accordance with the data previously obtained by Del Olmo et al. (2008) who registered a high
265 inhibitory activity of amidated lactoferrin and LFH against *P. fluorescens* ATCC 948. Recently, we
266 reported on the antimicrobial effect of LFH against psychrotrophic *Pseudomonas* spp. strains, including
267 *P. fluorescens*, isolated from HM Mozzarella cheese samples (Quintieri et al., 2012).
268
269 *3.3. Effect of lactoferrin hydrolysate on cheese blue discoloration of Mozzarella cheese disks*
270 The addition of LFH (50 mg/ml) to the GL of Mozzarella cheese disks inoculated with each blue
271 pigmenting strain did not develop any discoloration throughout cold storage (data not shown). In Fig.
272 2S the efficacy of LFH treatment on the 84095 strain is shown; this strain was selected for the
273 subsequent experiments.

3.4. Validation of LFH effect on the ball-shaped Mozzarella cheese

The efficacy of treatment was also verified on commercial Mozzarella cheeses dipped in GL amended with LFH and packed in sealed plastic under cold storage. To this purpose ball-shaped samples, obtained with chemically acidified curd were preferred because of their low microbial load. In spite of the blue indigo color, retrieved on inoculated microbially acidified Mozzarella cheese, this type of cheese turned from greenish reddish to bluish nuances (Fig. 3). This difference in pigmentation of the two kinds of inoculated Mozzarella was also reported by Cantoni and Bersani (2010). The inoculated cheese sample treated with LFH (trial D) did not show any pigmentation up to 14 days of cold storage (Fig. 3S); moreover, no significant differences in color (P = 0.350) were found between the LFH-treated and the uninoculated samples over time (Fig. 4).

3.4.1. Microbiological analyses

Microbiological analyses of GL from ball-shaped Mozzarella cheeses revealed that, at day 5, only mesophilic bacteria and pseudomonads counts, from the inoculated LFH-free GL samples (trial C) were ca. 2 log cycle significantly ($P = 1.10 \times 10^{-6}$) higher than those registered in LFH-treated (trial D) and both uninoculated samples (trials A or B; Fig. 5, left side). In the following days, these bacterial populations were no longer inhibited by LFH treatments (Figure 5, right side). These results are in accordance with those previously reported by Quintieri et al., (2012) and Quintieri et al. (2013b) demonstrating that LFH significantly delayed the growth of the autochthonous pseudomonads and coliforms throughout cold storage of commercial HM Mozzarella cheese samples.

Black colony counts from inoculated GL samples (trial C and D) did not change in the first three days of cold storage (4.30 log cfu/ml, on average; P = 0.825), whereas, starting from day 5, the black colony loads from inoculated LFH-free GL samples were steadily ca. one log cfu/ml higher (P = 1 x

297 10^{-13}) than those of LFH-treated samples (trial C and D, respectively; Fig. 5, left side). As expected, no
298 black colonies were found in the uninoculated trials A and B.
299 Blue full discoloration of Mozzarella cheese was observed on samples that were drained and
300 aerobically stored at 4 °C, following the procedure reported by the European Rapid Alert System for
301 Food and Feed (RASFF, 2010). No differences in total viable bacteria and presumptive pseudomonads
302 counts were found on PCA and PSA media, respectively (Fig. 5, right side). In contrast to this, the
303 black colony load, likely attributable to the inoculated strain *P. fluorescens* 84095, enumerated on
304 three-day LFH-treated cheese samples (trial D), was significantly ($P = 1 \times 10^{-15}$) lower than that found
305 in the samples of trial C (2.8 log cfu/g, on average; Fig. 5, right side**)**. As reported above for
306 uninoculated GL samples., no black colonies were found on the related cheese samples (trial A and B,
307 Fig. 5, right side). However, differently from that was observed in the samples from GL, *P. fluorescens*
308 84095 load dramatically increased in both un-treated and treated inoculated cheese samples starting
309 from day 5, but the cell counts in the former (trial C) were significantly 0.9 log cycles higher than the
310 latter ($P = 1.84 \times 10^{-11}$; Fig. 5, right side **)**. This discrepancy in growth kinetics of the strain 84095
311 could be associated with the well-known favorable aerobic conditions. Moreover, the partial increase in
312 *P. fluorescens* 84095 counts, found in cheese samples drained from GL amended with LFH after day 5
313 of cold storage, was also correlated to the concomitant reduction (ca. 80%) in the antimicrobial peptide
314 LfcinB content quantified by HPLC analysis (Fig. 6), as also previously reported by Quintieri et al.
315 (2012) under similar conditions. In spite of this drop in the LFH content, any blue discoloration was
316 registered on these drained Mozzarella cheese samples.
317 For the first time, these results showed that, under the experimental conditions used, the treatment of
318 HM Mozzarella cheese with LFH efficiently counteracted the chromatic spoilage of cheese throughout
319 storage time, even though it hampered the inoculated strain growth only in the first five days. Likewise,
320 Xu et al. (2010), assaying the inhibitory effects of LFH against one *Pseudomonas aeruginosa* strain,

showed that the treatment did not afford pyocyanin production and partially controlled the bacterial growth depending on the concentration of the hydrolysate applied. On the other hand, the intracellular indigoidine production was also previously reported (Starr et al., 1967) in the *nomen* species *Pseudomonas lemonnieri*, now re-named *P. fluorescens* biovar IV group that was supposed responsible for blue discoloration of HM Mozzarella cheese (Cantoni and Bersani, 2010) and a Latin-style fresh cheese (Martin et al., 2011) closer to that registered in the present work.

