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Lactic acid bacteria isolated from traditional Italian dairy products: activity against *Listeria monocytogenes* and modelling of microbial competition in soft cheese

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ABSTRACT

Several studies showed that lactic acid bacteria isolated from traditional cheeses produced in Calabria (Italy) inhibited in vitro the growth of different spoilage and/or pathogenic microorganisms. However, the activity of these autochthonous strains against *Listeria monocytogenes* has never been investigated. One hundred and fifteen lactic acid bacteria isolated from traditional Calabrian cheeses were screened for their technological characteristics and inhibiting action against *Listeria monocytogenes* in laboratory media. The anti-*Listeria* activity of representative strains was evaluated also in soft cheese during chilled storage. Bacterial interaction was studied using a competitive modelling approach based on Lotka-Volterra equations. Strains 29 (*Lactobacillus sakei*), 31 (*Lactobacillus plantarum* group), and 76 (*Lactobacillus plantarum* group) showed the best performances both in vitro and in soft cheese reducing the loads of *Listeria monocytogenes* from 0.5 to almost 1 Log CFU/g. Also considering their technological features, these strains could be used as adjunct cultures to improve the safety of finished products. The proposed competition model returned good predictions, especially after the application of the interspecific competition parameter β. This approach may be useful to understand the mechanisms of microbial competition in food.

1. Introduction

Dairy production has always been a cornerstone of the economy in Italy. Around thirty different varieties of traditional cheeses are manufactured in the region Calabria. These cheeses are characterized by the presence of different lactic acid bacteria (LAB) and this biodiversity contributes to secondary proteolysis and consequent development of important volatile components in finished products (De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2019; Pino et al., 2018; Randazzo, Pitino, Ribbera, & Caggia, 2010).

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes the human listeriosis. In cheese, presence and survival of this bacterium are related to the type of product, since some categories, e.g. soft and semisoft cheese, are more susceptible to its growth. Generally, pasteurized milk cheeses seem more subjected to the growth of *L. monocytogenes* given the absence, due to pasteurization, of the typical microbiota of raw milk that can play an antagonistic action on the pathogen. However, studies showed that there are no significative differences in the presence of *L. monocytogenes* between cheeses made from pasteurized or raw milk (Gérard, El-Hajjaji, Niyonzima, Daube, & Sindic, 2018; Martinez-Rios & Dalgaard, 2018). Since 1985, over thirty listeriosis outbreaks occurred worldwide involving different types of cheese. Outbreaks were caused, in most cases, by *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b (Martinez-Rios & Dalgaard, 2018).

An interesting approach to increase the safety of cheese is represented by the selection of adjunct cultures to contrast the growth of *L. monocytogenes*. LAB can inhibit growth of pathogenic microorganisms by reduction in pH and production of inhibiting compounds, including bacteriocins (Salomskiene et al., 2019). It has been demonstrated that LAB of dairy origin can exert an anti-*Listeria* activity during production and storage of different cheeses (Campagnollo et al., 2018; Morandi, Silvetti, Battelli, & Brasca, 2019; Ortolani, Yamazi, Moraes, Viçosa, &

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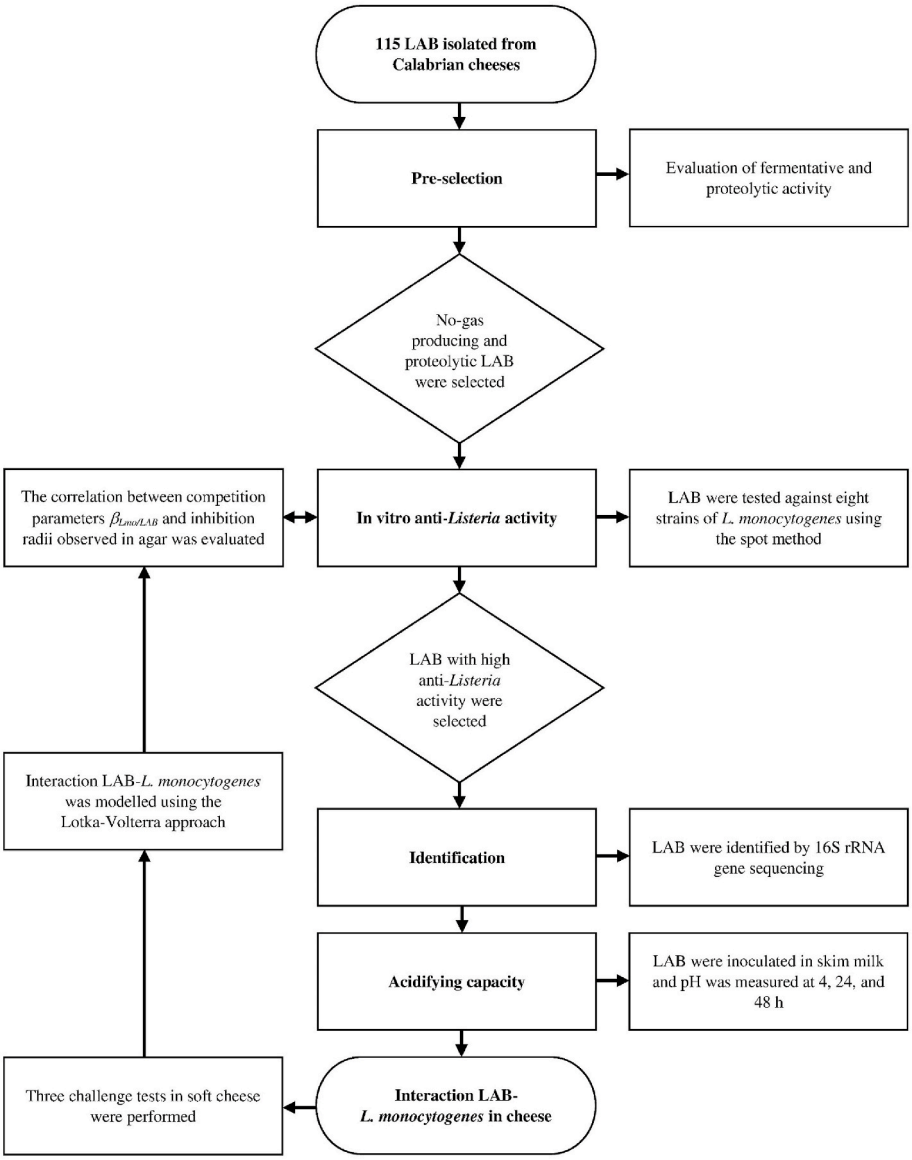

Fig. 1. Diagrammatic flowchart of the experimental steps.

Nero, 2010; Valero et al., 2014). Several LAB isolated from traditional Calabrian cheeses, including strains isolated from Pecorino del Poro and Caprino d'Aspromonte, inhibited in vitro the growth of *Escherichia coli*, *E. coli* O26, *E. coli* O157:H7, and *Salmonella* spp. (Caridi, 2002, 2003; Geria, Dambrosio, Normanno, Lorusso, & Caridi, 2013). Nevertheless, there are no studies on the activity, in vitro or in dairy products, of LAB isolated from typical Calabrian cheeses against *L. monocytogenes*.

