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Assessment of Probiotic Bacteria Isolated from Pharmaceutical probiotic Sachet

Ajit Kumar Dubedi Anal[1*], Saurabh N Singh[1], Sakshi Agarwal[2], Ramesh Kumar*[2], and Rubina Lawrence[1]

[1]AAI-DU Allahabad (U.P), India

[2]NRC on Litchi, Mushahari, Muzaffarpur, Bihar, India

Correspondance Mail: ajitnrcl@gmail.com

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ABSTRACT- Live microorganisms, have beneficial effects on their host's health, are called as probiotics. There are various possible sources to isolate these bacteria. In this studyp harmaceutical probiotic sachet is used as isolation source. The purpose of this study is to search the potentiality of probiotic bacteria and investigate the probiotic properties of isolates. Nine different samples of 3 brands of sachet were used for isolation of bacteria. Isolates were examined according to their probiotic properties. The probiotic characteristics like pH and Bile tolerance, Antagonistic activity and Antibiotic susceptibility of isolated bacteria Such as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* was done. Bile Tolerance and pH tolerance was determined with the help of the help of coefficient of growth inhibition if their coefficient of growth inhibition is less than 0.5 the organism was considered as the pH and Bile tolerance. The Strains of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* show best result at the pH Acidic to Neutral (5 to 7) and show a bile tolerance from 1-4 % bile. All the isolated bacteria show the maximum inhibition against *Staphyloccocus aureus* and minimum against *Salmonella typhi* by *Lactobacillus* Strains but *Bifidobacterium* show minimum against *Escheria coli.* Most isolates show resistance toward antibiotics. From this study it can be concluded that pharmaceutical probiotic products used in the study were showing satisfactory quality and potential probiotic strain.

Key words- Probiotic, *Lactobacillus, Bifidobacterium,* Sachet

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INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). *Lactobacillus* and *Bifidobacterium* are the commonly used probiotics (Kleerebezem and Vaughan, 2009) and are GRAS (Generally regarded as safe) for consumption (Salminen *et al*., 1998). The probiotic organisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract (Tuomola *et al.,* 2001). Moreover, probiotic strains antibiotic susceptibility should be investigated to assess their safety before their use as food additives (Parvez *et al*., 2006). Most of the LAB and *Bifidobacteria* naturally possess intrinsic resistance to wide range of antibiotics (Argyri *et al.* 2013; Saarela *et al*. 2000). The concept of probiotic used in different applications in a large variety of fields relevant for human and animal health. Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods containing microorganisms which have beneficial effects on the health of host. They can contain one or several species of probiotic bacteria, mainly from the genera *Lactobacillus* and *Bacillus* (McFarland and Elmer, 1997; Parvez *et al*., 2005; Hong *et al*., 2008). Most of products which used in human consumption are produced by fermented milk or given in powders or tablets. These capsules and tablets do not used for medicinal applications. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora. Lots of studies suggest that probiotics have beneficial effects on microbial disorders of the gut, but it is really difficult to show the clinical effects of such products. The probiotic preparations use for traveler‟s diarrhoea, antibiotic associated diarrhoea and acute diarrhoea which showed that they have positive therapeutic effect. Detailed studies are needed to establish their safety and probiotic potential. Most of the probiotic starter cultures are available in freeze-dried powder forms in sachets or capsules. Cryoprotectants are used to stabilize the membrane integrity of bacteria and to minimize the degrading effects during freeze drying (Forssten et al. 2011; Zarate and Nader- Macias 2006). The objective of this work was to asses the probiotic characteristic of different isolates and comparison of their potential probiotic properties like pH tolerance Bile Tolerance, Antimicrobial activity and Antibiotic Susceptibility pattern.

MATERIALS AND METHODS

Collection of Samples

For the study three different brand samples of pharmaceutical Probiotic Sachet were selected from local retailer medical shop of Allahabad city, Uttar Pradesh, India.The brands were designated as brand A, brand B, and brand C. These brand contains (*Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*) bacteria. These sachets were stored at 4^0C before working.

