CAHIERS MAGELLANES-NS
Volume 06 Issue 2
2024

ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
http://magellanes.com/

EFFICACY EVALUATION OF PROBIOTIC ISOLATES-A PRACTICAL APPROACH FOR THEIR HEPATOPROTECTIVE ACTION ON EXPERIMENTAL ANIMAL MODEL

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ABSTRACT
The study aimed to validate the probiotic properties of non-lactose fermentative bacteria through a range of experimental assessments which include acid and bile tolerance, cell adhesion, hydrophobicity and hepatoprotective nature. It also focuses to evaluate their efficacy using animal model based drug delivery system and post drug delivery analysis of vitals. Probiotics are commonly defined by their ability to confer health benefits through their interaction with the host's microbiota, often demonstrated by their ability to ferment lactose. However, this study focused on non-lactose fermentative strains, exploring their potential as probiotics. Laboratory tests included assays for adherence to intestinal epithelial cells, resistance to gastric acidity and bile salts. The efficacy was assessed using Sprague-Dawley animal model, where various doses of the bacterial strains were administered to laboratory animals, and subsequent health parameters were monitored. Results indicated that while the bacteria did not ferment lactose, they exhibited other probiotic characteristics such as high resistance to harsh gastrointestinal conditions and beneficial effects on gut health in animal models. These findings support the potential of non-lactose fermentative bacteria as probiotics, expanding the scope of probiotic candidates beyond traditional lactose-fermenting strains. Further studies are warranted to elucidate the mechanisms behind their beneficial effects and to evaluate their potential in human applications. All the 3 isolates *Lactobacillus plantarum (IS), Lactobacillus fermentum(IL) and Bacillus mojavensis (DF)* which were isolated and identified in our previous work are further processed in this study.

Key words: Probiotic, Lactose fermentation, Gut health, Hepato protection, Drug dosing, Lab animals

INTRODUCTION

The significance of probiotic organisms in mitigating liver toxicity, particularly in the context of methotrexate-induced damage is increasingly recognized. Probiotics, defined by their beneficial effects on host health, exhibit a range of critical attributes that enhance their therapeutic potential[1]. Among these, acid tolerance, bile tolerance, cell adhesion, hydrophobicity, and hepatoprotective ability are pivotal[2]. Acid and bile tolerance are essential for probiotics to survive and function effectively within the harsh conditions of the gastrointestinal tract, ensuring their presence in the gut where they can exert their protective effects [3]. Cell adhesion and hydrophobicity are crucial for the probiotics to adhere to the intestinal mucosa, which is essential for colonization and interaction with the host's microbiota [4]. Additionally, the hepatoprotective properties of probiotics are of particular interest in mitigating liver toxicity, as demonstrated in studies involving methotrexate-induced liver damage in rats[5]. Methotrexate, a common chemotherapeutic agent, often leads to oxidative stress and inflammation in the liver [6]. Probiotics with strong hepatoprotective capabilities can counteract these adverse effects, reducing liver damage and promoting overall hepatic health. This comprehensive understanding of probiotic attributes not only underscores their potential in therapeutic applications but also highlights

ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
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their role in liver health and disease management.

MATERIALS AND METHODS:

I. Acid tolerance assay

The acid tolerance of probiotic strains was assessed using a rigorous testing protocol designed to simulate the acidic conditions of the gastrointestinal tract [7]. Overnight nutrient broth cultures of the strains were inoculated at 1.0% into 100 ml of MRS broth, which was acidified to various pH levels using 0.1 N hydrochloric acid and adjusted to pH 2.0, 3.0, 4.0, and 7.0. The broths were then incubated at 37 °C, and the survival of the probiotic strains DF, IS, IL were evaluated at 2 and 4 hours. Post-incubation, survival was determined by plating on nutrient agar and incubating at 37 °C for 24 hours. This procedure allowed for a detailed analysis of each strain's resilience to acidic environments, providing valuable data on their potential efficacy in the human digestive system.

