“DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS”

Thesis Submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH

(Deemed-to-be -University)

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

Accredited ‘A+’ Grade by NAAC (3rd Cycle)

Placed in Category ‘A’ by MoE (GoI)

For the award of the degree of

Doctor of Philosophy

In the Faculty of Ayurveda

By

Dr.TONNI SANJEEV SHIVAPPA M.D (Ayu)

(Registration No: KLEU/Ph.D/D01217007

Under the Guidance of

Dr.RUDRAMMA. R. HIREMATH M.D (Ayu), Ph.D

& Co-Guidance of

Dr.NAYEEM KHATIB M.Pharma, Ph.D

KAHER’s SHRI.BMK AYURVEDA MAHAVIDYALAYA, SHAHAPUR, BELAGAVI.

2023

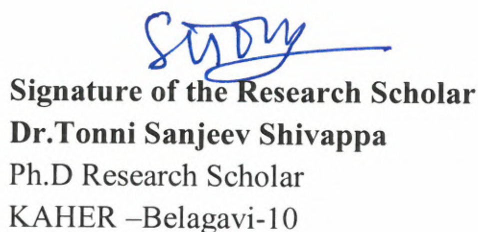
UNDERTAKING

I, **Dr.TONNI SANJEEV SHIVAPPA** hereby declare that the information and the data mentioned in my thesis entitled "**DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS"** belongs to me and is original.

I am aware of definition of plagiarism as detailed below:

- An act or instance of using or closely imitating the language and thoughts of another author without authorization and the representation of that author's work as one's own, as by not crediting the original author.
- A piece of writing or other work reflecting such unauthorized use or imitation.
- The deliberate or reckless representation of another's words, thoughts or ideas as one's own without attribution in connection with submission of academic work, whether graded or otherwise.

I hereby declare that the thesis prepared by me is original-one and does not involve plagiarism anywhere. In case at a later stage it is found that I have indulged in plagiarism, then I am solely responsible for the same and the Institution is at liberty to take any disciplinary action against me including cancellation of dissertation or any other penalties imposed by the University.



Date: 12.09.2023
Place: Belagavi

Signature of the Research Scholar
Dr.Tonni Sanjeev Shivappa
Ph.D Research Scholar
KAHER –Belagavi-10

PLAGIARISM REPORT

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Formerly known as KLE University)
(Deemed-to-be-University established u/s 3 of the UGC Act, 1956)
Accredited A+ Grade by NAAC (3rd Cycle) Placed in Category 'A' by MHRD (GoI)
JNMC Campus, Nehru Nagar, Belagavi-590 010, Karnataka State, India
☎: 0831-2444444 Web: http://www.kledeemeduniversity.edu.in E-mail: info@kledeemeduniversity.edu.in

Ref. No. KAHER/AA/23-24/D-218 8th August 2023

Sir,

The soft copy of Ph.D. research thesis of **Dr. Tonni Sanjeev Shivappa, Faculty of Ayurveda, KAHER, Belagavi** has been submitted for anti-plagiarism check at the office of the undersigned through "Turn-it-in" package. The scan has been carried out and the scanned output reveals a match percentage of **7%** which is within the acceptable limit of 10%.

To obtain the comprehensive report of the plagiarism test, research scholar can send a mail to **diracademic@kledeemeduniversity.edu.in** along with the Registration Number, Name of the Scholar, Name of Guide/Co-guide and title of the thesis.

Dr.(Mrs.) Roopa M. Bellad
Director, Academic Affairs

To,

Dr. Tonni Sanjeev Shivappa
Part-Time Ph.D. Scholar,
2017-18 Batch
KLE Shri. B.M. K. Ayurveda Mahavidyalaya,
Faculty of Ayurveda, KAHER
Belagavi.

