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Use of food grade acetic organic acid to prevent *Listeria monocytogenes* in mozzarella cheese

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ABSTRACT

Objective of the present study was to evaluate the ability of acetic acid to inhibit the growth of *L. monocytogenes* when added to artificial brine for mozzarella storage. *L. monocytogenes* inoculated in mozzarella packages were divided in ten series for the treatment with acetic acid at different concentrations: 6.25 mM (ac1), 12.49 mM (ac2), 18.375 mM (ac3), 24.98 mM (ac4), 49.96 mM (ac5), 75 mM (ac6), 99.92 mM (ac7), 133.2 mM (ac8) and 149.87 mM (ac9); moreover, a control series was prepared. All the samples were maintained at 8 °C and analysed at t0 and after 2, 5 and 10 days in triplicate. When applied to brine, acetic acid resulted effective only when its concentration was above 24.98 mM (ac4): in this case no growth was recorded during the sampling sessions. ac1, ac2 and ac3 series, also showed a lower growth with final increases of 2.69, 1.50 and 1.02 Log CFU/g, respectively. With concentrations above 49.96 mM (ac5), counts were always below the detection limit. pH values showed the acidifying effect of treatment exerted. The lowest concentrations of acetic acid with antimicrobial effect did not have significant repercussion on the sensorial characteristics of mozzarella cheese.

1. Introduction

The pathogenic microorganism *Listeria monocytogenes* is a real challenge in dairy production plants as it is psychrotrophic, resistant to a wide range of pH and high salt content, ubiquitous and very persistent in the environment. Listeriosis is a serious infection that may occur after eating contaminated food. Listeriosis outbreak investigations are frequently associated with processed and ready-to-eat RTE foods including dairy products (Jackson et al., 2011; De Castro et al., 2012; FIOD, 2015; Mani-Lopez, García, & López-Malo, 2012). According to EU Regulation (2073/2005) the concentration of *L. monocytogenes* should not exceed 100 cfu/g during the shelf-life of Ready to Eat (RTE) foods. In 2019, an EU baseline survey (EFSA, 2021) collected data about proportions of positive single samples from official sampling by Competent Authorities highlighting a prevalence at retail of this microorganism in soft and semi-soft cheeses of 0.70%.

Among soft cheeses, Mozzarella plays an important role owing to its wide diffusion (e.g. more than 90% of the Italian families are used to buy the product) (Assolatte, 2017). Mozzarella is a soft, unripened pasta filata cheese produced with pasteurized or unpasteurized bovine or buffalo milk. During production, milk acidification is applied before the addition of rennet; this could be obtained by adding food grade organic acids (the most used being citric or lactic acid), or by inoculating thermophilic starters cultures, which growth results in milk acidification.

Considering the production process of mozzarella cheese, the presence of the pathogen can be effectively contrasted by the high temperatures reached during milk pasteurization or the curd spinning step, when temperatures may reach 90–95 °C. Anyway, contamination may occur during or after the remaining cheese-making process. In a previous study, Greco et al. (2014) found a prevalence of *L. monocytogenes* of 24.4% in mozzarella cheese in a dairy plant in Lazio Region, due to an environmental contamination. Mozzarella cheese, thanks to its physical-chemical properties such as high moisture and pH close to neutrality may represent a suitable substrate for the growth of *L. monocytogenes*. In this context, the use of organic acids, allowed by the European Union legislation, could be advantageous in Ready to eat (RTE) foods such as fresh cheese to reformulate the product leading to a substrate less permissive for bacterial replication (Mitchell, 1961). Previous studies investigated Minimal Inhibitory Concentration (MIC) of lactic, acetic, citric and propionic acid for *L. monocytogenes* under conditions relevant to dairy products and some other studies reported MIC calculated directly in the cheese matrix (Coroller et al., 2005; van der Veen et al., 2008; Wemmenhove et al., 2016, 2018; Tirloni et al., 2019, 2021a, 2021b).

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Tirloni, Bernardi, Celandroni, et al. (2021) showed the efficacy of acetic acid at a concentration of 24.98 mM in preventing the growth of *L. monocytogenes* in broth through a 7 days period; when inoculated in cheese, *L. monocytogenes* was effectively inhibited by the same acid concentration at 4 °C, but when stored at 8 °C, a significantly lower growth was obtained only by doubling the acetic acid concentration (49.96 mM).

