Antifungal activity of lactic acid bacteria against phytopathogenic *Alternaria alternata* species and their molecular characterization

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Summary
Phytopathogenic filamentous fungi are responsible for spoilage of various food products, such as fruits or vegetables, causing significant economic losses. They are also able to produce several mycotoxins in infected plants and fruits, representing a serious risk for human and livestock health. Lactic acid bacteria (LAB) are considered natural antagonists of these dangerous microorganisms, thanks to the production of a large number of compounds with antifungal properties. In the present study, 45 LAB strains isolated from raw goats', cows' and camels' milk from various regions of Algeria, were tested for antifungal activity against five strains of the phytopathogenic, toxigenic and deteriorating fungal species *Alternaria alternata,* isolated from the stems, leaves, roots and fruits of tomatoes and carrots. Internal transcribed spacer polymerase chain reaction (ITS-PCR) analysis and 16S rDNA sequencing were used to characterize and identify the LAB isolates that showed various levels of inhibition of fungal growth. Two LAB strains, identified as *Enterococcus lactis* and *E. faecium*, had the strongest antifungal activity, suggesting a potential application in food technology as bio-preservatives against phytopathogenic and food-spoilage fungi.

Keywords
lactic acid bacteria; *Alternaria alternata*; internal transcribed spacer; antifungal; phytopathogenic fungi; food preservation

Various food products may be contaminated with a wide range of filamentous fungi, causing significant economic losses [1]. In addition to food spoilage, fungal contamination represents a serious risk for human health since strains from some genera such as *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium* are able to produce toxic secondary metabolites named mycotoxins [2]. *Alternaria alternata* is the most common species of the genus *Alternaria* in harvested fruits and vegetables and the most important species producing mycotoxins [3]. *Alternaria* species are known to be able to produce more than 70 phytotoxins, but only few have been chemically characterized and act as mycotoxins to humans and animals [4]. Some toxins such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and altertoxins (ATX) were described to induce harmful effects in animals, including fetotoxic and teratogenic effects [4]. Within *A. alternata*, seven different pathotypes have been identified to produce host-selective or host-specific toxins (HSTs) [5]. HSTs are secondary metabolites that cause damage only to the susceptible host and are released during conidium germination, before tissue penetration and mycelium production [5]. Because of their growth even at low temperatures, *Alternaria* species are responsible for deterioration of food products during transport and refrigerated storage [4].

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In recent years, increasing interest has been shown in biopreservation, i.e. the use of microorganisms or their antimicrobial metabolites in food preservation, due to consumer demand for a reduced use of chemicals in food and feed because of their health risks, such as indigestibility or allergies [2].

Lactic acid bacteria (LAB) are known to have a broad spectrum of antimicrobial properties. They are established as probiotics and have been widely used in food fermentation since a long time. This process contributes to the safety, stability, flavour and structure of foods. As food additives, LAB improve the microbiological safety of products without negatively altering the sensory characteristics of food.

To date, LAB are considered the best candidates for protection of a wide range of food products against spoilage fungi [1]. LAB can inhibit fungal growth or spore proliferation in food due to the production of several antifungal compounds such as organic acids, fatty acids, carboxylic acids, lactones, alcohols, hydrogen peroxide, diacetyl, CO_2, bacteriocins, protein compounds or cyclic dipeptides [6]. When mycotoxins are already produced by fungi in food, LAB can degrade or reduce their content through various mechanisms including adsorption on the cell wall and biodegradation [1]. There is a thorough knowledge of the antagonist effects of LAB on mycotoxigenic fungi associated with food contamination, but the studies focused specifically on *Fusarium, Aspergillus* and *Penicillium* species [2], while the number of published studies on LAB inhibition of *Alternaria* genus are few [7–9].

In the last years, the public need for high quality food without the addition of chemical preservatives, determined the search for new LAB strains that are able to control the fungal growth of phytopathogenic and mycotoxigenic species. Due to the limited studies on the antifungal activity of LAB against *Alternaria* species, the objective of this research was to test whether LAB strains isolated from various milk samples collected in three regions in Algeria, inhibit the growth of *A. alternata* strains. In particular, isolation, characterization and identification of *Alternaria* strains and LAB strains with antifungal properties were carried out.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

A total of 40 raw milk samples from camel (n = 15), cow (n = 15) and goat (n = 10) were collected during two years of the study (2014–2015) from three different regions of Algeria (Bechar, Oran and Relizane). After preparing the dilutions of milk, 1 ml of each dilution was used for in-depth seeding of the culture media selective for the growth of LAB, namely de Man, Rogosa and Sharpe medium (MRS; Condalab, Madrid, Spain) [10] and M17 (Condalab) [11], both liquid and solidified by adding 2 % agar, as described by the International Dairy Federation [12]. After incubation (30 °C, 24 h to 48 h), isolates were examined by microscopy to determine cell morphology and by Gram staining reaction. Further, they were tested for catalase activity. The shape, size and the reciprocal association of the cells were taken into account.