Cc to :

1. The Principal, KLE Shri. B.M. K. Ayurveda Mahavidyalaya, Belagavi
2. Dr. Rudramma R. Hiremath, Professor, KLE Shri. B.M. K. Ayurveda Mahavidyalaya, Belagavi Guide
3. Dr. N. A. Khatib, Associate Professor of Pharmacology, KLE College of Pharmacy, Belagavi-Coguide

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed-to-be-University)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
Re-Accredited 'A+' Grade by NAAC (3rd Cycle)
Placed in Category 'A' by MoE (GoI)

Copyright Declaration

We hereby declare that **KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA,** *shall have the rights to preserve, use and disseminate this thesis in print or electronic format for academic/research purpose.*

Signature	**Signature**
Name of Research Scholar	Name & Designation of Research Guide
Dr.Tonni Sanjeev Shivappa MD (Ayu)	**Dr.Rudramma R Hiremath** MD (Ayu), PhD.
Part – Time Ph.D Research Scholar	Prof & Head, Dept of Agadatantra
KAHER –Belagavi-10	KAHER's Shri.BMK Ayurveda Mahavidyalaya, Shahapur, Belagavi.
	HOD Department of Agada Tantra KAHER's Shri B.M.K. Ayurveda Mahavidyalaya, Shahapur, Belagavi.
Place: Belagavi	Place: Belagavi
Date: 12.09.2023	Date: 12.09.2023

© KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed-to-be-University)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
Re-Accredited 'A+' Grade by NAAC (3rd Cycle)
Placed in Category 'A' by MoE (GoI)

Declaration

I hereby declare that the thesis entitled **"DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS"** is a bonafide and original research carried out by me under the guidance of **Dr.Rudramma R Hiremath** M.D (Ayu), Ph.D Professor & Head, Department of Agadatantra, KAHER's Shri B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur Belagavi. *The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.*

Place: Belagavi
Date: 12.05.2023

Signature
Name & Address of the Research Scholar
Dr.Tonni Sanjeev Shivappa M.D (Ayu)
Part – Time Ph.D Research Scholar
Faculty of Ayurveda
KAHER's Shri.BMK Ayurveda
Mahavidyalaya, Shahapur, Belagavi.

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed-to-be-University)

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

Re-Accredited 'A+' Grade by NAAC (3rd Cycle)
Placed in Category 'A' by MoE (GoI)

Certificate

This is to certify that the thesis entitled "**DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS**" is a bonafide and genuine research carried out by **Dr.Tonni Sanjeev Shivappa** M.D (Ayu) under the guidance of **Dr.Rudramma R Hiremath** M.D (Ayu), Ph.D Professor & Head, Department of Agadatantra, KAHER's Shri. B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur Belagavi.

Place: Belagavi
Date: 12-09-2023

Signature
Name & Address of Dean
Dr.Prashant G Jadar M.D (Ayu), Ph.D
Dean,
KAHER's Shri.BMK Ayurveda
Mahavidyalaya, Shahapur, Belagavi.

DEAN
Faculty of Ayurveda
KAHER'S
Shri B.M.K. Ayurveda Mahavidyalaya
Shahapur, Belagavi-590003.

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH

(Deemed-to-be-University)

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

Re-Accredited 'A^{+}' Grade by NAAC (3rd Cycle)

Placed in Category 'A' by MoE (GoI)

Certificate

This is to certify that the thesis entitled "**DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS**" is a bonafide and genuine research carried out by **Dr.Tonni Sanjeev Shivappa** under the guidance of **Dr. Rudramma R Hiremath,** Professor and Head, Department of Agadatantra, KAHER's Shri. B.M.Kankanawadi Ayurveda Mahavidyalaya Shahapur, Belagavi.

Place: Belagavi

Date:

Signature

Dr.Suhas Kumar Shetty **M.D (Ayu), Ph.D**

Principal

KAHER's Shri.BMK Ayurveda

Mahavidyalaya, Shahapur, Belagavi.

PRINCIPAL
Shri B. M. Kankanawadi
Ayurved ... dyalaya
A Constituen... of KAHER
Shahapur, BELAGAVI-03.

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed-to-be-University)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
Re-Accredited 'A^{+}' Grade by NAAC (3rd Cycle)
Placed in Category 'A' by MoE (GoI)

Certificate

This is to certify that the thesis entitled "**DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS**" is a bonafide record of original research carried out by **Dr.Tonni Sanjeev Shivappa** for the award of degree of DOCTOR OF PHILOSOPHY IN FACULTY OF AYURVEDA under my supervision and guidance.