II. Bile tolerance assay:

To assess bile tolerance, the procedure followed the method outlined by Gilliland et al.[8]. Overnight cultures of the probiotic strains were grown for 16 ± 2 hours at 37°C, then centrifuged at 8,000 x g for 15 minutes at 4°C. The resulting pellet was resuspended in an equal volume of saline (0.85% NaCl). Fresh nutrient broth (NB) was prepared with and without 0.3% (w/v) ox bile. Each broth was inoculated with 250 µl (5%) of the cell suspension. Growth was monitored hourly by measuring the optical density (OD) at 650 nm. The time lag to reach the logarithmic growth phase in the presence of bile was used to determine bile tolerance. Strains were categorized based on the delay in growth compared to the control: resistant strains (delay ≤15 minutes), tolerant strains (15 < delay ≤40 minutes), weakly tolerant strains (40 < delay <60 minutes), and sensitive strains (delay ≥60 minutes). This classification helps in understanding the strains' ability to withstand bile stress, a key factor for their survival and effectiveness in the intestinal environment.

III. Cell surface Hydrophobicity assay

Cell surface hydrophobicity of isolated and procured strains of lactic acid bacteria was evaluated using the microbial adhesion to hydrocarbons (MATH) method as described by Raj Kumar Duary et al. (2011) [9]. Each bacterial culture was grown in MRS broth at 37°C for 24 hours. After incubation, the cells were collected by centrifugation and the pellets were washed twice with sterile normal saline. The initial absorbance (A0) of the cell suspension was measured at 600 nm and adjusted to 0.70 ± 0.02. In a clean, dry sterile tube, 5 mL of this suspension was mixed with 1 mL of either hexadecane or toluene. The mixture was vortexed for 2-3 minutes and then incubated at 37°C for 1 hour to allow phase separation. The lower aqueous phase was carefully removed, and the final absorbance (A1) was recorded at 600 nm.

The percentage of hydrophobicity (%H) was calculated using the formula:

$$\%H = \frac{A0 - A1}{A0 \times 100}$$

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DOI 10.6084/m9.figshare.2632574
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where A0 is the initial absorbance at 600 nm and A1 is the final absorbance at 600 nm. This procedure was repeated twice, and the average percentage hydrophobicity for each strain was calculated.

Cell adhesion assay:

The adhesion of indigenous lactic acid bacterial isolates was assessed using the method described by Jacobsen et al. (1999) [10]. Caco-2 and HT-29 cells were prepared in a monolayer and washed twice with 3 mL of phosphate-buffered saline (PBS). Two milliliters of minimal essential medium (MEM) without serum and antibiotics were added to each well and incubated at 37°C for 30 minutes to precondition the cells before bacterial inoculation. Bacterial cultures, each at a concentration of approximately 1 × 10^9 CFU, were suspended in 1 mL of MEM without serum and antibiotics and added to each well of the tissue culture plates. The plates were then incubated at 37°C in a 5% CO2 and 95% air atmosphere for 2 hours. Following incubation, the monolayers were washed five times with sterile PBS (pH 7.4) to remove any non-adherent bacteria. For microscopic examination of adhered lactic acid bacteria, the cells were analyzed to assess the extent of bacterial adherence.

IV. Evaluation of the hepatoprotective ability of Probiotic organisms against methotrexate induced liver toxicity in rats

Eight-week-old, clinically healthy, female Sprague Dawley rats (n=36), weighing between 170 and 200 grams, were randomly assigned to six groups (six rats per group) after a 7-day acclimatization period. They were housed in stainless-steel wire-mesh cages (three rats per cage) at a temperature of 24±2°C, 55% relative humidity, and a 12-hour light/dark cycle. The rats were given a standard diet and tap water. Each group received a specific treatment as shown in Table1 below.

The study builds on previous research by Abdelkader et al. (2023) [11]and Yucel et al. (2017)[12]. All treatments were administered to the groups according to the specifications in the table 1 below for a duration of 14 days. On day 15, the animals received a single dose of methotrexate (20 mg/kg). Twenty-four hours after the administration of methotrexate.

