Antagonistic activity of *Lactobacillus reuteri* strains isolated from different niches against food bacteria and its biopreservative application along with nisin in milk system

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Abstract: The objective of the present study was to isolate a potential reuterin producing *L. reuteri* strain and check its biopreservation efficacy along with nisin in milk system. A total of 35 lactobacilli isolates were obtained from 12 different sources which included faecal samples from infants, adult humans as well as rats and also human milk. Finally, 13 isolates could be confirmed as *Lactobacillus reuteri* on the basis of PCR. The 13 *L. reuteri* isolates were then screened for their antimicrobial activity in MRS media with or without glycerol supplementation. It was observed that the supernatant obtained from secondary fermentation from eight *L. reuteri* isolates in glycerol-supplemented media significantly inhibited the growth of majority of bacterial indicators, which was pH independent. Furthermore the supernatant from the same 8 isolates also exhibited significant antifungal activity against the tested fungal indicators. Based on the RAPD strain profile and reuterin based antimicrobial activity, *L. reuteri* strain 47 was selected as the most potent isolate for further study. The active units of reuterin in the supernatant of *L. reuteri* 47 were calculated to be 1600 AU/ml. In the milk system, the reuterin system significantly inhibited *E. coli*, but not *P. acidilactici*, nisin on the other hand had significant activity against *P. acidilactici* but not *E. coli*. The combination

of reuterin and nisin had synergistic effect against *L. monocytogenes*. Hence, the combination of reuterin and nisin can be utilized to target specific pathogens as well as to improve the overall safety of milk and milk products.

Keywords: Antibacterial, Antifungal activity, *Lactobacillus reuteri*, Milk system, Reuterin

Introduction

In the recent years, there is an increased demand of minimally processed safe foods with reduced levels of chemical preservatives (Da Costa et al. 2019). This has led to renewed interest in the natural antimicrobials particularly those that are derived from non-pathogenic, food grade Lactic Acid Bacteria (LAB). Many LAB species have been attributed GRAS status (Generally Recognized As Safe) by US-FDA and together with QPS (Qualified presumption of safety) status from EFSA, LAB possess a healthy and natural image (Pradhan et al. 2018). Basically, LAB is a group of fermentative bacteria that are naturally present in many of the food commodities (Ghanbari and Jami, 2013). Many of the LAB are also widely used as starter cultures in the food industry for the production of fermented foods, including dairy (yogurt, cheese), meat (sausages), fish, cereals (bread and beverages such as beer), fruit (malolactic fermentation processes in wine production), and vegetables (sauerkraut, kimchi, silage) (Calo-Mata et al. 2008). During their growth in the food products, LAB produces numerous antimicrobial metabolites such as organic acids, bacteriocins, diacetyl, fatty acids etc. that exert strong antagonistic activity against many closely related and unrelated microorganisms, including food spoilage organisms and pathogenic bacteria, thereby extending the shelf life of fermented food products.

The biopreservative potential of LAB is well known and many of the earlier reports (Rattanachaikunsopon and Phumkhachorn, 2010) have exclusively elaborated the various antimicrobial substances derived from LAB. Although, the major antagonistic effect of LAB is due to lowering of pH by the produced organic acids (lactic acid, acetic acid, formic acid etc.), a number of other metabolites also contribute to the overall biopreservative effect of the LAB (Egan et al. 2016). Among many of the antimicrobials

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produced by the LAB, the bacteriocins have received the most attention chiefly due to its stability, wide spectrum activity and minimum changes to food characteristics (Barbosa et al. 2017). Many bacteriocins from LAB have been isolated and characterized and also classified into different groups (Woraprayote et al. 2016). However only nisin and pediocin PA-1/AcH are approved to be used in foods, although other bacteriocins mix preparation such as ALTA™ and MICROGARD™ are also now commercially available (Goyal et al. 2018). The most limiting factor of bacteriocin activity is its inability to inhibit Gram negative bacteria which limits its antimicrobial action, thereby its application in food systems (Field et al. 2018). On the other hand, some other antimicrobials compounds have been reported from specific LAB organisms that show a wider spectrum of activity against various Gram positive and Gram negative bacteria as well as fungal microorganisms. One such of these antimicrobials having a broad antimicrobial spectrum have been reported from *L. reuteri* strains from diverse niches.

