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Technological traits and mitigation activity of autochthonous lactic acid bacteria from mediterranean fermented meat-products

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ABSTRACT

The production of safe and standardized fermented sausages with typical characteristics linked to traditional origin is highly desirable. The use of autochthonous starter cultures that provide peculiar flavor, texture and color to the fermented products, while maintaining the meat-product safe can be a feasible strategy for producers. In this study, 45 strains of *Latilactobacillus sakei* and 1 *Latilactobacillus curvatus* isolated from natural Mediterranean fermented sausages, were screened as potential protective cultures for their use in the fermented sausage industry. Technological properties, inhibitory activity towards *Escherichia coli* and *Listeria innocua* and the presence of genes coding for bacteriocins, were investigated. All tested strains showed an antagonistic effect by growing, while inhibiting the growth of target harmful microorganisms, in a strain-specific manner. At least one bacteriocin encoding genes was present in 25 strains, mainly sakacin X and sakacin P. The technological performances of the strains highlighted a great variability in the behavior, confirming the phenotypic diversity already reported for LAB species highly adapted to meat environment. Results highlight the potentiality of these strains to be used as protective starters in fermented meat products to improve food quality and microbiological safety, as well as giving peculiar characteristics to the final product.

1. Introduction

Food-borne diseases are a major cause of morbidity and mortality worldwide, causing up to 600 million cases of foodborne illness and 420,000 deaths per year (Lee & Yoon, 2021). This represents a substantial health burden for governments, which incur in greater expenditure to healthcare and medical expenses (Erdoğmuş et al., 2021; Faour-Klingbeil & Todd, 2020; Lee & Yoon, 2021). The ability of foodborne pathogens to grow, create biofilms and toxin production represent dangerous aspects connected to pathogenicity, outbreaks and affect consumers health (Janež et al., 2021; Kim & Kim, 2012). Consequently, there has been a growing demand from consumers in recent years for healthy and safe food (de Andrade et al., 2019; Gressier et al., 2020).

Manufacturing of standardized and safe food products, but still characterized by traditional and regional organoleptic and nutritional properties, represents a main issue for food companies, which aim to find a strategy to meet all the market requests (Gizaw, 2019). In this perspective, the use of autochthonous starter cultures can be a useful tool to achieve the production of safe and high quality traditional foods (Lorenzo et al., 2017). Moreover, indigenous starter cultures are known to often improve the organoleptic features of fermented products such as taste, texture and color (dos Santos Cruxen et al., 2019; Terzić-Vidojević et al., 2020).

Among processed foods, meat and meat products pose a significant challenge to food companies in ensuring safety of final products (Belloso Daza et al., 2022; Devleesschauwer et al., 2019). In fact, microbial contaminations of fresh and processed meats by various pathogenic and spoilage microorganisms has become a major issue for consumers health (Fegan & Jenson, 2018; Huffaker & Hartmann, 2021). In the last decades, these issues moved companies and researchers to broaden their

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knowledge about new control measures, such as bioprotectants and protective food cultures (Falardeau et al., 2021; Sameli & Samelis, 2022). Physical methods alone are frequently not sufficient to ensure the production of safe and reliable foods (Lahiri et al., 2022). A possible strategy is the incorporation of lactic acid bacteria (LAB), with the capacity to produce antimicrobial compounds, during the manufacturing process. Given their GRAS (generally recognized as safe) status, LAB are extensively employed in the meat industry as starter cultures to facilitate fermentation and/or as biocontrol agents, thereby addressing these concerns effectively (Patricia Castellano et al., 2017; Mathur et al., 2020; Raman et al., 2022)

LAB antimicrobial activity against foodborne pathogens and spoilage agents can be exerted by different metabolites produced during their growth such as organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins (Patricia Castellano et al., 2017; Chen et al., 2021; da Costa et al., 2019). Their inhibitory activity has been extensively studied in food matrices to evaluate the effect against the growth of pathogenic or spoilage microorganisms (Baillo et al., 2023; Ben Said et al., 2019; Danielski et al., 2022; Segli et al., 2021; Todorov et al., 2017; Xu et al., 2021). Several studies documented that a wide range of LAB strains, which include *Latilactobacillus sakei*, *Lactiplantibacillus plantarum*, *Ligilactobacillus animalis* and *Latilactobacillus curvatus*, can be used as effective bioprotective microorganisms in meat and meat products (Castellano et al., 2012; Jones et al., 2010; Li et al., 2016; Tirloni et al., 2014).

Food industries are continuously searching for autochthonous indigenous bacteria (LAB and Gram-positive catalase cocci), endowed with technological and antimicrobial features, that can be potentially used as new starter and, at the same time, as protective cultures in meat products (dos Santos Cruxen et al., 2019)

In the selection of potential LAB starter cultures, proper growth performances at different temperatures, even in the presence of high concentrations of NaCl and the consequent rapid pH drop in the meat matrix, are the most important technological characteristics (Nikodinoska et al., 2023). In addition, the contribution of candidate strains to the aroma profile formation is relevant for the sensorial acceptability and product recognizability (Carballo, 2021; Montanari et al., 2018).

In this study 45 *L. sakei* and 1 *L. curvatus* strains, isolated from spontaneously fermented sausages produced in the Mediterranean area and previously screened regarding their safety aspects (Barbieri et al., 2021; Bassi et al., 2022), were characterized for their ability to inhibit pathogenic microorganisms *in vitro* and in meat models. Moreover, the presence of genes related to the production of bacteriocins was assessed. The most promising strains were then analyzed for their technological properties. Growth kinetics at different salt concentrations and different temperatures were studied with the aim to exploit the microbial biodiversity of LAB populations in European fermented sausages and select new autochthonous starter cultures for traditional products manufacture.