Place: Belagavi

Date: 12.09.2023

Signature

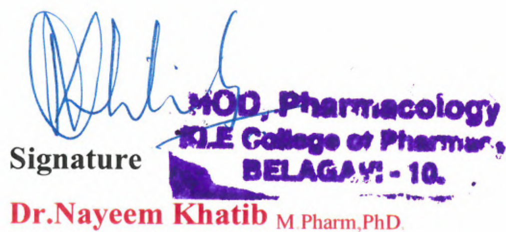
Dr.Rudramma R Hiremath **M.D (Ayu), Ph.D**
Prof & Head, Dept of Agadatantra
KAHER's Shri.BMK Ayurveda
Mahavidyalaya, Shahapur, Belagavi.

HOD
Department of Agada Tantra
KAHER's Shri B.M.K. Ayurveda
Mahavidyalaya, Shahapur, Belagavi.

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed-to-be-University)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
Re-Accredited 'A^{+}' Grade by NAAC (3rd Cycle)
Placed in Category 'A' by MoE (GoI)

Certificate

This is to certify that the thesis entitled "**DEVELOPMENT OF NEW AYUR-PROBIOTIC FORMULATION AND ITS EVALUATION IN EXPERIMENTAL ANIMALS**" is a bonafide record of original research carried out by **Dr.Tonni Sanjeev Shivappa** for the award of degree of DOCTOR OF PHILOSOPHY IN FACULTY OF AYURVEDA under my Co-guidance.



Place: Belagavi
Date:

Signature
Dr.Nayeem Khatib M.Pharm,PhD
Prof & Head, Dept of Pharmacology
KLE College of Pharmacy,
KAHER, Belagavi, Karnataka.

ACKNOWLEDGMENT

I am ever grateful to my guide Dr. Rudramma R Hiremath M.D(Ayu),Ph.D Prof & Head, Department of Agadatantra, KAHER's Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi for the magnitude of her guidance, encouragement throughout the study.

I express my deep sense of gratitude to my Co-Guide Dr.Nayeem Khatib M.Pharm,Ph.D Prof & Head, Department of Pharmacology, KLE College of Pharmacy, Nehru nagar, Belagavi for his non enervative timely guidance.

I express my deep sense of gratitude to Dr.Ashok Patil MD (Ayu), Ph.D, Professor and HOD, Department of Swasthavritta & Yoga for his complete cooperation, support and encouragement.

I wish to express my gratitude to Dr.Suhas Kumar Shetty MD (Ayu), Ph.D Principal & Medical Director of the institute, Dr.P.G.Jadar MD(Ayu), Ph.D Dean / Vice-Principal, Dr.B.S.Prasad MD(Ayu), Ph.D President, Board of Ayurveda, NCISM, NewDelhi, Dr.S.S.Jalalpure M.Pharm,Ph.D Principal, KLE College of Pharmacy, Dr.P.A.Patil, Dr.A.S.Ammanagi, Dr.Adavesh Holeyachi, Dr.Santosh Patil, Dr.Sunil Doamani, Dr.M.B.Gundkalle, Mr.Ajit Lingayat, Mr.S.B.Desai, Dr.Sandeep Sagare, Dr.Amal S C, Dr.Shruti Shet for their valuable suggestions regarding anti-microbial, analytical and experimental work.

My gratitude due to Dr. Amruta Kalyani, Dr.Vani Adake, Dr.Karuna Patil, Dr.Karishma, Dr.Sidhi Mutalik PrabhuDesai, Dr.Raktimabha, Dr.Arshia, Dr.Pooja, Dr.Alok, Dr.Astha, Dr.Arpit, Dr.Suma, Dr.Anju Yadav, Dr.Rudri, Dr.Jyoti K, Dr.Shashi Nayak, Dr.Srujana & Dr.Akankshya for their support in procedural work. I wish to express my gratitude to the authorities of KAHER for providing me all the requisite facilities to carry out this work.

Date:

Place: Belagavi **Dr.Tonni Sanjeev Shivappa**

LIST OF ABBREVIATIONS USED

- AAD : Antibiotic Associated Diarrhoea
- LAB : Lactic Acid Bacteria
- ABPM : Apakwa Bilwa Phala Majja
- ABPSD : Apakwa Bilwaphalasaara Dadhi
- FAO : Food Agriculture Organization
- WHO : World Health Organization
- FDA : Food & Drug Administration
- Ch.Sam : Charaka Samhita
- Su.Su : Sushruta Samhita
- C.F.U : Colony Forming Unit
- CPCSEA : Committee for the Purpose of Control and Supervision on Experimental Animals
- ˚C : Degree Centigrade
- gm : Gram
- hr : Hour
- I.A.E.C : Institute Animal Ethical Committee
- IP : Indian Pharmacopoeia
- K.g : Kilo gram
- LOD : Loss on Drying
- LD50 : Lethal dose 50
- mm : Millimeter
- % : Percentage
- ± : Plus-Minus Sign
- Q.S : Quantity Sufficient
- S : Sensitive
- Sl. No : Serial Number
- S.E : Standard Error
- S.D : Standard Deviation
- W.H.O : World Health Organization