L. reuteri is one of the important species of lactobacilli found as an autochthonous member in the gastrointestinal (GI) tracts of human and many other animals (Casas and Dobrogosz 2000). *L. reuteri* has also been shown to be a natural component of human milk (Mu et al. 2018). *L. reuteri* strains are often found in fermented foods (Vollenweider and Lacroix, 2004) as well as marketed as probiotic supplements. The strains of *L. reuteri* possess a unique property of anaerobically converting glycerol into a potent, broad-spectrum antimicrobial substance, termed 3-hydroxypropionaldehyde (3-HPA), which is also referred to as reuterin (Axelsson et al. 1989). Reuterin (3-hydroxypropionaldehyde) is an aldehyde with antimicrobial activity towards a broad spectrum of food-borne pathogens and spoilage organisms, including Gram-positive and Gram-negative bacteria, yeast, molds, and protozoa (Siedler et al. 2019). The compound has a high potential as a food biopreservative since it is water soluble, resistant to heat, active at a wide range of pH values and resistant to proteolytic and lipolytic enzymes (El-Ziney et al. 2000). The antimicrobial efficacy of reuterin has been demonstrated against many of the common food borne spoilage and pathogenic organisms such as *Listeria monocytogenes*, *E. faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli* in various food matrices such as milk, dairy products and cottage cheese (Arques et al. 2011). In all these studies, the reuterin system has been shown to have a higher antimicrobial activity than bacteriocins compounds and other non-bacteriocins antimicrobial compounds (Arqués et al. 2008). Therefore, the incorporation of reuterin in food products offers a superior alternative for improving the safety and quality of foods along with reduction in the addition of chemical preservatives (Vollenweider et al. 2003). Hence, the present work was carried to isolate potential reuterin producing *L. reuteri* strains from different niches and investigate its inhibitory role in milk system.

Materials and Methods

Bacterial strains and culture conditions

L. reuteri strain ATCC55730 from Biogaia (Sweden) was used as standard *L. reuteri* strain for the identification of *L. reuteri* isolates. The bacterial as well as fungal target strains used were *E. coli* K12, *Listeria monocytogenes* ATCC13932, *E. faecalis* NCDC135, *Serratia marcescens* ATCC 13880, *S. typhi* NCDC 113, *Yersinia enterocolitica* ATCC130715, *Klebsiella pneumonia* NCDC 138 and *Pediococcus acidilactici* LB42, and *Rhodotorula glutinis* NCDC 389, *Penicillium roqueforti* ATCC 10110 and *Aspergillus niger* NCDC 267, respectively, for the antimicrobial activity testing of *L. reuteri* isolates. All the bacterial cultures (excluding *P. acidilactici* LB42) were grown in Brain Heart Infusion Broth (BHI, Himedia, India) and *P. acidilactici* LB42 and *L. reuteri* strains were grown in deMann Rogosa Sharpe broth (MRS; Himedia, India) at 37°C for 24-48 hours. The fungal cultures were grown in Yeast extract Peptone Dextrose broth (YPD; Himedia, India) at 25°C for 3-5 days. The fungal cultures were maintained in YPD agar slant at 4°C, whereas the bacterial strains were maintained as 15% glycerol stock cultures at -20°C in BHI and MRS broth. The organisms were propagated twice before their use in the experiments.