Subsequently, the Gram-positive and catalase-negative isolates were sub-cultured in liquid and on solid MRS medium, as well as in liquid and on solid M17, until purification. From 7 to 10 isolated colonies were taken from the solid MRS medium or solid M17 and transferred to liquid MRS or liquid M17 and vice versa. The purity of the strains was verified by the appearance of the colonies (shape, colour, size) on solid medium, characteristic appearance of cultures in liquid medium and by microscopic examination.

Identification of lactic acid bacterial isolates

A total of 123 Gram-positive and catalase-negative bacteria were isolated and characterized by biochemical and physiological tests. The isolates with characteristics of LAB were chosen and screened for the antifungal activity. LAB strains which showed inhibition of the fungal growth were further characterized by molecular analyses as described below.

Biochemical and physiological characterization

Gram-positive and catalase-negative isolates were characterized by the following physiological and biochemical tests, as described by Merzouk et al. [13]: growth at 15 °C, 37 C and 45 °C in MRS broth for 5 days; growth at pH 6.5 and pH 9.6; growth in the presence of 4 % and 6.5 % NaCl; the fermentation type was determined by gas production from glucose in MRS broth with an inverted Durham tube; hydrolysis of arginine was tested in M16BPC medium prepared as described by Thomas [14]; Voges-Proskauer test was used to determine the production of acetoin from glucose. The ability to ferment 14 carbohydrates, including arabinose, ribose, xylose, galactose, fructose, mannitol, sorbitol, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose and esculin, was determined in a miniaturized preparation using microtitration plate (Th. Geyer, Rennin-

gen, Germany). Citrate utilization in the presence of glucose was analysed in Kempler and McKay medium prepared as described by KEMPLER and MCKAY [15]. Production of dextran from saccharose was determined using Mayeux Sandine Elliker medium (MSE; Biokar, Allonne, France) [16].

Molecular characterization of bacterial strains

DNA was extracted from bacterial strains grown on MRS agar at 30 °C for 24 h, following the method of RAINEY et al. [17] with some modifications. A single colony was dispersed in 400 μl of saline-EDTA buffer (0.15 mol·l^{-1} NaCl, 0.01 mol·l^{-1} ethylenediaminetetraacetic acid, EDTA; Sigma-Aldrich, Saint Louis, Missouri, USA). Then, 10 μl of a lysozyme solution (10 mg·ml^{-1}; Invitrogen, Carlsbad, California, USA) were added. The resulting preparation was vortexed and incubated at 37 °C for 30 min. Then, 5 μl of proteinase K (20 mg·ml^{-1}; Invitrogen) and 15 μl of 25 % sodium dodecyl sulfate (SDS, Invitrogen) were added, followed by incubation for 30 min at 55 °C. After centrifugation at 15000 ×*g* for 5 min, 400 μl of phenol (Merck, Darmstadt, Germany) were added to the preparation and the mixture was centrifuged at 15000 ×*g* for 5 min. The supernatant was collected in a new microtube and an equal volume of phenol-chloroform (4 : 1) (Merck) was added. Then, the preparation was mixed thoroughly, by inverting the microtube several times, and centrifuged. DNA precipitation was obtained by adding 1 ml of absolute ethanol (Sigma-Aldrich) at –20 °C to the supernatant and incubating at –80 °C for 1 h. After centrifugation at 4 °C for 10 min at 15000 ×*g*, the supernatant was discarded and the pellet was washed with 1 ml of cold (–20 °C) 70 % ethanol, dried and dissolved in 30 μl of Tris-EDTA (TE) buffer (Promega. Madison, Wisconsin, USA). RNase treatment was carried out by adding 1 μl of RNase (20 μg·ml^{-1}; Invitrogen) and incubating for 1 h at 37 °C. Finally, the samples were stored at –20 °C. The DNA extract was checked by electrophoresis in a 1 % agarose gel stained with ethidium bromide in Tris-Acetate-EDTA (TAE) buffer (Promega).

The amplification of internal transcribed spacer (ITS) and profiles analysis were carried out as described below:

Polymerase chain reaction (PCR) mixtures (final volume, 50 μl) contained 5 μl 10X Buffer, 2 μl $MgCl_2$ (50 mmol·l^{-1}), 4 μl deoxynucleoside triphosphate mixture (10 mmol·l^{-1}; all from Promega), 1 μl of each primer (12 pmol; Invitrogen), 5 U Taq polymerase (Promega) and 2 μl template DNA solution. The primer pair used was F1492 (5'-AAGTCGTAACAAGGTAACC-3') and R188 (5'-GGTACTTAGAGTTTTCAGTT-3') from Invitrogen following the protocol described by DE LEO et al. [18]. Amplification was performed in T-Personal Thermal Cycler (Biometra, Göttingen, Germany) using the following temperature programme: initial denaturation at 95 °C for 2 min, followed by 31 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min 30 s and a final extension at 72 °C for 7 min. The PCR products were visualized by electrophoresis on 2 % agarose gel. Profiles were examined using Kodak Digital Science 1d 2.0 software (Kodak, Rochester, New York, USA) and the analysis was carried out on the basis of the number and size of bands as compared to a 50 bp DNA ladder marker (Invitrogen). Strains with same ITS profile were clustered together and from each cluster, random strains were selected and identified by DNA extraction and 16S rDNA sequencing.

