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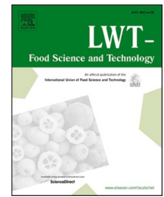

Effectiveness of lactic and acetic acids on the growth of *Listeria monocytogenes* and *Bacillus cereus* in primo sale fresh cheese

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ABSTRACT

In this study Brain Heart Infusion broth inoculated with *Listeria monocytogenes* with a concentration of acetic acid of 24.98 mM or a concentration of 44.40 mM of lactic acid did not determine the increase in absorbance in 7 days. A concentration of acetic acid of 24.98 mM and a concentration of 22.20 mM of lactic acid were effective against *Bacillus cereus* growth. Then, challenge tests on primo sale cheese were conducted to establish if these concentrations were efficient when applied to cheese. After inoculum with the pathogens (2 log CFU g^{-1}), cheese was dipped with acetic and lactic acid solutions. In a first trial, *L. monocytogenes* inoculated, showed the absence of significant differences in growth at 4 °C among the treated series (Ac1: acetic acid - 49.96 mM; Ac2: 24.98 mM; Lac1: lactic acid – 88.80 mM; Lac2: 44.40 mM) if compared to Control (CTRL) series (dipped with sterile water). At 8 °C, a significantly lower growth in Ac1 samples if compared to control ones and to all the treated series was observed ($P < 0.05$). The trial conducted inoculating *B. cereus* did not show any difference at 15 °C among samples treated with organic acids if compared to control series.

1. Introduction

The Gram-positive microorganism *Listeria monocytogenes* is a ubiquitous, intracellular pathogen, able to grow at low temperatures and easily adaptable to highly acidic or saline conditions; this microorganism has been also previously identified as causative agent in several foodborne outbreaks (Farber & Peterkin, 1991; Buchanan et al., 2017). Outbreaks due to the consumption of fresh cheese have been already reported, with *L. monocytogenes* stated as one of the predominant causative agents (Zottola & Smith, 1991; Oliver et al., 2005; Little et al., 2008; Martinez-Rios & Dalgaard, 2018). Also *Bacillus cereus*, is frequently associated to severe food poisoning episodes due to its ability to produce toxins like cereulide, cytotoxin K, haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE). *B. cereus* was already recognized as responsible of raw milk spoilage (Bartoszewicz et al., 2008) and its presence was also found in many dairy products, with prevalence from 2 to 52% (Adame-Gòmez et al., 2019; Spanu et al., 2016; Svensson et al., 2006; Wong et al., 1988; Zhao et al., 2020).

Consumers always request healthy 'fresh-like' food with a long shelf-life. The application of traditional thermal food processes or GRAS bio preservation is convenient, but not always possible in all the food processes or in some cases is not efficient. In particular, when considering the dairy industry, pathogens like *L. monocytogenes* or *B. cereus* are able to survive and multiply in raw materials but also in some typologies of cheeses (Tirloni et al., 2017, 2019b). In this context, the application of antimicrobial compounds such as food grade organic acids may play a role as additional hurdle to achieve a successful and effective growth inhibition. The application of organic acids may be suitable in Ready to eat (RTE) foods (Tirloni et al., 2020 a,b), with the aim to reformulate products or inhibit bacterial growth.

Microbial growth can be reduced in presence of undissociated organic acids that can pass through the bacterial cell membrane. When this happens, the organic acid dissociates in the cytoplasm, increasing hydrogen ion concentration in the cell. In order to re-establish internal pH, hydrogen ions are pumped out determining unfavourable conditions for the microorganisms (Mitchell, 1961).

Minimal inhibitory concentrations (MICs) of the undissociated forms of lactic, acetic, citric and propionic acid for *L. monocytogenes* have been investigated in previous studies (Conner et al., 1990; Chen & Shelef, 1992; Houtsma et al., 1993; Vasseur et al., 1999; Coroller et al., 2005; Van der Veen et al., 2008; Aryani et al., 2015), while very limited studies focused on the determination of MICs of undissociated acids under

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conditions relevant to dairy products (Coroller et al., 2005; van der Veen et al., 2008; Wemmenhove et al., 2016, 2018), and even fewer studies reported MIC calculated directly in the cheese matrix (Tirloni et al., 2019). For *B. cereus* very limited literature is reported about application of organic acids and the calculation of MIC (Jang et al., 2005; Stanojević-Nikolić et al., 2016).