ABSTRACT

In the present era, the concept of "**PROBIOTICS**" is inspiring in food and pharmaceutical industry. Probiotics are live bacteria that, if consumed in adequate amounts, provide the host with some health benefits. Probiotics like Lactobacillus and Bifidobacterium are most frequently utilized. Probiotics have been linked to anti-infection properties, a decrease in serum cholesterol, relief from lactose intolerance, and an improved balance of the intestinal microbial flora, which can treat diarrhoea in all its forms, including antibiotic-associated diarrhoea (AAD), traveller's diarrhoea, diarrhoea brought on by infection (both bacterial and viral forms), constipation, the synthesis of vitamins, and nutritional enhancement. About 5–30% of individuals get antibiotic-associated diarrhoea (AAD), either at the beginning of their antibiotic medication or for as long as two months after it has ended. So, one such drug in Ayurveda named Bilwa (**Aegle marmelos. Linn**), especially about its tender young fruit *(apakwa bilwa phala ie,)* which possesses *katu* (bitter), *tikta* (pungent), *kashaya* (astringent), *teekshna, snigdha* guna, *ushna* veerya and finest *samgrahika* (capable of absorbing fluids from the gastrointestinal tract), *deepaniya* (appetisers) and pacifies *kapha* and *vata dosha*[9,10,11]. *Apakwa bilwa phala* is having *marmelosin, luvangetin, aurapten, psoralen, marmelide & tannin* as an active ingradients. With considering all these supportive evidences, one such study is planned to develop "*Bilwaphala saara dadhi*" an Ayur-probiotic preparation which includes *apakwa bilwaphala majja in dadhi media.* And the same preparation will be used for evaluating its effect in Antibiotic Associated Diarrhoea (AAD) in the Wister rats.

Aim:

- Development and efficacy study of *an* Ayur-Probiotic (Bilvaphalasaara dadhi*)* preparation

Objectives:

- To develop an Ayur-Probiotic preparation and assessment of its bacterial load.
- To evaluate the efficacy of Ayur-Probiotic in Antibiotic associated diarrhoea in experimental animals.

Materials & Methods: Materials:

- o Curd
- o Un-ripened bilva (Aegle marmelos Linn) fruit (pulp)
- o Amoxicillin trihydrate
- o Sporlac sachets
- o Wistar rats

The Research study was carried out in two steps ie, Preparation & development of Ayur-Probiotic formulation & Experimental study of Ayur-Probiotic on Antibiotic Associated Diarrhoea (AAD) in Wistar rats.

I. Preparation of Ayur-Probiotic:

Ayur-Probiotic was prepared by Lyophilization of Apakwa bilwaphala majja with curd. This procedure was conducted in different ratio of combinations of Apakwa bilwaphala majja with curd. A lyophilized product was subjected to Phyto-chemical parameters, Physico-chemical parameters, organoleptic characteristics & Assessment of Lactobacillus bacteria. The analysis of Ayur-Probiotic was done at KAHER's BSRC Laboratory, Belagavi.

II. Experimental Study of Ayur-Probiotic:

Wistar rats were procured from Shri. Raghavendra Enterprises; Breeders & Suppliers of Animals (Govt. of India Reg. 841/b/04/CPCSEA) Vijaya Nagar,

Bengaluru, kept at an ambient temperature 25 ± 5°C and relative humidity 45-55% with 12 hours light/dark cycles as per CPCSEA Guidelines.

Wistar rats were acclimatized in the laboratory for one week and provided to access standard commercial feed pellets and clean water *ad libitum* throughout the experiment periods.

Study design:

48 Healthy Wistar rats of either sex weighing 150-180gm were selected for the study & divided into 8 groups with 6 rats in each group. Male & female animals were separated and each 3 animals were kept in polypropylene cages. All animals were marked as Head, Body & Tail for all the eight group animals. Initial weights of each animal were recorded. Potable drinking water & food pellets were provided daily.