The partial 16S rDNA gene was amplified following the protocol described by KRAKOVA et al. [19], using the universal primers F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and R1492 (5'-CGG CTA CCT TGT TAC GAC TT-3'; both Invitrogen). The purification and sequencing of PCR products were carried out by a commercial facility (Biofab, Rome, Italy). The closest relatives of LAB isolates were determined by comparison with rDNA gene sequences in the NCBI GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) and EMBL-EBI databases (European Molecular Biology Laboratory – European Bioinformatics Institute, Hinxton, United Kingdom) by Basic local alignment search tool (BLAST) search.

The sequences were aligned together with closely similar DNA sequences retrieved from GenBank. The phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis software Mega 7 [20] using the Neighbour-joining algorithm method and Kimura 2-parameter model, with 1000 bootstrap replications.

Isolation of fungi

A total of 27 samples from stems, fruits, leaves and roots of tomato and carrots with evident phenomena of fungal deterioration were collected during two years of the study (2014–2015) from Mostaganem region in Algeria. Fungal isolation was performed as described by SINCLAIR and DHINGRA [21]. Plant material showing brown or black lesions was cut into 2 cm pieces and the surface was sterilized in 0.1% (v/v) sodium hypochlorite solution for 2 min. These fragments were then transferred to Petri dishes containing potato dex-

trose agar medium (PDA; Oxoid, Basingstoke, United Kingdom) supplemented with streptomycin (150 mg·l^{-1}, Merck) and incubated for 5–8 days at 25 ± 2 °C. The dematiaceous fungal hyphomycetes with micro- and macro-morphological characteristics matching with the genus *Alternaria* were transferred to PDA medium and incubated at 25 ± 2 °C for 4 days. Isolation of pure cultures was carried out on the basis of macro-morphological cultural characteristics of *Alternaria* species [22] and using the monospore culture method [23]. Successive sub-cultures of the suspect fungal colonies on PDA medium were carried out until colonies of pure appearance were obtained.

Identification of *Alternaria* isolates

The description of the fungal isolates was based on the morphological characteristics (macroscopic and microscopic) of the colonies derived from the monospore culture, considered as "wild type". The identification of the selected isolates was carried out according to the macro- and micro-morphological characteristics described by Simmons [22].

To confirm the species identification, amplification and sequencing of ITS1, ITS2 and 5.8S rDNA was carried out as described below:

Genomic DNA was extracted from fungal strains cultured on PDA medium at 27 °C for 5 days. After the incubation time, approximately 1 cm^2 of the fungal material was transferred to a 2-ml microtube filled with glass beads (0.45–0.50 mm diameter) and 500 µl lysis buffer (Tris-HCl 0.1 mmol l^{-1}, pH 5.8; 10 mmol l^{-1} EDTA; 2 % SDS). This was followed by vortexing 4 times for 40 s. Then, 25 µl of 5 mol·l^{-1} NaCl was added and the preparation was vortexed 4 times for 40 s and centrifuged for 3 min at 15 000 ×*g*. The supernatant was transferred into a new microtube and 500 µl lysis buffer were added. Then, the mixture was vortexed 4 times for 40 s. After centrifugation for 3 min at 15 000 ×*g*, 400 µl of phenol (Merck) were added to the supernatant. The mixture was mixed thoroughly by inverting the microtube several times and centrifuged for 5 min at 15 000 ×*g*. The supernatant was transferred into a microtube and 400 µl of phenol-chloroform (4 : 1) were added, and mixed thoroughly by inverting the microtube several times, then centrifuged for 5 min at 15 000 ×*g*. A volume of 1 ml of absolute ethanol (–20 °C) was added to the supernatant and the microtube was inverted. The samples were placed at –80 °C for 1 h to precipitate DNA. After centrifugation at 4 °C for 10 min at 15 000 ×*g*, the supernatant was discarded and 1 ml of cold (–20 °C) 70 % ethanol was added to the pellet and centrifuged for 10 min at 15 000 ×*g*. The supernatant was removed, the pellet was dried and dissolved in 30 µl TE buffer. RNase treatment was carried out by adding 1 µl of RNase (20 µg·ml^{-1}) and incubating for 1 h at 37 °C. Finally, the samples were stored at –20 °C. The DNA extract was checked by electrophoresis in a 1% agarose gel stained with ethidium bromide in TAE buffer.

