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Antibiotic Sensitivity Pattern and Safety Evaluation of Isolated Strains of Fecal Origin *L. reuteri*

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Abstract

Forty isolates of *L. reuteri* from human infant fecal sample were previously isolated and identified in Microbial Metabolite laboratory of Dairy Microbiology Division, National Dairy Research Institute, Karnal. An initial attempt in context to study safety of 40 strains of *L. reuteri* was done in terms of decarboxylation of amino acids i.e. their ability to produce biogenic amines i.e. tyramine, histamine and puteriscine,antibiotic susceptibility. Physiological investigation of all the 40 *L. reuteri* strains for the presence of virulence characters revealed that incidence of such traits was not present as none of the isolate was found to possess β-hemolytic activity, DNAse activity and/or gelatinase activity. All 40 *Lactobacillus reuteri* strains from infant feces were subjected to antibiotic susceptibility testing using the agar diffusion method. All of them were sensitive to erythromycin (except LR1), rifampicin (except LR25), of loxocin (except LR15 and LR20), novobiocin (except LR1, LR6, LR15, LR21, LR22, LR23, LR25, LR26, LR34, LR 36 and LR38), bacitracin, chloramphenicol and clindamycin (except LR1 and LR25), but they were resistant to polymixin B (except LR15), gentamycin (except LR21), cefazolin, ampicillin (except LR15, LR20 and LR21), kanamycin, amikacin, vancomycin, cephalothin, cefuroxime (except LR5, LR9, LR34, LR38 and LR39).All the strains were found to be safe for human use.

Keywords: *Lactobacillus reuteri*, Reuterin, Response surface methodology, Optimization

Consumer demands have led to a renewed interest in the use of natural antimicrobials for food products. In the hurdle concept of food preservation, the combination of these antimicrobials as biopreservatives to achieve an enhanced level of product safety and stability has gained increased attention. In this regard, reuterin is a novel small antimicrobial compound that is produced as an intermediate metabolite during anaerobic fermentation of glycerol (Vu *et al.* 2017). It consists of an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde (Vu *et al.* 2017). Reuterin, produced by some strains of *Lactobacillus reuteri*, has shown antimicrobial activity against a range of food-borne pathogens and spoilage organisms, including Gram-positive and Gram-negative bacteria, yeasts, moulds and protozoa (Mishra *et al.* 2011; Mishra *et al*. 2012; Mishra *et al*. 2018a; Mishra *et al*. 2018b). In the present study safety of 40 strains of *L. reuteri* was evaluated in terms of decarboxylation of amino acids i.e. their ability to produce biogenic amines i.e. tyramine, histamine and puteriscine,antibiotic susceptibility etc.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Lactobacillus reuteri BPL-36 strain along with 39 previously isolated *L. reuteri* from human infant fecal sample (Mishra *et al.* 2012), was maintained and

propagated in MRS broth (Hi-Media labs, Mumbai, India). Bacterial strains were maintained as stock cultures at -80°C in MRS broth supplemented with 15% glycerol. The organisms were propagated twice before use in the experiments. The purity of all the bacterial cultures was always ascertained by Gram staining prior to use for any experiment.

Detection of Virulence Determinants

Hemolysis

The *Lactobacillus reuteri* strains were grown overnight in MRS medium at 37°C, and then streaked onto Blood Agar Base (HiMedia Laboratories Ltd., Mumbai) plates containing 5% of fresh calf blood obtained from Animal Health Complex of the Institute. The plates were incubated for 3 d at 37°C. The hemolytic reaction was recorded by observation of a clear zone of hydrolysis around the colonies (β-hemolysis), a partial hydrolysis and greening zone (α-hemolysis) or no reaction (γ-hemolysis)(Singroha *et al*. 2017).

DNase test

All the *Lactobacillus reuteri* strains were streaked on DNase agar medium (HiMedia Laboratories Ltd., Mumbai) to check production of DNase enzyme. Plates were incubated at 37°C for 48 h to observe for any appearance of pink colored colonies (Singroha *et al.* 2017).