Table 1: Drug delivery regimen for 6 groups of rats

S.No	Group Identification	Group Name	Treatment Specifications
1	Group A	Normal control	-
2	Group B	Disease Control	Methotrexate (20mg/Kg bw,i.p.)
3	Group C	Standard	Methotrexate (20 mg/kg bw, i.p.) + Silymarin (20 mg/Kg bw, p.o.
4	Group D	Test 1	Methotrexate (20 mg/kg bw, i.p.)+ Bacillus mojavenesis (20 mg/Kg bw, p.o.
5	Group E	Test 2	Methotrexate (20 mg/kg bw, i.p.) + Lactobacillus Plantarum (20 mg/Kg bw, p.o.)
6	Group F	Test3	Methotrexate (20 mg/kg bw, i.p.) + Lactobacillus fermentum (20 mg/Kg bw, p.o.)

The above table shows the details of planned drug delivery regimen for 6 different groups of rats.

CAHIERS MAGELLANES-NS
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RESULTS AND DISCUSSION

I. Based on all the test performed and analysis f their results the probiotic efficacy of the cultures can be summarized. Initially acid tolerance assay proved all the selected strains to be positive and have good acid tolerance indicating their suitability to be probiotic in nature. This was followed by bile tolerance assay whose results are furnished below:

Table 2: Bile salt tolerance of the selected bacterial strains

	OD of Culture 1 (DF)	OD of Culture 2 (IS)	OD of Culture 3 (IL)
Control	0.06	0.062	0.061
0 hr	0.0159	0.155	0.156
1 hr	0.215	0.262	0.214
2 hr	0.274	0.278	0.275
3 hr	0.388	0.396	0.394

The above table shows varied turbidity of the cultures upon exposing them to bile salts at for varied duration. All the 3 were positive for bile tolerance showing high tolerance rate.

II. **Hydrophobicity evaluation** of culture was performed and the result are summarized in table 3

Table 3: Summary of results obtained for Hydrophobicity assay:

Cell surface hydrophoicity								
	(DF)							
	OD 1	**OD 2**	**OD 3**	**Average**	**SD**	**Absorbance**	**Mean±SD**	**% Hydrophobicity**
Initial	0.691	0.689	0.688	**0.689**	**0.002**	0.63	**0.689±0.002**	**37%**
Final	0.431	0.438	0.425	**0.431**	**0.007**	0.372	**0.431±0.007**	
IL								
	OD 1	**OD 2**	**OD 3**	**Average**	**SD**	**Absorbance**	**Mean±SD**	**% Hydrophobicity**
Initial	0.798	0.785	0.779	**0.787**	**0.01**	0.728	**0.689±0.002**	**44%**
Final	0.442	0.438	0.445	**0.442**	**0.004**	0.383	**0.431±0.007**	
	(IS)							
	OD 1	**OD 2**	**OD 3**	**Average**	**SD**	**Absorbance**	**Mean±SD**	**% Hydrophobicity**
Initial	0.78	0.786	0.78	**0.782**	**0.003**	0.723	**0.689±0.002**	**82%**
Final	0.159	0.149	0.157	**0.155**	**0.005**	0.096	**0.431±0.007**	

Observation:

On the basis of the degree of adhesion to hydrocarbons, Tyfa et.al(2015) categorized and divided bacterial strains into three categories including srongly hydrophobic(>50%), moderately hydrophobic(20 to 50%) and hydrophilic (<20%).From the above results *Lactobacillus plantarum (IS)* is strongly hydrophobic and *Lactobacillus fermentum(IL) and Bacillus mojavenesis(DF*) are considered to be moderately hydrophobic.

