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Research Letter

RESEARCH LETTER – Food Microbiology

Biodiversity and dairy traits of lactic acid bacteria from foliage of aromatic plants before and after dehydration process monitored by a smart sensors system

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One sentence summary: Sage did not host lactic acid bacteria, but they were easily isolated from laurel in fresh and dehydrated form.

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ABSTRACT

The main hypothesis of this work was to evaluate the presence of lactic acid bacteria (LAB) intrinsically resistant to plant essential oils in sage (*Salvia officinalis* L.) and laurel (*Laurus nobilis*), for future applications in functional cheese production by addition of aromatic herbs. The effect of the drying process on the viability of LAB was evaluated with three biomass densities (3, 4 and 5 kg/m^2). The drying densities did not affect weight loss, but influenced the levels of LAB of sage and laurel. A total of 10 different strains of *Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus raffinosus* and *Leuconostoc mesenteroides* were identified from laurel, while sage did not host any LAB species. In particular, *L. mesenteroides* was the only species sensitive to the heat treatment. Only five strains, all enterococci, were resistant to at least one antibiotic, even though no strain showed gelatinase or haemolytic activity. The investigation on the technological traits useful in cheese making demonstrated that all LAB can be considered non starter LAB, because they were characterized by a slow acidification capacity (the pH was still above 6.00 after 3 d) and a very limited autolysis (the maximum decrease of the optical density at 599 nm was barely 0.2).

Keywords: drying; lactic acid bacteria; laurel; sage; smart system; dairy potential

INTRODUCTION

Recently, we are experiencing new ways to choose foods. In general, the majority of 'smart' consumers pays attention to health properties, absence of chemical additives, environmental implications and origin of raw materials. This trend led to an increase in the request of 'natural' foods, which are perceived as high quality products (Settanni and Moschetti 2014). The process of fermentation applied to several raw materials determines a given stability of the resulting foods which depends directly on the action of certain microorganisms, mainly lactic acid bacteria (LAB) and yeasts (Caplice and Fitzgerald 1999). This process represents a low cost biotechnology for preserving foods from deterioration and is based on the chemical compounds naturally produced by the fermenting microorganisms, such as primary metabolites and/or bacteriocins from LAB (Corsetti *et al.* 2008), and greatly improves the organoleptic characteristics, as well as, the nutritional value of the fermented products (Reis *et al.* 2012).

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Some fermented foods remain perishable, such as dairy products consumed fresh, even though LAB are still viable (Duarte *et al.* 2015). The use of chemicals to elongate the shelf life of these products is not advisable first of all for their health implications, such as carcinogenicity (Carnesecchi *et al.* 2020), but also because the undesired spoilage or even worse pathogenic microorganisms might acquire resistance (Xu and Lee 2001). Alternative natural agents to combat the undesired food spoilage/pathogenic bacteria are necessary to avoid the emergence of microbial agents resistant to chemical preservatives (Militello *et al.* 2011), in order to promote new biopreservation strategies.

Plants produce several molecules which are part of their defense against microbes (Kennedy and Wightman 2011). This makes some of these plants, such as aromatic herbs, used in food processing since ancient times. As a matter of fact, aromatic plants represent food ingredients useful not only to ameliorate the taste and flavor of the final products, but also to exert their antimicrobial properties (Gottardi *et al.* 2016; El-Sayed and Youssef 2019).

Drying is a process that prevents aromatic herbs from spoilage and decay through moisture removal, allowing the product to be stored for a long period with minimal deterioration (Nadian *et al.* 2015). The principle is to dry the vegetation waters with a forced flow of dry air, passed through the biomass spread over large surfaces, on one or more layers. Hot-air drying using convection ovens is a fundamental technology for postharvest preservation of aromatic and medicinal plants since it allows a fully controlled operation resulting to a desired end product; the most essential parameter influencing the quality of dried herbs is the temperature used (Rodriguez *et al.* 2013).

Plants like cinnamon, oregano, thyme etc., known to inhibit bacteria and fungi thanks to their essential oils (EOs) (Lopes, Pinto and Salgueiro 2017; Chimnoi *et al.* 2018; Farisa Banu *et al.* 2018; Merghni *et al.* 2018), are generally used in food formulations (Van Haute *et al.* 2016), including dairy products, also in view of their functional properties (Oraon *et al.* 2017; El-Sayed and Youssef 2019), because dairy products are not rich in bioactive ingredients (Bachmann 2001).

