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Oxalate Degradation Potential of Lactic Acid Bacteria Isolated from Traditional Fermented Milk Products, Human Vagina and Human Faecal Matter

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Abstract

Lactic acid bacteria (LAB) isolated from faecal matter of healthy human volunteers and traditional fermented milk samples such as dahi, buttermilk, lassi were identified and characterized by morphological, API identification and 16SrRNA sequencing. Selected isolates were studied for oxalate degradation potential using colorimetric assay kit (Sigma-MAK179). One human vaginal isolate was also included in the study. Study results revealed a higher LAB count in faecal matter as compared to fermented milk products. Faecal isolates mainly belonged to genera *Enterococcus* and *Lactobacillus*. Out of the 24 isolates studied, 11 isolates gave > 30% oxalate degradation and among them, five isolates gave > 50% degradation, which included *Enterococcus hirae* F8, *Weissella confusa* F9, *Enterococcus faecium* M11, *Lactobacillus helveticus* MTCC 5463 and *Lactobacillus rhamnosus* MTCC 25062. Oxalate degradation ability is found to be both species and strain specific. Fermented milk isolate *Enterococcus faecium* M11 gave highest oxalate degradation (69.7%) followed by vaginal isolate *Lactobacillus helveticus* MTCC 5463 (68.8%)and faecal isolate *Enterococcus hirae* F8 (68.4%).

Keywords: Lactic acid bacteria, oxalate degradation, fermented milk products, human volunteers, faecal matter

Kidney stone disease prevalence and its recurrence rates are increasing globally with limited options of effective drugs. The disease affects about 12% of the world population at some stage in their lifetime (Varma, 2015; Alelign and Petros 2018). In India, it affects nearly 2 million people every year and the life time risk is about 20% in those having this disorder (Saranya *et al*. 2017). Hyperoxaluria is considered the major risk factor for renal stones and the oxalate load in urine plays a vital role in calcium- oxalate stone formation even in normocalciuric patients (Campieri *et al*. 2001). Oxalate is found in a variety of foods such as sweet potato, chocolate, tea, etc. (Stewart *et al*. 2004; Holmes and Kennedy, 2000). Humans lack the enzymes needed to metabolize oxalate. It is therefore, excreted unchanged in the urine by the kidneys or eliminated in the faeces (Morton *et al*. 2002; Marengo and Romani, 2008; Kolandaswamy *et al*. 2009). Colon is the major site of oxalate absorption, with 3 to 5% of dietary oxalate being absorbed under normal conditions (Turroni *et al*. 2007). However, the increased absorption of oxalate-rich food in the gut could be dangerous, as this may lead to the formation of calcium oxalate stones.

The oxalate-degrading ability of several bacteria such as *Oxalobacter formigenes*, has therefore, been studied with a view to their possible use in the prevention of kidney stone disease. Besides *O. formigenes*, there are other enteric bacteria such as, *Enterococcus faecalis* (Hokama *et al*. 2000), *Providentia rettgeri* (Hokama *et al*. 2005), *Bifidobacterium infantis* (Campieri *et al*. 2001),

Lactobacillus and *Bifidobacterium spp* (Campieri *et al*. 2001; Lewanika *et al*. 2007; Turroni *et al*. 2007) that have shown their potential ability todegrade oxalate. A number of research studies have pointed towards the role Lactic Acid Bacteria (LAB) may play in utilizing the oxalate, hence potentially limiting its absorption from the intestinal lumen (Campieri *et al*. 2001; Lieske *et al*. 2005' Azcarate-Peril *et al*. 2006; Turroni *et al*. 2007; Anbazhagan *et al*. 2013; Liebman and Walukano, 2016). Such LAB species could possibly be used in a probiotic approach for prevention of kidney stone disease. Turroni *et al*. (2007) reported a range of oxalate degrading lactobacilli from pharmaceutical and dairy products and found significant oxalate degradation in *Lactobacillus acidophilus* and *Lactobacillus gasseri*. However, the number of identified oxalate degrading lactic acid bacterial species is limited. Traditional fermented milk products and fecal matter of healthy humans are considered to be rich sources of LAB. Hence taking into consideration, the LAB species diversity in the traditional fermented milk products and fecal matter of healthy humans, in the present study we tried to investigate the oxalate degradation ability of LAB isolates obtained from these sources. One human vaginal isolate of LAB previously isolated and characterized in our department was also included in the current study.

