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Research Paper

Evaluation of Effect of Non Steroidal Anti-Inflammatory Drugs on Growth of Probiotics

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Abstract: *Effect of various concentrations of NSAIDs (Non-steriodal anti-inflammatory drugs) on growth of commercial probiotic preparations (A, B, C, and D) were observed by agar well diffusion assay found that sample A followed by B found to be more resistant against all NSAIDs. Comparative evaluation of commercial probiotic preparations was carried out on the basis of physical parameter, antibiotic sensitivity, antimicrobial activity and hydrophobicity assay. Effect of temperature, pH and bile tolerance of samples were carried out in MRS medium and growth was determined by counting cfu value of appropriate dilution after 2h, 4h, and 24h. Antibiotic sensitivity was carried out by disc diffusion assay. Antimicrobial activity was tested using E. coli, S. aureus, P. mirabilis and E. ficalis by in-vitro agar well diffusion method and zone of inhibition was recorded after 24 h. Hydrophobicity assay were carried out using SAT and MATH techniques. Sample B was found to be the most promising probiotic product with respect to physical parameters and antimicrobial activity while sample D followed by B found to be more antibiotic resistant and higher hydrophobicity percentage.*

Keywords: Probiotics, bile salt, hydrocarbons, antibiotics, NSAIDs.

1. Introduction:

Gastrointestinal tract, oral cavity, upper respiratory tract, vagina and skin harbors complex microbiota of more than 1,000 different bacterial species with density of about 10^{14} bacterial cells. Out of it human gut contains 10 times more bacteria than entire body with over 400 known diverse bacterial species including probiotics (Neish AS, 2009). Most bacteria belong to the genera *Bacteroides, Clostridium, Fusobacterium Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, and Bifidobacterium*. Other genera, such as *Escherichia* and *Lactobacillus* are present to a lesser extent.

According to the definition by WHO and FAO, probiotics are live microorganisms which when administered in adequate amounts confers a health benefit on the host. (Schlundt, Jorgen, 2012). Probiotics are available to consumers mainly in the form of foods and as dietary supplements.

Probiotic microorganisms act through several interrelated mechanisms to promote health at molecular level (Thomas CM, *et. al.* 2010). They conquer potentially dangerous micro-organisms in the intestine, reducing the risk of infection or toxin mediated diseases. Additionally, probiotic promotes the function of the intestinal inner lining, enhancing its ability to act as a barrier to the entry of potentially dangerous micro-organisms, chemicals and suppress excessive inflammation. (Girardin M, *et. al.*2011). Probiotic therapy has been used to prevent or treat lactose intolerance, intestinal infection and diarrhea, gastritis and ulcers caused by *H. pylori*, colitis caused by excessive use of antibiotics, inflammatory bowel diseases and irritable bowel syndrome, lower the level of cholesterol (Kumar M., *et. al.*2010, Masood MI, *et. al.* 2011, Meijer BJ, *et. al.* 2011 and Whelan K, 2011). They are also proving instrumental in preventing colon cancer. (Denipote FG, *et. al.*2010 and Zhu Y,*et. al.* 2011).

NSAIDs have analgesic, antipyretic effects and also have anti-inflammatory effects in higher doses. Use of NSAID is associated with increased risk of gastrointestinal and cardiovascular effects mainly due to the inhibition of prostanoid biosynthesis. NSAIDs have many applications in pharmacokinetics and medicinal. Following administration of the broad-spectrum antibiotics and NSAIDs, there may be change in the levels of gut flora. NSAID ingestion may disrupt the homeostasis of intestinal flora and may induce the overgrowth of Gram-negative and anaerobic bacterial species.

2. Materials and Methods:

All media components were purchased from Hi-Media, India. All chemicals were purchased from Merck (India) and all were of A.R. grade.

Collection and maintenance of probiotic samples:

Probiotic samples were collected on the basis of their availability and sale inthe market. Four samples were chosen for experiment has following consortia.

a) Sample A: *Lactobacillus casei* shirota strain
b) Sample B: *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum, Saccharomyces boulardii, Streptococcus thermophilus*
c) Sample C: *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum, Saccharomyces boulardii, Streptococcus thermophiles, and Lactic acid bacillus.*
d) Sample D: *Sterptococcus faecalis* T-110 JPC, *Clostridium butyricum* TO-A, *Bacillus mesentericus* TO-A JPC, *Lactic acid bacillus*

All the samples were activated by successive growing in MRS (Man- Rogosa –Sharp) broth and incubated for 18-24 hrs before use at 37^{o} C.