Sample collection and bacterial isolation

The samples for *L. reuteri* isolation were collected locally from Karnal area, Haryana (India). Infant faecal samples, human milk, adult human faeces and rat faecal samples were collected with the help of sterile swabs in sample box containing MRS broth and immediately incubated at 37°C/2-3 h for primary enrichment. The samples were then serially diluted in sterile normal saline and appropriate dilutions were plated out in modified MRS medium. Since lactobacilli are known to be intrinsically resistant to antibiotic vancomycin, hence, vancomycin was added in media component to make it selective for lactobacilli growth. The plates were incubated at 37°C for 24-48 h. Typical cream coloured, rice shaped colonies were picked up randomly and transferred to MRS broth. After growth at 37°C for 24-36 h, the cultures were tested for purity and morphology by microscopic examination.

Identification of bacterial isolate

The isolated cultures were examined for their morphology by Gram staining and catalase reaction as per standard methods. The tentatively identified lactobacilli isolates based on Gram's staining and catalase test characteristics were further subjected to molecular characterization for confirmation of *L. reuteri* species. All the isolates were grown for 10 to 12 h in MRS broth at 37°C and the genomic DNA was extracted by the method of Pospiech and Neumann (1995). PCR was carried out using primer pair Lreu 1 (CAGACAATCTTTGATTGTTTAG) and Lreu4 (GCTTGTTGGTTGGGCTCTTC) specific against *L. reuteri* using

conditions as described by Song et al. (2000). The primers were custom synthesized from Sigma, New Delhi, India.

Preparation of Cell Free Culture Supernatants (CFCSSs)

The preparation of CFCSSs from isolated *L. reuteri* strains were carried out according to the method described by Luthi-Peng, 2002. Briefly, a single colony with typical morphology from the MRS agar plates was inoculated into MRS broth and incubated anaerobically (Gas pack system, Himedia, India) at 37°C for 20 h. After incubation, the culture was centrifuged (refrigerated centrifuge, Precision Biotek Instruments Pvt. Ltd., Delhi) at 4,000 x g for 10 min at 4°C. The supernatant was filter-sterilised through a 0.2 µm syringe filter (Millipore, India) to remove any remaining cells. The cell pellet was used for secondary fermentations to prepare supernatants with MRS supplemented with glycerol (360 mM). The supernatant obtained from secondary fermentation of glycerol-MRS was designated as MRS-Gly, while the supernatant derived from MRS broth was designated as MRSB. These supernatants were kept for a maximum of one month. Finally, the pH values of the above supernatants were determined using a portable pH meter (pH pen KS732, Shindengen; Japan). The pH adjustments of the supernatants were carried out by neutralizing them to pH 6.5 using 0.1 M sodium hydroxide at room temperature prior to testing their antimicrobial activity.

Antimicrobial activity of *L. reuteri* isolates

The agar-well diffusion method was used to check the antibacterial activity of CFCSSs derived from isolated cultures of *L. reuteri* as mentioned in Goyal et al. (2018). Briefly, 10⁶ cfu of target bacteria in its active growth were inoculated to 8 ml of melted and cooled (45°C) soft agar tubes (1% w/v) of BHI and poured on to the solidified and dried (one day at 37°C) TGE agar plates. The inoculated soft agar was allowed to properly solidify before punching out wells of 6mm diameter from agar plates with the help of cork borer. The agar buttons were removed and the base of the wells was sealed by molten soft TGE agar. The drilled wells in the agar plates were then filled with CFCSSs (100 µl) and the plates were kept at 4°C for 3-4 h to facilitate the diffusion of CFCSSs. The plates were then incubated at 37°C and the antimicrobial activity was recorded as the zone of inhibition extending laterally from the edges of wells.

The antifungal activity of CFCSSs was performed using agar overlay assay (Voulgari et al. 2010). Briefly, 5 µl of active cultures of *L. reuteri* isolates were spotted on MRS (with glycerol) agar plates and incubated for 48 h at 37°C under anaerobic conditions. Subsequently, an aliquot of 100 µl of fungal spores (corresponding to 10⁴ spores) were transferred to melted and cooled (45°C) YPD soft agar. The pH of soft agar was adjusted to 4.0 with sterile tartaric acid before pipetting the soft agar on to the culture agar plates. The soft agar was pipetted on the surface of petridish drop wise surrounding the culture spots. The soft agar was

allowed to solidify and the plates were incubated at 30°C for 3-5 days depending on the fungal growth rate. The antifungal activity of the culture was measured in terms of the diameter of zone of inhibition at the end of incubation period.

