Identification of the *Pseudomonas fluorescens* group as being responsible for blue pigment on fresh cheese

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ABSTRACT

New cases of blue cheese discoloration has led to recent research to identify the causal agent and factors that favor blue pigment appearing. Nonetheless, very few reports have described the source of contamination and the measurements to eradicate the microbiological source on cheese farms by determining the relation between blue discoloration on fresh cheese and the *Pseudomonas fluorescens* group. Thus, 60 samples from a cheese farm (cheese, equipment surfaces, tap water, and raw and pasteurized milk) were analyzed by phenotypical, MALDI-TOF, 16S rRNA sequencing and pulsed-field gel electrophoresis tests to determine the causal agent. The results obtained by pulsed-field gel electrophoresis with restriction enzymes *Xba*I and *Spe*I confirmed tap water as the initial contaminated source. The above-mentioned result was essential to avoid *Pseudomonas* contamination due to the most residual microorganisms being inactivated through a new disinfection program.

Key words: *Pseudomonas fluorescens*, cheese farm, fresh cheese, blue pigment, MALDI-TOF, pulsed-field gel electrophoresis

INTRODUCTION

Blue cheese discoloration has been revealed as a major concern because it affects some cheese varieties in different countries. In all cases, the causal agent was *Pseudomonas fluorescens* (Martin et al., 2011; Nogarol

et al., 2013; Cenci-Goga et al., 2014; Carrascosa et al., 2015) or other strains of the *P. fluorescens* group (Chierici et al., 2016; Carminati et al., 2019). These bacteria are included in the genus *Pseudomonas* (gram-negative, rod-shaped and ubiquitous bacteria), imply only a few nutritional requirements, and are described in different environments (soil, decomposing organic matter, atmospheric dust, vegetation, and water), but with a vast variety of animal and plant hosts (Anzai et al., 2000; Frapolli et al., 2007). This group of psychrotrophic *Pseudomonas* is associated with food deterioration, and has been related to the food trade and hygiene quality (Andreani et al., 2015b).

The special interest shown in the *P. fluorescens* complex and group (Garrido-Sanz et al., 2016) has grown after blue mozzarella occurred. It happened when Italian authorities reported a problem with mozzarella from Germany following consumer complaints in June 2010. They were based on how the product had turned blue after opening the packaging (RASFF, 2011). The microbiological analyses of the blue mozzarella samples revealed a *P. fluorescens* concentration of up to 10² cfu/g (Bogdanova et al., 2010). However, the presence of *Pseudomonas tolaasii* and *Pseudomonas libanensis* was confirmed, which also produce blue pigment (Chierici et al., 2016). Despite official laboratory analysis and health authorities having related these episodes to the presence of *P. fluorescens* in water, other reports question the relation between the blue color and the presence of the microorganism (Cenci-Goga et al., 2014).

Pseudomonas have been reported in not only these cheese types, but also in other cheeses such as fresh cheese on the Canary Islands (Spain; Carrascosa et al., 2015), fresh Burgos cheese on sale in a Spanish province (del Olmo et al., 2018), or Latin-style fresh cheese in New York (Martin et al., 2011), where contamination

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produced by fluorescent *Pseudomonas* strains has been described.

Regarding the source of contamination, the majority of blue cheese discoloration cases have reported the water used during the cheese-making process, cleaning water (Carrascosa et al., 2015), or biofilm formation on cheese equipment and pipeline surfaces (Martin et al., 2011). This means that once biofilm forms, *Pseudomonas* bacteria are more difficult to eliminate by the daily routine of taking hygiene measures for the surfaces that come into contact with raw milk (Ksontini et al., 2013), curd, whey, or cheese. Moreover, other environment elements such as temperature, carbon source, and growth of other bacteria on the medium, can affect the blue pigment production phenomenon (Chierici et al., 2016).

To reduce the frequency of contamination and to eliminate the niche populations for these organisms in food production and processing systems, different identification and tracking methods have been developed (Martin et al., 2011). However, the high genetic and genomic diversity within the *P. fluorescens* complex (Garrido-Sanz et al., 2016) found in cheese samples requires running different phenotypic tests, such as API 20 NE (Martin et al., 2011), MALDI-TOF (Hrabák et al., 2013), and distinct genotypic techniques, such as PCR (Scarpellini et al., 2004; Xu et al., 2017), 16S rRNA sequencing (Caputo et al., 2015; Cenci-Goga et al., 2014; Chierici et al., 2016; Garrido-Sanz et al., 2016), pulsed-field gel electrophoresis (PFGE; Martin et al., 2011; Carrascosa et al., 2015), and MLSA (Andreani et al., 2015a,b; Gomila et al., 2015; Chierici, 2016; Garrido-Sanz et al., 2016), to avoid making mistakes in their correct identification.

