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Insight into the role of lactic acid bacteria in the development of a novel fermented pistachio (*Pistacia vera* L.) beverage

Tiziana Di Renzo [a], Andrea Osimani [a,b], Serena Marulo [a], Federica Cardinali [b], Gianfranco Mamone [a], Cecilia Puppo [c], Antonela G. Garzón [d], Silvina R. Drago [d], Carmine Laurino [a], Anna Reale [a,*]

[a] Institute of Food Sciences, National Research Council (CNR-ISA), Via Roma 64, 83100, Avellino, Italy
[b] Dipartimento di Scienze Agrarie, Alimentari ed Ambientali (D3A), Università Politecnica delle Marche, via Brecce Bianche, 60131, Ancona, Italy
[c] CIDCA-UNLP-CONICET, 47 y 116 s/n, 1900, La Plata, Argentina
[d] Instituto de Tecnología de Alimentos, CONICET, Facultad de Ingeniería Química – Univerisdad Nacional del Litoral, 1° de Mayo 3250, 3000, Santa Fe, Argentina

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ABSTRACT

This study aimed to assess the fermentability of a pistachio-based beverage. The effects of lactic acid fermentation on the odor profile and the stability of the beverage were also assessed. To this end, a procedure for obtaining a pistachio beverage was developed, and the nutritional composition of the beverage was obtained. The beverage was characterized by a high content of fiber (2.9%), fat (4.6%), and protein (2%), and contained high amounts of K and Mg. For the experiment, 43 cultures of the following lactic acid bacteria were used: *Companilactibacillus paralimentarius*, *Companilactibacillus kimchi*, *Levilactibacillus brevis*, *Lactiplantibacillu pentosus*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus paraplantarum*, *Latilactobacillus curvatus*, *Leuconostoc pseudomesenteroides*, and *Furfurilactobacillus rossiae*. The cultures were evaluated for the following technological characteristics: i) growth and acidifying activity, ii) production of exopolysaccharides; iii) presence of histidine decarboxylase (*hdcA)* gene; iv) antimicrobial activity against *Listeria innocua*. The cultures were used in model pistachio-based fermentations to assess their ability to grow and acidify. The odor profile of the beverage after 24 h fermentation was assessed using an electronic nose system (E-nose). The pistachio-based beverage resulted an optimal substrate for lactic acid bacteria that reached loads between 8 and 10 log CFU/mL and acidified the beverage to pH values between 4 and 5.5. In the beverage, viable counts of all cultures remained above 8 log CFU/mL after 30 days of storage at 4 °C. The E-nose analysis represented a valid screening method to evaluate the effects of the fermentative activity of microbial starters on the qualitative characteristics of this novel food matrix.

1. Introduction

The pistachio plant (*Pistacia vera* L., family Anacardiaceae) is one of the oldest cultivated trees worldwide. Today, pistachios are produced in the United States (especially California), Iran, Turkey, China, Syria, Greece, Argentina, and Italy. Particularly in Italy, a pistachio cultivar (*Pistacia vera*, cultivar *Napoletana*, grafted on *Pistacia terebinthus*) of high quality is the typical one from Bronte (Sicily), that obtained in 2010 the Protected Designation of Origin (PDO) from the European Community (Commission Regulation No. 21/2010) in recognition of the special procedure for the cultivation and conservation of biodiversity (Jeffrey et al., 2018; Lucarini et al., 2020).

Pistachios are mainly used as snacks, either raw or roasted, and are an ingredient in the production of fermented meats, ice creams, breads, sauces, and pudding (Tomaino et al., 2010). Pistachios, like most nuts (almonds, hazelnuts, pine nuts, walnuts, etc.), seeds (sesame, flax, etc.), and legumes (soybean, peanut, etc.) have interesting nutritional properties due to the presence of unsaturated lipids, phenolic compounds, tocopherols, and phytosterols, which play an antioxidant, immune, and anti-inflammatory role, with beneficial effects on the prevention of cardiovascular diseases (Bullò et al., 2015; Grosso et al., 2015; Lucarini et al., 2020; Santini & Novellino, 2017). In addition, pistachios are also a source of protein, dietary fibre, minerals (magnesium, potassium, copper, selenium), and vitamins (folate, vitamin E and vitamin K) (Dreher,

* Corresponding author.
E-mail address: anna.reale@isa.cnr.it (A. Reale).

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2012; Gentile et al., 2007; Giuffrifda et al., 2006; Ryan et al., 2006; Saitta et al., 2009, 2014).

These nutritional characteristics make pistachios interesting and versatile for the production of high-value foods. More recently, the increase in food allergies and intolerances, as well as the demand for healthier plant-based products even as alternatives to dairy products, has led to the development of plant-based beverages, i.e. "ready-to-drink" foods based on nuts (almonds, hazelnuts, and walnuts), seeds (sesame and quinoa), legumes (soy, peanuts, and lentils), with more than 130 varieties of plant-based milk substitutes (PBMSs) just in Europe (Cardinali et al., 2021; Coda et al., 2017; Menezes et al., 2018; Muncey & Hekmat, 2021; Tsafrakidou et al., 2020; Verni et al., 2020). These new beverages have received great interest globally in response to major food consumption trends such as vegetarianism, veganism, and other health trends (Ranadheera et al., 2017). However, such beverages may have some low nutritional, quality and sensory deficiencies that can be improved with the use of lactic acid fermentation (Verni et al., 2020). Microbial fermentation (mainly that of lactic acid bacteria) can add value to these beverages, not only by stabilizing the product, but also by improving the taste and functional characteristics of the finished products (Ashaolu & Reale, 2020).

The use of lactic acid bacteria for producing fermented beverages has a long tradition. Indeed, cereal-based fermented beverages (e.g., boza, bouza, kvass, mahewu, seketeh, tapuy), produced with the aid of lactic acid bacteria, are part of the diet of many populations worldwide (Blandino et al., 2003). Moreover, numerous innovative fermented beverages based on non-cereal substrates have been developed exploiting selected lactic acid bacteria as starter cultures, including quinoa-based beverage, buckwheat beverage, and pea beverage (Boukid et al., 2021; Cardinali et al., 2021; de Souza et al., 2023). Of note, in order to obtain novel beverages with distinct techno-functional and sensory characteristics, each process should be well-designed taking into consideration the interaction between the fermenting microbial cultures and the substrate (de Souza et al., 2023). Hence, a case-by-case approach should be applied in the development of fermented beverages based on non-conventional substrates.