Liquefaction of gelatin

Gelatinase production by selected *Lactobacillus reuteri* isolates was studied by using TND agar containing 0.4% gelatin. Petriplates containing the medium were spot inoculated with the test culture and incubated at 37°C for 3 d. The petriplates were then flooded with saturated ammonium sulphate solution. Development of clear zones around the spots against opaque background indicated a positive reaction (Singroha *et al.* 2017).

Antibiotic Susceptibility

Patterns of resistance/susceptibility to antibiotics of strains of *L. reuteri* were studied by a disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA). A total number of 16 types of antibiotic discs (HiMedia Ltd, Mumbai, India): ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), cephalothin (30 μg), novobiocin (30 μg), bacitracin (10 μg), cefuroxime (30 μg), vancomycin (30 μg), rifampicin (30 μg), clindamycin (2 μg), kanamycin (30 μg), ofloxacin (5 μg), polymyxin B (300 units), cefazolin (30 μg), and amikacin (30 μg) were used.

Mueller Hinton agar (HiMedia, Mumbai, India) plates were poured and allowed to solidify. These were subsequently overlaid with 4 mL of Mueller Hinton soft agar tempered at 45ºC and seeded with 200 μL of the active cultures. Petriplates were allowed to stand at room temperature for 15 min and then the Himedia antibiotic discs were dispensed onto the agar surface using forceps under aseptic conditions. The agar plates were incubated at 37ºC aerobically for 24 h. Diameter (in mm) of the inhibition zone was measured using an antibiotic zone scale and results were expressed in terms of resistance, moderate susceptibility or susceptibility by comparing with the interpretative zone diameters provided in the Performance Standards for Antimicrobial Disk Susceptibility tests.

Assessment of Production of Biogenic Amines

The ability to produce various biogenic amines viz. tyramine and histamine by the decarboxylation of the tyrosine, histadine and putrescine, respectively, was evaluated by qualitative methods.

Qualitative estimation of biogenic amines production

As decarboxylation is accomplished by the rise in the pH hence decarboxylase activity was detected by means of suitable indicator added in the growth medium containing the amino acids to be investigated. The screening and qualitative estimation of amino acid decarboxylation activity of all the 40 *Lactobacillus reuteri* strains was done by using Improved medium as described by Bover-Cid and Holzapfel (1999), based on the colour change of

the indicator (bromocresol purple). All the *L. reuteri* strains along with both positive and negative culture (*E. faecalis* NCDC 114 and *L. lactis subspp. cremoris* NCDC 61, respectively) were sub cultured 5 times in MRS broth containing 0.1 % amino acids (i.e. tyrosine and histidine) and 0.005% pyridoxal-5- phosphate to promote enzyme induction before the actual screening test. Further, strains were inoculated in the improved broth medium and incubated for 4 d at 37ºC under anaerobic conditions in gas jar (GasPak, Himedia, India). The time for the production of violet color in the broth tube containing different amino acids was noted at an interval of 1 d for each culture. Strains were considered amino acids decarboxylase positive if color of the indicator turned yellow to violet in improved medium.

Determination of tyramine forming ability in plate media

During the above study as tyrosine was not soluble in the broth media, the tyrosine decarboxylase activity was assayed in Tyramine production Medium (TPM). Actively growing test cultures were spotted on preformed TPM plates and incubated for 4 d at 37°C under anaerobic conditions in gas jar and observed for solublization of tyrosine as indicated by clear zones below the grown cells.

RESULTS AND DISCUSSION

Detection of Virulence Determinants

DNAse activity, gelatinase activity and hemolysis activity

None of the *Lactobacillus reuteri* strains tested was found to be positive for DNAase activity, gelatinase activity and hemolysis (Table 1).

