Activity of probiotics from food origin for oxalate degradation

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Original Paper

Keywords: Probiotic, lactic acid bacteria, oxalate degrading bacteria

Posted Date: March 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-170748/v1

Version of Record: A version of this preprint was published at Archives of Microbiology on July 20th, 2021. See the published version at https://doi.org/10.1007/s00203-021-02484-3.

Abstract

There has been a concentrated attempt to introduce bacteria with oxalate degrading activity into the mammalian gut microbiota to enhance ecosystem function towards more effective oxalate degradation and prevent diseases. A few reports have studied Lactic acid bacteria (LAB) as promising intestinal oxalate degrading probiotic to treat kidney stone disease. In the present study, a total of 495 LAB isolates from different dairy products samples were primary characterized. Eighty-eight of the isolates belonged to genus *Lactobacillus* and were screened for their ability to degrade oxalate. Twenty-three of isolated lactobacilli showed oxalate degradation ability. According to the diameter of clear zone and the viable count, ten isolates were the most potent isolates for oxalate degradation. After the genotypic identification using 16s rDNA sequencing for these potent isolates, only seven strains were belonging to genus *Lactobacillus* spp. *Lactobacillus fermentum* (5 strains) and *Lactobacillus gastricus* (2 strains). These isolates were assessed for potential probiotic properties *in vitro* for further *in vivo* assessment. In conclusion, they exhibited good probiotic characters that suggested them to serve as good probiotic candidates for managing hyperoxaluria.

Introduction

Oxalic acid is a highly oxidized organic compound that is widely distributed in nature. It occurs widely in plants and animals. Oxalic acid and its oxalate salts occur in the blood (plasma) and urine of animals and humans. In human, part of the oxalate comes from consuming the oxalate-containing plant products in the shape of foods such as rhubarb, strawberries, beets, spinach, wheat bran, tea, chocolate, nuts, and coffee **(Duncan et al. 2002).** While, a certain oxalate amount also formed in the liver endogenously as an end-product resulting during the metabolism of glyoxylate, ascorbic acid and glycine **(Holmes and Assimos 1998).**

Due to the lack of enzymes in the human body that are needed for the metabolization of oxalate compound, the body deal with this potentially toxic compound in three ways: The first way is the elimination by absorption of these compound toward the urinary tract and excretion it with urine. The second way is the elimination in feces by combination of the oxalate toxic compound in the gut with calcium to form insoluble calcium oxalate. The third way is the microbial degradation by microbiota that found in the gastrointestinal tract (GIT). The amounts of oxalate and calcium are important factors that influence the rate of oxalate absorption and urinary excretion **(Campieri et al. 2001).** Oxalate high levels in human body can cause a wide range of medical pathologies such as hyperoxaluria, renal failure **(Hoppe et al. 2009)** and calcium oxalate urolithiasis **(Campieri et al. 2001)**. The calcium oxalate stones are one of the most common kidney stone, the highly prevalent and painful disease. One of the biggest challenges of the modern urology is the prevention of urinary calculi recurrence (**Tavasoli et al. 2020).**

Recent reports demonstrated that, using intestinal oxalate degrading bacteria is an appropriate probiotics solution to minimize the opportunity of kidney stone formation **(Okombo and Liebman 2010)**. Recently, probiotics have been served as a therapeutic agent for several diseases **(Gill and Guarner 2004)**. According to **FAO/WHO (2006),** Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'**.** It can be utilized in stabilizing gut microbiota, enhancement of the immune response and act as competitor against enteric pathogens **(Gu et al. 2008).** There is a lot of evidence that persons with kidney stone disease have an abnormal gastrointestinal tract (GIT) composition **(Stanford et al. 2020).**

Several studies have reported that, gut microorganisms utilizing the intestinal oxalate to maintain the oxalate homeostasis, while reducing the urinary oxalate excretion **(Allison et al. 1985; Ito et al. 1996)**. The oxalate degrading *Oxalobacter formigenes* is a Gram-negative bacterium, that uses intestinal oxalate as a sole carbon source in order to regulate the oxalate homeostasis. Other group of bacteria that recently have proved to be a vital resident of human intestinal ecosystem are Lactic acid bacteria (LAB), which have been used as probiotics due to their health promoting benefits to the host **(Gu et al. 2008).Turroni et al. (2007)** reported a range of oxalate degrading lactobacilli from dairy and pharmaceutical products and found significant oxalate degradation activity in *Lactobacillus acidophilus* and *Lactobacillus gasseri.*

The present study aims to isolate an efficient oxalate degrading *Lactobacillus* spp. from local dairy products and to evaluate the potential of probiotic characteristics *in vitro.*

Materials And Methods

Isolation, purification and identification of lactobacilli

Collection of samples

Different dairy product samples were collected from local markets such as: white cheese, karish cheese, creamy cheese, bramily cheese, labneh, yoghurt, stirred yoghurt (Zabado), crude and rayeb milk from different governorates (Cairo, Giza, Tanta, Dakahalia). All the collected samples were stored under refrigeration conditions for subsequent uses.

Isolation of *Lactobacillus* spp.