3.4.2. Molecular analyses

Sixty black colonies, isolated on PDA medium and picked from Mozzarella cheese samples (GL and cheese), revealed the same RAPD-PCR fingerprint of *P. fluorescens* 84095 (data not shown). Sequence and genetic analyses of one typed isolate confirmed it belonged to *P. fluorescens* suggesting that the assayed Mozzarella cheese samples were not contaminated by autochthonous blue-pigmenting strains.

3.4.3. Evaluation of Mozzarella cheese spoilage

GL of LFH-free Mozzarella cheese samples inoculated with *P. fluorescens* 84095 showed a free amino acid (FAA) content much higher than that found in the related uninoculated cheeses after 14 days of cold storage (836.7 µg Gly/ml and 36.27 µg Gly/ml, on average, respectively). These results were consistent with the disappearance from the outer part of the inoculated cheese samples of both α- and β-casein bands observed on Urea-PAGE starting from day 5 of cold storage. On the contrary, no casein hydrolysis occurred in LFH treated Mozzarella cheese samples inoculated with the strain 84095 (Fig. 7). In addition, the high level of FAA concentration of the related GL was similar to that found in the uninoculated and treated cheese samples (453 ± 74 µg Gly/ml) and was represented for more than 90% by peptides contained in LFH. These results were in accordance with those previously found for other pseudomonads spoiling Mozzarella cheese (Baruzzi et al., 2012) whose growth and proteolytic activity

345 were inhibited by the same LFH treatment (Quintieri et al., 2012; Quintieri et al., 2013b). Thus, the
346 double spoilage pattern (proteolysis and discoloration) caused by *P. fluorescens* 84095 could be
347 efficiently inhibited by the LFH treatment providing a more extended shelf-life of Mozzarella cheese.
348
349 *3.4.4. Extraction and analysis of indigoidine*
350 HPLC-MS analyses were performed on all 7 days-cold-stored samples from GL of Mozzarella cheese
351 samples in order to identify the blue pigment indigoidine. The analyses revealed the occurrence of the
352 colorless and reduced form of indigoidine, also known as leucoindigoidine (m/z = 251.0781), only in
353 the LFH-free inoculated GL samples (Figure 8, panels 1 and 2; Figure 9, panel 1 and 2). By applying
354 the XCalibur software for analyte identification, based on the accurate mass of the detected ions, the
355 elemental mass composition, corresponding to $C_{10}H_{11}O_4N_4$, was retrieved. In contrast, the MS
356 spectrum of LFH-treated samples, inoculated with *P. fluorescens* 84095 (trial D), recalled at the same
357 retention time, did not evidence a significant peak displaying the same accurate mass (Figure 8, panels
358 3-4). According to the polarity of this compound bearing two hydroxyl groups it is reasonable to expect
359 this form in the blue-colored-aqueous extract analyzed, whereas no trace of indigoidine was found in
360 the same extract probably due to the low solubility of this molecule in aqueous solution, as previously
361 reported (Cude et al., 2012; Reverchon et al., 2002).
362 As the ESI-Orbitrap-MS did not provide any evidence of peaks attributable to the oxidized (blue)
363 indigoidine in the cheese samples; a further indication of the presence of this pigment was obtained by
364 the spectrophotometric analysis of 7-days cold-stored HM Mozzarella acidified cheese extracts. Only
365 extracts from the cheese samples inoculated with *P. fluorescens* 84095 (trial C) showed an absorbance
366 peak at 582 nm (blue) after addition of concentrated HCl (Table 1), whereas NaOH addition caused the
367 pigment decomposition as demonstrated by shift of peak absorbance to 425 nm (Table 1).
368

4. Conclusions

Herein, for the first time, we reported that all autochthonous *P. fluorescens* strains displaying black colonies on PDA medium developed blue discoloration on Mozzarella cheese samples.

A novel approach was used to prevent this pigmentation on HM Mozzarella cheese; the addition to the GL of Mozzarella of a small amount of the peptic digested bovine lactoferrin (LFH), an approved food-grade protein containing the antimicrobial peptide LfcinB, led to counteract cheese blue discoloration. Moreover, a decrease in the load of the selected *P. fluorescens* pigment producer strain in the first four days of cold storage was observed; very likely, this antimicrobial effect allowed the prevention of blue discoloration of the LFH-treated cheeses, as also confirmed by the absence in the LFH-treated samples of the pigment leucoindigoidine, the reduced colorless form of indigoidine, detected, on the contrary, in the LFH-free Mozzarella samples. This compound, analysed by ESI-Orbitrap-based mass spectrometry, and directly associated to the blue Mozzarella cheese event, could be considered a chemical marker of the related spoilage microorganisms in cheese. Further studies are needed to elucidate the different pigmentation observed on the inoculated Mozzarella cheeses obtained by chemical or microbial acidified curds.

The strategy applied in this work could be extended throughout Mozzarella production to improve quality and shelf life of this fresh cheese without changing its production process.

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486

Tables

Table 1 Absorbance values registrated at 583 and 425 nm on acidified and alkalinized extracts from Mozzarella cheese, respectively.

Cheese samples*	Acidified extracts	Alkalinized extracts
A	0.033 ± 0.005	0.070 ± 0.008
B	0.038 ± 0.004	0.030 ± 0.006
C	0.960 ± 0.012	0.520 ± 0.025
D	0.102 ± 0.023	0. 013 ± 0.001

*A, uninoculated HM Mozzarella cheese in LFH-free governing liquid (GL); B, uninoculated HM Mozzarella cheese in GL amended with 50 mg/ml of LFH; C, HM Mozzarella cheese inoculated with *Pseudomonas fluorescens* 84095 in LFH-free GL; D, Mozzarella cheese inoculated with *P. fluorescens* 84095 in GL amended with 50 mg/ml of LFH. Values represent means ± SD (N = 3).

