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ENZYMATIC ACTIVITY AND ANTIBIOTIC RESISTANCE PROFILE OF *LACTOBACILLUS PARACASEI SSP. PARACASEI-1* ISOLATED FROM REGIONAL YOGURTS OF BANGLADESH

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ABSTRACT

Lactobacillus paracasei ssp. *paracasei-1* was identified from traditional yogurts of Khulna region, Bangladesh and its enzyme and antibiotic resistance profiles were determined. A commercially available API Zym kit was employed to determine the activities of 19 different enzymes. We found that *L. paracasei* ssp. *paracasei-1* showed strong activities for several enzymes, viz. leucine arylamidase, valine arylamidase, napthol-AS-BI-phosphohydrolase, β-galactosidase, α –Glucosidase, N-Acetyl- β- glucosaminidase while activities for other enzymes were absent. Antibiotic resistance profile was assessed by minimum inhibitory concentration (MIC) test for 61 major antibiotics and 4 antifungal agents obtained from commercial sources in MRS Agar media. The strain generally showed resistance to gram negative spectrum antibiotic while it showed susceptibility towards β-lactam antibiotic to gram positive spectrum antibiotic. The findings provide the therapeutic basis of using *L. paracasei* ssp. *paracasei-1* in finished food products.

Keywords: *Lactobacillus*, antibiotic resistance, enzyme profile, yogurt, MIC, 16s-23s rRNA

INTRODUCTION

Lactic acid bacteria (LAB) are widely distributed in nature comprising a wide range of genera and include a considerable number of species. These LABs are united by a constellation of morphological, metabolic and physiological traits. They are gram positive bacteria and their common characteristics are usually catalase negative, growth under microaerophilic to strictly anaerobic conditions and lactic acid production. These bacteria serve as the major component of starter culture in fermentation, especially for dairy products, and some of them occur naturally as a part of gastrointestinal microflora. *Lactobacillus* is one of the most important and well-studied genera of LAB in this regard. In raw milk and dairy products such as cheeses, yoghurts and fermented milks, lactobacilli are naturally present or added intentionally, for technological reasons **(Oskar *et al.* 2004)**. These bacteria are able to withstand food processing and can be found in finished products. Therefore, they constitute a large portion of the natural microflora in many fermented dairy and meat products. Moreover, due to the probiotic properties, lactobacilli are added to human or animal foodstuffs to exert beneficial effects **(Aguirre and Collins, 1993; Gasser, 1994; Gardiner *et al.*, 1998; Adams, 1999; Mannu *et al.*, 2000; Ouwehand *et al.*, 2002)**. They are also reported to show resistance to antibiotics. These resistance attributes are often fundamental to their survival and chromosomally encoded **(Curragh and Collins, 1992; Adams and Marteau, 1995; Charteris *et al.*, 1998; Salminen *et al.*, 1998)**. Furthermore, some lactobacilli are reported to transfer antibiotic resistance genes in plasmid-encoded mechanisms, as found in certain *L. fermentum*, *Lactobacillus plantarum* and *L. reuteri* strains **(Ishiwa and Iwata, 1980; Ahn *et al.*, 1992; Tannock *et al.*, 1994; Fons *et al.*, 1997)**. This has given rise to a concern that the use of microorganisms in food and feed supplements could serve as a basis for transmission of antibiotic resistance genes **(EFSA, 2008; Teuber *et al.*, 1999)**. Again, it was reported that the *Lactobacillus* genomes showed a considerable degree of auxotrophy for amino acids and/or other cellular building blocks mediated by a large array of transport functions **(Kleerebezem *et al.*, 2010)**. The genomes of different isolates were characterized by the functional groups representing their niche adaptation. This could be attributed to evolution of different enzyme activities for utlization of various carbohydrates, largely adapted to grow on lactose. It has been found that lactobacilli which are associated with intestional niche generally show a large capacity for sugar internalization and utilization throgh simple and complex enzyme mediated numerous sugar-uptake systems**(Kleerebezem and Vaughan, 2009)**. In addition, specific intestional adaptation is also apparent through the enrichment of mucus binding cell surface proteins and enzyme complexes that are predicted to be involved in carbohydrate degradation **(Boekhorst *et al.*, 2006 a, b; Siezen *et al.*, 2006, Sarah *et al.*, 2008)**. However, insights into biochemical characteristics of lactobacilli could be drawn from enzyme systems through their substrate specificities in given ecological niches. This could act as a complementary tool to molecular and biochemical approaches in identifying lactobacilli, aid in phylogenetic distribution. This could further increase its utilization in dairy industries and enhance the understanding on the influence of lactobacilli on bacterial metabolism and gut function **(Kleerebezem and Vaughan, 2009; Ling *et al.*, 1994)**. This will ultimately provide better understanding their roles during the fermentation process as well as facilitate in identifying particular properties that may be relevant to use of the organisms in starter cultures in an industrial set-up.