To isolate *Lactobacillus* spp., ten grams of each dairy product samples were added separately to 90mL of 0.9% sterile saline while cheese sample were emulsified in 2 % (w/v) sterile trisodium citrate and homogenized. Then, using sterile saline, serial dilution was prepared, and appropriate volume of each sample were spread on MRS agar (Oxoid) and incubated at 37^{o}C for 48h under anaerobic conditions. After pure isolates have been obtained, they subcultured twice overnight in MRS broth for characterization.

Characterization of the *Lactobacillus* isolates

Colony characteristics of the isolated bacteria were determined using parameters such as size, pigment, elevation, opacity, surface, edge and shape. Pure cultures were preliminarily characterized based on Gram's staining, catalase test, Glucose fermentation and acid production were performed according to **Gomathi et al. (2014).**

Screening of isolates for oxalate utilization

Agar well-diffusion method using calcium oxalate plates

To assess the oxalate utilization ability of the bacterial isolates, wells of 6mm diameter were prepared using corkborer in calcium oxalate plates. The well was inoculated with 0.1mL of overnight bacterial culture and incubated for 12h at 37^{o}C **(Campieri et al. 2001).** After incubation, bacteria with oxalate utilizing ability can form clear zone around the well due to oxalate decomposition. Zone diameter was measured.

Growth in oxalate enriched media

The bacterial count (cfu/mL) was performed for the selected isolates in MRS broth containing 20 mmol /L of calcium oxalate (MRS-ox). After incubation at 37°C for 72 h, bacterial count was assessed by plate count (cfu/mL) on MRS agar as described by **Murru et al. (2017).**

The combined effect between 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 hours then after that, each tube was serially diluted then plated on MRS agar containing 50 mmol /L calcium oxalate (MRS-ox) **(Murru et al. 2017).** The clear area around the bacterial colonies was an indication of oxalate degradation .Clear areas was recorded to determine the combined effect between the cultures**.**

Identification of the bacterial isolates

Phenotypic Identification of the isolates using API 50 CHL kit assay.

The isolated strains were identified using API 50 CHL (API system, BioMérieux, France) assay. Following the manufactures instructions. The Color reactions were recorded against a chart provided by the manufacture **(Conter et al. 2005)**. The results were analyzed using API WEB (Bio-Merieux).

Genotypic identification of the bacterial isolates Using 16s rDNA

Genomic DNA isolation

The potent *Lactobacillus* strains were identified genetically by sequencing of the 16S rDNA. DNA extraction was carried out using DNeasy Blood & Tissue Kit according to manufacturer instructions.

PCR amplifications

PCR amplification were carried out using 2 primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3'). The PCR amplification reaction was as fellow: 50 µl (5 µl of 10 × Dream Taq Green PCR buffer, 2 µl of each 10 µmol.dm^{-3} primer, 5µl of 2mmol.dm^{-3} dNTP, 0,3 µl Taq DNA polymerase and 0,5 µl of template DNA). The PCR reaction ran under the following conditions: 94^{o}C for 45 sec, 55^{o}C for 60 sec, and 72^{o}C for 60 sec.

Sequencing

The resulted PCR products were sequenced in 'Macrogen Company, South Korea'. The similarity and homology of the resulted 16S rDNA sequences were analyzed by comparing the sequences with known sequences available at the NCBI database using online BLAST search tools (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic trees were constructed using MEGA-X software.

Studying the probiotic properties for the isolates

Acid tolerance

Resistance of the lactobacilli strains to acidic pH was measured by growing the isolated bacteria in acidic MRS broth. Briefly, MRS broth was poured in test tubes and pH 7.0 (control), 4.0, 3.0 and 2.0 was adjusted with 1N HCl and 1N NaOH. Each isolate of lactobacilli was inoculated in each tube and incubated at 37^{o}C and growth was monitored using the plate count method, 1 ml of sample was taken after 0, 1, 2 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 were enriched with 0.0, 0,1, 0.3, 0.5 and 1.0% (w/v) of oxgall (Sigma), then inoculated with each bacterial culture. The growth was measured after incubation under anaerobic conditions at 37^{o}C for 48hrs using 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^{o}C. After incubation the culture were centrifuged at (5000rpm at 4^{o}C for 15 min), and the pH of supernatants was adjusted to 6.5 using 1M NaOH. These neutralized supernatants were tested

against indicator strains using well diffusion method (**De Vuyst et al. 2004)**. Wells with 0.5mm diameter were prepared in the nutrient agar plates previously inoculated with 100 µL of overnight pathogenic bacterial cultures. The neutralized supernatants were poured into the well incubated at 37 °C for 24 h. The antimicrobial activity was determined by measurement the inhibition zone diameter of around the well. The pathogenic strains were obtained from Dairy Microbiology Lab., National Research Centre.

Antibiotic susceptibility.

The antibiotic resistance activity of the selected bacterial strains was studied as described by the **Clinical and Laboratory Standards Institute(CLSI; Wayne, PA, USA) (Anandharaj and Sivasankari 2014).** The inhibition zone diameter was measured using an antibiotic zone scale. The obtained results were described in terms of susceptibility (S), Intermediate (I), or resistance (R). Results were compared with interpretative zone diameters described by Performance standards for Antimicrobial Disk Susceptibility tests **(CLSI 2009).** The susceptibility pattern was measured using 17 different antibiotic discs (Oxoid) were presented in **Table (6).**

Production of exopolysaccharides (EPS)

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

Resistance to different concentrations of 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^{o}C and viable counts were determined.