Although lactic acid bacteria are generally recognized as safe, their metabolic activity can produce unwanted substances, as biogenic amines (e.g., histamine), with negative impact on the safety of the fermented food (Ordóñez et al., 2016). Of note, the presence of histamine in food is associated with the so-called scombroid poisoning, whose symptoms include headaches, palpitations, and vomiting. In lactic acid bacteria, the production of histamine is encoded by a gene cluster that includes the *hdcA* gene, hence, the absence of this gene should be assessed in order to select lactic acid bacteria as starter cultures (Osimani et al., 2023).

To the authors' knowledge, there is little or no information in the scientific literature on the fermentation of pistachio-based beverage.

Therefore, the aim of the present work was to gain a first insight into the role of lactic acid bacteria in the development of a novel pistachio-based fermented beverage. First, a total of 43 lactic acid bacteria were identified by sequencing the 16S rRNA region and characterized for growth capacity, acidifying activity, ability to produce exopolysaccharides (EPS), *hdcA* gene detection, and antimicrobial activity against *Listeria innocua* used as a surrogate for Listeria monocytogenes (ANSES, 2019). Second, a protocol for the production of a pistachio beverage was developed. Subsequently, the lactic acid bacteria cultures were used in model-based pistachio fermentation tests to assess their fermentation behavior in the beverage and an electronic nose (E-nose) was used to differentiate the fermented samples based on their odor profile.

2. Materials and methods

2.1. Raw material

The Green Pistachio nuts (*Pistacia vera* L. Var. *Bronte*) of Bronte PDO were harvested in the year 2019 and purchased from Aroma Sicilia farm (Bronte, Southern Italy). Pistachio nuts used for the experiments consisted of shelled, unsalted, and unroasted, and had the following proximal composition: 6.0% moisture, 18.1% proteins, 56.1% lipids, 8.1% carbohydrates, 10.6% dietary fiber, and 1% ash.

2.2. Microbial strains

In this study, a total of 43 lactic acid bacteria isolated from different foodstuffs and belonging to the microbial culture collection of the Institute of Food Sciences - National Research Council (ISA-CNR, Avellino, Italy) were used. The strains were maintained as frozen stocks (in 50% glycerol v/v) and routinely propagated in DeMan Rogosa and Sharpe (MRS) medium (Oxoid, Milan, Italy), pH 6.8 for 24 h at 30 °C.

2.3. Lactic acid bacteria identification

DNA of lactic acid bacteria was extracted and amplified according to Osimani et al. (2015). Briefly a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) was used to evaluate the purity and quantity of the extracted DNA. DNAs were standardized to a final concentration of 100 ng/μL and subjected to PCR in a Mas-tercycler X50a (Eppendorf, Hamburg, Germany) using the universal prokaryotic primers 27f and 1495r. The obtained amplicons were then shipped to Azenta (Leipzig, Germany) for their purification and sequencing.

The raw sequences of the isolated lactic acid bacteria, obtained in FASTA format, were analyzed using the BLAST (Basic Local Alignment Search Tool) to compare the obtained sequences with the 16S rRNA sequences of type strains from GenBank DNA database (http://www.ncbi.nlm.nih.gov/).

2.4. Screening of growth performances and acidifying capacity

The growth kinetics and acidifying capacity were determined for each lactic acid bacteria culture. Briefly, bacterial suspensions were prepared in MRS broth and incubated at 30 °C overnight. Then, cultures were inoculated in MRS broth to reach an initial absorbance of 0.05 at 595 nm (OD_{595nm}) and incubated at 30 °C for 24 h. Optical density (OD_{595nm} measurements) and pH values were performed every hour for the first 10h, and then after 24 h of cultivation. MRS broth without inoculum was used as control.

The maximum specific growth rate (μmax) was obtained by plotting the natural logarithm of OD_{595nm} values against the incubation time and then fitting a linear regression model to the experimental data within the exponential growth phase. The slope of linear proportion of growth curves corresponds to the μmax.

The acidifying capability of the strains was assessed by pH decrease of the MRS medium (ΔpH, calculated as the pH difference at time zero and after 6 and 24 h). All experiments were done in triplicate and average values were taken.

2.5. EPS production of lactic acid bacteria

The 43 lactic acid bacteria were tested for the EPS production, based on the method reported by Hilbig et al. (2019) with some modifications. Briefly, lactic acid bacteria were thawed from cryosuspensions and cultured twice on MRS broth at 30 °C for 48 h. MRS agar supplemented with sucrose was used to promote the synthesis of homopolysaccharides (HoPS), whereas MRS agar supplemented with yeast extract, meat extract, galactose, and lactose was used to promote the synthesis of heteropolysaccharides (HePS). After the incubation at 30 °C for 48 h,

colonies were classified as positive when they showed a mucoid or a ropy appearance (capable of producing detectable filaments using a sterile toothpick).

2.6. Detection of the hdcA gene in lactic acid bacteria

The 43 lactic acid bacteria were screened for the presence of the *hdcA* gene. A PCR was carried out in a CFX Connect TM Real-Time System (BioRad Laboratories, Hercules, CA, USA) under the conditions already described by Belleggia, Ferrocino, Corvaglia, et al. (2022).

2.7. Antimicrobial activity of lactic acid bacteria

The 43 lactic acid bacteria were tested for antimicrobial activity against *Listeria innocua*, used as a surrogate for *Listeria monocytogenes* (ANSES, 2019). The antimicrobial activity of isolates was performed by following the agar well diffusion assay, described by Belleggia, Ferrocino, Corvaglia, et al. (2022).

