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Evaluation of Safety parameters of Pro BC Plus (*Bacillus coagulans*- LMG S-31876) in rats: Clinical and biochemical approach

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Abstract
The present study deals with the administration of *Bacillus coagulans* (LMG S-31876) 50 billion CFU/g (Colony Forming Unit/gram) on mice. The strain has been isolated from fermented rice. A study on acute and sub-acute toxicity of the strain was analyzed on the Wistar rats at the dose levels of 100-400 mg/kg body weight; the maximum dose volume was maintained at 20 ml/kg body weight for a consecutive period of 90 days. The objectives of the study were to assess the toxicological profile of the test item when administered by oral gavage to Wistar rats for a period of 90 consecutive days and assess the potential reversibility of any findings, and thereby provide data to support the use of the test item in humans. This study exhibited no significant clinical signs, body weight, haematology, or clinical chemistry changes before and after treatment as compared to the vehicle control group. Thereby, the test item is safe to be utilized as a probiotic product.

Keywords: bacteria, *Bacillus*, toxicity, clinical, mouse, food, safety, biochemical, probiotics, pathology

Introduction

Probiotics are often defined as live micro-organisms that when ingested/consumed in an adequate amount confers beneficial health effects on the host. The most utilized Probiotics are *Lactobacillus*, *Bifidobacteria*, and *Saccharomyces.* Probiotics extend beneficial effects to the host that includes (a) enhancing the immune system (b) treatment of diarrhoea and reducing bowel symptoms (c) reducing lactose intolerance. There are several effects of Probiotics on the host system that includes the enhancement of the immune system via modulating pathogen-induced inflammation via regulated receptor signalling pathways (Polk *et al*.,2011) [1]. Lactic acid bacteria are one of the most promising probiotic bacteria and these are considered as Generally Recognized as Safe (GRAS) (Donohue *et al*., 1996).

There have been previous studies on lactic acid strains, a combination of *Lactobacillus rhamnosus* and *acidophilus*, *Bifidobacterium lactis* that possess anti-infection and immune-enhancing properties (Gill *et al*., 1998). Some of the strains of *Bacillus coagulans* can survive extreme temperatures, acidity, and lower pH conditions that mimic the parameters of gastro-intestinal conditions,(Diaz *et al*., 2012) [4] similar results were obtained from Ganeden Bc 30. Toxicological assessment of the product is considered to be safe with 90-day sub chronic toxicity in the rat (Endres *et al*. 2009) [5].

The present study aims to access the toxicological profile of the *Bacillus coagulans LMG S-31876*. The administration of the test item was executed by oral gavage to Wistar rats for a consecutive period of 90 days. The observations that followed the test included general behavior, body weight changes, feed and water intake, biochemistry hematology, pathology, and histopathological changes in the control and test samples. These were used to access the potential reversibility of any findings and also to check the utilization of the test item in humans.

Materials and method

1. Test system

Male and female Wistar rats were procured from Adita Biosys Private Limited, Karnataka, India. Experiments were conducted following CPCSEA (Committee for the purpose of Control and Supervision of Experimentation on Animals) guidelines. The experimental animals were approved by IAEC (Institutional Animal Ethical Committee) and all the experiments were conducted following OECD guidelines (Rome *et al*. 1995) (Combes *et al*. 2004) [7]. Rats were acclimatized for seven days to laboratory conditions at a temperature of 22 ± 2°C, whereas relative humidity (30−60%) and 12 hrs light and dark phase.

2. Materials

The Probiotics product containing *Bacillus coagulans* (50 billion CFU/g) is stored at room temperature.

3. Toxicology studies

Animals were categorized into 6 groups that include G1-G6. The dose levels selected were 100, 200, and 400 mg/Kg body weight. The number of animals per group is denoted in table1.

