Isolation, *In vitro* Antibacterial Activity, Bacterial Sensitivity and Plasmid Profile of Lactobacilli

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ABSTRACT : The present research work was conducted to evaluate the beneficial effects as well as the safety aspects of lactobacilli as probiotic. Lactobacilli were isolated from poultry faecal samples, feed samples and from some known preparations procured from poultry feed manufacturers. *L. acidophilus* and *L. sporogenes* were tested for the antibacterial activity against four poultry pathogens viz. *Escherichia coli*, *Salmonella spp.*, *Proteus spp.* and *Pseudomonas aeruginosa*. Cell free supernatant (CFS) of *L. acidophilus* exhibited significantly higher antibacterial activity against *Salmonella spp.* at original pH (4.50±0.02). At the adjusted pH (6.50±0.02) significantly higher antibacterial activity was recorded against indicator organism except for *P. aeruginosa*. Likewise, *L. sporogenes* exhibited similar antibacterial activity at original as well as adjusted pH except for *E. coli*. Antibacterial activity against *E. coli* was significantly higher at adjusted pH than at original pH of CFS. The competitive exclusion of *E. coli* by lactobacilli over the intestinal epithelial cells (IEC) was checked. *L. acidophilus* strain I, which was of poultry origin, exhibited maximum attachment over IEC as compared to other three strains of non-poultry origin viz. *L. acidophilus* strain II, *L. sporogenes* strain I and II. Overall, *L. acidophilus* exhibited higher competitive exclusion as compared to *L. sporogenes*. All the lactobacilli of poultry origin were most sensitive to penicillin G, amoxycillin, ampicillin and chloramphenicol, least sensitive to sulphamethizole, ciprofloxacin, neomycin, norfloxacin and pefloxacin and resistant to metronidazole and nalidixic acid. The isolates from probiotic preparations were most sensitive to ampicillin, amoxycillin and tetracycline, least sensitive to sulphamethizole, norfloxacin, neomycin and ceftriazone and resistant to nalidixic acid and metronidazole. Eight of the multiple drug resistant lactobacilli isolates were studied for the presence of plasmids. Plasmids could be extracted from six isolates of lactobacilli. These plasmids could be responsible for bacteriocin production or for antibiotic resistance of the strains. The lactobacilli need further studies regarding their safety for use in the probiotic preparations. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 9 : 1336-1342)***

Key Words : Lactobacilli, Competitive Exclusion, Antibacterial Activity, Plasmid

INTRODUCTION

Probiotics are a group of food and feed products that are also called as direct fed microbials (DFM). The concept that lactobacilli might be useful in displacing and replacing harmful microorganisms on mucosal surfaces was presented a century ago. The competition among bacteria for nutrients and spaces contributes to the microbial composition of the ecosystem (Lebenthal and Lebenthal, 2002). Competitive exclusion (CE) is a term that has been used to describe the protective effect of the natural or native bacterial flora of the intestine in limiting the colonization of some bacterial pathogens. *Lactobacillus spp*. are one of the important component group of intestinal microflora which could afford the host animal not only with specific or non specific immunopotentitating capacity but with an antagonistic activities against pathogeneic bacteria (Yoon and Won, 2002; Byun et al., 2004). Gao and Meng (2004) postulated that lectins present on the surface of *Lactobacillus spp*. play a key role for their colonization in the intestinal mucus of host animals. Competitive exclusion provides an opportunity for protecting poultry against pathogenic bacterial colonization (Jeffrey, 1999). Probiotic bacteria produce a variety of substances that are inhibitory to both Gram positive and Gram-negative bacteria. These include organic acids, hydrogen peroxide and bacteriocins. These compounds may reduce not only the number of viable pathogenic organisms, but may also affect bacterial metabolism and toxin production (Rolfe, 2000). Recently, the drug resistance has been reported with probiotic strains (Katla et al., 2001). Since probiotics are added to different kinds of food or feed and intentionally grown into high numbers, such bacteria possibly could represent a potential source for spread of drug resistance. The fact that these strains may take up and transfer antibiotic resistance genes, both vertically (i.e. to related strains) and horizontally (i.e. to non-related strains) has posed questions on whether elevated risk may arise from the association of these strains with food fermentations and their use as probiotics. The present study was aimed to study *in vitro* antibacterial activity, antibiogram and plasmid profile of lactobacillus strains isolated from poultry faecal and feed samples and probiotic preparations.