Effect of Temperature:

Temperature tolerance of all samples was tested by inoculating overnight samples on MRS agar at 27^{o}C, 37^{o}C and 45^{o}C along with control dilution from each interval was transferred on MRS and incubated for 48 h. Growth of probiotic samples was monitored by counting CFU/mL.

Effect of pH:

Effect of pH on viability of strains was examined by inoculating them in MRS agar of different pH 2.5, 4.5, and 6.5 for 48 h at 37°C along with control and growth was monitored by counting cfu/mL.

Effect of Bile Salt:

Bile tolerance of the samples was examined by inoculating them in MRS agar containing oxgall (Central Drug House, New Delhi, India) at the concentrations of 0.2%, 0.3% and 0.5% incubated for 48 hrs. Growth of probiotics was monitored by counting cfu/mL.

Antibiotic Susceptibility:

A disc diffusion assay was performed to study antibiotic susceptibility of samples (Charteries, WP, *et. al.,* 1998 and Temmerman R, *et. al.,* 2002). The samples were inoculated in MRS agar using pour plate technique. The antibiotics were supplied in the form of dodeca discs (Hi-Media, India) which included nalixidic acid, netillin, nitrofurantoin, amikacin, ciprofloxacin, ceftazidime, co-trimoxazole, cefuroxime, oflaxacin, tetracycline, gentamicin, and amoxiclave. The samples were also inoculated on control plates (MRS agar without antibiotic discs) under identical conditions. The zones of inhibition were recorded after incubation at 37º C for 24 h.

Cell Surface Hydrophobic Assay:

(i) Salt Aggregation Test (SAT):

The hydrophobic characteristic of the bacterial strains was determined according to the method reported by Jonsson P. and Wadstrom T., (1984). Samples were grown in 10 mL of MRS broth at 37 °C for 16 h. Bacterial cells were harvested by centrifugation (3000 *g* for 15 min), washed twice with phosphate buffered saline (PBS) pH 7 and suspended in PBS at a concentration of 10^7 cells/mL. Bacterial cell suspensions (25 µL) were mixed with equal volumes of ammonium sulphate of various molarities (0.2–4.0 mol/L) on microscopic glass slides. The lowest concentration of ammonium sulphate giving a visible aggregation was scored as the SAT hydrophobicity value.

(ii) Microbial Adhesion to Hydrocarbon Test (MATH):

Cell surface hydrophobicity of isolates and standard culture was determined by microbial adhesion to hydrocarbons (MATH) method described by Geertsema-Doornbusch GI *et. al* (1993) using hexadecane, xylene and toluene as solvents. The isolates and standard cultures were grown in MRS broth for 16-18 h at 37ºC. Cultures were harvested by centrifugation (2000 *g*, 15 min, 25^0 C), washed twice in PUM buffer and finally suspended in the same buffer. The initial absorbance (A_0) at 600 nm of the suspension was adjusted to 0.70±0.02 units. Five ml of cell suspension in PUM buffer was dispensed in clean and dry round bottom test tubes followed by addition of one ml of hydrocarbon (hexadecane or toluene or xylene). The contents were vortexed for 2 min. The tubes were left undisturbed for 1 h at 37ºC to allow the phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and absorbance (A1) was recorded at 600 nm. Cell surface hydrophobicity in terms of per cent (H %) was calculated using the following formula.

$$H\ \% = (1 - A1/A_0)\ X\ 100$$

Antimicrobial Activity:

Antimicrobial activity was detected by using pathogenic target strains *Escherichia coli*, *Staphylococcus aureus* and *Protease mirabilis* were grown in nutrient broth. *Enterococcus ficalis* was grown successive sub cultured on MRS media.

Antimicrobial activity was determined by using *in-vitro* agar well diffusion method (Zinedine A. and Faid M., 2007). The activity of probiotic strains against test microorganisms mentioned earlier was recorded. The supernatant of overnight grown cultures of probiotic were prepared by centrifugation (3000 g for 15 min) and inoculated in the wells of nutrient agar where as test microorganisms were inoculated by pour plate technique. The inhibition zones were measured at the end of incubation period (24 h).

