Setup of a rapid method to distinguish among dead, alive, and viable but not cultivable cells of *Pseudomonas* spp. in mozzarella cheese

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ABSTRACT

Pseudomonas spp. is the main psychrotrophic genus involved in the spoilage of raw milk and more in general of dairy products, such as mozzarella cheese. The members of this bacterial species are able to produce heat-resistant proteolytic enzymes, determining the casein hydrolysis, and as a consequence, a reduction of the shelf life and sensory quality of the products. Therefore, the spoilage activity could be attributed not only to viable, but also to viable but noncultivable (VBNC) cells. For this reason, the setup of a non-culture-based method is useful for a rapid detection of cells that are still alive, but no longer cultivable, such as VBNC cells. Here we propose a method based on DNA or RNA content (or both) to reveal the presence of dead, alive, and VBNC cells belonging to the genus *Pseudomonas*. The obtained results clearly indicate the limits of the classical plating count overcome by molecular detection of *Pseudomonas* spp. through DNA and RNA analysis, enabling us to establish the presence of different states of the cells.

Key words: mozzarella cheese, *Pseudomonas* spp., quantification of dead, alive, and viable but noncultivable cells, retro-transcribed quantitative PCR

INTRODUCTION

Pseudomonas is the most common genus responsible for the spoilage of different types of foods of both plant and animal origin. All the species of this genus have simple nutritional requests and are characterized by a strong adaptability to different matrices due to the production of many enzymes (e.g., proteases, lipases, pectinases, lecithinase). The cells are easily inactivated by high temperatures, but their enzymes are heat stable and can cause food spoilage even after cell death (Chen et al., 2003; Marchand et al., 2009). Mozzarella

is a typical Italian cheese, the manufacturing process of which is characterized by the stretching of the curd, a treatment necessary to make the curd elastic (Ercolini et al., 2001; De Angelis et al., 2008). The contamination of mozzarella can occur at 2 production steps, generating different problems. When contamination occurs before the heat treatment, heat-stable enzymes can induce instability of the final product, determining changes in the hardness, meltability, and stretchability of mozzarella (Oommen et al., 2002). However, the most common contamination occurs after the heating process (Baruzzi et al., 2012). *Pseudomonas* spp. and other psychrotrophic bacteria represent the microbial spoilage contaminants of mozzarella (Baruzzi et al., 2012). The interest in the role of *Pseudomonas* spp., the *Pseudomonas fluorescens* species in particular, in spoiling of mozzarella increased in summer 2010 when the “blue mozzarella” occurred in Italy and then spread to many countries of the Europe (Nogarol et al., 2013; Andreani et al., 2014). Although several studies are available reporting the level of contamination of dairy products by the cultivable population of *Pseudomonas* spp. (Carraro et al., 2011; Conte et al., 2011; Martin et al., 2011), information related to the activity of viable but noncultivable (VBNC) *Pseudomonas* spp. populations in cheese is absent. The appearance of the VBNC microbial cells can be caused by multiple stresses (chemical or heat shock, osmotic stress, and dehydration) occurring during the production and the storage processes. In the VBNC state, the cells maintain most of their metabolic activities, thus affecting the quality and the shelf life of the product. Therefore, the aim of this work was the setup of a rapid method for the total detection and the quantification of *Pseudomonas* spp. in mozzarella cheese, discriminating among dead, alive, and VBNC cells.

MATERIALS AND METHODS

Sampling of Mozzarella Cheeses

Different batches of commercial mozzarella cheese samples were collected weekly from April 2012 to September 2013, directly from the dairy factory, im-

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mediately after production and before distribution. For challenging assay, samples of mozzarella were subjected to contamination with increasing concentrations of *P. fluorescens* DSM 50106^T cells, ranging from 1 to 6 log cfu⁻¹ of an overnight culture, washed, and resuspended in a sterile tryptone salt solution (0.85% wt/vol; Oxoid, Basingstoke, UK). The total count of the inoculum was determined by Bürker chamber microscopy quantification (Splittstoesser, 1992), and the concentration of the cultivable cells of the inoculum was determined by seeding on *Pseudomonas* Agar Base (Oxoid, Milan, Italy) supplemented with *Pseudomonas* CFC supplement (Oxoid). Plates were incubated at 35°C for 48 h.