Evaluation of experimental animal fecal/cecal sample:

Stool samples and caecal contents were obtained under aspetic circumstances to ascertain the

- ❖ Counts of microbial colonies (in CFU)
- ❖ Quantitating the various bacterial species (qPCR)
- ❖ CD (Clostridium difficile) toxin evaluation

The following organs' histo-pathological studies were conducted:

- ❖ Cecum, Anus, and Rectum

Results: Group-B has shown significantly less in water intake when compared to D, E, F, G & H groups ($P<0.001$). There was a relative decrease in water intake till 14th day ($p<0.05$) in all the groups observed but after the 21st day gradual increase was seen. Group-D (ie, Bilwa phala majja) showed increase in water intake when compared to Group- E, F, G & H ($p<0.001$). In comparison to group A, all the groups had relative food intake through day 30 (P 0.01). Up to the 21st day, there was a

significant decrease in food consumption; after that, there was a gradual rise in food intake in Group A (P 0.05). The Group-A (ie, Amoxicillin) Showed less increase in relative body weight when compared to other Groups may be due to Antibiotic induced diarrhoea till 30 days. Group-B, C, G & H did not exhibit significant body weight gain till 30th day. However the Group-D (ie, Bilwa phala majja) showed drastic reduction of weight (27.47%) from 7th to 14th day and gradual increase in weight seen till 30th day. Group E & F shows no significant difference from 7th to 14th day but after 14th day relative increase can be seen. Stool examination of all rats except Group-B (Control) showed watery diarrhoea on administration of amoxicillin after 7th day onwards.

Microbial colony count of organisms was done by serial dilution method. In 10^{-1} dilution, the control group, Bilwaphala majja and ayur-probitic group showed less count of microorganisms. Group-C (ie, Curd), group-G (ie, Amoxicillin+sporlac) showed moderate count but group-H (ie, Amoxicillin+Ayur-probiotic) showed optimum count of microorganism. This protects the microbial flora of the intestine by its classical properties.

Stool specimens were examined for C. difficile toxin presence, found that, all the groups except Control Group ie, Group-B ($P<0.0001$). It may be due to the presence of amoxicillin induced diarrhoea in other groups. qPCR showed Quantifying the different bacterial groups in all experimental groups such as Enterococcus faecalis, Bacillus subtilis & other organisms. Results of samples of all the groups are matching with respective strain no & suspected organisms. There was significant decrease in inflammation of caecum samples seen in the groups-C, D, E, F, G & H when compared to control group(B) ($P<0.01$) and group-G (ie, Antibiotic + Sporlac). Inflammation is reduced when compared to Group-E (Sporlac) and Group-H (ie,

Ayur-Probiotic) (P<0.005). The analysis of histo-pathological scoring in caecum, anus & rectum shown, Ayur-Probiotic formulation administered group-F & H reported improvement in villus height compared with the group-A. No remarkable changes seen in histo-pathological report of Ayur-Probiotic treated group-F & H group compared to those groups either treated with group-C (curd) or with group-D (Apakwa bilwaphala majja) which indicate that, effect of Ayur-Probiotic formulation alone in promoting mucosal healing to normal mucosa.

Discussion: On 31st day, during dissection of Group-C (Curd) animals, intestine of all the sacrificed animals shown more gaseous contents because of curd consumption. Intestine of sacrificed animals of Group-B & E, shown gaseous content because of Amoxycillin. Intestine of sacrificed animals of Group-H, shown hard and well-formed stool. Ayur-Probiotic had positive benefits on its host without altering the long-term microbiome, which was made feasible by the intestine's temporary colonisation. As a result, using an Ayur-probiotic during the use of antibiotics may safeguard gut homeostasis. The main outcome of this study is that giving experimental animals an Ayur-probiotic formulation dramatically reduces their probability of developing AAD (Antibiotic Associated Diarrhea). There was no significant improvement observed between the groups in the histo-pathological results of the ano-rectal samples of animals in relation to congestion, oedema, haemorrhage and inflammation and loss of intestinal glands. There was no significant improvement observed between the groups in the histo-pathological results of the caecum samples of animals in relation to congestion, oedema, haemorrhage, destruction of villi and mucosal ulcers except inflammation (P<0.0001).