2. Materials and methods

2.1. Strains and growth conditions

The 45 strains of *Latilactobacillus sakei* and 1 *Latilactobacillus curvatus* considered in this study are reported in Table 1, in relation to their isolation source (Bassi et al., 2022). Selected microorganisms were cultivated in MRS broth (Oxoid, Italy) for 48 h at 37 °C under anaerobic conditions. *Escherichia coli* ATCC 25922 and *Listeria innocua* UC8409, used to test the LAB inhibitory activity, were grown in BHI broth (Oxoid, Italy) overnight at 37 °C. After incubation, samples were stocked at −40 °C in MRS broth and BHI broth respectively, containing 20% glycerol (Carlo Erba, Italy) until the beginning of experiments.

Table 1
Strains of *Latilactobacillus* isolated from different naturally fermented Mediterranean sausages.

Isolation source (type of sausages and Countries)	Strains	Species
Salame Fabriano - producer 1 (Italy)	1M8	*L. sakei*
	1M24[a]	*L. sakei*
	1M51	*L. sakei*
Salame Fabriano - producer 2 (Italy)	2M7[a]	*L. sakei*
	2M9[a]	*L. sakei*
Salame Alfianello (Italy)	IAL8[a]	*L. sakei*
	IAL18	*L. sakei*
	IAL38	*L. sakei*
Traditional smoked salami with nitrates (Slovenia)	SN4	*L. sakei*
	SN34[a]	*L. sakei*
	SN58[a]	*L. sakei*
	SN63	*L. sakei*
	SN70	*L. sakei*
Traditional smoked salami without nitrates (Slovenia)	SWO10[a]	*L. sakei*
	SWO18	*L. sakei*
	SWO29	*L. sakei*
	SWO48	*L. sakei*
	SWO60	*L. sakei*
	SWO61[a]	*L. sakei*
Salchichón Alhendín (Spain)	ESA21	*L. sakei*
	ESA49	*L. sakei*
Salchichón Bérchules (Spain)	ESB2[a]	*L. sakei*
	ESB7	*L. sakei*
	ESB14[a]	*L. sakei*
	ESB24	*L. sakei*
	ESB53	*L. sakei*
	ESB60	*L. sakei*
	ESB67	*L. sakei*
Salchichón Écija (Spain)	ESE30[a]	*L. sakei*
	ESE41	*L. sakei*
	ESE67	*L. sakei*
Salchichón Olvera (Spain)	ESO8[a]	*L. sakei*
	ESO10	*L. sakei*
	ESO23[a]	*L. sakei*
	ESO38	*L. sakei*
	ESO47	*L. sakei*
	ESO65[a]	*L. sakei*
Chorizo Écija (Spain)	ECE2[a]	*L. sakei*
Chorizo Olvera (Spain)	ECO38[a]	*L. sakei*
Traditional unsmoked salami (Croatia)	HNS21	*L. sakei*
	HNS28	*L. sakei*
	HNS48[a]	*L. sakei*
	HNS55[a]	*L. curvatus*
Salami Zminjska Klobasica (Croatia)	HZK39[a]	*L. sakei*
	HZK42[a]	*L. sakei*
	HZK50	*L. sakei*

[a] strains selected for further technological characterization.

2.2. Inhibitory activity against E. coli ATCC25922 and List. innocua UC8409 with agar overlay assay

The agar overlay method was used to test the inhibitory capacity of the LAB strains against *E. coli* ATCC 25922 and *List. innocua* UC8409, selected as the non-pathogenic counterpart of Shiga toxin producing *Escherichia coli* (STEC) and *List. monocytogenes*, as previously described by Halder and colleagues with some modifications (Halder et al., 2017). Briefly, 10 μl of an overnight culture of the LAB microorganism to be tested, were spotted on MRS agar plates and incubated for 48 h at 30 °C under anaerobic conditions. After, each MRS plate was overlayed with 10 ml of BHI with the addition of 0.8% bacteriological agar (Oxoid) previously inoculated with 7 log CFU/ml of *E. coli* ATCC 25922 or *List. innocua* UC8409. After solidification of the overlayed agar medium, the plates were incubated at 30 °C for 48 h. The analysis was performed in triplicate. Once the incubation time has expired the diameter of the inhibition halos was measured as previously described by Shokryazdan et al. (2014). The results were analyzed using the following scale.

- (+++): diameter >4 cm;
- (++): diameter 2–4 cm;

- (+): diameter 0.5–2 cm;
- (−): no halo.

2.3. Inhibitory activity of Latilactobacillus sakei and Latilactobacillus curvatus against E. coli ATCC25922 and List. innocua UC8409 in sausage meat models

The fermented sausage (salami) meat model composition was reported in Table S1. Salami meat was finely minced in sterile conditions; then, 60 g were taken, supplemented with 12 ml of sterile water and pasteurized at 65 °C for 30 min. After, 180 ml of a molten 2% water-agar (Oxoid) solution were added to solidify the final mixture. The resulting mixture was mixed for 3 min and then filtered to remove particulate material. Following the recipe used in the industrial production, glucose (0.5 % of total weight; Carlo Erba), NaCl (3 % of total weight; Carlo Erba) and $NaNO_3$ (150 ppm; Carlo Erba) were added. Finally 2.5 ml of 1% solution of 2,3,5-triphenyltetrazolium chloride (MERC) was added to allow the enumeration of colonies (Beloti et al., 1999). The obtained medium was employed given its pH within the range of 5.5–6.