Isolation of Bacteria from sachet

1 grams of each sample were weighed aseptically and homogenized in 99 ml of sterile Ringer's solution .the sample was solubilized for about 5 min. then tenfold dilution up to 10^{-6} was prepared Pour plate technique was used to isolate the organisms. 1 ml aliquots of the samples were plated into MRS (Man, Rogosa and Sharpe) agar (pH 6.2) and Tripticase phyton yeast (TPY) agar (pH6.5) for *Lactobacillus* and *Bifidobacterium* respectively. . The plates were incubated at 37 °C for 24-48 h under anaerobic conditions (in anaerobe jar). After incubation, individual colonies were selected and transferred into sterile broth mediums. The isolates were purified by selecting colonies with streak plate technique.

Identification of isolates

Colony/culture characterization

All the isolates were speeded on MRS agar and TPY agar and incubated for 24 h at 37°C. Isolated colonies were examined for striking differences in size, shape, margin, elevation, consistency, texture, pigmentation which assist in identification of different group of micro organism. Morphological characterization shape, arrangement and gram's nature of the isolates were studied using gram's staining.

Biochemical Characterization

Different bio-chemical test was performed such as carbohydrate fermentation, Catalase Test, Oxidase Test, Motility Test and Nitrate Reduction test for identification.

Probiotic Characterizations

Acid Tolerance

Overnight cultures of lactobacilli strains and Bifidobacterium were added to MRS brothand TPY broth adjusted to pH 2 ,3,4,5,6,7 with 1 N HCl.. The broths were incubated for 6 h at 37°C. Cultural turbidity was hourly monitored at 620 nm with the help of spectrophotometer. Initial and final culture growth was measure against control broth.

Resistance to bile

To determine bile salt tolerance strains were grown overnight in MRS broth and TPY broth. 1% (v/v) overnight growth culture of each isolate was added into 10 mL of fresh MRS broth and TPY broth containing 1%,2%.3% and 4% (w/v) Bile (Sodium taurocholate) . The broths were incubated for 6 h at 37°C. Cultural turbidity was hourly monitored at 620 nm with the help of spectrophotometer.

Calculation of Coefficient of Growth Inhibition

The Coefficient of inhibition was calculated by using the following formula.

$$C\ Inh = \frac{(A\ 620nm Control - A620nm\ Bile/pH)}{A\ 620\ nm\ Control}$$

Here, $C\ Inh$ = Coefficient of Growth Inhibition

A620nm=Optical density at 620 nm

*If the Coefficient of Growth Inhibition is less than 0.5 the organism can be considered as pH /Bile tolerance.

Antimicrobial Activity

The antimicrobial activity was determined by the Agar well diffusion assay technique. The *lactobacilli* isolates and *Bifidobacterium* isolate were cultured in MRS broth and TPY broth respectively overnight and the pathogens were grown in Nutrient agar (NA) broth. The overnight of GIT Pathogens were spread onto the surface of nutrient agar plates. Wells of 6 mm diameter was cut from the agar plate using a stainless cork borer. 0.1 ml of CFS (cell free supernatant) obtained by centrifugation of the culture at 8000 rpm for 15 min was added into the wells. The plates were incubated at 37 °C for 24-48 h. The diameter of zone of inhibition around each well was measured. The pathogens tested include *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Bacillus cereus*. These GIT pathogenic bacterial strains were kindly provided from PG laboratory of Microbiology and Microbial Technology department of Allahabad agricultural Institute-Deemed University Allahabad (U.P).

Antibiotic sensitivity

Test all the isolates were inoculated and spreaded respective medias MRS Agar *for Lactobacillus* and TPY agar for *Bifidobacterium.* Antibiotic disc was placed in the center of the plates with the help of sterile forceps. All the plates were incubated at 37°C for 24 hours. The sensitivity was measured as a diameter of the zone of inhibition surrounding the disc and compared with CLSI standards**.**

Statistical analyses

All experiments in the present study were carried out in triplicates and the results indicate their mean values. The data recorded during the course of investigation analyzed statically using t-test, two way analysis of variance (ANOVA) and Data were analyzed at a 5% level of significance. The conclusion was drawn accordingly (Fisher and Yates, 1968).