Quantification of Cultivable *Pseudomonas* spp.

The mozzarella (100 g) and the governing liquid (100 mL) were homogenized within a sterile Stomacher bag, by using a Colworth 400 Stomacher (Seward, West Sussex, United Kingdom) for 5 min. Decimal dilutions in sterile tryptone salt solution (Oxoid) were prepared, and the concentrations of *Pseudomonas* spp. and total microorganisms were determined by spread technique on plates of CFC selective *Pseudomonas* Agar Base (Oxoid). Samples were also evaluated for the total microbial count by seeding on Tryptic Soy Agar (Difco, Detroit, MI). To allow the adaptation and growth of the stressed *Pseudomonas* spp. cells, a revivification step was set up. To this aim, 0.2 g of mozzarella cheese sample was incubated at 35°C for 4 h. Sixty-three mozzarella samples were analyzed. All the analyzes were carried out in triplicate.

DNA and RNA Extractions

One gram of mozzarella and governing liquid was used for the DNA and RNA extraction. Briefly, each sample was collected in a sterile stomacher bag and homogenized for 5 min using the Colworth 400 Stomacher. For DNA extraction, a QIAamp DNA Stool kit (Qiagen, Milan, Italy) was used according the manufacturer's instructions. After DNA quantification with a Smart Spec Plus Spectrophotometer (Bio-Rad Laboratories, Milan, Italy) and evaluation of 260/280 ratio, a solution of 1 ng/μL was prepared and used for the amplification. The repeatability of the DNA extraction was evaluated by means of 10 independent DNA analyses of mozzarella cheeses from the same production line batch.

Two milliliters of RNA Protector Bacteria Reagent (Qiagen) was used to prepare the samples. An RNeasy Plus Universal kit (Qiagen) was applied to extract RNA, followed by a treatment with amplification-grade

DNaseI (Sigma-Aldrich, Milan, Italy) to purify RNA and remove the residual DNA. Each sample was also subjected to a non-retro-transcribed quantitative PCR to evaluate the efficiency of the treatment. Subsequently, the RNA was quantified with Smart Spec Plus Spectrophotometer (Bio-Rad Laboratories), and 1 μg of RNA was used for the reverse transcription using an iScript cDNA Synthesis kit (Bio-Rad Laboratories) with random primers. The resultant cDNA was subjected to reverse transcription quantitative PCR (RT-qPCR).

PCR Experiments

The DNA and cDNA were subjected to qPCR analysis using the following primers specific for the genus *Pseudomonas*: P94F 5'-CGGACGGGTGAG-TAATGCCTAG-3', P649R 5'-CAGGAAATTCCAC-CACCCTCTACC-3' (Calisti, 2008). The amplified fragment of 550 bp belonged to the *Pseudomonas* spp. genus-specific portion of the *16S* rDNA gene. The PCR reactions were carried out in a final volume of 15 μL containing 7.5 μL of SSO Fast Eva Green Supermix (Bio-Rad Laboratories), 0.3 μM of each primer, and 5 ng of extracted DNA. The amplifications were performed in CFX96 Real-Time PCR System (Bio-Rad Laboratories), using the following program: initial denaturation step at 95°C for 3 min, amplification and quantification steps repeated for 40 cycles (95°C for 10 s, 62.4°C for 30 s, 72°C for 30 s). A mixture of all PCR reagents without DNA was used as a negative control. The response was considered positive if the amplification curve of the 2 replicates exceeded the fluorescence threshold line, which was positioned by a background-based algorithm calculated by the software.

Calibration Curves for *Pseudomonas* spp. Enumeration

Calibration curves were generated from plotting threshold cycle versus log₁₀ cells per milliliter. Different calibration curves were constructed for the assessment of population level by qPCR and RT-qPCR by using known quantities of genomic DNA and RNA of *P. fluorescens* DSM 50106^T, extracted from a 10-fold dilution series of cell pellets following a centrifugation step in a concentration range of 1 to 6 log cells/mL. The concentrations of the extracted nucleic acids were measured with Smart Spec Plus Spectrophotometer (Bio-Rad Laboratories). The results of qPCR and RT-qPCR were expressed as log₁₀ cfu per gram of total or alive *Pseudomonas* spp. cells.