Table 1: Represents the grouping of animals

Group	Treatment	Dose (mg/kg Bwt)	Concentration(mg/ml)	Treatment Period	No. of Animals		Animal numbers	
					Male	Female	Male	Female
G1	Vehicle Control	-	-	90	10	10	1-10	11-20
G2	Low dose	100	10		10	10	21-30	31-40
G3	Mid dose	200	20		10	10	41-50	51-60
G4	High dose	400	40		10	10	61-70	71-80
G5	Recovery Control	-	-	28	5	5	81-85	86-90
G6	Recovery Treated	400	40		5	5	91-95	96-100

4. Estimation of treatment procedures

During the studies, recovery and treated groups were administered with calculated doses as per body weight orally taken at a dosage of once a day in the forenoon season. In the case of G1 vehicle control, 1% gum gacaca suspension. The duration of the treatment was for a period of 90 days for the respective G1-G4 and recovery groups. Subsequently, after the completion of 90 days animals in the recovery group were observed for 28 days post-treatment for toxic effects (Barlow *et al*, 2002) [8].

5. Determination of physical parameters and their clinical signs

Animals were examined for the signs such as alteration in the skin, fur, eyes, secretions, and autonomic activity). Along with this, average feed consumption (feed-in and feed-out) was measured to estimate the consumption of food on weekly basis. Individual consumption of food was calculated and observed according to the parameters such as body weight, for this, the alteration in the weight was observed for day 1 and once a week till the study is completed. The other parameter such as water consumption was monitored weekly till the termination of the study (OECD guideline for testing of chemical no.39).

6. Analysis of clinical pathology and their examinations

Rats were fasted overnight with a water supply. 3 ml of blood was collected by puncturing the retro-orbital sinus plexus on the 90th day before the sacrifice. The sample was collected and sent to diagnostic labs for examination and hematology parameters such as RBC, Hb, total WBC count, neutrophil, lymphocytes, eosinophils, monocytes, packed cell volume, and platelets.

7. Estimation of biochemical parameters

Serum samples were separated from blood by centrifugation at 2500-3500 rpm for about 10-15 min. The serum was analyzed for analyzing triglycerides, lipoproteins, urea, creatinine, protein, albumin, SGOT, SGPT, and cholesterol.

8. Determination of pathology studies

Animals were sacrificed by cervical dislocation and subjected to necroscopy. Prominent organs/tissues were studied for the experiments and the tissues were dissected and trimmed out, these were preserved in 10% buffered formalin. These tissues were subjected to histopathological examination. Tissues (3-5 microns) were embedded in paraffin wax and stained with hematoxylin-eosin stain. These were subjected to microscopic examination and observed.

Statistical Analysis

The data that represents body weight, food consumption, and pathology and biochemical data estimations were analyzed statistically using One-Way ANOVA (Analysis of Variance) with the Bonferroni test for different treatment groups compared with Vehicle Control group data. All analyses and comparisons were evaluated at a 95% level of confidence ($p<0.05$).

Results

1. Analysis of parameters

The study was analyzed for the parameters such as body weight, feed, and water consumption. The changes in these parameters for the control and treated were found to be non-significant. The figures are represented in body weight, water intake, and feed intake.

2. Measurement of body weight

The weekly body weight measurements of male and female rats are represented in Figures 1 (A) and (B). Individual body weights for both male and female were recorded on day 1 of the treatment and once in a week (+1) till the termination of the study. Changes in both the control and treated groups and the body weight of all the animals of different treatment groups were found to be non-significant. The statistical analysis was found to be non-significant for all the groups. Endres *et al.*, 2009 [5] reported the mean body weight in 100mg/kg of the males was slightly lower than the control on the 50th day studied.