RESULTS AND DISCUSSION

A total of 6 isolates were obtained on MRS selective medium, and 3 on the TPY Selective medium. Among which 6 isolates 3 where *Lactobacilli acidophilus*, and 3 where *Lactobacillus rhamnosuson* MRS selective medium. On TPY Selective medium the 3 isoaltes of *Bifidobecterium bifidum* isolated by Morphological, Cultural and biochemical characterization. The isolates were Gram positive bacilli, single or in chain of few. The isolates did not show positive reaction to Catalase, Oxidase Motility and Nitrate reduction tests. In carbohydrate fermentation tests, the *Lactobacillus acidophilus* isolates reduced Fructose, Galactose, Glucose, Lactose, Sucrose, Mannose, Maltose but were failed to utilize Mannitol, Ribose and Arabinose. But in the case of

Lactobacillus rhamosus they reduced all the considered sugar (Fructose, Galactose, Glucose, Lactose, Sucrose, Mannose, Maltose, Mannitol, Ribose and Arabinose). *Bifidobacterium bifidum* showed the different pattern of sugar utilization they only reduced the Fructose, Galactose, Glucose, Lactose, Sucrose. Biochemically, all isolates were relatively homogenous and produced acid only and no gas production was observed.

This work is to evaluation the certain probiotic properties of *L. acidophilus* strains and *Bifidobacterium* strain important for their survival in Gastro Intestinal Tract (GIT) has been carried out**.**

Acid tolerance

For the characterization of probiotic strains they should survive in conditions of the gastrointestinal tract, So the survival at the the variable pH environment condition is necessary for the strains. The time from entrance to release from the stomach has been estimated to be approximately 90 min with further digestive processes requiring longer residence time (Berrada *et al.* 1991). Fig (1-3) showed that the the all studied isolates were sensitive from pH 2 to 4 at 6 h of incubation time. However in the case of *Lactobacillus acidophilus* strains [L.A (A), L.A (B) and L.A (C)] they show the sensitivity patter at pH 5 up to 4 h of incubation time. *Lactobacillus rhamnosus* showed the same time of pattern of growth up to pH 4. In the case of *Bifidobacterium bifidum* all strains are sensitive to up to pH 4. After pH 4 they showed Resistance to pH. All the studied isolates were showing inhibition at low pH environment. There are several reports that have same type of Ph tolerance patterns. The results of Bolin *et al.* (1997) indicated that the strains showed different survival abilities in the different pH rang 1.5 to 6.5. *L. acidophilus* strains B and V-74 showed better resistance to the acidic conditions than *L. acidophilus* CH-2 and CH-5. According to the Lankaputhra & Shah, (1995), Lankaputhra, *et al* (1996) Acidity is believed to be the most detrimental factor affecting growth and viability of *lactobacilli*, because their growth was down significantly below pH 4.5.

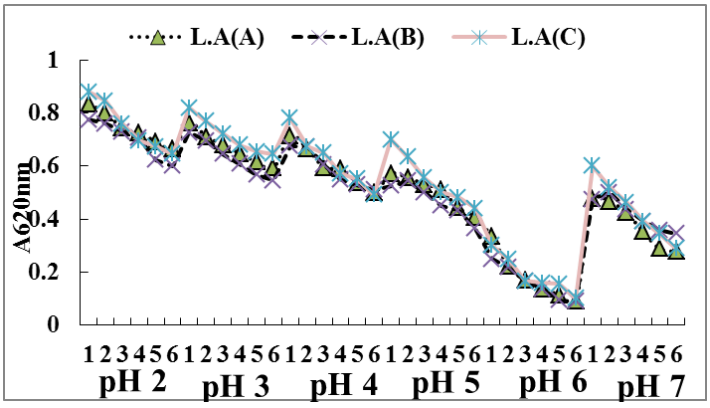

Fig 1: Coefficient of Growth Inhibition of *lactobacilli acidophilus* strains on MRS broth with different pH condition up to 6 hours