2.8. Pistachio beverage preparation

The procedure for preparing the pistachio-based fermented beverage is shown in Fig. 1. The beverage was obtained as described by Sánchez-Bravo et al. (2020) with some modifications. Briefly, 1 kg of Green pistachio nuts of Bronte PDO (shelled, unsalted, and unroasted) were washed, then dipped in tap water at room temperature (25 °C ± 2 °C) for 5 h. After hydration, the soaked nuts were drained and weighed. Then, hot water (80 °C) was added to the hydrated nuts to obtain a 1:5 (pistachios/water) ratio. The nuts were ground using a mixer (Thermomix TM31). Then, the mixture was filtered through a

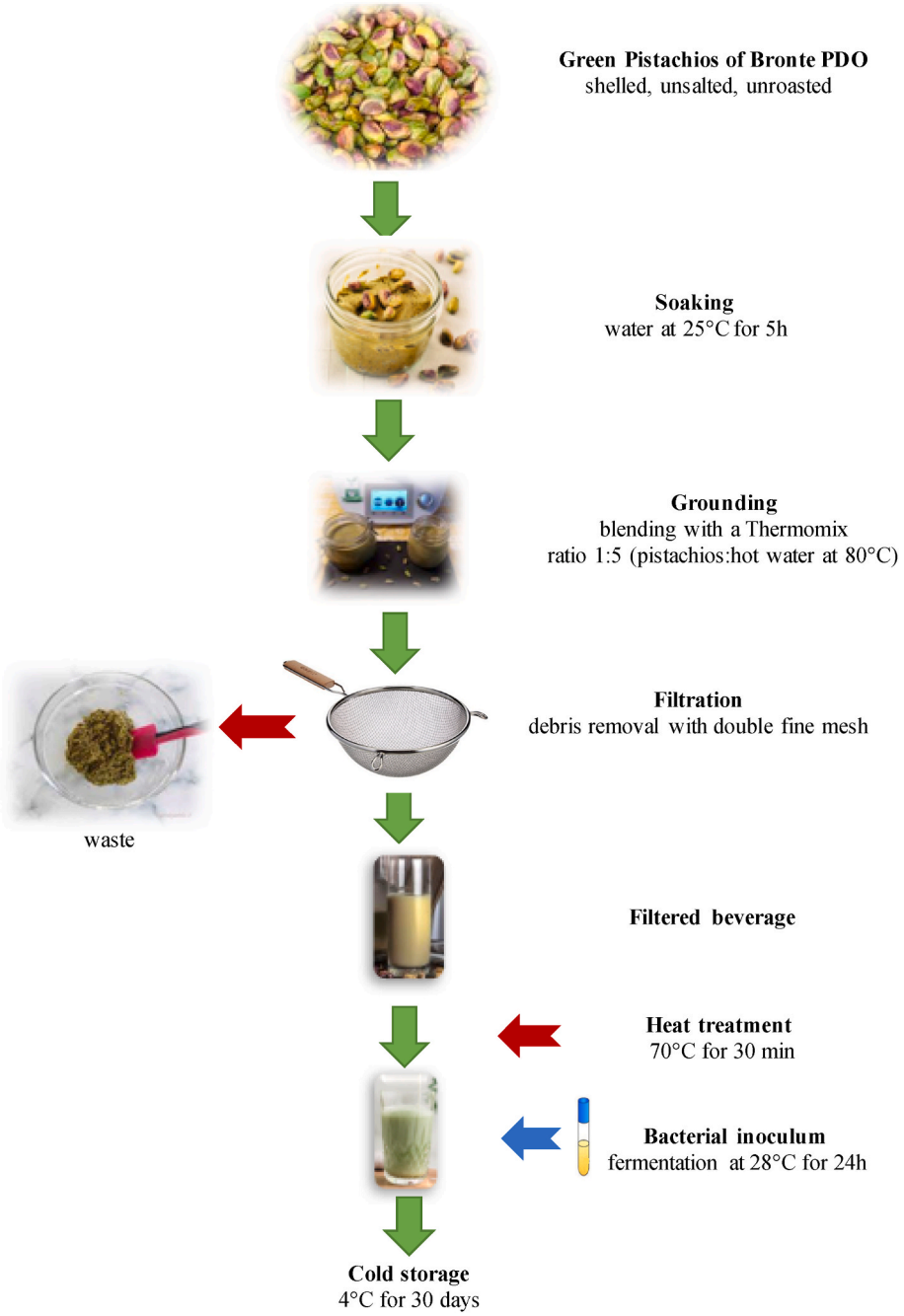

Fig. 1. Flow chart for the preparation of the fermented based-pistachio beverage.

double fine mesh strainer to remove solid particles and it was heat-treated at 70 °C for 30 min. The pistachio beverage was stored at 4 °C until microbial inoculum. Pistachio beverage was inoculated with the different lactic acid bacteria at a final concentration of about 6 log CFU/mL. Fermentation was carried out at 30 °C for 24h; after that, the beverages were stored at 4 °C for 30 days. The samples were analyzed for pH, microbial characteristics, and odors using an E-nose at time zero, after 24h fermentation, and after 30 days of storage.

2.8.1. Proximate composition of pistachio beverage

Moisture, fat (ether extract), protein, and ash contents were determined according to the AOAC methods (2000). Total dietary fiber was determined by AOAC 985.29 (2000) method using the Megazyme® commercial kit. Carbohydrates different from fiber were determined by difference. Samples were dry ashed and the mineral content was evaluated with an Analyst 300 atomic absorption spectrophotometer (Perkin Elmer IL 551, Instrumentation Laboratory, Norwood, Massachusetts, USA). The minerals analyzed were Fe, Zn, Ca, Cu, Mg (by flame atomic absorption spectroscopy), Na, and K (by flame photometry).

2.8.2. Microbiological analysis of the beverage

Pistachio beverage was analyzed before and after heat treatment, and at the end of cold storage for the presence of Enterobacteriaceae, fecal and total coliforms, enterococci, total mesophilic bacteria, yeasts and molds as described by Reale et al. (2009), and Pseudomonadaceae as reported by Belleggia et al. (2022b). The lactic acid bacteria count was determined using MRS medium agar supplemented with cycloheximide (0.1 g/L). Plates were incubated in anaerobiosis (AnaeroGen and AnaeroJar, Oxoid) at 30 °C for 48 h. Furthermore, the acidifying activity of the cultures in the pistachio beverage was evaluated by measuring pH at time zero and after 24 h fermentation with a pH-meter Crison (Model 507, Crison, Milan, Italy).