Predictive microbiology is an important tool to understand microbial dynamics in food and several models have been used to predict the interaction between LAB and *L. monocytogenes*. As example, empirical models based on the 'Jameson effect', resulting from the competition for nutrients and/or production of inhibitory compounds by LAB, describe the stop of growth of a bacterial population when the dominating one reaches the stationary phase and are widely used to predict microbial interaction in cheese as well as in other foods (Cadavez et al., 2019; Gimenez & Dalgaard, 2004; Mejlholm & Dalgaard, 2015; Østergaard, Eklöw, & Dalgaard, 2014). Another approach to describe microbial interaction is based on Lotka-Volterra equations that model the inter-specific competition between two populations according to their growth, including a term on the reduction of the growth rate of a group in relation to the population density of other competitors (Costa, Bover-Cid, Bolívar, Zurera, & Pérez-Rodríguez, 2019). Lotka-Volterra models have been successfully used to predict the behaviour of *L. monocytogenes* and LAB in meat, *Aeromonas* spp. and specific spoilage flora in seafood, and yeast-bacterium interactions in cheese (Dens, Vereecken, & Van Impe, 1999; Giuffrida, Valenti, Ziino, Spagnolo, & Panebianco, 2009; Giuffrida, Ziino, Valenti, Donato, & Panebianco, 2007; Mounier et al., 2008; Powell, Schlosser, & Ebel, 2004). The Lotka–Volterra approach allows to reproduce the competition between two bacterial populations describing different scenarios, e.g. mutual interaction and/or reduction or decline of only one population (Giuffrida et al., 2009). In addition, Lotka-Volterra models showed slightly better fit than the Jameson-based models in prediction of the behaviour of bacteriocin-producing *Lactobacillus sakei* and *L. monocytogenes* in fish fillets under modified atmosphere packaging (Costa et al., 2019).

The aim of the present work was to select and characterize LAB isolated from traditional dairy products manufactured in Calabria potentially usable as adjunct cultures against *L. monocytogenes* in cheese and to study their anti-*Listeria* activity in soft cheese during chilled storage using models based on Lotka-Volterra equations.

2. Materials and methods

Experimental steps are summarized in Fig. 1. Firstly, LAB isolated from traditional Calabrian cheeses were tested for their fermentative and proteolytic activity. Secondly, no-gas producing and proteolytic LAB were tested in vitro against *L. monocytogenes*; strains with the highest anti-*Listeria* activity were identified and their acidifying capacity was assayed. Thirdly, three challenge tests in soft cheese were performed to evaluate the antagonistic effect of representative LAB strains on *L. monocytogenes* in a finished product. Interaction between LAB and *L. monocytogenes* in cheese was studied using a competitive modelling approach based on Lotka-Volterra equations.

2.1. Pre-selection, anti-Listeria activity, identification, and acidifying capacity of LAB

2.1.1. Pre-selection

One hundred and fifteen LAB (Collection of the Laboratory of Microbiology, Department of Agriculture, *Mediterranea* University of Reggio Calabria, Italy) isolated from several Calabrian cheeses were previously identified at genus level according to Cogan et al. (1997). Briefly, strains were tested for their Gram and catalase reaction, shape by observation of overnight cultures with a phase contrast microscope Standard 20 (Carl Zeiss, Göttingen, Germany), growth in selective media, and biochemical features (Biolog Identification System, Biolog, Inc., Hayward, CA, USA). LAB were pre-selected evaluating their fermentative and proteolytic activity.

For the evaluation of fermentative activity, a cryobead (Microbank TM, Pro-Lab Diagnostics, Canada) of each strain was transferred from the frozen storage (−80 °C) in tubes containing 10 ml of sterile de Man, Rogosa and Sharpe (MRS) (VWR, Geldenaaksebaan, Leuven, Belgium) broth and an inverted Durham tube. Inoculated tubes were incubated at 30 °C for 48 h and strains exhibiting gas production (bubbles in the Durham tubes) were excluded from the following experimental steps.

The proteolytic activity was qualitatively evaluated spotting 0.1 ml of a pre-culture (30 °C for 48 h in MRS broth (VWR)) of each LAB on two media: i) skim milk agar (1.4% skim milk (VWR); 0.8% agar (VWR)), incubated at 37 °C for 24 h (El-Ghaish et al., 2010); ii) MRS-milk agar (Pereira, Crespo, & San Romao, 2001), consisting of MRS agar (Merck, Darmstadt, Germany) supplemented with 10% skim milk (VWR), incubated at 30 °C for 16 h in anaerobic conditions (Anaerogen™, Thermo Scientific, Oxoid, Basingstoke, USA). LAB that showed proteolytic activity in both media (clear/white halos around the inoculum spots) were selected for the following experimental steps.

2.1.2. Anti-Listeria activity in vitro

The anti-*Listeria* activity of LAB previously selected (see 2.1.1) was detected as described by Tirloni et al. (2014) with some modifications. LAB were transferred from the frozen storage (−80 °C) in tubes containing 10 ml of sterile MRS (VWR) broth and incubated at 30 °C for 24 h. Pre-cultures were diluted 10-fold in a PBS solution (phosphate buffered saline; pH 7.0) and 5 μl used to make spots (triplicate for each LAB) in MRS (Merck) agar plates, then incubated at 30 °C for 24 h in anaerobic conditions (Anaerogen™). Eight strains of *L. monocytogenes* were used for the assay: ATCC (American Type Culture Collection) 7644, ATCC 19112, ATCC 13932, ID (Identification Number) 1 from smoked salmon, ID 9 from fresh salmon, ID 13 of environmental origin, ID 212 from tomato rolls (Ready-To-Eat (RTE) food), ID 222 from stuffed artichokes (RTE food). *L. monocytogenes* strains were transferred from the frozen storage (−80 °C) in Brain Heart Infusion (BHI) (Biolife, Milan, Italy) broth and incubated at 30 °C overnight. An aliquot of 0.2 ml of each pre-culture was added to 5 ml of BHI soft agar (BHI broth Biolife; VWR agar 0.7%) maintained at 45 °C and then poured on MRS agar plates with LAB grown on the inoculum spots. Plates were incubated at 37 °C for 24 h and the mean radii of inhibition halos of each LAB against each *L. monocytogenes* strain were measured. Finally, the average of the mean radii of inhibition of each LAB against all *L. monocytogenes* strains was calculated.

2.1.3. Identification

DNA of LAB that exhibited the best anti-*Listeria* activity (see 2.1.2) was extracted by InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The identification was performed by 16S rRNA gene sequencing using fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3′) primers according to Martorana, Giuffrè, Capocasale, Zappia, and Sidari (2018). Then, the amplicons were purified and sequenced (Eurofins Genomics, Germany). The sequences were compared with those present at the National Centerfor Biotechnology Information (http://www.ncbi.nlm.nih.gov) using BLASTn (Altschul et al., 1997).

2.1.4. Acidifying capacity

Acidifying capacity of LAB previously selected and identified (see 2.1.1, 2.1.2, 2.1.3) was evaluated as described by Caridi (2003). Briefly, 50 ml of reconstituted skim milk (VWR) were inoculated with 1% (v/v) of each LAB pre-culture (30 °C for 48 h in MRS broth (VWR)) previously diluted in order to reach a concentration of 5 Log CFU/ml in milk. Tubes containing the inoculated milk were incubated at 30 °C and pH was measured (HI-121, Hanna Instruments, Woonsocket, RI, USA) at 4, 24, and 48 h after inoculation.