Aromatic plants or their extracts are traditionally used in cheese productions for their aromatic properties as well as their technological properties such as milk-clotting activity (Hayaloglu and Farkye 2011). Furthermore, some studies reported the ability of these plants to exert antimicrobial (Dupas *et al.* 2019) and/or antioxidant (Rashidinejad *et al.* 2012; Marchiani *et al.* 2016) activities acting as natural cheese preservatives.

In order to produce cheeses, LAB are essential during acidification of curd (starter LAB, SLAB) and for the ripening process (non starter LAB, NSLAB) (Settanni and Moschetti 2010). For this reason, when cheeses are added of aromatic herbs, their inhibitory compounds, especially EOs, do not have to interfere with LAB activity. Several recent studies focused on the use of plant extracts from thyme, rosemary and cumin as preservatives in cheese production, showing their ability to inhibit the main dairy pathogens while not affecting SLAB and NSLAB (Gammariello *et al.* 2008; Sadeghi *et al.* 2013; Moro *et al.* 2015). In general, LAB to be used as starter strains in cheeses with adjunct herbs should be tested for their resistance to aromatic plant EOs. In this work, we approached the selection of LAB resistant to plants EOs starting from two aromatic plants, sage (*Salvia officinalis* L.) and laurel (*Laurus nobilis*) as representative of herbaceous and tree plants, respectively.

With this in mind, the purpose of the present research was to select LAB resistant to plants EOs starting from two aromatic plants, sage (*Salvia officinalis* L.) and laurel (*Laurus nobilis*), in order to develop *ad hoc* starter culture(s) for future applications in raw milk cheese production in presence of these aromatic herbs. To this purpose, sage and laurel leaves were collected fresh and after dehydration process at three different drying densities and analysed for their LAB content. LAB isolates were then identified and characterized for their main dairy traits in view of their use as starter strains in functional cheese production. The dehydration process was realized using a low cost real time monitoring and control system designed by the authors.

MATERIALS AND METHODS

Plant material collection and dehydration monitoring system

The biomass samples of sage and laurel were taken at the experimental fields of the Department of Agricultural, Food and Forest Sciences, University of Palermo, Italy. Hot-air drying trials of the aromatic herb leaves were carried out in a dryer desiccator cabinet equipped with a smart sensors system prototype. The choice of the components to be used in the realization of the prototype of the biomass drying measuring system was based on low-cost, reliable and small-sized components. The smart sensors system structure consists of an Arduino Mega 2560 board, a load cell amplifier based on HX711, nine Siemens 7MH5102–1PD00 load cells with medium precision with a maximum capacity of 5 kg, a digital temperature and humidity sensor DHT22, a real-time clock DS3231, an OpenLog data logger, a transceiver Wireless Module ESP8266–05, power supply and external antenna. The data acquired were transmitted through Wi-Fi to a ThingSpeak account every 15 min in order to real time monitor the drying process. The drying temperature was maintained at 40°C since a higher temperature may result in a darkening of the samples due to non-enzymatic browning and leading to a loss of quality. Relative humidity was kept at 25% for the entire duration of the process. Three biomass densities (kg/m^2) were used for each species in the drying process. The trials, named 1, 2 and 3 with 0.3, 0.4 and 0.5 kg, respectively, corresponded to a biomass density of 3, 4 and 5 kg/m^2, respectively, were placed inside the shelves of the cabinet in perforated plastic boxes. Overall, about 30 kg of leaves (15 of sage and 15 kg of laurel) were taken and used to realize the different trials. Three replications for each trial were randomly arranged inside the drying chamber.

Enumeration and isolation of LAB

Aromatic plant leaves (10 g) were suspended into 90 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy), homogenized in a stomacher (BagMixer® 400; Interscience, Saint Nom, France) for 2 min at maximum speed (blending power 4) and then serially diluted keeping a dilution factor 1:10. Presumptive rod and coccus LAB were counted on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 mol/L) and on M17 agar, respectively. Both media were supplemented with cycloheximide (10 mg/mL) to inhibit fungal growth. Plates were incubated for 48 h at 30°C under anaerobic condition in hermetically sealed jars using the Anaerogen 3.5 L system (Oxoid, Hants, UK). Media and supplements were purchased from Microbiol Diagnostic (Catania, Italy). Plate counts were performed in triplicate.

After growth, at least 20 colonies of presumptive rod- and coccus-shaped LAB were randomly picked up from the highest

plated dilution. The isolates were purified by consecutive streaking on (MRS and M17) agar media. Gram-positive, catalase-negative and non-motile isolates were considered putative LAB and were stored at −80°C in 20% (v/v) glycerol stocks.

