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In vitro Assessment of Antioxidant and Antimicrobial Potential of *Lactobacillus gasseri* Strains Isolated from Human Milk and Infant Faeces

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Abstract

The present work demonstrates the antagonistic and antioxidant property of *Lactobacillus gasseri* LGS22 (MN258931), *Lactobacillus gasseri* MVS25(MN396621) isolated from human milk and *Lactobacillus gasseri* LBM220 (MN097539) isolated from exclusively breast-fed infant faeces. Antagonistic potential was assayed using agar well diffusion method against 17 food-borne and enteric pathogens. Further, in order to characterize the nature of inhibitory metabolites in culture supernatant, several preliminary experiments such as pH adjustment,treatment with protease, catalase and heat were performed. In addition, quantitative estimation of antimicrobial activity was performed using Time-kill assay against indicator pathogen *Sh. flexneri* type 2. Strains were further evaluated for antioxidant action such as scavenging of hydroxyl radical, DPPH radical and $ABTS^{+}$ radical. The capacity to resist 0.4 mM, 0.8mM and 1 mM hydrogen peroxide for 16 h was also studied. Results report the efficient inhibitory potential of *Lact. gasseri* strains with zone sizes ranging from moderate to very strong inhibition (10-22 mm) against most of the indicator pathogens employed in the current study. Also, inhibitory action of cell-free culture supernatant of isolates LBM220 and MVS25 when adjusted to pH 6.5 was significantly reduced, elucidating the production of organic acids, while isolate LGS22 produced hydrogen peroxide as inhibitory metabolite. *Lact. gasseri* isolates were found to significantly scavenge free radicals, with scavenging potential in range of (80.687- 26.552%). It is concluded that these three *Lact. gasseri* isolates may be explored more for their bio-potential as food additive with antioxidant and antimicrobial activities.

Keywords: *Lactobacillus* species, LAB, Infant faeces, Human milk, Antagonistic activity, Antioxidant activity

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INTRODUCTION

Recently, incidences of food-borne diseases are growing in most developing countries as food quality control is on little priority owing to inadequate funds. Food spoilage and enteric pathogens are responsible for high mortality and morbidity accounting to fifth principle determinant of death of all age groups worldwide[1]. Common among them are *Shigella spp., Salmonella spp.*, *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli*[2]. So far, several synthetic chemical antibiotics have been extensively employed as therapeutics. However, their safety and long-term consequences are highly doubted. In order to cater this need, use of *Lactobacillus spp.* and their metabolites were widely explored by food technologists/researchers owing to its diverse antimicrobial, preservation and therapeutic attributes. With development of probiotic research, role of *Lactobacillus spp.* in restoration of gastrointestinal microflora, alleviation of ilial inflammatory response,augmentation of gut immunological barrier functions and antagonistic ability clearly demonstrate their capacity to manage specific enteric diseases. Additionally, antimicrobial potential of these beneficial microbes and/or extracellular antimicrobial metabolites offers propitious prospects for their use in infant formula feeds, food preservation and veterinary medical supplements. So far, voluminous literatures are available that report the competence of genus *lactobacilli* in restraining infections due to both Gram-negative and Gram-positive spoilage and pathogenic bacteria[3,4]. Combined application of *Lactobacilli* and antibiotic(s) orally has shown to be effective in curbing enteric infections, with lesser antibiotics dose needed. Also, it may result in replenishment of the gastrointestinal microflora, ultimately benefitting the host and preventing antibiotics side effects [5]. Human milk is a genesis of commensal infant gastrointestinal tract microflora, thus it is obvious that the microbial composition of infant faeces reflects that of breast milk[6]. Species of genus *Lactobacilli*, such as *Lact. acidiphilus*, *Lact. rhamnosus*, *Lact. fermentum*, *Lact. gasseri* and *Lact. plantarum* are among the most prevailing species in the gastrointestinal tract of the infant and they help in alleviation of several common enteric infections, though mechanism of their action is limited. Studied mechanisms include production of inhibitory metabolites, competitive exclusion of pathogenic microbes in the gut, strengthening of the intestinal barrier and modulation of host immune system[7]. Earlier, Toba et al. (1991)[8] reported antimicrobial property of *Lact. gasseri* LA39 from infant stool sample owing to its production of bacteriocin, gassericin A. Majority of commercial probiotics available nowadays have been of human or food/dairy origin. Although literatures voicing and assuring antimicrobial activity of *lactobacilli* are available, only limited researches uncover the antimicrobial efficacy of breast milk *lactobacilli*. In lieu of the above facts, present work was outlined to study the potency of CFCS of *Lactobacillus gasseri* strains exclusively isolated from breast milk and infant faeces against wide range of human and food-borne pathogenic bacteria.

