

Study of the population dynamics of *Listeria monocytogenes* and *Pseudomonas fluorescens* in buffalo mozzarella by means of challenge testing

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Abstract

Campania's buffalo mozzarella is a greatly appreciated cheese in Italy and worldwide. From a microbiological standpoint, it is a highly perishable food and potentially at risk of contamination by pathogens such as *Listeria monocytogenes* (*L. monocytogenes*). The present paper reports the results of a challenge test carried out to assess the population dynamics of *L. monocytogenes*, alone and in presence of *Pseudomonas fluorescens* (*P. fluorescens*), in buffalo mozzarella. For this purpose buffalo mozzarella samples were contaminated with *L. monocytogenes* alone or combined with *P. fluorescens*. In samples where *L. monocytogenes* was inoculated alone, the bacterial load remained unchanged. On the contrary, in samples contaminated with *L. monocytogenes* and *P. fluorescens*, the growth of *L. monocytogenes* increased.

Introduction

Over 1600 types of cheese are produced worldwide; their characteristics vary according to the geographical area of production and local traditions. Stretched curd cheeses made from buffalo milk are typical products of southern Italy. Amongst these cheeses, the *mozzarella di bufala campana* (Campania's buffalo mozzarella) takes pride of place. Owing to the production process, this cheese is a highly perishable foodstuff and a possible source of food poisoning.

In recent years, an increase in cases of abnormal coloration of fresh stretched curd

cheeses has been reported in Italy (Cantoni *et al.*, 2003). The cause of these colorations has been attributed to a biovariant of *Pseudomonas fluorescens* (*P. fluorescens*) that is able to produce blue pigments (biovar IV). A further characteristic of *P. fluorescens* is its ability to produce biofilm. *Listeria monocytogenes* (*L. monocytogenes*), which also produces a biofilm, can exploit the biofilms produced by *P. fluorescens* on work surfaces and on the products themselves, thereby becoming more resistant not only to adverse environmental conditions but also to some of the disinfectants commonly used for environmental sanitation (Saá Ibusquiza *et al.*, 2012; Wuertz *et al.*, 2004; Norwood and Gilmour, 1999, 2001; Pan *et al.*, 2006; Kastbjerg and Gram, 2009). *L. monocytogenes* is able to survive for long periods in various ecological niches, including work surfaces in stables as well as and in dairy and cheese-making facilities (Porsby *et al.*, 2008).

Human listeriosis is mainly (95-99%) a foodborne disease (Scallan *et al.*, 2011; Kovacevic *et al.*, 2013). Human infections are caused by ingestion of contaminated foodstuffs, which allow the growth of *L. monocytogenes*. In humans with normal immune defenses the infective dose is believed to be 10⁴ colony forming unit (CFU)/g *L. monocytogenes*, while in immunocompromised subjects it can even be lower than 10³ CFU/g (Ooi and Lorber, 2005). From 2008 to 2011, cases of human listeriosis in Europe remained stable at a rate of about 1400 per year (Denny and McLauchlin, 2008; EFSA, 2013). Over the last 30 years, several outbreaks of food poisoning due to cheeses contaminated by *L. monocytogenes* have been reported (EFSA, 2013). *L. monocytogenes* can survive in substrates containing up to 16% of salt, and can even double in fresher, less-mature substrates and those containing less salt (Oliver *et al.*, 2009; Fretz *et al.*, 2010).

The aim of the present study was to investigate the growth of *L. monocytogenes* associated to *P. fluorescens* in buffalo mozzarella, by means of a series of assays and experimental inoculations of these two bacteria, alone or in combination.

Materials and Methods

The challenge test was carried out in accordance with a protocol designed by ANSES 2008 guidelines (ANSES, 2008). One hundred and twenty eight mozzarella cheeses were collected, each weighing 100 g and belonging to a single production batch. The detection of *L. monocytogenes* and the enumeration of *P. fluorescens* were immediately evaluated on samples as reported below. Samples were indivi-

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dually immersed in a conservation liquid inside single packages, then they were contaminated with three different strains of *L. monocytogenes* and three strains of *P. fluorescens* – one standard strain (ATCC 13932) and two *wild* strains isolated from dairy products [one in the *culturable* (V) phase, *i.e.* live and able to replicate, and the other in the *viable-but-not-culturable* (VBNC) phase, *i.e.* live but unable to replicate]. The contamination was performed by immersion of each sample in solutions of bacteria at room temperature (22±1°C) for two min. The bacterial loads of the solutions were 10 and 100 CFU/mL respectively. After contamination, the samples were stored at a temperature of 4±1°C for the first three days, and were then stored at 12±1.5°C until the end of the experiment (day 11; T11).

The detection of *L. monocytogenes* and enumeration of *L. monocytogenes* and *P. fluorescens* as well as the chemical-physical analyses [pH value and water activity (a_w) value] were performed after 1, 3, 6, 9 and 11 days of storage (times T1, T3, T6, T9 and T11). Each test was performed in duplicate. For the microbiological assays, the following methods were performed: detection of *L. monocytogenes* by means of the VIDAS® (bioMérieux, Marcy l'Etoile, France) technique; *L. monocytogenes* count by means of UNI EN/ISO 11290-2:2005 (ISO, 2005); *P. fluorescens* count by means of ISO/TS 11059 IDF/RM 225:2009 (ISO, 2009). pH and a_w were detected through the use of pH-meter Knick 911 (Crami Group, Pero, Italy) and Fast-lab (GBX, Dublin, Ireland) devices, according to the manufacturer's instructions.