In the present study, efforts have been undertaken to construct profiles of antibiotic resistance along with enzyme activity of *L. paracasei ssp. paracasei-1*. This isolate was previously isolated from a traditional yogurt from Bangladesh **(Hoque *et al.*, 2010)** and subsequently identified as *L. paracasei ssp. paracasei-1* using exclusive biochemical studies including sugar fermentation test (**Islam *et al.*, 2012**). Additionally, a molecular technique utilizing PCR amplification of 16s-23s intergenic spacer regions with specific primers was employed to ascertain the identity of *L. paracasei* ssp. *paracasei-1*.

MATERIAL AND METHODS

Maintenance and culture of microorganisms

The cultures of LAB were maintained at 4°C in MRS broth. The *Lactobacillus* isolate was obtained from Biochemistry and Molecular Biology Laboratory of Biotechnology & Genetic Engineering Discipline, Khulna University, Bangladesh. This *Lactobacillus spp*. was previously isolated from a traditional yogurt of Bangladesh. Media preparation, cultivation of these bacteria and their subsequent storage were carried out in the similar method as described by **Islam *et al.* (2012)** and **Hoque *et al.* (2010)**.

Identification of *L. paracasei* ssp. *paracasei-1*

Preliminary identification of the isolate based on morhological and physiological characteristics was carried out as described by **Hoque *et al.* (2010)**. Subsequently, they were subjected to identification depending on biochemical

parameters and designated as *L. paracasei* ssp. *paracasei-1* (**Islam *et al.*, 2012**). Furthermore, for molecular identification of this isolate was conducted using amplification of 16s-23s rRNA gene spacer regions in this study. For this purpose, two pair of primers (Lp1F: sequences **5'->3'**GGGGATCACCCTCAAGCACCCT and Lp1R: sequences **5'->3'** GCGTCAGCGGTTATGCGATGC) and (Lp9F: sequences **5'->3'** TCTGACGGAAACCTGCACACACG and Lp9R: sequences **5'->3'** CTTGCGTCAGCGGTTATGCGA) specific for the 16s-23s spacer region of *L.paracasei* were selected. Primers were designed by using the primer tool from GenBank. The GenBank accession numbers of 16S-23s spacer sequences is U32964 for *L. paracasei* (**Tisala *et al.*, 1996**). Chromosomal DNA of *L. paracasei* was isolated by using the protocol described by **Andrea *et al.*, (2001)**. The PCR reactions were carried out in a thermocycler (Eppendorf AG, Hamburg, Germany) with a final volume of 10 µl composed of 2.7µl dH_2O, 2µl template DNA, 1µl forward primer, 1µl reverse primer, 1µl dNTPS (250µM), 1µl reaction buffer (10x), 0.3µl $MgCl_2$ (1.5 mM), 1µl Taq DNA polymerase (2U). The reagents for PCR amplification were purchased from Bioneer Corp., USA unless otherwise stated.

The amplification cycle was consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 57°C for 1 min, elongation or extension at 72°C for 2 min, after the end of 30 cycles a final elongation at 72°C for 5 min. Then the reaction mixture was kept at 4°C for 24 h. The amplified products were analyzed at 2% agarose gel stained in 2.5% ethidium bromide, visualized and documented.

Enzyme activity

L. paracasei ssp. *paracasei-1* was assayed for 19 different enzymes in accordance to manufacturer's protocol (API Zym kit, BioMe´rieux, France). Briefly, the cells from a single colony grown on MRS agar medium were suspended in API suspension medium to a density of 5 McFarland. Sixty-five µl of the sample was added to each cupule and the test strips were incubated for 4 h at 37° C. Following incubation, 1 drop of ZYM A (API; tris-hydroxymethyl-aminomethane, hydrochloric acid, sodium lauryl sulphate, H_2O) and ZYM B (API; fast blue BB, 2-methoxyethanol) were added to each cupule. The gallery was kept in the dark for 5 minutes and then exposed under a 500-watt lamp for 20 seconds to prevent non-specific yellowing of the coloring reagent. The reactions were then graded 0-5, depending on the intensity of color compared with representations on a color chart.