When enteric pathogens proliferate excessively the intestinal tract, they induce endotoxin accumulation in the blood, thereby causing significant oxidative stress[9], leading to cell membrane damage, protein denaturation and incorrect DNA replications. Oxidative stress is correlated with initiation of multiple disorders such as cancer, lung injury, diabetes and atherosclerosis. Although, human body is endowed with repair systems and enzymatic antioxidant defences such as glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) as well as non-enzymatic antioxidant defences such as to copherols, thioredoxin, glutathione, ascorbic acid to protect against oxidative damage[10]. Sometimes they are insufficient to cater to it efficiently. Therefore, numerous synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene are consumed to resist damage, though their usage is highly questioned as they may cause cancer and liver damage[11]. Thus, recently, focus on developing safer and effective natural antioxidant agents is gaining momentum[12]. So far, several studies have reported the potential role of *lactobacilli* as antioxidant. *Lact. gasseri* NLRI-312 isolated from stool sample of Korean infant was found to reduce H_2O_2 induced cellular lipid and DNA damage[13]. Similarly, *Lacto. gasseri* 4M13 isolated from infant faeces displayed high DPPH and ABTS scavenging ability[14]. Thus, *Lact.*

gasseri strains were also tested for scavenging potential of DPPH, $ABTS^{+}$, Hydroxyl free radicals as well as resistance to hydrogen peroxide.

MATERIALS AND METHODS

Microbial cultures and culture media

For the purpose of isolation, 12 human milk samples and 18 breast-fed infant feaces samples were aseptically collected. All samples were cultured in *lactobacillus* selective De Man Rogosa Sharpe (MRS) medium (Himedia, India) at 37°C under anaerobic condition (80% N_2, 10% CO_2, 10%H_2) generated by anaerobic system (Anoxomat III, USA) for 48 h. A total of 162 isolates from human milk samples and 230 isolates from infant faecal samples, were isolated.Based on preliminary antimicrobial and antioxidant (by DPPH free radical method) screening of the isolates, the three potential strains which displayed significant inhibitory and scavenging capacity, were selected. Further identification was done using MALDI-TOF, while confirmation was done by 16S rRNA sequencing. Sequences were submitted in GenBank under accession number: *Lactobacillus gasseri* LBM220 (MN097539) from infant faeces and *Lactobacillus gasseri* LGS22 (MN258931) and *Lactobacillus gasseri* MVS25(MN396621) from human milk. The study was approved by institutional ethical review board of Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow (Ref. No. 2784/RMLIMS/2018). The strains were stored for short-term period at 4°C and for long-term period at -80°C with 5% skim milk. All pathogenic strains employed in present study were procured from Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow and aerobically cultured in Brain heart infusion broth (BHI)(Oxoid, UK). Clinical strains used were *Salmonella typhimurium*, *Salmonella paratyphi* T (H), *Salmonella typhi* T (H), *Serratia fecaria, Corynebacterium diphtheria*, *Shigella boydii*, *Listeria monocytogenes,Shigella sonnei*, *Shigella dysenteriae*, *Staphylococcus aureus* ATCC 25923, *Shigella flexneri* type2*,* and ATCC indicator strains used were *Enterococcus faecalis* ATCC-51299, *Klebseilla oxytoca* ATCC-700324,*Proteus vulgaris* ATCC-6380, *Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-27853, *Klebseilla pneumonia* ATCC 700602.

Antagonistic activity

Antimicrobial activity of *Lact. gasseri* strains against seventeen toxic enteric pathogens was determined as described by Rastogi et al*.,* (2019)[15]. Both isolates were anaerobically cultured in MRS broth for 48 h, followed by centrifugation at 12000xg for 15 min at 4°C. Supernatants were collected and filter sterilised using 0.2µ cellulose acetate paper to remove residual bacterial cells. The cell-free culture supernatants (CFCS) were then used to determine the antibacterial activity. Also, all indicator pathogens were aerobically grown overnight in BHI broth at 37°C. 20 ml BHI soft agar containing test pathogen (about 10^8 CFU/ml) was plated and was kept to solidify. Wells of 6 mm diameter were made and filled with 80 µl of CFCS. Plates were kept for 2 hours at 4°C for rapid diffusion and then at 37°C overnight. Each plate was examined for inhibitory halo zones after incubation. The inhibitory activity was interpreted as very strong (>17 mm), strong(14-24mm), intermediate (10-13mm) and weak (7-9mm) as reported by Sirichokchatchawan et al*.,*(2018)[16]. Assay was carried out in three replicates and results are expressed as mean of inhibition zones± SD.

To elucidate the effect of different treatments on antagonistic potential of CFCS of *Lact. gasseri* strains

Effect of various treatments on inhibitory action of CFCS of *Lact. gasseri* strains was examined according to Yu et al.,(2015)[17] with minor changes. Agar well diffusion assay was employed against test pathogen *Sh. flexneri.* Untreated CFCS served as control.