2.2. Interaction between LAB and L. monocytogenes in soft cheese

2.2.1. Production of soft cheese

Soft cheese was produced at the Pilot Plant of Food Science and Technology, Department of Veterinary Sciences, University of Messina (Italy). Cow's milk was pasteurized at 72 °C for 15 s. Milk was heated until 45–50 °C and a 25% citric acid solution used to reduce the pH to 5.4–5.5. Subsequently, rennet (Clerici, Cadorago, Italy) was added after the required dilution (1:10) in sterile deionized water. When a complete curd setting was observed (45 min), this was cut, drained (20 min) and NaCl (2% w/w) was added.

2.2.2. Strains and pre-cultures preparation

Three LAB - LAB 29 (*Lactobacillus sakei*), LAB 31(*Lactobacillus plantarum* group), LAB 76 (*Lb. plantarum* group) - and three *L. monocytogenes* (ATCC 7644, ATCC 13932, ATCC 19112) were used for experimental trials.

L. monocytogenes and LAB were transferred from the frozen storage (−80 °C) in Tryptic Soy Yeast Extract broth (TSYEB) (Biolife) and MRS broth (Merck), respectively, both incubated at 25 °C overnight. To adapt bacteria to the temperature of the subsequent challenge tests, strains were afterwards pre-cultured in TSYEB and MRS broth at 15 °C for two days and then diluted to the desired concentration.

2.2.3. Challenge tests and storage conditions

Three challenge tests (CT) were performed: i) CT1 (LAB 31 and *L. monocytogenes*); ii) CT2 (LAB 76 and *L. monocytogenes*); iii) CT3 (LAB 29 and *L. monocytogenes*).

Cheese was inoculated (1% v/w) with *L. monocytogenes* (cocktail of ATCC 7644, ATCC 13932, and ATCC 19112 in equal proportion) and LAB to obtain a concentration of 3 Log CFU/g and 5 Log CFU/g, respectively, since LAB usually represent the dominating microbiota in cheese. Practically, all the cheese produced was firstly inoculated with *L. monocytogenes* and instantly divided in 4 batches; three of these were immediately inoculated with selected LAB and the remaining one represented the control (*L. monocytogenes* in monoculture). Inoculation was performed adding gradually an adequate amount of each diluted bacterial culture in cheese and mixing it using sterile spoons to obtain a homogenous distribution of cells in the final product. Inoculated cheese was divided in aliquots of 50 g in sterile plastic containers and incubated

Table 1
Outcomes of the 30 LAB strains active against *L. monocytogenes* in vitro.