Agar-salami medium was cooled at 50 °C and then poured on sterile 25-well plates (Thermo Fisher Scientific). Then, 30 μl of a 6 log CFU/ml concentrated culture of each *Latilactobacillus* (Table 1) were spotted separately in each well. Subsequently the agar-salami medium was poured, and the inoculum was homogenized with a sterile loop. After solidification of the media, overnight cultures of *List. innocua* UC8409 with a microbial cell load of $8x10^8$ CFU/ml and *E. coli* ATCC 25922 with a concentration of $1x10^9$ CFU/ml, were serially diluted 7 times with saline solution. Then, 30 μl of each obtained diluted microorganism was spotted on the surface of 1 agar-salami well, previously inoculated with *L. sakei* or *L. curvatus*. As a positive control the agar-salami medium was poured on sterile 25-well plates without the addition of any *Latilactobacillus*; after solidification 30 μl of each previously prepared dilution of *List. innocua* and *E. coli* were spotted on the medium surface separately. The plates, containing 8 wells per sample, were first incubated at 37 °C for 24 h under anaerobic conditions followed by other 24 h at room temperature in aerobic conditions. Same conditions were maintained also for positive controls, without adding any *Latilactobacillus* strain. The analysis was performed in triplicate. The results obtained were expressed as the logarithmic reduction of growth when compared to the positive control. pH values for each tested strain were measured and was expressed as the mean of eight growing wells.

2.4. Gene-specific PCR for the detection of genes coding for bacteriocins

The presence of genes related to the production of bacteriocins was investigated for the 45 strains of *L. sakei* and one *L. curvatus*. DNA was extracted from LAB strains using the NucleoSpin® Tissue (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturers instructions in order to perform the gene-specific PCR test for the detection of genes encoding bacteriocins. Extracted DNA was PCR processed according to previous protocols (Barbosa et al., 2014; Dortu et al., 2008; Fontana et al., 2015). The presence of genes coding for Curvacin A (*curA*), Sakacin P (*sakP*), Sakacin Q (*sakQ*), Sakacin G (*sakG*), Sakacin Tα (*sakTα*), Sakacin Tβ (*sakTβ*), Sakacin X (*sakX*) was detected. PCR was performed on a total volume of 25 μl containing 12.5 μl of PCR Master Mix, 2X (Promega, Germany), 1.25 μl of each primer at concentration of 10 μM, 8 μl of nuclease free water (Promega, Germany) and 2 μl of DNA. We used two different PCR profiles to detect genes for different 21 bacteriocins; primers and PCR conditions are described in Table 2. The amplified products were separated in 1.5% agarose gel and visualized by Sybr-Safe staining. Positive controls (2 μl of a reference strain) were also included in the amplification runs. DNA from *L. curvatus* M05 was used as positive control of *SakQ, SakP, SakG, SakT, SakT* e *SakX* amplification, while *L. curvatus* 705 and R212 as positive control of *CurA* detection.

2.5. Growth performances in presence of different salt concentrations at different incubation temperatures

Based on results obtained from their antimicrobial activity, 19 *L. sakei* and 1 *L. curvatus* were chosen to perform further technological analyses (strains highlighted with * on Table 1). LAB strains were evaluated for their growth performances in MRS broth in relation of different salt concentrations (0%, 2.5% and 5% NaCl) at 20 °C and at different incubation temperatures (10 °C, 20 °C and 30 °C) in the absence of salt. They were pre-cultivated in MRS broth for 24 h at 30 °C and then inoculated to a final concentration of 5 log CFU/ml into the different media for further analyses. During incubation, their growth was monitored through the variation of optical density at 600 nm, measured with an UV-VIS spectrophotometer 6705 UV–Vis (Jenway, Stone, UK). The analysis was performed in triplicate.

2.6. Predictive microbiology models

The collected data used for the model fitting, elaborated through predictive microbiology models, were the means of three replicates of each sampling time. In this context, Gompertz equation (1), as modified by Zwietering et al. (1990), was used to model them with Statistica 8.0 software (StatSoft Inc.):

$$OD_{600} = A \bullet e^{-e^{\left(\left(\frac{\mu_{max} \bullet e}{A}\right) \bullet (\lambda - t) + 1\right)}} \quad (1)$$

where A represent the maximum OD_{600} value reached (OD_{600}), μ_{max} is

Table 2
Primers and PCR profiles used for the detection of bacteriocin coding genes.

Target bacteriocin	Primer Name	Sequence (5′-3′)	Size (bp)	PCR Profile	Reference
Sakacin P	SakP-F	GAA(T/A)T(A/G)(C/A)(A/C)ANCAATTA(C/T)(A/C)GGTGG	124	94°Cx5′, 35 x (94°Cx30 ‘’, 50°Cx45″, 72°Cx1′), 72°Cx7′	Dortu et al. (2008)
	SakP-R	GGCCCAGTTTGCAGCTGCAT			
Sakacin T	SakTα-F	TCGGTGGCTATACTGCTAAACA	160	94°Cx5′, 35x (94°Cx 30″,50°Cx45″,72°Cx1′), 72°Cx7	Macwana and Muriana (2012a)
	SakTα-R	TGTCCTAAAAATCCACCAATGC			
Sakacin T	SakTβ-F	AAGAAATGATAGAAATTTTTGGAGG	151	94°Cx5′,35x (94°Cx 30″,50°Cx45″,72°Cx1′), 72°Cx7	
	SakTβ-R	TGTGAAATCCAATCTTGTCCTG			
Sakacin Q	SakQ-F	GAA(T/A)T(A/G)(C/A)(A/C)ANCAATTA(C/T)(A/C)GGTGG	130	94°Cx5′, 35x (94°Cx30 ‘’, 50°Cx45″, 72°Cx1′), 72°Cx7′	Dortu et al. (2008)
	SakQ-R	TACCACCAGCAGCCATTCCC			
Sakacin X	SakX-F	AGCTATGAAAGGTATTGTCGGG	156	94°Cx5′, 35x (94°Cx 30″,50°Cx45″,72°Cx1′), 72°Cx7′	Macwana and Muriana (2012b)
	SakX-R	TAAGATTTCCAGCCAGCAGC			
Sakacin G	SakG-F	GTAAAAATTATTTAACAGGAGG	492	94°Cx5′, 35 x (94°Cx30 ‘’, 50°Cx45″, 72°Cx1′), 72°Cx7′	Dortu et al. (2008)
	SakG-R	TTAGTGCTTTTTTATCTGGTA			
Curvacin A	CurA-F	GTAAAAGAAITAAGTATGACA	171	94°Cx5′, 35 x (94°Cx30 ‘’, 50°Cx45″, 72°Cx1′), 72°Cx7′	Remiger et al. (1996)
	CurA-R	ITACATTCCAGCTAAACCACT			