Fermentation of carbohydrates

The carbohydrates fermentation pattern of the isolated strains was analyzed using commercial API 50 CH system (bio Merieux, Marcyl' Etoile, France). Following the manufactures instructions.

Results And Discussion

Isolation, purification, and identification of lactobacilli

32 different dairy products were collected from local markets such as: (2) White cheese, (5) Karish cheese (cottage cheese), (3) Creamy cheese, (2) Bramily cheese, Labneh, (6) Yoghurt, (6) Stirred yoghurt (Zabado), (4) raw milk and (4) Rayeb milk from different governorates (Cairo, Giza, Tanta, Mansoura).A total of 495 isolates were isolated from the different collected dairy product samples. After primary characterization of them the experimental data cleared that among which 88 isolates were of lactobacilli. In MRS agar plates, the isolates showed different morphological appearance:(i) small white spindle shaped with sharply ends sub-surface convex with entire margin (ii) large creamy white spindle shaped with sharply ends colonies sub-surface convex with entire margin.

In addition, the isolates (88) were found Gram -positive, short to medium long rod shaped, non- spore forming bacteria under the light microscope. All of them were found catalase negative, able to ferment glucose and form a clear zone in 0.5% CaCO3 plate. This clear zone is created due to disappearance of the turbidity originally created by the lower solubility of calcium carbonate. However, as the lactic acid bacteria grow, they produce lactic acid that lower the pH and this in turn increases the solubility of calcium carbonate. Therefore, as the calcium carbonate dissolves that particular area becomes clear which indicates growth of the colony.

Screening of isolates for oxalate utilization

Agar well-diffusion method using calcium oxalate plate

Oxalate degrading ability for the presumptive isolates was assessed *in vitro* using calcium oxalate plate method. The isolates showed high degree of variability in oxalate degradation. Some isolates were unable to grow on MRS-Ox, others able to grow on MRS-Ox without clear zone around the colonies but only 23 isolates were able to grow with a clear zone around the colonies indicating that these isolates possess the oxalate degrading enzyme. It is worthy to mention that 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).** In the same point, **Chamberlain et al. (2019)** alluded to the confirmation of the oxalate-degrading ability of *L. gasseri* and *L. acidophilus* in the presence of other preferred carbon sources measuring *in vitro* ^{14}C-oxalate consumption via liquid scintillation counting.

As shown in **Table 1,** by comparing the diameter of the clear zone around the colonies the strains can be differed as (+++ve, ++ve and +ve)**. Turroni et al. (2010)** reported that, maximum oxalate degradation ability was measured in the isolates of *L. salivarius* AB11 (62.59%), *L. fermentum* TY12 (58.3%) and five strains of *L. fermentum*. These results demonstrated that oxalate degradation activity was both species and strain specific. Also, **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 capability for oxalate degradation. It is clear that some isolates showed a better growth in the presence of oxalate MRS-ox broth than MRS broth as following: L.O.L07, K1.O.L09, C.O.L10, C.O.L15, Z.O.L03, R.O.L09, K2.O.L02, C.O.L.06, K3.O.L07 and Y.O.L03. Among

the tested isolates, Z.O.L03had the best growth in the presence of oxalate as the increase of log count reached 0.24.In this respect, **Campieri et al. (2001)** tested *L. acidophilus* for oxalate degradation in oxalate containing media and the results demonstrated a five-fold increase in population density. The growth of other isolates was not enhanced in the presence of oxalate .Particularly, *Lactobacillus spp.* is known as" generalist oxalotrophs" which do not depend entirely on oxalate as a sole carbon and energy source and can ferment other substrates for growth **(Sadaf et al. 2017).**These potent isolates were assed for their combined effect.

The combined effect between the most potent isolates on oxalate utilization

Table 2 shows the effect of different combinations between the most potent isolates on the oxalate degradation. From the results it is clear that the combination between (i)-Z.O.L03and K2.O.L02, (ii)- K3.O.L07 and K2.O.L02 increased the diameter of the clear zone around the colonies which indicated increasing in oxalate degradation. These findings are in harmony with those obtained by **Lieske et al. (2005; 2010)** Who mentioned that a mixed probiotic "Oxadrop" (VSL Pharmaceuticals), which contains *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) pointed out that the differences in persistence between inoculated populations and natural oxalate-degrading populations suggest that other microbe-microbe metabolic interactions facilitate the persistence of the oxalate-degrading function when dietary oxalate becomes scarce. In addition, given the ecological interactions that occur within the gut microbiota in response to dietary oxalate, the use of whole communities adapted to oxalate degradation may be a more effective strategy for transferring oxalate-degrading function than other similar strains. Using whole communities ensures that all bacteria that maintain the oxalate-degrading function are present, increasing the probability of continued existence within the new community, even without an oxalate-rich diet or continued inoculation.

Identification of the bacterial isolates.