RAPD-PCR of *L. reuteri* isolates

Random amplified polymorphic DNA (RAPD)-PCR was performed on the *L. reuteri* isolates with antimicrobial activity to determine the genetic relatedness and exclude replicates among the isolates. A total of 5 random RAPD primers (Weiss5, Weiss1, 275, OPPGandOPAP-1) were first tested for RAPD band pattern, out of which OPAP-1 (AACTGGCCCC)(Singh et al. 2017) was selected based on reproducibility and number of bands. RAPD-PCR was carried out following standard PCR protocol and the PCR amplified products obtained were electrophoresed on 1.5 % agarose gels at low voltage of 60V for 3 h (Singh et al. 2017).

Activity units of Cell Free Culture Supernatant (CFCSS)

The active units of CFCSS of the most potent culture was quantified by MIC method using microtiter plate method against *E. coli* K12 as described by Chung et al. (1989). Overnight grown culture of *E. coli* K12 was harvested, washed twice with phosphate buffer (pH 7.2, 50 mM), suspended in the same buffer, and diluted to an A₄₂₀ of 0.2 OD measured using spectrophotometer. This suspension was diluted 1/10, which corresponds to about 1x 10⁶ cfu/ml. The diluted suspension (0.1 ml) was used to inoculate 0.2 ml of serial dilutions of CFCSSs diluted in Muller-Hinton medium (Himedia, India Laboratories). The microplate was incubated at 37°C for 12 h and examined accordingly for growth. The active units in the CFCSSs was defined as the reciprocal of the highest dilution that did not permit visible growth of the indicator strain and expressed as Arbitrary Units (AU).

Antimicrobial activity in milk system

The inhibitory action of reuterin alone (CFCSS) or in combination with a bacteriocin (nisin) was finally tested in milk system against some model food microorganisms viz. *E. coli* K12, *Pediococcus acidilactici* LB42 and *Listeria monocytogenes* ATCC 13932. These indicator organisms were selected since *E. coli* is sensitive to reuterin, while LB42 strain is nisin sensitive while *Listeria* is sensitive to both the tested antimicrobial substances, hence this would allow testing the synergistic effect between the two antimicrobials and also whether the drawback of one can be fulfilled by another.

Stock solutions of nisin (10000 IU/ml) (Nisaplin, Danisco, India) were prepared in 0.02 N HCl and stored at -20°C. The stock solution of nisin was diluted accordingly and 100 IU/ml was added into the milk system. The CFCSSs was then added at an estimated final activity of 16 AU/ml. Cells (of the target bacteria) of the early stationary phase were harvested and 100 µl were separately

inoculated into flasks containing 100 ml of reconstituted skim milk (11% total solids). The seeded milk with each bacterial strain with no addition of reuterin or nisin served as control. All the flasks were then subsequently incubated at 37°C for 24 h. The microbiological counts of the entire flask were determined at 0 h and subsequently after 4 and 24 h on BHI and MRS medium agar plates.

Statistical analysis

The data obtained in this study was presented as mean $\pm$ standard deviation and analyzed statistically with the GraphPad Prism 5.0 software. The statistical significance among different antimicrobials and its combination in milk system was compared by one way ANOVA following Tukey's post hoc test and considered significant at $P < 0.05$.

Results and Discussion

Isolation and Identification of *L. reuteri* strains

Lactic acid bacteria are natural inhabitants of nutrient rich niches, in particular *L. reuteri* are common residents of the gastrointestinal tract (GIT) of diverse mammals such as humans, pigs, mice, rats and many species of birds (Frese et al. 2011). *L. reuteri* have been isolated from the gastrointestinal tract of human, pigs etc. (Hou et al. 2015). Therefore, we obtained a number of samples comprising of infant faeces, adult faeces, breast milk and rat faeces in an attempt to obtain some potential strains of *Lactobacillus reuteri* with broad spectrum antimicrobial activity.