Table 1: Detection of virulence determinants in *L. reuteri* isolates

Sl. No.	Isolates No.	*Gelatin Production*	*Haemolysin Production*	DNase Production
1	LR 1	—	γ	—
2	LR 2	—	γ	—
3	LR 3	—	γ	—
4	LR 4	—	γ	—
5	LR 5	—	γ	—
6	LR 6	—	γ	—
7	LR 7	—	γ	—
8	LR 8	—	γ	—
9	LR 9	—	γ	—
10	LR 10	—	γ	—
11	LR 11	—	γ	—
12	LR 12	—	γ	—
13	LR 15	—	γ	—
14	LR 16	—	γ	—
15	LR 17	—	γ	—
16	LR 18	—	γ	—
17	LR 19	—	γ	—
18	LR 20	—	γ	—
19	LR 21	—	γ	—
20	LR 22	—	γ	—
21	LR 23	—	γ	—
22	LR 24	—	γ	—
23	LR 25	—	γ	—
24	LR 26	—	γ	—
25	LR 27	—	γ	—
26	LR 28	—	γ	—
27	LR 29	—	γ	—
28	LR 30	—	γ	—
29	LR 31	—	γ	—
30	LR 32	—	γ	—
31	LR 33	—	γ	—
32	LR 34	—	γ	—
33	LR 35	—	γ	—
34	LR -BPL-36	—	γ	—
35	LR 37	—	γ	—
36	LR 38	—	γ	—
37	LR 39	—	γ	—
38	LR 40	—	γ	—
39	LR 41	—	γ	—
40	LR 42	—	γ	—

No zone of hydrolysis = — Zone of Hydrolysis = + Strong haemolysis = β Weak haemolysis = α No haemolysis = γ Very weak α-haemolysis = [α]

Assessment of Production of Biogenic Amines

Biogenic amines are organic basic compounds produced in different kinds of food, including dairy products, due to the decarboxylase activity of some of the microorganisms. Several toxicological problems are related with the ingestion of food containing high levels of biogenic amines. Histamine and tyramine have vasoactive and psychoactive properties, while the diaminesputrescine and cadaverine may potentiate the toxicity of the cited amines. Moreover, they are also known to be potential precursors of carcinogenic nitrosamines when nitrosable agents are present in food (Bover-Cid and Holzapfel, 1999).

In the present study safety of 40 strains of *L. reuteri* was evaluated in terms of decarboxylation of amino acids i.e. their ability to produce biogenic amines i.e. tyramine, histamine and puteriscine. Assessment of biogenic amines productions was done by qualitative (decarboxylating medium) method.

Qualitative screening for decarboxylase activity

All the 40 strains of *L. reuteri* were initially screened for amino acid decarboxylase activity towards two amino acids viz. tyrosine and histidine using improved medium (Bover-Cid and Holzapfel, 1999).

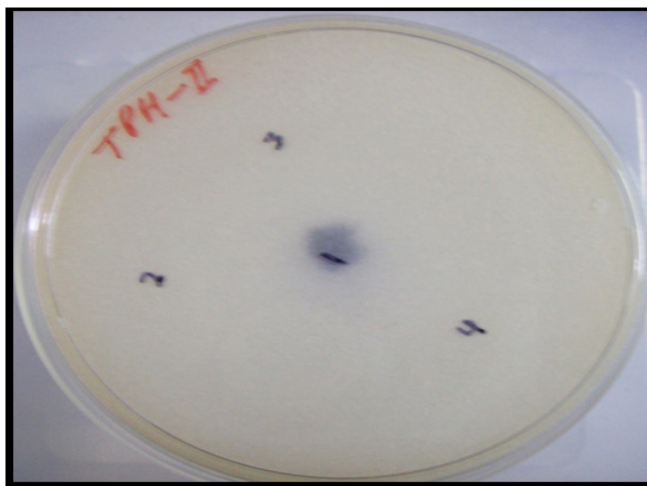

Fig. 1: Biogenic amines production test on Tyrosine Medium (TPM)

1. E. faecalis NCDC 113 (Positive control), 2- LR-BPL-36, 3- LR- 34, 4- LR- 37

All forty strains were found to be negative for histidine decarboxylase activity as indicated by no colour change of medium from yellow to purple within 72 hrs in response of the indicator to a pH shift. The pH change is dependent of the production of the more alkaline BA from the amino acids initially included in the medium.