One of the most widespread procedures for phylogenetic testing is 16S ribosomal RNA, which is based on a small ribosomal subunit sequence. Nonetheless, this procedure is controversial for either its poor resolution when similarly related bacteria are identified (Fox et al., 1992) or recombination and lateral gene transfer events (Boucher et al., 2004). For this reason, running a multilocus sequence analysis (MLSA) frequently overcomes the difficulties of 16S rRNA-inferred phylogenies (Gevers et al., 2005) and has been reported as a better guarantee than 16S rRNA in the genus *Pseudomonas* (Kämpfer and Glaeser, 2012), in which the sequence of 3 concatenated housekeeping genes (*gyrB*, *rpoD*, *rpoB*), along with 16S rRNA sequencing, has worked well to provide a suitable phylogeny of the *P. fluorescens* group (Mulet et al., 2012). Therefore, the MLSA tree has established the presence of 14 main groups according to the tree topology (Garrido-Sanz et al., 2016).

Genus *Pseudomonas* shows different extracellular pigments, and phenazines represent the most important

one. A distinctive trait of *P. fluorescens* and *P. aeruginosa* is the secretion of soluble pyocyanin pigment (El-fouly et al., 2015), which contributes to its survival and is produced profusely in media with a low iron content. Iron is an essential nutrient for *P. fluorescens* to grow (Jayaseelan and Ramaswamy, 2014).

Unlike other pigments, such as pyocyanine, pyoverdine, fluorescein, pyorubin and pyomelanin, which are produced by other *P. fluorescens* strains (Andreani et al., 2015b), very few studies are available in the literature on the blue discoloration identified in cheeses. Thus, only the most recent studies have investigated the characteristics of this discoloration on mozzarella cheese contaminated by *P. fluorescens* strains (Caputo et al., 2015) and have shown the presence of the uncolored parental compound of indigoidine (leuco-indigoidine). It is a blue diazadiphenoquinone pigment (CAS number 2435–59–8; chemical formula: $C_{10}H_8N_4O_4$) synthesized by different microorganisms. This finding could make indigoidine responsible for mozzarella blue pigment instead of pyocyanine.

The aim of this work was to identify the source of *P. fluorescens* contamination on a cheese farm and to determine the relation between the blue discoloration onto fresh cheese and the *P. fluorescens* identified by phenotype and genotype testing. In addition, new detergent and disinfection protocols were implemented to reduce the *P. fluorescens* contamination risk.

MATERIALS AND METHODS

The collected fresh cheeses were manufactured artisanally on a cheese farm by enzymatic coagulation, and were salted in brine. These cheeses were made from pasteurized goat (60%) and cow (40%) milk. Fresh cheese had a shelf life of 12 d, and was hand-packed at 5°C in plastic bags. The daily cheese production was 300 kg/d using a monitoring clean-in-place system (Hazard Analysis Critical Control Point, **HACCP**). Several new actions and controls were performed on tap water and cheese farm equipment, such as maintaining chlorine in tap water at 1.5 ppm, increasing the water temperature (45°C) of the initial rinse, using acid foam detergent and peracetic acid on open surfaces, and adding peracetic acid to cleaning in place. *Pseudomonas* sp. surface controls were also applied as a new weekly routine.

Sample Collection

Samples were collected in those fresh cheese batches where blue discoloration was identified. This situation had alerted producers about the cheese alteration made

on the cheese farm a few (5–6) days ago. These first cheeses formed part of the samples retained for the quality controls on the cheese farm.

To isolate and identify the causal agent of blue discoloration in fresh cheese, 60 samples were collected from an artisanal cheese farm located in North Tenerife, Canary Islands (Spain). The identification methodology described by Carrascosa et al. (2015) was applied with some significant variations, such as the MALDI-TOF test for *Pseudomonas* identification and collecting samples from equipment surfaces.

