Activity of probiotics from food origin for oxalate degradation

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Received: 28 January 2021 / Revised: 6 July 2021 / Accepted: 8 July 2021 / Published online: 20 July 2021
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Abstract
Kidney stones composed of oxalate are a significant health problem. It has been suggested that modification of the intestinal microbiota to reduce the amount of oxalate in the digestive system could be an effective treatment. There have been several studies into the use of lactic acid bacteria for the degradation of intestinal oxalates. We isolated 88 lactic acid bacteria strains from a range of dairy products, and screened for their ability to degrade oxalate. Using the oxalate-degrading Enzymatic Activity Index and the viable cell counts, five strains of *Lactobacillus fermentum* and two strains of *Lactobacillus gastricus* were identified as having strong oxalate degradation abilities, and were further investigated. All seven strains were able to tolerate acid (pH 4 and 3), bile salts (0.3%), phenol (0.3%), and to produce exopolysaccharides. They were resistant to a wide range of antibiotics. Among these strains, *Lactobacillus fermentum* NRAMJ5 and *Lactobacillus gastricus* NRAMJ2 were, therefore, good candidates as probiotics for managing hyperoxaluria.

Keywords Probiotic · Lactic acid bacteria · Kidney stones · Oxalate-degrading bacteria

Introduction
High levels of oxalate in the human body can cause a wide range of pathologies, such as hyperoxaluria, renal failure (Hoppe et al. 2009) and calcium oxalate urolithiasis (Campieri et al. 2001). Calcium oxalate stones are one of the most common types of kidney stone, a highly prevalent and painful disease. One of the biggest challenges of modern urology is the prevention of the recurrence of urinary calculi (Tavasoli et al. 2020).

Oxalic acid, also known as ethanedioic acid, is a highly oxidized, toxic organic compound. It is a white to colorless crystalline solid, widely distributed in nature, which occurs widely in plants, animals, and humans. In humans, as the end products of metabolism, oxalic acid and its oxalate salts are present in the blood plasma and urine. Some of the oxalate compounds in the human body come from consuming a variety of foods derived from oxalate-containing plant products, such as rhubarb, strawberries, beets, spinach, wheat bran, tea, chocolate, nuts, and coffee (Duncan et al. 2002). Some oxalate amount is derived from the endogenous metabolism of glyoxylate, ascorbic acid, and glycine in the liver (Holmes and Assimos 1998).

The human body lacks the enzymes necessary for metabolizing oxalate compounds. The body, therefore, deals with this potentially toxic compound in three ways. They may be absorbed via the urinary tract and excreted in the urine. Toxic oxalate compound in the gut may be compounded with calcium to form insoluble calcium oxalate, which can be eliminated in the feces. The third method of elimination is via microbial degradation by the microbiota found in the gastrointestinal tract (GIT). The amounts of oxalate and calcium are important in the rate of oxalate absorption and urinary excretion (Campieri et al. 2001).

Recent studies have indicated that using oxalate-degrading probiotic bacteria as a dietary supplement may help to manage hyperoxaluria and to minimize the opportunity for the formation of kidney stones (Okombo and Liebman 2010). Several other studies have indicated that probiotics may be valuable for the treatment and prevention of a variety of diseases, such as gastrointestinal disorders, cancer, and liver disease (Gill and Guarner 2004). Probiotics can also help to stabilize the gut microbiota and enhance the immune Brackenfort.

Communicated by Erko Stackebrandt.

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response, and can act against enteric pathogens (Gu et al. 2008). According to the FAO/WHO (2006), probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.”

The gut microbiota has been shown to consume oxalate to maintain oxalate homeostasis, leading to a reduction in urinary oxalate excretion (Allison et al. 1985; Ito et al. 1996). There is growing evidence that individuals with kidney stone disease have abnormal gut microbiota (Stanford et al. 2020). The Gram-negative bacterium Oxalobacter formigenes has been studied for its ability to utilize intestinal oxalate as a sole carbon source with which to regulate oxalate homeostasis (Duncan et al. 2020). Turroni et al. (2007) have studied Lactobacillus species isolated from different dairy and pharmaceutical products, and found that two strains, Lactobacillus acidophilus and Lactobacillus gasseri, showed significant oxalate degradation activity.

In this study, we aimed to isolate an efficient oxalate-degrading Lactobacillus sp. from local dairy products, and to evaluate its probiotic characteristics in vitro.