Figure captions

Fig. 1. Graphical scheme of the experimental plan carried out in the present work.

Fig. 2. Clustering of *Pseudomonas fluorescens* strains on the basis of different pigmented colonies developed on King A, King B and potato dextrose agar. Clusters: I, black; II and III, black and green; IV, green and fluorescent; V, fluorescent; VI, green; VII, no pigmented colonies.

Fig. 3. Mozzarella cheese, produced by chemical or microbial acidification, inoculated with *Pseudomonas fluorescens* 80095 and incubated at 4 °C for 5 days (A). Relative color differences between both kinds of cheese; the bars represent the averages of CIE ΔE distance metric ± standard deviations.

Fig. 4. CIE distance metric ΔE values representing the average color differences of Mozzarella cheese samples stored in governing liquid (GL) at 4°C for 14 days between each experimental cheese sample (B, C, and D) and the control (A). C, cheese samples inoculated with *Pseudomonas fluorescens* 84095; D, cheese samples inoculated with *P. fluorescens* 84095 and treated with LFH; B, cheese samples treated with 50 mg/ml of pepsin-digested bovine lactoferrin (LFH); A, uninoculated and LFH free-cheese samples. Differences among ΔE values higher than 2.5 (Fisher's LSD; at 95% confidence interval) separate significantly different means. The CIE Lab coordinates L*, a*, b*of sample A varied from 92.49 ± 88.87, -2.35 ± -1.48, 12.36 ± 15.96.

Fig. 5. Effect of treatment of pepsin-digested bovine lactoferrin (LFH) and storage time on bacterial populations of ball-shaped Mozzarella samples incubated at 4 °C for 14 days. PCA, total mesophilic bacteria; PSA, presumptive pseudomonads ; PDA, presumptive *Pseudomonas fluorescens* 84095 (growing as black colonies) from governing liquid (GL) sampled from HM Mozzarella cheese packs incubated at 4 °C for 14 days (**left side**) and from Mozzarella cheese collected (three replicates) at each time assay and aerobically kept at 4 °C for 12 h (**right side**). **A**, uninoculated samples without LFH; **B**, uninoculated samples amended with LFH; **C**, inoculated samples without

519 LFH; **D**, inoculated with LFH. The values represent means with SD (error bars). No black colonies
520 were found on PDA from A and B trials.

521 **Fig. 6.** Reduction in lactoferricin B (LFcinB) concentration in Mozzarella cheese packs, stored at 4
522 °C for 14 days and containing governing liquid (GL) supplemented with 50 mg/ml of pepsin-
523 digested bovine lactoferrin (LFH). Trial B: uninoculated samples; trial D: samples inoculated with
524 *Pseudomonas fluorescens* 84095. The values represent means with SD (error bars).

525 **Fig. 7.** Urea-PAGE pattern of caseins from the outer part of Mozzarella cheese stored at 4 °C for 14
526 days. A: uninoculated samples; B, samples inoculated with *Pseudomonas fluorescens* 84095
527 without bovine lactoferrin hydrolyzed by pepsin (LFH); C, samples inoculated with *P. fluorescens*
528 84095 and treated with 50 mg/ml of LFH. α-, β-casein (Sigma-Aldrich) were used as standards.

529 **Fig. 8.** Overlay of full HR-MS chromatograms and extracted ion chromatograms filtered on the
530 accurate mass of leucoindigoidine from the governing liquid (GL) samples. In the upper panels are
531 reported (1) the full mass chromatogram and (2) the extracted ion chromatogram filtered on the
532 accurate mass of leucoindigoidine (m/z = 251.0781) of 3 kDa GL filtrate of the samples inoculated
533 with *Pseudomonas fluorescens* 84095 (trial C). In the lower panels are shown the full scan MS (3)
534 and extracted ion (4) chromatograms referred to the analysis of the inoculated and pepsin-digested
535 bovine lactoferrin (LFH) treated samples (trial D).

536 **Fig. 9.** Comparison of two HR-mass spectra extracted in the time window 1.80-1.95 min by
537 applying a mass accuracy of 5 ppm, corresponding respectively to the inoculated (1) and inoculated
538 and LFH-treated (2) Mozzarella cheese samples.