Following NSAIDs were used for the study

NSAIDs	Concentration range
Ibuprofen	0.9 – 4.0 mg
Diclofenac	1.5 – 7.5 mg
Aspirin	6.0 – 12 mg
Nimesulide	3.0 – 6.0 mg
Lornoxicam	0.9 – 1.8 mg

Effect of NSAIDs:

Effect of various NSAID on the growth of probiotic samples was tested using agar well diffusion assay. Probiotic samples were grown in MRS broth overnight at 37^0C and spread on MRS agar. Various concentrations of NSAIDs dissolved in appropriate solvent was added to wells and plates were incubated at 37^o C for 24 h and zone of inhibition was recorded.

3. Results:

Effect of Temperature:

Probiotic sample B exhibited ability to grow well at all the temperatures (27^o C, 37^o C, and 45^o C) with 94.47, 95.31, and 90.44 % of survival respectively as compared to all other samples. By considering the physiological temperature of human body and the optimum temperature obtained, all further experiments were carried out at 37^o C.

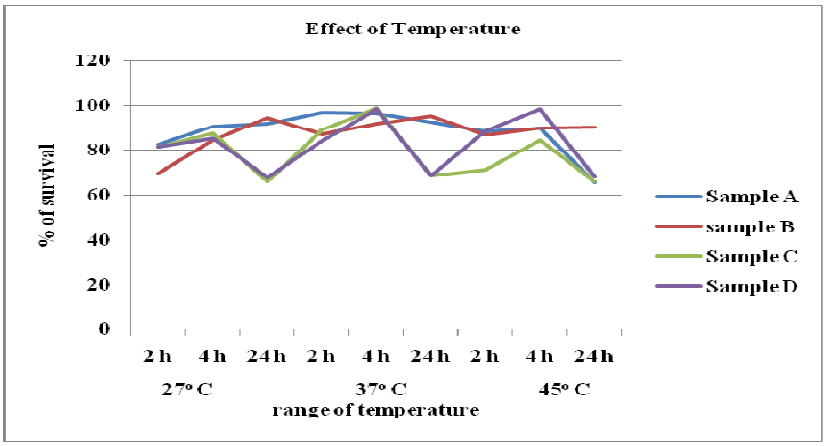

Figure 1: Effect of various temperatures on the growth of probiotic samples (A, B, C, and D)

Effect of pH:

On the basis of percent survival, sample B was found to be most tolerant to pH 4.5 and 6.5 with 93.45 % and 99.68% respectively. **(Figure 2)** However, growth gradually decreases at pH 2.5 from 69.83 % (at 2h) to 0 % (24 h). Percent survival of Sample D was 22.72 % at pH 2.5 after 24 h followed by sample C (18.88 %) was more significant. Thus, probiotic samples C and D were more tolerant to pH 2.5 as compared to sample A and B.

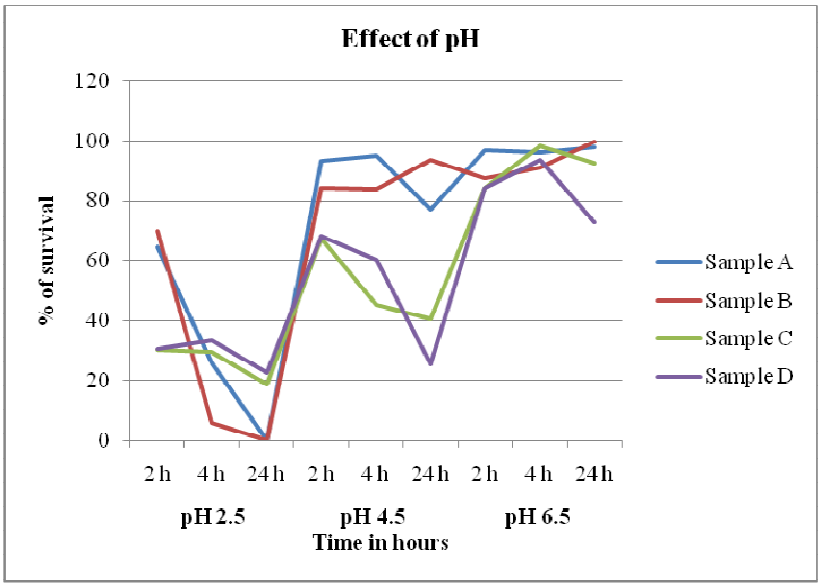

Figure 2: Effect of different pH on growth of probiotics samples (A, B, C and D)

Bile Tolerance:

Exposure to bile salt at various concentrations (0.2%, 0.3% and 0.5%) indicated that with increase in concentration of bile salt, growth of probiotic samples decreases **(Figure 3).** Sample B was more tolerant to all bile salt concentrations showing almost 100 % survival at all the concentrations of bile salt.