The aim of the present study was to investigate at which concentrations of lactic and acetic acid *L. monocytogenes* and *B. cereus* were not able to replicate in broth for a possible application to cheese production. In a second stage, challenge tests with *L. monocytogenes* and *B. cereus* inoculated separately onto the surface of an Italian fresh cheese (primo sale cheese) were also conducted, to evaluate the ability of the two organic acids (lactic and acetic acids) to inhibit the growth of these two pathogens when dipped on the cheese; this approach could be extremely useful and interesting in the inhibition of the growth of food borne pathogens in cheese industry.

2. Materials and methods

2.1. MICs determination

A strain of *L. monocytogenes*, isolated from a fresh dairy product (strain MS12209), provided by DTU Food (Danish Technical University, DK), and a reference strain of *B. cereus* (ATCC 14579) were considered for this trial. Stocks were kept frozen at −80 °C in Microbank Cryogenic vials (Pro-Lab Diagnostics U.K., Merseyside, UK). From each stock culture, a loopful was transferred to 10-mL tubes of Brain Heart Infusion broth (BHI) (Oxoid, Basingstoke, UK) with pH 7.2 and incubated at 37 °C for 24h. Cultures were harvested in late exponential growth phase defined as a relative change in optical density (OD) of 0.05–0.2 at 540 nm (Jenway 6105, Staffordshire, UK). Cell concentrations of these precultures were determined by microscopy at 1000× magnification (Motic, B310, Wetzlar, Germany), considering approximate that one cell per field of view corresponded to a concentration around 10^6 CFU mL^{-1} (Adams & Moss, 2000). Bacterial suspensions were diluted to a concentration of 10^4 CFU mL^{-1}. Afterwards, aliquots of 0.1 mL of each strain suspension were separately inoculated into 10-mL tubes containing BHI added with lactic (code 252476, Sigma Aldrich, Steinheim, Germany) or acetic acid (code 1005706, Sigma Aldrich, Steinheim, Germany) solutions, reaching a final bacterial concentration of about 100 CFU mL^{-1}. The acid concentrations tested were 2.78 mM, 5.55 mM, 11.10 mM, 22.20 mM and 44.40 mM for lactic acid, and 1.25 mM, 2.50 mM, 5.00 mM, 12.49 mM and 24.98 mM for acetic acid; a control series (BHI without acid addition) was also prepared.

At the time of inoculation (t_0), OD was measured and the tubes were then incubated at 37 °C in duplicate. At fixed times (24, 48, 72, 96 h from inoculation), OD was newly measured. Blank (not inoculated) broth series for each trial were also prepared. pH of each series was measured at t_0.

2.2. Challenge tests

Specific challenge tests were performed on primo sale cheese, a fresh cheese made from cows' milk, characterized by an early stage of maturation. The trials were carried out to establish if the MICs determined in section 2.1 for undissociated acetic and lactic acid on *L. monocytogenes* and *B. cereus* were efficient when applied to cheese matrix.

Primo sale cheese samples were purchased on the first day after production; all the samples came from the same producer and, for each trial, belonged to the same production batch. The composition of the product, taken from the nutritional label, was (upon 100 g of product): fats: 17 g, carbohydrates: 3.2 g, proteins: 13 g, salt: 0.65 g. Prior to inoculation, slices were obtained, in order to standardize their weight (8 g).

The samples used for microbial challenge tests were inoculated with *L. monocytogenes* (strain MS12209) or *B. cereus* (ATCC 14579). As described in the previous section, the frozen strains were transferred to BHI and incubated at 37 °C for 24h. In order to pre-adapt the cells to the environmental conditions of each of the challenge tests, cultures were subsequently re-inoculated in BHI broth and then incubated at different temperatures (4 and 8 °C for *L. monocytogenes*, 15 °C for *B. cereus*) depending on the storage temperature of the challenge test. Cultures were harvested in late exponential growth phase, and cell concentrations were determined by microscopy (see section 2.1). Pre-cultures of individual isolates were diluted in sterile saline water (0.85% NaCl). 20 μL aliquots of the suspensions were spread onto the surface of the cheese to obtain the starting concentration around 2 log CFU g^{-1} after inoculation on the product. The small volume used assured to exert a negligible impact on the product characteristics (EURLm, 2019).