Cheese	LAB	Inhibition radii against *L. monocytogenes*[a,b]								
		ATCC 19112	ATCC 13932	ATCC 7644	ID 1	ID 9	ID 13	ID 212	ID 222	AVG value[b,c]
Buffalo Mozzarella	163	6.8 ± 0.8 ab	6.0 ± 0.5 ab	11.0 ± 1.0 fghi	6.0 ± 0.0 ab	4.8 ± 0.3 a	6.7 ± 0.3 ab	5.8 ± 0.8 a	9.0 ± 0.0 bcdefg	7.0 ± 2.0 k
Caprino d'Aspromonte	5	10.3 ± 0.6 fghi	9.0 ± 1.1 fghijk	7.8 ± 1.0 a	7.5 ± 1.0 bcdefg	8.8 ± 1.5 ghi	8.5 ± 1.0 cdefg	8.2 ± 1.0 cdef	8.7 ± 0.6 bcdef	8.6 ± 0.9 ghij
Caprino d'Aspromonte	25	8.3 ± 0.6 bcde	8.3 ± 0.3 cdefgh	10.3 ± 0.6 efgh	10.0 ± 0.9 ijk	7.8 ± 0.3 defgh	6.7 ± 0.8 ab	9.5 ± 0.0 efghi	8.8 ± 1.3 bcdefg	8.7 ± 1.2 ghi
Mozzarella	127	7.0 ± 1.0 abc	8.3 ± 1.1 cdefgh	13.3 ± 1.0 kl	9.5 ± 0.5 hij	7.2 ± 0.8 cde	8.7 ± 0.6 defg	10.2 ± 1.3 hij	10.3 ± 0.6 hijkl	9.3 ± 2.0 defgh
Mozzarella	146	7.3 ± 0.6 abc	6.7 ± 1.1 abc	10.0 ± 0.9 cdefg	7.7 ± 0.6 cdefg	5.5 ± 0.9 ab	6.3 ± 0.6 a	7.0 ± 0.5 abc	7.5 ± 0.9 ab	7.3 ± 1.3 k
Mozzarella	151	6.5 ± 0.9 a	6.8 ± 1.0 abcd	8.7 ± 0.6 abcde	6.8 ± 0.8 abcd	6.8 ± 0.3 bcd	7.8 ± 1.2 abcdef	6.3 ± 0.6 ab	7.0 ± 0.0 a	7.1 ± 0.8 k
Mozzarella	152	7.2 ± 0.3 abc	5.5 ± 0.5 a	8.3 ± 1.2 abcd	6.2 ± 0.6 abc	5.7 ± 0.6 ab	7.3 ± 1.5 abcd	7.0 ± 1.0 abc	7.7 ± 0.6 abc	6.9 ± 1.0 k
Mozzarella	153	6.8 ± 1.2 ab	7.0 ± 0.0 abcde	8.5 ± 0.9 abcde	5.7 ± 0.6 a	6.5 ± 0.5 bcd	7.8 ± 0.8 abcdef	7.3 ± 0.8 abcd	9.2 ± 0.3 cdefgh	7.4 ± 1.1 jk
Pecorino del Poro	1	9.3 ± 0.8 def	8.0 ± 0.5 cdefg	9.7 ± 1.0 abcdefg	7.7 ± 0.3 cdefg	6.2 ± 0.8 abc	9.5 ± 1.3 fghi	9.7 ± 1.7 efghij	10.0 ± 1.0 fghijk	8.8 ± 1.3 ghi
Pecorino del Poro	3	10.3 ± 1.0 fghi	8.5 ± 0.5 defghi	9.8 ± 1.1 bcdefg	8.0 ± 1.2 defgh	6.8 ± 1.2 bcd	10.7 ± 1.5 ijk	8.7 ± 0.3 cdefgh	9.7 ± 1.7 efghij	9.1 ± 1.3 efghi
Pecorino del Poro	6	7.8 ± 1.1 abcd	8.8 ± 1.0 fghijk	10.0 ± 1.0 cdefg	8.7 ± 1.0 fghij	11.2 ± 1.3 k	9.8 ± 1.3 ghij	9.7 ± 1.5 efghij	10.3 ± 1.2 ghijk	9.5 ± 1.1 cdefgh
Pecorino del Poro	7	10.0 ± 1.0 fghi	9.7 ± 1.0 hijkl	9.3 ± 1.0 abcdef	8.5 ± 1.3 efghi	8.8 ± 1.3 ghi	9.5 ± 1.5 fghi	8.0 ± 1.7 bcde	11.3 ± 1.2 klmn	9.4 ± 1.0 cdefgh
Pecorino del Poro	14	9.2 ± 0.3 def	7.5 ± 1.1 bcdef	8.0 ± 0.9 ab	7.2 ± 1.5 abcdef	8.7 ± 0.3 fghi	6.7 ± 0.6 ab	7.7 ± 1.5 bcd	8.3 ± 1.7 abcde	7.9 ± 0.8 ijk
Pecorino del Poro	15	9.3 ± 0.6 def	8.5 ± 1.0 defghi	10.3 ± 1.2 efgh	6.7 ± 0.6 abcd	7.2 ± 0.6 cde	8.3 ± 0.6 bcdefg	8.3 ± 0.3 cdefg	9.7 ± 0.3 efghij	8.5 ± 1.2 hij
Pecorino del Poro	16	9.5 ± 1.1 def	8.5 ± 0.5 defghi	10.3 ± 1.1 efgh	9.3 ± 0.6 hij	8.3 ± 0.8 efghi	7.8 ± 0.8 abcdef	8.5 ± 0.5 cdefgh	10.2 ± 0.8 fghijk	9.1 ± 0.9 efghi
Pecorino del Poro	17	9.7 ± 1.2 efg	10.3 ± 0.6 jkl	8.2 ± 0.3 abc	11.2 ± 1.0 kl	7.3 ± 0.6 cdef	8.2 ± 0.8 bcdefg	10.2 ± 0.8 hij	11.0 ± 1.5 ijklm	9.5 ± 1.4 cdefgh
Pecorino del Poro	19	10.2 ± 0.6 fgh	8.3 ± 1.0 cdefgh	11.3 ± 1.2 ghij	7.8 ± 1.0 defgh	7.2 ± 0.3 cde	8.0 ± 1.0 abcdef	8.8 ± 0.3 defgh	8.2 ± 1.0 abcde	8.7 ± 1.4 ghi
Pecorino del Poro	20	12.0 ± 1.1 i	9.7 ± 1.0 hijkl	10.7 ± 0.6 fghi	10.2 ± 0.8 jk	9.2 ± 0.8 hij	10.5 ± 0.5 hijk	11.3 ± 1.5 jkl	10.2 ± 0.8 fghijk	**10.5 ± 0.9** abcd
Pecorino del Poro	22	12.0 ± 1.0 i	10.2 ± 0.3 ijkl	10.0 ± 0.0 cdefg	8.5 ± 0.5 efghi	8.5 ± 0.5 efghi	8.2 ± 0.3 bcdefg	11.0 ± 1.0 ijk	10.3 ± 0.3 ghijk	9.8 ± 1.4 cdefg
Pecorino del Poro	23	9.2 ± 0.6 def	9.5 ± 0.9 ghijkl	10.2 ± 0.8 defg	7.0 ± 0.0 abcde	7.7 ± 0.8 defg	9.3 ± 1.6 efghi	8.7 ± 0.6 cdefgh	8.7 ± 0.3 bcdef	8.8 ± 1.0 fghi
Pecorino del Poro	24	9.7 ± 1.0 efg	9.2 ± 0.6 fghijk	10.7 ± 0.6 fghi	7.3 ± 0.8 bcdefg	8.3 ± 0.3 efghi	8.5 ± 0.0 cdefg	8.0 ± 0.0 bcde	9.5 ± 0.5 defghi	8.9 ± 1.1 fghi
Pecorino del Poro	26	10.3 ± 1.2 fghi	10.2 ± 0.8 ijkl	10.8 ± 0.8 fghi	8.8 ± 0.3 ghij	8.5 ± 0.9 efghi	11.8 ± 1.4 k	10.7 ± 0.6 ijk	10.7 ± 1.0 hijkl	**10.2 ± 1.1** bcde
Pecorino del Poro	28	8.7 ± 1.0 cdef	8.7 ± 0.6 efghij	12.3 ± 1.2 ijkl	7.7 ± 0.8 cdefg	6.8 ± 0.8 bcd	7.7 ± 0.8 abcde	8.7 ± 0.6 cdefgh	8.0 ± 0.0 abcd	8.6 ± 1.7 ghij
Pecorino del Poro	29	11.7 ± 1.2 hi	10.5 ± 0.5 kl	11.0 ± 1.0 fghi	9.5 ± 0.5 hij	9.7 ± 1.2 ij	11.3 ± 1.5 jk	13.0 ± 1.0 lm	12.7 ± 1.3 n	**11.2 ± 1.3** ab
Pecorino del Poro	30	12.0 ± 1.0 i	11.0 ± 1.0 l	13.2 ± 0.3 jkl	10.2 ± 0.3 jk	10.3 ± 1.3 jk	10.5 ± 0.5 hijk	13.3 ± 0.6 m	12.2 ± 0.8 lmn	**11.6 ± 1.3** a
Pecorino del Poro	31	11.3 ± 1.5 ghi	10.2 ± 0.8 ijkl	14.2 ± 0.6 l	12.3 ± 1.2 l	9.7 ± 1.3 ij	11.5 ± 0.5 jk	12.0 ± 1.5 klm	12.3 ± 1.0 mn	**11.7 ± 1.4** a
Pecorino di Mammola	78	8.7 ± 1.2 cdef	9.5 ± 1.0 ghijkl	12.2 ± 1.0 hijk	10.2 ± 1.2 jk	8.8 ± 0.3 ghi	9.8 ± 1.4 ghij	10.0 ± 1.0 ghij	11.2 ± 0.3 jklmn	**10.0 ± 1.2** bcdef
Pecorino di Mammola	79	9.5 ± 0.5 def	10.3 ± 0.6 jkl	10.0 ± 1.0 cdefg	9.5 ± 1.2 hij	6.5 ± 0.5 bcd	6.8 ± 0.3 abc	8.8 ± 1.0 defgh	11.3 ± 1.2 klmn	9.1 ± 1.7 efghi
Pecorino di Mammola	80	8.3 ± 0.8 bcde	7.0 ± 0.5 abcde	12.3 ± 1.2 ijkl	8.2 ± 0.8 defgh	6.5 ± 0.9 bcd	8.8 ± 1.0 defgh	9.8 ± 1.0 fghij	10.7 ± 0.6 hijkl	9.0 ± 1.9 efghi
Smoked Ricotta	76	9.2 ± 0.8 def	11.0 ± 1.7 l	12.2 ± 1.3 hijk	10.0 ± 1.0 ijk	8.8 ± 1.3 ghi	10.8 ± 0.3 ijk	11.0 ± 1.3 ijk	12.3 ± 0.6 mn	**10.7 ± 1.3** abc

[a] Average ± Standard Deviation of three spots (mm).
[b] Values followed by different small letters in the same column are significantly different ($p < 0.05$).
[c] Average of the inhibition radii (mm) against *L. monocytogenes* (ATCC 7644, ATCC 19112, ATCC 13932, ID 1, ID 9, ID 13, ID 212, ID 222) ± Standard Deviation. AVG values of the strain with the highest anti-*Listeria* activity are highlighted in bold.

at 15 °C in a cold room. Data loggers (iButton, Econorma, San Vendemiano, Italy) were positioned in the room to check the temperature fluctuations.