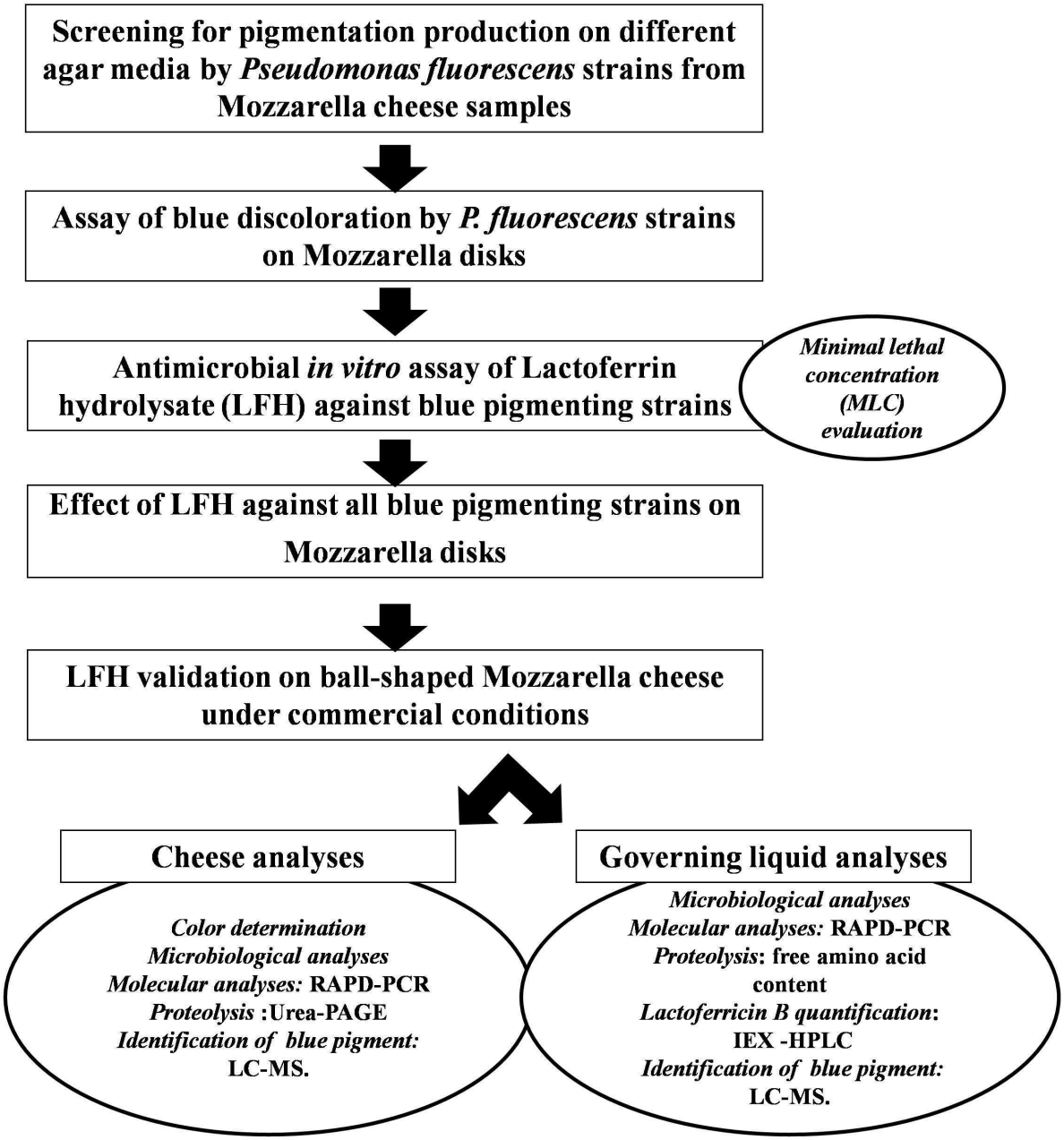
539

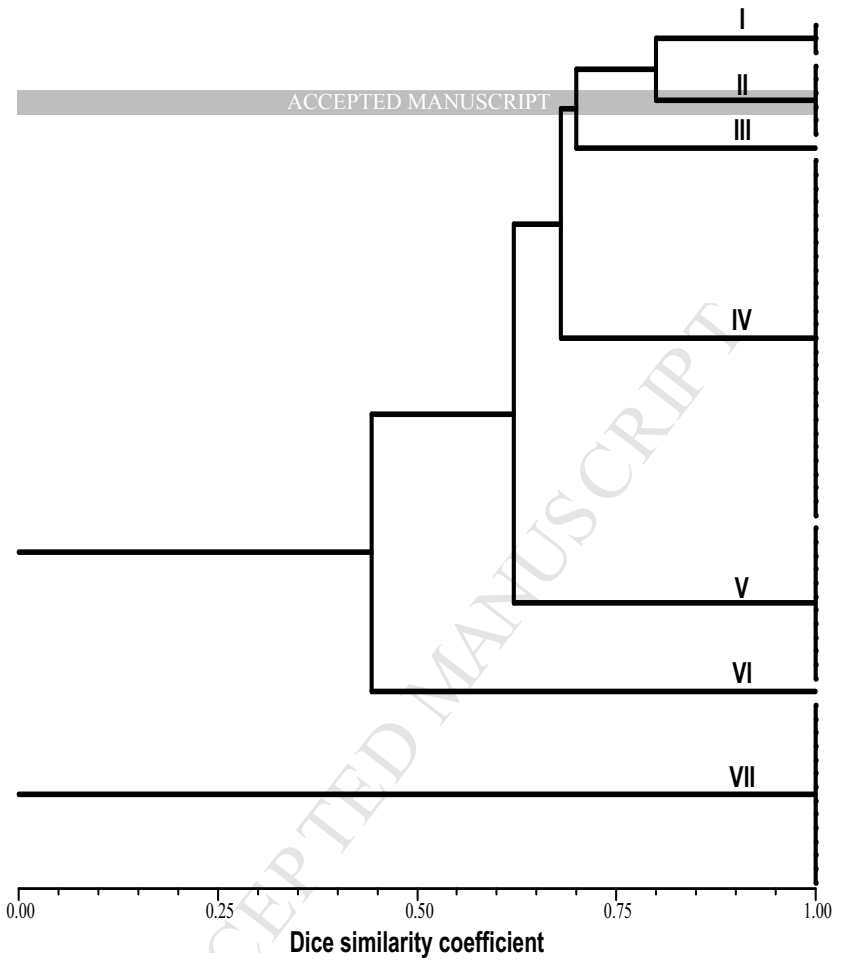
SUPPLEMETARY DATA

Fig. 1S. Mozzarella cheese-disks inoculated with *Pseudomonas fluorescens* strains. Within each lane from top to down: **1,** uninoculated cheese samples (three replicates); **2,** 14765, 85884 and 93361; **3**, 84080, 139019 and 84091; **4**, 88482/3, 15620 and 84095.