Sampling was carried out in 3 different months (January, March, May) when 20 samples were taken each month. Five sample types were tested on 5 occasions: fresh cheese, equipment surface (milk tank, curd vat, table), tap water, raw milk, and pasteurized milk. All the surfaces that came into contact with milk, curd, or cheese were cleaned and disinfected routinely, according to a disinfection program that forms part of a self-monitoring HACCP (Carrascosa et al., 2012). Surface samples were taken by sterile sponges (Nasco Meat Sampling Kit, with 25 mL buffered peptone water and a Whirl-Pak Speci-Sponge bag, Whirl-Pak, Madison, WI), and represented different cheese making process steps, such as cheese vats, curd vat, table, and raw milk tank. Finally, one additional control by traditional microbiology was carried out to confirm the presence of the *P. fluorescens* group on fresh cheese for 2 more months.

Culture, Identification and Determination of the Blue Phenotype. All the collected samples were analyzed to detect *P. fluorescens*. Cheese samples (25 g) were collected under aseptical conditions. Six serial dilutions were carried out and subsequently pipetted onto a plate of Cetrinide agar (Cultimed, 416256, Barcelona, Spain) following the protocol by Anderson and Pascual, (2000). The raw and pasteurized milk samples (10 mL) were processed as indicated above. Water was analyzed according to the ISO 16266:2006 protocol by a vacuum pump.

Then samples were cultured on cetrinide agar plates, and the green coloration and the fluorescent yellow-green coloration in UV light were identified. To obtain isolated colonies, they were sown by streaking on successive cetrinide agar plates. These isolates were cultured on MacConkey agar (Cultimed, 413779) to help identification. *P. fluorescens* on MacConkey agar showed a negative result (unable to degrade lactose, no color change according to the pH indicator neutral red), as well as colorless to pink colonies. All the gram stain, catalase +, oxidase +, H₂L medium + (Hugh and Leifson, 1953), oxidative + colonies were identified as *Pseudomonas* group members and were subsequently confirmed by the additional analysis.

All the cheeses were evaluated to determine the blue pigment production by streak plating onto mascarpone agar according to Cantoni et al., (2001), with confirmation after incubation at 4°C and 14°C in 2 mL of mozzarella preserving fluid (Chierici et al., 2016). Pure cultures were stored at –80°C in tryptic soy broth with 20% glycerol (Sigma-Aldrich, St. Louis, MO) until total identification.

The API 20NE system was applied following the manufacturer's instructions, but this was used only during the first sampling. A profile was obtained after 24 to 48 h of incubation, and only those colonies identified as excellent (99.9% identification and a t-value of 0.75) or very good species (99.0% identification and a t-value of 0.5; Bosshard et al., 2006) were further analyzed. This test was applied only to the January sampling because it was not possible to identify the *P. fluorescens* group. Therefore, it was not performed during the next sampling.

MALDI-TOF Analyses Identification

The MALDI-TOF (AutoFlex II system, Brüker Daltonik, Bremen, Germany) was used to categorize bacterial species from direct colonies. This process was followed as previously reported by Seng et al., (2013) and bacterial species were directly identified from one bacterial colony. Each deposit was covered with 2 mL of matrix solution (saturated α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). The MALDI Biotyper 3.0 software (Matsumura et al., 2014) was used to observe the 100 first peaks of each spectrum to our previously updated MALDI-TOF database. Any identification made by MALDI-TOF was classified as follows: (1) an isolate was considered correctly identified at the species level using MALDI-TOF if 2 spectra had scores $\geq 2,000$; (2) uncertain identified isolates at the species level, but accurate classifications at the genus level, including multiple choice in the same genus (scores 1,999–1,700), were classified without doubt by the MALDI-TOF analysis of 2 additional spectra; (3) a posterior MALDI-TOF identification was run with 4 spectra to identify unsatisfied species during the MALDI-TOF period. Only a minor discrimination between 2 species or more was made, including the correct species that required additional tests ($<1,700$; Li et al., 2014; Seng et al., 2013).

Genotyping

16S rRNA Sequencing. An approximate 1,400-bp fragment of the 16S rRNA gene was PCR-amplified using the previously described primers (Lau et al., 2006). The resultant product was sequenced and compared

with known 16S rRNA gene sequences in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) by the Basic Local Alignment Search Tool (Altschul et al., 1990).