the maximum OD_{600} increase rate in exponential phase (h^{-1}) and λ is the lag phase (h). pH in MRS broth was measured with pH-meter Basic 20 (Crison Instruments). The initial pH of the growth medium was 5.9 ± 0.07.

2.7. Statistical analysis

Parameters resulting from the Gompertz model fitting for all the strains were further elaborated using the statistical software R (R Core Team, 2020). The analyses were performed by using "boxplot" function. For each parameter considered, the data were also explored to highlight significant differences in relation to temperature or NaCl concentration. With these purposes the data were analyzed through a one-way ANOVA model by addition of "lme4" (Bates et al., 2015) and "emmeans" (Lenth et al., 2018) packages in software R. All statistical differences were considered significant at a level of $P \leq 0.05$ using the Bonferroni test.

3. Results and discussion

3.1. Inhibitory activity with agar overlay assay against E. coli ATCC 25922 and List. innocua UC8409

This study aims to test the ability of different LAB strains, belonging to *L. sakei* and *L. curvatus* species, isolated from spontaneously fermented sausages produced in the Mediterranean area, in inhibiting pathogens that could be considered as possible contaminants in meat products. This approach was meant to identify the most inhibiting wild strains. To assess this potential, antimicrobial activity of the 46 selected autochthonous LAB strains (45 *L. sakei* and 1 *L. curvatus*) was evaluated against *E. coli* ATCC 25922 and *List. innocua* UC8409 as candidate microorganisms. Data reported in Table 3 show that all LAB inhibited the growth of *E. coli* and *List. innocua* at different levels and that the inhibition grade was strain-dependent. In fact, as expressed by the results of the inhibition halo tests, *E. coli* was the most sensible microorganism to the mitigation effect exerted by LAB. To support this outcome, 14 out of 45 *L. sakei* strains created an inhibition zone between 2.4 and 3.2 cm, while 30 out of 45 *L. sakei* strains generated an inhibition zone between 1.6

Table 3
Inhibition halo in medium plates of the 46 LAB against *E. coli* and *List. innocua*. Inhibition zones: (+++) > 4 cm; (++) 2–4 cm; (+) 0.5–2 cm; (- no halo). Log reduction of *E. coli* and *List. innocua* and final pH in meat model used for the assays. Data are reported as the mean of three replicates for each sample.

Strains	Species	Target microorganisms Inhibition zones		Target microorganisms Log_{10} CFU reduction		Final pH	
		E. coli ATCC 25922	*List. innocua* UC8409	*E. coli* ATCC 25922	*List. innocua* UC8409	*E. coli* ATCC 25922	*List. innocua* UC8409
1M8	*L. sakei*	(++)	(+)	1	1	4.16	4.15
1M24	*L. sakei*	(++)	(+)	4	4	3.71	3.71
1M51	*L. sakei*	(++)	(+)	4	3	3.63	3.63
2M7	*L. sakei*	(++)	(+)	3	2	3.94	3.96
2M9	*L. sakei*	(++)	(+)	4	4	3.72	3.68
IAL8	*L. sakei*	(+)	(+)	3	2	4.07	4.10
IAL18	*L. sakei*	(+)	(+)	1	0	4.32	4.08
IAL38	*L. sakei*	(+)	(++)	2	3	3.69	3.65
SN4	*L. sakei*	(++)	(+)	3	1	4.36	4.36
SN34	*L. sakei*	(++)	(+)	4	4	3.72	3.70
SN58	*L. sakei*	(++)	(+)	4	4	3.89	3.85
SN63	*L. sakei*	(++)	(+)	2	1	3.89	3.94
SN70	*L. sakei*	(++)	(+)	2	1	4.03	3.99
SWO10	*L. sakei*	(++)	(+)	4	4	3.65	3.70
SWO18	*L. sakei*	(++)	(+)	2	1	4.11	4.11
SWO29	*L. sakei*	(++)	(+)	3	2	3.75	3.74
SWO48	*L. sakei*	(++)	(++)	3	3	3.70	3.68
SWO60	*L. sakei*	(++)	(+)	2	2	3.91	3.90
SWO61	*L. sakei*	(++)	(+)	3	2	3.84	3.94
ESA21	*L. sakei*	(++)	(+)	4	3	3.85	3.85
ESA49	*L. sakei*	(++)	(+)	3	3	3.79	3.82
ESB2	*L. sakei*	(++)	(++)	4	4	3.83	3.83
ESB7	*L. sakei*	(+++)	(+++)	2	1	4.03	4.07
ESB14	*L. sakei*	(++)	(+)	4	4	3.69	3.70
ESB24	*L. sakei*	(++)	(+)	3	3	3.82	3.85
ESB53	*L. sakei*	(++)	(+)	1	1	3.98	4.19
ESB60	*L. sakei*	(++)	(+)	3	2	3.97	4.17
ESB67	*L. sakei*	(++)	(+)	4	2	3.95	4.06
ESE30	*L. sakei*	(++)	(+)	3	2	3.87	3.85
ESE41	*L. sakei*	(++)	(+)	2	1	3.97	4.03
ESE67	*L. sakei*	(++)	(+)	2	3	4.05	4.01
ESO8	*L. sakei*	(++)	(+)	2	3	4.04	4.00
ESO10	*L. sakei*	(++)	(+)	2	3	4.04	4.04
ESO23	*L. sakei*	(++)	(++)	2	3	4.01	3.98
ESO38	*L. sakei*	(++)	(+)	1	2	3.90	3.93
ESO47	*L. sakei*	(+)	(+)	3	2	3.93	3.92
ESO65	*L. sakei*	(+)	(+)	3	3	3.86	3.89
ECE2	*L. sakei*	(+)	(+)	3	1	3.87	3.91
ECO38	*L. sakei*	(++)	(+)	4	1	3.64	3.83
HNS21	*L. sakei*	(+++)	(+++)	6	4	3.86	3.84
HNS28	*L. sakei*	(+++)	(+++)	5	3	3.83	3.89
HNS48	*L. sakei*	(+++)	(+++)	7	5	3.77	3.78
HNS55	*L. curvatus*	(+++)	(+++)	2	3	4.12	4.14
HZK39	*L. sakei*	(+++)	(+++)	1	3	4.29	4.13
HZK42	*L. sakei*	(+++)	(+++)	4	3	3.91	4.04
HZK50	*L. sakei*	(+++)	(+++)	2	1	4.00	4.10