2.8.3. Evaluation of pistachio beverage samples by E-nose

The odor profiles from the headspace of pistachio beverage samples were carried out using a commercial portable E-nose (PEN 3, Airsense Analytics Inc., Schwerin, Germany, including the Win Muster software). The E-nose consists of a sampling unit and a gas detection system equipped with an array of 10 metal oxide semiconductors (MOS) sensors (Supplementary Table S1) to offer selectivity toward various volatile classes, as reported by Shi et al. (2020). The analyses were carried out on the pistachio beverages fermented for 24h with the 43 different cultures of lactic acid bacteria. A pistachio beverage without inoculum (Ctr, control sample) was used in comparison. For sample preparation, 5 mL of each sample were put in 45 mL airtight glass vials and sealed with a screw cap with Poly (1,1,2,2-tetrafluoroethylene) (PTFE)/silicone septum. To reach the headspace equilibrium, each vial was kept at 30 °C for 30 min and analyzed at 22 °C ± 2 °C and 50% ± 5% relative humidity (RH). During the measurement time, the gas headspace was injected into the E-nose for 80 s at 400 mL/min. Analyses were conducted in five technical replicates for each biological sample and data were collected at each second. E-nose data were collected by the pattern recognition software (WinMuster, v.1.6., Airsense Analytics GmbH, Germany) and the average of each sensor response in the range from 70 to 75 s (area under the curve) was used for statistical data analysis.

2.9. Statistical analysis

The chemical and microbiological analyses were carried out in triplicate. Mean values and standard deviation were calculated. Analysis of variance was performed to determine significant differences ($p < 0.05$) between means. Graphs were obtained using SYSTAT 13.0 for Windows (Systat Software Inc., Richmond, CA, USA). The data obtained from the sensor array of the E-nose were analyzed by principal component analysis (PCA) using the free software Tanagra, version 1.4, (Rakotomalala, 2005). The software was free-downloaded by https://tanagra.software.informer.com/download/#downloading (Accessed 2022).

3. Results and discussion

The results of lactic acid bacteria identification and technological characterization are reported in Table 1.

The strains analyzed for the study were 5 *Levilactobacillus brevis*, 3 *Companilactobacillus kimchii*, 24 *Lactiplantibacillus plantarum*, 5 *Lactiplantibacillus paraplantarum*, 1 *Lactiplantibacillus herbarum*, 1 *Latilactobacillus curvatus*, 2 *Companilactobacillus alimentarius*, 1 *Furfurilactobacillus rossiae*, 1 *Leuconostoc pseudomesenteroides*. The maximum growth rate (μmax) of the selected lactic acid bacteria varied from 0.120 to 0.208 (h^{-1}). The strains with the highest rates belonged to *L. plantarum* species, such as *L. plantarum* PC4 (0.208 ± 0.018), PG2 (0.201 ± 0.022) and PP5 (0.199 ± 0.021). On the other hand, the species with the lowest growth rate was *L. brevis* (0.120 ± 0.008).

The results of the screening of lactic acid bacteria for EPS production showed that most of the strains were unable to produce EPS, except for *Leuc. pseudomesenteroides* PD4 and *L. plantarum* PI1 strains. Microbial EPS are produced outside cells and released into the surrounding environment. For the producer cell, EPS act as a carbon reserve and water-binder, thus enabling the producer microorganism to withstand harsh conditions such as heat, desiccation, osmotic, and acidic stress (Reale et al., 2020). Moreover, EPS protect the producing microbial cell from the effect of antibiotics, poisonous compounds, and from phagocytosis by the host immune system. EPS (in form of homopolysaccharides or heteropolysaccharides) produced by lactic acid bacteria have attracted further attention as they can act as gelling agents, emulsifiers, stabilizers, water-binders, and viscosifying agents (Daba et al., 2021). It is known that *Leuc. pseudomesenteroides* is considered an important producer of microbial EPS (e.g., α-D-glucans) (Pan et al., 2022), being this microorganism capable to convert sucrose to dextran with potential positive applications in the food industry, such as improvement of softness in bakery products, prevention of crystallization, improvement of moisture retention and increase of viscosity in confectionary or ice cream, as well as adjuvant, emulsifying, and stabilizing effects (Farinazzo et al., 2020; Korcz & Varga, 2021). Of note, the novel fermented pistachio beverage herein studied did not contain added sugars (e.g., sucrose), hence, the sucrose-independent EPS production showed by *Leuc. pseudomesenteroides* isolate PD4 can be particularly attractive in obtaining a sucrose-free beverage. Regarding *L. plantarum*, EPS produced by this lactic acid bacterium showed important technological characteristics that include the improvement of texture and rheological properties of dairy products, as well as prebiotic and antioxidant effects (e.g., scavenging of reactive oxygen species and reduction of lipid peroxidation) (Korcz & Varga, 2021).

It is noteworthy that the techno-functional features of EPS vary according to their monosaccharide composition and process conditions (e.g., pH of the fermented matrix) (Yalmanci et al., 2022). Hence, in order to optimize the production of EPS by the isolates herein tested, the monosaccharide characterization should be performed.

None of the tested cultures showed antimicrobial activity against *L. innocua*. Indeed, no halo of inhibition was observed in the agar-well diffusion assay for any of the strains tested. The production of antimicrobial compounds (e.g., bacteriocins) by lactic acid bacteria is widely reported in the scientific literature (Perez et al., 2022). However, the ability of lactic acid bacteria to produce such molecules is strictly strain-dependent and is mediated by the expression of many genes involved in the processing of precursor peptides, secretion, autoimmunity, and regulation of their production (Perez et al., 2022).

The result of *hdcA* gene detection performed on lactic acid bacteria showed that no culture tested positive for the target gene. The *hdcA* gene of Gram-positive bacteria encodes for the pyruvoyl-dependent decarboxylase of the amino acid histidine whose decarboxylation leads to the production of histamine (Hilbig et al., 2019). The absence of the *hdcA*

Table 1
Identification and technological characteristics of the lactic acid bacteria strains used in the study.