Pulsed-field Gel Electrophoresis. Pulsed-field gel electrophoresis was used to establish any relatedness among the cheese *Pseudomonas* isolates obtained in all the sample collections, with the water *Pseudomonas* isolates from the second sampling (March), which were previously identified as *P. fluorescens* by 16S rRNA sequencing with blue discoloration on cheese in the laboratory. This unique tap water *Pseudomonas* isolate was included in the analysis. The PFGE was performed following the standard Centers for Disease Control and Prevention PulseNet protocol for enteric pathogens (Ribot et al., 2006). Briefly, these bacteria were cultured on tryptone soya agar plates overnight at 32°C. Later they were fixed in 1% agarose plugs (SeaKem Gold agarose, Cambrex, Rockland, ME), lysed, and washed. Genomic DNA was digested with a primary restriction enzyme (*Xba*I, Roche Diagnostics, Mannheim, Germany) for 2 h at 37°C following the manufacturer's instructions. A second restriction enzyme (*Spe*I, Merck KGaA, Darmstadt, Germany) was used to verify that the 2 strains had indistinguishable patterns.

The size separation of the restricted DNA fragments was performed in 1% agarose gels (Bio-Rad, Hemel Hempstead, UK) in 0.5× TBE buffer at 14°C by the CHEF MAPPER system (Bio-Rad). The obtained images were interpreted with the Info-QuestFP software (Bio-Rad). Isolates were designated distinct PFGE types if any change was observed. A dendrogram was generated by Dice coefficient and the unweighted pair group method with arithmetic averages. Therefore, the API 20NE, MALDI-TOF and 16s rRNA sequencing tests were used to confirm the *Pseudomonas* species isolated from different samples.

RESULTS

The results of the phenotypic and genotypic identifications are shown in Table 1. The 60 isolates were obtained from one cheese farm as follows: 15 isolates from each different sampling, such as fresh cheese, surface, raw milk, pasteurized milk, and tap water. *Pseudomonas* spp. were found in both the spoiled and unspoiled fresh cheese samples, but *P. fluorescens* was detected only in the cheese with blue discoloration and in the tap water from the cheese-making plants (Table 1).

All the collected samples displayed growth on Cetrimide agar, except for the pasteurized milk samples and 16 samples from different surfaces (6 samples from the January sampling, 6 from March, and 6 from May). The first sampling (January) gave the following results on Cetrimide agar: cheese and raw milk > 10⁵ cfu/g,

whereas surface and tap water < 10³ cfu/g after 6 d at 6°C. However, the next sampling results (March and May) were lower than those in the first sampling. The observation of green fluorescence under UV light on Cetrimide agar demonstrated *Pseudomonas* spp. in the analyzed samples. The isolates obtained from pure cultures were identified as gram-negative, catalase-positive, and oxidase-positive rods, and were assigned as the *P. fluorescens* complex by API 20NE strips (Table 1). These bacteria showed green fluorescent pigment under UV in mascarpone agar and mozzarella government liquid, but produced no blue pigment on Cetrimide, except for one blue cheese sample collected in January and another one in May. When these bacteria were spread on the fresh cheese stored at 6°C for 8 d, only sample numbers 27, 31, 32TF (tap water from March), 8TF (blue cheese from January), and 49TF (blue cheese from May) produced blue pigment.

Despite the absence of blue pigment on the Cetrimide plates from the sampled cheeses and surfaces, the counts of these cheeses after opening packaging were > 10⁵ cfu/g (after storage at 6°C for 6 d), which suggests postpasteurization contamination.

The identification of the first sampling (January) by API 20NE found that most isolates 9/16 (56.25%) were assigned to *P. fluorescens*; 3/16 (18.75%) to *P. aeruginosa*, 2/16 (12.5%) to *P. aureofaciens* and 2/16 (12.5%) to *Serratia marcescens*. Nonetheless, the identification coincidence (only *Pseudomonas*) between API 20NE and MALDI TOF was 16/16 (100%) at the genus level and 3/14 (21.45%) at the species level during this sampling (Table 1).

The identification results obtained during the 3 samplings by the MALDI-TOF and 16s ARN tests were as follows: 14 and 20 samples were identified, respectively, as *P. fluorescens*; 5 and 2 samples as *P. aeruginosa*; 6 and 0 samples as *Pseudomonas tolaasii*; 0 and 7 samples as *Pseudomonas gessardii*; 3 and 1 samples as *Pseudomonas proteolytica*; 2 and 0 samples *Pseudomonas synxantha*; 4 and 0 samples as *Pseudomonas libanensis*; 3 and 3 samples as *Pseudomonas koreensis*; 3 and 2 samples as *Pseudomonas putida*; 1 and 0 samples *Pseudomonas lutea*; 0 and 1 samples as *Pseudomonas protegens*; 1 and 1 samples as *Pseudomonas chlororaphis*; 0 and 2 as *Pseudomonas alcaligenes*; 0 and 1 samples as *Pseudomonas rhodesiae*.

