

Screening of oxalate degrading lactic acid bacteria of food origin

Nicoletta Murru,¹ Giuseppe Blaiotta,²
 Maria Francesca Peruzi,¹
 Serena Santonicola,¹
 Raffaelina Mercogliano,¹ Maria Aponte²

¹Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples; ²Department of Agricultural Sciences, University of Naples Federico II, Portici (NA), Italy

Abstract

A screening for oxalate degrading abilities was initially carried on within Lactic Acid Bacteria cultures of different food origin. Seventy-nine strains were drop-inoculated onto MRS agar plates containing calcium oxalate. By comparing colonies diameters, 31 strains were used to inoculate, in parallel, MRS and MRS modified by sodium oxalate addition. Differences in the strains' growth were assessed by colony forming unit counts. For two strains, the growth in oxalate enriched medium was significantly higher; while, for eleven strains an opposite behaviour was recorded. Two strains – probiotic *Lactobacillus rhamnosus* LbGG and *Enterococcus faecalis* 59 – were chosen. The first strain appeared to be able to metabolize oxalate more efficiently than the other tested cultures, while strain 59 appeared unable to gather advantage by oxalates and, indeed, appeared to be inhibited by the salt presence in the medium. Outcomes revealed that higher glucose concentrations may favour oxalates utilization. In MRS with oxalate, but without glucose, citrate was completely metabolized. Evaluation along time confirmed that the oxalate degradation is more significant in presence of glucose. Outcomes may represent a good start for the development of a safe and even probiotic culture able to lower the oxalates content of food.

Introduction

Oxalate is a toxic compound ubiquitous in the plant kingdom and widely consumed in normal human diets as a component of fruits, vegetables, grains and nuts (Hodgkinson, 1977). The normal daily intake of oxalate ranges from 70 to 920 mg, but strongly increases in vegetarians (Turroni *et al.*, 2007). Oxalic acid is a highly oxidized organic compound and is a strong chelator of cations, especially Ca²⁺.

These properties result in limited possibilities for its catabolism and also make oxalate toxic, especially for mammals. The accumulation of oxalic acid may lead to several pathologic conditions, including hyperoxaluria, urolithiasis, renal failure, cardiomyopathy and cardiac conductance disorders (Turroni *et al.*, 2007).

The current therapeutic drug treatment options for primary hyperoxaluria and/or the dietetic regimen for patients with secondary hyperoxaluria do not always lead to adequate success, e.g. sufficient reduction of urinary oxalate excretion. Hence, new treatment options are clearly needed. In addition, an oxalate-free diet is difficult to achieve and would probably be deficient in essential micronutrients. The discovery of oxalate-degrading bacteria in the human gastro intestinal tract has opened the way to several investigations regarding their potential role in reducing the urinary excretion of oxalic acid (Giardina *et al.*, 2014). Treatment with oxalate degrading bacteria could be such a new therapeutic option in patients with both form of hyperoxaluria. The most studied oxalate-degrading bacterium is *Oxalobacter (O.) formigenes*, a Gram-negative strictly anaerobic bacterium, found in the colon of humans and other vertebrates (Weese *et al.*, 2004). The pathway for oxalate utilization in *O. formigenes* involves two enzymes: formyl-CoA transferase and oxalyl-CoA decarboxylase (Turroni *et al.*, 2007) responsible for the conversion in CO₂ and formate further metabolized and excreted via faeces. Treatments with *O. formigenes* have been proposed for patients with either absorptive and/or dietary, hence secondary, hyperoxaluria: colon recolonization by *O. formigenes* may lead to up a 50% reduction of urinary oxalate excretion (Hatch *et al.*, 2011; Jiang *et al.*, 2011).

Apart from *O. formigenes*, strains within *Bifidobacterium* and *Lactobacillus (Lb.)* genera are known to efficiently metabolize oxalate in dogs (Gnanandarajah *et al.*, 2012). As matter of fact, several surveys were focused on the search of oxalates degradation abilities in Lactic acid Bacteria (LAB) strains (Hokama *et al.*, 2000; Hoppe *et al.*, 2005; Lieske *et al.*, 2005; Turroni *et al.*, 2007; Murphy *et al.*, 2009; Ren *et al.*, 2011). Anbazhagan *et al.* (2013) generated a recombinant *Lb. plantarum* strain able to efficiently degrade oxalate up to 90%, against a 15% of not modified bacteria. Since the use of genetically modified organisms is not allowed, a search for oxalate degrading activities within LAB of food origin was attempted.