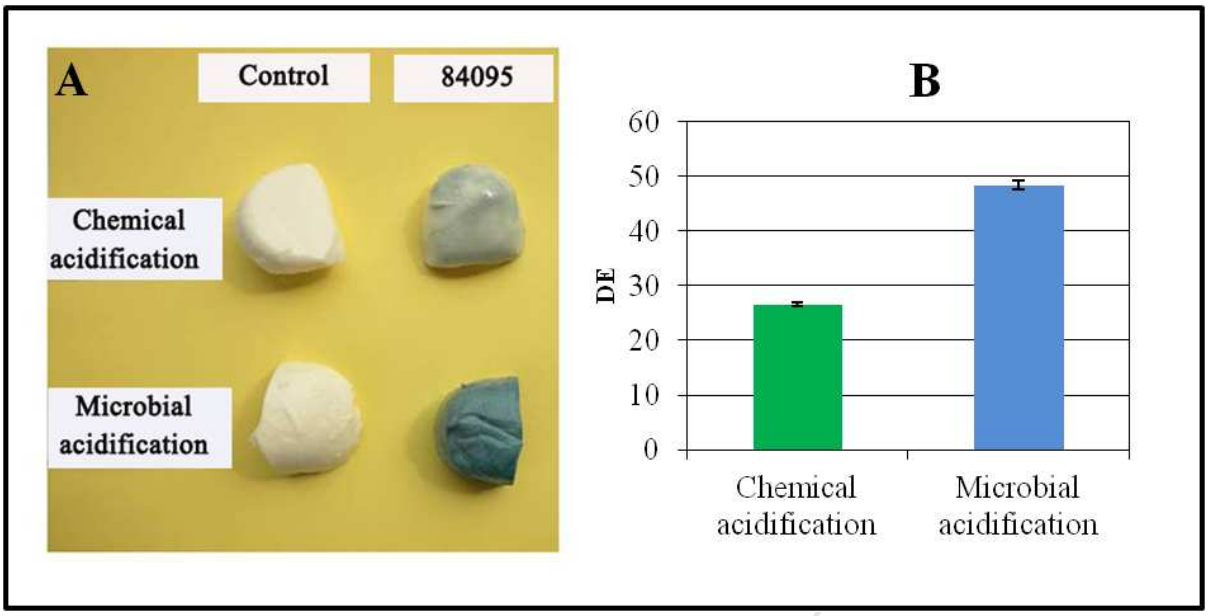
Fig. 2S. Effect of pepsin-digested bovine lactoferrin (LFH) or lactoferricin B (LfcinB) on blue discoloration of Mozzarella cheese-disks inoculated with *Pseudomonas fluorescens* 84095 and incubated at 4 °C for 5 days. **1**, untreated cheese; **2,** cheese amended with LFH (50 mg/ml); **3,** cheese amended with LfcinB (10 mg/ml). Uninoculated and untreated cheese samples (control) samples are on the right side (closed box). All experiments were carried out in triplicate.

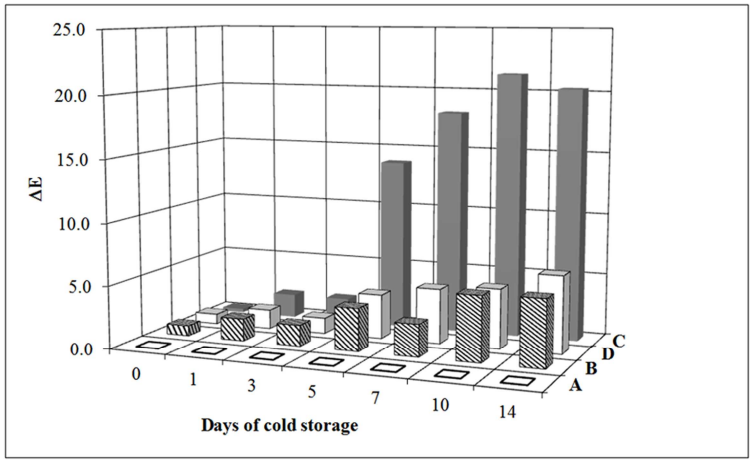
Fig. 3S. Effect of treatment of pepsin-digested bovine lactoferrin (LFH) and storage time on blue discoloration of ball-shaped Mozzarella cheese samples incubated at 4 °C for 14 days. **A**, uninoculated Mozzarella cheese; **B**, uninoculated Mozzarella cheese in governing liquid (GL) amended with 50 mg/ml of pepsin-digested bovine lactoferrin (LFH); **C**, Mozzarella cheese inoculated with *Pseudomonas. fluorescens* 84095 and stored in LFH-free GL; **D**, Mozzarella cheese inoculated with *P. fluorescens* 84095 and stored in GL amended with 50 mg/ml of LFH.