In addition, the 16S rRNA test was used to confirm the MALDI-TOF identification results by the *P. fluorescens* complex and group (Garrido-Sanz et al., 2016), whereas this test identified 33.3% of the similarity range by identical species; 52.7% (19.04% + 33.3%) of similarity by species, which belonged to the same group; 88.41% (35.71% + 52.7%) of similarity by species, which belonged to the *P. fluorescens* complex. Finally,

Table 1. Phenotypic and genotypic results of the 44 samples collected on a cheese farm in Tenerife

Phenotypic results (<i>Pseudomonas</i> spp.)											Genotypic results		
Sample	Source	Blue pigment			Green pigment			Complex/ group*	MALDI-TOF	Score	16S RNA	Complex/ group**	PFGE ⁵
		Cet ¹	Ch ²	MP ³	Cet	Ch	UV						
First sampling													
1TF	Cheese	-	-	-	+	+	+	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas putida</i>	2.098	<i>P. putida</i>	a ⁶	-
2TF	Cheese	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>P. putida</i>	2.075	<i>P. putida</i>	a ⁶	-
3TF	Cheese	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>Pseudomonas koreensis</i>	1.975	<i>P. koreensis</i>	a ⁸	-
4TF	Cheese	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>P. korensis</i>	1.901	<i>P. koreensis</i>	a ⁸	-
5TF	Curd vat	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>Pseudomonas proteolytica</i>	2.180	<i>P. proteolytica</i>	a ⁸	-
6TF	Milk tank	-	-	-	+	+	+	<i>Pseudomonas aureofaciens</i>	<i>P. lutea</i>	1.690	<i>Pseudomonas rhodesiae</i>	b ⁶	-
7TF	Cheese	-	-	-	-	-	-	<i>Serratia marcescens</i>	<i>Serratia liquefaciens</i>	2.190			-
8TF	Cheese	+	+	+	+	+	+	<i>P. fluorescens</i>	<i>P. fluorescens</i>	2.050	<i>P. fluorescens</i>	a ⁸	+
9TF	Curd vat	-	-	-	+	+	+	<i>P. aureofaciens</i>	<i>P. proteolytica</i>	1.880	<i>Pseudomonas gessardii</i>	b ⁸	-
10TF	Milk mixture	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>Pseudomonas synzantha</i>	1.985	<i>P. fluorescens</i>	b ⁸	-
11TF	Cow milk	-	-	-	-	-	+	<i>S. marcescens</i>	<i>S. marcescens</i>	2.140			-
12TF	Cow milk	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>P. korensis</i>	1.992	<i>P. koreensis</i>	a ⁸	-
13TF	Water	-	-	-	+	+	+	<i>P. fluorescens</i>	<i>P putida</i>	1.839	<i>Pseudomonas plecoglossicida</i>	b ⁶	-
14TF	Water	-	-	-	+	+	+	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	2.262	<i>P. plecoglossicida</i>	b ⁶	-
15TF	Water	-	-	-	+	+	+	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	1.936	<i>Pseudomonas alcaligenes</i>	b ⁶	-
16TF	Water	-	-	-	+	+	+	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	1.825	<i>P. fluorescens</i>	a ⁸	-
Second sampling													
21TF	Cheese	-	-	-	+	+	+	-	<i>Pseudomonas tolaasii</i>	1.970	<i>P. fluorescens</i>	b ⁸	-
22TF	Cheese	-	-	-	+	+	+	-	<i>P. tolaasii</i>	1.980	<i>P. fluorescens</i>	b ⁸	-
23TF	Cheese	-	-	-	+	+	+	-	<i>P. synzantha</i>	1.910	<i>P. fluorescens</i>	b ⁸	-
24TF	Curd vat	-	-	-	+	+	+	-	<i>P. fluorescens</i>	2.040	<i>P. gessardii</i>	b ⁷	-
25TF	Milk tank	-	-	-	+	+	+	-	<i>P. fluorescens</i>	2.200	<i>P gessardii</i>	b ⁷	-
26TF	Cheese	-	-	+	+	+	+	-	<i>P. fluorescens</i>	2.050	<i>P. fluorescens</i>	a ⁸	-
27TF	Water	+	-	-	+	+	+	-	<i>P. fluorescens</i>	2.031	<i>P. fluorescens</i>	a ⁸	+
28TF	Cheese	-	-	-	+	+	+	-	<i>P. fluorescens</i>	2.021	<i>P. fluorescens</i>	a ⁸	-
29TF	Cheese	-	-	-	+	+	+	-	<i>P. fluorescens</i>	2.201	<i>P. fluorescens</i>	a ⁸	-
30TF	Cheese	-	-	-	+	+	+	-	<i>P. fluorescens</i>	2.101	<i>P. fluorescens</i>	a ⁸	-
31TF	Water	-	-	-	+	+	+	-	<i>Pseudomonas tolaasii</i>	1.613	<i>P. fluorescens</i>	b ⁸	+
32TF	Water	-	-	-	+	+	+	-	<i>P. tolaasii</i>	1.818	<i>P. fluorescens</i>	b ⁸	+
33TF	Water	-	-	-	+	+	+	-	<i>P. fluorescens</i>	1.948	<i>P. gessardii</i>	b ⁷	+
34TF	Water	-	-	-	+	+	+	-	<i>Pseudomonas chlororaphis</i>	1.93	<i>P. chlororaphis</i>	a ⁸	-
Third sampling													
41TFP	Cheese	-	-	-	+	+	+	-	<i>P. tolaasii</i>	1.997	<i>P. fluorescens</i>	b ⁸	-
42TFP	Cheese	-	-	-	+	+	+	-	<i>Pseudomonas libanensis</i>	1.954	<i>P. fluorescens</i>	b ⁸	-
43TFP	Cheese	-	-	-	+	+	+	-	<i>P. fluorescens</i>	1.94	<i>P. fluorescens</i>	a ⁸	-
44TFP	Cheese	-	-	-	+	+	+	-	<i>P. libanensis</i>	2.028	<i>P. gessardii</i>	b ⁷	-
45TFP	Cheese	-	-	-	+	+	+	-	<i>P. libanensis</i>	1.977	<i>P. gessardii</i>	b ⁷	-
46TFP	Cheese	-	-	-	+	+	+	-	<i>P. proteolytica</i>	1.73	<i>P. fluorescens</i>	b ⁷	-
47TFP	Water	-	-	-	+	+	+	-	<i>P. libanensis</i>	2.07	<i>P. gessardii</i>	b ⁷	-