2.2.4. Microbiological and chemical analyses

At each sampling point, a container with 50 g of cheese for each CT and for the control series was used for microbiological and chemical analyses. Analyses were performed in duplicate every 24 h, until the stationary phase for *L. monocytogenes* was observed. For microbiological

analysis, 10 g of cheese were diluted 10-fold in physiological saline peptone (PS) (0.85% NaCl (Biolife); 0.1% Bacto-Peptone (Biolife)), homogenized for 30 s (Stomacher® 400 Circulator; International PBI s.p.a., Milan, Italy) and dilutions (1:10) were prepared in tubes of PS. Count of LAB was performed in MRS (Merck) agar plates, incubated at 30 °C for 48 h in anaerobic atmosphere (Anaerogen™). *L. monocytogenes* was enumerated on agar *Listeria* according to Ottaviani and Agosti (ALOA®, Merck) with *Listeria* agar Enrichment-Supplement (Chromocult®, Merck), incubated at 37 °C for 48 h.

The pH of soft cheese was determined with a pH-meter (HI90023CW - Hanna Instruments – with electrode Mettler Toledo InLab 427) and a_w was measured by a water activity-meter (AquaLab 4 TE Duo, Decagon Devices, USA). Quantification of organic acids was performed as described by Bevilacqua and Califano (1989). After the extraction, analysis was conducted using a Knauer HPLC Smartline Pump 1000, equipped with Knauer Smartline UV detector 2600 set at 210 nm and a 20-μL Rheodyne injection valve. The system was coupled with an Acclaim OA5 column (4 mm i.d. × 250 mm length × 5 μm particle size). Mobile phase was 100 mM Na_2SO_4 acidified to 2.65 pH with methane sulfonic acid CH_3SO_3H at 30 °C; flow rate was 0.6 mL/min. Before analysis, samples were filtered in a 0.45-μm PTFE syringe filter Supelco. Dry matter of cheese was quantified after 24 h at 105 °C.

2.2.5. Modelling

A predictive model based on Lotka-Volterra equations, according to Dens et al. (1999), Giuffrida et al. (2009), Giuffrida et al. (2007), Powel et al. (2004), was generated to model the activity of LAB on *L. monocytogenes*. The following system of equations was used as a primary model.

$$\frac{dN_{Lmo}}{dt} = \mu_{maxLmo}\, N_{Lmo}\, \frac{Q_{Lmo}}{1+Q_{Lmo}}\, (1 - \frac{N_{Lmo} + \beta_{Lmo/LAB}\, N_{LAB}}{N_{max\ Lmo}}) \quad (1a)$$

$$\frac{dQ_{Lmo}}{dt} = \mu_{maxLmo}\, Q_{Lmo} \quad (1b)$$

$$\frac{dN_{LmLAB}}{dt} = \mu_{maxLAB}\, N_{LAB}\, \frac{Q_{LAB}}{1+Q_{LAB}}\, (1 - \frac{N_{LAB} + \beta_{LAB/Lmo}\, N_{Lmo}}{N_{max\ LAB}}) \quad (2a)$$

$$\frac{dQ_{LAB}}{dt} = \mu_{maxLAB}\, Q_{LAB} \quad (2b)$$

Where N_{Lmo} and N_{LAB} are the concentrations (Log CFU/g) of *L. monocytogenes* and LAB at time *t*, respectively; μ_{maxLmo} and μ_{maxLAB} are the maximum growth rates (h^{-1}) of *L. monocytogenes* and LAB, respectively; N_{maxLmo} and $N_{max\ LAB}$ are the maximum populations densities (Log CFU/g) of the two populations; $\beta_{Lmo/LAB}$ and $\beta_{LAB/Lmo}$ are the interspecific competition parameters of LAB on *L. monocytogenes* and vice versa; Q_{Lmo} and Q_{LAB} represent the physiological state of the two populations (Baranyi & Roberts, 1994).

To solve Eqs. 1 (a-b), μ_{maxLmo} was calculated at each variation in environmental parameters and product characteristics (temperature (T), pH, a_w, lactic acid) using the secondary model proposed by Le Marc et al. (2002). Eqs. 2 (a-b), instead, were solved using the secondary model proposed by Wijtzes, Rombouts, Kant-Muermans, Van't Riet, and Zwietering (2001). Initial values of Q_{Lmo} and Q_{LAB} (Q_0) were calculated fitting the predictive growth curves obtained by the resolution of Eqs. 1 (a-b) and 2 (a-b) to the observed curves of *L. monocytogenes* and LAB in the control samples, setting $\beta = 0$ since concentrations of *L. monocytogenes* were always higher than those observed for LAB and an anti-*Listeria* activity by LAB could not be hypothesized. N_{max} values were set equal to the real ones, while μ_{maxLmo} and μ_{maxLAB} were obtained by solving the secondary models according to changes in environmental parameters and product characteristics during the specific experiment. Q_0 and N_{max} were used to obtain the predictive curves for *L. monocytogenes* in co-culture with LAB, using data on T, pH, a_w, and lactic acid to calculate the maximum growth rates. $\beta_{LAB/Lmo}$ (Eq. (2a)) was set equal to 0 for the aforementioned reasons, while $\beta_{Lmo/LAB}$ (Eq. (1a)) was calculated for each experiment by fitting the predictive curves to those observed, setting the parameter as a variable to be modified according to the minimization of the Root Mean Squared Error.

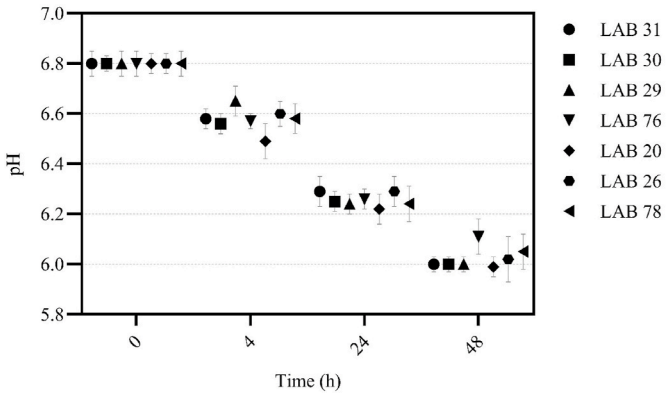

Fig. 2. Acidifying capacity of LAB with the highest activity against *L. monocytogenes* in vitro. Error bars indicate the standard deviation of two replicates.

The model consisting of Eqs. 1 (a-b) and 2 (a-b) was solved using the average of environmental parameters, product characteristics, and $\beta_{Lmo/LAB}$ values to reproduce mean curves for *L. monocytogenes* and LAB in the three CT.

The correlation between $\beta_{Lmo/LAB}$ values and the radii of inhibition halos against *L. monocytogenes* (ATCC 7644, ATCC 13932, ATCC 19112) by LAB 29, LAB 31, and LAB 76 observed in agar (see 2.1.2) was evaluated with a linear regression test.

2.3. Statistical analysis and curve fitting

Data on anti-*Listeria* activity of LAB in vitro, bacterial counts and changes in product characteristics of soft cheese during storage (see 2.1.2 and 2.2.4) were analyzed using StatGraphics Centurion XVI (StatPoint Technologies, Inc., USA) according to Fisher's LSD (Least Significant Difference) ($p < 0.05$). Equations (see 2.2.5) were fitted using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA). GraphPad Prism version 8.2.1 (GraphPad Software, San Diego, California, USA) was used to make the graphs.