Phenotypic grouping, genotypic differentiation and identification of LAB

All isolates were phenotypically characterized as described by Gaglio *et al.* (2014) in order to obtain an initial grouping. In particular, putative LAB were grouped on the basis of cell morphology, spatial distribution of the cells, growth at 15 and 45°C, heat resistance (60°C for 30 min), hydrolysis of arginine and aesculin, acid production from carbohydrates and CO_2 production form glucose. For coccus isolates, sub-grouping included also the determination of the ability to grow at pH 9.2 and in the presence of NaCl (6.5 g/L).

All overnight grown LAB isolates were subjected to the DNA extraction by means of the DNA-SORB-B kit (Sacace Biotechnologies Srl, Como, Italy) following the instructions provided by the manufacturer. Cell extracts were used as templates for Polymerase Chain Reaction (PCR).

Random amplified polymorphic DNA (RAPD)-PCR analysis with primers M13, AB106 and AB111 was applied for LAB biotyping. PCRs were performed applying the protocol described by Gaglio *et al.* (2017). RAPD profiles were analyzed using the Gelcompare II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium). All LAB showing different RAPD-PCR profiles were also analyzed by 16S rRNA gene sequencing for identification. PCR reactions were performed as described by Weisburg *et al.* (1991). DNA fragments were visualized and amplicons of about 1600 bp were subsequently purified and sequenced by Eurofins Genomics (Ebersberg, Germany).

The identity of the bacterial sequences was determined by comparison with the sequences available in GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) and Ez-Taxon-e (http://eztaxon-e.ezbiocloud.net/) databases. The last database compares a given sequence to those of type strains only. The multiplex PCR assay based on the *sodA* gene reported by Jackson, Fedorka-Cray and Barrett (2004) was applied to confirm the identity of *Enterococcus* species.

Safety aspects of LAB

All LAB strains were characterized for their susceptibility to different antimicrobial compounds commonly used for the treatment of human and animal infections (Gaglio *et al.* 2016). The test was performed by the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2017). Thirteen antimicrobial compounds belonging to the 10 following families: penicillins [penicillin G (P- 10 UI), ampicillin (AMP-10 μg) and amoxicillin/clavulanic acid (AMC-30 μg)]; glycopeptides [vancomycin (VA-30 μg)]; macrolides [erythromycin (E-15 μg)]; tetracyclines [tetracycline (TE-30 μg)]; fluoroquinolone [ciprofloxacin (CIP-5 μg) and levofloxacin (LEV-5 μg)]; phenicols [chloramphenicol (C-30 μg)]; streptogramins [quinupristin/dalfopristin (QD-15 μg)]; oxazolidinones [linezolid (LZD-30 μg)]; aminoglycosides [high level gentamicin (CN-120 μg) and high-level streptomycin (STR–300 μg)]; were tested.

After incubation at 30°C for 18–24 h, each strain was classified as susceptible (S), intermediate (I) or resistant (R) based on the inhibition zone diameters according to the CLSI guidelines (CLSI 2017).

The minimum inhibitory concentration (MIC) was determined for each IR or R strain by the broth microdilution method according to the CLSI (CLSI 2017). *Enterococcus faecalis* ATCC 29212 was used as quality control strain.

Each IR or R strain on a given antimicrobial was investigated for the detection of antimicrobial resistance genes. The genes investigated were: *erm*(A), *erm*(B), *erm*(C) for resistance to macrolide, lincosamide and streptogramin B; $cat_{(pC221)}$ for resistance to chloramphenicol; *gyr*A and *par*C for resistance to fluoroquinolone.

Gelatinase production was determined as reported by Lopes, Simões and Tenreiro (2006) depositing a drop of each strain on a plate containing Gelatin Agar. Haemolytic activity was assessed by streaking the cultures on blood agar (Microbiol Diagnostic), after anaerobic incubation at 30°C for 24–48 h (Gaspar, Crespo and Lopes 2009). The haemolytic reactions were classified as total or β-haemolysis, partial or α-haemolysis and absent or γ-haemolysis.

Dairy potential of LAB

All LAB strains susceptible to antibiotics were evaluated for acidification and autolytic kinetics, diacetyl, exopolysaccharide and antimicrobial compound production.

The kinetics of acidification was determined in 100 mL of whole fat UHT milk (Conad, Mantova, Italy) inoculated with 1% (v/v) l suspension from the overnight cultures after centrifugation at 5000 × *g* for 5 min, washing and re-suspension in Ringer's solution (Cruciata *et al.* 2018). Inoculated milks were incubated at 30°C and the pH was measured every 2 h until 8 h, and after 24, 48 and 72 h.