Continued

Table 1 (Continued). Phenotypic and genotypic results of the 44 samples collected on a cheese farm in Tenerife

Sample	Source	Phenotypic results (<i>Pseudomonas</i> spp.)							Genotypic results					
		Blue pigment			Green pigment				MALDI-TOF	Score	16S RNA	Complex/ group**	PFGE ⁵	
		Cet ¹	Ch ²	MP ³	Cet	Ch	UV	API 2-NE ⁴						Complex/ group*
48TFP	Cheese	-	-	+	+	+	+	+	-	<i>P. tolaasii</i>	2.003	<i>P. fluorescens</i>	b ⁸	-
49TFP	Cheese	+	+	+	+	+	+	+	-	<i>P. fluorescens</i>	1.957	<i>P. fluorescens</i>	a ⁸	+
50TFP	Cheese	-	-	+	+	+	+	+	-	<i>P. fluorescens</i>	1.951	<i>P. fluorescens</i>	a ⁸	-
51TF	Water	-	-	-	+	+	+	+	-	<i>P. aeruginosa</i>	2.343	<i>P. aeruginosa</i>	a ⁶	-
52TF	Water	-	-	-	+	+	+	+	-	<i>P. aeruginosa</i>	2.036	<i>P. alcaligenes</i>	b ⁶	-
53TF	Water	-	-	-	+	+	+	+	-	<i>P. aeruginosa</i>	2.105	<i>P. aeruginosa</i>	a ⁶	-
54TF	Water	-	-	-	+	+	+	+	-	<i>P. fluorescens</i>	1.91	<i>Pseudomonas protegens</i>	b ⁶	-

¹Cet = presence of blue or green pigment on cetrimide agar.²Ch = presence of blue or green pigment on fresh cheese.³MP = presence of blue pigment in mozzarella preserving fluid (Chierici et al., 2016).⁴API 2-NE = phenotypic test.⁵PFGE = pulsed-field gel electrophoresis.⁶Not included in the *Pseudomonas* complex (Garrido-Sanz et al., 2015).⁷Both species belong to different groups of *Pseudomonas* (Garrido-Sanz et al., 2016).⁸Both species belong to the same groups of *Pseudomonas* (Garrido-Sanz et al., 2016).