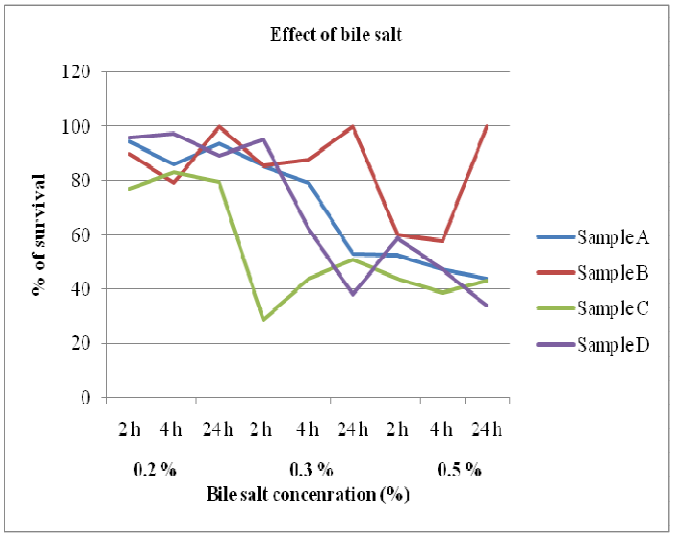

Figure 3: Effect of different concentrations of bile salt on the growth of probiotic samples (A, B, C, and D)

Antibiotic Susceptibility:

Response of probiotic samples towards various antibiotics are summarized in **Table 1** indicated that sample D followed by B are more resistant to all antibiotics while sample C is more susceptible. The control plate showing confluent growth under identical conditions was used to compare the results of agar disc diffusion assay.

Table 1: Antibiotic susceptibility of probiotic samples

Antibiotics (Symbol)	**Concentration (mcg)**	**Zone of inhibition (diameter, in mm)**			
		Sample A	**Sample B**	**Sample C**	**Sample D**
Co-Trimoxazole (COT)	25	17	18	34	0
Amoxyclav (AMC)	30	24	18	30	24
Gentamicin (GEN)	10	14	14	36	14
Tetracyclin (TE)	30	36	26	48	30
Ofloxacin (OF)	5	16	14	36	22
Cefuroxime (CXM)	30	28	25	40	10
Ceftazidime (Ca)	30	14	16	11	0
Ciprofloxacin (Cf)	5	22	18	39	22
Amikacin (Ak)	30	12	16	30	0
Nitrofurantonin (Nf)	30	18	22	30	10
Netilin (Nt)	30	20	16	38	0
Nalidixic acid (Na)	30	0	10	16	0

Hydrophobicity Assay:

The hydrophobic characteristics of the bacterial surface of sample was determined by salt aggregation test (SAT) and cell surface hydrophobicity. Lowest concentration of ammonium sulphate giving aggregation was 0.2 M **(Table 2).** Maximum hydrophobicity percentage with different hydrocarbons (hexadecane, 45% and toluene, 42%) was observed in sample D followed by sample B showing 34% with xylene. **(Table3)**

Table 2: Salt aggregation test

Concentration of ammonium sulphate (M)	**Concentration of ammonium sulphate giving aggregation**			
	Sample A	Sample B	Sample C	Sample D
0.2 M	+	+	+	+
0.3 M	+	+	+	+
0.4 M	+	+	+	+
0,5 M	+	+	+	+

Where, +: Positive, ammonium sulphate giving aggregation with samples

Table 3: Cell surface hydrophobicity

Hydrocarbon	**Hydrophobicity percentage (%)**			
	Sample A	**Sample B**	**Sample C**	**Sample D**
Hexadecane	7 %	32 %	34 %	45 %
Xylene	5 %	34 %	----	29 %
Toulene	3 %	26 %	----	42 %

Antimicrobial Activity:

Supernatant of probiotic samples A and B exhibited an antimicrobial activity against all target pathogens used except *E. ficalis*, while sample D showing against all the target strains was quite significant.