and 2.4 cm. On the other hand, *List. innocua* was inhibited to a minor extent with only 8 strains of *L. sakei* out of 45 able to form an inhibition zone comprised between 2.6 and 3.4 cm and 34 strains that create inhibition zones of 1.6–2.4 cm. The only L. *curvatus* isolated from a Croatian salami Zminjska Klobasica showed good inhibition performance creating an inhibition zone >4 cm against both pathogens. Similarly, a total of 7 *L. sakei* strains, 6 isolated from Croatian salami Zminjska Klobasica and one present in Spanish Salchichón Alhendín, demonstrated the highest inhibitory effect (+++) against both microorganisms. Low differences between inhibitory performance of *L. sakei* and *L. curvatus* are supported by the literature where it has been also reported that these two microorganisms, traditionally bounded to meat products (Hugas, 1998), are phylogenetic and metabolically close (Lopez-Arvizu et al., 2021).

3.2. Inhibition of *E. coli* ATCC 25922 and *List. innocua* UC8409 by LAB strains in fermented sausage model

Results of *in vitro* agar overlay assay, although promising, can only suggest the ability of viable cells to inhibit the growth of pathogenic microorganisms. For this reason, an inhibition assay on agar-salami media was assessed. This medium was prepared starting from pork meat to maintain the same nutritional and physicochemical characteristics of the real product. Meat batter purchased from a local market and used in this model was selected to allow the growth of LAB and target microorganisms to be inhibited, mimicking the processes that take place in the real product. The mitigation activity of *Latilactobacillus* strains was assessed by comparing the growth and the inhibition of the target microorganisms with and without the addiction of the LAB cultures. The inhibition rate was expressed as log CFU reduction. The results reported in Table 3 indicated that all the strains were able to inhibit the growth of selected microorganisms to some extent, except for the strain IAL18 that did not cause any reduction of *List. innocua.* Data also confirmed that *E. coli* showed higher sensitivity to LAB inhibition with respect to *List. innocua*, with 12 strains of *L. sakei* able to limit the growth of *E. coli* of at least 4 log CFU in the meat model. This potential for inhibition of growth of *E. coli* harbored by *L. sakei* was already demonstrated against STEC O157:H7 in a simulated meat fermentation medium at 26 °C and 2% w/v glucose (Papathomopoulou & Kotzekidou, 2009). Three strains from the same Slovenian salami, namely HNS21, HNS28 and HNS48 inhibited respectively 5, 6 and 7 log CFU/ml of *E. coli*. Experiments conducted by other researchers evidenced that in biofilm formations, *L. sakei* resulted in more than six log reduction in the *E. coli* counts when compared to controls, even if this effect could not be attributed to bacteriocin production (Gómez et al., 2016).

On the other hand, only 8 strains of LAB were able to reduce 4 log CFU of *List. innocua* with only one strain (HNS48) able to reduce *List. innocua* microbial cell load of 5 log CFU/ml. Different experiments recently investigated the effect of different compounds derived from LAB as bioprotectants in food models. For example, Incili et al. (2023) tested the effect of paraprobiotics deriving from *Pediococcus acidilactici* to inhibit *E. coli* O157:H7, *Salmonella typhimurium* and *List. monocytogenes* in meatballs. They reported that the concentration of paraprobiotics should be 10 times higher in food with respect to *in vitro* to achieve the same inhibitory effect, and that this phenomenon is probably due to the matrix effect. In our experiments we obtained a sensible reduction of pathogens by using live microorganisms, which suggests their efficacy in increasing the safety of processed meat (Kürşad İncili et al., 2023). In Pisano et al. (2022), an inhibitory effect of LAB against *List. monocytogenes* is reported, where *Lactiplantibacillus plantarum* and *Lactococcus lactis* produced an inhibition zone of >4 mm in cheese agar (Pisano et al., 2022). By-products of LAB metabolism can allow the inhibition of undesired microorganisms, determined by means of a synergistic effect between different bioactive compounds and an adverse environment for the growth of harmful microbes. Among all bio-protectant acid compounds, lactic acid seems to be particularly effective (Barcenilla et al., 2022; Parlindungan et al., 2021). In fact, the highest reductions in log CFU values were achieved by the strains that caused the greater drop in pH values (Table 3). From our results, it was also possible to notice that the strains with best inhibitory performances against *E. coli*, caused the most relevant decrease of *List. innocua* concentrations. Nevertheless, lower values of *Listeria* reduction achieved in our experiments are supported also by literature. As an example, Wang et al. (2015), reported the necessity of an increased contact time between *List. monocytogenes* and lactic acid, with respect to *Salmonella* spp. and *E. coli*, to achieve the same level of inactivation.

