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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.

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

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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 analyzed 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 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 ($\lambda = 300–400$ 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

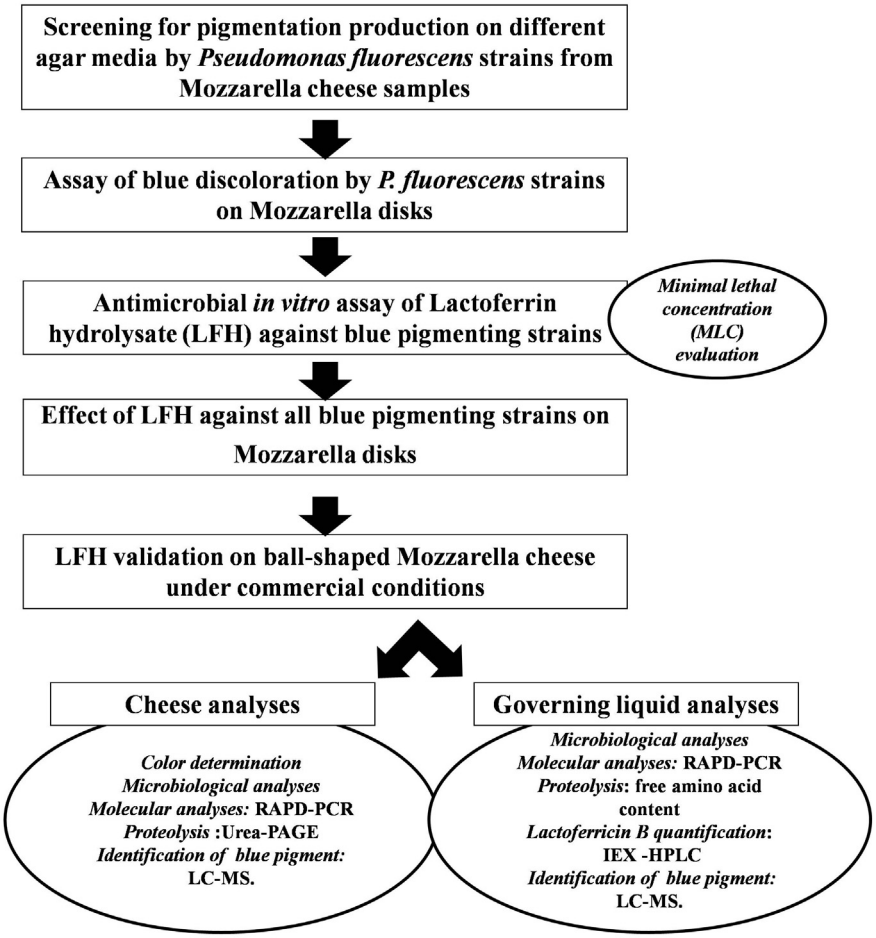

Fig. 1. Graphical scheme of the experimental plan carried out in the present work.