Effect of pH on antagonistic potential- CFCS of *Lact. gasseri* strains was adjusted to pH 6.5 using sterile 1N sodium hydroxide, followed by agar well diffusion assay using treated CFCS in order to determine the outcome of pH change on its inhibitory ability.

Effect of catalase on antagonistic potential- To ascertain the antimicrobial activity due to hydrogen peroxide, 0.5 mg/ml catalase (Himedia, India) enzyme was added to CFCS of *Lact. gasseri* strains, followed by agar well diffusion assay to observe the change in inhibitory potential. Effect of protease on antagonistic potential- To assess the inhibitory activity due to bacteriocin

and/ or inhibitory peptides, CFCS of *Lact. gasseri* strains was treated with 1 mg/mL protease (Himedia, India) for 1 h. Thereafter, the enzyme was denatured at 60°C for 20 min, followed by agar well diffusion assay to observe the alterations in inhibitory activity.

Effect of high temperature on antagonistic potential- *Lact. gasseri* strains' CFCS was subjected to high temperature (60°C for 1 h), and then tested for its antibacterial potential as described above using this treated CFCS.

Time-kill assay with CFCS of *Lact. gasseri* strains on indicator pathogen -*Sh. flexneri*

Time-kill assay was performed by culturing *Sh. flexneri* with CFCS of *Lact. gasseri* strains as outlined by Zhang et al.(2011)[18] with some modifications. To perform, 500 µl of *Sh. flexneri* (10^8 cfu ml^{-1}) was mixed with 5 ml each CFCS as mentioned above, viz. CFCS (pH 6.5), CFCS (protease treated), CFCS (catalase treated), CFCS (High temperature treated) and MRS broth (pH 6.5; as control), followed by aerobic incubation at 37°C. Aliquots were removed after predefined intervals (t = 0, 2, 4, 8,16h), serially diluted and each dilution plated on BHI to assess the viability of pathogens after incubation. The assay was carried out in three replicates and results reported as mean of log cfu ml^{-1}±SD.

Assay antioxidant potential of *Lact. gasseri* strains

DPPH free radical-scavenging ability

2,2-diphenyl-1-picrylhydrazyl(DPPH; Himedia, India) radical scavenging capability of *Lact. gasseri* LBM220 was measured as described by Nithya et al.(2013)[19]. Briefly, 48 h anaerobically grown *Lact. gasseri* strains were centrifuged (12000xg, 10 min, 4°C) to collect culture supernatant. Two ml DPPH solution (6 mg/100ml of methanol) was added to 500µl of culture supernatant to prepare test solution. Blank comprised of 2 ml methanol in 500µl of supernatant. Positive control used was L- ascorbic acid (Himedia, India). Both test and control tubes were thoroughly vortexed and kept in dark for 25 min. Absorbance (517 nm) was recorded using Nano Drop Spectrophotometer(DS-11, Denovix, USA).

DPPH radical scavenging %= {1-[A_{517} sample-A_{517}blank/A_{517} control]} x 100

$ABTS^+$ radical scavenging ability

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)($ABTS^+$) radical scavenging ability of *Lact. gasseri* strains were assayed by a method given by Shi et al.,(2019)[20] with some changes. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Himedia, India) stock solution was prepared and kept overnight in the dark at 25°C. Thereafter, $ABTS^+$ solution was adjusted to 0.700±0.05 absorbance (735 nm) with methanol. Culture supernatant (100µl) was added to 3.0 ml $ABTS^+$ solution, followed by measuring absorbance at 735 nm. L-ascorbic acid was used as positive control. Blank was prepared by mixing 100µl methanol or MRS broth. Percentage scavenging was calculated as follows:

$ABTS^+$radical cation scavenging %= {1-[A_{734} sample-A_{734}blank/A_{734} control]} x 100

Hydroxyl radical scavenging ability

The capacity of *Lact. gasseri* strains to scavenge hydroxyl radical was evaluated according to modified protocol of Achuthan et al.,(2012)[21]. *Lact. gasseri* strains were anaerobically grown in MRS broth at 37°C for 48 h and centrifuged(1200xg, 10 mins, 4°C) to obtain culture supernatant. For generation of hydroxyl radicals, 1 ml 1,10-phenanthroline(Himedia), 1.5 ml phosphate buffer saline(pH 7.4), 1 ml (0.01% v/v) $H_2O_2$and 1ml $FeSO_4$were mixed in the solution. Briefly, 1.0 ml culture supernatant was added to solution containing generated hydroxyl radical, followed by 30 min incubation at 25°C, thereby recording absorbance (536 nm) using NanoDrop spectrophotometer. Positive control was L-ascorbic acid. The results were expressed as follows:

% hydroxyl radical scavenging = {1-[A_{536} sample-A_{536}blank/A_{536} control-A_{536}blank]} x 100

Where, A_{536} sample is the absorbance of test solution in presence of Culture supernatants, A_{536} control is the absorbance in presence culture supernatant but where H_2O_2 in test solution was replaced with double distilled water and A_{536} blank is the absorbance without culture supernatant in test solution which is replaced with MRS broth.