Resistance to bile

There are several reports that suggest that bile tolerance is one of the important parameters to consider any lactic acid bacteria as probiotic and the tolerance to bile allows lactic acid bacteria to survive in the small intestine. When bacteria was supplemented with bile the cellular homestasis disruptions causes the dissociation of lipid bilayer and integral protein of their cell membranes, resulting in leakage of bacterial content and ultimately cell death.

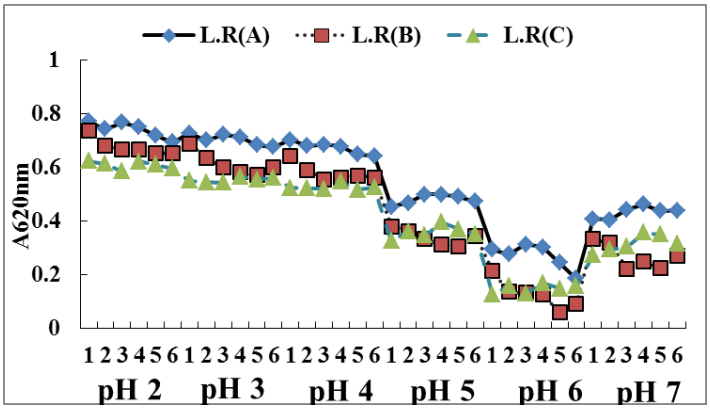

Fig. 2: Coefficient of Growth Inhibition of *lactobacilli rhamnosus* strains on MRS broth with different pH condition up to 6 hours

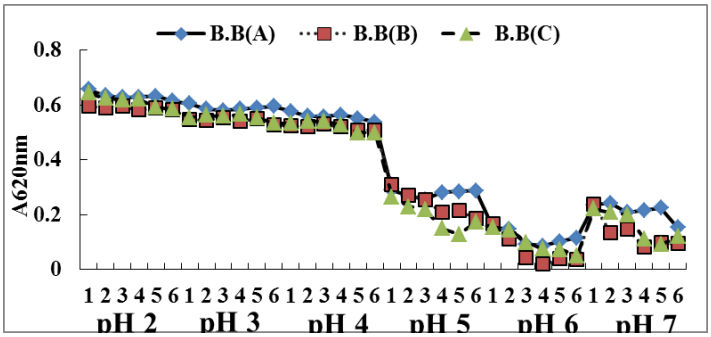

Fig 3: Coefficient of Growth Inhibition of *Bifidobacterium* strain on TPY broth with different pH condition up to 6 hours

In this present study the bile tolerance of the selective bacteria was performed. After analyzing the coefficient of growth inhibition, it can be concluded that strains of the *L. acidophilus*, *L. rhamosus* and *B.bifidum* strains was considered as bile tolerant up to 4% (Table 1) because their growth inhibition coefficient was less than 0.5 (Gopal *et al*., 1996). But in the case of L.A(C) it was found that the coefficient of growth inhibition was excided from 0.5 at 4% concentration. In *L.rhamnosus* strains the L.R (A) showed sensitive toward 4% bile.the *B.bifidum* strain B.B(B) and B.B(C) showed same type of pattern of bile tolerance.

According to the Buck and Gilliland (1994) the tolerance to bile of the *L. acidophilus* isolated from faeces. None of the bacterial isolates showed higher tolerance to bile in comparison with the model strain of *L. acidophilus* ATCC 43121. The growth level of absorbance for all strains ranged from 2 to 2.8 h on the MRS medium supplemented with 0.3 ox-

gall. *L. acidophilus* ATCC 43121 tolerated bile much better and this strain was found to grow faster than the remaining examined strains but this strain was isolated from the intestinal chyme of pigs and cannot be applied in the human diet. Banch *et al*., (2001) said that the DSM 20215 and 20239 strains of the B.bifidum bacteria can be considered as "strains moderately sensitive to the effect of bile".