Table 4: Antimicrobial activity of overnight grown sample supernatant

Pathogenic strains	**Zone of inhibition (diameter, in mm)**			
	Sample A	**Sample B**	**Sample C**	**Sample D**
E. coli	13	14	----	13
P. mirabilis	17	18	11	11
S. aureus	17	19	16	15
E. ficalis	----	----	----	12

Effect of Non-Steroidal Anti-Inflammatory Drugs on Probiotics

i) Ibuprofen:

Percent inhibition of all the probiotic samples increases with increase in concentration of ibuprofen. Sample C more susceptible followed by sample D, sample B more resistant to ibuprofen concentration (0.9 – 4.5 mg) (Figure 4).

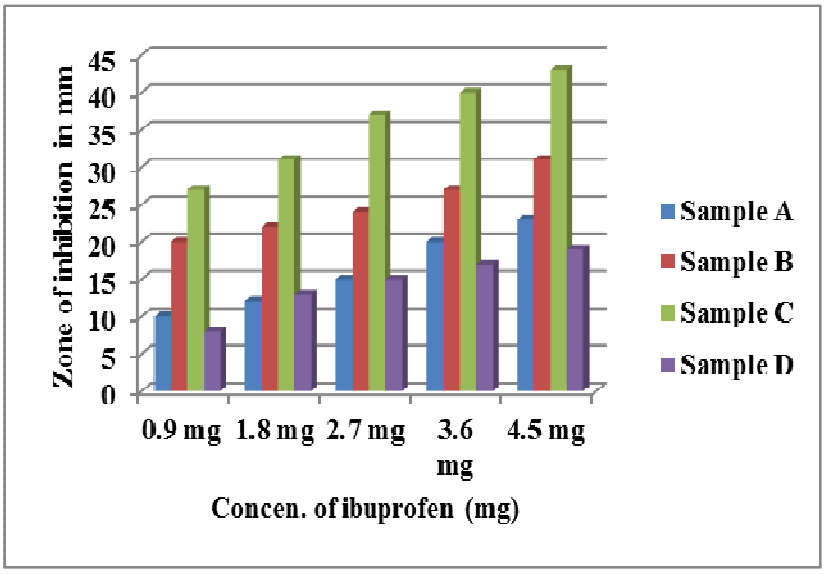

Figure 4: Effect of ibuprofen on growth of probiotic culture

ii) Effect of Diclofenac on Probiotic Samples:

Sample A was found to be more resistant to diclofenac at 7.5 mg while sample C more susceptible as concentration increases from 1.5 mg to 7.5 mg (Figure 5).

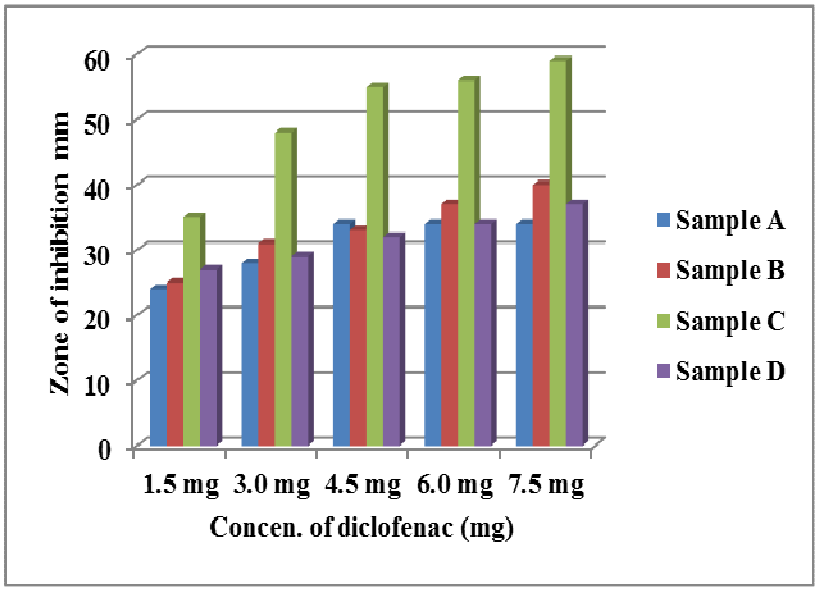

Figure 5: Effect of diclofenac on growth on probiotic culture

iii) Effect of Aspirin on Probiotic Samples:

As concentration of aspirin increases from 6.0 mg – 12 mg, percentage of inhibition also increases. Sample A showing resistance at higher concentration (12 mg) used while sample D is more susceptible (from 6.0 mg to 12 mg) (Figure 6).