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ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
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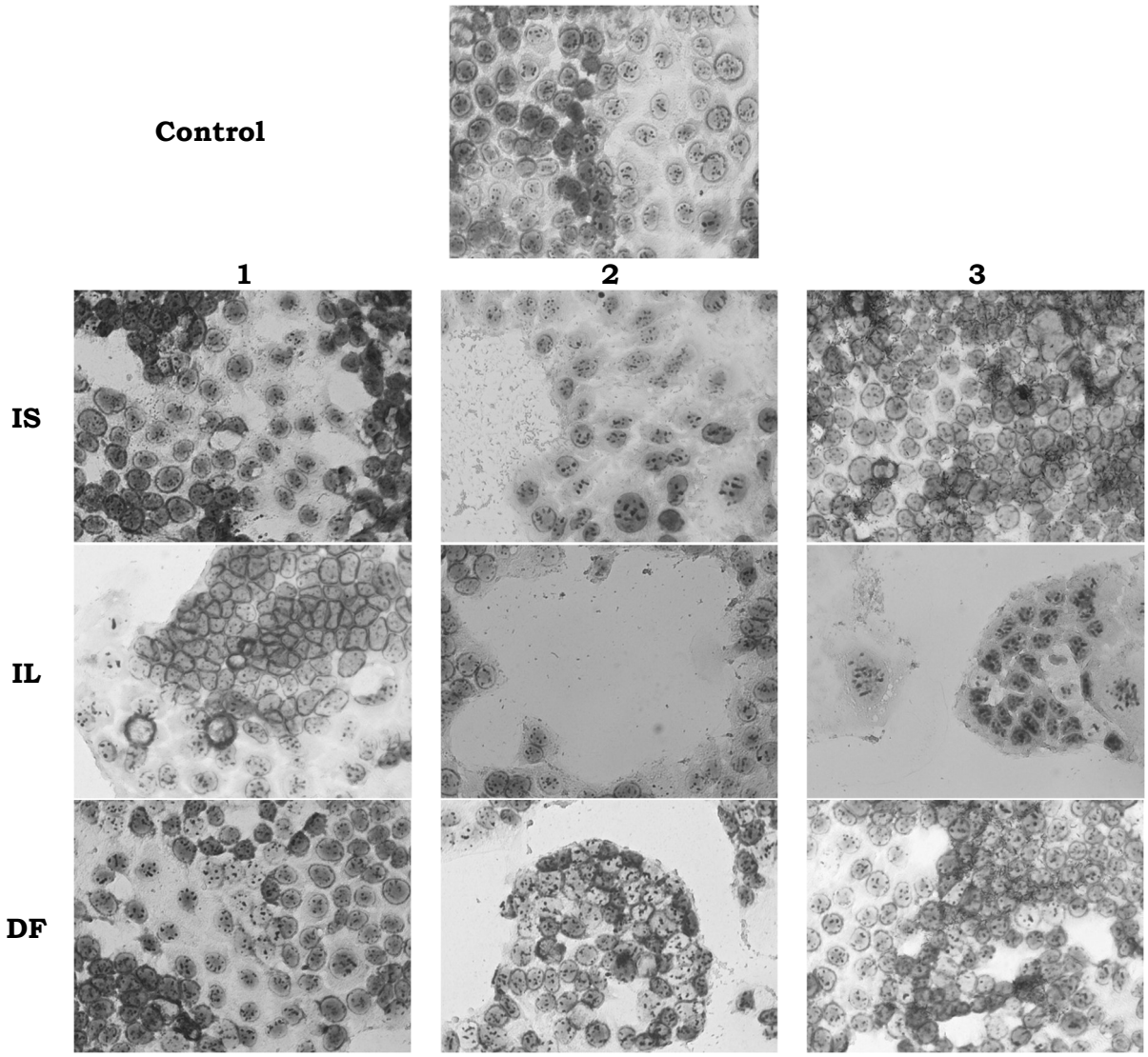
III. Figure 1 : Adhesion of Bacterial cultures on Caco-2 cell culture



From the above images of Cell adhesion study it can be observed that Control and DF has shown no adhesion. Whereas IS has shown strong adhesive property as compared with IL.