In a previous study concerning strains (n = 37) of *L. sakei*, *L. curvatus*, *Leuc. mesenteroides* and *E. durans* isolated from spontaneously fermented sausages, 37.5% of the strains showed antagonism against potential pathogen microorganisms but the inhibition in the majority case was due to competitive exclusion rather than production of bacteriocins, which was found only in 2 strains (5.4%) of *L. sakei* (Fuka et al., 2020).

3.3. Presence of bacteriocin genes

To further characterize the isolated strains, the presence of genes coding for bacteriocins was investigated. Results showed that 25 strains out of 46 possessed almost one bacteriocin-producing gene, and specifically, 14 strains harboring one gene and 11 strains showing the presence of more than one (Table 4). Two strains (HZK39 and HZK42) deriving from a Slovenian smoked salami appeared to be particularly interesting for the presence of four different genes coding for bacteriocins. In detail, HZK39 strain showed the presence of *spp*A, *spp*Q, *sak*T and *sak*X genes, while HZK42 possessed *sap*A, *spp*A, *sak*G and *sak*X, confirming the preliminary results obtained in the agar overlay assay and in the salami-food model. Conversely, among the 46 strains analyzed, 21 strains did not exhibit any genes encoding bacteriocins. Among the LAB strains investigated in this study, *sakX* (present in 20 out of 25 strains) and *sppA* (present in 12 out of 25 strains) were the most observed genes associated with bacteriocin production.

To have more coverage against possible other altering and especially pathogenic bacteria, the production of bacteriocins has become an increasingly important criterion in selection (Chikindas et al., 2018; Oliveira et al., 2018). The presence of genes to produce different bacteriocins is reported in the literature (Alvarez-Sieiro et al., 2016; Cintas et al., 2001; Masafumi Noda et al., 2018) and is bound to the ability of strains to face competition with bacteria from other genera. Moreover, it is reported that genes coding for bacteriocins are often carried on plasmids, which can be acquired by microorganisms, supporting the ability of different genera to produce the same bacteriocins (Lozo et al., 2021).

3.4. Growth and acidification performances of selected LAB strains under different NaCl conditions

Considering the isolation sources, the inhibitory activity against pathogens and the presence of genes coding for bacteriocins, 20 strains were chosen for further technological characterization (Table 1). In particular, the growth of 19 *L. sakei* and one *L. curvatus* strains at different temperatures and NaCl concentrations was determined by monitoring the changes in optical density (OD_{600}). For each condition and strain, the experimental data were modelled using the Gompertz equation. The estimated parameters are reported in Table S2, together with the maximum pH decrease. To highlight the variability among strains, Fig. 1 reports Box and Whisker plots concerning the distribution of the parameter estimates in relation to NaCl concentration and the pH decrease. The presence of significant differences ($P \leq 0.05$) was tested with one-way ANOVA. Concerning the parameter *A*, a significant diminution of the median (the thick line inside the box) was observed in relation to salt (the predicted OD_{600} was 1.96 at 0%, 1.82 at 2.5% and 1.60 at 5%). Three strains were considered outliers when cultivated in the absence of salt, due to their low *A* final level (SN34, SWO10 and

Table 4
Presence of genes detected by PCR and coding for bacteriocins in LAB strains object of the study.

Strains	Species	Bacteriocin genes						
		curA = *sap*A	*spp*A	*spp*Q	*sak*G	*sak*T	*sak*X	**Tot**
1M8	*L. sakei*							
1M24	*L. sakei*						X	1
1M51	*L. sakei*							
2M7	*L. sakei*				X		X	2
2M9	*L. sakei*							
IAL8	*L. sakei*							
IAL18	*L. sakei*							
IAL38	*L. sakei*							
SN4	*L. sakei*							
SN34	*L. sakei*						X	1
SN58	*L. sakei*		X				X	2
SN63	*L. sakei*							
SN70	*L. sakei*							
SWO10	*L. sakei*							
SWO18	*L. sakei*							
SWO29	*L. sakei*						X	1
SWO48	*L. sakei*							
SWO60	*L. sakei*							
SWO61	*L. sakei*						X	1
ESA21	*L. sakei*		X				X	2
ESA49	*L. sakei*							
ESB2	*L. sakei*		X				X	2
ESB7	*L. sakei*		X					1
ESB14	*L. sakei*							
ESB24	*L. sakei*						X	1
ESB53	*L. sakei*							
ESB60	*L. sakei*						X	1
ESB67	*L. sakei*						X	1
ESE30	*L. sakei*						X	1
ESE41	*L. sakei*							
ESE67	*L. sakei*						X	1
ESO8	*L. sakei*		X	X			X	3
ESO10	*L. sakei*							
ESO23	*L. sakei*							
ESO38	*L. sakei*							
ESO47	*L. sakei*						X	1
ESO65	*L. sakei*			X			X	2
ECE2	*L. sakei*							
ECO38	*L. sakei*	X	X				X	3
HNS21	*L. sakei*		X					1
HNS28	*L. sakei*		X					1
HNS48	*L. sakei*		X					1
HNS55	*L. curvatus*	X					X	2
HZK39	*L. sakei*		X	X		X	X	4
HZK42	*L. sakei*	X	X		X		X	4
HZK50	*L. sakei*		X	X				2

ESB2). The same strains were characterized by the lowest final *A* at NaCl 2.5%, while at the higher salt level (5%) the lower value for this parameter were observed in the strains SN34 and SN58. As expected, the values of μ_{max} decreased with the increase of salt concentration (median values 0.161, 0.105, 0.045 h^{-1} at 0, 2.5 and 5%, respectively). The strain SN34 presented the best performance at 5% of salt concentration and was considered as an outlier, but it was characterized by high μ_{max} also in the other conditions (Table S2). Concerning λ, the increase was particularly relevant when NaCl concentration was 5% (median at 24.35 h, compared with 13.48 h at 0% and 15.89 h at 2.5%). In absence of salt, the strains SWO61, ECE2 and HNS48 presented the shorter λ estimates. The data concerning the pH value after incubation, reflected the growth extent as determined through OD_{600}; in other words, the pH decrease with respect to the initial value (approx. 5.9) was inversely proportional to the *A* value estimated for growth. In particular, ANOVA did not reveal differences among the strains tested at 0 and 2.5% (median values of pH decreases −1.86 and −1.75 units, respectively), while the strains grown at 5% showed a significant lower value (median −1.63).