3. Results

3.1. Pre-selection, anti-Listeria activity, identification, and acidifying capacity of LAB

3.1.1. Pre-selection

Ninety-seven of the 115 strains tested did not produce gas. Sixty-three out 97 no-gas producing strains exhibited proteolytic activity in both media and they were selected for the antagonistic activity assay in vitro.

3.1.2. Anti-Listeria activity in vitro

Thirty LAB (47.6% of the 63 strains tested) showed antagonistic action against *L. monocytogenes* (Table 1). Seven LAB (23.3% of the 30 antagonist LAB) exerted high antagonistic activity with final averages of inhibition radii (AVG values) ≥ 10 mm. Nine LAB (30.0% of the 30 antagonist LAB) exhibited AVG values from 9.0 ± 1.9 to 9.8 ± 1.4 mm. Eight LAB (26.7% of the 30 antagonist LAB) showed AVG values from 8.5 ± 1.2 to 8.9 ± 1.1 mm. Six LAB (20.0% of the 30 antagonist LAB) showed AVG values from 6.9 ± 1.0 to 7.9 ± 0.8 mm.

3.1.3. Identification

The sequences of the 7 LAB with the highest antagonistic activity (see

Table 2
Count of LAB and *L. monocytogenes* in soft cheese at time zero (h 0) and at the end of storage (h 137.5).

CT	LAB	h 0 (Log CFU/g; Avg. ± SD)[a,b]			h 137.5 (Log CFU/g; Avg. ± SD)[a,b]		
		LAB	*L. monocytogenes* (with LAB)	*L. monocytogenes* (monoculture)	LAB	*L. monocytogenes* (with LAB)	*L. monocytogenes* (monoculture)
1	31-*Lb. plantarum* group	5.3 ± 0.5 a	3.5 ± 0.1	3.5 ± 0.1	7.8 ± 0.5 a	5.6 ± 0.3 a	6.5 ± 0.1
2	76-*Lb. plantarum* group	4.8 ± 0.4 b	3.5 ± 0.0	3.5 ± 0.1	7.6 ± 0.2 b	6.0 ± 0.3 b	6.5 ± 0.1
3	29-*Lb. sakei*	5.0 ± 0.1 c	3.5 ± 0.1	3.5 ± 0.1	7.9 ± 0.3 c	5.8 ± 0.0 c	6.5 ± 0.1

[a] Average ± Standard Deviation of two replicates.
[b] Values followed by different small letters in the same column are significantly different ($p < 0.05$).

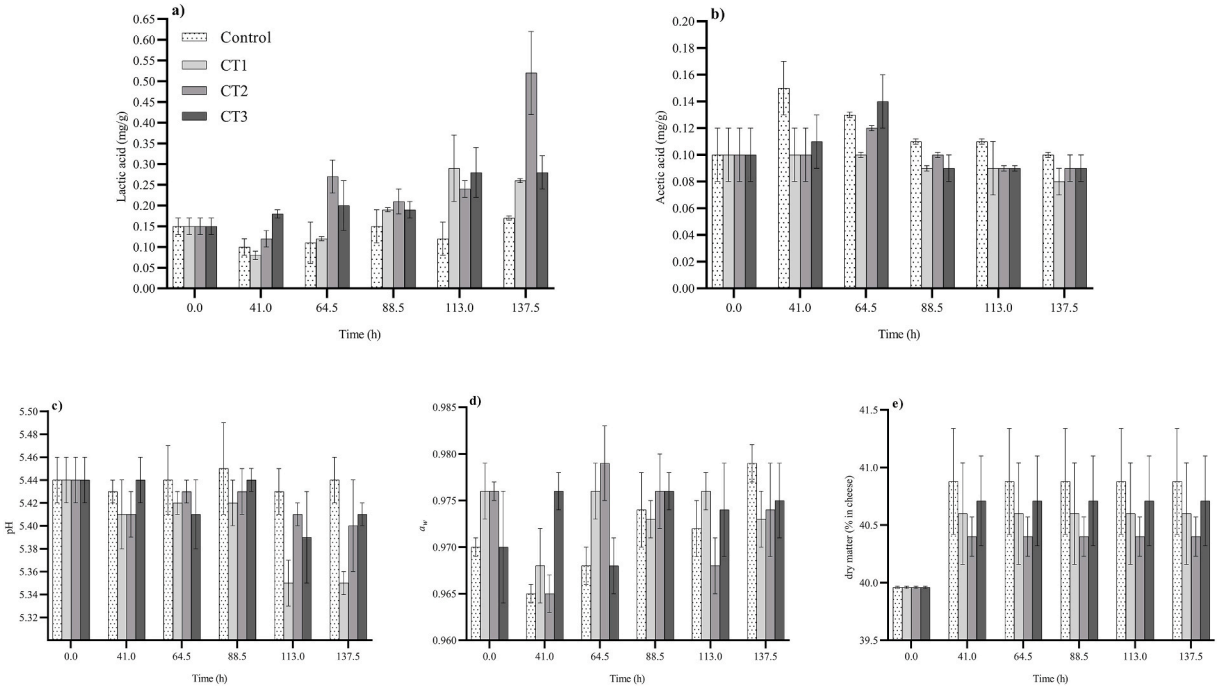

Fig. 3. Changes in lactic acid (a), acetic acid (b), pH (c), a_w (d), and dry matter (e) of soft cheese during storage at 15 °C. Error bars indicate the standard deviation of two replicates. The legend in the first image is valid for all graphs.

3.1.2 and Table 1) obtained with the 16S rRNA scored high percentage of similarity (99–100%) with those deposited in the GenBank DNA database. LAB 20, 26, 30, 31, 76, and 78 belonged to the *Lb. plantarum* group, while LAB 29 belonged to *Lb. sakei* species.

3.1.4. Acidifying capacity

The 7 strains with the highest anti-*Listeria* activity and previously identified (see 3.1.2 and 3.1.3) displayed low acidifying capacity (Fig. 2). LAB 20 showed the best acidifying activity with a pH of 5.99 ± 0.04 after 48 h. The pH values for the other LAB after 48 h were between 6.0 ± 0.02 (LAB 29, 30, 31) and 6.11 ± 0.07 (LAB 76).

3.2. Interaction between LAB and L. monocytogenes in soft cheese

Between the 7 LAB that showed the highest anti-*Listeria* activity in vitro, identified, and tested for the acidifying capacity (see 3.1.2, 3.1.3, 3.1.4), three representative strains were chosen for challenge test trials (LAB 29, 31, 76). Among the 6 strains belonging to *Lb. plantarum* group (LAB 20, 26, 30, 31, 76, 78), LAB 31, isolated from Pecorino del Poro cheese, was selected because represented the strain with the best anti-*Listeria* activity in vitro. LAB 76, isolated from Smoked Ricotta, was also tested in soft cheese to evaluate if there could be differences in the activity against *L. monocytogenes* between strains belonging to the same group but isolated from different products. In addition, LAB 29, identified as *Lb. sakei* and isolated from Pecorino del Poro cheese, was chosen to check if strains belonging to different species but isolated from the same cheese could have different inhibiting activity on *L. monocytogenes*.

In co-culture, *L. monocytogenes* reached concentrations of 5.6 ± 0.3 Log CFU/g in CT1, 6.0 ± 0.3 Log CFU/g in CT2, and 5.8 ± 0.0 Log CFU/g in CT3 after 137.5 h. In control samples, *L. monocytogenes* reached a concentration of 6.5 ± 0.1 Log CFU/g at the same time (Table 2).