The objective of the present study was to evaluate the ability of acetic acid to inhibit the growth of *L. monocytogenes* when added to brine used for mozzarella preservation; the information obtained could be useful for the management of the potential presence of food borne pathogens by mozzarella producers.

2. Materials and methods

2.1. Challenge test

2.1.1. L. monocytogenes strain preparation

A strain of *L. monocytogenes* (strain MS12209), previously isolated from a fresh dairy product and provided by DTU Food (Danish Technical University, DK), was considered for this trial. The strain was maintained at −80 °C in Microbank Cryogenic vials (Pro-Lab Diagnostics U.K., Merseyside, UK). From the stock culture, a loopful was transferred to Brain Heart Infusion broth (BHI) (Oxoid, Basingstoke, UK) with pH 7.2 and incubated at 37 °C for 24 h. In order to pre-adapt the cells to the environmental conditions of each of the challenge tests, culture was subsequently re-inoculated in BHI broth and then incubated at 8 °C for 4 days (EURLm, 2021). The culture was 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 concentration of this pre-culture was determined by microscopy at 1000x magnification (Motic, B310, Wetzlar, Germany), considering one cell per field corresponding to a concentration close to 10^6 CFU mL^{-1} (Adams and Moss, 2000).

2.1.2. Experimental plan

Specific challenge tests were performed on mozzarella cheese. The trials were carried out to establish if the MICs determined by Tirloni, Bernardi, Celandroni, et al. (2021) for acetic acid on *L. monocytogenes* were efficient when applied to mozzarella cheese matrix.

Mozzarella cheese samples were obtained on the first day after production; all the samples came from the same producer and belonged to the same production batch. The composition of the product, taken from the nutritional label, was (upon 100 g of product): fats: 18 g, carbohydrates: 1.1 g, proteins: 17 g, salt: 0.5 g. Each package was constituted from 16 to 18 mozzarella cherries (each one weighing 7/8 g) immersed in an aliquot of 140 mL of preserving brine. The brine was analysed for the determination of pH (as described below) and organic acids (critric, acetic and lactic) by the HPLC method described by Tormo and Izco (2004), to evaluate an eventual combined effect with the added acetic acid used or the trial. Brine pH was 6.4, while organic acids concentrations were all below the detection limit (63 mg/kg, 47 mg/kg and 16 mg/kg) for citric, acetic acid and lactic acid, respectively.

Each mozzarella cherry was taken from the original package and transferred in a sterile container where was immersed in around 10 mL of sterile solution composed by sterile water and NaCl (1%). The samples were divided in ten series, that were added with acetic acid solutions. The series were: Ac1 (6.25 mM), Ac2 (12.49 mM), Ac3 (18.375 mM), Ac4 (24.98 mM), Ac5 (50 mM), Ac6 (75 mM), Ac7 (99.92), Ac8 (133.2 mM), Ac9 (149.87 mM) and Control (brine without acid addition).

2.1.3. Product inoculation and sampling

Each package was then inoculated with *L. monocytogenes* (strain MS12209): the bacterial suspension was diluted in sterile saline water (0.85% NaCl). 100 μL aliquots of the suspensions were spread in the brine of each package in order to obtain a starting concentration around 2 log CFU g^{-1}. The inoculum volume (1% of the total weight of the sample) was chosen to assure a negligible impact on the product characteristics, according to European Guidelines for Challenge tests with *L. monocytogenes* (EURLm, 2021).

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

The trial was performed at 8 °C. During storage, temperature was recorded by data loggers (Testo, Saveris, Milan). Samples were analysed at t0 and after 2, 5 and 10 days from inoculation, in triplicate.

Each sample was made by a whole cherry, that 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 with Palcam Selective Supplement (Scharlab, Barcelona, E) and incubated at 37 °C for 48 h. An increase of +0.5 Log CFU g^{-1} in the mean value was used to discriminate growth or absence of growth in the product (EURL, 2021). pH was measured at each sampling time in triplicate by using a pH meter (Amel, Milan, I).

2.2. Sensorial analysis

After the challenge test, a sensorial panel test was conducted, involving eight non-trained panellists; the series with the two lowest acetic acid concentrations exerting an inhibitory activity towards *L. monocytogenes* were compared to the control series. Each panellist was asked to describe the smell and the taste of the cherries, and to indicate the presence of an eventual difference among the series.