Table 4 and 5: Summary of adhesion score and percent adhesion

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Average	SD	Adhesion category
IS	131	134	143	153	140	153	147	161	145	128	126	132	132	137	132	140	152	135	122	135	138.90	10.30	Strongly adhesive
IL	45	46	46	48	47	52	46	42	47	42	46	45	48	47	52	46	65	64	72	45	49.55	8.04	Adhesive
DF	24	30	23	15	24	0	15	18	16	0	21	23	33	21	25	30	0	3	30	19	18.50	10.42	Non adhesive

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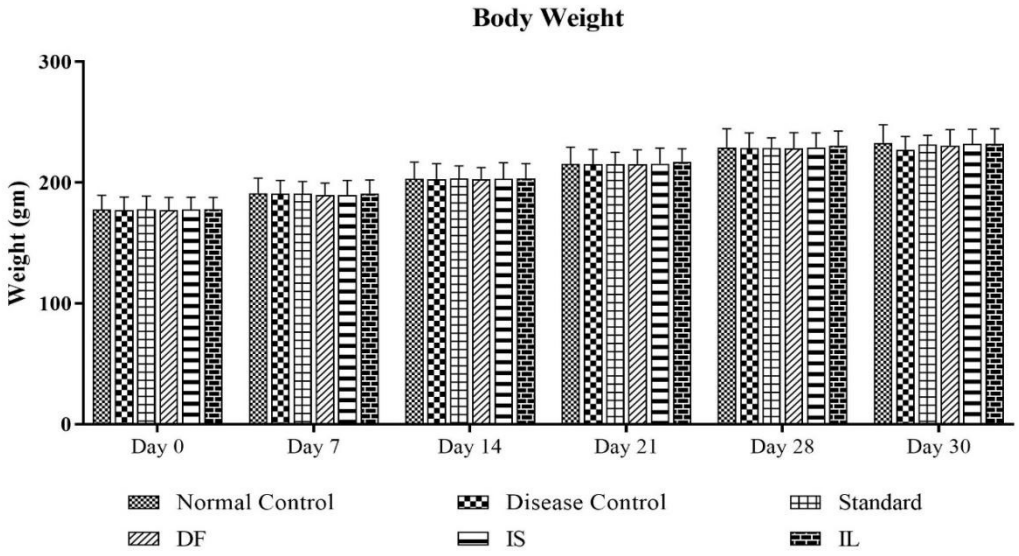
	Initial Count			Final Count			Percent Adhesion			Average
	1	2	3	1	2	3	1	2	3	
IS	1.04 X 10^9	1.07 X 10^9	1.02 X 10^9	4.8 X 10^7	6.9 X 10^7	6.3 X 10^7	4.62	6.45	6.18	5.75±0.99^{bccc}
IL	1.09 X 10^9	1.06 X 10^9	1.04 X 10^9	4.2 X 10^7	4.1 X 10^7	4.4 X 10^7	3.85	3.87	4.23	3.98±0.21^{acc}
DF	1.06 X 10^9	1.07 X 10^9	1.12 X 10^9	1.4 X 10^7	1.7 X 10^7	1.2 X 10^7	1.32	1.59	1.07	1.33±0.26^{aaabb}

a ($p<0.05$), aa ($p<0.01$), aaa ($p<0.001$) when compared to IS; b ($p<0.05$), bb ($p<0.01$), bbb ($p<0.001$) when compared to IL; c ($p<0.05$), cc ($p<0.01$), ccc ($p<0.001$) when compared to DF.

From the above table 4 and 5 it can be summarized that the cell adhesion assay has been done in triplicate where initial and final count was taken for individual bacterial isolates. The percent adhesion of IS isolate has shoen strongly adhesive as compared with IL and DF was non-adhesive.

IV. Series of results obtained for Drug dosage regimen on selected rats are furnished below:
A) Figure 2: Effect of treatments on changes in body weight



NC: Normal control, DC: Disease control. Values are expressed as Mean±SD.

Based on the above graph following observations can be made: Weekly body weight changes were recorded and it was observed that there were no significant change observed in all groups when compared to each other.