Detection of antimicrobial activity

Isolates of *Lactobacillus* and *Bifidobacterium* collected from probiotic sachet were screened for antimicrobial activity against *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Bacillus cereus* using ager well diffusion assay. The result of *L.acidophilus* showed that the maximum activity was observed against *S.aureus* (27.5 mm) by L.A © strain while minimum activity was observed against *S .typhi* (7.5 mm) by L.A (B). But in the case of *L. rhamnosus* the maximum activity was observed against *S. aureus* (31.5 mm) by L.R (B) strain while minimum activity was observed against *E. coli* (7.5 mm) by L. R (C*). Bifidobacterium* strain B. B (B) showed maximum activity against *S.aureus* (32.50 (16.00 mm). Ozbas and Aytac (1998) said that *Lactobacillus acidophilus* exert antagonistic effect on the growth of pathogens such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Yersinia enterocolitica* and *Clostridium perfrigens.* According to Mishra and lanbert (1996) probiotic bacteria enhance resistance against intestinal pathogens via antimicrobial mechanism; these include competitive colonization and production of organic acid, such as lactic acid and acetic acid, bacteriocin and production of organic solvent, H_2O_2. Anand *et al.* (1984) reported that *B.bifidum* strains inhibit the growth of *B. cereus*, *Salmonella typhi*, *Shigella dysenteriae*, *E.coli*, *Micrococcus flavus*, *Staphylococcus aureus*, and *Pseudomonas fluorescence* effectively.

Bile	**Duration**	***Lactobacillus acidophilus***			***Lactobacillus rhamnosus***			***Bifidobacterium bifidum***		
Bile (%)	**Time (h)**	**L.A (A)**	**L.A (B)**	**L.A (C)**	**L.R (A)**	**L.R (B)**	**L.R (C)**	**B.B (A)**	**B.B (B)**	**B.B (C)**
1%	1	0.119	0.025	0.240	0.113	0.166	0.150	0.070	0.238	0.244
	2	0.133	0.100	0.250	0.021	0.136	0.159	0.120	0.181	0.229
	3	0.021	0.083	0.222	0.111	0.111	0.043	0.116	0.170	0.220
	4	0.058	0.098	0.178	0.089	0.083	0.132	0.130	0.125	0.130
	5	0.057	0.133	0.155	0.070	0.061	0.111	0.142	0.117	0.222
	6	0.037	0.090	0.135	0.084	0.127	0.122	0.153	0.113	0.224
2%	1	0.142	0.150	0.280	0.204	0.190	0.150	0.210	0.333	0.444
	2	0.155	0.217	0.288	0.170	0.181	0.159	0.219	0.295	0.416
	3	0.106	0.187	0.296	0.203	0.133	0.043	0.209	0.276	0.380
	4	0.137	0.215	0.303	0.178	0.070	0.132	0.217	0.229	0.358
	5	0.115	0.094	0.275	0.157	0.122	0.111	0.183	0.235	0.314
	6	0.111	0.109	0.254	0.152	0.163	0.122	0.193	0.226	0.310
3%	1	0.285	0.175	0.400	0.318	0.285	0.200	0.315	0.500	0.555
	2	0.288	0.239	0.384	0.255	0.250	0.227	0.317	0.477	0.541
	3	0.276	0.208	0.370	0.277	0.222	0.195	0.279	0.446	0.500
	4	0.254	0.215	0.339	0.267	0.229	0.264	0.282	0.416	0.490
	5	0.250	0.169	0.344	0.245	0.183	0.259	0.265	0.411	0.462
	6	0.222	0.181	0.322	0.237	0.254	0.245	0.250	0.369	0.431
4%	1	0.476	0.450	0.520	0.568	0.500	0.500	0.473	0.642	0.688
	2	0.466	0.500	0.519	0.553	0.477	0.500	0.463	0.590	0.666
	3	0.466	0.479	0.500	0.574	0.444	0.478	0.465	0.553	0.640
	4	0.450	0.490	0.500	0.535	0.437	0.490	0.434	0.520	0.603
	5	0.423	0.471	0.482	0.508	0.408	0.481	0.428	0.490	0.574
	6	0.425	0.454	0.474	0.457	0.454	0.456	0.423	0.452	0.568