2.3. Statistical analysis

Data obtained from *L. monocytogenes* counts (expressed as the log CFU g^{-1} difference between each sampling time and t0) 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. The threshold value for statistical significance was set at $P < 0.05$.

3. Results and discussion

Use of organic acids in food industries as component of the recipes or as added preservatives is a common approach, as food grade organic acids are cheap, easily managed, and can exert a fast and effective in terms of antibacterial and antifungal activity (Hinton & Corry, 1999, pp. 285–296; Barmpalia et al., 2004; Mani-Lopez, García, & López-Malo, 2012, Tirloni et al., 2020). Moreover, their use in cheese production is accepted by the international rules, as several organic acids are recognized as safe (GRAS) by the Food and Drug Administration and are registered as food additives by the European Union regulations. The use of acetic acid is allowed by EU Reg. 1333/2008 (registered as E260) in the production of all the fresh cheeses with the "*quantum satis*" approach, owing to their safe use in many foodstuffs (EC, 2008).

Organic acids are defined as weak acids, showing better antimicrobial activity when pH of the matrix is below their pKa; the relatively high pKa value of acetic acid (4.76) allows the presence of a sufficient proportion of undissociated molecule in dairy products, whose production process often includes the growth of fermentative microorganisms. In any case, the relative efficacy of an organic acid is influenced by many factors related to the complex food substrate, that can act as a buffer and can bind organic compounds (Samelis & Sofos, 2003).

Minimal inhibitory concentrations (MICs) of the undissociated form of acetic acid for *L. monocytogenes* have been investigated in the past (Conner et al., 1990; Coroller et al., 2005; Vasseur et al., 1999), but very few studies investigated the MICs under conditions relevant to dairy products or calculated the MIC directly in the cheese matrix (Coroller et al., 2005; Tirloni et al., 2019; van der Veen et al., 2008; Wemmenhove

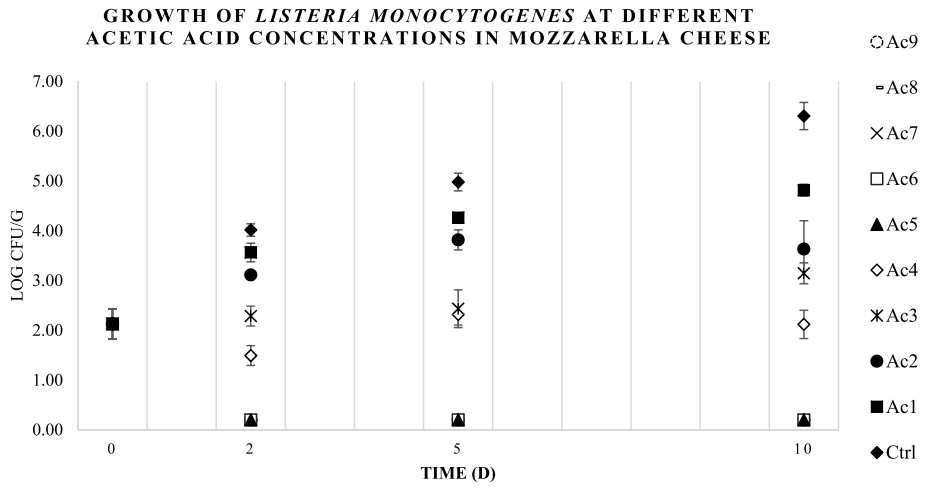

Fig. 1. *L. monocytogenes* counts obtained in mozzarella cheese during the challenge test performed at 8 °C. The acetic acid concentrations tested were 6.25 mM (Ac1), 12.49 mM (Ac2), 18.375 mM (Ac3), 24.98 mM (Ac4), 50 mM (Ac5), 75 mM (Ac6), 99.92 mM (Ac7), 133.2 mM (Ac8) and 149.87 mM (Ac9); Ctrl = control samples (without acetic acid).