Materials and methods

Isolation and purification of lactobacilli

Samples of dairy products, including white cheese, Karish cheese, creamy cheese, Bramily cheese, labneh, yogurt, stirred yogurt (Zabado), and crude and Rayeb milk from the Cairo, Giza, Tanta, and Dakahalia governorates were collected from local markets. All samples were stored under refrigeration for subsequent use.

To isolate Lactobacillus spp., 10 g of each dairy product sample was added to 90 mL of 0.9% sterile saline. Cheese samples were emulsified in 2% (w/v) sterile trisodium citrate, and homogenized. Using sterile saline, serial dilutions were prepared, an appropriate volume of each sample was spread on MRS agar (Oxoid, Basingstoke, UK), and incubated at 37°C for 48 h under anaerobic conditions. After pure isolates were obtained, they were subcultured twice overnight in MRS broth for characterization.

Screening of isolates for oxalate utilization

Agar well-diffusion method using calcium oxalate plates

To assess the oxalate utilization ability of the bacterial isolates, 6 mm diameter wells were prepared using a cork borer in calcium oxalate plates. Each well was inoculated with 0.1 mL of overnight bacterial culture and incubated for 12 h at 37°C (Campieri et al. 2001). After incubation, bacteria with the ability to utilize oxalate form clear zones around the well in which they have been incubated, due to their decomposition of the oxalate. The diameters of the zones were measured, and oxalate-degrading enzyme indexes were calculated as described by Afriani et al. (2018) using the following formula.

Enzymatic Activity Index = diameter of hydrolysis zone/diameter of colony.

Growth in oxalate enriched media

Bacterial counts (cfu/mL) were carried out for selected isolates, in MRS broth containing 20 mmol/L of calcium oxalate (MRS-Ox). After incubation at 37°C for 72 h, bacterial count for each isolate was assessed by plate count (cfu/mL) on MRS agar, as described by Murrur et al. (2017).

Combined effect of the most potent isolates on oxalate utilization

The Lactobacillus isolates able to degrade oxalate were inoculated, separately and in combination, into MRS broth tubes (El-shafei et al. 2008). The tubes were incubated at 37°C for 48 h, each tube was serially diluted up to 10⁻⁷, and the bacteria were plated on MRS agar containing 50 mmol/L calcium oxalate (MRS-Ox) (Murrur et al. 2017). The clear areas around the bacterial colonies were measured, and the oxalate-degrading enzyme indexes were calculated, to determine the combined effect among the cultures.

Identification of bacterial isolates

Phenotypic identification of the isolates

The colony characteristics of the bacteria, such as size, pigment, elevation, opacity, surface, edge and shape, were measured. Pure cultures were preliminarily characterized based on Gram staining, catalase tests, glucose fermentation, and acid production, using the methods published by Gomathi et al. (2014). Furthermore, the strains were identified using an API 50 CHL (API System, bioMérieux, France) assay, following the manufacturer’s instructions. The color reactions were recorded against a chart provided by the manufacture (Conter et al. 2005). The results were analyzed using API WEB (bioMérieux).

Genotypic identification of the bacterial isolates using 16S rDNA sequencing

The potent Lactobacillus strains were genetically identified using 16S rDNA sequencing. DNA extraction was carried out using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

PCR amplification was carried out using two primers (27F 5′-AGAGTTTGATCCTGCGTG-3′; 1492R 5′-GGT
TACCTTGTACAGCTT-3'). The PCR amplification reaction was as follows: 50 µL (5 µL of 10× DreamTaq Green PCR buffer (Thermo Fisher Scientific, Waltham, MA), 2 µL of each 10 µmol.dm\(^{-3}\) primer, 5 µL of 2 mmol.dm\(^{-3}\) dNTP, 0.3 µL Taq DNA polymerase and 0.5 µL of template DNA). The PCR reaction was run under the following conditions: denaturation step at 94 °C for 5 min followed by 35 cycles of amplification (94 °C for 45 s, 55 °C for 30 s, and 72 °C for 60 s) and a final extension step at 72 °C for 5 min. The PCR amplified products were run on 1.5% agarose gels with TAE buffer containing ethidium bromide, and bands of the expected sizes were excised and purified using Montage PCR Clean up kit (Millipore, Burlington, MA) according to the manufacturer’s directions. The purified products were then shipped for sequencing by Macrogen, Inc. (Seoul, Korea). The similarity and homology of the 16S rDNA sequences were analyzed by comparing the sequences with known sequences available at the NCBI database using online BLAST search tools. A phylogenetic tree was constructed using MEGA-X software (Kumar et al. 2018) and evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The final phylogenetic tree involved 13 nucleotide sequences and all bacterial 16S rRNA gene sequences obtained were submitted to GenBank via BankIt at the NCBI webpage with unique accession numbers. The sequences of the isolates were deposited in the GenBank database with accession numbers MT712170 to MT712176, and MT731344.