Strain	Species	Accession no.	% identity	Growth rate (h^{-1})	EPS production		Antimicrobial activity	*hdcA* gene	Reference
					Sucrose dependent	Sucrose independent			
PA1	*Companilactobacillus kimchi*	NR_025045	100%	$0.145 \pm 0.012^{e,f}$	–	–	–	–	NI
PA2	*Companilactobacillus kimchi*	NR_025045	100%	$0.157 \pm 0.014^{d,e,f}$	–	–	–	–	NI
PA4	*Levilactobacillus brevis*	NR_044704	99.05%	$0.167 \pm 0.018^{b,c,d,e,f}$	–	–	–	–	NI
PA5	*Levilactobacillus brevis*	NR_116238	99.51%	$0.193 \pm 0.020^{a,b,c,d}$	–	–	–	–	NI
PA6	*Levilactobacillus brevis*	NR_044704	98.94%	$0.173 \pm 0.022^{a,b,c,d,e}$	–	–	–	–	[31]
PA7	*Levilactobacillus brevis*	NR_116238	98.64%	0.120 ± 0.008^{g}	–	–	–	–	NI
PC4	*Lactiplantibacillus plantarum*	NR_117813	99.51%	0.208 ± 0.018^{a}	–	–	–	–	NI
PD2	*Levilactobacillus brevis*	NR_044704	99.40%	$0.176 \pm 0.011^{b,c,d}$	–	–	–	–	NI
PD3	*Lactiplantibacillus plantarum*	NR_117813	99.03%	$0.156 \pm 0.010^{d,e,f}$	–	–	–	–	NI
PD4	*Leuconostoc pseudomesenteroides*	NR_040814	98.56%	$0.131 \pm 0.011^{f,g}$	+	+	–	–	[31]
PD5	*Lactiplantibacillus plantarum*	NR_113338	99.75%	0.141 ± 0.009^{f}	–	–	–	–	NI
PD6	*Lactiplantibacillus plantarum*	NR_113338	98.73%	$0.163 \pm 0.014^{c,d,e,f}$	–	–	–	–	NI
PE2	*Lactiplantibacillus plantarum*	NR_042394	99.64%	$0.171 \pm 0.013^{b,c,d}$	–	–	–	–	NI
PE3	*Lactiplantibacillus plantarum*	NR_042394	84.74%	$0.165 \pm 0.011^{c,d,e}$	–	–	–	–	NI
PE4	*Lactiplantibacillus plantarum*	NR_113338	84.74%	0.169 ± 0.004^{d}	–	–	–	–	NI
PE5	*Lactiplantibacillus plantarum*	NR_117813	99.64%	$0.165 \pm 0.012^{c,d,e}$	–	–	–	–	NI
PG1	*Lactiplantibacillus plantarum*	NR_117813	99.30%	$0.173 \pm 0.021^{a,b,c,d,e}$	–	–	–	–	NI
PG2	*Lactiplantibacillus plantarum*	NR_117813	98.90%	$0.201 \pm 0.022^{a,b}$	–	–	–	–	NI
PG3	*Companilactobacillus alimentarius*	NR_025045	84.81%	$0.164 \pm 0.013^{c,d,e}$	–	–	–	–	NI
PG4	*Companilactobacillus alimentarius*	NR_025045	99.74%	$0.166 \pm 0.010^{c,d,e}$	–	–	–	–	[31]
PI1	*Lactiplantibacillus plantarum*	NR_117813	99.02%	$0.141 \pm 0.012^{e,f}$	+	–	–	–	[31]
PI2	*Lactiplantibacillus herbarum*	NR_145899	83.44%	$0.182 \pm 0.022^{a,b,c,d}$	–	–	–	–	NI
PI3	*Lactiplantibacillus plantarum*	NR_104573	99.79%	$0.190 \pm 0.012^{a,b}$	–	–	–	–	NI
PI4	*Lactiplantibacillus plantarum*	NR_104573	99.17%	$0.183 \pm 0.032^{a,b,c,d,e}$	–	–	–	–	NI
PN1	*Lactiplantibacillus paraplantarum*	NR_025447	99.49%	$0.180 \pm 0.026^{a,b,c,d,e}$	–	–	–	–	NI
PN3	*Lactiplantibacillus paraplantarum*	NR_025447	99.67%	$0.178 \pm 0.016^{a,b,c,d}$	–	–	–	–	NI
PN4	*Lactiplantibacillus paraplantarum*	NR_025447	99.73%	$0.170 \pm 0.011^{c,d}$	–	–	–	–	NI
PO1	*Latilactobacillus curvatus*	NR_113334	99.13%	$0.177 \pm 0.006^{b,c,d}$	–	–	–	–	NI
PO2	*Lactiplantibacillus paraplantarum*	NR_025447	87.62%	$0.161 \pm 0.013^{c,d,e,f}$	–	–	–	–	NI
PP1	*Lactiplantibacillus plantarum*	NR_113338	100%	$0.135 \pm 0.014^{f,g}$	–	–	–	–	NI
PP2	*Lactiplantibacillus paraplantarum*	NR_025447	99.91%	$0.183 \pm 0.024^{a,b,c,d}$	–	–	–	–	NI
PP5	*Lactiplantibacillus plantarum*	NR_115605	99.29%	$0.199 \pm 0.021^{a,b}$	–	–	–	–	NI
PP6	*Lactiplantibacillus plantarum*	NR_115605	99.29%	0.140 ± 0.011^{f}	–	–	–	–	NI
PR1	*Lactiplantibacillus plantarum*	NR_113338	99.38%	0.196 ± 0.012^{a}	–	–	–	–	NI
PR2	*Lactiplantibacillus plantarum*	NR_113338	99.38%	$0.197 \pm 0.014^{a,b}$	–	–	–	–	NI
PR3	*Lactiplantibacillus plantarum*	NR_113338	99.50%	$0.167 \pm 0.012^{c,d,e}$	–	–	–	–	NI
PT1	*Lactiplantibacillus plantarum*	NR_115605	99.38%	$0.185 \pm 0.024^{a,b,c,d}$	–	–	–	–	NI
PT5	*Lactiplantibacillus plantarum*	NR_115605	99.38%	$0.194 \pm 0.022^{a,b,c,d}$	–	–	–	–	NI
PT6	*Lactiplantibacillus plantarum*	NR_117813	98.97%	$0.185 \pm 0.031^{a,b,c,d,e}$	–	–	–	–	NI