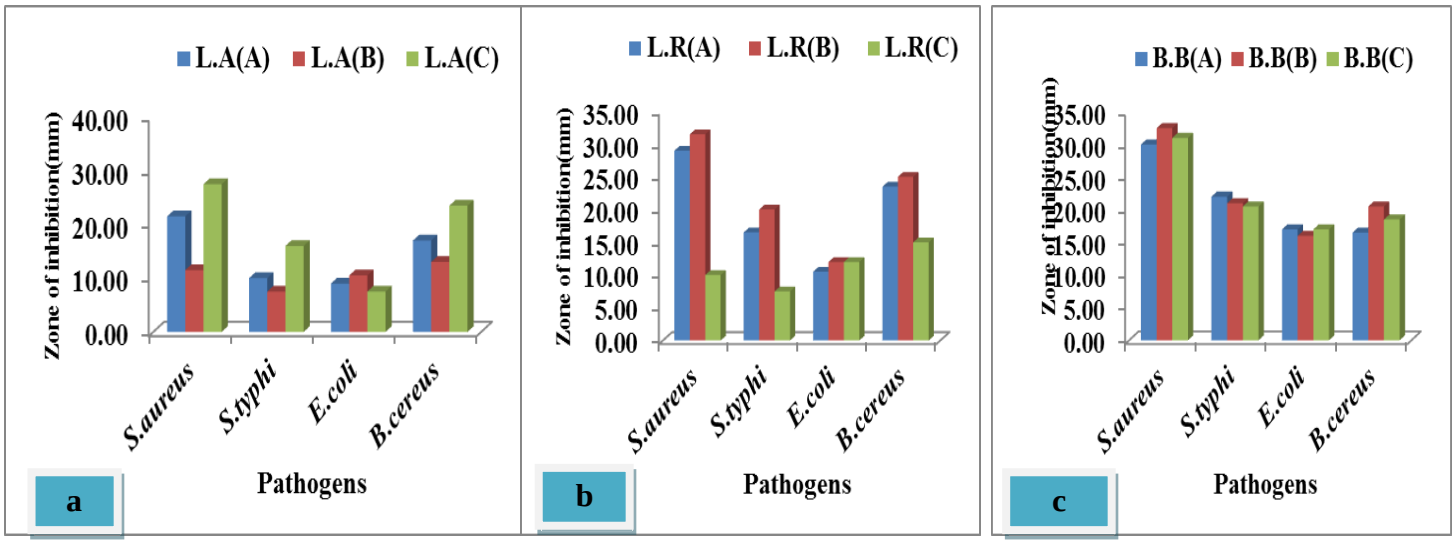

Fig 4 (a): Antagonistic activity of different isolates

(a) *Lactobacillus acidophilus* (b) *Lactobacillus rhamnosus* (c) *Bifidobacterium bifidum* against pathogens