Determination of antibiotic resistance profile by minimum inhibitory concentration (MIC) test

MIC profiles of *L. paracasei* ssp. *paracasei-1* were determined against 61 antibiotics and 4 antifungal agents according to manufacturer's instructions (HiMedia, India). Briefly, the 0.5 McFarland standard equivalent bacterial cultures were inoculated by spread plate technique in MRS agar media. MIC combs (Hi-Media, India) were spotted over the inoculums and subsequently incubated for 16 h at 37° C. The results with the lowest concentration of inhibition were listed for representation and interpreted as susceptible, intermediate and resistant according to standard specifications of CLSI, designed for antibiotics testings **(CLSI, 2012)**.

RESULTS AND DISCUSSION

The isolate was identified as *L. paracasei* ssp. *paracasei-1*

MRS agar media facilitated the isolation of LAB from other bacteria which was in good agreement with other findings related to isolation of *lactobacillus* from milk and milk products (**Hoque *et al.*, 2010**, **Coeuret *et al.*, 2003**). The isolates were identified as *Lactobacillus* based on morphological, physiological and biochemical attributes. They formed small, circular, white-creamy colored colonies; were gram positive, rod shaped non-motile, catalase negative and non-spore forming. Apart from that, they were able to coagulate milk and tolerate a number of inhibitory substances in medium such as NaCl and phenol in varying concentrations. The results from sugar fermentation patterns denoted the isolated *Lactobacillus spp.* as *L. paracasei* ssp. *paracasei-1* with 95% confidence which was similar to the findings reported by **Islam *et al.*, 2012.** The selective amplification of 16s-23s rRNA intergenic region using two specific primer pairs (Lp1F and Lp1R) and *(*Lp9F and Lp9R), as shown in Figure 1 ascertained the molecular identification of this *L. paracasei* ssp. *paracasei-1*. Agarose-gel electrophoresis revealed that PCR amplified products generated bands of 146 and 190 bp lengths with two different primer pair. These lengths are in exact concordance to *L. paracasei* ssp.

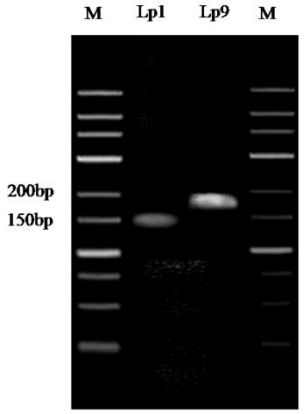

Figure 1 PCR amplification of 16s-23s internal spacer region of *Lactobacillus* isolate using species-specific primers. M: GeneRuler™ Low Range Ladder (MBI Fermentas); Lp1: PCR amplification product (146bp) using primer pair Lp1F and Lp1R; Lp9: PCR amplification product (190 bp) using primer pair Lp9F and Lp9R.

Most of the enzymes activities of *L. paracasei* ssp. *paracasei-1* were observed related with nutrient absorption and metabolism

The results of enzymatic activity of *Lactobacillus paracasei* ssp. *paracasei-1* are shown in Table 1. It possessed strong activity for leucine arylamidase, valine arylamidase, napthol-AS-BI-phosphohydrolase, β-galactosidase, α –Glucosidase, N-Acetyl- β- glucosaminidase (mean activity 4-5) while activity of other ezymes were absent in this isolate. Among them, leucine and valine arylamidase as well as β-galactosidase are related to nutrient absorption and metabolism. High level of β-glucuromidase in clinically pathogenic strains like *E. Coli* and *Clostridium* has been reported (**Gadelle *et al.*, 1985; Hawksworth *et al.*, 1971**). No harmful enzyme activity such as β-glucuronidase was observed in the enzyme activity profile of *L. paracasei* ssp. *paracasei-1*.

LAB with low β-glucuronidase is considered to inhibit the growth of β-glucuronidase-positive pathogenic or harmful bacteria in the gut by formation of antimicrobial substances or by competition with other microbes for adhesion sites and nutrients (**Silva *et al.*, 1987**; **Islam and Haziyamin, 2012**). Thus the isolate *L. paracasei* ssp. *paracasei-1* may have therapeutic potential in the intestinal environment when used in dairy products because it may produce less toxic compounds released by β-glucuronidase reaction on benign substrates in the colon.