Diacetyl production was determined in 1 mL of whole fat UHT milk inoculating the strains as reported above. The tubes after 24 h of incubation at 30°C were added with 0.5 mL of 1% (w/v) α-naphthol and 16% (w/v) KOH. The generation of diacetyl was confirmed by the appearance of a red ring at the top of the tube (King 1948).

The autolysis was determined as reported by Mora *et al.* (2003) on washed cells transferred into a 50 mmol/L potassium phosphate solution at pH 6.5. The decrease of optical density (OD) was measured using a ScanReady Microplate photometer P-800 (Life Real Biotechnology Co., Ltd, Hangzhou, China) at 2, 4, 6, 8, 24, 48 and 72 h.

Exopolysaccharide (EPS) production was assessed depositing a drop of each strain on MRS medium modified for the sugar composition: 50 g/L of sucrose in place of 20 g/L of glucose (Van der Meulen *et al.* 2007). In case of EPS production, the colonies grown at the optimal temperature developed a mucoid consistence after 6 d.

The antimicrobial activity of LAB was tested by the agar-spot deferred method (ASDM) and the well diffusion assay (WDA) described by Schillinger and Lücke (1989) as modified by Corsetti *et al.* (2008) using *Listeria innocua* 4202, *Listeria monocytogenes* ATCC 19114 and *Lactobacillus sakei* LMG 2313 as indicator strains. The active supernatants were treated for 2 h at 37°C with three proteolytic enzyme [proteinase K (12.5 U/mg), protease B (45 U/mg) and trypsin (10.6 U/mg)] (Sigma-Aldrich) diluted in phosphate buffer (pH 7.0) at the concentration of 1 mg/mL. The remaining activity was quantified with a second WDA (Settanni *et al.* 2005).

Statistical analyses

The results of microbial loads were subjected to one-way analysis of variance (ANOVA). Pair comparison of different dried

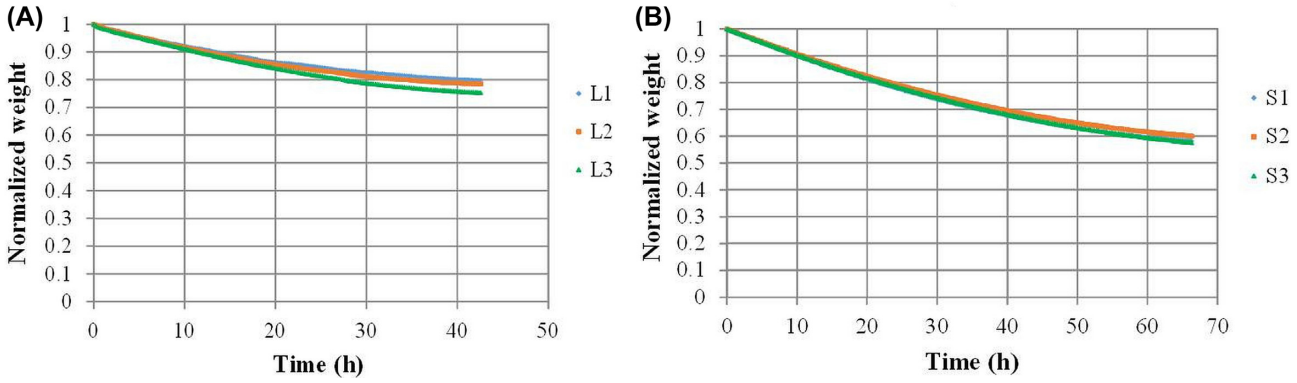


Figure 1. Curves of weight decrease normalized to the initial weight of the biomass as a function of time dehydration process. A, laurel; B, sage. Abbreviations: L, laurel; S, sage; L1 and S1, laurel and sage biomass density 3 kg/m^2; L2 and S2, laurel and sage biomass density 4 kg/m^2; L3 and S3, laurel and sage biomass density 5 kg/m^2. Results indicate mean of three determinations.