ITS region was amplified by PCR using universal primers ITS1 and ITS4, which are specific for fungal ITS1, ITS2 and 5.8S rRNA genes [24]. The reaction mixture contained 0.48 mmol·l^{-1} of each primer (Invitrogen), 25 µl of MyTaq Mix 2x (Bioline, London, United Kingdom) and 2 µl of template DNA solution in the total reaction volume of 50 µl. PCR was performed with the following programme: 5 min denaturation at 95 °C, followed by 35 cycles of a 1 min at 94 °C, 1 min at 55 °C and 90 s at 72 °C, and final extension was run at 72 °C for 10 min in T-Personal Thermal Cycler. The PCR products were analysed in a 1.5% agarose gel stained with ethidium bromide. Purification and sequencing of PCR products were performed by a commercial facility (Biofab). The closest relatives of isolates were determined by comparison with rDNA gene sequences in the NCBI GenBank and EMBL-EBI databases by BLAST search.

Antifungal activity assay

A total of 45 bacterial isolates, identified as LAB by biochemical and physiological analyses, were screened for antifungal activity against 5 strains of *A. alternata* isolated from deteriorated vegetables. After 18 h of incubation, LAB strains were streaked in two lines of 2 cm apart on MRS agar and incubated at 30 °C for 48 h. Then, a section of fungal strain aged 5 days was deposited in the centre of the agar plates and incubated at 30 °C. After 3 days, the diameter of the fungal colonies was measured and compared with a control, which was a fragment of a five-day-old fungal colony deposited in the center of the agar plate without LAB [25]. The percentage of growth inhibition (I) was calculated as follows:

$$I = \frac{R_w - R_t}{R_w} \times 100 \quad (1)$$

where R_w is the maximum radial distance grown by phytopathogenic fungus in the control without LAB and R_t is the radial distance grown by phytopathogenic fungus in the direction of the antagonist (in centimetres) [26]. All the experiments were carried out in triplicate and repeated three times.

Nucleotide sequence accession numbers

The 16S rDNA gene sequences of nine bacte-

rial isolates were deposited to NCBI databases and are available under accession numbers from MT672309 to MT672316 and MT703828. Fungal ITS rDNA gene sequences were deposited to NCBI databases and are available under accession numbers MT661476 to MT661480.

RESULTS AND DISCUSSION

Phenotypic characterization of bacterial isolates

LAB are Gram-positive, non-sporulating, air and acid tolerant, organotrophic, fermentative rods or cocci producing lactic acid, the major metabolic end product of carbohydrate fermentation [1]. They have a ubiquitous distribution, being usually present in (fermented) dairy products, meat, vegetables, soil, water and also in the gastrointestinal and urogenital tracts of humans and animals [27]. Recently, LAB have received a great interest in the field of food preservation, due to their ability to produce a plethora of antifungal metabolites and to remove mycotoxins from food and feed [1].

In the present study, 123 Gram-positive and catalase-negative bacteria were isolated from raw cows', goats' and camels' milk samples. Among them, a total of 45 strains were identified as LAB as a result of morphological, biochemical and physiological analyses. They were Gram-positive, catalase-negative and non-sporulating. Colonies were small, whitish, transparent, smooth, lenticular and regular on solid medium. The cells had a form of coccobacilli or cocci, being arranged in small chains or as diplococci.

The 45 presumable LAB strains were further subdivided into three groups (Tab. 1):

- Group 1: 19 homofermentative strains, arginine dihydrolase (ADH) positive, acetoin-negative, citrate-positive, dextrane-negative, growing at 45 °C, at pH 6.5 and with 4.0 % NaCl, but not at pH 9.6 and with 6.5 % NaCl. They did not ferment sorbitol and melibiose.
- Group 2: 18 homofermentative strains, ADH-positive, acetoin-positive, citrate-positive, dextrane-negative, growing at 45 °C, at pH 6.5 and with 4.0 % NaCl, but not at pH 9.6 and with 6.5 % NaCl. They did not ferment arabinose, sorbitol, trehalose and raffinose.
- Group 3: 8 heterofermentative strains, ADH-positive, acetoin-negative, citrate-positive, dextrane-negative, growing at 45 °C, at pH 6.5 and with 4.0 % NaCl, but not at pH 9.6 and with 6.5 % NaCl. They fermented all the sugars tested.

Following the recommendations of CARR et al. [28] and KHEDID et al. [29], the three groups could be assigned to the species *Lactococcus lactis* subsp. *lactis* (Group 1), *Lactococcus lactis* subsp. *diacetylactis* (Group 2) and *Weissella cibaria* (Group 3).

Tab. 1. Morphological, physiological and biochemical characteristics of lactic acid bacteria isolated from Algerian raw cows', goats' and camels' milk.