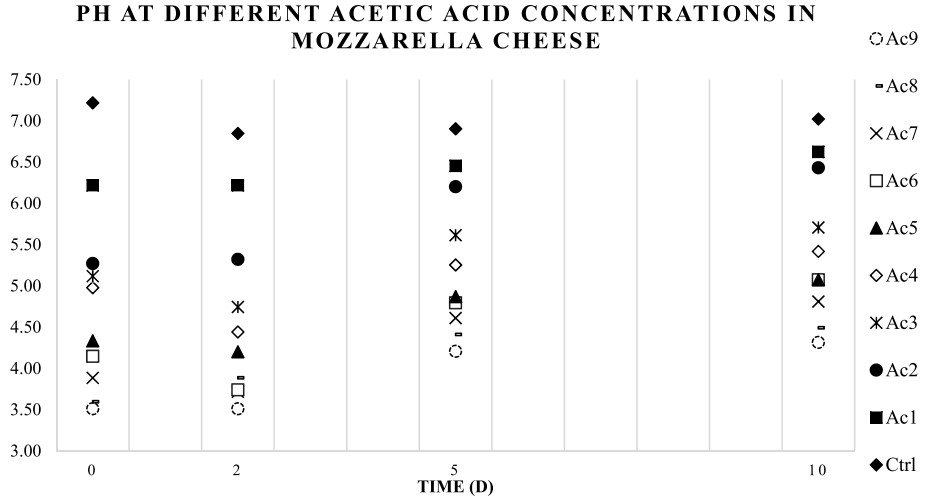

Fig. 2. *pH* values obtained in mozzarella cheese during the challenge test performed at 8 °C. The acetic acid concentrations tested were 6.25 mM (ac1), 12.49 mM (ac2), 18.375 mM (ac3), 24.98 mM (ac4), 50 mM (ac5), 75 mM (ac6), 99.92 mM (ac7), 133.2 mM (ac8) and 149.87 mM (ac9); Ctrl = blank samples.

et al., 2016, 2018).

In a previous study, *L. monocytogenes* inoculated in brain heart infusion broth added with acetic acid at a concentration of 24.98 mM didn't show any growth (increase in optical density of the broth) through a 7 days' trial at 37 °C. When the same acid concentration was used for dipping "primo sale" fresh cheese inoculated with *L. monocytogenes*, it showed to be insufficient to slow the growth of the pathogen if compared to a "control" cheese, whereas a significantly lower growth was obtained by the same treatment with a solution where the acid concentration was doubled (49.96 mM) (Tirloni, Bernardi, Celandroni, et al., 2021). In another study (Tirloni, Bernardi, & Stella, 2021), higher concentrations of acetic acid (149.88 mM) were needed to determine a significantly lower growth of *Pseudomonas brenneri* on fresh "Primo sale" cheese stored at 6 °C for 96 h, if compared to the control series.

The present study was carried out to evaluate the effect of the addition of food grade acetic acid on *L. monocytogenes* growth in mozzarella cheese, by inoculating the pathogen in the brine where the cheese is constantly immersed for the whole shelf life. This method of acid addition could be of easy application, during mozzarella cheese packaging. In control samples an evident growth of the pathogen was recorded in mozzarella cheese samples (a theoretical rate of about 0.4 Log CFU/g/day), with an increase of 4,18 Log CFU/g after 10 days at 4 °C (Fig. 1 and Table 1). The addition of acetic acid to the brine showed a dose-dependent inhibiting effect on *L. monocytogenes* growth. Considering the whole data, a significantly higher growth was observed

Table 1
L. monocytogenes increase/decrease obtained in mozzarella cheese during the challenge test performed at 8 °C. The acetic acid concentrations tested were 6.25 mM (Ac1), 12.49 mM (Ac2), 18.375 mM (Ac3), 24.98 mM (Ac4), 50 mM (Ac5), 75 mM (Ac6), 99.92 mM (Ac7), 133.2 mM (Ac8) and 149.87 mM (Ac9); control samples (without acetic acid).

Δ (log CFU/g)	T2-T0	T5-T0	T10-T0
Ac9	<-1,43	<-1,43	<-1,43
Ac8	<-1,43	<-1,43	<-1,43
Ac7	<-1,43	<-1,43	<-1,43
Ac6	<-1,43	<-1,43	<-1,43
Ac5	<-1,43	<-1,43	<-1,43
Ac4	−0,64	0,19	−0,01
Ac3	0,16	0,31	1,02
Ac2	0,98	1,69	1,50
Ac1	1,43	2,13	2,69
Control	1,89	2,85	4,18

in the control series if compared with all the series except for Ac1 and Ac2 (P = 0.093 and 0.103, respectively). Anyway, lower growth was observed just in Ac1 series (6.25 mM), that showed a total increase of 2,69 Log CFU/g: in this case, an initial growth was observed (resulting in an increase of about 1.5 Log CFU/g in the first two days of storage), followed by a less pronounced growth phase (0.70 Log CFU/g increase between day 2 and day 5, and only 0.55 Log CFU/g in the remaining five days). Such trend could be justified by the gradual growth of lactic acid bacteria on mozzarella surface and in the brine during the storage, thus exerting a synergic antagonistic effect towards the pathogen (see Fig. 2).