Concerning the effect of temperature on growth kinetics, the parameters of the Gompertz equation were estimated (Table S2) and their distributions are reported in Fig. 2. The value of *A* was not significantly different at 20 and 30 °C (median OD_{600} 1.96 and 1.93, respectively), while at 10 °C it was lower (median 1.83). In addition, a *L. sakei* strain (CO38) did not grow at the lower temperature. Noteworthy, the estimates at 10 °C were characterized by a greater variability, as demonstrated by the variability coefficient (CV) reported in Table S2. Three strains (SN34, SWO10 and SB2) presented the lower *A* values, independently on the temperature (the outliers at 20 °C).

Temperature had a marked effect on median values of μ_{max} which significantly decreased passing from 30 to 10 °C (0.268, 0.161 and 0.034 h^{-1}, respectively).

The length of λ showed an increase from 30 to 20 °C, passing from 6.87 h to 13.48 h that, resulted not significant according to ANOVA. At 10 °C the median value was 49.38 h with a strain with no growth and strain ESO23 characterized by an extremely long λ (147.28 h), considered as outlier. As already observed for the effect of salt, the final pH decrease obtained at the end of fermentation was related to the values of *A*. The final pH observed was not significantly different at 20 and 30 °C (the median pH decrease was −1.86 and −1.92 respectively), while this value at 10 °C was −1.50.

In general, the selection of new starter cultures involves strain isolated from traditional products spontaneously fermented because they are adapted to the environmental and process conditions (Pereira et al.,

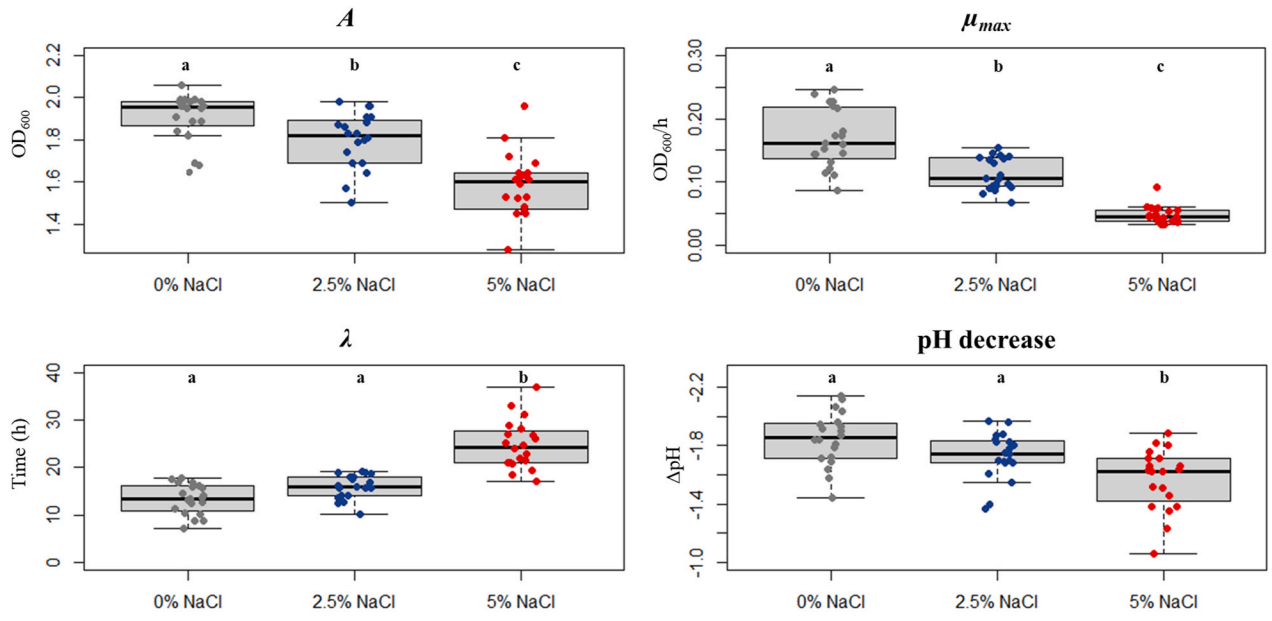

Fig. 1. Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (A, μ_{max} and λ) of strain growth kinetics at different salt concentrations (0%, 2.5% and 5%). Final pH decrease under different conditions is also reported. In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ±1.5 times the box height. For each salt concentrations, the Box and Whisker represent the results obtained for 20 strains considered.