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MATERIALS AND METHODS

The feed and faeces of poultry birds were collected

from commercial poultry farms around Nagpur in sterilized polythene bags. The samples were collected from both layers and broilers.

Isolation of lactobacilli

The isolation of lactobacilli was done as per the method described by Ahn et al. (2002). The faecal samples were collected aseptically using sterile swabs. The swabs with faecal samples were put in acidified MRS broth containing 0.2% (w/v) sodium azide and were incubated at 37°C for 48 h in microaerophillic condition. Each sample culture was then streaked on MRS agar plates. Typical colonies grown on MRS agar plates were picked and checked for purity on the same medium. The identification of lactic acid bacterial isolates was made as per Bergey's manual (Kandler and Weiss, 1986). Similarly feed samples and probiotic preparations were also processed for isolation of lactobacillus.

***In vitro* antibacterial activity**

The antibacterial spectrum of cell free supernatant (CFS) of *L. acidophilus* and *L. sporogenes* was studied against poultry pathogens maintained in the department viz. *E. coli*, *Proteus spp., Salmonella spp.* and *P. aeruginosa* employing agar diffusion method. The antibacterial activity of the isolates was determined using CFS obtained from culture grown in MRS broth. Lactobacilli were grown in 50 ml MRS broth at 37°C for 48 h. Broth cultures of the lactobacilli were centrifuged at 10,000 rpm for 45 min. The CFS was collected aseptically and pH was recorded. The CFS was divided into two parts, one part was kept as such and the pH of the other part was adjusted to 6.5 with 1 N NaOH. The CFS was then sterilized by passing through a sterile membrane filter (0.45 µm). The sterilized porcelin beads were charged with CFS and were placed on the Muller and Hinton's plates seeded with indicator organism. Plates were then incubated at 37°C for 18 h without inversion. Formation of a clear zone of inhibition around the beads was taken as an evidence of antibacterial activity against the indicator microorganisms. The diameter of the zone of inhibition around the beads, if any, was measured in mm.

***In vitro* competitive exclusion**

Preparation of intestinal epithelium cells (IEC) : Suspension of IEC of chicken was prepared as per the method of Mayra- Makineh et al. (1983) with slight modifications. Briefly, the fresh poultry intestines were collected in chilled normal saline solution (NSS). The duodenum was cut into 1×4 cm pieces. The epithelium tissue samples were held in NSS at 4°C for 1 h to loosen the surface mucus and washed several times to remove the mucus. Surface of the epithelium tissues was scrapped off with the edge of a sterile microscope slide and the epithelial cells were suspended in NSS. The suspension was kept at 4°C for 30 min to let the mucus settle down. The supernatant containing epithelial cells was collected, suspended in NSS and was centrifuged at 7,000 rpm for 20 min. The washing was done three times to remove any remaining mucus. The epithelial cell pellet was resuspended in NSS. The live epithelial cells were counted by trypan blue dye exclusion test. The epithelial cell concentration was adjusted to 8.8×10^5 $ml^{-1.}$

Preparation of lactobacilli and E. coli : Each overnight culture broth of lactobacilli and *E. coli* cells was centrifuged at 6,000 rpm for 15 min at 4°C. Supernatant was discarded and the cell pellet was resuspended in NSS. The bacteria were then counted in RBC counting chamber of Neubaur's slide and both the bacterial cell suspensions were adjusted to 1.5×10^8 cfu ml^{-1}.

Adhesion of lactobacilli and *E. coli* to the IEC

To study adhesion of lactobacilli and *E. coli* over IEC principally method of Fuller (1978) was adopted. A 100 µl of each bacterial cell suspension was added separately to 400 µl of IEC suspension so as to get approximately ratio of 50:1. The tubes containing cell mixture were rotated at 20 rpm/min at 37°C for 30 min. Smears were prepared at 15 and 30 min of incubation. Adhesion of the bacterial cells to the IEC was examined using light microscopy (×1,500) after Gram staining.