B) Changes in food consumption capacity:
A constant weight of 100gms of feed was weighed and given to all groups and remaining quantity of the food was weighed after 24 hr. There were no any significant changes observed in food consumption when compared to each other. The results obtained are plotted in a graph and shown below:

ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
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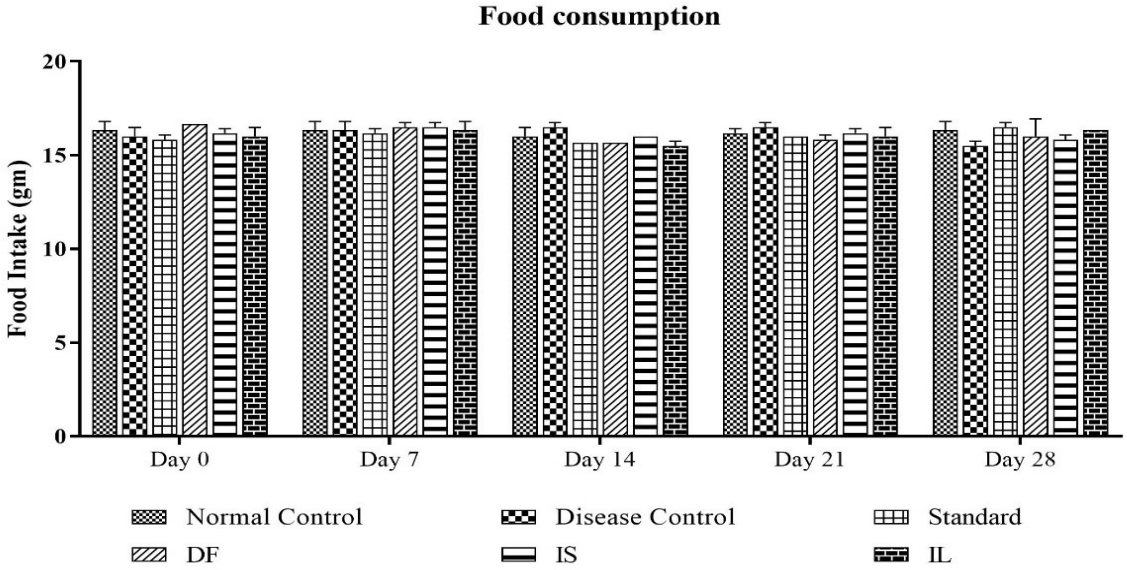
Figure 3: Drug delivery and its effect on food consumption



NC: Normal control, DC: Disease control. Values are expressed as Mean±SD.
Based on the graph it can be summarized that there is no considerable change in the food intake capacity of rats.

C) Effect on organ weight: The relative organ weight of vital organs such as liver, kidneys, spleen, heart, adrenals, lungs and gonads was evaluated at the end of the study. There was no significant change observed in relative organ weights of Liver, Spleen, Heart, Adrenals and Gonads when compared to disease control.

Table 6: Effect of drug dose on organ weight of rats

Groups	Liver	Lung	Heart	Spleen	Kidney	Adrenals	Gonads
NC	3.33±0.70	0.70 ± 0.18	0.36±0.03	0.21±0.05	0.76±0.14	0.19±0.03	2.13±0.16
DC	4.00±0.12	0.62±0.11	0.35±0.08	0.21±0.08	0.94±0.08	0.18±0.02	2.11±0.22
STD	3.71±0.74	0.68±0.13	0.37±0.06	0.24±0.07	0.75±0.11	0.18±0.02	2.12±0.27
DF	3.57±0.51	0.69±0.13	0.38±0.06	0.23±0.05	0.78±0.11	0.17±0.02	2.19±0.25
IS	3.26±0.42	0.67±0.08	0.37±0.07	0.20±0.07	0.74±0.16	0.18±0.02	2.16±0.26
IL	3.18±0.41	0.66±0.07	0.36±0.07	0.20±0.07	0.73±0.13	0.19±0.03	2.16±0.17

In accordance to the table 6 the organ weights of the sacrificed organism after drug dosage of different ratios exhibit no change. Thus there is no effect of drug on organ weight.