Screening for pigmentation production on different agar media by *Pseudomonas fluorescens* strains from Mozzarella cheese samples
Assay of blue discoloration by *P. fluorescens* strains on Mozzarella disks
Antimicrobial *in vitro* assay of Lactoferrin hydrolysate (LFH) against blue pigmenting strains
Minimal lethal concentration (MLC) evaluation
Effect of LFH against all blue pigmenting strains on Mozzarella disks
LFH validation on ball-shaped Mozzarella cheese under commercial conditions
Cheese analyses
Color determination
Microbiological analyses
Molecular analyses: RAPD-PCR
Proteolysis :Urea-PAGE
Identification of blue pigment: LC-MS.
Governing liquid analyses
Microbiological analyses
Molecular analyses: RAPD-PCR
Proteolysis: free amino acid content
Lactoferricin B quantification: IEX -HPLC
Identification of blue pigment: LC-MS.

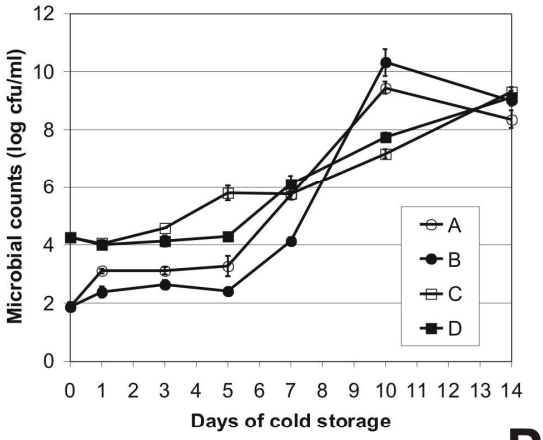


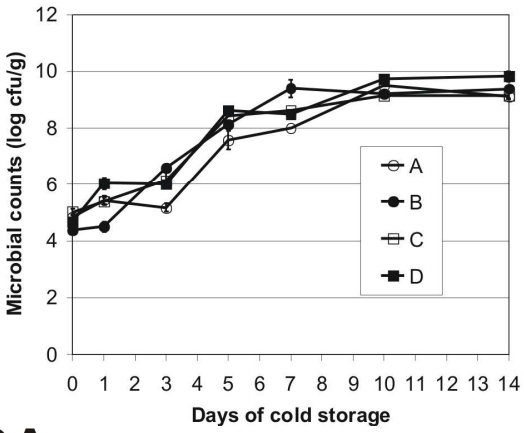
Cluster	Strains
I	84084, 84091, 84095
II	84089, 93361, 94158, 101709, 139019, 15620
III	84085.1
IV	84085.2, 84094.1, 84094.2, 35123, 84080, 32199, 32188, 85882, 85881, 28238, 27801.1, 22856, 27424, 22866.1, 22862, 14763, 6973.1, 14768, 130121, 6975.2, 105824, 104335, 94124, 35129, 90330, 90328, 88482.1
V	84082.1, 35127, 27432, 22866.2, 4330, 1780, 120462, 120465, 90329, 88482.4, 199, 84082.2
VI	87552
VII	85884, 84025, 20309, 35124, 32196, 27802.2, 27803, 32179, 32183, 91984, 88482.3, 27822.1, 22864.1, 91980