	Group 1	Group 2	Group 3
Number of isolates	19	18	8
Gram stain reaction	G+	G+	G+
Spores formation	–	–	–
Catalase activity	–	–	–
CO_2 from glucose	–	–	+
NH_3 from arginine	+	+	+
Growth at temperature			
15 °C	+	+	+
37 °C	+	+	+
45 °C	+	+	+
Growth at pH			
6.5	+	+	+
9.6	–	–	–
Growth in a medium with NaCl			
4.0 %	+	+	+
6.5 %	–	–	–
Production			
Dextrane from saccharose	–	–	–
Acetoin from glucose	–	+	–
Citrate utilization	+	+	+
Sugar fermentation			
Arabinose	+	–	+
Ribose	+	+	+
Xylose	+	+	+
Galactose	+	+	+
Fructose	+	+	+
Mannitol	+	+	+
Sorbitol	–	–	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	–	+	+
Saccharose	+	+	+
Trehalose	+	–	+
Raffinose	+	–	+
Esculin	+	+	+
Species identification*	*L. lactis* subsp. *lactis*	*L. lactis* subsp. *diacetylactis*	*Weissella cibaria*

* – species identification following key identification by CARR et al. [28] and KHEDID et al. [29].

Molecular analysis of bacterial strains

Sixteen LAB strains which showed an antagonist activity against the phytopathogenic fungal

Tab. 2. DNA fragments obtained by amplification of the internal transcribed spacer rDNA region.

Strain	Number of bands	Fragment length [bp]	ITS-PCR Group
BL2, BL4.6, BL4.10, BL4.13, BL4.19, BL4.25, BL10, BL14, BL14.2, BL14.16, BL16.19, BL27, BL31, BL35	3	900 800 700	A
BL4.18	1	700	B
BL16.16	2	700 600	C

ITS-PCR – internal transcribed spacer polymerase chain reaction.

species *A. alternata* were further characterized by molecular analyses. In particular, ITS-PCR was applied to detect inter- and intra-species differences at the genus or species level [30, 31]. In detail, ITS-PCR was applied to analyse strains BL16.19, BL14, BL14.2, BL4.10, BL4.18 belonging to the phenotypic Group 1, strains BL2, BL4.6, BL31, BL4.19, BL4.13, BL4.25, BL16.16, BL14.16 belonging to the phenotypic Group 2, and strains BL35, BL10 and BL27 belonging to the phenotypic Group 3.

Three PCR profiles were obtained after amplification of ITS followed by separation of the PCR products by electrophoresis. ITS-PCR patterns showed from 1 to 3 reproducible bands ranging from 600 bp to 900 bp. According to the number of the bands obtained and their molecular weights, strains BL35, BL10, BL2, BL4.6, BL31, BL4.19, BL16.19, BL14, BL14.2, BL27, BL4.13, BL4.25, BL14.16 and BL4.10 clustered in the same profile with three bands of 700 bp, 800 bp and 900 bp (Group A) while strain BL4.18 and strain BL16.16 clustered in two different profiles with one band of 700 bp (Group B) and two bands of 600 bp and 700 bp (Group C), respectively. The band of 700 bp was common to all 16 strains (Tab. 2).

The molecular analyses demonstrated that the homofermentative strains assigned by biochemical and physiological analyses to *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *diacetylactis* generated the same ITS profile (ITS-PCR profile A) with the exception of the strain BL4.18 (ITS-PCR profile B). Representative strains from each ITS-PCR profile were identified by 16S rDNA gene sequence analysis and compared to the known sequences in GenBank and EBI-EMBL databases. Results are shown in Tab. 3.

LAB isolates belonging to the Group A were identified as *Enterococcus faecium* (strains BL35, BL14 and BL14.2) with sequence similarity from 98.6 % to 100 %, and *Enterococcus lactis* (strains BL2, BL4.6, BL4.13 and BL10) with sequence similarity from 99.7 % to 100 %. The phylogenetic analysis based on 16S rDNA carried out on strains belonging to the Group A compared with four closely similar DNA sequences retrieved from GenBank supported these results, showing the existence of two very closely related clusters: one cluster grouped the strains identified as *E. faecium* (BL35, BL14 and BL14.2), while the other cluster grouped the strains identified as *E. lactis* (BL2, BL4.6, BL4.13 and BL10; Fig. 1). The strain BL4.18 (Group B) was identified as *Lactobacillus plantarum* (sequence similarity 99.7 %).

The heterofermentative strain BL16.16 had another ITS-PCR profile C and the comparison with 16S rDNA gene sequences in the NCBI GenBank resulted in a very low percentage of similarity (80.6 %), which did not allow identification at species level. For this reason, further analyses are being carried out to clarify the identification of this strain.

Differences in classification of LAB by biochemical and physiological analyses versus 16S rDNA sequencing has been already highlighted by Ruiz Rodríguez et al. [32]. In fact, the choice of appropriate identification methods for LAB is controversial. In general, with a few

Tab. 3. Representatives of lactic acid bacteria strains identified by partial sequencing of 16S rDNA.