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ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
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V. Effect on hematology of the rats

a. WBC: The WBC count was found to be decreased in Disease Control ($p<0.001$) when compared to Normal Control. Whereas, in standard ($p<0.001$) and IS ($p<0.001$) the WBC count was significantly increased as compared to Disease Control

b. Lymphocytes: There was no significant change observed in lymphocyte count in all groups when compared to Disease control or Normal control groups.

c. Neutrophils: There was no significant change observed in Neutrophil count in all groups when compared to Disease control or Normal control groups.

d. RBC: There was no significant change observed in RBC count in all groups when compared to Disease control or Normal control groups.

e. Hemoglobin: There was no significant change observed in haemoglobin content in all groups when compared to each other.

f. HCT: There was no significant change observed in HCT content in all groups when compared to each other.

g. Platelets: There was no significant change observed in platelet count in all groups when compared to each other.

In case of Lymphocytes, Neutrophils, RBC, Hemoglobin, HCT, Platelets, PDW and PCT there were no any statistically significant changes observed in comparison with Disease Control.

Table 7: Summary of the various cell counts and their variation

Group	WBC (10^3/µl)	LYM (10^3/µl)	MID (10^3/µl)	NEUT (10^3/µl)	RBC (10^6/µl)	HGB (g/dl)	HCT (%)	PLT (10^3/µl)	PDW (%)	PCT (%)
NC	7.79± 1.16	5.67± 1.67	0.48± 0.04	1.65± 0.59	5.66± 0.28	12.25± 0.63	33.95± 1.95	237.50 ± 28.58	10.35± 2.86	0.13± 0.02
DC	1.05± 1.09###	0.72± 0.81	0.10± 0.15	0.17± 0.21	7.03± 2.31	15.05± 4.90	37.30± 12.50	186.00 ± 55.48	9.93± 2.18	0.10± 0.08
STD	6.63± 1.02***	4.58± 1.21	0.37± 0.13	1.64± 0.60	6.21± 0.77	13.00± 0.71	40.03± 7.13	198.33 ± 41.88	10.99± 2.56	0.19± 0.14
DF	1.07± 0.53	0.92± 0.42	0.03± 0.05	0.12± 0.09	6.90± 1.07	14.92± 1.96	35.93± 5.23	187.00 ± 34.50	8.92± 0.80	0.08± 0.05
IS	4.70± 1.43***	2.98± 1.06	0.34± 0.11	1.38± 0.55	6.49± 1.05	13.55± 1.55	43.87± 8.26	246.67 ± 31.95	10.90± 1.21	0.32± 0.17
IL	1.53± 0.74	1.27± 0.50	0.08± 0.07	0.27± 0.21	6.21± 0.32	13.30± 0.60	31.39± 1.60	190.00 ± 37.39	8.17± 0.85	0.08± 0.04

ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
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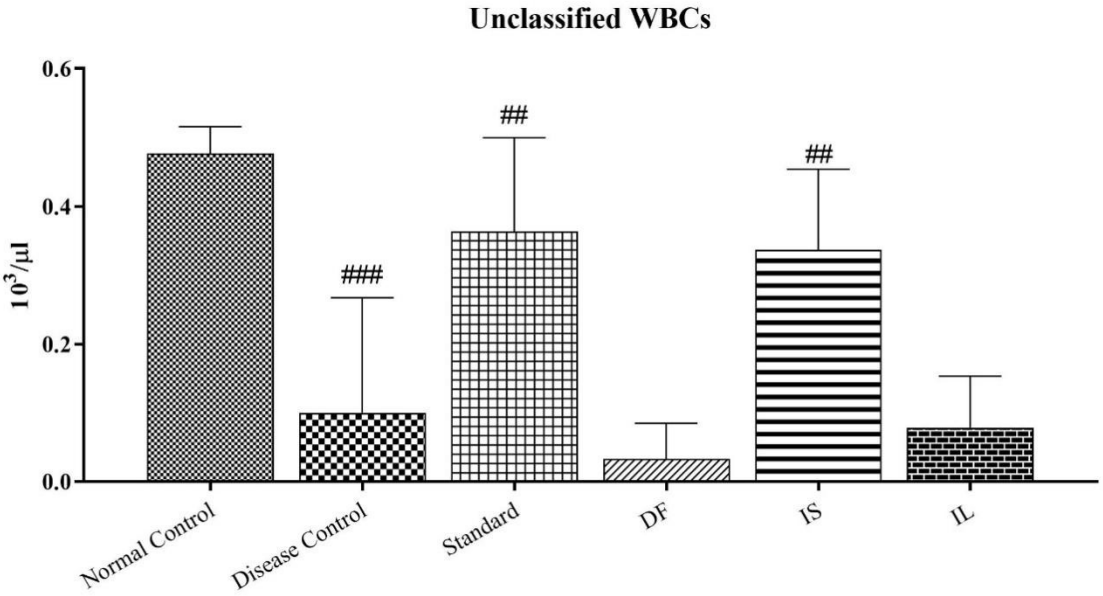
Based on the above table it can be observed that The WBC count was found to be decreased in Disease Control ($p<0.001$) when compared to Normal Control. Whereas, in standard ($p<0.001$) and IS ($p<0.001$) the WBC count was significantly increased as compared to Disease Control