*Comparison between API and MALDI-TOF, with "a" being the same species and "b" being different species in both identification methods.

**Comparison between MALDI-TOF and 16S RNA, with "a" being the same species and "b" being different species in both identification methods.

the PFGE test was also run with the 2 isolates from cheese and the other 3 from tap water (Figure 1), which were the only isolates that produced blue pigment on fresh cheese surfaces in the laboratory (8TF, 49TF and 27, 31, 32 TF). These isolates produced identical patterns, which suggests that they may share a recent common ancestor.

DISCUSSION

The results obtained herein could explain both the source of environmental contamination and the causative microorganism of the blue discoloration found on a cheese farm in Tenerife.

One of our work objectives was to determine the ability of the phenotypic and MALDI-TOF tests to identify of *P. fluorescens* at the group level. The comparison of both methods provided a coincidence of 42/42 strains (100%) at the genus level and poor discrimination at species level 10/20 *P. fluorescens* strains (50%). Other clinical microbiology studies have concluded that MALDI TOF is well able to identify a large number of bacteria (Seng et al., 2013) and yeasts (Dhiman et al., 2011), including rare species. Nonetheless, if we merely considered the identified strains to the *P. fluorescens* complex or at the group level, but not at the species level, we would obtain a coincidence of 37/42 at the complex level, and one of 22/42 at the group level, respectively. These results obtained by a simple protocol (phenotypic test and MALDI-TOF) could be enough to deal with the identification and origin of blue discoloration on cheese farms.

Previous clinical microbiology studies have shown a higher coincidence between MALDI-TOF and 16S rRNA. Legarraga et al., (2013) reported a correct identification for 91% (245/269) at the species level and for 95.7% (269/281) at the genus level. Identical results have also been described by Li et al., (2014), who revealed a higher 92% coincidence at the species level and 94% at the genus/species level. These results suggest that some factors inherent to aerobic or anaerobic bacteria could exist (Legarraga et al., 2013; Wieser et al., 2012). MALDI-TOFF only includes 20 species of *Pseudomonas* genus in its database, which could limit its identification ability. Nevertheless, all the species herein identified are included in the database. The MALDI-Biotyper Compass reference library is steadily updated according to precise quality-controlled procedures. Currently, it presents 7,311 reference spectra of 2,509 species from 433 bacteria and fungi genera. The (Bruker) MALDI-TOF MS technology has become a useful instrument for identifying bacteria in the clinical microbiology of laboratories given its accurate, inexpensive and rapid performance efficiencies.

Figure 1. Pulsed-field gel electrophoresis (PFGE) Xbai and SpeI results from Tenerife cheese (8TFP, 18TFP), water (16TFP) and Gran Canaria cheese (6GC, 7GC).

Regarding the contamination source, the results of the present study could be compatible with the idea of Nogarol et al. (2013), for whom affected raw samples could be the most believable cause of cross-contami-

nation. Hence the contaminated raw milk and water used for cheese production could lead to contaminated working equipment. This last factor was likely the contamination source because *Pseudomonas* spp. was

Figure 2. Flowchart of the *Pseudomonas* spp. control in cheese farm.

isolated from the tap water supply network, but not in pasteurized milk. This feature could be explained by the water supplied to the cheese farm being stored in a cistern to which no chlorine was added (assessed level < 0.2 ppm). By taking in account the relation that links biofilm formation, the discoloration production in *P. fluorescens* strains and the presence of bacteria in food manufacturing environments (Rossi et al., 2018), contaminated water could spread to all equipment surfaces (pipeline, mold, curd vats, tables). In addition, produced biofilms could remain on surfaces for 4 mo, and could even survive the cleaning and disinfection plan set up to eliminate *Pseudomonas* bacteria (Carrascosa et al., 2015). Similar results have been shown for different cheese types (Leriche et al., 2003), in which the source of contamination was traced back to the water supply used for brining cheese. The same applies to fresh Latin-style cheese, where biofilm formation in an agitator track was the source of contamination (Martin et al., 2011).

Finally, the PFGE test revealed that 2 isolates from cheese and 3 from water had identical patterns, which suggests that these isolates shared a recent common ancestor. This could support the hypothesis that tap water might be the main source of contamination. Although numerous *P. fluorescens* strains have already been identified from mozzarella with blue discoloration (Nogarol et al., 2013), the association between *P. fluorescens* strains and mozzarella off-color has not yet been proven. This test showed enough reliability to confirm the relation between *Pseudomonas* found in water tap and on fresh blue cheese. Therefore, these

findings would suggest that tap water without chlorine would be the source of contamination.