Resistance to Hydrogen peroxide

Ability of *Lact. gasseri* strains to resist hydrogen peroxide was carried out as elucidated by Li et al.,(2012)[22]with minor changes. *Lact. gasseri* strains were anaerobically grown in MRS broth at 37°C for 48 h. Thereafter, 900 µl of MRS broth supplemented with 1% (v/v) 0.4 mM, 0.8 mM and

1.0 mM hydrogen peroxide (H_2O_2; Himedia, India) was inoculated with 100µl of test strain culture, followed by 16h anaerobic incubation at 37°C. The culture growth was estimated by recording absorbance(600 nm). Control was MRS culture broth without H_2O_2 supplement. Assay was carried out in triplicates and results given as mean±SD.

Statistical analysis

All the experiments were performed in three parallel replicates and the results are given as mean ± standard deviation of triplicate values.

RESULTS

Antimicrobial activity

The capacity of all three *Lact. gasseri* isolates to antagonise the growth of 17 pathogens was determined in the present study. Culture supernatants of all studied *Lact. gasseri* strains inhibited almost all indicator pathogens to varying levels, with inhibition zones ranging in size from 9-22 mm as presented in Fig. 1. Infant faeces isolated strain LBM220 exhibited strongest inhibition against *E.coli* ATCC-25922 with inhibition halo of 19 mm, followed by *Sh. flexneri* (17 mm), *Salm. typhimurium* and *Sh. sonnei* (16 mm), while minimum inhibitory zone was observed for *Staph. aureus* and *L. monocytogenes* (6 mm). While human milk isolated strains displayed superior antimicrobial activity with greater inhibitory zone sizes. Isolate LGS22 displayed maximum inhibition for *Sh. boydi* and *Sh. flexneri* (21 mm), followed by *Salm. typhimurium* and *Entero. faecalis* ATCC-51299 (18 mm). While strain MVS25 exhibited maximum inhibition against *Sh. sonnei* (22 mm), *Salm. typhi*(T) (19 mm), followed by *Sh. flexneri*, *Coryne diphtheria*, *Pr. vulgaris* and *Salm. typhimurium* (inhibitory zone size of 18 mm). Results obtained in the present study clearly elucidate the fact that *Lact. gasseri* strains from human origin are effective in inhibiting growth of human and food-borne pathogenic microbes.

Effect of multiple treatments on antimicrobial activity of CFCS of *Lact. gasseri* LBM220

On adjusting the CFCS of *Lact. gasseri* isolates to pH 6.5, both LBM220 and MVS25 showed dramatic reduction in antimicrobial activity, clearly suggesting that the antimicrobial ability was pH-dependent, while LGS22 showed significant inhibitory zones. Similarly, CFCS of both LBM220 and MVS25 isolates when tested for the production of hydrogen peroxide using catalase enzyme, resulted in no significant elimination of its antibacterial activity, while LGS22 displayed reduction in its inhibitory potential. This strongly