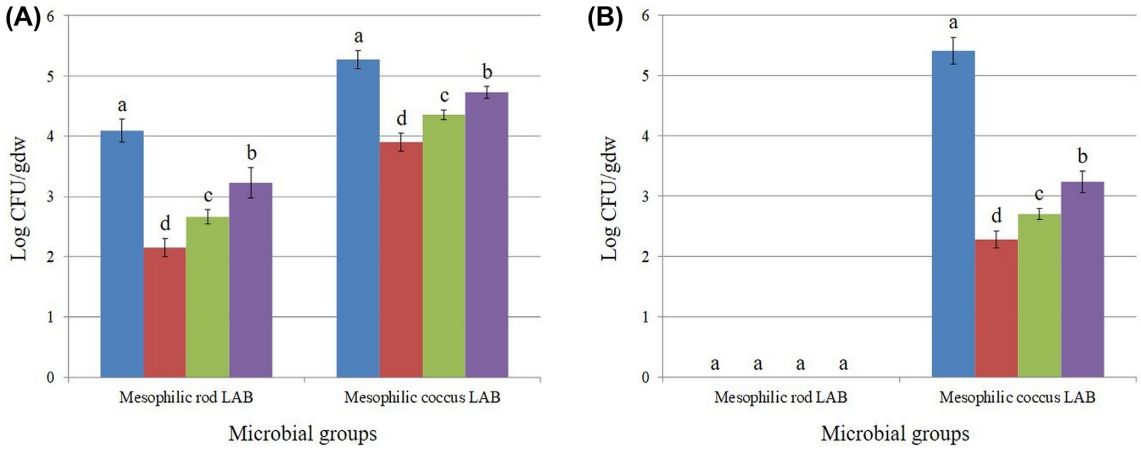


Figure 2. Microbial loads (Log CFU/gdw) of aromatic herbs subjected to drying. A, laurel; B, sage. Microbial groups: blue rectangles, untreated; red rectangles, biomass density 3 kg/m^2; green rectangles, biomass density 4 kg/m^2; purple rectangles, biomass density 5 kg/m^2. Results indicate mean values and standard deviation of three plate counts.

means was achieved by student's *t*-test at $P < 0.05$. the analysis was performed using XLStat software version 7.5.2 for excel (Addinsoft, New York).

RESULTS

Dehydration process

A sample of leaves was brought to the laboratory for drying in stove at 105°C for the determination of the dry weight of each species. The dry weight of sage was 27.2 ± 0.6% and that of laurel 45.3 ± 0.5%. The difference in the dry weight found for the two species is due to the different structural characteristics of the biomass processed.

Fig. 1 shows the progress of the dehydration process for the aromatic herbs carried out at three biomass densities (trial 1 = 3 kg/m^2; trial 2 = 4 kg/m^2 and trial 3 = 5 kg/m^2), in terms of normalized weight decrease compared to the initial weight. The curves of the three biomass densities did not show statistically significant differences for both species.

The whole process had a total duration of 42 h for laurel (Fig. 1A) and 65 h for sage (Fig. 1B). The sage weight decreased by 20% in the first 20 h of the process while the further 20% was lost during the following 35 h. The process was completed with the loss of an additional 3% of water in the last 10 h.

With regards to laurel, the weight of the biomass decreased by 10% in the first 10 h of the process while a further 10% was lost in the following 20 h. The process was completed with the loss, in the last 12 h, of an additional 2% of water for trials L1 and L2 and 5% for trial L3.

At the end of the process the moisture present in sage and laurel biomasses were 30 and 33%, respectively.

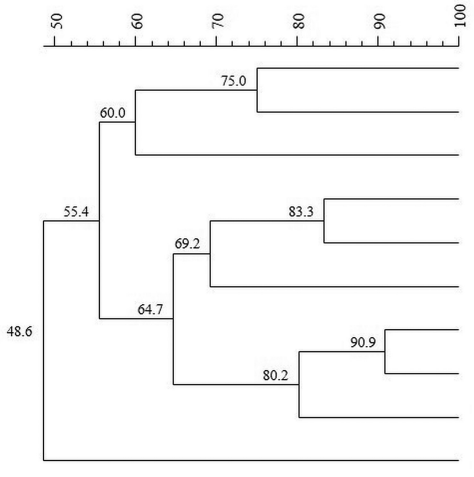
Levels of cultivable LAB

The results of viable counts of the rod- and coccus-shaped LAB of laurel and sage samples just after harvest and at the end of drying process are reported as Log CFU per g of dry weight (dw) in Figure 2. Statistical significant differences ($P < 0.0001$) were found for rod- and coccus-shaped LAB in laurel samples (Fig. 2A) and for coccus LAB in sage samples (Fig. 2B). With the exception of rod LAB in sage, below the detection limit (Fig. 2B) independently on the biomass density applied, the other LAB object of investigation were detected after drying, and their levels increased with biomass density. The highest levels of LAB (5.27 Log CFU/gdw) were registered for coccus LAB in untreated laurel samples.