**Probiotic properties of the isolates**

**Acid tolerance**

The resistance of the lactobacilli strains to acidic pH was measured by growing the bacteria in acidic MRS broth. Briefly, MRS broth was poured into test tubes and the pH was adjusted with 1 N HCl and 1 N NaOH to 7.0 (control), 4.0, 3.0, and 2.0. Each *Lactobacillus* isolate was inoculated into each tube and incubated at 37 °C. Growth was monitored using the plate count method, with 1 mL of sample taken after 0 h, 1 h, 2 h, and 3 h (Awan and Rahman 2005).

**Bile tolerance**

The ability of isolated strains to survive in the presence of bile salts was measured as described by Dunne et al. (2001). Briefly, MRS broth was enriched with 0.0%, 0.1%, 0.3%, 0.5%, and 1.0% (w/v) of ox gall (Sigma-Aldrich, St. Louis, MO), then inoculated with each bacterial culture. Growth was measured after incubation under anaerobic conditions at 37 °C for 48 h, using the plate count method (Awan and Rahman 2005).

**Antimicrobial activity**

To measure the antimicrobial activity of the *Lactobacillus* strains, the bacteria were incubated in MRS broth overnight at 37 °C. After incubation the cultures were centrifuged at 4000 g at 4 °C for 15 min, and the pH of the supernatant was adjusted to 6.5 using 1 M NaOH. These neutralized supernatants were tested against indicator strains using a well-diffusion method (De Vuyst et al. 2004). Wells of 0.5 mm diameter were prepared in nutrient agar plates previously inoculated with 100 µL of overnight cultures of pathogenic bacteria. The neutralized supernatants were added to the well, and incubated at 37 °C for 24 h. The antimicrobial activity was determined by measurement of the diameter of the inhibition zones around the wells. The pathogenic strains were obtained from the Dairy Microbiology Lab., National Research Centre, Egypt.

**Antibiotic susceptibility**

The antibiotic resistance activity of the selected bacterial strains was studied as described by the Clinical and Laboratory Standards Institute (CLSI 2009; Anandharaj and Sivasankari 2014). The diameter of the inhibition zone was measured using an antibiotic zone scale. The results were described in terms of sensitive (susceptible) (S), intermediate (I), or resistant (R). The results were compared with the interpretation of the zone diameters described by the Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI 2009). The susceptibility pattern measured using 17 different antibiotic disks (Oxoid) is presented in Table 5.

**Production of exopolysaccharides (EPS)**

The bacterial strains were grown on MRS agar supplemented with 100 g/L sucrose. The plates were incubated at 37 °C for 48 h under anaerobic conditions. After incubation, the colonies were tested by touching them with a sterile metal loop, and the formation of slime was observed (Herrero et al. 1996).

**Resistance to phenol**

Resistance to phenol was measured using MRS agar supplemented with different concentrations of phenol: 0%, 0.1%, 0.2%, 0.3%, and 0.5% (Yadav et al. 2007). Plates were incubated anaerobically for 48 h at 37 °C, and viable counts were determined.
Fermentation of carbohydrates

The carbohydrate fermentation patterns of the strains were analyzed using a commercial API 50 CH system (bioMérieux), following the manufacturer’s instructions.

Results and discussion

Isolation and purification of lactobacilli

Thirty-two different dairy products were collected from local markets; two samples of white cheese, five Karish (cottage) cheeses, three creamy cheeses, two Bramily cheeses, six Labneh, six yogurts, six stirred yogurts (Zabado), four raw milks, and four Rayeb milks from the Cairo, Giza, Tanta, and Mansoura governorates. A total of 495 isolates were isolated from the collected dairy product samples and primarily characterized using Gram’s stain, catalase tests, glucose fermentation, and acid production tests to select lactobacilli colonies. Only 88 isolates out of 495 were characterized as lactobacilli and selected to examine their ability to degrade oxalate.