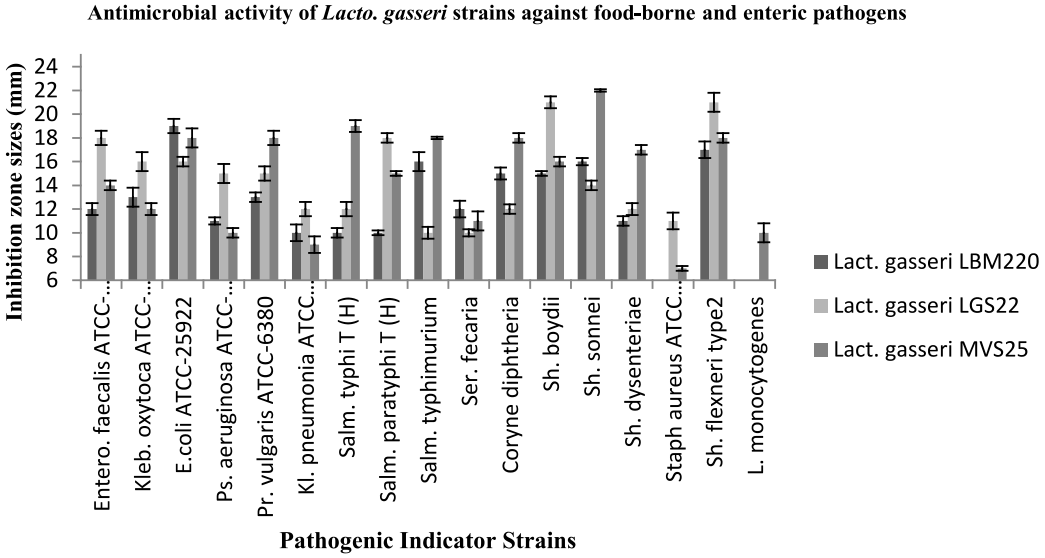

Fig. 1. Antimicrobial activity of *Lact. gasseri* strains against toxic food-borne and enteric pathogens
The antimicrobial activities of *Lact. gasseri* strains against seventeen enteric pathogens were evaluated and presented by measuring the diameters of growth inhibition zones around the well as mean ± SD from three replications.

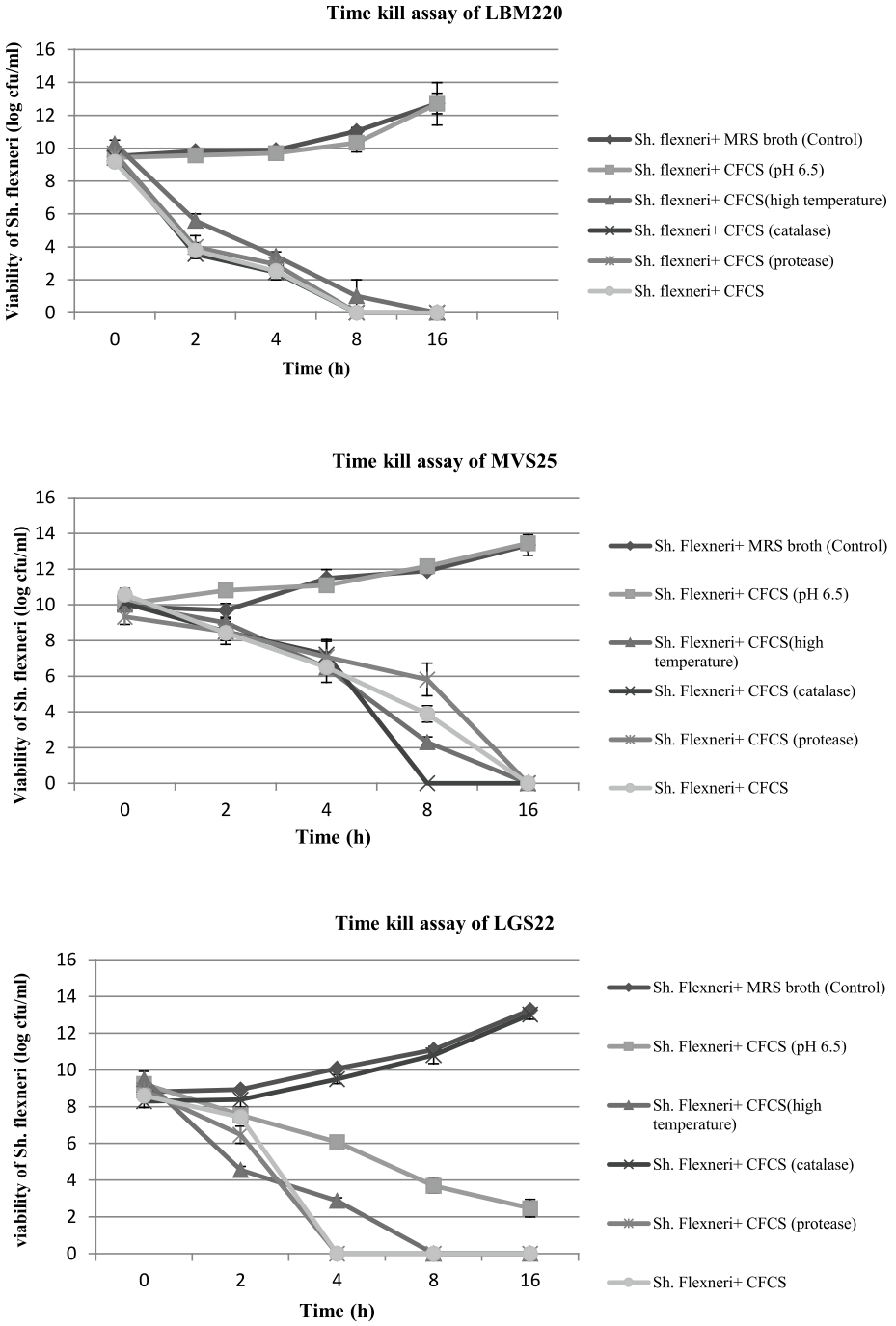

Fig. 2(A-C). Evaluation of all three *Lact. gasseri* strains (LBM220, MVS25 and LGS22) cell-free culture supernatant (CFCS) kill assay on pathogen *Sh. flexneri* by the contact time.
Sh. flexneri was incubated in untreated CFCS, CFCS neutralized to pH 6.5, CFCS protease treated, CFCS catalase treated, CFCS heat treated and sterile MRS broth (pH 6.5) as control. Aliquots were removed at 0h, 2h, 4h, 8h and 16h. Serially diluted and plated on BHI to determine colony counts in log cfu ml^{-1}. Each value is expressed as mean ± SD (n=3)