Correspondence: Maria Aponte, Department of Agricultural Sciences, University of Naples Federico II, via Università 100, 80055 Portici (NA), Italy.
 Tel: +39.081.2539398.
 E-mail: aponte@unina.it

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Materials and Methods

Seventy-nine LAB strains were drop-inoculated onto MRS (Oxoid, Basingstoke, UK) agar plates containing 50 mmol/L of calcium oxalate. After incubation at 30°C for 48 hours, the presence of clear areas around to bacterial colonies was considered indication of oxalate degradation.

Selected strains were tested for sodium oxalate degradation in MRS broth containing 20 mmol/L of sodium oxalate (MRS-ox) as described by Ren *et al.* (2011). Isolates were cultured twice in MRS and then used to inoculate in parallel MRS and MRS-ox broth. After incubation at 37°C for 72 h, bacterial loads were assessed by drop counts on MRS agar. After centrifugation at 12,000 rpm for 15 min, supernatants were examined by high performance liquid chromatography (HPLC) according to Blaiotta *et al.* (2012). Analysis was performed on samples diluted in mobile phase 1:5 (vol/vol) and filtered by AcroDisc millipore (0.2 µm) by system (Gilson 307 Series HPLC system; Gilson, Middleton, WI, USA) fitted with column (MetCarb68 Hcolumn 6.5×300 mm, Varian) in an oven thermostated at 65°C. Columns were eluted at 0.4 mL/min by a solution 0.25 N H₂SO₄ in ultrapure water. A refractometer (RID 133, Gilson) was used as detector. Standards were used for quantification of different sugars and acids in the samples. Each experiment was performed in triplicate. For two strains, trials were repeated with MRS-ox broths with different glucose concentrations (0, 5 or 10 g/L). After 6, 24,

30, 48, 54 and 72 hours of incubation at 37°C, the bacterial populations were quantified by MRS counts and O.D. determination, while oxalate concentrations were assessed by HPLC.

Results

MRS agar containing calcium oxalate did not allow a clear discrimination of oxalate-degrading strains. All 79 LAB cultures grew on the agar plates and in no case the development of a clear halo surrounding colonies was evident (data not shown). By comparing the diameter of the colonies, 31 strains were selected (Table 1). Cultures were used to inoculate, in parallel, MRS-ox, namely MRS modified by sodium oxalate addition, and unmodified MRS. CFU

counts were used to compare strains. Around 68% of the strains presented a higher growth in MRS broth base than in MRS-ox, but such difference proved to be significant ($P < 0.05$) for 11 strains (*Enterococcus* spp. 11, 16, 26 and 59, *Ec. durans* 17, *Ec. faecalis* 62, *Lb. johnsonii* La1, *St. thermophilus* DSM8713 and DSM20617, *Lb. reuteri* Bio and *Lb. casei* Lbc496). Similarly, 11 strains showed a better growth in MRS-ox, but the difference was significant for just two strains: *Lb. rhamnosus* LbGG and *St. salivarius* DSM20479 (Table 1).

Strains LbGG, DSM 20479 and RBT739, all characterized by a better growth in presence of oxalate and *Ec. faecalis* 59, showing an opposite behaviour were selected for further experiments. CFU counts confirmed previous outcomes, while the oxalate degradation, as revealed by

HPLC analyses proved to be more efficient in strain LbGG rather than in RBT739 (Figure 1). Strain 59 showed a reduced consumption of sugar, followed by a lower production of lactic acid. The influence of the glucose on the utilization of sodium oxalate was evaluated by repeating the same set of trials in MRS-ox with decreasing glucose concentrations, namely half content (5 g/L) and without (0 g/L) glucose (Figure 2). The presence of glucose seemed to exert a substantial effect on the oxalate consumption, since in broth without glucose, oxalate consumption was similar for both strains (Figure 2).

The analysis in MRS-ox with different glucose concentration was then repeated, by monitoring strains along incubation up to 72 hours (Figure 3). Cultures turbidity showed the same trend in the broth culture with 5 and 10 g/L of glucose, with a higher

Table 1. Growth in MRS and MRS-added of sodium oxalate of strains used in this study.