Competitive exclusion of *E. coli* by lactobacilli

In vitro competitive exclusion of *E. coli* by lactobacilli over chicken IEC was studied. Suspension of both, *E. coli* and lactobacillus (100 µl, each) was mixed with 400 µl of epithelial cell suspension and the tubes containing the cell mixture were incubated at 37°C for 30 min and were rotated at 20 rpm/min. Smears were prepared after 15 and 30 min of incubation. Adhesion of the bacterial cells to the epithelium cells was examined using light microscopy (×1,500) after Gram's staining.

***In vitro* bacterial sensitivity of isolated strains of lactobacilli**

In vitro bacterial sensitivity of isolated strains of lactobacilli was studied by disc diffusion method (Bauer et al., 1966). The bacterial sensitivity was studied against the nineteen antibiotics procured from Hi-Media Ltd., Mumbai.

Plasmid profile of lactobacillus strains : Plasmid profile of the selected lactobacillus strains was done as per the method described by Frere (1994) with slight modifications. Briefly, *Lactobacillus* strains were harvested from 1.5 to 10 ml of culture (O.D. 660 nm from 1 to 1.4) by centrifugation, suspended in 300 µl of cold solution I (10 mmol L^{-1} EDTA,

Table 1. *In vitro* antibacterial activity of cell free supernatant of Lactobacilli against indicator organisms

Indicator organisms	Zone of inhibition (mm)			
	L. acidophilus		*L. sporogenes*	
	Original pH	Adjusted pH	Original pH	Adjusted pH
E. coli	$6.33^{a}\pm0.49$	$5.17^{b}\pm0.54$	$7.00^{B}\pm0.44$	$8.33^{A}\pm0.33$
Salmonella sp	$8.50^{bB}\pm0.49$	$6.32^{bA}\pm0.33$	5.57±0.56	6.50±0.49
Proteus sp	$6.66^{a}\pm0.49$	$6.17^{b}\pm 0.48$	7.00±0.44	6.17±0.40
P. aeruginaosa	$5.43^{aB}\pm0.21$	$1.20^{aA}\pm0.14$	7.50±0.56	6.33±0.62

Small letter exhibit significance column wise ($p<0.01$).
Capital letter exhibit significance row wise ($p<0.05$).

50 mmol L^{-1} Tris, pH 7.5, 100 µg L^{-1} RNase A) and transferred in a micro centrifuge tube and vortexed for 30 sec to 1 min on a Topmix vortex, at maximum speed. Three hundred µl of solution II (0.2 mol L^{-1} NaOH, 1% SDS) was added. After 5 min at room temperature, 300 µl of solution III (2.55 mol L^{-1} potassium acetate, pH 4.8) was added. The tube was then centrifuged at 10,000 g for 15 min. Supernatant was transferred to a second micro centrifuge tube and proteins were extracted with 600 µl of a mixture of 25:24:1 (v/v) phenol- chloroform- isoamylic alcohol (PCI). After gentle inversions, the mixture was centrifuged at room temperature for 5 min at 10,000 g. The aqueous phase above the white protein interface was collected into a microcentrifuge tube, and the plasmid DNA precipitated with one volume of isopropanol and pelleted by centrifugation at 4°C for 10 min at 15,000 g. The isopropanol was carefully removed and the DNA pellets were dried. Plasmid DNA was dissolved in 20 µl of 10 mmol L^{-1} Tris, 1 mmol L^{-1} EDTA pH 7.5 (TE) and examined by agarose submarine gel electrophoresis.

Statistical analysis

Averages and significant errors were calculated using standard statistical methods. Analysis of antibacterial activity was done by applying Student's t-test and CRD. However, statistical analysis for competitive exclusion was carried out by applying Nested design (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

A total of 58 faecal and 8 feed samples of poultry were processed for isolation of *Lactobacillus*. Fifty three isolates of lactobacilli were obtained from faecal samples and four from feed samples. Three each of *L. acidophilus* and *L. sporogenes* were isolated from known preparations used as probiotics in poultry feed. In the present study, the frequency of isolation of *L. gasseri* was highest (26.31%) from faecal samples followed by *L. fermentum* (12.28%) and *L. plantarum* (10.52%). *L. acidophilus* was recovered at moderate frequency (5.26%), whereas, *L. jensenii*, *L. leichmanni* and *L. casei* recovered least (1.75%) each. Earlier also Gilliland et al. (1975) and Ahn et al. (2002) reported isolation of *L. acidophilus* from faecal samples of chicken. Similarly, Jin et al. (1996a), Schneitz et al. (1993) and Miyamoto et al. (2000) isolated lactobacilli from the intestine, crop and cecum and cloaca of chickens, respectively.