Table 1 Assays for enzyme activity in *Lactobacillus paracasei* ssp. *paracasei-1*

No.	Enzyme Assayed For	*Lactobacillus paracasei* ssp. *paracasei-1*
1	Control	(0)
2	Alkaline phosphatase	- (0)
3	Esterase (C4)	- (2)
4	Esterase lipase	- (2)
5	Lipase	- (1)
6	Leucine arylamidase	+ (5)
7	Valine arylamidase	+ (5)
8	Cystine arylamidase	- (2)
9	Trypsin	- (1)
10	α-Chymotrypsin	- (1)
11	Acid phosphatase	- (2)
12	Napthol-AS-BI-phosphohydrolase	+ (4)
13	α –Galactosidase	- (0)
14	β-Galactosidase	+ (5)
15	β –Glucuronidase	- (0)
16	α –Glucosidase	+ (4)
17	β –Glucosidase	- (1)
18	N-Acetyl- β- glucosaminidase	+ (5)
19	α –Mannosidase	- (0)
20	α –Fucosidase	- (1)

Enzyme activities were measured in a 0-5 scale, where 0 represents no activity and 5 represents maximum activity respectively.

Antibiotic resistance profiles of *L. paracasei* ssp. *paracasei-1*

The results of MIC test against 61 antibiotics and 4 antifungal agents are listed in Table 2. *Lactobacillus paracasei* ssp. *paracasei-1* was resistant against Amikacin, Cefepime, Fusidic Acid, Gentamicin, Kanamycin, Lomefloxacin, Nalidixic Acid, Neomycin, Nitrofurantoin, Polymixin B, Streptomycin, Sulphadiazine, Sulphafurazole, Sulphamethizole, Sulphamethoxypyridazine, Sulphaphenazole, Tobramycin, Vancomycin and all four antifungal agents. The

isolate was suceptible to β-lactam antibiotics such as penicillin or ampicillin, to gram-positive antibiotic such as erythromycin and to braod spectrum antibiotic such as chloramphenicol or tetracyclin. On the other hand, *L. paracasei* ssp. *paracasei-1* was resistant to gram negative spectrum antibiotic nalidixic acid, aminoglycoside antibiotic kanamycin and streptomycin and cephalosporin antibiotic cefepime. The resistnace of gram-negative spectrum antibiotic nalidixic acid seems to be a common feature in lactobacillus strains (**Lara-villoslada *et al.*, 2007; Zhou *et al.*, 2005**). Resistance against aminoglycosides such as neomycin, kanamycin, streptomycin and gentamicin has been observed frequently among lactobacilli (**Coppola *et al.*, 2005; Danielson and Wind, 2003; Zhou *et al.*, 2005**). In addition, resistance against vancomycin, a glycopeptide antibiotic was observed which was agreement with previous findings that certain species of *Lactobacillus* genus were inherently resistant (**Lara-villoslada *et al.*, 2007**).

Study on the antibiotic resistance of LAB has seen a dramatic rise in the last decade due to three key reasons. Firstly, there is the possibility of exchange of resistance factors with other microorganisms, particularly the pathogenic ones. Secondly *Lactobacilli* have been reported as etiological agents, albeit in few cases, of endocaditis that can be controlled by antibiotic therapy. Lastly, the use of *Lactobacilli* as probiotic supplement in cases of gastrointestinal disorders depend on the knowledge of their antibiotic resistance to reinforce the concomitant antibiotic therapy **(Lee and Wong, 1998)**. Our findings provide a basal prediction on the behaviour of this isolate against antibiotic therapies. Additional questions could arise whether such microbial culture should be deliberately released in the food chain, although intra-and interspecies transfer of resistance properties need to be proven for this isolate.