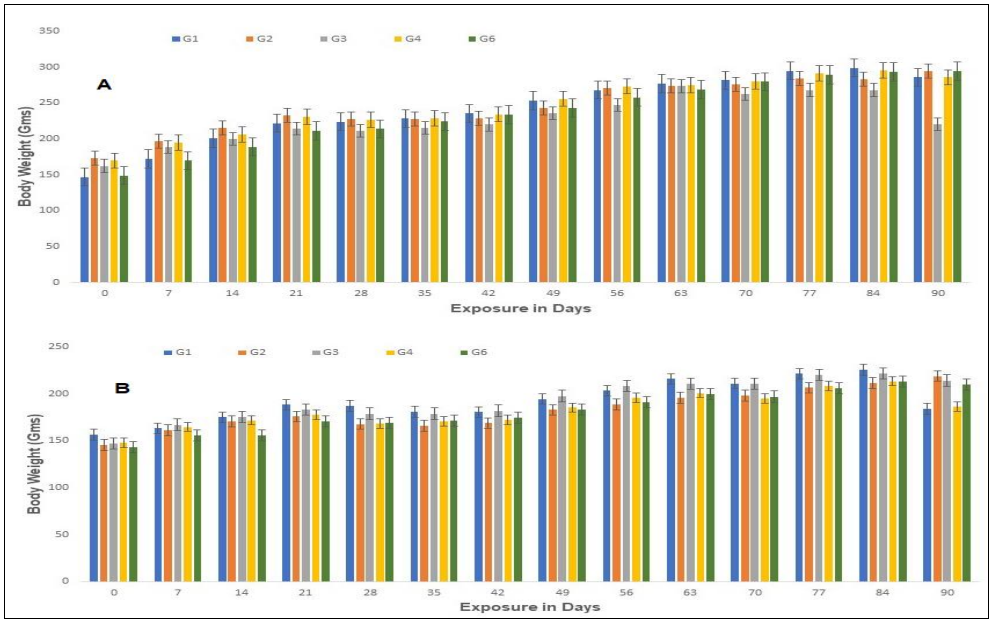

Fig 1: (A) Depicts a 90-day sub-acute toxicity study in rats. Influence on body weight in rats (males n=10)
(B) Represents a 90-day sub-acute toxicity study in rats. Influence on body weight in rats (female n=10)

3. Analysis of water consumption

The average weekly water intake is represented in Figure 2(A) and (B). Individual water consumption was monitored each week till the experiment was terminated. The changes in the water intake were recorded periodically and found to be non-significant.