Antibacterial activity of lactobacilli

The results indicate that *L. sporogenes* possesses significant antibacterial activity against the indicator organisms used in the study (Table 1). The antibacterial activity was statistically at par at both original as well as adjusted pH, except in case of *E. coli*, where the antibacterial activity was significantly lower (7.00±0.44 mm) at original pH in contrast to the observed at adjusted pH (8.33±0.33 mm). At original pH antibacterial activity of CFS of *L. acidophilus* was significantly higher against *Salmonella spp.* and *P. aeruginosa* in contrast to other indicator organisms (Table 1). However, at adjusted pH it was significantly higher against all indicator organisms except that of *P. aeruginosa*. Present results indicate that in addition to pH i.e. acid production, other factors are also contributing significantly towards antibacterial activity of lactobacilli. Prasad and Gandhi (1987), Ehrmann et al. (2002) recorded maximum antibacterial activity against various poultry pathogens at original pH (3.2) of CFS and decreased activity with an increase in pH. Similarly, the pH dependent antibacterial activity of milk culture filtrate of *L. acidophilus* was also reported (Jin et al., 1996b). Various factors contributing to antibacterial activity of CFS of lactobacilli have been postulated. pH independent antibacterial activity of *Lactobacillus spp.* observed against indicator organisms may be attributed to lactocidin production (Prasad and Gandhi, 1987; Ehrmann et al., 2002) and bacteriocin production as suggested by Surono (2003).

***In vitro* competitive exclusion (CE)**

The rate of attachment of two strains of *L. acidophilus* over IECs significantly increased with period of incubation (data not shown). Overall, *L. acidophilus* strain I of poultry origin exhibited significantly higher (7.02±0.18) attachment than that of non poultry origin strain II (4.37±0.16). Ahn et al. (2002) also confirmed the host specific adherence of the

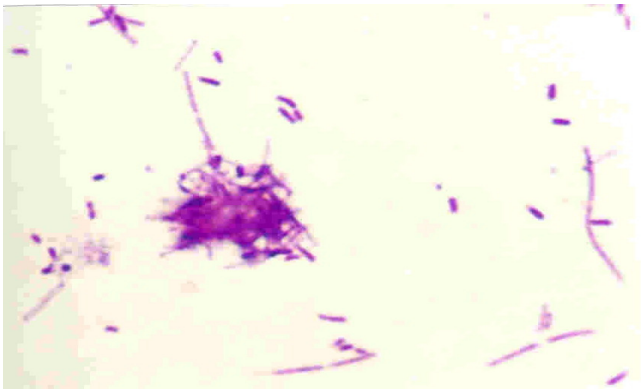

Figure 1. Adhesion of *L. sporogenes* strain I and *E. coli* to the intestinal epithelium cells of chicken.

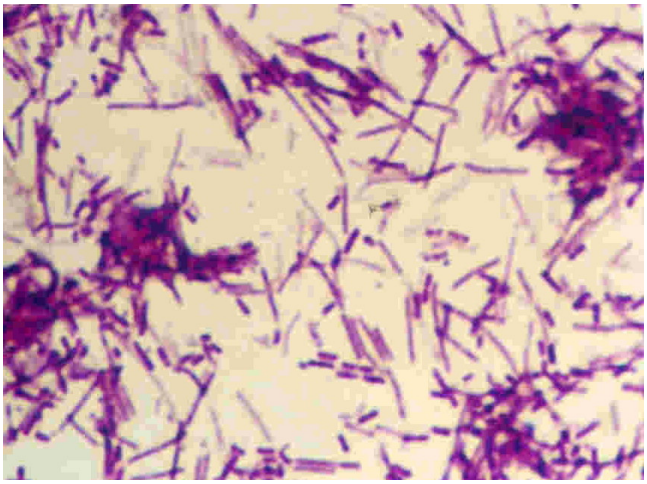

Figure 2. Adhesion of *L. sporogenes* strain II and *E. coli* to the intestinal epithelium cells of chicken.

lactobacilli. Overall, average rate of attachment for strain I and II of *L. sporogenes* was statistically at par (3.67±0.17 and 3.59±0.18). The average frequency of attachment of *E. coli* over IEC was recorded to the tune of 3.96±0.14 and 4.63±0.14 at 15 and 30 min of incubation, respectively.