The repeatability of the assessment of population level by qPCR and RT-qPCR was evaluated carrying

out 3 independent analyzes of mozzarella cheeses from the same production line batch. From each sample, 10 DNA and 7 RNA extractions and quantifications were carried out. To quantify the dead and VBNC cells, we evaluated the total DNA and RNA of artificially noncontaminated mozzarella samples and mozzarella samples inoculated with increasing known concentrations of overnight culture of *P. fluorescens* DSM 50106^T. The quantification of *Pseudomonas* spp. by qPCR represented the sum of dead, cultivable, and VBNC cells, whereas the quantification of *Pseudomonas* sp. by RT-qPCR indicated the sum of cultivable and VBNC cells. Therefore, the difference between qPCR and RT-qPCR data represented the dead *Pseudomonas* spp. cells.

Statistical Analysis

An ANOVA was carried out to determine differences among mean quantities of *Pseudomonas* spp. cells for each sample. Amplification efficiencies and detection sensitivities among different experiments were investigated: slopes of the calibration curves were calculated by performing a linear regression analysis. Amplification efficiency (*E*) was estimated by using the slope of the calibration curve and the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency generated a slope of -3.32 .

Blue Mozzarella Spoilage Assay

Different samples of mozzarella cheeses from the same production line batch were inoculated with $2.30 \log \text{ cfu/g}$ of *P. fluorescens* DSM 50106^T blue pigment producer. Noninoculated samples from the same production line batch were used as controls. All samples were incubated at 4, 12, and 25°C, and the count of *Pseudomonas* spp. was performed on selective *Pseudomonas* medium after 0, 7, 17, and 29 d of incubation.

RESULTS AND DISCUSSION

Molecular Detection and Quantification of *Pseudomonas* spp.

To quantify the *Pseudomonas* spp. cells in food samples, we generated calibration curves (3 independent experiments) starting from serially diluted cells in tryptone salt solution. We extracted DNA and quantified them by qPCR. We found the linearity range to be from 1 to 7 log cells/mL; the efficiency was 85.33% and the determination coefficient (R^2) was 0.986 (Figure 1). We extracted DNA from mozzarella samples subjected

Figure 1. Calibration curve for DNA quantification, showing the linear relationship between the threshold cycle (CT) values and log cells for serially diluted DNA obtained from an overnight *Pseudomonas fluorescens* DSM 50106^T culture. Color version available online.

and nonsubjected to a revivification treatment at 35°C for 4 h. The obtained data indicated that the revivification did not modify the amount of *Pseudomonas* spp. detected by qPCR: $5.13 \pm 0.01 \log \text{ cfu/g}$ without revivification and $5.06 \pm 0.10 \log \text{ cfu/g}$ with revivification. Therefore, the latter step was not used anymore. We obtained the good repeatability of DNA extraction by carrying out independent analysis of 2 mozzarella samples from the same production line batch. From each sample, we conducted 10 DNA extractions and quantifications by qPCR. This analysis confirmed a good repeatability of the method: the amount of *Pseudomonas* spp. cells quantified by qPCR among samples in the same analysis (intra-analysis), and between 2 different analyses (inter-analysis), did not show statistically significant differences.

To set up a rapid method to highlight the presence of VBNC cells, we extracted the total RNA from mozzarella samples and used it in RT-qPCR experiments. It is well known that RNA detection is an indicator for monitoring viable bacteria (Oliver, 2010) because its half-life is about 3 to 5 min (Conway and Schoolnik, 2003), and continued gene expression by noncultivable cells is considered a reliable indicator of bacterial cell viability (Lleó et al., 2000; Oliver, 2010). Previous studies based on RT-PCR demonstrated that a variety of genes, including those for 16S rRNA synthesis, were expressed in noncultivable bacterial cells (Yaron and Matthews, 2002). Therefore, as for DNA analysis, we generated calibration curves (in triplicate), and assessed the repeatability of RT-qPCR experiments (data not shown). The linearity range was from 1.7 to 6.7 log cells/mL, the efficiency was 82.86%, and the determination coefficient (R^2) was 0.982 (Figure 2).