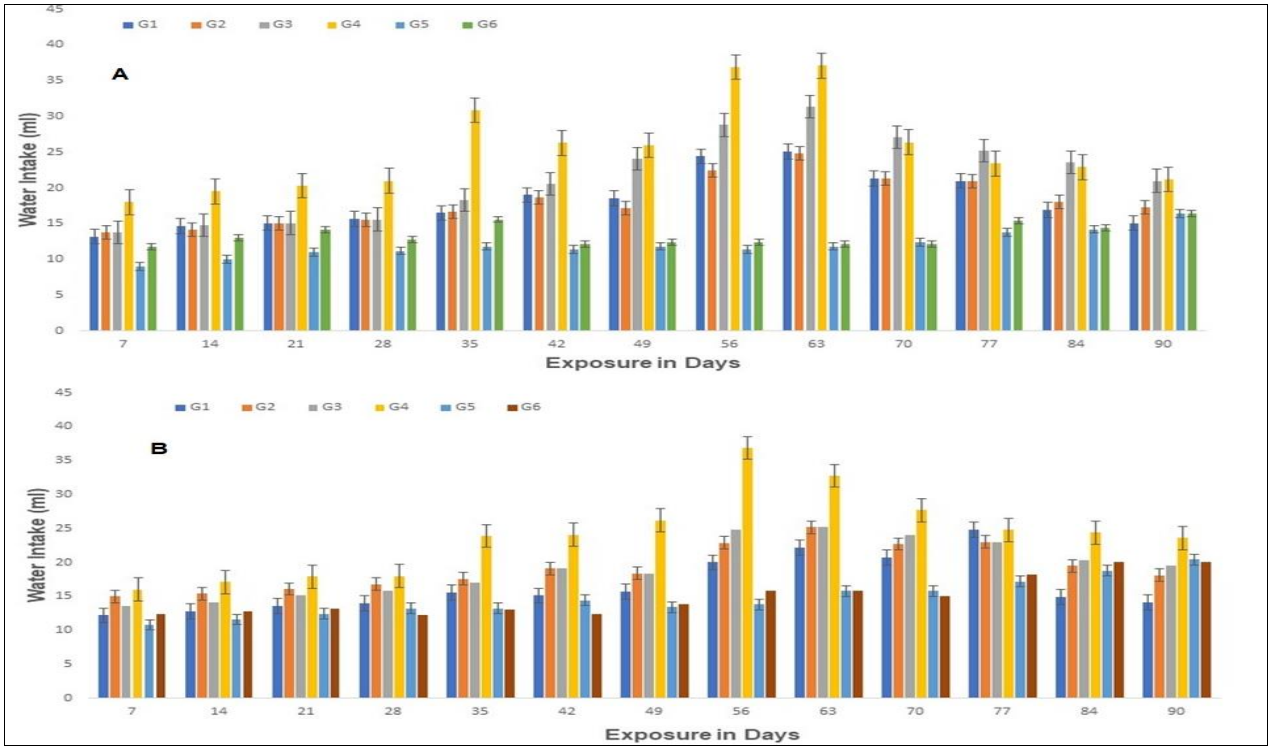

Fig 2: (A) Depicts 90 days sub-acute toxicity study in rats. Influence on average water intake in rats (males n=10)
(B) Representation of 90 days sub-acute toxicity study in rats. Influence on average water intake in rats (female n=10)

4 Analysis of feed consumption

The weekly average feed consumption (g/animal/day) of male and female are represented in figures 3(A) and (B). Cage-wise food consumption was measured weekly once. Feed-in and feed-out measurements were evaluated to estimate the average feed consumption by each group of animals every week. Individual feed consumption was calculated and recorded. There were no changes after the treatment observed for feed consumption, irrespective of sex, and all treatment groups