Screening of isolates for oxalate utilization

Agar well-diffusion method using calcium oxalate plates

The oxalate-degrading ability of the presumptive isolates was assessed in vitro using the calcium oxalate plate method. The isolates showed a high degree of variability in oxalate degradation. Some isolates were unable to grow on MRS-Ox, while others were able to grow on MRS-Ox without a clear zone around the colonies. Only 23 isolates were able to grow and produce a clear zone around the colonies, indicating that these isolates possessed the oxalate-degrading enzyme (Fig. 1). The microbial enzymes formyl-CoA transferase and oxalyl-CoA decarboxylase have been identified in some, but not all, oxalate-degrading gut bacteria, including those from the genera Enterococcus and Lactobacillus (Miller and Dearing 2013). Chamberlain et al. (2019) confirmed the oxalate-degrading ability of L. gasseri and L. acidophilus in the presence of other preferred carbon sources by measuring in vitro 14C-oxalate consumption via liquid scintillation counting.

A semi-quantitative method was used to measure the Enzymatic Activity Index. Table 1 shows the measured diameter (mm) of clear zones around the colonies, and the Enzymatic Activity Indexes of the different isolates. C.O.L.15 and R.O.L.09 isolates had the highest Enzymatic Activity Index, indicating the highest oxalate degradation ability. Turroni et al. (2010) reported that the maximum oxalate degradation ability was measured in isolates of L.
salivarius AB11 (62.59%), L. fermentum TY12 (58.3%), and five strains of L. fermentum. These results demonstrate that oxalate degradation activity is both species and strain specific. Murphy et al. (2007) reported that oxalate utilization among probiotics in vitro was interspecies dependent.

Growth in oxalate enriched media

Viable counts were used to compare species that had the capability of oxalate degradation. Some isolates showed better growth in the presence of oxalate MRS-Ox broth than in MRS broth: K1.O.L09, C.O.L10, C.O.L15, Z.O.L03, R.O.L09, K2.O.L02, C.O.L06, K3.O.L07, and Y.O.L03. Among the tested isolates, Z.O.L03 had the best growth in the presence of oxalate. Campieri et al. (2001) tested L. acidophilus for oxalate degradation in oxalate-containing media, and observed a fivefold increase in population density. The growth of other isolates was not enhanced in the presence of oxalate. Lactobacillus spp. are known as “generalist oxalotrophs”, which do not depend entirely on oxalate as a carbon and energy source, but can ferment other substrates for growth (Sadaf et al. 2017). These potent isolates were assed for their combined effect.

Combined effect of the most potent isolates on oxalate utilization

Table 2 shows the effect of different combinations of the most potent isolates on the oxalate-degrading Enzymatic Activity Index. From the results it is clear that the combination between (i)-Z.O.L03 and K2.O.L02 (ii)-K2.O.L02 and K3.O.L07 increased the Enzymatic Activity Index to 6.3 and 6.9, respectively. These findings are in harmony with those obtained by Lieske et al. (2005, 2010), who reported that; the mixed probiotic “Oxadrop” (VSL Pharmaceuticals, Gaithersburg, Maryland, USA), which contain L. brevis, L. acidophilus, B. infantis, and S. thermophilus, reduced urinary oxalate levels in patients with a history of calcium oxalate urolithiasis. Miller and Dearing (2013) indicated that microbe-microbe interactions between the inoculated bacteria and natural oxalate-degrading populations enhance the oxalate-degrading function when dietary oxalate becomes scarce.

Identification of the bacterial isolates

Phenotypic identification of the isolates

On MRS agar plates, the isolates showed a distinct morphological appearance, being small white spindle-shaped cells with sharp ends, convex sub-surface, and entire margins, and forming large creamy white spindle-shaped colonies with sharp ends, convex sub-surface, and entire margins. The 88 isolates were Gram-positive, short to medium long rod shaped, non-spore forming bacteria under the light microscope. All of them were catalase negative, able to ferment glucose and to produce acid. On the other hand, results from the API 50 CH test kits and the API software database were used to identify the ten isolates (L.O.L07, K1.O.L09, C.O.L10, C.O.L15, Z.O.L03, R.O.L09, K2.O.L02, C.O.L06, K3.O.L07, and Y.O.L03). The different isolates varied in their utilization of carbohydrate sources, according to API 50 CHL assays. The results indicated that only three isolates, L.O.L07, K1.O.L09 and C.O.L10, did not belong to the genus Lactobacillus. Isolates C.O.L15 and Z.O.L03 were found to be two different strains of Lactobacillus sp.1, while R.O.L09, K2.O.L02, C.O.L06, K3.O.L07, and Y.O.L03 were found to be five different strains of Lactobacillus sp.2. These results agree with those obtained by several researchers who focused on the isolation and characterization of lactic acid bacteria from raw milk and fermented dairy products (Tserovska et al. 2002; Lee et al. 2011; Pringsulaka et al. 2012).