The results of CE indicate non significant effect of period of incubation on ratio of attachment of lactobacilli and *E. coli* over IEC (data not shown). No significant difference was recorded between the CE of *E. coli* by both the strains of *L. acidophilus*. On the contrary, *L. sporogenes* strain II exhibited significantly higher CE in comparison to strain I (Figures 1 and 2). In general CE of *E. coli* by *L. acidophilus* (1:0.97±0.06) was significantly higher than that of *L. sporogenes* (1:1.24±0.11). The present findings are in accordance with those of Todorikil et al. (2001), Ahn et al. (2002) and Bae et al. (2003) who recorded that lactobacilli possess antiadhesion activity against *E. coli* and *Salmonella* species. Similar results were reported by Weinack et al. (1981) and Hakkinen and Schneitz (1999) in the experiments conducted *in vivo*. They found lactobacilli significantly protect chickens challenged with *Salmonella spp.* and *Clostridium jejuni*. Muralidhara et al. (1977) also reported competitive attachment between lactic acid bacteria and coliforms in the intestinal tract of broilers.

Studies on bacterial sensitivity of lactobacilli isolates

The safety of probiotic cultures must be carefully evaluated. One safety aspect is the transfer or acquisition of antibiotic resistance. Because non-pathogenic enteric bacteria may also be a source for resistance genes that can spread to potentially pathogens, hence, surveillance activities should include non-pathogenic as well as

Table 2. Bacterial sensitivity test lactobacilli isolated from poultry feed and faeces

Antibiotic (concentration mcg/disc)	Zone of inhibition (mm) (sensitivity zone)			Total number of isolates (percentage)	
	Minimum	Maximum	Average	Sensitive isolates	Resistant isolates
Ampicillin (10)	11	29	17.96	55 (96.49)	2 (3.5)
Amoxycillin (10)	13	32	22.14	56 (98.24)	1 (1.75)
Chloramphenicol (30)	13	37	22.74	54 (94.74)	3 (5.26)
Ciprofloxacin (30)	27	30	28.5	2 (3.5)	55 (96.49)
Ceftriaxone (30)	14	27	19	26 (45.61)	31 (54.38)
Cefalexin (30)	11	38	15.95	43 (75.44)	14 (24.56)
Cloxacillin (10)	10	33	17.88	36 (63.16)	21 (36.84)
Furazolidone (50)	13	31	21.16	6 (10.53)	51 (89.47)
Gentamicin (50)	13	21	12.72	22 (38.59)	35 (61.40)
Metronidazole (5)	-	-	-	0	57 (100)
Neomycin (30)	13	15	14	2 (3.5)	55 (96.49)
Nalidixic acid (30)	-	-	-	0	57 (100)
Norfloxacin (10)	15	15	15	2 (3.5)	55 (96.49)
Penicillin-G (10)	15	40	25.64	56 (98.24)	1 (1.75)
Pefloxacin (5)	20	23	21.5	2 (3.5)	55 (96.49)
Streptomycin (10)	12	20	13.7	10 (17.54)	47 (82.45)
Sulphamethizole (10)	13	13	13	1 (1.75)	56 (98.24)
Tetracycline (10)	16	40	26.6	5 (8.77)	52 (91.22)
Trimethoprim (30)	13	31	19.14	8 (14.04)	49 (85.96)

Table 3. Bacterial sensitivity test of lactobacilli isolated from known lactobacilli preparations