LAB strain	Closest relative sequences	Sequence similarity [%]
BL2	*Enterococcus lactis* MN560018.1	100.0
BL4.6	*Enterococcus lactis* MN560018.1	99.7
BL4.13	*Enterococcus lactis* MN560018.1	100.0
BL4.18	*Lactobacillus plantarum* MT573825.1	99.7
BL10	*Enterococcus lactis* MN560018.1	99.7
BL14	*Enterococcus faecium* MH236332.1	98.6
BL14.2	*Enterococcus faecium* MH236325.1	100.0
BL16.16	*Enterococcus* sp. JN173076	80.6
BL35	*Enterococcus faecium* MT573707.1	99.8

LAB – lactic acid bacteria.

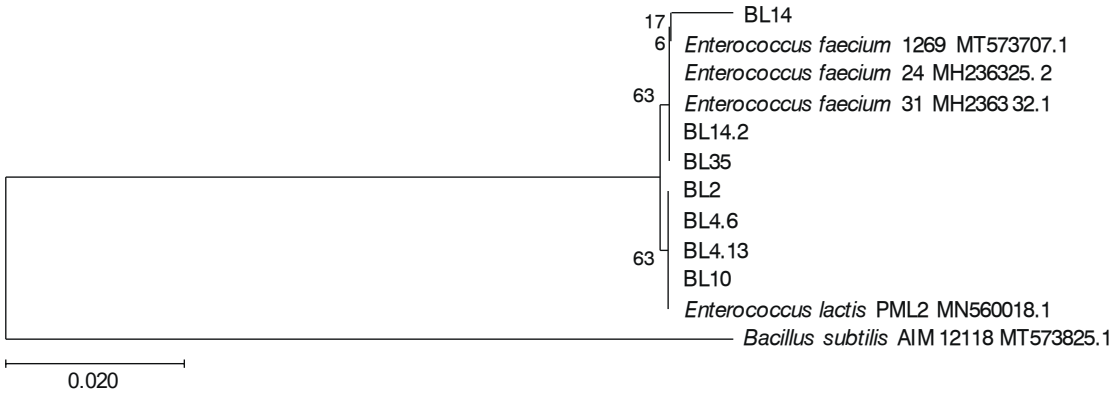

Fig. 1. Neighbor-joining tree based on the 16S rDNA sequences of *E. faecium* and *E. lactis* strains.

exceptions, a phenotypic test could be suitable for clinical isolate identification, while for food isolates, molecular analyses are considered the most sensitive and reliable approach [32]. Based on 16S rDNA sequencing, the majority of strains that showed antifungal activity belonged to the genus of *Enterococcus* with the two species of *E. faecium* and *E. lactis.*

The presence of the genus *Enterococcus* in raw cows', goats' and camels' milk was already reported by previous studies [33–35]. The species belonging to the genus *Enterococcus* represent a large part of the autochthonous flora of the gastrointestinal tract of mammals [36] and they may contaminate raw milk through intestinal or environmental contamination. For example, direct contact between the milking parlour and bedding area promotes contamination of milk by *E. faecium* [34]. After contamination, *Enterococcus* species become an important part of the fermented food microflora due to their ability to survive in harsh conditions such as extreme pH, temperatures and salinity [36]. *E. lactis* was first isolated from sour milk products, fresh ewes' milk and raw milk cheese as an atypical *Enterococcus* strain and it was later described by MORANDI et al. [37] as a new species belonging to genus *Enterococcus*.

Alternaria alternata isolation and identification

Twenty-seven fungal isolates were collected from plants with similar symptoms of foliar blight disease. They affected leaves, stems and fruits. The cultural and morphological characteristics of five isolates were very similar to those described by SIMMONS [22]. Conidiophores growing alone or in small groups produced spores in chains. Conidiospores were large, with longitudinal and transverse septa and their apex had beak-like appearance, typical for conidia of *Alternaria alternata* and related species (Fig. 2). The identification of the isolates was confirmed by molecular analysis. Amplification and sequencing of the rDNA ITS region and subsequent comparison in EMBL-EBI database confirmed the phenotypic identification of 5 fungal isolates (07MY, 17MM, 19MY, 32MA, 37MY) as *Alternaria alternata* species (percentage of similarity ≥ 99.4 %). The closest relative sequences and percentage of sequence similarity of each isolate is shown in Tab. 4.

Alternaria genus includes plant-pathogenic spe-

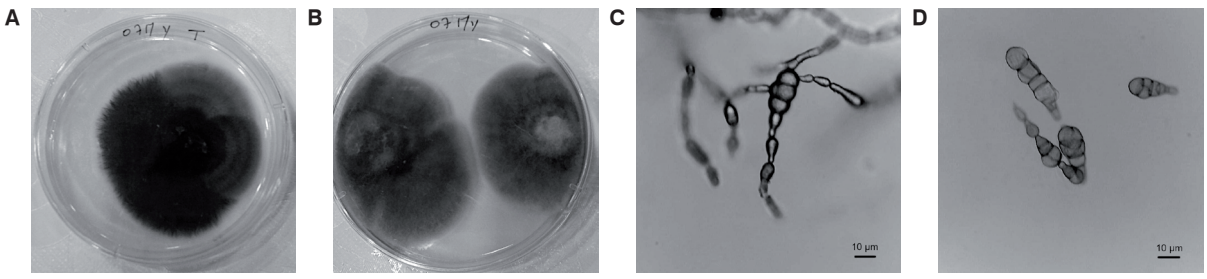

Fig. 2. Morphological characteristics of *Alternaria alternata* isolates.