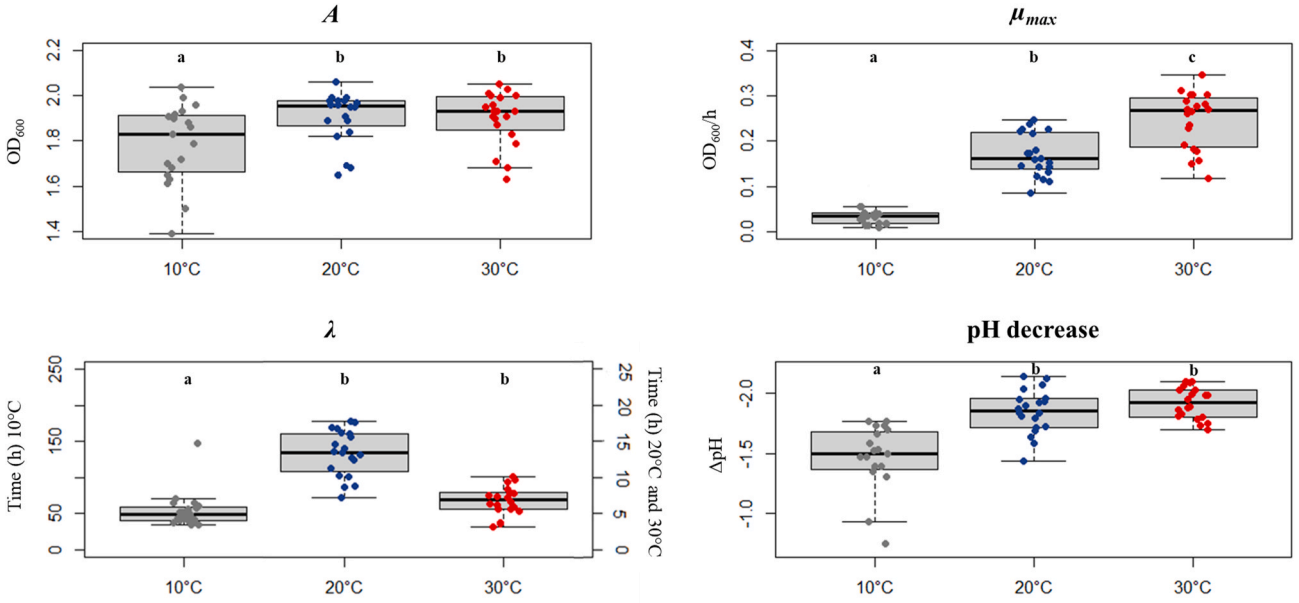

Fig. 2. Box and Whisker plots that represent the distribution of parameters estimated by Gompertz equation (A, μ_{max} and λ) of strain growth kinetics at different incubation temperatures (10 °C, 20 °C and 30 °C). Final pH decrease in the different conditions is also reported. In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ±1.5 times the box height. For each temperature, the Box and Whisker represent the results obtained for 20 strains considered.

2020). In any case, the ability of new candidate strains as starter cultures to compete with the wild microbiota must be tested under the operating conditions that characterize the production of fermented sausages (García-Díez & Saraiva, 2021). In this perspective, the most important environmental parameters can be considered are NaCl concentration and fermentation temperature.

The data indicated positive performances of the strains in terms of growth at low temperature (with the exception of the strain ECO38 that was not able to grow at 10 °C) and a good aptitude to multiply at 5% of salt, confirming the data reported for other *L. sakei* strains by Montanari et al. (2018), who tested the strain performances even at 5 °C and 8% of salt (Montanari et al., 2018). Results similar to those obtained in this work in relation to NaCl concentration and process temperature were also reported by Franciosa et al. (2022) for strains of *L. sakei* from Salame Piemonte spontaneously fermented.

In addition, Ammor et al. (2005) showed that 97% of *L. sakei* strains,

isolated from traditional dry sausages, grew at 4 °C, while 55% of them was able to grow in the presence of 6.5% of salt (Ammor et al., 2005). The increases of the variability under the most restrictive conditions, highlights the importance of these screening tests in order to select candidates for their use as potentially new starter cultures.

4. Conclusions

The purpose of this work was to identify potential autochthonous starter cultures and/or bio-protective food cultures. The results concerning antimicrobial activity revealed that all tested strains exhibited inhibitory effects, with strain-dependent differences. Among the most active strains, 24 *L. sakei* and the unique *L. curvatus* strains showed the presence of at least one bacteriocin encoding genes, prevalently sakacin X and sakacin P. These strains exhibited the highest antimicrobial activity *in vitro* and demonstrated an equivalent inhibitory potential in the meat model. The most intriguing strains were selected for further technological analyses to assess their performances under varying salt concentrations and incubation temperatures. The strain behavior exhibited significant variability, highlighting the phenotypic diversity previously reported for LAB species well-adapted to the meat environment.

According to Leroy and Praet (2015), the characteristics of traditional fermented meats should not be considered as monolithic entities. In recent decades, research has moved towards the development of procedures that meet the demands for safety but also for the typicality of these products in a logic of innovation-through-tradition. This perspective also includes the development of more sustainable and economical processes. The search for new starter cultures is undoubtedly an important step above all to respond to these questions while maintaining the distinctiveness and recognizability of the final product, maintaining cultural continuity with the tradition.

Based on the results achieved in this study, a number of LAB strains exhibiting favorable technological characteristics and significant antimicrobial potential were identified. These strains hold promising potential application as starter or bio-protective cultures in meat-based food systems, including fresh or fermented sausages. Their utilization has the potential to enhance food quality, ensure microbiological safety, and impart distinctive attributes to the final product.

CRediT authorship contribution statement

Giovanni Milani: Methodology, Formal analysis. **Giulia Tabanelli:** Writing – original draft, Supervision, Conceptualization. **Federica Barbieri:** Methodology, Formal analysis, Data curation. **Chiara Montanari:** Writing – original draft, Data curation. **Fausto Gardini:** Writing – review & editing, Conceptualization. **Mireya Viviana Belloso Daza:** Formal analysis. **Vincenzo Castellone:** Writing – original draft. **Marianna Bozzetti:** Methodology. **Pier Sandro Cocconcelli:** Writing – review & editing. **Daniela Bassi:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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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.2024.115861.

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