Antibiotic (concentration, mcg/disc)	Zone of inhibition (mm) (sensitivity zone)			Total number of isolates (percentage)	
	Minimum	Maximum	Average	Sensitive isolates	Resistant isolates
Ampicillin (10)	11	25	16.66	6 (100)	0
Amoxycillin (10)	15	28	21.33	6 (100)	0
Chloramphenicol (30)	15	18	16.25	4 (66.66)	2 (33.33)
Ciprofloxacin (30)	16	23	19.00	4 (66.66)	2 (33.33)
Ceftriaxone (30)	21	27	24.00	2 (33.33)	4 (66.66)
Cefalexin (30)	15	16	15.66	3 (50.00)	3 (50.00)
Cloxacillin (10)	17	20	19.00	4 (66.66)	2 (33.33)
Furazolidone (50)	14	27	21.25	4 (66.66)	2 (33.33)
Gentamicin (50)	12	20	15.40	5 (83.33)	1 (16.66)
Metronidazole (5)	-	-	-	0	6 (100)
Neomycin (30)	13	14	13.50	2 (33.33)	4 (66.66)
Nalidixic acid (30)	-	-	-	0	6 (100)
Norfloxacin (10)	16	18	17.00	2 (33.33)	4 (66.66)
Penicillin-G (10)	24	32	27.60	5 (83.33)	1 (16.66)
Pefloxacin (5)	19	24	21.00	3 (50.00)	3 (50.00)
Streptomycin (10)	16	18	16.66	3 (50.00)	3 (50.00)
Sulphamethizole (10)	12	12	12.00	1 (16.66)	5 (83.33)
Tetracycline (10)	15	21	17.00	6 (100)	0
Trimethoprim (30)	16	28	21.60	5 (83.33)	1 (16.66)

pathogenic bacteria (Katla et al., 2001). So far no much information is available as far as antibiogram of lactobacilli used as probiotics are concerned. Hence present investigation was undertaken and results are presented in Table 2. In the present study, all the isolates of lactobacilli recovered from poultry faeces and feed were most sensitive to penicillin G and amoxycillin (98.24%, each), which is in accordance with the findings of Charteris et al. (1998) and Bae et al. (2002). As shown in Table 3, lactobacilli isolates from probiotic preparation were highly (100%) sensitive to ampicillin, amoxycillin and tetracycline followed by gentamicin, penicillin, and trimethoprim (83.33% each). All the isolates recovered from poultry faeces, feed and probiotic preperation exhibited absolute resistance to metronidazole and nalidixic acid (Table 2). Strains of lactobacilli used as probiotics also showed multiple antibiotic resistance, therefore, it is important to select strains without any highly transferable antibiotic resistances or special virulence mechanisms (Klein et al., 2000).

Plasmid profile

Attempts were made to study the plasmid profile of lactobacilli isolates exhibiting multiple drug resistance. *L. acidophilus* (3) and *L. sporogenes* (1) strains isolated from probiotic preparations and *L. acidophilus* (1), *L. gasseri* (1) and *L. casei* (2) strains isolated from poultry faeces and feed were investigated from plasmid profile. Out of eight strains, plasmid could be extracted from six isolates. Earlier, Clewell et al. (1974) introduced $pAMB_1$ a broad- host-range conjugative plasmid conferring macrolide resistance into lactobacilli. Later on, conjugal transfer of a drug resistance plasmid into *L. casei* has been documented (Gibson et al., 1979). Successful conjugal transfer of this plasmid into *L. casei* (Gibson et al., 1979), *L. reuteri* (Vescovo et al., 1983; Luchansky et al., 1989) and *Lactobacillus spp.* (Romero and McKay, 1985) has also been described. Other workers have shown that lactobacilli can act as donors in inter and intergeneric mating on solid media (Cocconcelli et al., 1985; Tannock, 1987; Luchansky et al., 1989) and in the intestinal tract of gnotoxenic mice (Morelli et al., 1988).

From the present study we can conclude that lactobacilli are present in good numbers in the poultry faeces. *L. acidophilus* and *L. sporogenes* exhibit a significant antibacterial activity against poultry pathogens. The antibacterial activity of CFS of *L acidophilus* was pH independent except for *Salmonella spp.* and *P. aeruginosa* where as that of *L. sporogenes* for *E. coli*. *L. acidophilus* of poultry origin exhibited maximum competitive exclusion against *E. coli* as compared to *L. sporogenes*. The lactobacilli from poultry origin as well as those used as probiotic in poultry feed are highly resistant to commonly used antibiotics. These multiple drug resistant lactobacilli were found to harbor plasmid. Transfer the antibiotic resistance to related and non-related species, vertically and horizontally may occur due to such multiple drug resistant lactobacilli. To examine this aspect of lactobacilli used as probiotic further studies are recommended.

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