A, B – colonies of the fungus grown on potato dextrose agar medium after 4 days of incubation at 25 °C, C – hyphae with conidiophores, D – spores observed under light microscope.

Tab. 4. Fungal strains isolated in this study.

Fungal strain	Closest relative sequences	Sequence similarity [%]
07MY	*Alternaria alternata* MN822658	99.8
17MM	*Alternaria alternata* MT573464	100.0
19MY	*Alternaria alternata* KT356738	99.4
32MA	*Alternaria alternata* MN481948	100.0
37MY	*Alternaria alternata* MN093376	99.4

cies, which may affect crops in the fields or cause harvest and postharvest decay of plant products. In particular, the development of new taxonomic tools has shown that *A. alternata* is predominant in several crops, including fruits and vegetables, causing substantial losses in their yield [3]. In addition, it is known that *A. alternata* is able to produce several mycotoxins in infected plants and/or fruits, which may seriously compromise the quality of processed food products and are also a risk for food safety [3].

Antifungal activity assay

LAB isolates were screened for antifungal activity against five strains of *A. alternata* using the confrontation method. After incubation, the vertical and horizontal diameters of the LAB-inhibited mycelium were measured. The test revealed varying antifungal activity of bacterial strains, with percentages of inhibition of the fungal growth between 0 % and 100 %. Results are shown in Tab. 5 and Fig. 3.

It was observed that LAB strain BL2 *E. lactis* and LAB strain BL35 *E. faecium* had the strongest antifungal activity, with a percentage of inhibition of 100 % against four *A. alternata* strains (07MY, 19MY, 17MM, 37MY) and three *A. alternata* strains (07MY, 17MM and 37MM), respectively (Fig. 4). Strain BL2 showed a minor inhibition activity (60 %) only against 32MA *A. alternata* strain, while BL35 had a minor inhibition activity against 32MA (20 %) and 19MY (62.5 %) *A. alternata* strains.

LAB isolates BL4.6 (*E. lactis*), BL27, BL16.19 and BL4.19, inhibited 100 % of the growth of only one *A. alternata* strain (37MM, 32MA, 17MM and 37MM, respectively). Minor percentages ranging from 13.1 % to 66.6 % of inhibition were observed in the other cases.

The remaining LAB isolates BL14 (*E. faecium*), BL14.16, BL4.13 (*E. lactis*), BL4.25, BL10 (*E. lactis*), BL14.2 (*E. faecium*), BL4.10, BL31, BL16.16 and BL4.18 (*Lb. plantarum*) affected the fungal growth with percentages which did not exceed 66.7 %. In particular the strain BL14.2 showed the weakest antagonistic activity, with percentages of inhibition of the fungal growth

Tab. 5. Antifungal activity of 16 lactic acid bacteria strains isolated from raw cows', goats' and camels' milk against five *A. alternata* strains.

LAB strain	*Alternaria alternata* strain				
	07MY	32MA	19MY	17MM	37MY
Enterococcus lactis BL2	+++	++	+++	+++	+++
Enterococcus lactis BL4.6	++	+	++	+	+++
Enterococcus sp. BL4.10	++	+	++	+	+
Enterococcus lactis BL4.13	++	+	++	++	+
Lactobacillus plantarum BL4.18	++	+	+	+	+
Enterococcus sp. BL4.19	++	+	+	++	+++
Enterococcus sp. BL4.25	+	+	+	+	+
Enterococcus lactis BL10	++	+	++	+	++
Enterococcus faecium BL14	++	+	++	+	+
Enterococcus faecium BL14.2	+	–	+	+	+
Enterococcus sp. BL14.16	++	+	+	++	++
Enterococcus sp. BL16.16	++	+	++	++	++
Enterococcus sp. BL16.19	++	+	++	+++	+
Enterococcus sp. BL27	++	+++	+	+	+
Enterococcus sp. BL31	+	+	+	+	+
Enterococcus faecium BL35	+++	+	++	+++	+++

LAB – lactic acid bacteria.
(–) – no visible inhibition, (+) – weak antifungal activity with inhibition rate between 13.3 % and 38.9 %, (++) – intermediate antifungal activity with inhibition rate between 40 % and 70 %, (+++) – strong antifungal activity with inhibition rate ≥ 70 %.