Strain	Source	Taxon	CFU/mL	
			MRS	MRS-ox
8	Ricciardi <i>et al.</i> (2014)	<i>Ec. hirae</i>	7.85±0.08	7.54±0.16
11	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	7.56±0.17	6.46±0.14
15	Ricciardi <i>et al.</i> (2014)	<i>Ec. durans</i>	8.00±0.10	8.07±0.15
16	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	8.28±0.17	7.07±0.19
17	Ricciardi <i>et al.</i> (2014)	<i>Ec. durans</i>	7.78±0.18	6.53±0.13
24	Ricciardi <i>et al.</i> (2014)	<i>Ec. hirae</i>	8.65±0.12	8.52±0.11
26	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	8.43±0.12	6.54±0.17
30	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	6.46±0.08	6.96±0.17
31	Ricciardi <i>et al.</i> (2014)	<i>Ec. durans</i>	7.63±0.08	7.24±0.10
35	Ricciardi <i>et al.</i> (2014)	<i>Ec. hirae</i>	8.70±0.16	8.30±0.16
36	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	8.35±0.11	8.40±0.12
51	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	8.24±0.19	8.65±0.17
59	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	8.77±0.19	6.76±0.13
61	Ricciardi <i>et al.</i> (2014)	<i>Enterococcus</i> spp.	8.10±0.18	7.48±0.17
62	Ricciardi <i>et al.</i> (2014)	<i>Ec. faecalis</i>	8.10±0.13	7.00±0.14
La1	Nestlé, Vevey, Switzerland	<i>Lb. johnsonii</i>	8.28±0.17	7.18±0.14
YIT9029	Yakult Honsha Co. Ltd., Tokyo, Japan	<i>Lb. casei</i> Shirota	8.26±0.20	8.75±0.18
DN-114 001	Danone, Paris, France	<i>Lb. casei</i>	8.50±0.21	8.86±0.12
DSM20479	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany	<i>St. salivarius</i>	7.26±0.20	8.57±0.16
DSM8713	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany	<i>St. thermophilus</i>	8.03±0.17	6.50±0.16
DSM20617	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany	<i>St. thermophilus</i>	8.45±0.19	7.38±0.23
LbGG	Blaiotta <i>et al.</i> (2012)	<i>Lb. rhamnosus</i>	7.77±0.21	8.53±0.24
VT1	Blaiotta <i>et al.</i> (2012)	<i>Lb. rhamnosus</i>	8.69±0.18	8.88±0.23
RBT739	Blaiotta <i>et al.</i> (2012)	<i>Lb. rhamnosus</i>	8.38±0.14	8.83±0.15
RBM526	Blaiotta <i>et al.</i> (2012)	<i>Lb. rhamnosus</i>	7.84±0.10	7.83±0.14
OM13	Aponte <i>et al.</i> (2012)	<i>Lb. pentosus</i>	7.78±0.16	7.77±0.20
Lbc491	Aponte <i>et al.</i> (2008)	<i>Lb. casei</i>	7.50±0.17	7.60±0.19
Lbc496	Aponte <i>et al.</i> (2008)	<i>Lb. casei</i>	8.46±0.19	7.58±0.13
Bio	BioGaia AB, Stockholm, Sweden	<i>Lb. reuteri</i>	7.22±0.14	5.26±0.20
ATCC393	American Type Culture Collection	<i>Lb. casei</i>	8.12±0.21	8.03±0.15
Lyofast LC4P1	Sacco, Cadorago (CO), Italy	<i>Lb. casei</i>	8.68±0.23	8.28±0.13

CFU, colony forming units; MRS-ox, MRS-added of sodium oxalate. Strains code, species identity and source are reported.

level of turbidity in broth cultures with the highest glucose concentration (data not shown). LbGG showed a humble growth in broth without glucose as revealed by CFU counts and pH monitoring (Figure 3). The cells number increased after the first 24 hours of incubation, afterward lactobacilli loads started to drop. Actually, populations decline in MRS without glucose started at 24 hour, while in the media with sugar, the drop begins after 48 hours. The increase of LAB populations was of almost one log for trials glucose-added. pH trends confirmed LAB counts evolution. HPLC analysis revealed an higher lactic acid production as long as the concentration of glucose increases. On the other hand, the acid acetic production registered a fluctuating trend along time.