Comparison of the Counting of *Pseudomonas* spp. in Mozzarella Cheese by Standard Plating and by Cultivation-Independent PCR-Based Methods

We quantified *Pseudomonas* spp. in mozzarella cheese samples by standard plating on selective medium and by a cultivation-independent qPCR approach with and without the addition of known concentrations of *P. fluorescens* DSM 50106^T cells. By qPCR, we detected *Pseudomonas* spp. in all the samples (A to G, derived from batch of production between April and September 2012 and 2013; Table 1), whereas we revealed these bacteria only in samples challenged with *P. fluorescens* DSM 50106^T when cultivated on a selective medium. In all analyzed samples, the total concentration of *Pseudomonas* spp. was higher in mozzarella samples produced in summer than those measured in the spring samples. This kind of difference probably reflected a lower quality of the raw milk produced in summer compared with spring, as previously observed by other authors (Barron et al., 2001; Caridi et al., 2003; Alonso et al., 2011). Moreover, the population level in the noninoculated sample, quantified by qPCR, was very high and was different ($P < 0.05$) from the plate count, suggesting the presence of VBNC, dead cells, or both (Table 1). Interestingly, after the expiration date, sample A, not challenged with *Pseudomonas* spp. cells, showed a viable *Pseudomonas* spp. concentration lower than the detection limit (<1 log cfu/g), even though it appeared visually deteriorated in terms of exfoliation and loss of texture. The absence of growth on tryptic soy agar medium allowed us to exclude the presence of the other alternative microorganisms (data not shown). However, these degrading effects could be due to the presence of VBNC cells, their residual enzymatic activities, or both, as previously reported in other food samples. In fact, *Pseudomonas* spp. produces heat-resistant proteases that hydrolyze casein and decrease the shelf life of dairy products (Marchand et al., 2009; Arslan et al., 2011).

Figure 2. Calibration curve for RNA quantification, showing the linear relationship between the threshold cycle (CT) values and log cells for serially diluted RNA obtained from an overnight *Pseudomonas fluorescens* DSM 50106^T culture. Color version available online.

The overall data obtained by comparing the standard plating procedure and qPCR clearly showed the limit of the traditional plate count technique for the detection of *Pseudomonas* spp. in mozzarella cheese.

With the aim to establish the amount of the VBNC *Pseudomonas* spp. cells, we quantified the *Pseudomonas* spp. in nontreated mozzarella samples (H-O, from the same batch of production, Table 2), and after inoculation with known concentrations of an overnight culture of *P. fluorescens* DSM 50106^T, the predominant species reported as responsible for spoilage of milk and dairy products (Marchand et al., 2009). We counted the *Pseudomonas* spp. cells by qPCR and RT-qPCR approaches (Table 2). By RT-qPCR, we detected a measurable amount of VBNC cells in mozzarella samples negative for *Pseudomonas* spp. quantification by the standard plating method (Table 2). Moreover, in mozzarella samples that showed a measurable amount of *Pseudomonas* spp. by standard plating methods, we quantified the VBNC by subtracting RT-qPCR data from the plate count data. This value was 1.94

Table 1. Quantification of *Pseudomonas* spp. in mozzarella cheese: comparison of plate count and quantitative PCR analysis results¹

Sample	Spring time analysis			Sample	Summer time analysis		
	Plate count	qPCR	Significance		Plate count	qPCR	Significance
A (0)	$<1 \pm 0.00$	3.26 ± 0.72	**	H (0)	$<1 \pm 0.00$	5.57 ± 0.47	**
B (1)	1.59 ± 0.41	3.68 ± 0.18	**	I (1)	1.91 ± 0.29	5.86 ± 0.24	**
C (2)	2.66 ± 0.33	4.10 ± 0.24	**	J (2)	3.02 ± 0.25	5.89 ± 0.03	**
D (3)	3.67 ± 0.44	4.13 ± 0.24	*	K (3)	3.82 ± 0.44	6.27 ± 1.19	**
E (4)	4.44 ± 0.29	4.97 ± 0.58	NS	L (4)	4.81 ± 0.30	5.96 ± 0.03	**
F (5)	5.61 ± 0.61	6.06 ± 0.82	NS	M (5)	5.60 ± 0.44	6.60 ± 0.28	*
G (6)	6.29 ± 0.04	7.07 ± 0.58	NS	N (6)	6.70 ± 0.43	7.40 ± 0.25	NS

¹A to N represent samples of mozzarella of the 2 batches collected in spring and summer. The inoculum size (log cfu/g) of *Pseudomonas fluorescens* DSM 50106^T is reported in parentheses. All data are expressed as log cfu/g $\pm$ SD.