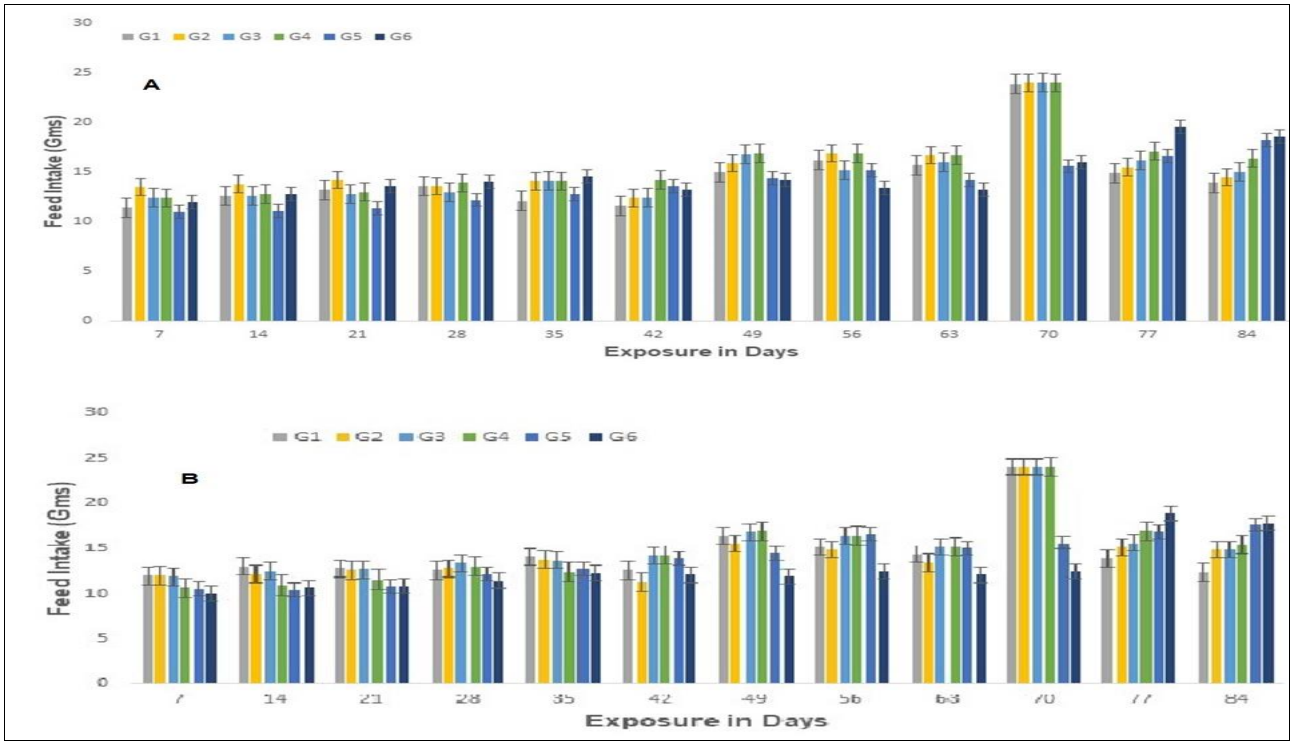

Fig 3: (A) Represents a 90-day sub-acute toxicity study in rats. Influence on average feed intake in rats (male n=10)
(B) Depiction of 90 days sub-acute toxicity study in rats. Influence on average feed intake in rats (female n=10)

5. Clinical Pathology

5.1 Estimation of Biochemical analysis

The biochemistry parameters are presented in Tables 2 and 3. The statistical analysis exhibits significant changes for 400 mg/kg (G4) for Cholesterol and Urea contents when compared to the control. Creatinine content was significantly different in G4 and recovery groups while the serum, protein content was different in G4, as compared to the control group. Although some of the parameters were found to be significantly changed in variance analysis with a confidence level of 95% criteria, the variations could not be associated with treatment-related effects. As the changes were not biologically significant and values ranged within the normal physiological limits for individual parameters, the final concluding biological interpretation will be a non-significant variation. As all the parameters recorded fall within the normal range, the priority of consideration stands in support of a final conclusion as biologically non-significant.

Table 2: Represents 90 days sub-acute toxicity study in rats, biochemistry parameters in rats (male n=10) CHL – Cholesterol, TGL – Triglycerides, HDL – High-Density Lipoprotein LDL – Low-Density Lipoprotein, UR – Urea, CR – Creatinine, PR – Protein, ALB – Albumin, OT – SGOT, PT – SGPT

Male										
Control/normal (G1)	**Cholesterol**	**TGL**	**HDL**	**LDL**	**Urea**	**Creatinine**	**P r**	**Albumin**	**OT**	**PT**
MEAN	84.3301485	97.6347	15.3747	51.011	39.9756	0.6307809	6.45068	1.94684	206.105	95.2836
SD	16.2295478	36.8602	3.26422	15.2956	5.47545	0.0527046	0.69001	0.35103	61.6579	31.8354
STD ERROR	5.13223365	11.6562	1.03224	4.8369	1.73149	0.0166667	0.2182	0.11101	19.4979	10.0672
400 mg/kg (G4)										
MEAN	81.7309842	110.023	15.2388	45.4213	38.6121	0.6425828	6.57463	1.82081	157.73	64.869
SD	42.0294681	47.3493	7.65173	22.5517	20.67	0.3479073	3.50357	0.96064	85.7041	37.3952
STD ERROR	21.0147341	23.6747	3.82587	11.2758	10.335	0.1739537	1.75179	0.48032	42.852	18.6976
Recovery Group (G6)										
MEAN	168.2	104.14	13.72	133.6	42.94	0.58	7.68	2.18	285.62	114.1
SD	13.01	38.73	3.37	9.21	10.05	0.04	0.69	0.16	140.19	53.81
STD ERROR	5.82	17.32	1.51	4.12	4.5	0.02	0.31	0.07	62.7	24.06