The chromatographic analysis confirmed a higher oxalate consumption in presence of the maximum glucose concentration. Citric acid was fully metabolized in the medium without glucose and its consumption decreased with the increase of glucose concentration.

Discussion

In the present study, a screening for oxalates degrading activity was carried out by considering 79 LAB strains known for probiotic features or isolated from foods and evaluated for functional capabilities during previous surveys (Aponte *et al.*, 2008, 2012; Blaiotta *et al.*, 2012; Ricciardi *et al.*, 2014). Strains exhibiting a better growth on MRS agar plates enriched in oxalates were further investigated. According to outcomes, most of the strains showed a low oxalate degrading capacity. Among the tested strains, *Lb. rhamnosus* GG exhibited the highest percentage of oxalate consumption, above all in the presence of glucose. To authors' knowledge, this is the first time that this popular probiotic strain has been investigated for such feature. Results even proved a correlation between the presence of sugars and the rate of oxalate consumption, thus underlining that the availability of glucose might provide an input for the oxalate metabolism as already pointed out by Ren *et al.* (2011). Without glucose, both LbGG and *Ec. faecalis* 59, isolated from Pecorino di Carmasciano cheese (Ricciardi *et al.*, 2014) fully metabolized citrate and, above all, utilized oxalate at the same level. In other words, LbGG proved to degrade less efficiently the salt, while *Ec. faecalis*, that was chosen for its inability to degrade oxalate, exhibited an attitude comparable to that recorded for LbGG.

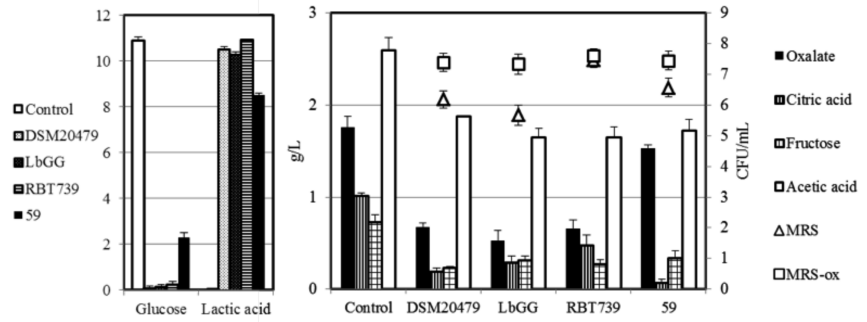


Figure 1. Oxalate degrading ability of *Lb. rhamnosus* strains LbGG and RBT739, *St. salivarius* DSM 20479, and *Ec. faecalis* 59 after 24 h of incubation. Evolution of reducing sugars, organic acids by high performance liquid chromatography and of microbial loads in MRS and MRS-added of sodium oxalate are reported. Un-inoculated broth served as control.

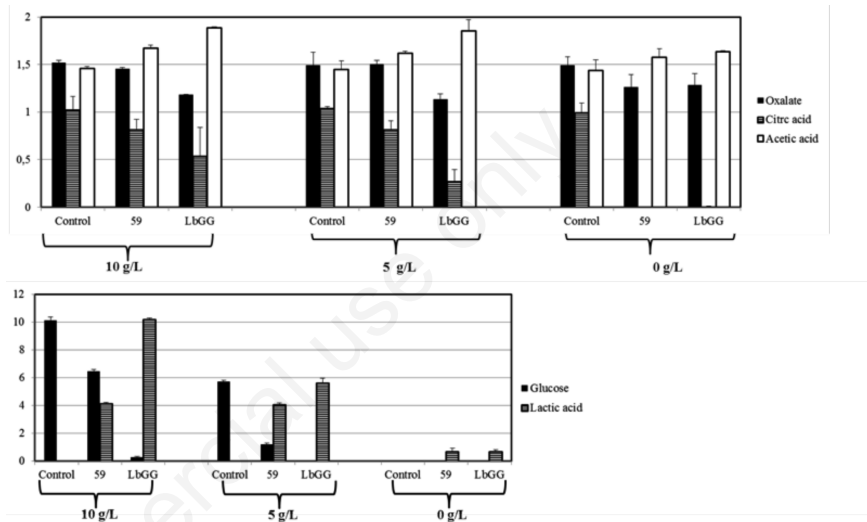


Figure 2. Oxalate degrading ability of *Lb. rhamnosus* LbGG and *E. faecalis* 59 in MRS-added of sodium oxalate with decreasing glucose concentrations (10, 5, 0 g/L). Content of glucose, lactic, acetic and citric acids by high performance liquid chromatography are reported. Un-inoculated broth served as control.