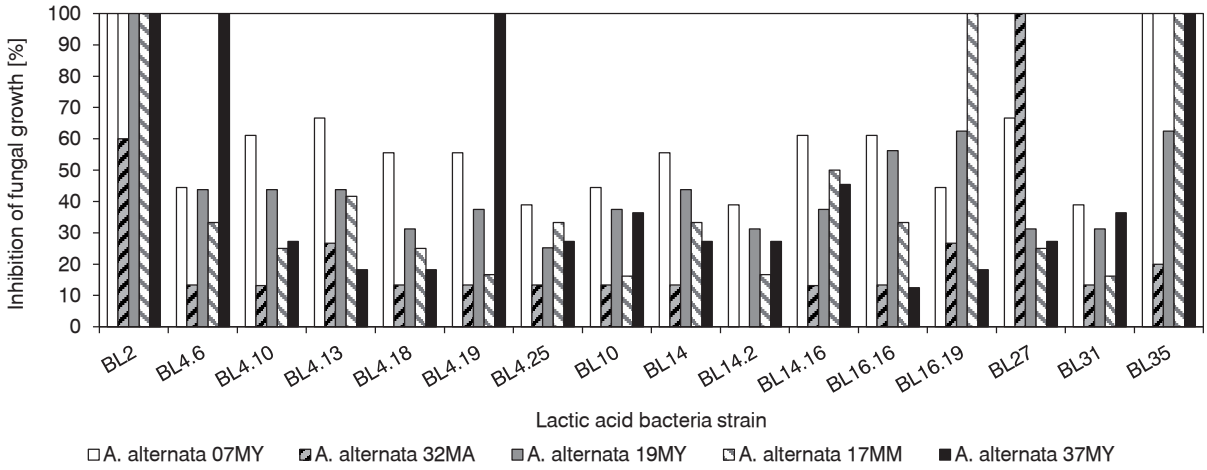

Fig. 3. Inhibition of five phytopathogenic *Alternaria alternata* strains by 16 strains of lactic acid bacteria.

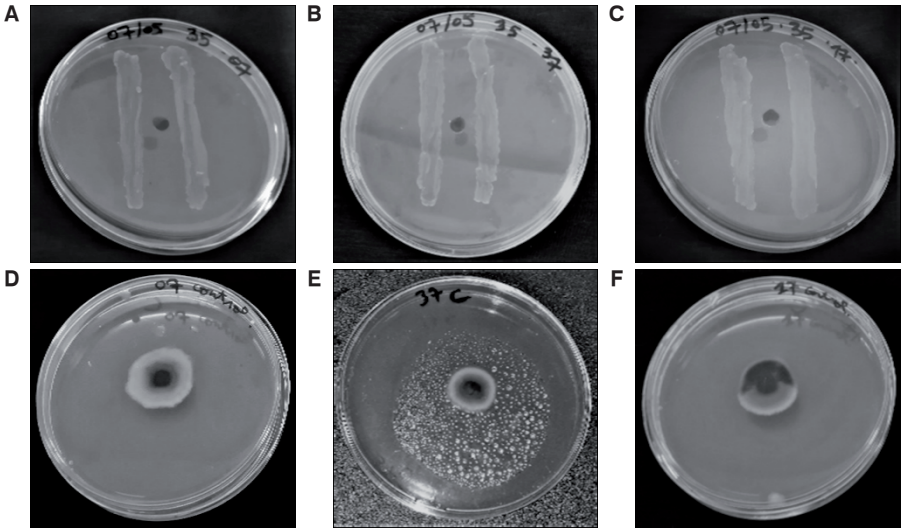

Fig. 4. Antifungal activity of *Enterococcus faecium* BL35 against *Alternaria alternata* strains.

A – *A. alternata* 07MY, B – *A. alternata* 37MY, C – *A. alternata* 17MM. D, E, F – controls.

between 0 % and 38.9 %. LAB strains BL2 and BL35 identified as *E. lactis* and *E. faecium*, respectively, have showed a very good antagonistic efficiency against the majority of *A. alternata* strains, evidenced by the total inhibition of fungal growth.

These findings are in agreement with other studies that demonstrated good antifungal activity of *E. faecium* for different phytopathogenic and spoilage fungi [31], including *A. alternata* [9]. The inhibition of filamentous fungi was also evidenced for *E. lactis*, antimicrobial properties of which were found to be connected with the production of a bacteriocin [38]. On the contrary, the strain BL4.18 identified as *Lb. plantarum*, the species having been reported in the literature as one of the most studied species for its antifungal properties [1], showed only a weak antifungal activity against *A. alternata* strains in this study.

CONCLUSIONS

In this study, we showed that raw cows', goats' and camels' milk can be a source for isolation of LAB strains able to inhibit the growth of the phythopatogenic and food spoilage fungus *A. alternata*, which is also known to produce various mycotoxins harmful to humans and animals. In particular, the strains *Enterococcus faecium* BL35 and *Enterococcus lactis* BL2 showed strong inhibi-

tory effects. Further investigations will be carried out to describe the mechanism and/or the nature of compounds involved in fungal growth inhibition.

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