MATERIALS AND METHODS

Isolation of LAB

Fecal samples were collected from ten healthy individuals (mean age of 23–40) who had not taken antibiotics and probiotics at least for the past three months. Samples were collected in sterile container, kept in ice box, transported to laboratory within 1-2 h and processed immediately. Fermented milk products such as *dahi*, *chaas*, *lassi*, *shrikhand* were collected from in and around the areas of Anand district, Gujarat, India and used to isolate LAB. For the isolation, desired dilutions of samples were prepared and plated on MRS agar medium. The plates were incubated at 37ºC for 48-72h. Typical colonies of LAB were picked up and streaked on the MRS agar for further purification.

Characterization of isolates

Pure isolates were studied for catalase reaction, morphological characteristics and ability to grow in the presence of potassium oxalate and milk fermentation abilities. The isolates were subjected to identification and characterization using Analytical Profile Index kits. Further confirmation of identity of the isolates at molecular level has been done through 16S rRNA sequencing. Additionally, one vaginal isolate (*Lactobacillus helveticus* MTCC 5463) already preserved in the Dairy Microbiology department was also included in the study.

Evaluation of oxalate degradation potential of LAB isolates

In order to know the growth curve of isolates in the presence of potassium oxalate, active isolates were inoculated in MRS medium containing10mM potassium oxalate and incubated at 37ºC for 72h. Growth was measured at different time intervals (0, 8, 24, 32, 48, 72h) as Optical Density at 600nm. Further confirmation of oxalate degradation by LAB isolates has been carried out using colorimetric assay kit (Sigma-MAK179). For the same a standard curve has been generated and the active cultures were inoculated into MRS broth containing 10mM Potassium oxalate and incubated at 37°C for 24h. After 24 h, culture tubes were subjected to centrifugation at 10,000rpm/10min to collect the supernatant. The supernatant was used for estimation of oxalate concentration by colorimetric assay method (Sigma-MAK179), taking absorbance at 450 nm using spectrophotometer. Oxalic acid concentrations were measured both graphically (obtained from standard curve formula) and theoretically (from standard O.D) and the mean of concentrations were calculated to obtain final oxalate concentration. From this, percent oxalate degradation was measured using the formula given below:

$$\% \text{ Oxalate degradation} = \frac{\text{Oxalate conc. in KOX control} - \text{Oxalate conc. in supernatant}}{\text{Oxalate conc. in KOX control}} * 100$$

RESULTS AND DISCUSSION

A total of 10 fecal and 10 fermented milk samples were used for isolation of LAB. Human fecal matter and traditional fermented dairy products are considered good sources of lactic acid bacterial cultures. LAB count of fecal samples were higher than that found in fermented milk products. The average LAB count of fecal samples varied between 8-10 logcfu/g of fecal matter. For fermented milk products it varied between 5-8 log cfu/g of the products. A total of 36 (fecal isolates = 21 and fermented milk isolates =15) Gram positive, catalase negative isolates were picked up. All the isolates were capable of fermenting milk. LAB isolates from both faecal and fermented milk samples mainly belonged to genera *Lactobacillus* and *Enterococcus*. Faecal enterococci isolates belonged to species *Enterococcus hirae*, but species diversity was observed in case of *Lactobacillus* strains and the species included *Lactobacillus ruminis, Lactobacillus plantarum, Lactobacillus oris* and *Weissella confusa.*

Preliminary growth studies of the isolates in the presence of different concentrations of Potassium oxalate showed that some of the isolates were unable to grow in its presence. Hence, these isolates were not taken for further study. Selected 24 isolates only were taken for further growth studies and oxalate degradation studies. The identity of these isolates with their accession numbers are shown in Table 1. Growth curve studies of fecal isolates in MRS medium containing 10mM Potassium oxalate exhibited wide variation (Figs 1 and 2). Some isolates entered into stationary phase after 8h of incubation while some others entered into stationary phase after 24h of incubation and few isolates were poor growers. While in case of isolates from fermented products, almost all of them showed good growth rate and remained in log phase up to 24h.