(A)

Characters	Clusters				
	1	2	3	4	5
Morphology	C	C	C	C	C
Cell disposition	sc	sc	sc	sc	sc
Growth:					
15°C	+	+	+	+	+
45°C	-	+	+	+	+
pH 9.2	-	+	+	+	+
6.5% NaCl	+	+	+	+	+
Resistance to 60°C	-	-	-	-	-
Hydrolysis of:					
arginine	-	+	+	+	-
aesculin	-	+	+	+	+
Acid production from:					
arabinose	+	+	+	+	+
ribose	+	+	+	+	+
xylose	+	+	+	+	+
fructose	+	+	+	+	+
galactose	+	+	+	+	+
lactose	+	+	+	+	+
sucrose	+	+	-	+	+
glycerol	+	+	+	-	+
CO_2 from glucose	+	-	-	-	-

(B)



Strain	Phenotypic group	Species	Acc. No.
VA10	2	*E. faecium*	MT116074
VA15	2	*E. faecium*	MT116075
VA35	2	*E. faecium*	MT116076
VA36	4	*E. mundtii*	MT116077
VA17	4	*E. mundtii*	MT116078
VA46	5	*E. raffinosus*	MT116079
VA12	4	*E. mundtii*	MT116080
VA14	4	*E. mundtii*	MT116081
VA18	3	*E. faecium*	MT116082
VA3	1	*L. mesenteroides*	MT116083

Figure 3. Differentiation of LAB isolates from laurel. A, phenotypic grouping. B, dendrogram obtained with combined RAPD-PCR patterns. Abbreviations: C, coccus; sc, short chain. *E.*, *Enterococcus*; *L.*, *Leuconostoc*.

Isolation, grouping and genetic identification of LAB

After enumeration, 121 colonies were collected from the agar media used to count LAB populations. All cultures were microscopically inspected and separated into 104 cocci and barely 17 rods. After preliminary characterization, only 57 cocci, all isolated from laurel samples, resulted Gram-positive and catalase-negative. The phenotypic differentiation of the presumptive LAB isolates allowed their separation into five groups (Fig. 3A), while RAPD analysis showed the presence of 10 different strains (Fig. 3B).

Table 1. Distribution of LAB species within laurel samples.

Species	L0	L3	L4	L5
E. faecium	■	■	■	■
E. mundtii	■	■	■	■
E. raffinosus	■			■
L. mesenteroides	■			

Abbreviations: L0, untreated; L3, biomass density 3 kg/m^2; L4, biomass density 4 kg/m^2; L5, biomass density 5 kg/m^2; *E.*, *Enterococcus*; *L.*, *Leuconostoc*.

Table 2. Characteristics of the LAB strains studied.

Strain	Species	Antimicrobial resistance phenotype[a]	Antimicrobial MIC (μg/ml)			Antimicrobial genes	Gelatinase activity	Type of haemolysis
			E	CIP	C			
VA10	*E. faecium*	E-CIP	2	2	n.e.	n.d.	–	γ
VA15	*E. faecium*	CIP	n.e.	2	n.e.	n.d.	–	γ
VA18	*E. faecium*	CIP	n.e.	2	n.e.	n.d.	–	γ
VA12	*E. mundtii*	n.d.	n.e.	n.e.	n.e.	n.d.	–	γ
VA14	*E. mundtii*	n.d.	n.e.	n.e.	n.e.	n.d.	–	γ
VA17	*E. mundtii*	n.d.	n.e.	n.e.	n.e.	n.d.	–	γ
VA35	*E. faecium*	n.d.	n.e.	n.e.	n.e.	n.d.	–	γ
VA36	*E. mundtii*	E	2	n.e.	n.e.	n.d.	–	γ
VA46	*E. raffinosus*	C-QD	n.e.	n.e.	32	*cat*(pC221)	–	γ
VA3	*L. mesenteroides*	n.d.	n.e.	n.e.	n.e.	n.d.	–	γ

[a]P, penicillin; AMP, ampicillin; VA, vancomycin; E, erythromycin; TE, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin; C, chloramphenicol; QD, quinupristin-dalfopristin; L, linezolid; CN, gentamicin; STR, streptomycin. Abbreviations: *E.*, *Enterococcus*; *L.*, *Leuconostoc*; n.d., not detected (value < detection limit of method); n.e. (not evaluated).

The analysis by 16S rRNA gene sequencing indicated that the LAB community of laurel was mainly represented by the species *Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus raffinosus* and *Leuconostoc mesenteroides*.