suggests that only LGS22 produce hydrogen peroxide as inhibitory metabolite. Further, when CFCSs of *Lact. gasseri* strains were treated with protease to determine whether their killing ability was due to bacteriocin and/or peptidal in nature, results showed no significant reduction in their antagonistic ability. Likewise,inhibitory activity of CFCSs of *Lact. gasseri* strains remained largely resistant to high temperature treatment.

Time-kill assay with CFCS of *Lact. gasseri* strains on indicator pathogen *Sh. flexneri* type 2

To further confirm the results obtained by agar-well diffusion method for *Lact. gasseri* isolates, time kill assay was also performed which demonstrated significant inhibitory activity on *Sh. flexneri* type 2 when co-cultured for 16 h. The viability of *Sh. flexneri* after contact with CFCS, CFCS (pH 6.5), CFCS (catalase), CFCS (protease) and MRS broth (control) was assessed for five consecutive time intervals (t= 0,2,4,8,16 h) and results displayed in Fig. 2. It was observed that after 2h, the survivability of indicator pathogen decreased drastically by almost 4-5 log cfu ml^{-1} in presence of *Lact.gasseri* CFCSs, clearly elucidating its strong kill effect. When *Lact.gasseri* CFCSs were neutralized to pH 6.5, negligible killing was recorded for both LBM220 and MVS25. Similarly, when CFCS was treated with catalase, only isolate LGS22 displayed no significant killing effect. However, after 16 h of incubation*, Sh. flexneri* was found to be non-viable in presence of CFCS treated with either protease or high temperature. Whereas the control, MRS broth (pH 6.5) displayed no viability loss.

Antioxidant activity

The capacity to scavenge free radicals is

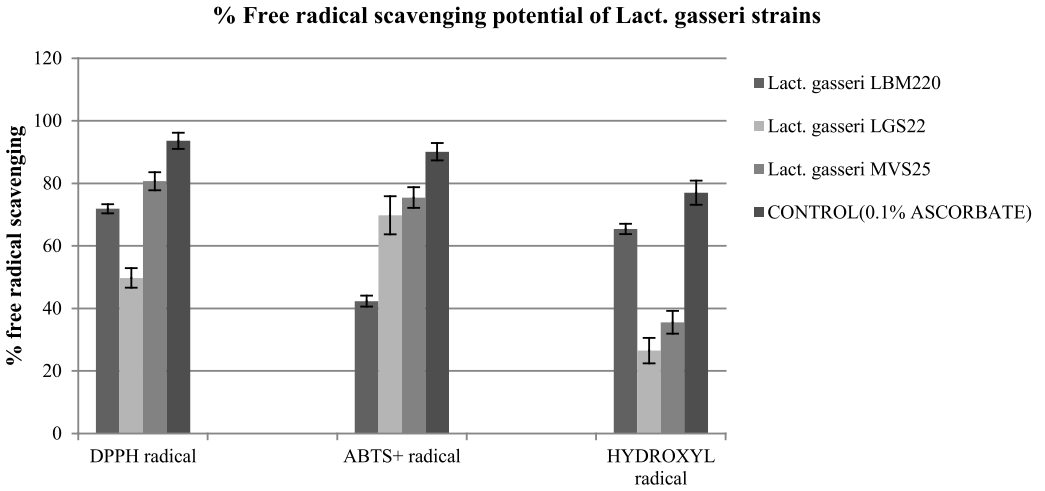

Fig. 3. Percentage free radicals scavenging potential of *Lact. gasseri* strains from infant faeces and human milk
Scavenging effect on hydroxyl radicals, DPPH free radicals and ABTS+ radicals by culture supernatant of Lact. gasseri strains incubated at 25°C . Each value is expressed as mean ± S.D. (n = 3)

Table 1. Resistance of *Lact. gasseri* strains at different hydrogen peroxide concentrations