Table 1: Lactic acid bacteria isolates used in the study

Sl. No	Isolate code	Identified species	NCBI accession number/MTCC No.
1	F1*	*Aerococcus viridans*	—
2	F2	*Enterococcus hirae*	MG696184
3	F3	*Enterococcus hirae*	MG696185
4	F5	*Lactobacillus ruminis*	MG696186
5	F6*	*Aerococcus viridans*	—
6	F7	*Lactobacillus plantarum*	MG696187
7	F8	*Enterococcus hirae*	MG696188
8	F9	*Weissella confusa*	MG696189
9	F10	*Enterococcus hirae*	MG696190
10	F11*	*Lactococcus lactis*	—
11	F12	*Enterococcus hirae*	MG696191
12	F15*	*Enterococcus avium*	—
13	F16*	*Enterococcus faecalis*	—
14	F17	*Lactobacillus oris*	MG696192
15	M5	*Lactobacillus fermentum*	KU366365
16	M11	*Enterococcus faecium*	KU366367
17	M16	*Lactobacillus paracasei*	KU366368
18	M38	*Lactobacillus plantarum*	KU366371
19	MK16	*Enterococcus faecium*	MF351740
20	NK10	*Lactobacillus rhamnosus*	KR732326/ MTCC 25062
21	NS4	*Lactobacillus rhamnosus*	KJ156963/ MTCC 5945
22	NS6	*Lactobacillus rhamnosus*	KJ156964/ MTCC 5946
23	V3	*Lactobacillus helveticus*	GQ253959/ MTCC 5463
24	MK30*	*Lactococcus lactis*	—

**Identification using Analytical profile Index kits, molecular identification is remaining. All other isolates identified by 16S rRNA sequencing.*

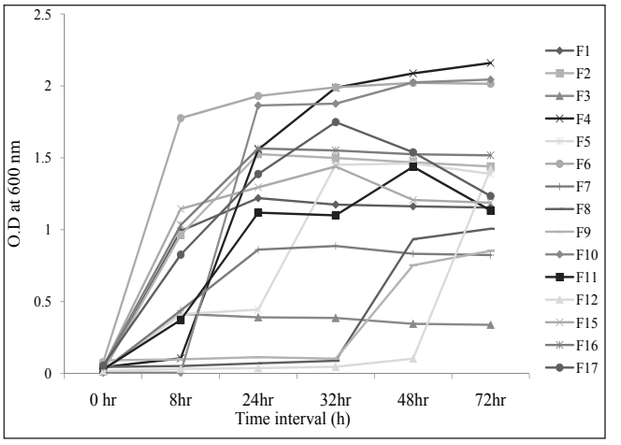

Fig. 1: Growth pattern of fecal isolates in MRS medium containing 10mM Potassium oxalate

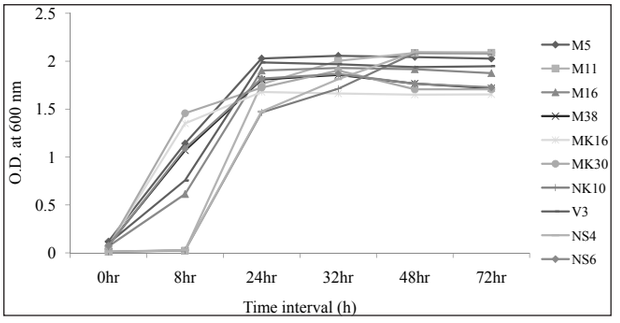

Fig. 2: Growth pattern of fermented milk isolates and vaginal isolate in MRS medium containing 10mM Potassium oxalate

Study on oxalate degradation by LAB isolates using colorimetric assay kit (Sigma-MAK179) revealed high degree of variation in the oxalate degradation ability of the isolates (Fig. 3). Out of the 24 isolates studied, 11 isolates gave more than 30% oxalate degradation. Among these, five isolates gave more than 50% degradation which included *Enterococcus hirae* F8 (68.4%), *Weissella confusa* F9 (61.4%), *Enterococcus faecium* M11 (69.7%), *Lactobacillus helveticus* MTCC 5463 (68.8%), and *L. rhamnosus* MTCC 25062 (56.5%) (Fig. 3).