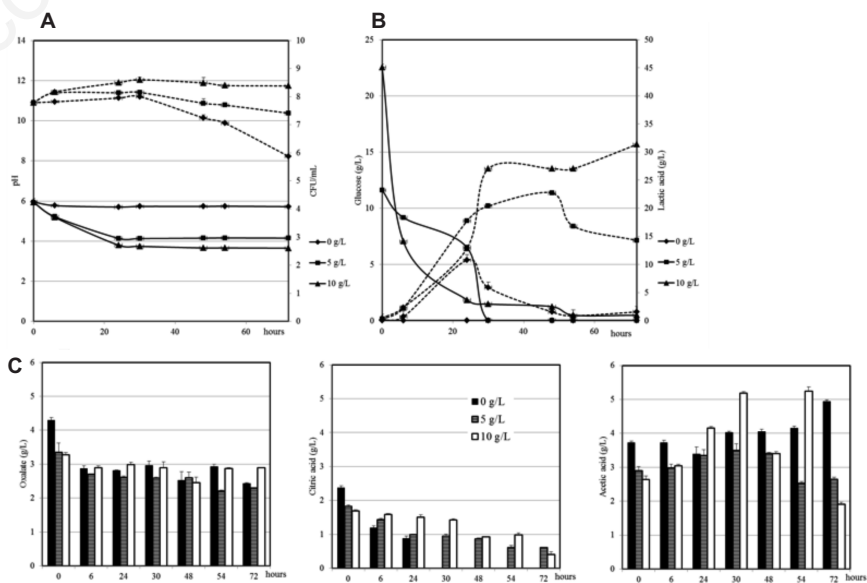


Figure 3. Evolution of pH (continuous lines) and MRS counts (dashed lines) (A); glucose (continuous lines) and lactic acid (dashed lines) (B); oxalic, citric and acetic acids (C) of *Lb. rhamnosus* GG in in MRS-added of sodium oxalate with decreasing glucose concentrations (10, 5, 0 g/L) during 72 h of incubation.

Conclusions

Many reports are currently focused on oxalates degrading capacity of *O. Formigenes* (Knight *et al.*, 2013). However, its safety issue and health benefits are not as well established as in LAB. The research of different microorganisms able to express the same degrading-capacities led to investigate on several LAB strains mainly referable to *Enterococcus* spp and *Lactobacillus* spp (Hokama *et al.*, 2000; Turroni *et al.*, 2007). *Bifidobacterium* and *Lactobacillus* strains are commonly used in dairy and pharmaceutical probiotic preparation and are generally recognised as safe for human consumption (Fao, 2002; Iqbal *et al.*, 2014). However, all studies highlighted in LAB cultures a minor oxalate degrading capacity if compared *O. formigenes* performances, for two main reasons: LAB prefer other substrates to obtain energy (Ren *et al.*, 2011), and the genes encoding for the synthesis of oxalate degrading enzymes are less efficient than those reported in *O. formigenes* (Turroni *et al.*, 2007). Basing on outcomes obtained in the present survey, *Lb. rhamnosus* GG, never evaluated before for this feature, may express an interesting potential for the degradation of oxalate. Since LbGG is well known for its ability to survive and to tolerate adverse storage conditions in dairy products (Alampreese *et al.*, 2005), fresh-cut apple (Alegre *et al.*, 2011), maple sap (Khalf *et al.*, 2010), chestnut purees and mousses (Blaiotta *et al.*, 2012; Romano *et al.*, 2014), and emmer beverages (Coda *et al.*, 2011), new perspectives may be hypothesised for the production of probiotic food, with particular emphasis on fermented vegetable foods, with reduced oxalates content and endowed with LbGG, thus likely to promote an oxalate reduction in the human organism, beyond the beneficial and therapeutic effects commonly associated to the ingestion of such probiotic strain.

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