Isolates	Optical density at 600 nm			
	0 mM H_2O_2 (Control)	0.4mM H_2O_2	0.8mM H_2O_2	1mM H_2O_2
Lact. gasseri LBM220	0.974±0.07	0.554±0.08	0.097±0.05	0.028±0.06
Lact. gasseri LGS22	1.022±0.04	0.945±0.07	0.086±0.02	0.045±0.04
Lact. gasseri MVS25	0.625±0.05	0.451±0.04	0.127±0.07	0.091±0.03

Growth of test strains were evaluated by recording optical density at a wavelength of 600 nm after 16 h anaerobic incubation at 37°C and results expressed as mean±SD (n=3)

another valuable functional attribute of studied *Lact. gasseri* strains suggestive of their antioxidant property. In present study, antioxidant activity of our cultures were determined by various assays in order to establish authenticity, such as scavenging of DPPH, $ABTS^{+}$ and hydroxyl radicals. In addition, the capacity to resist hydrogen peroxide was also studied. In presence of these free radicals, the culture supernatants of the studied strains displayed varying degree of scavenging potential, the results for which are presented in Fig. 3. For DPPH free radical, highest scavenging capacity was recorded for MVS25 (80.687%±2.89), followed by LBM220 (71.884%±1.46). Highest free radical scavenging for $ABTS^{+}$ was observed for MVS25 (75.474%±3.3); while LGS22 displayed (69.8%±6.1). Highest hydroxyl free radical scavenging was observed for LBM220 (65.432%±1.65). *Lact. gasseri* isolates when tested for resistance to 0.4 mM, 0.8 mM and 1mM hydrogen peroxide for 16 h, displayed significant viability at 0.4mM; while viability declined drastically with increase in hydrogen peroxide concentration as reflected from the optical density (OD) values presented in Table 1.

DISCUSSION

Nowadays, application of *Lactobacillus* strains from human origin with superior antimicrobial potential is gaining momentum in formulation of natural therapeutics. The CFCSs of *Lacto. gasseri* strains isolated from breast milk and infant faeces in the present study were tested for their antagonistic ability against wide range of Gram-negative and Gram-positive pathogens. The results of present study displayed significant strain-specific inhibitory activity of all three *Lact. gasseri* isolates against test pathogens employed in the current study. Among gram-positive test pathogens,*Staph. aureus* and *L.monocytogenes* displayed significant resistance towards CFCS of both LBM220 and LGS22. Similar to our findings, Jose et al. (2015)[23]also reported inability of *lactobacilli* culture supernatants in retarding the growth of Listeria species. In addition, Serrano-Nino et al., (2016)[24], also reported no inhibition of breast milk *lactobacilli* against *L. monocytogenes* and *Staph. aureus.* In the study, *Kleb. pneumonia* and *Ser. fecaria* were only moderately inhibited by all three strains. Interestingly, very few studies reported inhibitory activity of CFCS of human milk *lactobacilli* against gram negative *Ser. fecaria.* Similar to our results, CFCS of *Lact. casei* and *Lact. brevis* from breast milk failed to inhibit *Kleb. pneumonia;* while significantly inhibiting *Sal. typhi, Sh. flexneri* and *P. aeruginosa* (Sharma et al. 2017)[25]. The CFCSs of all three isolates strongly antagonise both *Sal. typhimurium* and *Sal. enterica* serovar *Typhi* in our study. Besides this, we also observed strong to moderate inhibitory activity of *Lact. gasseri* strains against *Pr. vulgaris* and *P. aeruginosa*, both of which are important opportunistic pathogens in immuno-compromised and hospitalized patients[26]. Gram positive test strains *Sh. flexneri*, *Coryn. diphtheria* and *Sh. boydii* also displayed significant sensitivity against CFCS of all three strains. However, the activity among strains is highly variable.

Breastfeeding is the copious source of *lactobacilli* species to the infant gut, which tends to ameliorate neonatal gastrointestinal microbial composition of *lactobacilli*. These species exert numerous biological functions, important among them is inhibition of toxic pathogenic microbes[27]. Although, the mechanism underlying antimicrobial activity by these species remain largely to be determined, probable among them is either competition for nutrients or epithelium adhesion, coaggregation of pathogens and/or production of inhibitory metabolites that include hydrogen peroxide, organic acids and bacteriocins that affect not only the toxic pathogen survivability, but may also affecttoxin production or bacterial metabolism[7]. In order to determine the inhibitory metabolite, CFCS was treated with catalase, pH adjustment, protease enzyme and high temperature. All three *Lacto. gasseri* culture supernatants varied in their antagonistic pattern on account of different treatments. Both *Lact. gasseri* LBM220 and *Lact. gasseri* MVS25 displayed sharp decline in antimicrobial activity when pH was adjusted to 6.5, while *Lact. gasseri* LGS22 showed disappearance of inhibitory activity on catalase treatment. These results strongly suggest the exocellular secretion of organic acids such as lactic acid and acetic acid of *Lact. gasseri* LBM220 and *Lact. gasseri* MVS25. Olivares et al., (2006)[28] also reported antimicrobial activity of *Lact. fermentum* CECT5716 owing to its production of high amount of lactic acid. Study of Tsai et al., (2019)[29], reported