Phenotypic identification of the isolates using API 50 CHL kit assay

Results from the API 50 CH test kits and API software database identified 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)**.** There was a variation in carbohydrates sources utilization of the API CHL 50 systems by the different isolates. The results cleared that only three (3) isolates L.O.L07, K1.O.L09, C.O.L10 were not belonging to the genus *Lactobacillus.* Also, C.O.L15 and Z.O.L03 isolates were found as (2) 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 as five (5) different strains of *Lactobacillus* sp.2. These results agree with those obtained by several researcheres, 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

The genotype identification of 16s rDNA using two universal primers shown in **Table 3.** These data suggested that R.O.L09, K2.O.L02, C.O.LO6, K3.O.L07, Y.O.L03 isolates were identified as *Lactobacillus fermentum*. Also, C.O.L15, Z.O.L03 isolates were identified as *Lactobacillus gastricus* using genotypic identification. The sequences of the isolates were deposited in the GeneBank database with accession numbers MT712170 to MT712176 and MT731344.

Phylogenetic Analysis Based on 16S rDNA Gene

The 7 selected *Lactobacillus* isolates NRAMJ1, NRAMJ2, NRAMJ3, NRAMJ4, NRAMJ5, NRAMJ6 and NRAMJ7 were identified genetically by sequencing of 16S rDNA. The bacterial DNA was extracted, amplified, sequenced and aligned with identified sequences in the Gene bank database to measure the similarity score of the obtained sequences using online BLAST tool (http://www.blast.ncbi.nlm.nih.gov/Blast). The obtained results showed a high similarity of the 16S rDNA sequences for the NRAMJ1, NRAMJ4, NRAMJ5,NRAMJ6 and NRAMJ7isolates with *Lactobacillus fermentum* with homology and identity reached 100%, 99.27%, 100.00%, 99.77% and 100% respectively, the results confirmed high similarity of the 16S rDNA sequences for NRAMJ2,NRAMJ3 with *lactobacillus gastricus* with homology (98.24 and %97.25%).The partial 16S rDNA sequences from the *Lactobacillus* isolates identified as *Lactobacillus fermentum* NRAMJ1,*Lactobacillus gastricus*NRAMJ2, *Lactobacillus gastricus*NRAMJ3, *Lactobacillus fermentum* NRAMJ4, *Lactobacillus fermentum* NRAMJ5, *Lactobacillus fermentum* NRAMJ6, and *Lactobacillus fermentum* NRAMJ7 with accession numbers MT712170, MT712171, MT712173, MT731344, MT712174, MT712175 and MT712176 respectively. The phylogenetic tree was constructed using MEGA-X program and presented in **Figure 1(Kumar et al. 2016)** and neighbour-joining method **(Saitou and Nei 1987)**.

Study of the probiotic properties for the strains

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

Acid tolerance

Figure 2 shows the ability of the 7 *Lactobacillus* strains to tolerate different acidic pH values 2, 3, 4 and 7 as control tested in MRS medium. Tolerance level of all species to acidic environment was variable. At pH 4 and 3, some strains showed a slight reduction between 0.1-0.7 log unit *Lactobacillus fermentum* NRAMJ1 and *Lactobacillus gastricus* NRAMJ2 after 3hrs of incubation. However, *Lactobacillus gastricus* NRAMJ3, *Lactobacillus fermentum NRAMJ5* and *Lactobacillus fermentum NRAMJ7* strain were the most resistant at pH 4 and 3 and their viable count increased. In addition, the results cleared that *Lactobacillus fermentum NRAMJ6* showed a slight reduction in the count after 1 and 2 hrs of incubation but recovered again after 3 hrs of incubation. Also, all the strains were able to save viable counts upon 6 log CFU/ml in pH 4 and 3 after 3 hours of incubation. At pH2, all the isolated strains did not survive at this acidic pH and their viable numbers showed a sever reduction after 3 hrs of incubation but all the strains can survive for 60 min except *Lactobacillus*

fermentum NRAMJ6 lost its viability after 30 min**.** In addition**,** *Lactobacillus fermentum NRAMJ5* was the only strain which exhibited a high survival after 3 hrs. of exposure to acidic pH 2**.** On the other hand, at pH4 all the strains showed good survival as the count of the strains increased by time. These results are in the same line of other lactobacilli found by **Corcoran et al. (2005)** who mentioned that, *L. rhamnosus* GG displayed 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 decrease in concentration to very low levels after only 30 min of exposure to simulated gastric juice (pH 2.0). Also**, Charteris et al. (1998),** pointed out that lactobacilli can tolerate pH 2.0 for several minutes.

Bile tolerance

One important characteristic of the *Lactobacillus* strains is the bile salt tolerance, by which they survive and grow in the upper small intestine, since bile salts disorganize the structure of the cell membrane **(Bao et al. 2010).** The survival of the seven *Lactobacillus* strains tested on MRS agar medium supplemented with different concentrations of bile salts (oxgall) (0.1 , 0.3 , 0.4, 0.5 and 1 %) and without as control after incubation at 37 ^{o}C for 48 hrs are presented in **Table 4.**The data cleared that all the *Lactobacillus* strains were able to survive at the different concentration of bile salts and still save the viable counts upon 6 log CFU/ml which is required for its probiotic property .Also, by increasing the concentrations of the bile salts the viable count decreased gradually. It has been reported that, some probiotic avoids this problem by production of bile salt hydrolase (BSH), which can hydrolyze conjugated bile salts to decrease their toxicity **(Guo et al. 2019).**