In recent years, different studies have worked to shed light on the causal agent and pigment structure that cause blue cheese discoloration (Andreani et al., 2015b; Caputo et al., 2015; Chierici et al., 2016). The first studies indicated that *P. fluorescens* was the main microorganism of color cheese with blue pigment (Cantoni et al., 2001; Martin et al., 2011; del Olmo et al., 2018). Interestingly, current studies have reported that different *P. fluorescens* group species are responsible for blue discoloration in mozzarella (Chierici, 2016; Carminati et al., 2019) and that resilient strains *P. fluorescens* remain in dairy factory environments (Nogarol et al., 2013). This issue might be influenced by different factors, including nutrient availability (Aswathanarayan and Vital, 2014), growth conditions (Rossi et al., 2016), and the attached surface (Marchand et al., 2012). Other factors such as low incubation temperature have also been found to strongly influence the biofilm formation ability of *P. fluorescens*. In contrast, high temperatures boost adherence capacity. Cenci-Goga et al. (2014) showed that the cold chain did not seem to be a major barrier to grow well in the preservation liquid of mozzarella cheese at refrigeration temperatures.

Finally, a plan was proposed to reduce blue discoloration in fresh cheese and its enormous economic loss (Figure 2). The first measure included increasing the chlorine concentration in tap water to 1.5 ppm as the chloramine gradient reduces the presence of the mixed *P. aeruginosa* / *Klebsiella pneumoniae* biofilm (de Beer et al., 1994). This elevation also diminishes phenotypic

Figure 3. Flowchart showing the previous (A) and new (B) cleaning disinfection procedure of the open surfaces. *Alkaline and acid detergents should be alternatively used.

Figure 4. Flowchart showing the cleaning and disinfection procedure of the CIP (clean in place) surfaces. (A) Previous and (B) new cleaning and disinfection program for removing *Pseudomonas* from milk and curd circuit *Alkaline and acid detergents should be alternatively used.

variation, which leads to biofilm formation and resistance to chlorine, acid, osmotic and oxidative stresses. A new cleaning and disinfection program, composed of an enzymatic detergent (Enzivix 30, Proquimia S.A., Spain), followed by the application of chlorinated alkaline foam detergent-disinfectant and an oxidizing disinfectant based on peracetic acid (Asep 150, Proquimia S.A., Spain), was used on all open surfaces (Figure 3) and clean-in-place surfaces (Figure 4). These measures reduced *Pseudomonas* spp., and blue cheese discoloration completely disappeared. This plan was essential for reducing biofilm formation as the most residual microorganisms were inactivated through disinfection. Similar recommendations have been made by Verran et al. (2016). Other authors prefer to add a starter culture as a type of hurdle technique to reduce the risk of pathogen growth and spoilage (Martin et al., 2011). Nonetheless in our study, these starter cultures were rejected because they could affect fresh cheese taste, as reported by Carrascosa et al. (2015). To improve the control of *Pseudomonas* on cheese farms, further microbiology controls, such as pasteurized milk, surfaces, and fresh cheese, were included in the laboratory routine. These results were all negative for the following 2 mo.

CONCLUSIONS

Given the relevance of blue discoloration in mozzarella and fresh cheeses, it is necessary to shed light on the current phenotypical and genotypical features of the *P. fluorescens* group, the storage conditions of fresh cheeses, and their control on cheese farms. Therefore, the implementation of specific measures for the early detection of *Pseudomonas* spp. by controls made on surfaces, final products and water should also be

included as normal microbiological routines. The application of specific hygiene and disinfection plans would drastically reduce *pseudomonas* counts and avoid new blue discoloration on fresh cheeses. Maintaining the cold chain does not seem an issue for *Pseudomonas* spp. contamination; these microorganisms are able to grow well even at refrigeration temperatures, as blue discoloration appears on the first day of contamination. The combination of phenotype and MALDI-TOF tests suitably identified the complex *P. fluorescens* level. Nevertheless, it proved less efficient for determining isolated species, which could be enough to find causal agents and to introduce corrective measures to avoid huge economic losses. Further studies are necessary to determine *P. fluorescens* behavior on cheese farms and the nature of blue pigment, and to obtain an accurate identification to help to understand the role of this phenomenon and how it can be prevented.

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