From the plated MRS samples (with vancomycin), a total of 113 random colonies were picked based on colonial morphology (cream colour, oval shape), and used for further analysis. Based on the phenotype screening test (standard Gram staining procedure and catalase test), a total of 35 isolates were found to be Gram positive rods and catalase negative and were presumed to be *Lactobacillus* cultures. Further, the *L. reuteri* confirmation was carried out for the 35 tentative *Lactobacillus* isolates with species specific PCR using primer pair Lreu 1/Lreu 4. Thirteen isolates out of the 35 tested isolates gave a specific amplification product of 303bp for *L. reuteri* specific PCR (Fig. 1). Earlier, it has been reported that *L. reuteri* is one of the most abundant species present in the gut of pigs, rodents, and chickens, (Abbas et al. 2000), however the prevalence of *L. reuteri* is much lower in human adults, where the species is only occasionally found (Walter 2008). Similarly in our study no *L. reuteri* isolate could be obtained from human adult feces, and out of the final 13 confirmed *L. reuteri* isolates, 10 were from breast milk, 2 from infant faeces and 1 from rat faeces. The 13 PCR confirmed isolates of *L. reuteri* were then subjected to further analysis.

Antimicrobial activity of Cell Free Culture Supernatants (CFCS) against selected indicator organisms

Some strains of *L. reuteri* can produce reuterin from anaerobic conversion of glycerol (Siedler 2019). Reuterin is a potent antimicrobial compound that has a wide spectrum of antimicrobial activity surpassing various kinds of bacteria, fungi, protozoa etc. (Axelsson et al. 1989). However, the reuterin producing capacity is highly variable among different strains of *L. reuteri*

Table 1 Antimicrobial activity of *L. reuteri* isolates against Gram negative and positive bacterial indicators by agar well diffusion assay

<i>L. reuteri</i> isolates	Gram Negative						Gram Positive	
	EC	ST	YE	KP	SM	LM	EF	LB42
11	+++ ¹	+++	+	+++	+++	+	-	-
31	-	-	-	-	-	-	-	-
47	+++	+++	+++	+++	++	++	+	-
48	+	+	+++	+++	+	++	-	-
49	+++	+++	++	+++	+++	+	+	-
68	-	-	-	-	-	-	-	-
71	-	-	-	-	-	-	-	-
74	-	+	++	++	+	+	-	-
78	-	-	++	++	-	++	-	-
79	-	++	++	+	+++	+	-	-
81	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-
7/R	++	++	+++	+++	+++	+	-	-
Nisin	-	-	-	-	-	++	+++	+++

¹Zone of inhibition: +++ : >15 mm, ++: 10-15 mm, + : < 10 mm.

EC= *Escherichia coli*, ST= *Salmonella typhi*, YE= *Yersinia enterocolitica*, KP= *Klebsiella pneumonia*; SM= *Serratia marcescens*; LM= *Listeria monocytogenes*; EF= *Enterococcus faecalis*; LB42= *Pediococcus acidilactici*

Fig. 1 Species specific PCR of isolated *Lactobacillus reuteri* strains using Lreu-1 and Lreu-4 primer pair; Lane M: 100 bp DNA Marker; L1: *L. reuteri* ATCC 55730; L2: 47; L3: 49; L4: 68; L5: 74; L6: 78 L7: 7/R.