Antimicrobial activity

Antimicrobial activity of the seven *Lactobacillus* strains against some enteric pathogens presented in **Table 5**.The indicator pathogens included Gram-positive bacteria such as *B. subtilius , B. cereus* and *Listeria monocytogenes* , Gram-negative bacteria such as *Pseudomonas aeruginosa, Salmonella sp.* and *Yersinia sp.* , yeasts (Saccharomyces cerevisiae and *Candida albicans*) and filamentous fungi (*Aspergillus niger*). The results showed that *Lactobacillus fermentum NRAMJ5* was the most potent strain on the basis of inhibition zone against all tested indicators. Also, it is clear that all the strains 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 it. In addition, both *Lactobacillus fermentum* NRAMJ1 and *Lactobacillus fermentum NRAMJ7* strains were unable to affect the growth of *Salmonella sp.* All strains did not have antimicrobial activity against *B.cereus* except *Lactobacillus fermentum NRAMJ5. Lactobacillus fermentum NRAMJ4, Lactobacillus gastricus* NRAMJ2 and *Lactobacillus fermentum NRAMJ5* strains had antibacterial activity against *B. subtilius* and *P. aeruginosa.* In conclusion, the most sensitive tested organisms were *A. niger, S. cerevisiae* and *Yersinia.* The results showed that the isolated *Lactobacillus* strains had strong antimicrobial activity against pathogenic Gram- negative bacteria than Gram- positive bacteria. The diameter range of the inhibition zones ranged from 0.5 to 3 mm. for *Salmonella* sp. and *Yersinia* sp. while the diameter range of the inhibition zones was from 0.5 to 1.5 mm. for *B. subtilius* and *B. cereus.* Also, it was obvious that *Salmonella* sp. and *Yersinia* sp. were more sensitive to the metabolites of *Lactobacillus* sp.

In this context, **De Vuystet al. (2004)** mentioned that *L. rhamnosus* GG strain most probably produces an agent with antimicrobial activity that is active *in vitro* towards *Salmonella typhimurium*. Also, pointed out 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.

In addition, **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.* Moreover, the results showed a strong antifungal activity for the isolates as the diameter range of the inhibition zone was from 1.5 to 4.5 mm. These results are in harmony with **Tropcheva et al. (2014)** who mentioned that isolated strains of *L. brevis* KR3, *L. brevis* KR4 and *L. brevis* KR51 completely suppress the growth of *Aspergillus awamori, Penicillium claviforme,* and *Aspergillus niger* and showed antimicrobial activity against *Candida albicans.* A few reports demonstrated that *Lactobacillus* spp. have a strong anti-*Candida* activity **(Atanassova et al. 2003; R€onnqvist et al. 2007).** Therefore, these isolates isolated 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 native microbiota of the human intestine is an excellent precondition for horizontal genes transfer that coded antimicrobial resistance properties with the aid of mobile genetic elements **(Teuber et al. 1999).** Thus, antibiotic-resistance screening for starter and probiotic cultures now tends to become systematic to eliminate the possibility of acquired resistance. Antibiotic susceptibility of the seven *Lactobacillus* strains were tested using antibiotic discs diffusion method on Mueller-Hinton agar medium presented in **Table 6.** The results showed that all the strains were sensitive to Cefuroxime, Ampicillin \Sulbactam, Clindamycin, Ampicillin, Rifampin, Gentamycin, Chloroamphenicol, Tetracyclins, Amoxicillin and Cefoperazone. However, all the isolates resisted Ciprofloxain except for *Lactobacillus gastricus* NRAMJ2. The obtained results are in accordance with **Georgievaa et al. (2015)** who reported that all tested *Lactobacillu*s strains were susceptible toward ampicillin, gentamicin, erythromycin and tetracycline.On the other hand, *Lactobacillus fermentum* NRAMJ6 and Lactobacillus gastricus NRAMJ2were the only strains that resisted Cephalexin while the other strains were sensitive to it.

Production of extrapolysaccharides (EPS)

The *Lactobacillus* strains were tested for EPS production on MRS agar medium containing 100 g /L sucrose. The polysaccharide slime formed by all the strains by touching them with a metal loop and measured the formation of slime. The results were presented in **(Table 7)**. All the strains were able to produce EPS. These EPS represent safe additives for novel food formulations and may have applications in nonfood products **(Crescenzi 1995).** In this study the results showed that all the *Lactobacillus* isolates were positive for production of exopolysaccharide and these results were in line with **Li et al. (2014)** who produced *in vitro* of exopolysaccharides (EPS) of 3 strains of *Lactobacillus helveticus* MB2-1. In addition, **Oleksy and Klewicka (2016)** reported that

Lactobacillus spp. synthesized exopolysaccharides (EPS), including both homo- and hetero-polysaccharides, which play an important role in the production of fermented foods. Furthermore, the capsular structure of exopolysaccharide plays a potential role in potentiating strains from unfavorable environment.

Resistance to different concentrations of phenol

Gut microbiota can deaminate the aromatic amino acids derived from dietary proteins to form phenols. These resulted 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 at different concentrations of phenol (0.1, 0.2, 0.3, 0.5%) and without phenol as control after incubation at 37^{o}C for 48 hrs are presented in **Figure 3.** The results showed that all the tested strains had the ability to resist concentrations 0.1, 0.2 and 0.3 % of phenol. However, by increasing the concentration of phenol the count of the strains decreased but still the counts upon 6 log CFU/ml. Also, the results showed that all the isolates were unable to resist concentration 0.5 % of phenol. In this study, the results showed that all the isolates were able to resist concentration of phenol 0.1 up to 0.3 but by increasing the concentration to 0.5 all the isolates failed to resist this concentration. The results are in the same line with **Forhad et al. (2015)** who mentioned that at 0.1% concentration of phenol all the isolates showed higher level of tolerance whereas in 0.3% they were moderate but in 0.4% tolerance was much lower.