3.2.1. Changes in product characteristics during storage

Data on changes in product characteristics and the relative significant differences of the mean values for each experiment are reported in Fig. 3 and Fig. 1SM, respectively. Major increases in lactic acid concentration at the end of storage were observed in CT2 with 0.52 ± 0.10 mg/g in cheese, followed by CT3 with 0.28 ± 0.04 mg/g, and CT1 with 0.26 ± 0.00 mg/g. A significant difference was observed only between CT2 and control samples. Levels of acetic acid remained stable for all challenge tests, with significant differences between CT1 and control samples. A slight reduction in pH compared to the initial value was observed in all challenge tests at the end of storage, with significant differences between CT1 and control samples. No significant differences

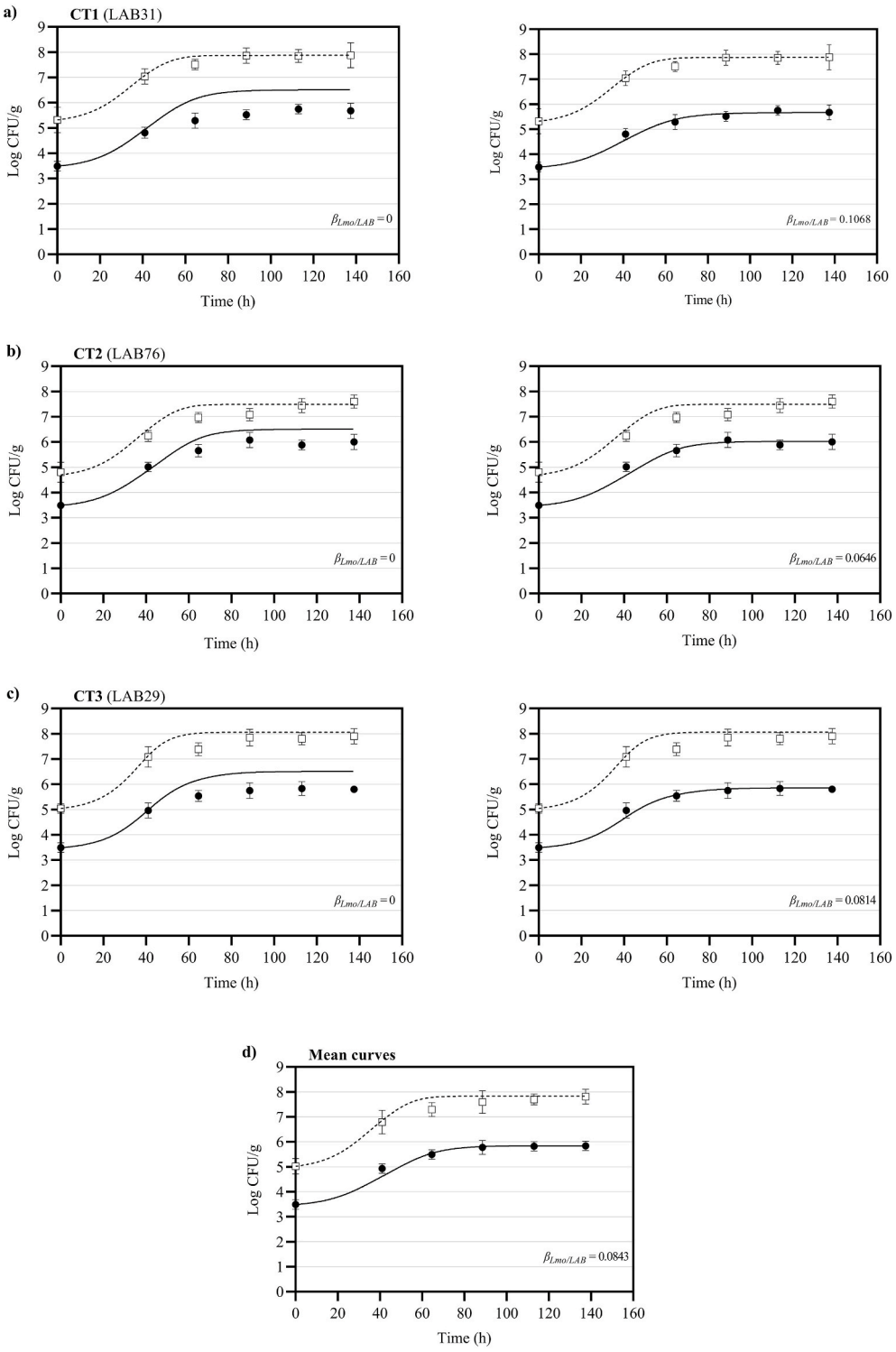

Fig. 4. Observed (□ LAB; ● *L. monocytogenes*) and predicted (— LAB; — *L. monocytogenes*) growth curves in CT1 (a), CT2 (b), and CT3 (c) before (on the left) and after (on the right) the individuation of the interspecific competition parameter $\beta_{Lmo/LAB}$ (values of $\beta_{Lmo/LAB}$ are included in the graphs). In Fig. 4d are reported the mean fitted growth curves of the three experiments (CT1, CT2, CT3) and the mean value of $\beta_{Lmo/LAB}$. Error bars indicate the standard deviation of two replicates.

in a_w values were observed between experiments, while the only difference in dry matter was between CT2 and control samples.

3.2.2. Modelling

The primary values of Q_0 were 0.05 for *L. monocytogenes* and 0.03 for LAB. N_{max} used to solve Eqs. 1 (a-b) and 2 (a-b) were 6.51 and 7.83 Log CFU/g for *L. monocytogenes* and LAB, respectively. In control samples (Fig. 2SM), other indigenous LAB, probably residing in the dairy environment, grew until a concentration of 4.3 Log CFU/g but no inhibition of *L. monocytogenes* was observed. In CT1, *L. monocytogenes* stopped its growth when LAB 31 approached the maximum population density (Fig. 4a). A best fit of growth curves was obtained after the quantification of the parameter $\beta_{Lmo/LAB}$ (Fig. 4a). Similar behaviour was observed in CT2 (LAB 76) (Fig. 4b) and CT3 (LAB 29) (Fig. 4c) with a best fit

Table 3
Inhibition radii (mm) of LAB 29 (*Lb. sakei*), LAB 31 (*Lb. plantarum* group), LAB 76 (*Lb. plantarum* group) against *L. monocytogenes* and relative interspecific competition parameters.

	LAB 29	LAB 31	LAB 76
Inhibition radii against *L. monocytogenes* (mm) [a]	11.06 ± 0.6	11.89 ± 2.0	10.78 ± 1.5
$\beta_{Lmo/LAB}$	0.0814	0.1068	0.0646

[a] Average ± Standard Deviation of three spots.