After the inoculum, the samples were kept for 30 min to allow bacterial adhesion to the cheese surface. Then, they were divided in five series for each microorganism, to be dipped with different organic acid solutions:

- For L. *monocytogenes*: acetic acid, Ac1: 49.96 mM; Ac2: 24.98 mM; lactic acid, Lac1: 88.80 mM; Lac2: 44.40 mM; CTRL: sterile distilled water.
- For *B. cereus*: acetic acid, Ac1: 49.96 mM; Ac2: 24.98 mM; lactic acid, Lac2: 44.40 mM; Lac3: 22.20 mM; CTRL: sterile distilled water.

The concentrations of undissociated organic acids were estimated with Equation (1):

Undissociated organic acid (mM)= (organic acid (mM))/(1+ 〖10〗 ^(pH-pKa)) (1)

The applied pKa values were 4.76 and 3.86 for acetic acid and lactic acid, respectively.

Blank (non-inoculated) sample series, intended for pH determination, were also prepared, using the same organic acid concentrations.

Dipping of each sample was performed in Petri dishes filled with the acid solution/distilled water; then, all the samples were put on single sterile Petri dishes and incubated at selected temperature conditions: the trials were performed at 4 and 8 °C for *L. monocytogenes* and at 15 °C for *B. cereus*. During storage, temperature was recorded by data loggers. During the challenge test performed at 4 °C, the samples were analysed at t_0 and after 2, 5, 8 and 10 days from inoculation. At 8 °C, the samplings were performed at t_0 and after 1, 2, 3, 5, 6 and 8 days from inoculation. At 15 °C, samples were analysed at t_0 and after 1, 2, 3 and 4 days from inoculation.

At each sampling time, the following analyses were performed in duplicate: the whole cheese sample was 10-fold diluted in pre-chilled sterile saline and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made with pre-chilled sterile saline. *L. monocytogenes* was enumerated by spread plating on Palcam Agar added Palcam Selective Supplement and incubated at 37 °C for 48 h. *B. cereus* was enumerated by spread plating onto PEMBA agar, and incubated at 37 °C for 48 h. *B. cereus* spores were also enumerated by pasteurizing the serial dilution at 80 °C for 10 min, and then by spread plating onto PEMBA agar. All the media were supplied from Scharlab (Barcelona, E). An increase of +0.5 Log CFU g-1 was used to discriminate growth and no growth in the product (EURLm, 2019).

Surface pH of the non-inoculated samples was measured at each sampling time in duplicate by using a pH meter (Amel, Milan, I).

2.3. Sensorial analysis

A panel test composed by twenty non trained panellists was conducted on non-inoculated primo sale cheese dipped with acids (acetic acid, Ac1: 49.96 mM; Ac2: 24.98 mM; lactic acid, Lac1: 88.80 mM; Lac2:

Table 1
Absorbance of L. *monocytogenes* and *B. cereus* in broth at 37 °C in presence of different concentrations of lactic and acetic acids.