Fermentation of carbohydrates

Fermentation of different carbohydrates was examined using API 50 CH system for the seven *Lactobacillus* strains presented in **Table 8.** In this study, all the isolates were able to ferment 9 different carbohydrates, i.e., glucose, galactose, fructose, mannose, ribose, N-acethyl-glucosamine, salicin and lactose indicating that they are able to grow in variety of habitats utilizing different type of carbohydrates.

Conclusion

In this study, seven strains of *Lactobacillus* spp. were isolated from different dairy products. These strains showed oxalate degrading ability. The isolated seven strains were identified as *Lactobacillus fermentum* (5 strains) and *Lactobacillus gastricus* (2 strains) by the 16S rRNA gene. Additionally, these seven strains are probiotic candidates that respectively possess a good probiotic property due to tolerance to acidity and bile salts. Also, they exhibited strong antagonistic effect against tested pathogens and absence of transferable antibiotic resistance. Improving the added value of these probiotic strains by using them for prophylaxis of calcium oxalate stone disease in rat model will be focus of our next study.

Declarations

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

Author contributions NS performed the experiments and NS, BE, NM, NT, MI wrote the manuscript; NS, BE, NM, NT, MI performed the study and analyzed the data. NS, BE, NM, NT, MI guided the experiments and revised the manuscript.

Compliance with ethical standards

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

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

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Tables

Table 1 Screening of the isolates for oxalate utilization.

No.	Isolate Code	Clear zone	Log cfu/ml	
			MRS	MRS-OX
1	L.O.L07	+++	7.95	7.90
2	K1.O.L01	++	8.25	8.10
3	K1.O.L03	++	8.00	8.07
4	K1.O.L08	+	8.22	7.92
5	K1.O.L09	+++	7.79	7.91
6	C.O.L01	+	8.27	8.17
7	K2.O.L06	+	8.22	7.94
8	C.O.L10	+++	8.41	8.63
9	C.O.L15	+++	8.36	8.47
10	C.O.L01	+	7.38	7.19
11	Z.OL03	+++	8.36	8.60
12	Z.O.L05	++	8.27	8.30
13	Z.O.L06	+	7.25	7.01
14	R.O.L03	+	7.83	7.60
15	R.O.L09	+++	8.03	8.20
16	R.O.L10	++	9.71	9.53
17	K2.L01	+	8.44	8.34
18	K2.O.L02	+++	8.27	8.36
19	K2.O.L03	++	8.60	8.49
20	K2.O.L05	+	8.43	8.27
21	C.O.L06	+++	8.30	8.38
22	K3.O.L07	+++	7.53	7.54
23	Y.O.L03	+++	8.13	8.21

MRS: De Man ,Rogosa and Sharpe medium

MRS-OX: De Man ,Rogosa and Sharpe supplemented with calcium oxalate medium

Table 2 The combined effect between the isolates on oxalate utilization.

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.LO6	+++	+++	+++	++	++	+	+
C.O.L15	+++	+++	+++	++	++	++	++
Z.O.L03	++	+++	+++	+++	+	+	
K2.O.L02	++	+	+	+	++	+++	+
R.O.L09	++	+++	+++	+	++	++	+
K3.O.L07	+	++	+++	+++	+++	+	
Y.O.L03	+	+	+	+	++	+	+++

+,++,+++ referred to the clear areas around the colonies

Table 3 The genotypic identification of the isolates using 16s rDNA

No.	Code of the isolate	Genotypic Identification	Max identity %	GeneBank Accession No.
1	C.O.LO6	*Lactobacillus fermentum NRAMJ1*	100	MT712170
2	C.O.L15	*Lactobacillus gastricus NRAMJ2*	98.24	MT712171
3	Z.O.L03	*Lactobacillus gastricus NRAMJ3*	97.25	MT712173
4	K2.O.L02	*Lactobacillus fermentum NRAMJ4*	99.22	MT731344
5	R.O.L09	*Lactobacillus fermentum NRAMJ5*	100	MT712174
6	K3.O.L07	*Lactobacillus fermentum NRAMJ6*	99.77	MT712175
7	Y.O.L03	*Lactobacillus fermentum NRAMJ7*	100	MT712176

Table 4 Survival of the tested *Lactobacillus* strains in different concentrations of bile salt after incubation at 37 ºC for 48 hrs.

l	Control (no bile salt)	Growth (log cfu/ml) at different bile salt concentrations (%)				
		0.1	0.3	0.4	0.5	1
cillus fermentum NRAMJ1	9.38	9.27	9.23	9.07	9.03	8.92
cillus gastricus NRAMJ2	9.23	9	9	8.91	8.73	7.63
cillus gastricus NRAMJ3	9.04	8.88	8.87	8.77	7.95	6.69
cillus fermentum NRAMJ4	9.18	8.90	8.77	8.73	8.20	8.14
cillus fermentum NRAMJ5	9.36	9.25	9.23	9.14	8.60	8.40
cillus fermentum NRAMJ6	9.14	8.92	8.85	8.83	8.51	8.07
cillus fermentum NRAMJ7	9.12	9.07	9.07	8.99	8.90	8.83

Table 5 Antimicrobial activity of *Lactobacillus* strains tested toward some indicator pathogenic strains

Bacterial strain	Indicators strain								
	B. subtilis	*P. aeruginosa*	*B cereus*	*Salmonella sp.*	*C. albicans*	*S. cervisiae*	*Yerseniasp.*	*L. monocytogenes*	
	Diameter of inhibition zone (cm)								
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

ble 6 Antibiotic resistance profiles of the tested *Lactobacillus* strains after incubation at 37 ºC for 48 hrs.