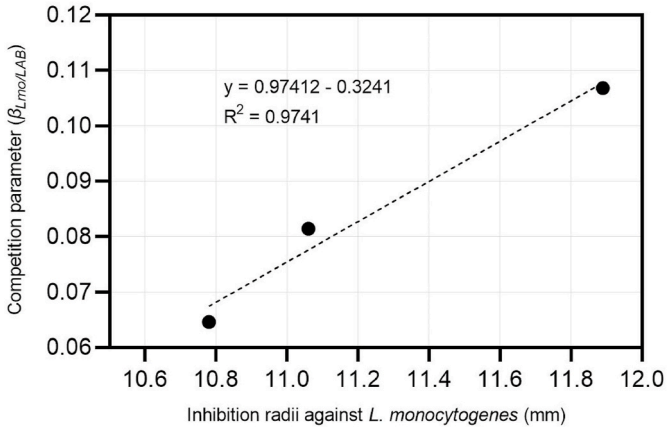

Fig. 5. Linear regression test between the size of inhibition radii against *L. monocytogenes* by LAB 29 (*Lb. sakei*), LAB 31 (*Lb. plantarum* group), and LAB 76 (*Lb. plantarum* group) observed in agar and values of the relative interspecific competition parameters.

obtained after the individuation of $\beta_{Lmo/LAB}$.

The mean fitted growth curves of the three experiments concerning *L. monocytogenes* and LAB are reported in Fig. 4d.

Outcomes of the linear regression test highlighted a correlation between the inhibition radii against *L. monocytogenes* observed in agar and the $\beta_{Lmo/LAB}$ values obtained during the experiments in cheese using the Lotka-Volterra model (Table 3, Fig. 5).

4. Discussion

The selection of adjunct cultures for cheese production should consider the characteristics of the finished products but, in general, some features are always desirable. An adjunct culture should be characterized by low CO_2 production to prevent gassy defects in cheese (Ortakci, Broadbent, Oberg, & McMahon, 2015). However, adjunct cultures should displayed low acidifying capacity especially during the first steps of cheesemaking to avoid a further acidification than that caused by the starter culture; this is crucial to prevent an over-acidification of the curd with consequent negative effects on physico-chemical properties and sensory acceptability of cheese (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). A strong proteolytic activity, instead, can be considered as a positive element, given its importance during the maturation of many fermented dairy products (De Pasquale et al., 2019; Pino et al., 2018; Randazzo et al., 2010). The strains selected in the present work showed all the characteristics listed above and they are therefore suitable for use as adjunct cultures for cheese production.

In the present study, the anti-*Listeria* activity of LAB isolated from traditional cheeses produced in Calabria was assayed in vitro and outcomes showed that different strains can inhibit growth of *L. monocytogenes* in laboratory media. The assay was conducted with the spot method, a commonly used technique for evaluating the activity of LAB against pathogenic and/or spoilage microorganisms. However, this method can sometimes lead to misleading results and "false inhibition", probably due to production of organic acids by LAB. Spelhaug and Harlander (1989) reported that *Lactococcus lactis* LM0230, a non-bacteriocin producing LAB, produced inhibition zones of 7 mm against *L. monocytogenes* using MRS as bottom and BHI as overlay agar. In the present experiments, many of the tested LAB produced inhibition halos largely above 7 mm (see 3.1.2 and Table 1) and their low acidifying activity was demonstrated in skim milk (see 3.1.4 and Fig. 2) and in soft cheese (see 3.2.1 and Fig. 3).

Selected LAB exerted a slight effect on growth of *L. monocytogenes* in soft cheese during chilled storage with a reduction of *L. monocytogenes* loads of 0.5 Log CFU/g during CT2 (LAB 76, *Lb. plantarum* group), 0.7 Log CFU/g in CT3 (LAB 29, *Lb. sakei*), and almost 1 Log CFU/g in CT1 (LAB 31, *Lb. plantarum* group) (Table 2). Production of organic acids and drop in pH constitute important inhibiting factors for growth of *L. monocytogenes* in food (Martinez-Rios, Gkogka, & Dalgaard, 2019; Mejlholm & Dalgaard, 2015). In the pH range of Gouda cheese (5.2–5.6) the average MIC of undissociated lactic acid was 5.0 mM, while the average MIC of undissociated acetic acid was 19.0 mM (Wemmenhove, van Valenberg, Zwietering, van Hooijdonk, & Wells-Bennik, 2016). In fresh Ricotta, the growth of *L. monocytogenes* was inhibited in 7 days at 15.2 °C with 8.8 mM of undissociated acetic acid (initial pH of 5.27), while no growth was observed with 14.7 mM of lactic acid (Tirloni, Stella, Bernardi, Dalgaard, & Rosshaug, 2019). In the present experiments, LAB produced low levels of organic acids with a minimal reduction in pH during cheese storage, so the observed inhibition was not attributable to these factors. An explanation for the observed inhibition could be production of bacteriocins by LAB, considering that dairy-related strains of *Lb. plantarum* and *Lb. sakei* produce bacteriocins active against *L. monocytogenes* (Malheiros, Cuccovia, & Franco, 2016; Mills et al., 2011; Pei, Li, Han, & Tao, 2018).

The modelling approach based on Lotka-Volterra equations confirms its potential to understand and parametrize bacterial competition in food. The proposed model considers the substrate complexity, the environmental conditions, and the interaction between LAB and *L. monocytogenes.* Particularly, the last aspect is guaranteed by the β parameters (specific of the Lotka-Volterra approach) that express the competition of a species against the other one. Generally, the intensity of this competition is directly proportional to the β value size. Furthermore, according to Eqs. (1a) and (2a) (see 2.2.5), the competition of a species against the other one is correlated to the growth of each populations. For this reason, in the present study, $\beta_{LAB/Lmo}$ (parameter that expresses the antagonism of *L. monocytogenes* against LAB) was set equal to 0 since it is well recognized that *L. monocytogenes* does not inhibit the growth of LAB, whereas the parameter $\beta_{Lmo/LAB}$ was obtained through fitting techniques (observed curves against predicted by modifying the β value). This approach allowed to obtain a better reproduction of growth curves with consequent reliability and robustness of the model (see 3.2.2 and Fig. 4a, b, c). In addition, the good reproduction of growth curves obtained by the mean growth values (Fig. 4d) shows the general suitability of the model for the prediction of microbial competition dynamics.

The linear regression test highlighted a correlation between β values obtained in cheese and the antagonistic activity of LAB in agar (Table 3, Fig. 5).This finding further emphasized the importance of β, as this can reliably relate the antagonistic action of LAB observed in cheese with the activity observed in vitro. This also means that with measurement of inhibition radii obtained using the spot method (see 2.1.2) we can predict the effect of LAB on *L. monocytogenes* and the behaviour of the two populations in cheese.

5. Conclusion

LAB isolated from traditional cheeses manufactured in Calabria (Italy) reduced the growth of *L. monocytogenes* in vitro and in cheese and they could be used as adjunct cultures for cheese production. The

interspecific competition model based on Lotka-Volterra equations returned good predictions and allowed to generate mean curves for LAB and *L. monocytogenes* in soft cheese during chilled storage. This modelling approach could be useful to better understand the mechanisms of microbial interaction in food and for the quantitative risk assessment process.

Declaration of competing interestCOI

None.

CRediT authorship contribution statement

Felice Panebianco: Conceptualization, Investigation, Methodology, Writing - original draft. **Filippo Giarratana:** Investigation, Methodology, Writing - review & editing. **Andrea Caridi:** Formal analysis, Resources, Writing - review & editing. **Rossana Sidari:** Investigation, Methodology. **Alessandra De Bruno:** Investigation, Methodology. **Alessandro Giuffrida:** Formal analysis, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110446.

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