Lactic Acid – *Listeria monocytogenes*		T0	T24	T48	T72	T96
44.40 mM	average	**−0,043**	**−0,048**	**−0,050**	**−0,049**	**−0,054**
	std. dev.	0,042	0,049	0,050	0,049	0,037
22.20 mM	average	−0,078	−0,087	−0,105	−0,111	0,064
	std. dev.	0,042	0,033	0,050	0,054	0,320
11.10 mM	average	−0,015	−0,016	−0,025	0,142	0,960
	std. dev.	0,012	0,011	0,018	0,004	0,371
5.55 mM	average	0,008	−0,008	−0,024	0,246	1,576
	std. dev.	0,037	0,033	0,040	0,117	0,053
2.78 mM	average	−0,013	−0,008	−0,024	0,449	1,706
	std. dev.	0,052	0,031	0,042	0,098	0,105
CTRL (0 mM)	average	0,086	0,086	0,060	0,557	1,674
	std. dev.	0,020	0,041	0,006	0,047	0,069
Lactic Acid – *Bacillus cereus*		**T0**	**T24**	**T48**	**T72**	**T96**
44.40 mM	average	−0,032	−0,013	−0,050	−0,036	−0,008
	std. dev.	0,001	0,037	0,013	0,006	0,073
22.20 mM	average	**−0,037**	**−0,020**	**−0,061**	**−0,073**	**−0,040**
	std. dev.	0,074	0,097	0,093	0,103	0,062
11.10 mM	average	−0,021	−0,014	−0,026	0,026	0,025
	std. dev.	0,094	0,073	0,094	0,056	0,106
5.55 mM	average	0,000	0,014	−0,028	0,099	0,065
	std. dev.	0,030	0,035	0,048	0,028	0,045
2.78 mM	average	−0,036	−0,024	−0,061	0,133	0,048
	std. dev.	0,031	0,047	0,042	0,068	0,023
CTRL (0 mM)	average	0,021	0,009	−0,006	0,223	1,703
	std. dev.	0,055	0,006	0,034	0,037	0,002
Acetic Acid – *Listeria monocytogenes*		**T0**	**T24**	**T48**	**T72**	**T96**
24.98 mM	average	**0,024**	**0,038**	**0,015**	**0,014**	**0,007**
	std. dev.	0,011	0,004	0,006	0,011	0,001
12.49 mM	average	0,005	0,000	−0,028	−0,008	1,042
	std. dev.	0,004	0,008	0,003	0,005	0,021
5.00 mM	average	0,065	0,092	0,071	0,300	1,110
	std. dev.	0,037	0,064	0,053	0,162	0,048
2.50 mM	average	0,057	0,061	0,033	0,391	1,511
	std. dev.	0,092	0,069	0,082	0,093	0,002
1.25 mM	average	0,037	0,040	0,016	0,422	1,423
	std. dev.	0,013	0,024	0,034	0,039	0,373
CTRL (0 mM)	average	0,086	0,086	0,060	0,557	1,674
	std. dev.	0,020	0,041	0,006	0,047	0,069
Acetic Acid – *Bacillus cereus*		**T0**	**T24**	**T48**	**T72**	**T96**
24.98 mM	average	**−0,003**	**0,009**	**−0,022**	**−0,028**	**−0,031**
	std. dev.	0,073	0,059	0,061	0,060	0,075
12.49 mM	average	−0,038	0,013	−0,050	−0,045	0,116
	std. dev.	0,028	0,032	0,042	0,030	0,006
5.00 mM	average	0,004	0,037	−0,019	0,010	0,036
	std. dev.	0,029	0,023	0,001	0,017	0,006
2.50 mM	average	−0,006	−0,006	−0,016	0,098	0,081
	std. dev.	0,002	0,034	0,005	0,040	0,004
1.25 mM	average	0,079	0,072	0,075	0,246	0,130
	std. dev.	0,061	0,042	0,029	0,095	0,011
CTRL (0 mM)	average	0,021	0,009	−0,006	0,223	1,703
	std. dev.	0,055	0,006	0,034	0,037	0,002

44.40 mM) vs control samples (non treated). Each panellist was asked the questions: did you find any difference among the samples? If yes, explain which ones.

2.4. Statistical analysis

Data obtained from *L. monocytogenes* and *B. cereus* counts (expressed as the log CFU g^{-1} difference between each sampling time and t_0) were submitted to 2-way univariate ANOVA in SAS (version 9.1, 2016; SAS Institute Inc., Cary, NC) to reveal eventual differences among the treatments. Threshold values for statistical significance were set at $P < 0.05$ and $P < 0.01$.

3. Results and discussion

3.1. Determination of MICs for L. monocytogenes and B. cereus

In the present study, the absorbance of broths inoculated with *L. monocytogenes* was measured showing if different concentrations of lactic and acetic acids were effective against the growth of the microorganism. The results are reported in Table 1. Our experiments highlighted that at 37 °C, BHI with a concentration of acetic acid of 24.98 mM did not support growth of *L. monocytogenes* within 7 days. The same effect was obtained with BHI with a concentration of 44.40 mM of lactic acid. Moreover, BHI added with a concentration of acetic acid of 24.98 mM did not support the growth of *B. cereus* within 7 days at 37 °C. In the same conditions, BHI with a concentration of 22.20 mM of lactic acid did not support the growth of the microorganism.

3.2. Trial 2: challenge test – L. monocytogenes

The first trial (Fig. 1a), carried out to identify the effect of the addition of acetic or lactic acids on *L. monocytogenes* growth in primo sale cheese, showed the absence of significant differences among the treated series (samples dipped with two concentrations of the organic acid) and the control one at 4 °C. Nevertheless, a slight reduction in the pathogen counts was observed in Ac1 samples (49,96 mM of acetic acid), leading to a significant difference of 1.74 Log CFU g^{-1} in *L. monocytogenes* counts at t_{10} (last sampling time), if compared to CTRL series ($P < 0.05$). A slightly lower growth rate was also detected in the other treated series, leading to differences in *L. monocytogenes* counts at t_8 of 1.13, 0.53 and 0.25 Log CFU g^{-1} for Lac2 (44,40 mM), Lac1 (88,80 mM) and Ac2 samples (24,98 mM), respectively ($P < 0.05$).