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	S	R	S	S	S	R	S
Lactobacillus gastricus NRAMJ2	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
Lactobacillus gastricus NRAMJ3	S	S	S	-	-	S	S	S	S	S	R	S	S	S	R	S
Lactobacillus fermentum NRAMJ4	S	S	s	S	S	S	S	S	S	S	R	S	S	S	R	S
Lactobacillus fermentum NRAMJ5	S	S	S	R	I	S	S	S	S	S	R	S	S	S	S	S
Lactobacillus fermentum NRAMJ6	R	S	s	S	S	S	S	R	S	S	R	S	S	S	-	S
Lactobacillus fermentum NRAMJ7	S	S	s	S	R	S	S	S	S	S	R	S	S	S	R	S

Nalidixic acid (NA 30µg/ml), Norfloxacin (Nor 10µg/ml), Tetracyclins (TE 30µg/ml), Amoxicillin\clavulinic acid (AMC 20\ 10µg/ml), Clindamycin (DA 2µg/ml), Ampicillin \Sulbactam (SAM 20µg/ml), Cefuroxime (CXM 30µg/ml), Gentamycin(CN 10µg/ml), Ampicillin(Amp 10µg/ml), Kanamycin (K 30µg/ml), Cephalexin (CL 30µg/ml), Chloroamphenicol (C 30µg/ml), Ciprofloxain(CIP 5µg/ml), Vancomycin (VA 30µg/ml), Amoxicillin (Ax 25µg/ml), Rifampin(RA 5µg/ml), Cefoperazone (CEP 75µg/ml).

R=Resistance, I=Intermediate, S=Sensitive, - = no growth

ble 7 Exopolysaccharide production by different *Lactobacillus* strains

Lactobacillus strain	Exopolysacchride production
Lactobacillus fermentum NRAMJ1	+
*Lactobacillus gastricus*NRAMJ2	+
*Lactobacillus gastricus*NRAMJ3	+
Lactobacillus fermentum NRAMJ4	+
Lactobacillus fermentum NRAMJ5	+
Lactobacillus fermentum NRAMJ6	+
Lactobacillus fermentum NRAMJ7	+

(+) The strain produce EPS , (-) The strain not produce EPS

ble 8 Fermentation of different carbohydrates of *Lactobacillus* strains using API 50CH

	Carbohydrate																
Lactobacillus strain	GLY	ERY	DAR	RIB	ADO	GAL	GLU	FRU	MNE	SBE	INO	MAN	SOR	MDM	MDG	NAG	SAL
L. fermentum NRAMJ1	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-	+	+
*L. gastricus*NRAMJ2	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-	+	+
*L. gastricus*NRAMJ3	-	-	-	+	-	+	+	+	+	-	_	+	-	-	+	+	+
L. fermentum NRAMJ4	-	-	-	+	-	+	+	+	+	-	_	_	-	-	-	+	+
L. fermentum NRAMJ5	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-	+	+
L. fermentum NRAMJ6	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-	+	+
L. fermentum NRAMJ7	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-	+	+

Figures

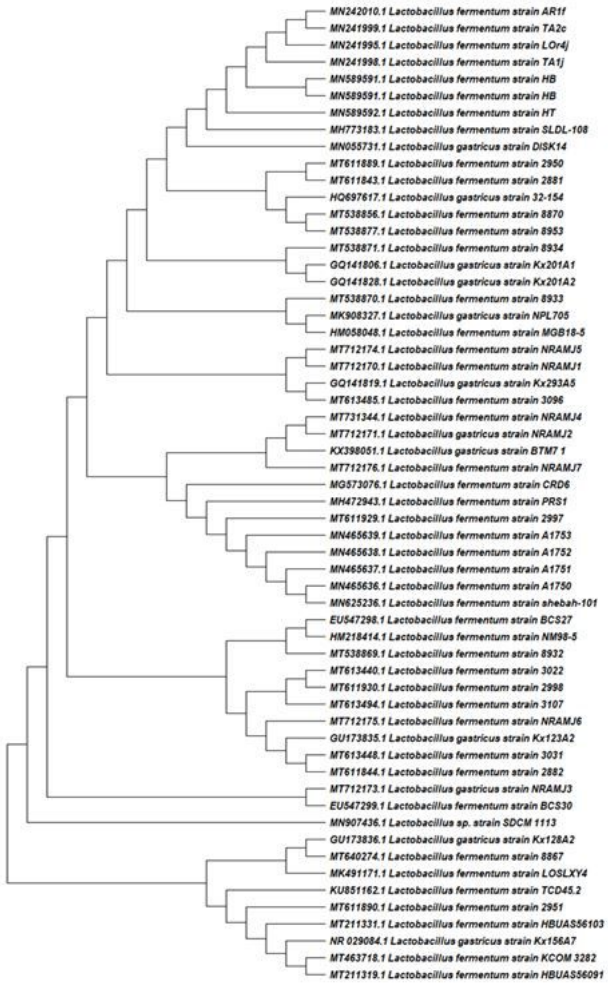

Figure 1

Constructed phylogenetic tree using Neighbour-Joining method to match the 7 bacteria (NRAMJ1, NRAMJ2, NRAMJ3, NRAMJ4, NRAMJ5, NRAMJ6 and NRAMJ7) to already published sequences