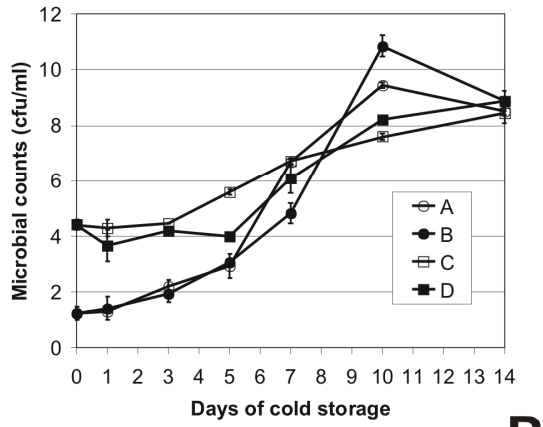


PCA


Microbial counts (log cfu/ml)
Days of cold storage
A
B
C
D


Microbial counts (log cfu/g)
Days of cold storage
A
B
C
D

PSA


Microbial counts (cfu/ml)
Days of cold storage
A
B
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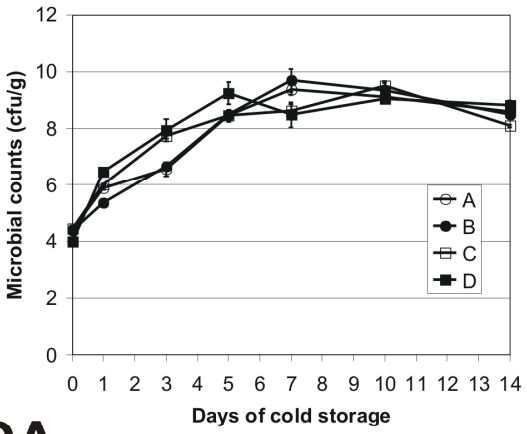
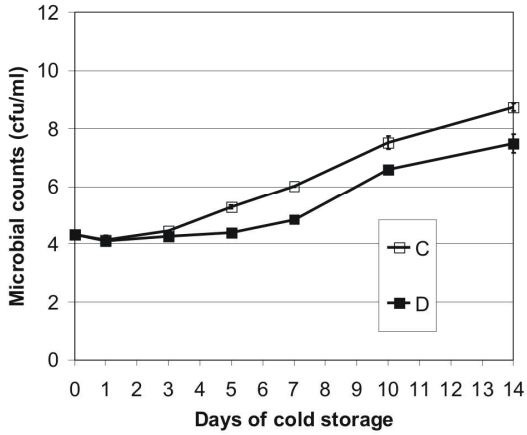
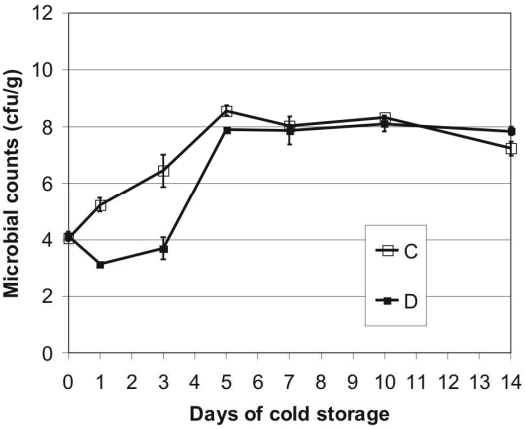
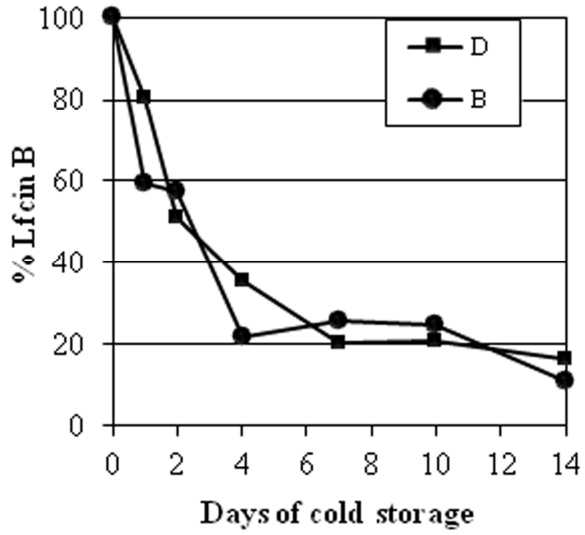
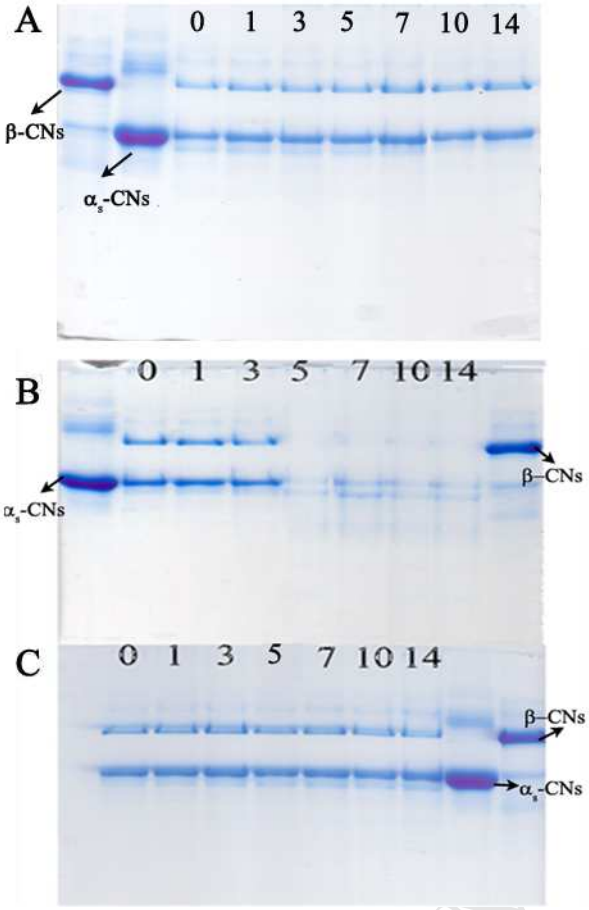