VI. MID variation after drug regimen

Mid-range absolute count, which is the combined value of white blood cells (WBCs) that are not classified as lymphocytes or granulocytes. MID cells include less common and rare cells, such as monocytes, eosinophil, basophils, blasts, and other precursor white cells that fall within a specific size range.

Figure 4: Variations in the unclassified WBC's



It was observed that in Disease control group MID levels were increased significantly ($p<0.001$) as compared to NC. Whereas in case of Standard ($p<0.01$) and IS ($p<0.01$) the MID levels were significantly increased as compared to DC.

VII. Biochemical analysis of serum to assess liver function

Table 8: Summary of the variation in SGOT and SGPT values

Groups	SGOT (IU/L)	SGPT(IU/L)
NC	94.48 ±3.58	34.33 ±3.14
DC	170.68 ±16.36	82.68 ±23.34
Standard	120.84 ±47.64	45.75 ±17.83
DF	140.28 ±12.30	62.09 ±12.65
IS	128.84 ±22.97	49.83 ±17.20
IL	129.22 ±8.26	51.29 ±14.78

SGOT is a part of a liver function test and therefore it was evaluated to know the liver damage. Statistical

ISSN:1624-1940
DOI 10.6084/m9.figshare.2632574
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analysis revealed that there was a significant ($p<0.001$) increase in SGOT levels after methotrexate administration in Disease Control group animals as compared to Normal Control thereby confirming the induction of liver damage. In treatment groups, Standard ($p<0.05$); significant decrease in the SGOT levels were found in comparison with Disease Control animals and DF, IS, IL also decrease in SGOT levels in comparison with disease control but statistically non-significant results were observed.

SGPT levels were evaluated to determine the extent of liver damage after exposure to methotrexate in probiotic treated rats. It was observed that the methotrexate administration leads to a significant increase in levels of SGPT in the Disease Control group in comparison with Normal Control ($p<0.01$). whereas in Standard ($p<0.05$), IS ($p<0.05$) and IL ($p<0.05$) treatments have shown significant decrease in SGPT levels due to prior administration of probiotic in comparison with Disease Control group. SGPT levels were decreased in DF group also in comparison with Disease control but values were statistically non-significant

CONCLUSION

The primary objective of this study was to develop a safe and effective probiotic with strong hepatoprotective properties. We characterized the isolated bacterial strains for their probiotic capabilities through tests for acid and bile tolerance, cell adhesion, and cell surface hydrophobicity. All strains demonstrated significant tolerance to acid and bile, as well as notable hydrophobicity. To assess the hepatoprotective activity of the probiotics, we used the methotrexate-induced acute liver injury model in rats which is a well-established method for evaluating liver protection . This model accurately mimics acute liver disease pathogenesis in humans . Elevated serum levels of SGOT and SGPT, hepatic metabolic enzymes, indicated liver damage. The subsequent reduction in these enzyme levels confirmed the hepatoprotective effects of our probiotic isolates. Based on these results, the isolated probiotic bacteria show promise as candidates for inclusion in nutraceutical formulations aimed at liver health.

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