Genotypic identification of the isolates using 16S rDNA

Genotype identification of the bacteria was performed using 16S rDNA with two universal primers. The results suggested that isolates R.O.L09, K2.O.L02, C.O.L06, K3.O.L07, and Y.O.L03 were Lactobacillus fermentum. Isolates C.O.L15 and Z.O.L03 were identified as Lactobacillus gastricus.

Table 2 The combined effect between the isolates on oxalate degrading enzymatic activity indexes

| Bacterial isolates | C.O.L06 | C.O.L15 | Z.O.L03 | K2.O.L02 | R.O.L09 | K3.O.L07 | Y.O.L03 |
|--------------------|---------|---------|---------|----------|---------|----------|---------|
| C.O.L06           | 5       | 2.6     | 4.2     | 2.5      | 4.4     | 5.2      | 2       |
| C.O.L15           | 4.2     | 7.5     | 3.6     | 4.7      | 4.1     | 2.8      | 4       |
| Z.O.L03           | 2.8     | 3       | 5.8     | 6.3      | 3.3     | 6.3      | 2.3     |
| K2.O.L02          | 4.5     | 2.2     | 6.5     | 2        | 3       | 6.9      | 2.5     |
| R.O.L09           | 4.2     | 3.1     | 3.8     | 2.4      | 7.5     | 3.5      | 1.3     |
| K3.O.L07          | 2.9     | 4       | 2.1     | 4.9      | 6       | 6.7      | 2.2     |
| Y.O.L03           | 1       | 2.3     | 1.4     | 1.6      | 4.5     | 2.2      | 7       |
Phylogenetic analysis based on 16S rDNA

Seven selected *Lactobacillus* isolates NRAMJ1, NRAMJ2, NRAMJ3, NRAMJ4, NRAMJ5, NRAMJ6, and NRAMJ7 were identified genetically by 16S rDNA sequencing. The bacterial DNA was extracted, amplified, sequenced, and aligned with identified sequences from the GenBank database, and the similarity of the sequences was calculated using the online BLAST tool (http://www.blast.ncbi.nlm.nih.gov/Blast). The results showed that the 16S rDNA sequences of the NRAMJ1, NRAMJ4, NRAMJ5, NRAMJ6, and NRAMJ7 isolates had high similarity with *Lactobacillus fermentum*, with identity scores of 100%, 99.27%, 100.00%, 99.77%, and 100%, respectively. The results confirmed a high similarity of the 16S rDNA sequences for the NRAMJ2 and NRAMJ3 isolates with *Lactobacillus gastricus*, with identity 98.24% and 97.25%, respectively. A phylogenetic tree was constructed using the MEGA-X program and presented in Fig. 2.

Probiotic properties of the strains

To select the most potent *Lactobacillus* candidates for the use as probiotics, strains showing a high degree of oxalate degradation were selected for probiotic assessment.

Acid tolerance

Figure 3 shows the ability of the seven *Lactobacillus* strains to tolerate different acidic pH values. pH values of 2, 3, and 4, with pH 7 as the control, were tested in MRS medium. The tolerance of all species to acidic environments was variable. At pH 4 and 3, *Lactobacillus fermentum* NRAMJ1 and *Lactobacillus gastricus* NRAMJ2 showed a slight reduction, between 0.1 and 0.7 log unit, after 3 h of incubation. However, the strains *Lactobacillus gastricus* NRAMJ3, *Lactobacillus fermentum* NRAMJ5, and *Lactobacillus fermentum* NRAMJ7 were the most resistant at pH 4 and 3, and their viable count increased. *Lactobacillus fermentum* NRAMJ6 showed a slight reduction in viable count after 1 and 2 h of incubation, but recovered after 3 h of incubation. Also, all the strains were able to save viable counts upon 6 log CFU/mL in pH 4 and 3 after 3 h of incubation. At pH 2, all the isolated strains did not survive at this acidic pH and their viable numbers showed a severe reduction after 3 h of incubation but all the strains can survive for 60 min except *Lactobacillus fermentum* NRAMJ6 lost its viability after 30 min. *Lactobacillus fermentum* NRAMJ5 was the only strain which exhibited high survival after 3 h of exposure to an acidic pH 2. These results are in agreement with those of other lactobacilli investigated by Corcoran et al. (2005), who found that, *L. rhamnosus* GG had the highest survival rate up to 90 min of exposure to gastric juice (pH 2.0), while *Lactobacillus paracasei* NFBC 338 was the poorest survivor, with a decrease in concentration to very low levels after only 30 min. Charteris et al. (1998) found that lactobacilli can tolerate pH 2.0 for several minutes.