Table 2 Antifungal activity of *L. reuteri* isolates against fungal indicators by agar overlay assay

Fungal indicators	<i>L. reuteri</i>												
	11	31	47	48	49	68	71	74	78	79	81	82	7/R
<i>A. niger</i>	+++	+	+++	-	+	-	++	-	+	+++	++	-	+
<i>P. roqueforti</i>	+++	++	+++	+	++	+	++	++	++	+++	++	+	+++
<i>R. glutinis</i>	+++	++	+++	++	++	++	++	++	++	+++	++	++	+++

+ Less than 20 mm; ++ 20-35 mm; +++Greater than 35 mm

due to variations in gene expression of associated enzymes (Spinler et al. 2008). We screened the 13 identified *L. reuteri* strains for its antimicrobial activity against 8 different bacterial indicators that included important food pathogens. Both the neutralized as well as non-neutralized forms of CFCS derived from MRS broth fermented with glycerol supplementation (MRS-Gly) as well as without glycerol supplementation (MRSB) were used to test the antimicrobial activity. The antimicrobial activity was observed in both MRS-Gly as well as MRSB, however the inhibitory effect of MRSB was not evident in its pH adjusted counterparts. *L. reuteri* being a LAB secrete organic acids such as lactic acid, acetic acid etc. as a primary fermentation product. These are strong antimicrobial agents particularly in a relative low pH environment, which may have contributed the antimicrobial activity observed in case of MRSB, since the activity could not be replicated in its pH neutralized counterpart. On the other hand, CFCS (neutralized) from MRS-Gly derived from 8 strains out of the 13 identified *L. reuteri* strains exhibited good antibacterial activity against majority of the indicator organisms (Table 1 and Fig. 3). The activity observed in this case can be attributed to reuterin action since reuterin is active at wide range of pH values including neutral pH (Garde et al. 2016). The highest antimicrobial activity was observed in the neutralized CFCS derived from *L. reuteri* strain 11, 47, 49 and 7R. The CFCS of these *L. reuteri* strains were able to inhibit both Gram negative as well as Gram positive bacterial indicators. Moderate activity was seen in *L. reuteri* strain 48, 74, 78 and 79, while very negligible or no

activity was observed in *L. reuteri* strain 31, 68, 71, 82 and 81 strains. However, none of the *L. reuteri* isolates had any significant inhibitory effect against *Enterococcus faecalis* and *Pediococcus acidilactici*. This is not surprising as it has been reported that reuterin affects lactic acid bacteria to a lesser degree and relatively higher concentration of reuterin is required to inhibit the growth of LAB (Axelsson et al. 1989).

Screening of *L. reuteri* isolates for antifungal activity

The most important attribute of reuterin system is its wide spectrum antimicrobial activity, which gives it a very important prospect of being an excellent biopreservative molecule. In addition to inhibiting a wide range of bacterial indicators, the reuterin system has also been shown to demonstrate wide antifungal activity (Schnurer and Magnusson, 2005). Here, the antifungal activity of the *L. reuteri* cultures as well as its supernatant was checked by agar overlay and spot on lawn assay on mMRS and MRS media, respectively. Out of the two methods, the agar overlay assay using *L. reuteri* culture streak in mMRS media was found to be more suitable for assessing the antifungal activity of *L. reuteri* strains, hence the antifungal activity testing of the test strains was carried out with the agar overlay assay. From the agar overlay assay, it was observed that the indicator organism *Rhodotorula glutinis* was heavily inhibited by all the *L. reuteri* isolates and very low growth of the fungal spores was observed in the agar plates containing the *L. reuteri* cultures,

Fig. 2 Antimicrobial activity of CFCSs of *L. reuteri* strains and nisin against different indicator strains (Gram positive and Gram negative) by agar well assay

although, we could observe proper growth of *R. glutinis* in the agar plate without any *L. reuteri* culture spots (Table 2). Similarly, *Penicillium roqueforti* also showed little growth in the presence of most of the isolated *L. reuteri* cultures. The mold *A. niger* was the most resistant fungus to the inhibitory activity of *L. reuteri* isolates and only strain 47, 79 and 11 showed significant inhibition, while other strains showed little or no inhibition against *A. niger*. Earlier, it has been demonstrated by Vimont et al. 2019, that reuterin at concentration 6.9 mM can display strong fungicidal activity against a host of food fungi including the fungal indicators tested in this study. The concentration reported is generally formed in the common method of reuterin production by *L. reuteri* strains. Hence, we can presume that the isolated *L. reuteri* strains in the study were able to produce enough reuterin in the medium in order to inhibit the growth of tested fungal indicators.