(*continued on next page*)

Table 1 (*continued*)

Strain	Species	Accession no.	% identity	Growth rate (h^{-1})	EPS production		Antimicrobial activity	*hdcA* gene	Reference
					Sucrose dependent	Sucrose independent			
PU2	*Companilactobacillus kimchii*	NR_025045	99.73%	0.176 ± 0.012 ^{b,c,d}	–	–	–	–	NI
PY4	*Lactiplantibacillus plantarum*	NR_113338	99.65%	0.188 ± 0.011 ^{a,b}	–	–	–	–	NI
PV2	*Lactiplantibacillus plantarum*	NR_117813	99.56%	0.161 ± 0.008 ^{d,e,f}	–	–	–	–	NI
PV4	*Furfurilactobacillus rossiae*	NR_029014	100%	0.172 ± 0.002 ^{c,d}	–	–	–	–	NI

NI = new identification.
Values were obtained from three replicates and shown as the means ± standard deviation. Different letters (a,b,c,d,e,f,g) in each column indicate significant differences.

gene in the analyzed isolates represents a positive trait in order to select them as potential starter cultures for the production of the experimental pistachio-based beverage.

Fig. 2 (panel A) shows the results of the acidifying ability of lactic acid bacteria after 6 and 24h of fermentation. Significant differences were found among the lactic acid bacteria species and strains in their ability to reduce pH, which became more pronounced during incubation. After 6h of incubation, lactic acid bacteria determined a reduction in the pH of the substrate between 0.8 and 1.5 units. On the other hand, after 24 h it was possible to distinguish a highly acidifying group (16 strains), which reached a ΔpH of approximately 3, and a moderately acidifying group, which reached a ΔpH of approximately 2 (27 strains). The selection of starter cultures according to the acidification rate is one of the main selection criteria, as it can significantly influence the characteristics of the end products.

Fig. 2 (panel B) shows the distribution of lactic acid bacteria species according to the acidification ability after 24h. A wide heterogeneity was observed. The species *F. rossiae*, *L. herbarum*, *C. alimentarius* and *C. kimchii* produced the highest substrate acidification. Within the species *L. plantarum*, highly and moderately acidifying cultures were identified. The cultures belonging to the species *L. brevis*, *L. paraplantarum*, *L. curvatus* and *L. pseudomesenteroides* had moderate acidifying activity, reaching a pH value of about 4 after 24h of fermentation. Therefore, the cultures were used in fermentation trials in a model-pistachio beverage.

The beverage, produced as described in Fig. 1, was microbiologically analyzed before and after heat treatment. Before heat treatment, discrete amounts of Enterobacteriaceae (5.3 log CFU/mL), total and faecal coliforms (3.7 and 2.8 log CFU/mL, respectively), enterococci (3.0 log CFU/mL), total mesophilic aerobes (6.1 log CFU/mL), eumycetes (4.6 log CFU/mL), and Pseudomonas spp. (2.8 log CFU/mL) were detected. In the raw material, lactic acid bacteria were <1 log CFU/mL. These data were in agreement with other authors who have pointed out how pistachios are possible sources of food contamination because, although increased good hygiene practices are particularly recommended for pistachios production, the harvesting and processing of pistachios could lead to unsatisfactory hygiene traits (Al-Moghazy & Pulvirenti, 2014). The high load of microorganisms mentioned resulted not only from the raw material, but also from contamination during the various production steps of the beverage (maceration of the pistachios in water, homogenization, filtration, etc.) (data not shown). Therefore, considering the high amount of microbial contamination found in the beverage produced in this study, a microbiological decontamination treatment is recommended. Heat treatment at 70 °C for 30 min restored the beverage. In fact, for all microbial groups, the count was below 1 log CFU/mL, thus indicating the safety of the beverage. The chemical

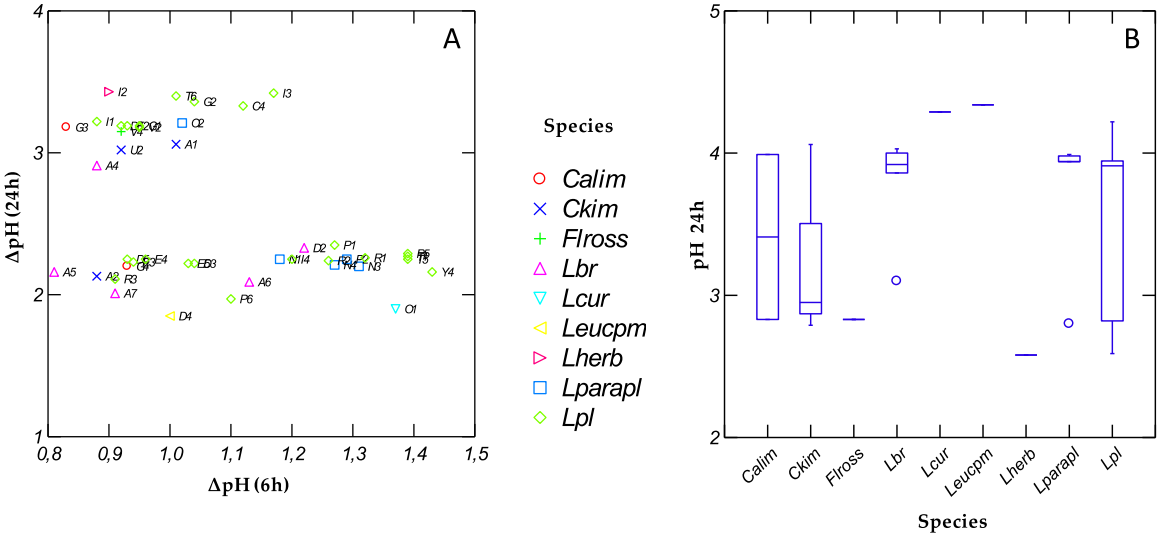

Fig. 2. Distribution of lactic acid bacteria cultures on the basis of acidifying capacity after 6 and 24h of incubation at 30 °C. Results are reported as pH variation (ΔpH) between time zero and 6 and 24h (panel A). Box plot diagram showing the distribution of strains, separated by species according to the acidifying ability (panel B). Calim, *Companilactobacillus alimentarius*; Ckim, *Companilactobacillus kimchi*, Flross, *Furfurilactobacillus rossiae*; Lbr, *Levlilactobacillus brevis*; Lcur, *Lactobacillus curvatus*; Leucpm, *Leuconostoc pseudomesenteroides*; Lherb, *Lactobacillus herbarum*; Lparapl, *Lactoplantibacillus paraplantarum*; Lpl, *Lactiplantibacillus plantarum*. Outliers are marked with circle (o).