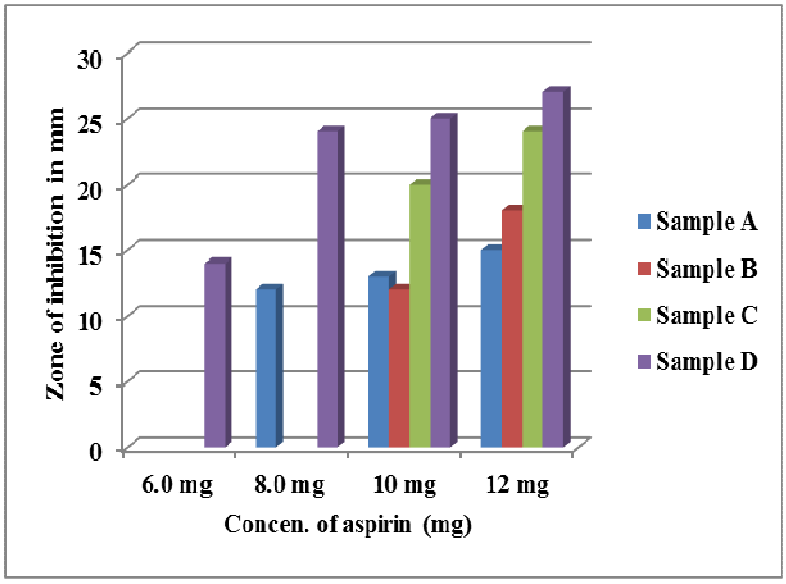

Figure 6: Effect of aspirin on growth of probiotic culture

iv) Effect of Nimesulide on Probiotic Samples:

Sample A found to be most tolerant to the higher concentration i. e. 6.0 mg but sample C followed D found to be very susceptible at all concentration i. e. 4.0 - 6.0 mg (Figure 7).

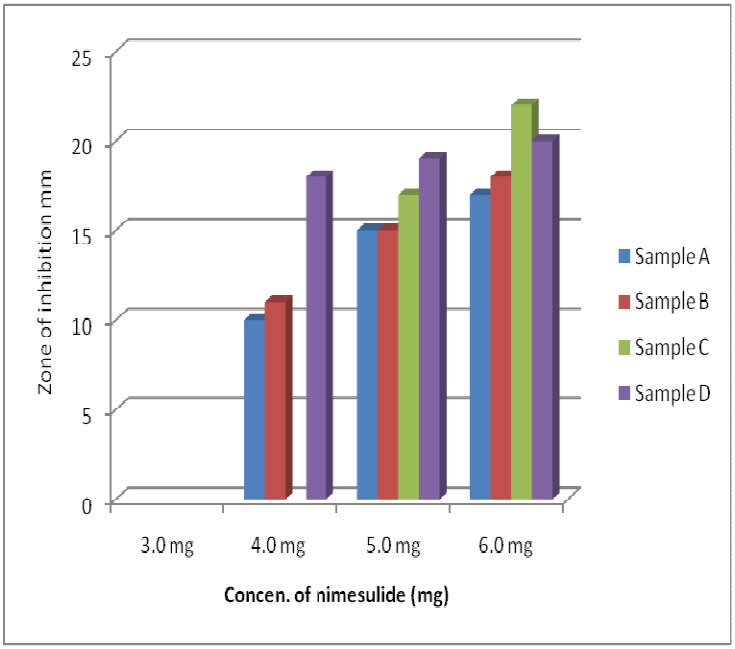

Figure 7: Effect of nimesulide growth of probiotic culture

v) Effect of Lornoxicam on Probiotic Samples:

Lornoxicam inhibited the growth of probiotic sample D at low concentration (0.9 mg) as compared to other samples while sample A found to be resistant at concentration of 1.8 mg (Figure 8).