An effect of dipping on primo sale surface pH was evident (Fig. 2a): taking the whole experimental period, a significant difference was evidenced between CTRL and the other series ($P < 0.01$). A stronger acidification effect of dipping with acetic acid was shown, coupled with a dose-dependent effect of both organic acids, obtaining the following values at the end of the trial (t_{10}): 6.15 (CTRL), 5.70 (Ac1), 5.90 (Ac2), 5.63 (Lac1) and 5.99 (Lac2) (Fig. 2a).

At 8 °C, a significantly lower growth of *L. monocytogenes* (Fig. 1) was detected in Ac1 (49,96 mM) samples if compared to control ones and to all the other treated series ($P < 0.05$). Indeed, no evident growth of *L. monocytogenes* was revealed in Ac1 series until t_8, achieving a difference in the counts equal to 3.40 Log CFU g^{-1} if compared to control samples. At the same conditions, Ac2 and Lac1 samples showed a significantly lower growth if compared to CTRL and Lac2 ($P < 0.05$). These data showed both the effect of acid concentration and the difference between the two organic acids tested.

A higher activity of acetic acid has been described (Barmpalia et al., 2004), thanks to its higher pKa value (4.76 vs 3.86 of lactic acid), but the relative efficacy of the specific organic acids is influenced by several factors linked to the complex food substrate (Samelis & Sofos, 2003). In this study, the concentration of the undissociated form was evidently higher for acetic acid, thus justifying the observed effect. The undissociated fraction of acetic acid tested (3.6 mM in Ac1 series, at the pH of the product) was very low if compared to the MIC reported by other authors (Coroller et al., 2005; Le Marc et al., 2002; Mejlholm & Dalgaard, 2009; Tirloni et al., 2019; Wemmenhove et al., 2016). This could be due to differences in the substrates considered (different pH, food matrix).

The determination of pH of the samples stored at 8 °C (Fig. 2b) confirmed the acidifying effect of all the dipping treatments, with a significant difference between the CTRL samples and all the other series ($P < 0.01$). As for the trial performed at 4 °C, Ac1 treatment resulted in significantly lower pH values than the other treatments ($P < 0.05$) (Fig. 2b).

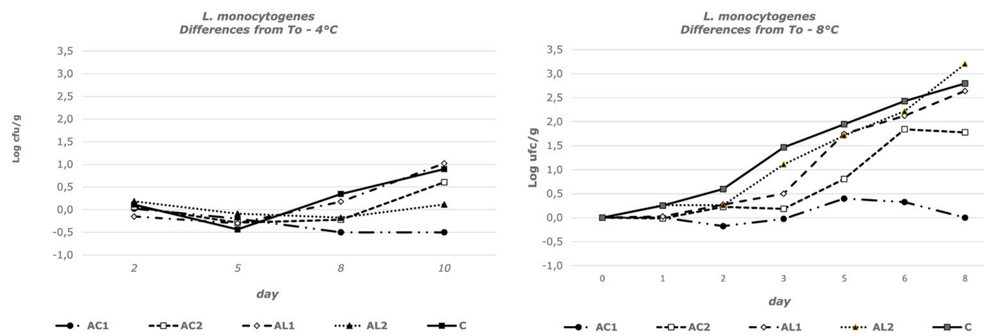

Fig. 1. *L. monocytogenes* counts obtained in primo sale cheese during the challenge test performed: a) at 4 °C, and b) at 8 °C. Dipping: Ac1: acetic acid, 49.96 mM; Ac2: acetic acid, 24.98 mM; Lac1: lactic acid, 88.80 mM; Lac2: lactic acid, 44.40 mM; CTRL: distilled water.