The distribution of these species among laurel samples is reported in Table 1. *Enterococcus faecium* and *E. mundtii* were the only two LAB species found before and after drying and they were identified for the three different biomass densities employed (3, 4 and 5 kg/m^2). *Leuconostoc mesenteroides* was only detected in untreated laurel while, *E. raffinosus* was also detected after drying when the biomass density was 5 kg/m^2.

Safety aspects of LAB

The results of antimicrobial resistance, virulence and cellular toxicity are reported in Table 2. A total of five LAB out of ten identified displayed resistance to at least one of the antibiotics tested. In particular, *E. faecium* VA10 was resistant to erythromycin/ciprofloxacin, *E. raffinosus* VA46 to chloramphenicol/quinupristin-dalfopristin, *E. faecium* VA15 and VA18 to ciprofloxacin and *E. faecalis* VA36 to erythromycin. These five enterococci were subjected to the MIC determination by microdilution assay (Table 2). The results of this analysis confirmed the classification obtained from the disk diffusion method. The results related to the detection of antimicrobial resistance genes by PCR showed that *cat*(pC221) gene, associated with chloramphenicol resistance, was found in *E. raffinosus* VA46. The genes associated with the resistance to macrolide, lincosamide, streptogramin B (*E. faecium* VA10 and *E. mundtii* VA36) and fluoroquinolone (*E. faecium* VA10, VA15 and VA18) were not found.

Regarding gelatinase and haemolytic activity none of the strains tested was scored positive.

Technological traits of LAB

The five LAB strains susceptible to antibiotics and belonging to the species *E. faecium*, *E. mundtii* and *L. mesenteroides* were technologically characterized and the results are shown in Table 3. Regarding the acidification kinetics and autolysis, all strains showed a slow decrease of these parameters during the incubation. In particular, the pH of milk was above 6.00, while the autolysis ranged from 0.756 to 0.891 after 72 h. Only the strain *L. mesenteroides* VA3 was scored positive for diacetyl production. *E. faecium* VA35 was the only strain that displayed antimicrobial activity against *Ls. monocytogenes* and *Ls. innocua*.

DISCUSSION

Plant habitat represents a dynamic environment where the structure of the microbial communities is affected by several factors, including the interactions among the different populations (Araújo *et al.* 2002). Plant bacteria play different role, including the control of pathogens increasing the defence of the plants towards diseases (Berg and Hallmann 2006). Plant microbiomes are influenced also by plant secondary metabolites (Schmidt *et al.* 2014). Among these compounds, EOs play active roles in controlling several microorganisms, including fungi and bacteria (Fonseca *et al.* 2015; Kim *et al.* 2016). Aromatic plants are rich in EOs (Reichling *et al.* 2009) and for this reason, bacteria living in association with medicinal and aromatic herbs have to be tolerant to EOs synthesized by the plants (Tiwari *et al.* 2010). LAB are generally present in different plant tissues (Nguyen-The and Charlin 1994; Minervini *et al.* 2015; Alfonzo *et al.* 2017; Pontonio *et al.* 2018) even though their numbers are not high compared to other bacterial populations. They have been also isolated from aromatic plants such as *O. vulgare* (Pontonio *et al.* 2018). With this in mind, this work was carried out to investigate on the presence of LAB in two aromatic plants, sage and laurel, in order to isolate strains to be used in functional cheese production by addition of aromatic herbs rich in phenolic compounds that have to be resistance to plant EOs.

In order to evaluate the effect of the drying process on the viability of LAB, sage and laurel were investigated before and after drying. In particular, three different drying densities were applied and, regarding weight loss, no differences were registered. However, the drying density influenced the levels of both LAB cocci and rods of laurel and those of LAB cocci in sage, since their levels increased with biomass density. LAB rods were not detected in sage. So far, there are no works available in literature on the levels of LAB in fresh sage. Some works reported the presence of different bacterial groups in dried herbs including sage (Vitullo *et al.* 2011), but no data on LAB are available. This is the first work showing data on the levels of LAB in laurel.

After the isolation of LAB, all cultures from sage were found to be positive to catalase test and, for this reason, no more

Table 3. Technological characteristics of LAB isolated from laurel samples.