On TPM, the *L. reuteri* strains did not show any zones of clearance i.e. were negative for tyrosine decarboxylase activity. Tyrosine decarboxylation activity on TPM is shown in Fig. 1.

Antibiotic Susceptibility

One of the safety considerations in bioprotection studies is the verification that a potential bioprotective strain does not contain transferable resistance genes. Therefore, all 40 *Lactobacillus reuteri* strains from infant feces were subjected to antibiotic susceptibility testing using the agar diffusion method.

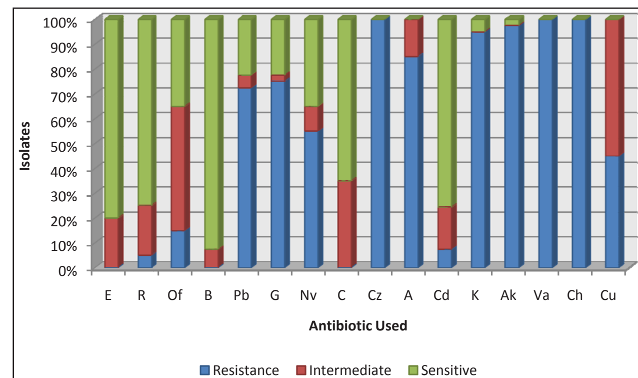

Fig. 2: Antibiogram of selected L. reuteri isolates against narrow and broad range antibiotics.

E- Erythromycin; R- Rifampicin; Of- Oflaxocin; B- Bacitracin; Pb-Polymyxin B; G-Gentamycin; Nv- Novobiocin; C- Chloramphenicol; Cz-Cefazolin; A- Ampicillin; Cd- Clindamycin; K- Kanamycin; Ak-Amikacin; Va- Vancomycin; Ch-Cephalothin; Cu- Cefuroxime.

All of them were sensitive to erythromycin (except LR1), rifampicin (except LR25), ofloxocin (except LR15 and LR20), novobiocin (except LR1, LR6, LR15, LR21, LR22, LR23, LR25, LR26, LR34, LR 36 and LR38), bacitracin, chloramphenicol and clindamycin (except LR1 and LR25), but they were resistant to polymixin

B (except LR15), gentamycin (except LR21), cefazolin, ampicillin (except LR15, LR20 and LR21), kanamycin, amikacin, vancomycin, cephalothin, cefuroxime (except LR5, LR9, LR34, LR38 and LR39) (Fig. 2).

Apart from potential pathogenicity of LAB, the presence of antibiotic resistance genes is a safety problem. Antibiotic resistance genes are widespread, not only in pathogenic bacteria but also among non-clinical isolates, probably due to the constant selective pressure caused by the extensive use of antibiotics. The transfer of such resistance genes from intrinsic bacteria to pathogenic bacteria may lead to transfering of antibiotic resistances in human, potentially impacting worldwide on human health. LAB carrying antibiotic resistance genes are therefore undesired for daily consumption (Sharma *et al*. 2017).

Considering these data, the antibiotic resistances which were observed for the strains in this study were considered to be intrinsic or natural resistances. Conversely, the strains of *L. reuteri* except LR 1.did not contain any of the transferable, acquired resistances that are known to occur among LAB and include resistances towards chloramphenicol and erythromycin.

CONCLUSION

This study has provided some evidence for the safety status of these novel strains of *L. reuteri*. But before that, a thorough investigation of these strains for their toxicological effects (if any) on animal models and human volunteers is required. Though it was beyond the scope of the present investigation, their exploitation as protective cultures in food and dairy industry would no doubt make an interesting and useful study. The studies in this work also serve as a contribution towards the validation of the bio protective culture status claimed for these novel strains.

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Compliance with Ethics Requirements: This article does not contain any studies with human or animal subjects. Additional informed consents were obtained from all the parents of infants from whom fecal samples were collected for isolation of lactic acid bacteria.

Conflict of Interest: Santosh Kumar Mishra and R. K. Malik declare that they have no conflict of interest.

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