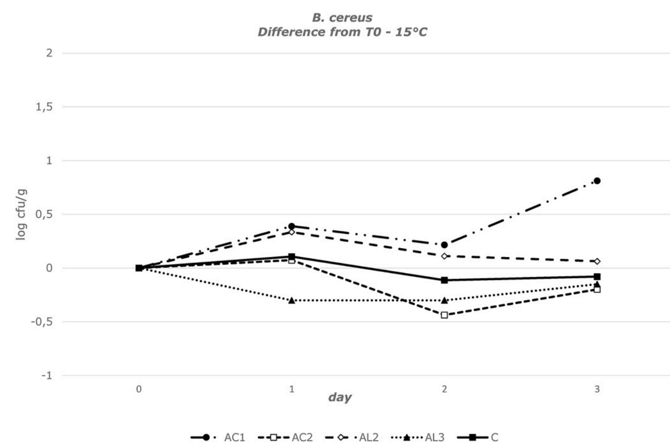

Fig. 2. pH values measured in primo sale cheese during the challenge test performed: a) at 4 °C, and b) at 8 °C. For the legend, see Fig. 1.

In these trials, a conditioning of the substrate with food grade organic acids demonstrated to be a potential useful intervention for the inhibition of the replication of *L. monocytogenes*. Also Millet et al. (2006), established that low pH and the presence of short-chain organic acids had an inhibitory effect on the growth of this pathogen in Dutch-type cheeses such Gouda, Edam and Maasdam made from pasteurized milk. Moreover, in a previous study, Tirloni, Nauta, et al. (2020) used the same approach in order to condition a RTE fish based product with three different organic acid solutions (acetic acid - benzoic acid + acetic acid + lactic acid - lactic acid + sodium acetate), finding an effective treatment that allowed to decrease the concentration of *L. monocytogenes*.

3.3. Trial 2: challenge test – *B. cereus*

B. cereus growth did not show any significant difference among treated and control series during storage at 15 °C (Fig. 3). Basically, no growth on the substrate was revealed in any of the series. This effect could be due to a combination of environmental factors, mainly temperature (15 °C is near the lower growth limit for most *B. cereus* strains), while pH values were variable but always permissive, from 6.4 (t_0) to 6.2/6.3 (t_4) without any particular trend in all the series during the trial (Fig. 4). The nature of this inhibition should be further studied as several other factors may also concur (superficial moisture, presence of natural microflora, etc.) (Holzapfel et al., 1995; Tirloni et al., 2014). Inhibition of *B. cereus* in dairy foods has already been reported in milk medium, in Gouda cheese, in Brie and in Taleggio cheese (Little & Knøchel, 1994; Rukure & Bester, 2001; Tirloni et al., 2017; Wong et al., 1988).

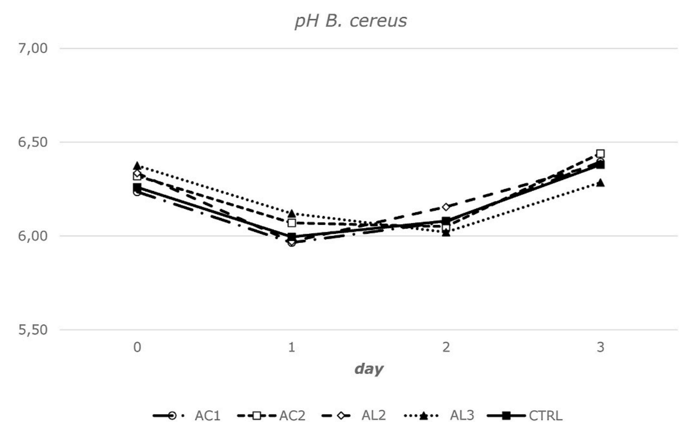

Fig. 4. pH values measured in primo sale cheese during the challenge test performed at 15 °C. For the legend, see Fig. 3.

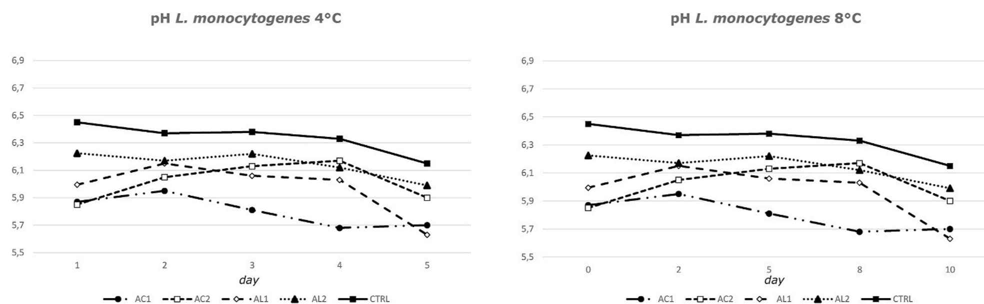

Fig. 3. *B. cereus* counts obtained in primo sale cheese during the challenge test performed at 15 °C. Dipping: Ac1: acetic acid, 49.96 mM; Ac2: acetic acid, 24.98 mM; Lac2: lactic acid, 44.40 mM; Lac3: lactic acid, 22.20 mM; CTRL: distilled water.