* $P < 0.05$; ** $P < 0.01$; NS: $P > 0.05$.

Table 2. Quantification of the viable but noncultivable (VBNC) *Pseudomonas* spp. population in mozzarella cheese¹ using culture, quantitative (q)PCR, and reverse transcription (RT) qPCR

Sample; inoculum	Plate count (culturable cells)	DNA (qPCR) (dead, culturable, and VBNC cells)	RNA (RT-qPCR) (culturable and VBNC cells)	Dead cells (log DNA – log RNA)	VBNC cells (log RNA – log culturable)
O (0)	<1 ± 0.00	4.40 ± 0.2	1.94 ± 0.1	4.40 ± 0.3	1.94 ± 0.1
P (1)	1.81 ± 0.2	4.56 ± 0.2	2.32 ± 0.1	4.56 ± 0.3	2.16 ± 0.4
Q (2)	2.79 ± 0.1	4.81 ± 0.1	2.94 ± 0.3	4.80 ± 0.4	2.42 ± 0.2
R (3)	4.11 ± 0.1	5.78 ± 0.3	4.13 ± 0.2	5.77 ± 0.4	2.78 ± 0.4
S (4)	4.91 ± 0.2	5.29 ± 0.2	4.93 ± 0.1	5.04 ± 0.3	3.58 ± 0.4
T (5)	5.91 ± 0.3	6.23 ± 0.0	5.91 ± 0.2	5.94 ± 0.2	3.79 ± 0.3
U (6)	6.95 ± 0.2	7.17 ± 0.1	6.96 ± 0.1	6.75 ± 0.2	5.32 ± 0.3

¹O to U represent samples of mozzarella of the one batch of production. The inoculum size (log cfu/g) of *Pseudomonas fluorescens* DSM 50106^T is reported in parentheses. All data are expressed as log cfu/g ± SD.

log cfu/g in sample A, and increased by increasing the *P. fluorescens* DSM 50106^T inoculum size in the other samples (Table 2).

Counting of the Blue Pigment Producer *P. fluorescens* in Mozzarella Cheese

During a controlled shelf-life experiment, we quantified *Pseudomonas* spp. in mozzarella samples, challenged or not with 2.3 log₁₀ cfu/g of *P. fluorescens* DSM 50106^T, by standard plating procedure. In the control samples, noninoculated with *P. fluorescens* DSM 50106^T, *Pseudomonas* spp. naturally present in mozzarella showed different trends based on the incubation temperature: at 4°C it was still at a lag phase after 7 d, whereas at 12°C this phase was absent, and the microbial growth was detected starting from d 7 of incubation. We did not detect *Pseudomonas* spp. after incubation at 25°C, even if after 3 d the mozzarella was deteriorated. In the inoculated sample, the *Pseudomonas* spp. concentration was immediately detectable, and it increased in time regardless of incubation temperature. At 25°C we observed the blue spoilage after only 2 d, whereas 7 and 17 d were necessary at 12°C and 4°C, respectively (data not shown). Moreover, we observed the blue coloration in all contaminated samples when *Pseudomonas* spp. concentration reached about 8 log cfu/g in accordance with Sechi et al. (2013). We subjected samples stored at 4°C and 12°C to qPCR and RT-qPCR after 17 d of incubation, even if the blue color appeared in a sample challenged with *P. fluorescens* DSM 50106^T and stored at 4°C (data not shown). The results obtained, by standard plating and by qPCR and RT-qPCR (Table 3), clearly indicated that the “blue” spoilage was related to a *Pseudomonas* spp. population close to 8 log₁₀ cfu/g: we detected a *Pseudomonas* spp. at a count of 8.75 log₁₀ cfu/g in a blue-pigmented commercial mozzarella sample obtained from the market. The reason for the blue pigmentation is still unknown, and it was reported that