RAPD analysis of *L. reuteri* strains

A total number of five arbitrary primers were tested with RAPD-PCR with DNA extracts from pure cultures of 8 *L. reuteri* isolates showing antimicrobial activity. The RAPD primer, OPAP-1 for *L. reuteri* was able to produce more than four scorable bands and was selected for further analysis. The profile generated from RAPD-PCR of the 8 *L. reuteri* cultures generated between 8 to 10 DNA bands ranging from 100 to >1500 bp (Fig. 3). The strains isolated from infant faeces (Lane 2) showed similar pattern as did the strains isolated from human milk (Lanes 4, 5, 7, and 8), although variation in the antimicrobial activity was observed between the strains. Previously, Singh et al. (2017) also reported that the strain types resulting from RAPD-PCR do not necessarily segregate based on metabolic activity. On the contrary, the strain (Lane 1) isolated from rat gave distinct RAPD profile from other strains from human milk. Although, Ganzle and Yogel, (2003) have previously described that not all *L. reuteri* strains from different sources could be discriminated on account of their RAPD-PCR patterns with one arbitrary primer, we were able to detect the

Fig. 3 RAPD profiles of *L. reuteri* strains showing antimicrobial activity generated against RAPD primer OPAP-1. Lanes M: 100 bp DNA ladder; L1: 7/ R; L2: 11; L3: 74; L4: 49; L5: 79; L6: 47; L7: 78; L8: Lb48

a. *E. coli* K12

b. *Pediococcus acidilactici* LB 42

c. *Listeria monocytogenes*

Fig. 4 Log counts (cfu/ml) of Gram-positive and Gram-negative pathogens in milk without antimicrobial agents(C), with reuterin, with nisin and their combination(R+N) after incubation at 37°C for 4 h and 24h.

strain replicates and overall 4 group or strain type was obtained from the tested 8 *L. reuteri* strains.

Activity units of the most potent *L. reuteri* strain 47

Based on the antimicrobial (antibacterial and antifungal) activity and RAPD profile, *L. reuteri* 47 was selected as the most potent isolate and used for further study. The active units of reuterin

present in the cell free culture supernatant (CFCS) of the *L. reuteri* 47 was calculated by MIC determination against *E. coli* K12 by microtitre plate assay. Serial dilutions of CFCS of *L. reuteri* 47 were prepared in microtitre plate and activity unit of CFCS was obtained against defined number of cells of *E. coli* in the microtiter plate. After overnight incubation at 37°C, growth was observed in the fifth well pertaining to 16x dilution of the stock CFCSs. No visible growth was observed in the fourth dilution (pertaining to

8x dilution), accordingly the activity unit of the CFCS was tabulated as 1600 AU/ml.

Antibacterial activity of reuterin from *L. reuteri* 47 alone or in combination with nisin in milk system

As mentioned earlier, biopreservation of processed foods is an attractive area particularly using metabolites of LAB due to its safe nature. However, much work has been focussed on the proteinaceous bacteriocins which due to its cationic nature are unable to penetrate the LPS layer of Gram negative bacteria and reach the target site i.e. cell wall (Field et al. 2018). However, Gram negative bacteria such as coliforms and other members of enterobacteriaceae family consist of the major spoilage and pathogenic microflora in food products including milk and milk products. Contrary to the bacteriocins, the reuterin system acts by modifying the thiol groups in proteins and small molecules of the target microorganisms, therefore, are able to inhibit a wide spectrum of both Gram positive and negative bacteria including fungal targets (Siedler et al. 2019). However, reuterin has been demonstrated to have weak effect on LAB, probably due to its antioxidant system, hence, a combination of reuterin and nisin was tested in this study for best antimicrobial action. Furthermore, the antimicrobial action of these molecules have been observed to display variable antimicrobial action in different food systems, possibly due to interactions with food components, hence it is very important to test the antimicrobial action of any antimicrobial system in the real food system.