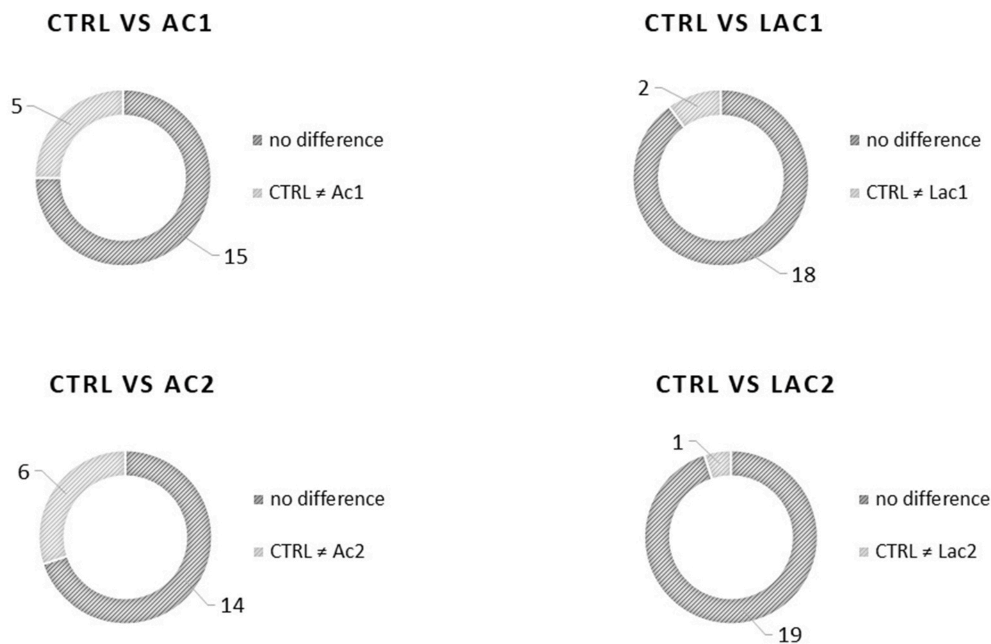

Fig. 5. Results obtained from sensorial analyses performed on treated and control samples.

3.4. Sensorial analysis

The concentrations of lactic acids tested (lactic acid, Lac1: 88.80 mM; Lac2: 44.40 mM) did not have a repercussion on the sensorial characteristics of primo sale cheese (Fig. 5): in particular, treated samples were not distinguished from the original cheese by 18/20 (90%) and 19/20 (95%) of the panellists, respectively. The concentrations of acetic acids tested (Ac1: 49.96 mM; Ac2: 24.98 mM) had a very small impact on the sensorial characteristics of primo sale cheese (Fig. 5): in particular, treated samples were not distinguished from the original cheese by 15/20 (75%) and 14/20 (70%) of the panellists, respectively. Thus, only 5 people recognized a difference between control series and Ac1, the highest concentration tested for acetic acid. In all the cases, the difference referred by the panellists, was very negligible.

4. Conclusions

The surface of primo sale cheeses is characterized by conditions suitable for the growth of microorganisms. Contaminations during production are difficult to avoid with the presence of spoilage organisms or ubiquitous pathogens that should be considered a potential concern. Addition of preservative can prolong shelf life: in the present study the potential inhibitory activity of organic acids dipping as final surface treatment for primo sale cheese was investigated with promising effects especially of acetic acid against *L. monocytogenes*. When defining an application protocol, the producer should balance the need to reach growth inhibition and to minimize the effect on the sensorial characteristics of the product. Further studies should be planned, also considering combination of different interventions, like the use of mixtures of different organic acids/salts.

CRediT authorship contribution statement

Erica Tirloni: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Cristian Bernardi:** Writing – review & editing. **Francesco Celandroni:** Methodology, Data curation, Writing – review & editing. **Emilia Ghelardi:** Methodology, Data curation, Writing – review & editing. **Simone Stella:** Data curation, Formal analysis, Investigation, Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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