Bile tolerance

One important characteristic of the *Lactobacillus* strains is their tolerance to bile salts. Bile salts disorganize the structure of the cell membrane (Bao et al. 2010), so this tolerance allows the bacteria to survive and grow in the upper small intestine. The survival after incubation at 37 °C for 48 h of the seven *Lactobacillus* strains tested on MRS agar medium supplemented with 0.1%, 0.3%, 0.4%, 0.5%, and 1% bile salts (oxgall), with no bile salts in the
control, are presented in Table 3. All the *Lactobacillus* strains were able to survive in the different concentrations of bile salts and still produce viable counts of 6 log CFU/mL, as required for them to be probiotic. With increasing concentrations of bile salts the viable count gradually decreased. It has been reported that some probiotics avoid this problem by producing bile salt hydrolase, which can hydrolyze conjugated bile salts to decrease their toxicity (Guo et al. 2019).

### Table 3 Survival of the tested *Lactobacillus* strains in different concentrations of bile salt after incubation at 37 °C for 48 h

| Bacterial strain           | Control (no bile salt) | Growth (log cfu/mL) at different bile salt concentrations (%) |
|---------------------------|------------------------|---------------------------------------------------------------|
|                           |                        | 0.1   | 0.3   | 0.4   | 0.5   | 1     |
| *Lactobacillus fermentum* | 9.38                   | 9.27  | 9.23  | 9.07  | 9.03  | 8.92  |
| *Lactobacillus gastricus* | 9.23                   | 9     | 9     | 8.91  | 8.73  | 7.63  |
| *Lactobacillus*            | 9.04                   | 8.88  | 8.87  | 8.77  | 7.95  | 6.69  |
| *Lactobacillus*            | 9.18                   | 8.90  | 8.77  | 8.73  | 8.20  | 8.14  |
| *Lactobacillus*            | 9.36                   | 9.25  | 9.23  | 9.14  | 8.60  | 8.40  |
| *Lactobacillus*            | 9.14                   | 8.92  | 8.85  | 8.83  | 8.51  | 8.07  |
| *Lactobacillus*            | 9.12                   | 9.07  | 9.07  | 8.99  | 8.90  | 8.83  |

**Antimicrobial activity**

The antimicrobial activity of the seven *Lactobacillus* strains against some enteric pathogens is presented in Table 4. The indicator pathogens included Gram-positive bacteria such as *B. subtilis, B. cereus*, and *Listeria monocytogenes*; Gram-negative bacteria such as *Pseudomonas aeruginosa, Salmonella* *sp.*, and *Yersinia* *sp.*; the yeasts *Saccharomyces cerevisiae* and *Candida albicans*; and the filamentous fungus *Aspergillus niger*. The results showed that *Lactobacillus fermentum NRAMJ5* was the most potent strain, on the basis of inhibition zone, against all tested indicators. All strains
### Table 4  Antimicrobial activity of *Lactobacillus* strains tested toward some indicator pathogenic strains

| Bacterial strain | Indicator strains | B. subtilis | P. aeruginosa | B cereus | Salmonella sp. | C. albicans | S. cerevisiae | Yersinia sp. | A. niger | L. monocytogenes |
|------------------|-------------------|-------------|---------------|----------|----------------|-------------|--------------|-------------|---------|-----------------|
| *Lactobacillus fermentum* NRAMJ1 | -ve | -ve | -ve | -ve | 1.5 | 3.5 | 0.5 | 1.5 | -ve |
| *Lactobacillus gastricus* NRAMJ2 | 1 | 1 | -ve | 1.2 | 3 | 3.2 | 1 | 4 | 1 |
| *Lactobacillus gastricus* NRAMJ3 | -ve | -ve | -ve | 2.4 | -ve | 3 | 1.5 | 4.5 | 1.5 |
| *Lactobacillus fermentum* NRAMJ4 | -ve | -ve | -ve | 3 | 2 | 4 | 1.5 | 2 | -ve |
| *Lactobacillus fermentum* NRAMJ5 | 1 | 1 | 1.8 | 2.3 | 1.5 | 3 | 2.5 | 1.5 | 1 |
| *Lactobacillus fermentum* NRAMJ6 | 1.5 | 1.5 | -ve | 2 | 1.2 | 3.5 | 1.2 | 3.5 | 1.9 |
| *Lactobacillus fermentum* NRAMJ7 | 1 | -ve | -ve | -ve | 0.5 | 2.5 | 1.5 | 3.5 | -ve |