composition of the obtained pistachio beverage is shown in Table 2. In more detail, the beverage was characterized by a high content of fiber, fat proteins, and minerals such as K and Mg and low amounts of Na. The protein content of the sample was similar to that reported in a pistachio beverage by Sánchez-Bravo et al. (2020) (1.7%). Moreover, the pistachio beverage had higher protein and fiber content than other commercial drinks such as rice (0.1% and 0%, respectively), oat (0.7% and 0.8%) and almond (1.3% and 1%) (Aydar et al., 2020). As also highlighted by Kocyigit et al. (2006), pistachio nuts have higher amount of fiber than walnut, hazelnut, peanuts, pecans, and macadamia nuts. Additionally, pistachio beverage contained higher amount of Ca than similar cereals beverages (Silva et al., 2020).

Then, the beverage was inoculated with each selected culture and fermented at 30 °C for 24h. The initial pH of the beverage was 6.53. Almost all lactic acid bacteria showed a good degree of acidification recording pH values between 4.06 (isolate P6) and 4.87 (isolate T6) after 24h of fermentation (Fig. 3). Only three isolates caused a slight acidification of the beverage which reached pH values of 5.5 (A7) and 5.36 (O1, R3). All the tested cultures showed an increase in load after 24h fermentation. In particular, most of the cultures recorded load values above 9.0 log CFU/mL, whereas some cultures recorded values between 7.9 and 9.0 log CFU/mL (A7, D3, G3, G4, R3, T6). These data have demonstrated how pistachios represent a good substrate for the growth of lactic acid bacteria, in accordance with other authors who have found that vegetables matrices such as lentil, chickpea, quinoa, and coconut extracts are an optimal substrate for the growth of the same microbial group (Di Renzo et al., 2018; Mesquita et al., 2020; Verni et al., 2020).

Furthermore, the viable counts of all tested cultures in the beverage remained above 8 log CFU/mL after 30 days of storage at 4 °C (data not shown), thus also demonstrating a protective effect of the pistachio matrix on the viability of the microbial cells. This protective effect could have been exerted by the high amount of fat occurring in the beverage (about 3%). As ascertained by other authors, in fact, high fat content, as in cheese or yogurt, shows positive effects on the viability of microbial cells, mainly due to good buffering capacity (Matouskova et al., 2021; Reale et al., 2019; Sagheddu et al., 2018). Moreover, no undesirable microorganisms were found after 30 days in all the beverage samples (data not shown), thus also highlighting the inhibitory effect of the acidification on the microbial contaminants.

The fermented beverages were analyzed with E-nose to assess the effects of the fermentation activity of the different tested cultures. The olfactory fingerprint data of the 43 cultures obtained by E-nose allowed to distinguish different aromatic patterns to select the isolates on the basis of more objective parameters for further analysis (Fig. 4). The data set, composed of 2200 observations and 10 features, has been investigated by PCA. The first two principal components (PC) expressed 83.34% of the total variance and allowed a good discrimination of the different beverages. The samples, as determined by the two PCs (factors), were located in different zones of the plane. A few samples were positively associate with PC1 and PC2 (upper right section of the graph) and were mainly influenced by sensors S2, S6 and S8. A few samples were positively associated to PC1 and negatively associated to PC2 (lower right section of the graph) and were mainly influenced by sensors S4, S7, S9 and S10. Most of the samples were negatively associated with PC1 (lower and upper left section of the graph) and were mainly influenced by sensors S1, S3 and S5. In detail, the control sample (situated in the upper left section of the graph) differed from the other fermented beverages highlighting the influence of the lactic fermentation on the odor composition of the beverages. From the PCA analysis, it was possible to identify three main groups of beverages according to the odors derived from the fermentation activity of the strains used. The first group (located in the upper left section of the graph) was closest to the control sample and included mainly beverages fermented with the species *L. brevis*, *L. curvatus*, and *L. paraplantarum*. A second group, the largest, was in the lower sections of the graph and included beverages produced mainly with the species *L. plantarum*, *C. alimentarius*, *C. kimchi*, and *F. rossiae*. The third group included only *L. pseudomesenteroides* D4 strain, which differed strongly from the control and from all the other samples. The E-nose analysis allowed a qualitative discrimination of the beverages fermented by the tested lactic acid bacteria species. As already evidenced by Seesaard and Wongchoosuk (2022), e-nose have been successfully applied for quality evaluation of fermented food and beverage due to its fast analysis, low operating costs, and simplicity. Our study provided a fast-screening method using E-nose system to evaluate the effect of the fermentation activity of numerous microbial starters on the odor characteristics of pistachio beverages. Target cultures were selected from each identified group (about 6 isolates in total) for further studies on the assessment of proteolytic activity, volatile organic compound composition, and nutrient bioaccessibility of the fermented beverages.

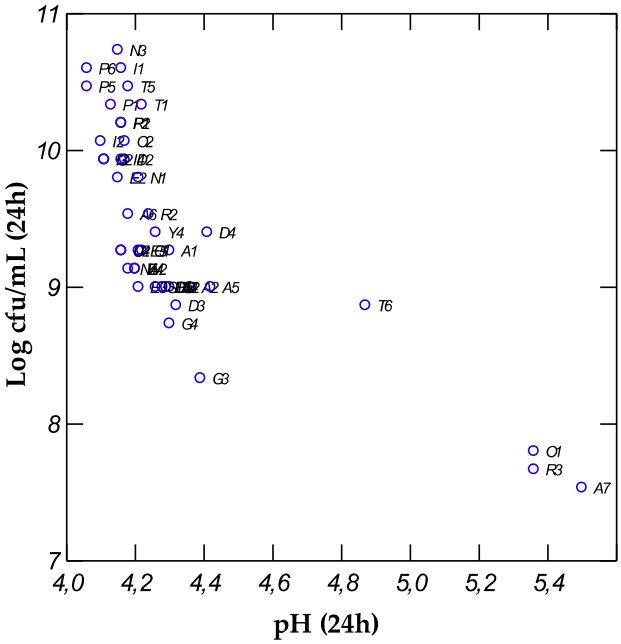

Fig. 3. Distribution of selected lactic acid bacteria on the basis of acidifying ability (pH) and growth ability (log CFU/mL) in the beverage after 24h of incubation ad 30 °C.