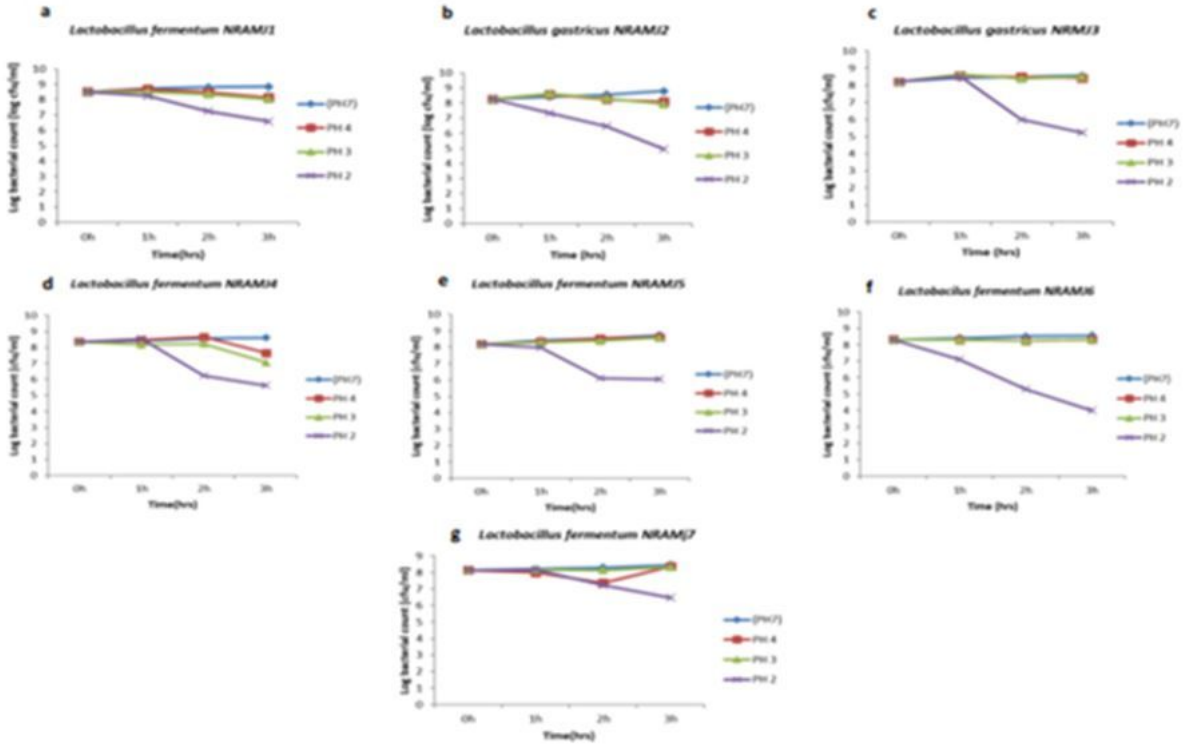

Figure 2

FiFigure 2 Tolerance of Lactobacillus strains to different pH values during incubation at 37 oC for 48

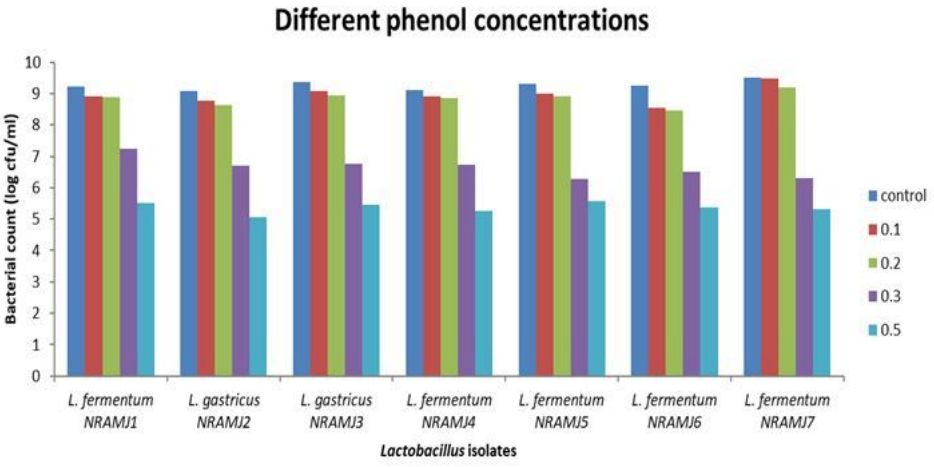

Figure 3

Survival of the tested Lactobacillus strains in different concentrations of phenol after incubation at 37 oC for 48 hrs