Similar results, with stronger activity, were obtained in Ac2 (12.49 mM) also in these cases, the final increase in *L. monocytogenes* count (1,50 Log CFU/g) was the result of an initial active growth through the first two days (about 1 Log CFU/g increase), a further lower growth phase (0,70 Log CFU/g between days 2 and 5) and a slight decrease in the final part of the trial (−0.19 Log CFU/g). A significantly lower growth (P = 0.027) was recorded for Ac3 series (18.375 mM), with a final increase of about 1 Log CFU/g.

Acetic acid resulted effective in contrasting completely bacterial growth only when its concentration reached 24.98 mM (Ac4 series): this concentration can be thus considered as the MIC for *L. monocytogenes* in the substrate tested. With concentrations above 50 mM (ac5 series) a decrease in *L. monocytogenes* counts was observed starting from day 2, with the concentrations always below the detection limit of 1 Log CFU/g. An early conditioning in the first stages is important for the conditioning of the substrate.

The determination of pH of the samples showed the acidifying effect of treatment exerted, with a significant difference between the CTRL samples and all the other series (P < 0.01). Ac9 treatment resulted, as expected, in a significantly lower pH values than the other series (pH ac9 series at T0 = 3.51). A slight increase was recorded from T0 to the subsequent sampling sessions in mainly all the series.

For sensorial trial ac4 and ac5 were taken in consideration as they resulted to be the series with the two lowest acetic acid concentrations exerting an inhibitory activity towards *L. monocytogenes*. No differences between ac4 and control samples were perceived by the panelists, considering both smell and taste of the product. The higher concentration of acetic acid (ac5) did not have a repercussion on the sensorial characteristics of mozzarella cheese for 50% of panelists, while 37.5% found differences towards control samples in the taste of the product, but not related to “acidic” perception; the same panelists didn’t perceive any difference in the smell of the samples. One panelist clearly distinguished treated samples from the original cheese with a very “acidic” perception for both smell and taste and the rejection of the product. Thus, the ac4 series appeared to be the most promising approach combining the antimicrobial efficacy and the negligible sensorial effect.

4. Conclusions

The surface of fresh cheeses like mozzarella is characterized by very permissive conditions for growth of microorganisms including *L. monocytogenes*. This study showed the potential inhibitory activity of acetic acid when added to preservative liquid of mozzarella cheese. This treatment can be applied as a part of an integrated hygiene plan and may contribute in the extension of the shelf life. Further studies should be conducted, also considering the sinergic effect of different techniques, like the use of mixtures of different organic acids/salts.

CRediT authorship contribution statement

Erica Tirloni: Conceptualization, Methodology, Investigation, Writing – original draft. **Bernardi Cristian:** Data curation, Writing – review & editing. **Simone Stella:** Conceptualization, Methodology, Investigation, Writing – original draft.

References

Assolatte.(2017). Available at: https://mercati.assolatte.it/201709/04_I-valori-di-consumo-dei-Latticini.html. (Accessed 12 April 2022).

Barmpalia, I. M., Geornaras, I., Belk, K. E., Scanga, J. A., Kendall, P. A., Smith, G. C., & Sofos, J. N. (2004). Control of Listeria monocytogenes on frankfurters with antimicrobials in the formulation and by dipping in organic acid solutions. *Journal of Food Protection, 67*, 2456–2464. https://doi.org/10.4315/0362-028x-67.11.2456

Conner, D. E., Scott, V. N., & Bernard, D. T. (1990). Growth, inhibition, and survival of Listeria monocytogenes as affected by acidic conditions. *Journal of Food Protection, 53*(8), 652–655.

Coroller, L., Guerrot, V., Huchet, V., Le Marc, Y., Mafart, P., Sohier, D., & Thuault, D. (2005). Modelling the influence of single acid and mixture on bacterial growth. *International Journal of Food Microbiology, 100*, 167–178. https://doi.org/10.1016/j.ijfoodmicro.2004.10.014

Ec (European Community). (2008). Regulation (EC) No 1333/2008 of the European parliament and of the council of 16 december 2008 on food additives. *Official Hournal of the Euroean Union L, 354*, 16–33.