antimicrobial ability of, *Lact. acidophilus RY2, Lact. salivarius MM1 and Lact. paracasei En4* isolated from infant faeces against enteropathogens. Apart from their role in effecting pathogen's metabolism and toxin production; secretion of organic acid metabolites leads to reduction in the pH of the micro environment, rendering obstruction to the growth of pathogens that are susceptible to acidic conditions. Also, organic acids tend to permeabilize the outer membrane of gram-negative microbes, thereby alleviating gastrointestinal infections[30]. *Lact. gasseri* LGS22 was found to secrete hydrogen peroxide as exocellular inhibitory metabolite. Further, time kill assay confirmed the results of agar well diffusion study. As no viability loss of *Sh. flexneri* was observed on pH adjustment in case of LBM220 and MVS25, same was recorded for LGS22 in presence of catalase treated CFCS. Results also indicate sharp decline in log cfu ml^{-1} values of *Sh. flexneri* after 16h co-incubation with CFCS (untreated) of all three strains. Taken together, results elucidate the effective role of *Lact. gasseri* strains from human origin in mitigation of gastrointestinal infections as they release antimicrobial metabolites, majorly organic acids and hydrogen peroxide that have broad antimicrobial spectrum.

The present research also strongly indicates the application of all three *Lact. gasseri* strains as natural antioxidant, as they significantly scavenged free radicals (80.6-26.5%). Thus, determining the possible role of these strains in preventing the progress of several degenerate disorders caused due to oxidative stress. Oxidative stress occurs owing to disturbance in antioxidant-prooxidant balance in the cell, resulting in increased intracellular levels of free radicals that cause protein denaturation, DNA hydroxylation, lipid peroxidation, and apoptosis. In presence of DPPH free radical, culture supernatant of *Lact. gasseri* MVS25 displayed highest scavenging ability of up to 80.69%. Our results are in concordance with previous research by Gao et al*.,* (2013)[31]. Whereas, Ding et al*.,* (2017)[32]have reported highest DPPH scavenging activity to be 39.3% among 23 *Lactobacilli* studied. Also, *Lact. delbrueckii subsp. bulgaricus* F17 displayed only 22.57% DPPH scavenging (Lee et al*.,* 2005)[33]. Among the reactive oxygen species, hydroxyl radicals are the most reactive radical that lead to oxidative damage of adjoining biomolecules. Induction of oxidative damage in biomolecules due to accumulation of hydrogen peroxide indirectly enhances the production of hydroxyl radicals through iron-catalyzed Fenton reactions, which can be prevented by antioxidant agents. Thus, elimination of hydroxyl radical is crucial. Our results demonstrated that *Lact. gasseri* LBM220 have significantly high hydroxyl scavenging ability of 65.4% as compared to previous reports on *Lact. plantarum* of 53% (Shi et al*.,* 2019)[20] and *Lact. delbrueckii subsp. bulgaricus* F17 of 59% (Lee et al*.,* 2005)[35]. Hydrogen peroxide, relatively feeble oxidant, is highly diffusive with extended lifetime, thereby contributing significantly to oxidative stress either directly or as hydroxyl radical precursor. Thus, resistance to hydrogen peroxide was studied for *Lact. gasseri* isolates, by recording the culture growth in presence of 0.4 mM, 0.8mM and 1.0mM H_2O_2 for 16h, our results displayed significant resistance in 0.4 mM H_2O_2 concentration. The results are consistent with previous reports by Shi et al*.,*(2019)[20]. So far, very few studies reported the contribution of *Lact. gasseri* strains specifically isolated from human milk in neutralising oxidative stress molecules.

CONCLUSION

Findings from the present study report that novel *Lactobacillus gasseri* strains isolated from breast milk and infant faeces may find application as valuable autochthonous antimicrobial and antioxidant natural agent in food and gut matrix. However this is a preliminary study and more detailed work with prudent validation of the cytotoxic effects in *in vivo* models is required.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

SR performed all the experiments in the laboratory and was a major contributor in writing the manuscript. VM provided the laboratory facility and analysed the results. AS conceptualised the study, analysed the results of the experiments and evaluated the manuscript. All authors have read and approved the final manuscript.

ETHICS STATEMENT

The study was approved by institutional ethical review board of Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow (Ref. No. 2784/RMLIMS/2018). Written informed consent was taken from each volunteer.

DATA AVAILABILITY

The datasets used and/or analysed during the current study are available from corresponding author on reasonable request.

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