Table 3: Represents 90 days subacute toxicity study in rats, biochemistry parameters in rats (female n=10)

Female										
Control/normal (G1)	**Cholesterol**	**TGL**	**HDL**	**LDL**	**Urea**	**Creatinine**	**Pr**	**Albumin**	**OT**	**PT**
MEAN	94.55	49.67	13.87	70.65	46.95	0.83	6.73	1.91	159.24	51.01
SD	25.05	18.49	4.61	18.19	9.7	0.17	0.5	0.22	70.56	18.98
STD ERROR	8.35	6.16	1.53	6.06	3.23	0.05	0.16	0.07	23.52	6.32
400 mg/kg (G4)										
MEAN	102.08	63.28	15.7	74.26	48.55	0.79	7.27	2.06	175.56	52.6
SD	28.15	28.04	4.07	23.72	12.77	0.11	0.8	0.26	53.88	13.44
STD ERROR	8.9	8.87	1.28	7.5	4.04	0.03	0.25	0.08	17.04	4.25
Recovery Group (G6)										
MEAN	159.74	140.8	11.52	116.2	28.12	0.54	7.04	2.22	253.88	103.24
SD	2.54	29.87	0.93	2.17	2.67	0.05	0.11	0.16	18.76	16.53
STD ERROR	1.14	13.36	0.42	0.97	1.19	0.02	0.05	0.07	8.39	7.39

5.2 Determination of hematology parameters

The hematological parameter evaluations of male and female rats are presented in Tables -4 and 5. There were no significant changes in males of the treatment group in comparison to the control and recovery group of animals respectively. However, groups G1 and G4 show a significant difference in neutrophil count.

Table 4: Depiction of 90 days sub-acute toxicity study in rats (hematology parameters in rats, male n=10), here Hb represents Hemoglobin, RBC – RED BLOOD CORPUSCLES
TC – TOTAL WBC COUNT, P – Polymorph/neutrophil, L – Lymphocyte, E – Eosinophil, M – Monocyte, PLT – Platelet, PCV – PACKED CELL VOLUME

MALE									
Control/Normal (G1)									
	Hb	**RBC**	**TC**	**P**	**L**	**E**	**M**	**Plt**	**PCV**
MEAN	13.0572	7.99813	14136.2	27.785	52.7044	0.67363	1.52072	647981	46.4998
SD	0.97775	0.36286	6256.23	4.87739	7.94145	0.82327	0.94868	67965.5	3.34139
STD ERROR	0.30919	0.11475	1978.39	1.54236	2.51131	0.26034	0.3	21492.6	1.05664
400mg/Kg (G4)									
MEAN	13.4385	8.18669	13140	29.799	54.2613	0.67363	0.93675	689919	47.4722
SD	0.80664	0.18257	6230.28	7.28469	6.94102	0.82327	0.94281	181598	3.31698
STD ERROR	0.25508	0.05774	1970.19	2.30362	2.19494	0.26034	0.29814	57426.5	1.04892
Recovery Group (G6)									
MEAN	16.22	9.22	11880	32.2	65.6	0.6	1.6	930600	51
SD	0.91	0.26	6647.33	1.48	1.82	0.89	0.55	109999	3.74
STD ERROR	0.41	0.12	2972.78	0.66	0.81	0.4	0.24	49193.1	1.67

Table 5: Depiction of 90 days sub-acute toxicity study in rats (hematology parameters in rats, female n=10)