EURL Lm (European Union Reference Laboratory for Listeria monocytogenes). (2021). *EURL Lm technical guidance document on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to Listeria monocytogenes*. Version 4 of 1 July 2021.

European Centre for Disease Preventionand Control EFSA – ECDC. (2021). The European union one health 2020 zoonoses report. *EFSA Journal, 19*(12), 6971.

Greco, S., Tolli, R., Bossù, T., Rodas, E. M. F., Di Giamberardino, F., Di Sirio, A., Vita, S., De Angelis, V., Bilei, S., Sonnessa, M., Gattuso, A., & Lanni, L. (2014). Case of contamination by Listeria monocytogenes in mozzarella cheese. *Italian Journal of Food Safety, 3*, 1708, 2014.

Hinton, M. H., & Corry, J. E. L. (1999). *The decontamination of carcass meat. Poultry meat science*. Oxon: Cabi Publishing. https://doi.org/10.4315/0362-028X-53.8.652

Mani-Lopez, E., García, H. S., & López-Malo, A. (2012). Organic acids as antimicrobials to control Salmonella in meat and poultry products. *Food Research International, 42*, 713–721.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature, 191*, 144–148.

Samelis, J., & Sofos, J. N. (2003). Organic acids. In S. Roller (Ed.), *Natural antimicrobials for the minimal processing of foods* (pp. 98–132). Cambridge, UK: Woodhead Publishing.

Tirloni, E., Bernardi, C., Celandroni, C., Ghelardi, E., & Stella, S. (2021). Effectiveness of lactic and acetic acids on the growth of Listeria monocytogenes and Bacillus cereus in primo sale fresh cheese. *LWT, 151*, Article 112170. https://doi.org/10.1016/j.lwt.2021.112170

Tirloni, E., Bernardi, C., Rosshaug, P., & Stella, S. (2019). Potential growth of Listeria monocytogenes in Italian mozzarella cheese as affected by microbiological and chemical-physical environment. *Journal of Dairy Science, 102*(6), 4913–4924. https://doi.org/10.3168/jds.2018-15991

Tirloni, E., Bernardi, C., & Stella, S. (2021). Pseudomonas spp.: Are food grade organic acids efficient against these spoilage microorganisms in fresh cheeses? *Foods, 10*(4), 891. https://doi.org/10.3390/foods10040891, 2021.

Tirloni, E., Nauta, M., Vasconi, M., Di Pietro, V., Bernardi, C., & Stella, S. (2020). Growth of Listeria monocytogenes in ready-to-eat "shrimp cocktail": Risk assessment and possible preventive interventions. *International Journal of Food Microbiology, 334*, Article 108800. https://doi.org/10.1016/j.ijfoodmicro.2020.108800

Tormo, M., & Izco, J. M. (2004). Alternative reversed-phase high-performance liquid chromatography method to analyse organic acids in dairy products. *Journal of Chromatography A, 1033*, 305–310.

van der Veen, S., Moezelaar, R., Abee, T., & Wells-Bennik, M. H. J. (2008). The growth limits of a large number of Listeria monocytogenes strains at combinations of stresses show serotype- and niche-specific traits. *Journal of Applied Microbiology, 105*, 1246–1258. https://doi.org/10.1111/j.1365-2672.2008.03873.x

Vasseur, C., Baverel, L., Hebraud, M., & Labadie, J. (1999). Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of Listeria monocytogenes. *Journal of Applied Microbiology, 86*, 469–476. https://doi.org/10.1046/j.1365-2672.1999.00686.x

Wemmenhove, E., van Valenberg, H. J. F., van Hooijdonk, A. C. M., Wells-Bennik, M. H. J., & Zwietering, M. H. (2018). Factors that inhibit growth of Listeria monocytogenes in nature-ripened gouda cheese: A major role for undissociated lactic acid. *Food Control, 84*, 413–418. https://doi.org/10.1016/j.foodcont.2017.08.028

Wemmenhove, E., van Valenberg, H. J., Zwietering, M. H., Van Hooijdonk, A. C. M., & Wells-Bennik, M. H. J. (2016). Minimal inhibitory concentrations of undissociated lactic, acetic, citric and propionic acid for Listeria monocytogenes under conditions relevant to cheese. *Food Microbiology, 58*, 63–67. https://doi.org/10.1016/j.fm.2016.03.012