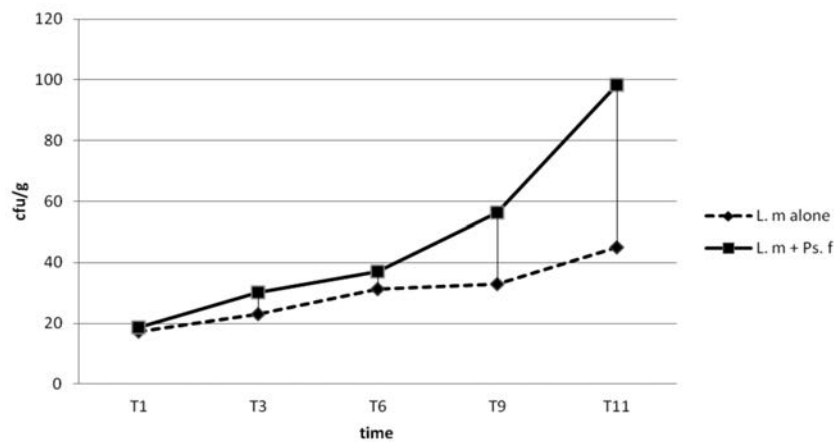


Figure 1. Enumeration of *Listeria monocytogenes* alone and combined with *Pseudomonas fluorescens*: average of the results globally observed.

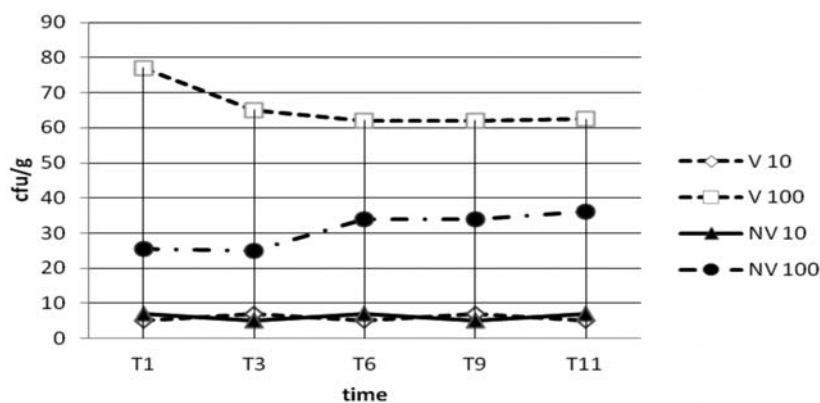


Figure 2. Enumeration of *Listeria monocytogenes* alone: all types of inoculums. V, culturable; NV, viable-but-not-culturable; 10 and 100, amount of inoculums (colony forming unit).

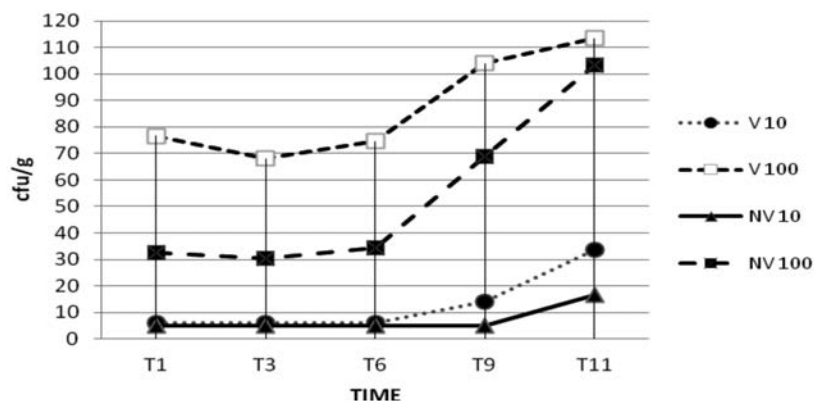


Figure 3. Enumeration of *Listeria monocytogenes* in samples contaminated jointly with *Pseudomonas fluorescens*: all types of inoculums. V, culturable; NV, viable-but-not-culturable; 10 and 100, amount of inoculums (colony forming unit).

Results and Discussion

The results obtained are reported in Figures 1, 2 and 3. *L. monocytogenes* was detected in all analysed samples, though sometimes at loads below the detection limit of the EN ISO 11290-2:2005 technique (ISO, 2005). In the samples where *L. monocytogenes* was inoculated alone, the bacterial load of both forms (V and VBNC) remained substantially unchanged (Figure 2). By contrast, in those samples where *L. monocytogenes* was inoculated jointly with *P. fluorescens*, the growth of the latter influenced the growth of the former (Figure 3). In these last samples, *L. monocytogenes* growth increased starting from T6 of the experiment.

None of the samples analysed showed modification in sensory properties or in the occurrence of improper pigmentations related to contamination by *P. fluorescens*. The pH of the cheeses tested became slightly more acidic, moving from initial values of 5.41 to values of 5.34 pH U at the end of the 11th day of conservation. Finally, the value of a_w in the samples analysed declined slightly, from initial values of 0.983 to final values of 0.978.

Conclusions

Campania's buffalo mozzarella is a fresh cheese with a short shelf-life and values of pH and a_w which, in theory, are insufficient to curb the growth of a pathogen such as *L. monocytogenes*. Nevertheless, the results of our tests seem to indicate that the chemical and physical properties of this product, as well as other factors not considered in this study, are able to curb the growth of *L. monocytogenes*, provided that the contaminating bacterial load is low or very low (around 10 CFU/g or less). The growth of the pathogen remained below 0.5 degrees log of load between the value estimated on day 1 (T1) and at the end of the test (T11). The growth of *L. monocytogenes* appears to be positively influenced by the co-presence of *P. fluorescens* (Figure 1), thus revealing the role of *P. fluorescens* not only as spoilage bacteria, but also as an indirect source of risk for human beings.

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