Microbial counts (cfu/g)
Days of cold storage
A
B
C
D

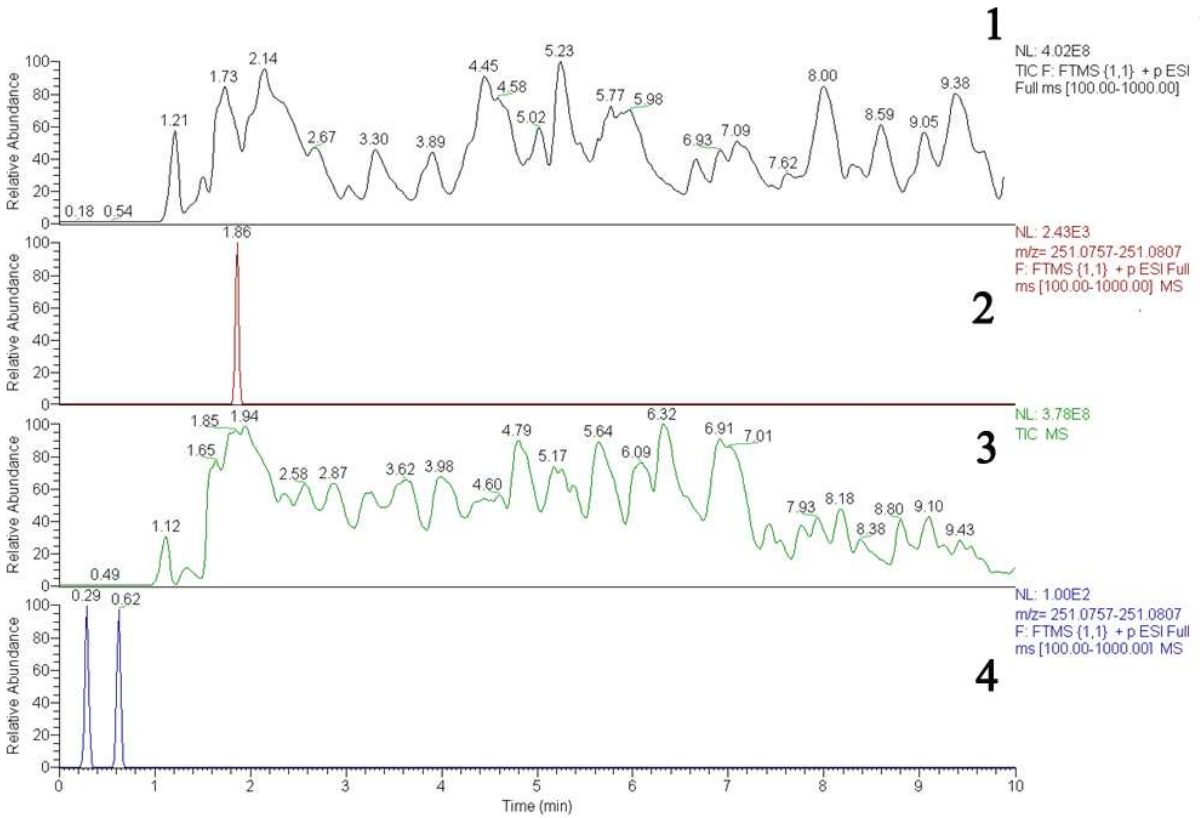
PDA

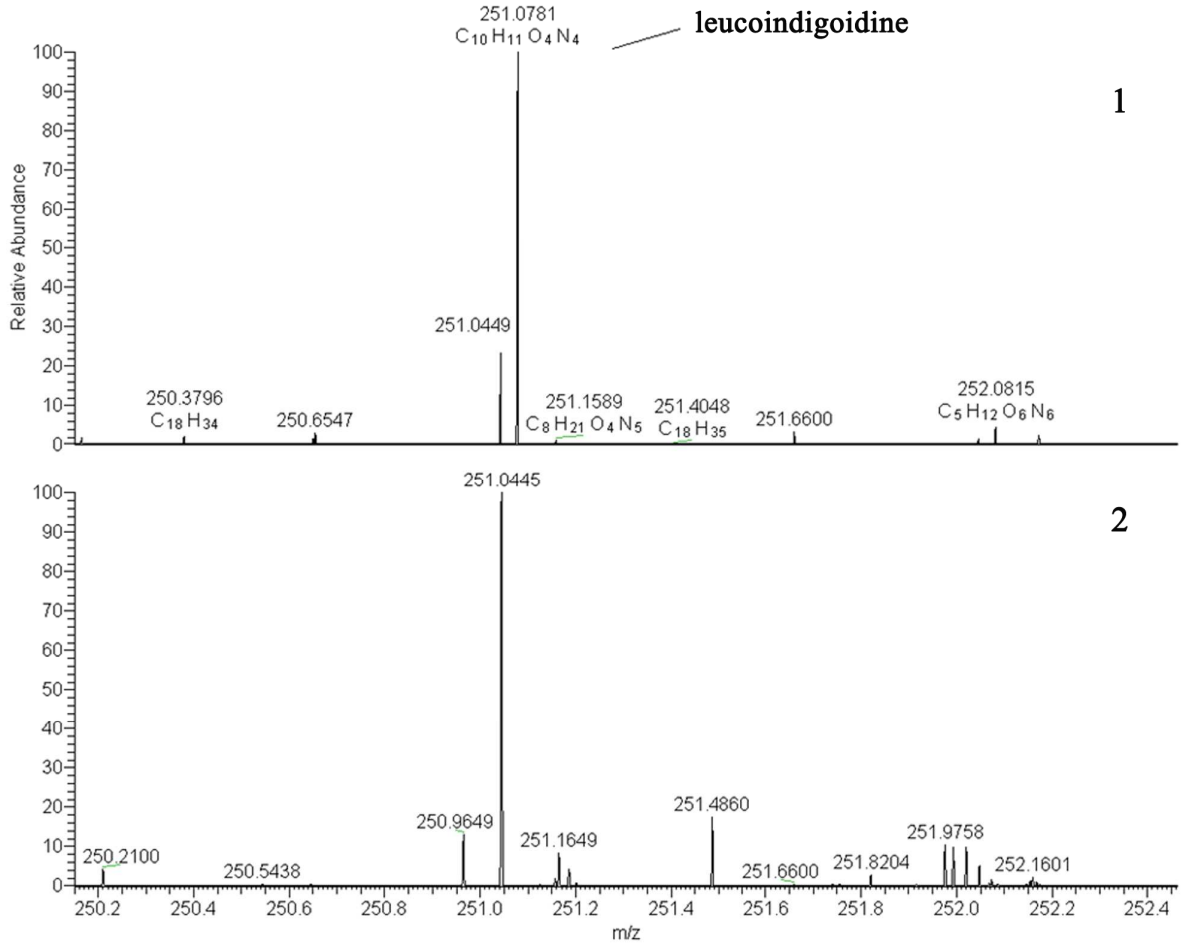

Microbial counts (cfu/ml)
Days of cold storage
C
D


Microbial counts (cfu/g)
Days of cold storage
C
D









Highlights

- Ten out of 64 *Pseudomonas fluorescens* strains developed cheese blue discoloration.
- Lactoferrin hydrolysate counteracted Mozzarella blue discoloration.
- LFH treatment delayed *Pseudomonas fluorescens* growth in Mozzarella cheese.
- Leucoindigoidine was detected by mass spectrometry only in pigmented Mozzarella.
- Leucoindigoidine pigment was not retrieved in LFH-treated Mozzarella cheese