Table 2 Antibiotic resistance profile of *L. paracasei* ssp. *paracasei-1*

Antibiotic Name	Range (µg)	MIC value	Antibiotic Name	Range (µg)	MIC value
Amikacin	A: 256 - 0.1 B: 4 - 0.001	256	o-trimoxazole	A: 240 - 0.01 B: 4-0.001	60
Amoxycillin	A: 240 - 0.01 B: 4 - 0.001	0.5	Erythromycin	A: 240 - 0.01 B: 4 - 0.001	0.1
Amoxyclav	A: 240 - 0.01 B: 4 - 0.001	5	Fusidic Acid	A: 240 - 0.01 B: 30 - 0.001	240
Ampicillin	A: 256 - 2 B: 2.048- 0.016	0.256	Gatifloxacin	A: 64 - 0.01 B: 2 - 0.001	4
Azithromycin	A: 128 - 0.01 B: 2 - 0.0001	0.1	Gentamicin	A: 240 - 0.01 B: 5 - 0.001	128
Azlocillin	A: 240 - 0.01 B: 16 - 0.001	0.01	Kanamycin	A: 240 - 0.01 B: 30 - 0.001	240
Aztreonam	A: 240 - 0.01 B: 2 - 0.0001	5	Levofloxacin	A: 240 - 0.01 B: 5 - 0.005	2
Benzyl Penicillin	A: 256 - 2 B: 2.048-0.016	8	Lincomycin	A: 240 - 0.01 B: 30 - 0.001	3
Carbenicillin	A: 512 - 0.01 B: 32 - 0.01	4	Linezolid	A: 240 - 0.01 B: 8 - 0.001	0.5
Cefalexin	A: 240-0.01 B: 30-0.001	1	Lomefloxacin	A: 240 - 0.01 B: 4 - 0.001	120
Cefazolin	A: 240 - 0.01 B: 4 - 0.001	1	Methicillin	A: 240 - 0.01 B: 4 - 0.001	0.5
Cefdinir	A: 240 - 0.01 B: 4 - 0.001	10	Minocycline	A: 240 - 0.01 B: 4 - 0.001	2
Cefepime	A: 240 - 0.01 B: 4 - 0.001	256	Mupirocin	A: 240 - 0.01 B: 30 - 0.001	30
Cefotaxime	A: 240 - 0.01 B: 30 - 0.001	8	Nalidixic Acid	A: 240 - 0.01 B: 8 - 0.001	240
Cefpirome	A: 240 - 0.01 B: 30 - 0.001	30	Neomycin	A: 240 - 0.01 B: 30 - 0.001	120
Ceftazidime	A: 240 - 0.01 B: 30 - 0.001	7.5	Nitrofurantoin	A: 240 - 0.01 B: 30 - 0.001	60
Ceftriaxone	A: 240 - 0.01 B: 30 - 0.001	7.5	Norfloxacin	A: 240 - 0.01 B: 8 - 0.001	4
Chloramphenicol	A: 240 - 0.01 B: 8 - 0.001	0.5	Ofloxacin	A: 64 - 0.01 B: 8 - 0.001	32
Ciprofloxacin	A: 240 - 0.01 B: 2 - 0.001	2	Oxacillin	A: 256 - 2 B: 2.048-0.016	2.048
Clarithromycin	A: 240 - 0.01 B: 16 - 0.001	0.1	Pefloxacin	A: 240 - 0.01 B: 30 - 0.001	60
Clindamycin	A: 240 - 0.01 B: 8 - 0.001	0.1	Piperacillin/Tazobactum	A: 240 - 0.01 B: 30 - 0.001	5
Colistin	A: 240 - 0.01 B: 30 - 0.001	-	Polymixin B	A: 240 - 0.01 B: 32 - 0.001	240
Pristinomycin	A: 240 - 0.01 B: 30 - 0.001	0.1	Sulphamethoxypyridazine	A: 240 - 0.01 B: 30 - 0.001	240
Rifampicin	A: 240 - 0.01 B: 32 - 0.001	1	Sulphaphenazole	A: 240 - 0.01 B: 30 - 0.001	240
Roxithromycin	A: 240 - 0.01 B: 30 - 0.001	0.1	Teicoplanin	A: 240 - 0.01 B: 1 - 0.001	5
Sparfloxacin	A: 64 - 0.01 B: 2 - 0.001	0.5	Tetracycline	A: 240 - 0.01 B: 5 - 0.01	1
Streptomycin	A: 240 - 0.01 B: 30 - 0.001	120	Ticarcillin	A: 240 - 0.01 B: 16 - 0.001	0.1
Sulfasomidine	A: 240 - 0.01 B: 30 - 0.001	-	Tobramycin	A: 240 - 0.01 B: 16 - 0.001	240
Sulphadiazine	A: 240 - 0.01 B: 30 - 0.001	240	Trimethoprim	A: 240 - 0.01 B: 32 - 0.001	10
Sulphafurazole	A: 240 - 0.01 B: 30 - 0.001	240	Vancomycin	A: 240 - 0.01 B: 4 - 0.001	128
Sulphamethizole	A: 240 - 0.01	240			

	B: 30 - 0.001			
Antifungal Agents				
Amphotericin B	A: 32 - 0.25 B: 0.256-0.002	32	Ketoconazole	A: 32 - 0.25 B: 0.256 - 32 0.002
Fluconazole	A: 256 - 2 B: 2.048 -0.016	256	Itraconazole	A: 32 - 0.25 B: 0.256 - 32 0.002

S= susceptible (MIC ≤ 4 µg/ml), I= intermediate (MIC= 8-32 µg/ml), R= Resistant (MIC≥64 µg/ml); A & B denotes individual strip.

CONCLUSION

It could be concluded that insight into phenotypic properties of probiotic strain could serve a basis for functional food ingredients as dietary supplements towards the welbeing of the consumers. This information is particularly important for the development of functional and comparative approaches to unravel the in situ functionality of these probiotic strains in the gastro-intestinal tract of human. These advances could ultimately be utilized towards the development of novel and designer probiotics with a predestined impact on human health.

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