-ve: no inhibition zone
had strong antimicrobial activity against A. niger, S. cerevisiae, and Yersinia sp. Lactobacillus gastricus NRAMJ3 was unable to inhibit the growth of C. albicans, while Lactobacillus gastricus NRAMJ2 showed the largest inhibition zone against C. albicans. Both Lactobacillus fermentum NRAMJ1 and Lactobacillus fermentum NRAMJ7 were unable to affect the growth of Salmonella sp. No strains had antimicrobial activity against B. cereus, except for Lactobacillus fermentum NRAMJ5. Lactobacillus fermentum NRAMJ4, Lactobacillus gastricus NRAMJ2, and Lactobacillus fermentum NRAMJ5 had antibacterial activity against B. subtilis and P. aeruginosa. In summary, the most sensitive organisms were A. niger, S. cerevisiae, and Yersinia. The Lactobacillus strains had stronger antimicrobial activity against pathogenic Gram-negative bacteria than against Gram-positive bacteria. The diameter of the inhibition zones ranged from 0.5 to 3 mm. for Salmonella sp. and Yersinia sp., while the diameter of the inhibition zones was from 0.5 to 1.5 mm. for B. subtilis and B. cereus. Salmonella sp. and Yersinia sp. were more sensitive to the metabolites of Lactobacillus sp.

De Vuyst et al. (2004) found that L. rhamnosus strain GG probably produces an agent with antimicrobial activity that is active in vitro toward Salmonella typhimurium. These researchers also found that several Lactobacillus strains produce antimicrobial, low molecular mass, heat-stable, proteinaceous compounds, so-called bacteriocin-like peptides, with a broad inhibitory spectrum, including both Gram-positive and Gram-negative bacteria.

Heredia-Castro et al. (2017) recorded that crude extracts of Lactobacillus fermentum displayed a strong inhibitory activity against S. aureus, E. coli, L. innocua, and S. cholerae. The results showed a strong antifungal activity of the isolates, as the diameter range of the inhibition zone was from 1.5 to 4.5 mm. These results are in agreement with those of Tropcheva et al. (2014) who found that the strains L. brevis KR3, L. brevis KR4, and L. brevis KR51 completely suppress the growth of Aspergillus awamori, Penicillium claviforme, and Aspergillus niger, and show antimicrobial activity against Candida albicans. A few reports have shown that Lactobacillus spp. have strong anti-Candida activity (Atanassova et al. 2003; Reonqvist et al. 2007). Therefore, the isolates identified in our study are recommended for use as starter cultures in the food industry, co-cultures, or bioprotective cultures, to improve food safety and quality or as probiotic therapeutics for clinical practice.

**Antibiotic susceptibility**

The close contact between Lactobacillus spp. and the human intestinal flora is an excellent precondition for the transfer of antimicrobial resistance genes, with the assistance of transposable elements (Teuber et al. 1999). Thus the selection of antibiotic-resistant probiotic cultures and starters is important to eradicate opportunities for the development of acquired resistance. The antibiotic susceptibilities of the seven Lactobacillus strains were tested using the antibiotic disk diffusion method on Mueller–Hinton agar medium, and the results are presented in Table 5. All of the strains were sensitive to cefuroxime, ampicillin, clindamycin, ampicillin, rifampin, gentamycin, chloramphenicol, tetracycline, amoxicillin, and cefoperazone. However, all of the isolates were resistant to ciprofloxacin, except for Lactobacillus gastricus NRAMJ2. These results are in accordance with those of Georgieva et al. (2015), who reported that all the Lactobacillus strains they tested were susceptible to gentamicin, ampicillin, erythromycin, and tetracycline. Lactobacillus fermentum NRAMJ6 and Lactobacillus gastricus NRAMJ2 were the only strains that were resistant to cephalixin.

**Table 5** Antibiotic resistance profiles of the tested Lactobacillus strains after incubation at 37 °C for 48 h

| Lactobacillus strain | Antibiotic resistance |
|----------------------|-----------------------|
|                      | AMC | CXM | SAM | NA | Nor | DA | Amp | CL | RA-5 | CN | CIP | C | TE | AX | VA | CEP |
| Lactobacillus fermentum NRAMJ1 | S   | S   | S   | R  | R   | S   | S   | S   | S   | R   | S   | S   | R   | S   |     |
| Lactobacillus gastricus NRAMJ2 | S   | S   | S   | S   | S   | S   | S   | R   | S   | S   | S   | S   | S   | S   | S   |
| Lactobacillus gastricus NRAMJ3 | S   | S   | S   | S   | S   | S   | R   | S   | S   | S   | S   | S   | S   | S   | S   |
| Lactobacillus fermentum NRAMJ4 | S   | S   | S   | S   | S   | S   | S   | S   | R   | S   | S   | S   | R   | S   | S   |
| Lactobacillus fermentum NRAMJ5 | S   | S   | S   | S   | R   | I   | S   | S   | S   | R   | S   | S   | S   | S   | S   |
| Lactobacillus fermentum NRAMJ6 | R   | S   | S   | S   | S   | S   | R   | S   | S   | S   | S   | S   | S   | S   | S   |
| Lactobacillus fermentum NRAMJ7 | S   | S   | S   | R   | S   | S   | S   | S   | R   | S   | S   | S   | S   | R   | S   |