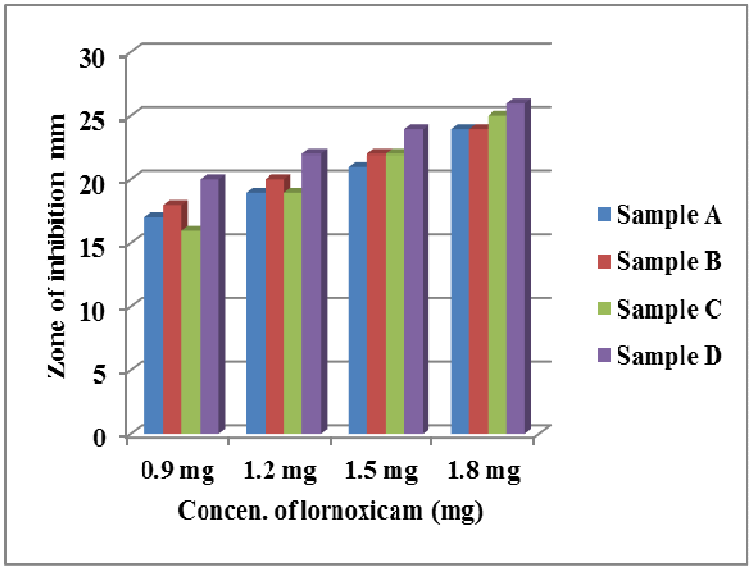

Figure 8: Effect of lornoxicam growth of probiotic culture

4. Conclusion:

Comparative *in vitro* evaluation of all samples was carried out for their survival in GIT. All samples were able to grow at 27° C, 37° C and 45° C. Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant to survive in the human gastrointestinal tract (Berrada N, 1991). All the four samples could tolerate pH 2.5 for 4 h while sample C and D could survive for 24 hrs were very significant. Acid tolerance reported by Jacobsen *et. al.* (1999) who recorded survival of probiotic at pH 2.5 for 2 h. Bile tolerance is an important characteristic of probiotic microorganisms. A concentration of 0.3% bile salt closely appropriates the bile level found in the gastrointestinal tract (Goldin BR., 1992). Our results revealed that the samples were viable at different concentrations of bile salt and showed maximum growth at 0.2% bile. At higher concentrations of bile salt (0.3% and 0.5%) sample B was found to be more bile tolerant followed by A, C and D. Many reports recorded the bile tolerance of *lactic acid bacteria* (LAB). However, majority of them demonstrated bile tolerance upto 0.3%concentration (Diana Drasksle *et. al.,* 2004, Liong and Shah 2005, Mcauliffe *et al.*2005, Hoque MZ, *et al* 2010). The determination of antibiotic susceptibility of a bacterial strain is an important prerequisite prior to considering it safe for human and animal consumption. The all four samples were subjected to antibiotic susceptibility test. Sample D was found to be resistant to COT, Ca, Ak,, Na, Nt. Sample B found to be resistant to AMC, GEN, OF, Cf, TE while sample C was sensitive to all antibiotics used. SAT hydrophobicity value giving a visible aggregation at concentration of 0.2 M ammonium sulphate while Hydrophobicity by using SAT test reported by Jonsson P. *et al* 1984and Diana Drasksler *et. al.,* 2004 which showed aggregation of probiotic strain at 0.2 M ammonium sulphate. Our results are in line and scored 0.2 M ammonium sulphate as SAT hydrophobicity value. Hydrophobicity by MATH indicated that sample D was able to adhere to solvents such as hexadecane (45%) and toluene (42%). Our results are in accordance with Geertsema-Doornbusch *et. al.* (1993) and Duary *et al*. (2011)

Antimicrobial activities of probiotics showed that most of target pathogens were inhibited by the culture supernatant of sample A and B except *E. ficalis*. Sample C found to be effective against only *P. mirabilis* and *S. aureus.* Jacobsen *et. al*.(1999) and Gauri Dixit *et.al.* (2013) reported weak zone of inhibition against *S. aureus*. None of them could strongly inhibit *E. coli*. Hence our results of sample A and B showing inhibitory activity against all test microorganisms are very promising, thereby emphasizing its probiotic characteristics.

Effect of NSAIDs indicated that sample A was more resistance to aspirin, nimesulide and lornoxicam while sample D is more susceptible by agar well diffusion assay. Sample C was found to be susceptible to increasing concentrations of nimusulide and diclofenac. Sample D found to be resistant to ibuprofen. In conclusion, probiotic sample B was found to be superior probiotic product while sample A followed by B found to be more resistant against all NSAIDs was more resistant to various NSAIDs used.

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