Strains	Species	pH/autolysis[a]								Diacetyl production	EPS production	Bacteriocin-like inhibitory activity[b] Indicator strains[c]			
		0 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h			19 114	4202	2313	
VA35	*E. faecium*	6.74/0.997	6.74/0.997	6.71/0.997	6.70/0.995	6.47/0.995	6.45/0.857	6.33/0.857	6.32/0.856	–	–	2.5 ± 0.17	1.66 ± 0.10	–	
VA12	*E. mundtii*	6.74/0.999	6.72/0.996	6.70/0.996	6.66/0.983	6.62/0.980	6.48/0.810	6.34/0.794	6.22/0.793	–	–	–	–	–	
VA14	*E. mundtii*	6.75/0.998	6.70/0.998	6.66/0.998	6.62/0.992	6.60/0.990	6.48/0.902	6.34/0.818	6.22/0.756	–	–	–	–	–	
VA17	*E. mundtii*	6.72/0.999	6.67/0.998	6.65/0.998	6.61/0.994	6.59/0.994	6.51/0.835	6.44/0.833	6.26/0.830	–	–	–	–	–	
VA3	*L. mesenteroides*	6.75/0.996	6.74/0.996	6.74/0.993	6.71/0.991	6.70/0.990	6.57/0.916	6.40/0.892	6.24/0.891	–	+	–	–	–	

[a]Results of pH and autolysis indicate mean values of two independent experiments.
[b]With of the inhibition zone (millimeters). Results indicate mean ± standard deviation of three independent experiments.
[c]Bacterial species: *Listeria monocytogenes* ATCC 19 114; *Listeria innocua* 4202; *Lactobacillus sakei* 2313.
Abbreviations: *E.*, *Enterococcus*; *L.*, *Leuconostoc*; EPS, exopolysaccharide.

characterized. These findings are consistent with earlier studies reporting that LAB are rare contaminants in spices and dried herbs (Baxter and Holzapfel 1982; Säde, Lassila and Björkroth 2016). However, the search of LAB in fresh aromatic plants is not very common (Pontonio *et al.* 2018). All isolates from laurel were processed because they showed the main phenotypic traits of LAB as being catalase-negative Gram positive bacteria. Basically, the putative LAB isolated from MRS and M17 showed similar characteristics and when analyzed by RAPD-PCR they shared almost the same polymorphic profiles, indicating that the two media, although at different levels, allowed the growth of the same bacteria. Considering the highest levels registered for LAB cocci, we can affirm that MRS allowed the growth of bacteria grown well in M17. This finding confirmed the observation of a previous study by Settanni *et al.* (2012) who noticed that LAB cocci are able to develop on MRS, probably due to the low pH of this medium after acidification with lactic acid.

A total of 10 different strains were identified and allotted into *E. faecium*, *E. mundtii*, *E. raffinosus* and *L. mesenteroides*. Enterococci grow well in M17 medium and are also able to develop on MRS and this explains why the same RAPD patterns were shown by bacteria grown in M17 and MRS. Among the species identified, *L. mesenteroides* is commonly allotted into the group of LAB of dairy interest (Settanni and Moschetti 2010) while enterococci are responsible of the typicality of certain cheeses (FoulquiéMoreno *et al.* 2006) and commonly isolated from cheeses (Franciosi *et al.* 2008; Gaglio *et al.* 2014), even though their antibiotic resistance and virulence genes (Gaglio *et al.* 2016) are negative traits for their use in cheese production.

The distribution of the species among the samples showed how drying process affected LAB biodiversity. *Leuconostoc mesenteroides* did not tolerate the heat treatment, since it was isolated only from fresh laurel leaves. *Enterococcus raffinosus*, was isolated also after drying, but only when the density was high (5 kg/m^2), while no difference among treatment and drying density was registered for *E. faecium* and *E. mundtii*. Both species are generally found associated to vegetables (Bennik *et al.* 1998; Ponce *et al.* 2008) and *E. mundtii* was also identified from the aromatic plant oregano (Pontonio *et al.* 2018).

In view of their use in cheese production all bacteria were analyzed for their safety aspects. Only five strains, all enterococci, were resistant to at least one antibiotic, while none of the ten LAB showed gelatinase or haemolytic activity. The investigation on the technological traits useful in cheese making demonstrated that all LAB can be considered NSLAB.

In conclusion, the selection of LAB resistant to aromatic plant EOs based on the isolation from sage and laurel barely allowed the identification of NSLAB strains. Further screenings including different aromatic herbs are necessary to isolate SLAB. Works are also necessary to evaluate the interaction among the chemical compounds released by the herbs and the aromatic impact due to LAB in cheeses. Moreover, the strong reduction of LAB viability due to the dehydration process suggests to analyse LAB content of aromatic plants before the application of drying processes. In this work we provided a clear explanation to the common lack of LAB in dried herbs.

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