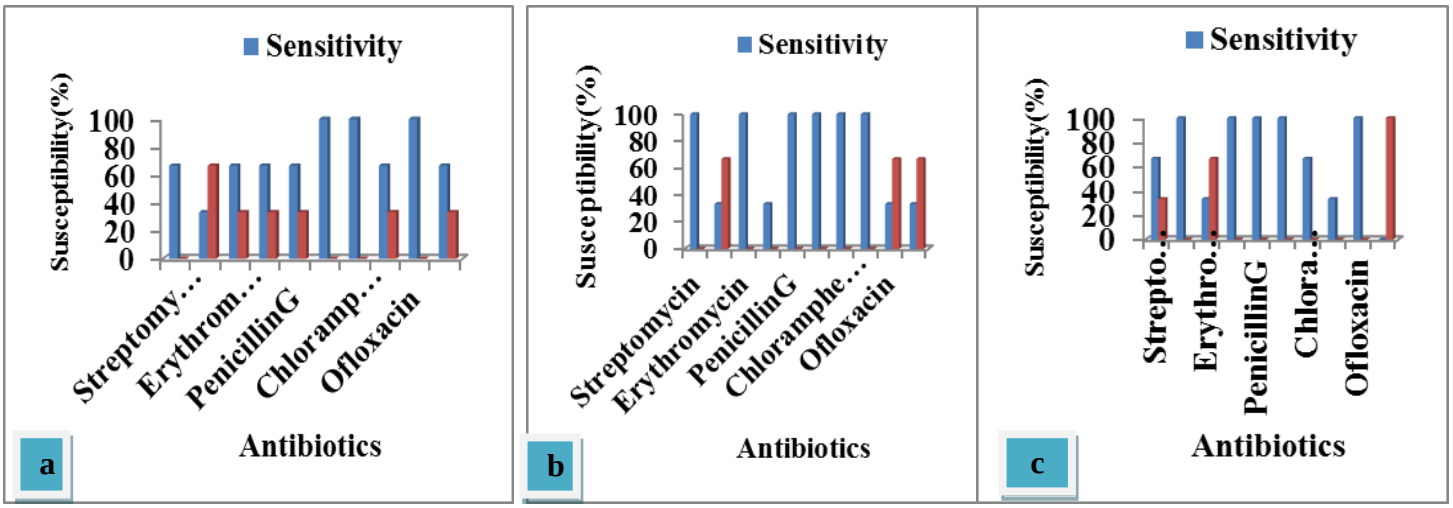

Fig. 5: Antibiotic susceptibility of different isolates

(a) *Lactobacillus acidophilus* (b) *Lactobacillus rhamnosus* (c) *Bifidobacterium bifidum*

Antibiotic sensitivity

Out of three strains of *L. acidophilus*, two strains L.A(B,) and L.A(C) showed a multiple drug resistance (MDR) pattern to various antibiotics. From the fig of Antibiotic Susceptibility pattern the L.A(A) of Sachet A and L.A(C) of sachet C showed good antibiotic resistance property than L.A(B).same type of result was observed in the case of *L. rhamnosus* where L.R(a) and L.R(C) showed good antibiotic resistance activity in comparison to the L.R(B) strain.In the case of *Bifidobacterium bifidum* B.B (C) of sachet (C) showed good antibiotic resistance property in comparison to B.B (B) of sachet (B). Goderska and Czarnecki (2007) reported that the *L.acidophilus* bacteria as one of the species commonly accepted as probiotic turned out quit effective in preventing aliments causined by the application of ampicillin, neomycin and amoxicillin. Goderska and Czarnecki (2007) said that the DSM 20456 strain of *B .bifidum* turned out to be most sensitive to 12 out of 16 tested antibiotics. Lim *et al.*, 1993 studied with 4 strains of *B. bifidum* showed marked differences among strains in sensitivity Pencillin, Chlorophenicol, Oxytertracyclin, Neomycin, and Streptomycin. According to the FAO/WHO (2002) working group recommended determining the antibiotic resistance probiotic strains because probiotic strain could accomplish one antibiotic therapy. In this aspect the antibiotic susceptibility of each selective strain is very important.

CONCLUSIONS

From the above studied it can be concluded that various *Lactobacillus* and *Bifidobacterium bifidum* isolated strains do exist in the sample pharmaceutical probiotic sachet, the isolates be exploited as a probiotic after investigating its beneficial characteristics. The isolate fulfills the

required character for a *Lactobacillus* sp., and *Bifidobacterium bifidum* such as tolerance to conditions such as acidic (pH), Bile, Production of extracellular antibacterial substance that inhibits pathogenic test organisms and resistant to various test antibiotics. Therefore from this study it is considered that the all isolate can be potential use as probiotic organism and safe for consumption.

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