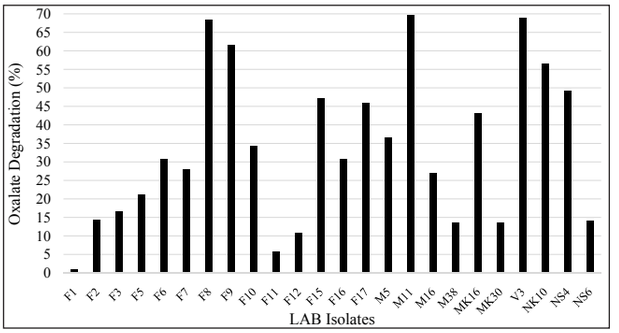

Fig. 3: Oxalate degradation (%) by LAB isolates using colorimetric assay kit (Sigma-MAK179)

The highest was seen in case of *Enterococcus faecium* M11 (69.7%) which was isolated from fermented milk product *dahi*. This was followed by vaginal isolate *Lactobacillus helveticus* MTCC 5463 (68.8%) and faecal isolate *Enterococcus hirae* F8 (68.4%). Campieri *et al.* (2001) investigated *in vitro* oxalate degradation in *Lactobacillus* species, they reported good oxalate degradation by *L. acidophilus* strain, in comparison to *L. brevis* and *L. plantarum* which demonstrated a modest ability to degrade oxalate. Turroni *et al*. (2007) in their study reported that *Lactobacillus acidophilus* and *Lactobacillus gasseri* showed significant oxalate degradation in 5mM oxalate whereas other strains showed less oxalate consumption, especially *Lactobacillus salivarius* which showed 20% oxalate degrading ability. In a study reported by Gomathi *et al.* (2014), the maximum oxalate degradation was detected in isolates of *L. salivarius* AB11 (62.59%) and *L. fermentum* TY12 (58.3%) and all five strains of *L. fermentum* sp. differed in their oxalate degrading ability. Interestingly, the current study has also shown such a trend. Between the three *Lactobacillus rhamnosus* species (NK10, NS4, NS6) a significant variation was observed in their oxalate degradation ability. Such a trend was observed in case of *Enterococcus hirae* strains (F2 (14.3%), F3(16.6%), F8(68.4%), F10(34.2%), F12(10.7%) also. These results showed that oxalate degradation is both species and strain specific. Murphy *et al*. (2009) reported that oxalate utilization among probiotics *in vitro* was interspecies dependent. Among the identified oxalate degrading LAB strains, *Weissella confusa* and *Weissella cibaria* have the ability to degrade oxalate between 40 and 50%. In the current study, *Weissella confusa* F9 showed an oxalate degradation of 61.4%. Giardina *et al*. (2014) investigated 11 strains of lactic acid bacteria (Lactobacilli and Bifidobacteria), for their capability to degrade oxalate by mean of RP-HPLC-UV method and identified four promising bacterial strains *viz*., *Lactobacillus plantarum* PBS067, *Lactobacillus acidophilus* LA-14, *Bifidobacterium breve* PBS077, *Bifidobacterium longum* PBS078. Murru *et al.* (2017) in their study on the oxalate degradation potential of LAB from food origin reported that *Lactobacillus rhamnosus* LbGG strain showed a more promising oxalate metabolism in comparison to other strains tested. Almost all the studies regarding the oxalate degradation potential of LAB strains revealed moderate effect in comparison to oxalate degradation potential of *O. formigenes* which is a known oxalotroph, where as LAB are said to be general oxalotrophs. Also the genes encoding for the synthesis of oxalate degrading enzymes are reported

to be less efficient in LAB compared to those in *O. formigenes* (Turroni *et al*. 2007).

CONCLUSION

Current study results demonstrated that LAB strains, irrespective of their source of isolation, posses oxalate degradation ability which is both species and strain specific. Such oxalate degrading LAB strains could possibly be used to reduce the levels of oxalate in the gut in a probiotic approach for prevention or treatment of kidney stone disease.

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