Female									
Control/Normal (G1)									
	Hb	**RBC**	**TC**	**P**	**L**	**E**	**M**	**Plt**	**PCV**
MEAN	12.92	7.75	13082.6	25.12	56.53	0.63	1.46	677210	47.06
SD	1.59	0.49	7806.72	4.76	5.19	0.7	0.86	136730	5.08
STD ERROR	0.53	0.16	2602.24	1.58	1.73	0.23	0.28	45576.6	1.69
400mg/Kg (G4)									
MEAN	13.16	7.84	15145.2	20.69	56.59	0.94	0.84	715468	47.79
SD	1.26	0.6	7554.07	7.84	13.78	1.05	0.87	67321.9	4.49
STD ERROR	0.39	0.19	2388.8	2.48	4.36	0.33	0.27	21289	1.42
Recovery Group (G6)									
MEAN	14.92	8.78	8800	28.6	68	0.4	1	757400	49.8
SD	1.36	0.29	1850.68	6.91	10.32	0.55	0.71	160863	5.43
STD ERROR	0.61	0.13	827.65	3.09	4.62	0.24	0.32	71940	2.43

5.3 Analysis of histology and neurological evaluation

The histology of the vital organ is represented in Figures 4 A and B provided in the supplementary information. The neurological evaluation and functional observations were conducted during the last week of the experiment period for all animals. During this study period, no abnormal changes were observed for various parameters of neurological evaluation and functional observations of the animals across all the treatment groups (Table S1).

Discussion

Probiotics have gained significant attention over some time and are required as a supportive treatment along with conventional therapeutics (TA *et al*. 2010) [9]. Probiotics are available as dietary ingredients. This research activity deals with comprehensive clinical tests that comprise biochemical, histology, and histopathological parameters. All the animals were observed for general behavior, mortality, and neurological reflexes during the study period. No abnormal behavior was observed in the animals used for the study. None of the animals died during the study period. The animals were normal and healthy. None of the toxic symptoms related to CNS, ANS, CVS, and GIT were observed in the animals. Similar, results were visible in a study reported by Carmichael *et al*., 2006 [13]. Administration of formulation did not influence the growth in rats as evidenced by the insignificant differences in body weights between control and treated groups. The growth pattern was normal in rats during the study period as evident from regular assessment. On certain days the body weights decreased and increased. There was no significant difference between the control and treated groups. The food consumption was comparable between the control and treated groups and the difference between the control and treated was not significant. The food consumption in the animal was in accordance with the growth pattern observed. The water consumption in the control and treated group are comparable and statistically, it is not significant. All biochemical parameters studied were in the normal range in all groups such as control, low, medium, and high dose. The differences observed in the control and various test groups were statistically insignificant. Similarly, Barton *et al*. 2006 [13, 14] reported various measurements of toxicity. Minor differences were observed and were well within the normal ranges. The administration of formulation did not influence the clinical chemistry parameters. The hematological parameters studied in the control and other treated groups were in the normal range. The formulation of *Bacillus coagulans* 50 billion cfu/g oral administration did not alter the hematological parameters in the treated groups. The differences observed in the control and various test groups were statistically insignificant. Minor differences were observed and were well within the normal ranges. No abnormal changes were observed for the organs in the control and treated group animals (Macroscopic study). The organs such as lungs, liver, kidney, heart, spleen, ovaries/testes, and brain appeared to be normal. The wet weight of all organs expressed as percent body weights were comparable between control and other treated groups.

Conclusion

The present study of *Bacillus coagulans* LMG S-31876 studied at 50 Billion cfu/g" administered orally did not influence any of the above parameters in rats in the 90 days chronic toxicity study in the doses studied. There were no other significant, treatment-related adverse effects on body weights, food consumption, organ weights, hematology, and clinical chemistry parameters of animals in all groups when compared to the Vehicle control group. There were no test material-related histopathological changes at G4in both sexes. The recovery group was observed for 28 days and no significant observations were made and the results were comparable to the normal group. Hence, the NOAEL (No-Observed Adverse Effect Level) for the present study is 400 mg/kg body weight in both sexes.

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Author's Contribution

RK: Conceptualization
SM: Design, Execution
AM: Execution, implementation

Conflict of interest

Authors declare no conflict of interest

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