In this study, the inhibitory action of reuterin (16 AU/ ml), nisin (100 IU/ml) and their combination on certain bacterial indicators viz. *E. coli* K12, *P. acidilactici* LB42 and *Listeria monocytogenes* were tested in sterile milk system as shown in Fig. 4. In case of *E. coli* K12, as anticipated the presence of reuterin in milk had a significant bacteriostatic effect on the growth of *E. coli*, which did not demonstrate any net increase in counts even after 24 h of incubation at 37°C (Fig. 4a). On the other hand, nisin had negligible effect and the growth of *E. coli* in its presence was similar to the control milk sample with no additive. Furthermore, no synergistic effect was seen when both the antimicrobials were mixed, rather nisin had a slight hindering effect on the reuterin action and the numbers increased after an initial reduction. Conversely, *Pediococcus acidilactici* LB42 is a bacteriocin sensitive strain (Goyal et al. 2018) and as expected the inhibition was also demonstrated in the milk system wherein LB42 counts decreased initially on nisin exposure and no net growth was observed after 24 h incubation (Fig. 4b). However no effect of reuterin was observed on LB42. The synergistic effect could only be seen in case of *L. monocytogenes*. It is well known that both nisin and reuterin have antagonistic activity against *L. monocytogenes* (Arques et al. 2011) which was also seen here in the milk system. In case of reuterin, the activity observed was more bacteriostatic since there was no significant inhibition after 4 h and even after 24 h the counts did not variably increase. On the other hand,

nisin has bacteriocidal effect on *L. monocytogenes* since the count had significantly decreased (Fig. 4c) after initial 4 h incubation, thereafter the growth resumed. The increase of *L. monocytogenes* cells after 24 h incubation maybe attributed to the regrowth of resistant cells.

Previously, numerous attempts have been made to prepare combinations of nisin with other antimicrobial compounds such as ascorbic acid and EDTA (Sangcharoen et al. 2017; Khan et al. 2015), plant essential oils and ascorbic acid (Campion et al. 2017), EDTA and lysozyme (Bhatia and Bharti, 2015) etc. in order to overcome the drawback of LAB bacteriocins. Although, these formulations were successful in inhibiting Gram negative organisms also, the application of most of these antimicrobials is limited either due to its chemical nature, production cost or interference with the sensory properties of the final product. In this regard, reuterin offers a suitable alternative due to the GRAS status of the producing species, besides being active against a wide target range and physical conditions of temperature, pH etc. along with the absence of any sensory characteristics. In the present study, reuterin and nisin were able to display synergistic effects against *L. monocytogenes*, besides overcoming the drawbacks of one another against other target microorganisms in milk system. Apart from displaying efficacy in milk system reuterin have also been positively tested against *Listeria monocytogenes* in cottage cheese and UHT skim milk (El-Ziney and Debevere, 1998), besides controlling *Clostridium* spp. spores growth in dairy products (Avila et al. 2014). The target site of reuterin system is different from that of nisin. Due to the differences in target sites of reuterin and nisin, the combination can cause multiple cell injuries in the target cell that could inhibit even the resistant strains and also prevent the emergence of resistance (Arques et al. 2011). However, very limited studies have attempted to study the efficacy of these antimicrobial substances against the mixed microflora that exist in natural food products rather than spiked food samples. Hence, even in case of the reuterin and nisin combination tested in the present study, it is important to check the activity in the natural mixed microbial population that exists in different foods before building applications for specific food systems.

Conclusions

In the present study, reuterin and nisin in the tested concentration could effectively inhibit their individual targets, in addition to showing a synergistic activity against a common target such as *Listeria monocytogenes* in milk system. Hence, it may be concluded here that reuterin can form effective combination with bacteriocins such as nisin in order to target different microbial food contaminants. The suitability of the combination should also be tested in different food matrices in order to utilise as an effective biopreservative. However, further studies needs to be carried out on its toxicity and also efficacy against the natural

microflora persisting in different food systems in order to use the combination as an effective food biopreservative.

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