Table 2
Proximate composition of pistachio beverage.

Parameter	g/100 mL
Proteins	2.0 ± 0.0
Lipids	4.6 ± 0.3
Total Dietary Fibre	2.9 ± 0.0
Moisture	90 ± 0.3
Ash (minerals)	0.31 ± 0.00
Total carbohydrates (different from fibre)	0.21 ± 0.00
Minerals	**ppm (mg/L)**
Ca	124.7 ± 1.0
Zn	1.6 ± 0.1
Na	4.3 ± 0.3
K	924.6 ± 20.1
Fe	4.5 ± 0.2
Cu	1.1 ± 0.0
Mg	169.1 ± 9.0

4. Conclusions

The results obtained in the present study are intended as a starting point for the development of a novel fermented pistachio beverage as a feasible alternative to dairy-based products that could be suitable for different targeted consumer groups, including lactose-intolerant or

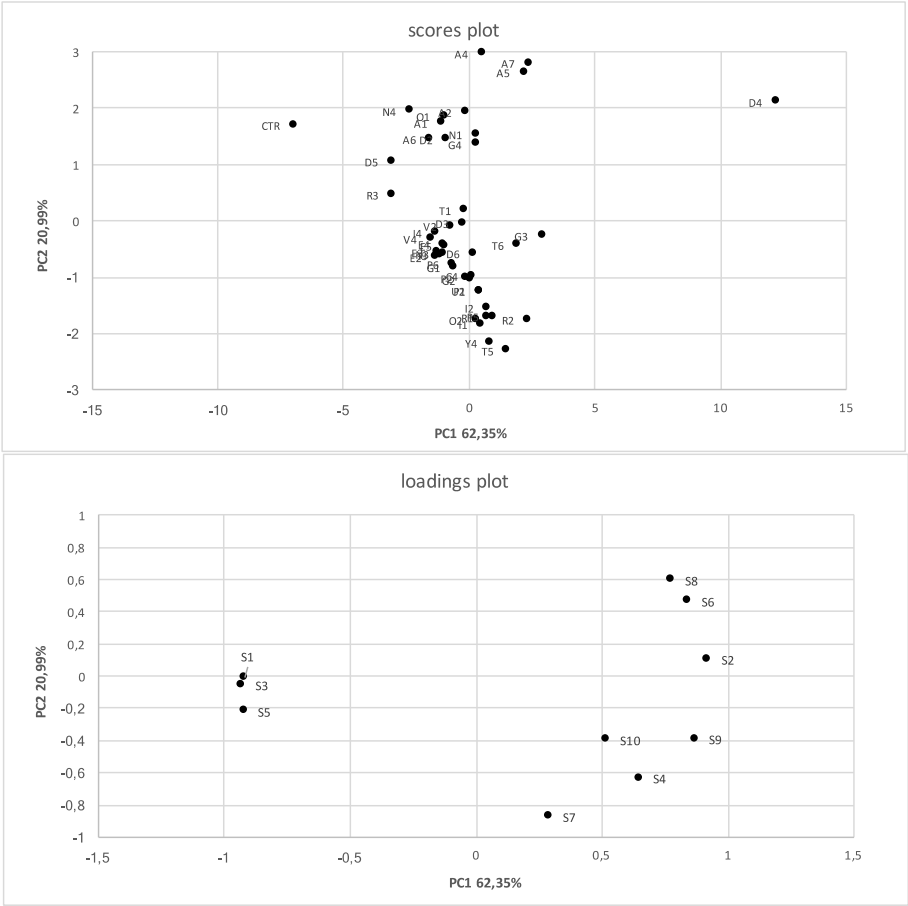

Fig. 4. Principal component analysis (PCA) of odors that mainly differentiated the beverages inoculated with different lactic acid bacteria.

vegetarians. In this study, the suitability of lactic acid bacteria to ferment a pistachio-based matrix was studied and a protocol for obtaining a fermented pistachio-based beverage has been developed. Pistachio proved to be an optimal matrix for the growth of lactic acid bacteria. Most of the selected lactic acid bacteria achieved high viable counts (approximately 9 log CFU/mL) after 24h fermentation at 30 °C and maintained a high viability (about 8 log CFU/mL) in the beverage during the entire supposed shelf-life (30 days) at 4 °C. The effects of lactic acid bacteria fermentation evaluated by analyzing the odor profile of the beverage using the E-nose may represent a valid screening method to assess the effect of the fermentative activity of microbial starter cultures on the qualitative characteristics of such food matrix. Further investigations are underway to explore more in depth the sensory characteristics of the obtained novel pistachio fermented beverage and to determine the level of acceptance of the product by consumers, and also to assess potential health benefits. Moreover, fermentation parameters should also be optimized in order to obtain a viable technology for manufacturing the beverage at large scale production.

Credit author statement

Tiziana Di Renzo: Conceptualization, Writing original draft, Writing—review and editing. **Andrea Osimani**: Conceptualization, Data curation, Methodology, Writing original draft, Writing—review and editing. **Serena Marulo**: Formal analysis. **Federica Cardinali**: Formal analysis. **Gianfranco Mamone**, Conceptualization. **Cecilia Puppo**: Methodology. **Antonela G. Garzòn**: Data curation, investigation. **Silvina R. Drago**: Methodology. **Carmine Laurino**: Formal analysis. **Anna Reale**: Conceptualization, Data curation, Methodology, Funding acquisition, Project administration, Writing original draft, Writing—review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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.fbio.2023.102802.

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