Nalidixic acid (NA 30 µg/mL), Norfloxacin (Nor 10 µg/mL), Tetracycline (TE 30 µg/mL), Amoxicillin-clavulanic acid (AMC 20/10 µg/mL), Clindamycin (DA 2 µg/mL), Ampicillin-Subbactam (SAM 20 µg/L), Cefuroxime (CXM 30 µg/mL), Gentamycin (CN 10 µg/mL), Ampicillin (Am 10 µg/mL), Kanamycin (K 30 µg/mL), Cefalexin (CL 30 µg/mL), Chloramphenicol (C 30 µg/mL), Ciprofloxacin (CIP 5 µg/mL), Vancomycin (VA 30 µg/mL), Amoxicillin (Ax 25 µg/mL), Rifampin (RA 5 µg/mL), Cefoperazone (CEP 75 µg/mL)

R resistant, I intermediate, S sensitive, — no growth
Production of extrapoly saccharides (EPS)

The *Lactobacillus* strains were tested for EPS production on MRS agar medium containing 100 g/L sucrose. The polysaccharide slime was assessed by touching their colonies with a metallic loop. These EPS appear to be safe additives for food and non-food manufacture (Crescenzi 1995). All of the *Lactobacillus* isolates tested in this study were positive for production of exopolysaccharide, as in the results of Li et al. (2014) who produced EPS of 3 strains of *Lactobacillus helveticus* MB2-1 in vitro. Oleksy and Klewicka (2016) reported that *Lactobacillus* spp. synthesized EPS, including both homo- and hetero-polysaccharides, which have significant utility in the production of fermented foods, with their structure protecting the strains from harsh conditions.

Resistance to phenol

The gut microbiota can deaminate the aromatic amino acids derived from dietary proteins to form phenols. These phenol compounds can suppress the growth of LAB. Therefore, phenol resistance by probiotics is critical factor for their survival in the mammalian gut (Xanthopoulos et al. 2000). The survival of the seven *Lactobacillus* strains, after incubation at 37 °C for 48 h at phenol concentrations of 0.1%, 0.2%, 0.3%, and 0.5%, and without phenol as control, are presented in Fig. 4. All of the strains tested could resist phenol concentrations of 0.1%, 0.2%, and 0.3%. However, with increasing concentration of phenol the count of the strains decreased, but the counts were still up to 6 log CFU/mL. None of the isolates were able to resist a concentration of phenol of 0.5%. The results are in agreement with those of Forhad et al. (2015), who found that at a 0.1% concentration of phenol all of the isolates showed a high level of tolerance, at 0.3% the tolerance was moderate, but at 0.4% tolerance was much lower.

Fermentation of carbohydrates

The ability of the seven *Lactobacillus* strains to ferment different carbohydrates was examined using the API 50 CH system. In this study, all of the isolates were able to ferment nine different carbohydrates: glucose, galactose, fructose, mannose, ribose, N-acetyl-glucosamine, salicin, and lactose, indicating that they were able to grow in variety of habitats, utilizing different type of carbohydrates.

Conclusions

In this study, seven strains of *Lactobacillus* spp. were isolated from different dairy products. These strains showed oxalate-degrading ability. The seven strains isolated were identified as five strains of *Lactobacillus fermentum* and two strains of *Lactobacillus gastricus*, using 16S rRNA gene sequencing. These seven strains are candidate probiotics, and have good probiotic properties due to their tolerance to acidity and bile salts. The isolates exhibited strong antagonistic effects against the pathogens tested, and had an absence of transferable antibiotic resistance. The focus of our next study will be the improvement of the value of these probiotic strains, using them for the prophylaxis of calcium oxalate stone disease in a rat model.
Author contributions NS performed the experiments and all authors wrote the manuscript, analyzed the data. BE, NM, NT, MI guided the experiments and all authors revised the manuscript.

Funding The authors would like to thank the National Research Centre, Egypt for funding.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

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