the blue phenotype is a strain-dependent character (Andreani et al., 2014). Interestingly, the incubation of mozzarella cheese at 25°C for 2 d seemed to mask the growth of VBNC *Pseudomonas* spp. cells already present in the sample (data not shown), whereas a lower storage temperatures (4°C and 12°C) allowed detection of *Pseudomonas* spp. colonies at d 17 of incubation. Nevertheless, it is worthwhile to mention that also at a storage temperature of 4°C and 12°C, a large fraction of *Pseudomonas* spp. cells already present in the sample belonged to the VBNC population (Table 3). On the other hand, the decreasing amount of VBNC *Pseudomonas* spp. cells (until the nondetectable value) in the sample inoculated with a standardized amount of *P. fluorescens* DSM 50106^T, and stored at 4° and 12°C, could be explained as follows: (1) the partial revivification of the VBNC cells, cultivable upon resuscitation probably due to the addition of metabolically active *P. fluorescens* cells, as previously reported for *Vibrio* spp. (Ayrappetyan et al., 2014); (2) the loss of competition, during the storage process, between the *Pseudomonas* spp. population already present in mozzarella and the additionally inoculated with *P. fluorescens* DSM 50106^T.

CONCLUSIONS

The genus *Pseudomonas* is known to be an important spoilage organism of foods (Caldera and Franzetti, 2013), in particular of raw milk. It can deteriorate foods, producing anomalous colors, developing biofilms, and despite its sensitivity to the heat treatment, releasing extracellular heat-stable enzymes (Marchand et al., 2009; Arslan et al., 2011). For this reason, it is important to rapidly detect and quantify cultivable, dead, and VBNC cells belonging to this genus to prevent deterioration and spoilage of food products (Gunasekera et al., 2003). Therefore, the development of a non-culture-dependent method should be useful to demonstrate the presence of alive, dead, and VBNC

Table 3. Quantification of *Pseudomonas* spp. by standard plating and by qPCR and RT-qPCR in mozzarella cheese after 17 d of controlled shelf life experiment¹

Sample	Storage temperature (°C)	Sample color	Plate count	DNA (qPCR)	RNA (RT-qPCR)	Dead cells	VBNC cells
A	4	Normal	2.74 ± 0.1	3.95 ± 0.2	3.89 ± 0.1	3.06 ± 0.3	3.86 ± 0.2
B	4	Blue	8.76 ± 0.1	8.79 ± 0.1	8.76 ± 0.2	7.61 ± 0.3	0.12 ± 0.3
C	12	Normal	3.91 ± 0.2	4.01 ± 0.1	3.93 ± 0.2	3.24 ± 0.3	2.58 ± 0.4
D	12	Blue	7.96 ± 0.1	8.30 ± 0.2	7.96 ± 0.1	8.03 ± 0.3	0.00 ± 0.2
E	Cold chain during sale	Blue	8.75 ± 0.2	8.97 ± 0.1	8.94 ± 0.2	7.79 ± 0.3	8.49 ± 0.4

¹A and C, mozzarella samples of 2 different batches of production. B and D, respective samples A and C contaminated with 2.3 log cfu/g of *Pseudomonas fluorescens* DSM 50106^T. E is mozzarella from the trade market. All data are expressed as log cfu/g ± SD.

²VBNC = viable but noncultivable.

cells. In this study, we set up a rapid method for the detection and the quantification in mozzarella cheese of live, dead, and VBNC cells of *Pseudomonas* spp., the most abundant psychrotrophic contaminant bacteria in this Italian cheese. This experimental approach allows the operator to get a rapid sample quantification in less than 4 h. Moreover, the application of the method to samples of mozzarella contaminated with *P. fluorescens*, producer of the blue pigment, enabled us to confirm a clear relationship between the amount of viable *Pseudomonas* spp. and the development of the blue spoilage, visible when *Pseudomonas* spp. population is close to 8 log cfu/g of cheese. This result is in accordance with the previous studies (Sechi et al., 2013; Cenci-Goga et al., 2014) where the authors correlated the appearance of the anomalous blue coloration of mozzarella cheese to a specific *P. fluorescens* cell number, thus confirming the reliability of the method here described.

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