Conclusion: In the present study, Ayur-Probiotic formulation treatment shown significant gastro-intestinal protective effect in AAD (Antibiotic Associated

Diarrhoea) in experimental animals. Hence, it could be novel therapeutic formulation in clinical practice & antibiotic associated damage can be prevented by using this formulation.

Keywords: Apakwa bilwaphala majja, Dadhi, Lyophilisation, Ayur-Probiotic, Lactobacillus, Antibiotic Associated Diarrhoea

TABLE OF CONTENTS

Sl. No.		Particulars	Page No.
1.	**1.1**	**ABSTRACT**	**xii**
	1.2	**INTRODUCTION**	**1-3**
	1.3	**BACKGROUND**	**4-6**
2.		**AIM & OBJECTIVES**	**7**
3.		**REVIEW OF LITERATURE:** • Probiotic: • Bilwa: • Cow Milk (Go-Ksheera): • Curd (Dadhi): • Lyophilization:	**8-65**
4.		**JUSTIFICATION**	**66**
5.		**MATERIAL AND METHODS**	**67-92**
	5.1	Formulation Development Study	
	5.2	Quality Control Study	
	5.3	Experimental Study	
6.		**OBSERVATION AND RESULTS**	**93-188**
	6.1	Formulation development study results	
	6.2	Quality Control study results	
	6.3	Experimental study results	
7.		**DISCUSSION**	**189-197**
8.		**CONCLUSION**	**198**
9.		**SUMMARY**	**199-202**
10.		**LIMITATIONS OF THE STUDY**	**203**
11.		**RECOMMENDATIONS FOR FURTHER STUDY**	**204**
12.		**BIBLIOGRAPHY**	**205-232**
		ANNEXURES	**233-303**

LIST OF TABLES

Sl.No	Contents	Page.No
1.	Table no.1 showing nutritional value of Bilwaphala (per 100 g)	**35**
2.	Table.no.2 showing the properties of *pakwa and apakwa bilwa phala*	**36**
3.	Table no.3 showing list of the properties of Bilva in Brihattrayi	**36**
4.	Table no.4 showing Pharmacological Activity of Aegle marmelos Corr.	**40**
5.	Table.no.5 showing nutritional value of cow milk (per 100 gm)	**44**
6.	Table.no. 6 showing the characteristics of dadhi as stated by Bruhatrayee	**49**
7.	Table.no.7 showing dadhi as a Hetu (Aetiology)	**50**
8.	Table.no.8 showing the presence of Guna and Karma of Dadhi in the disease prognosis	**51**
9.	Table.no.9 Showing the relationship among Guna and Rogakaratva	**52**
10.	Table.no.10 showing relevance between Karma & Rogakaaratva	**52**
11.	Table.no.11 showing Lyophilized sample in different ratio of curd and *Bilva phala majja*	**71**
12.	Table no.12 showing the results of organoleptic characters of the Lyophilized samples	**71**
13.	Table no.13 showing the results of Phyto-chemical analysis of the samples	**72**
14.	Table.no.14 showing the results of Physico-Chemical properties of the Lyophilized samples	**72**
15.	Table.no.15 showing the ratio of curd and *apakwa bilva phala majja*	**73**
16.	Table.no.16 showing observation & result of samples assessed	**75**
17.	Table.no.17 showing the ratio of curd and apakwa Bilva phala majja	**77**
18.	Table.no.18 showing observation & results of the samples assessed	**79**
19.	Table.no.19 showing quantity of curd & *apakwa bilwaphala majja*	**80**

20.	*Table.no.20 showing the dose schedule for – Acute toxicity study*	**87**
21.	Table.no.21 showing experimental animal group details	**89**
22.	Table.no.2**2** showing analysis results of cow milk sample	**93**
23.	*Table.no.23 showing analysis results of curd sample*	**94**
24.	Table.no.24 showing authentication of Apakwa Bilwa phala	**95**
25.	Table.no.25 showing analytical study results of Apakwa Bilva Phala	**95**
26.	Table.no.26 showing the result of Phyto-chemical analysis of the Lyophilized sample	**96**
27.	Table.no.27 showing the result of Physico-Chemical properties of the Lyophilized sample	**97**
28.	Table.no.28 showing the result of organoleptic characters of the Lyophilized sample	**97**
29.	Table no.29 showing observations of five animals throughout the acute toxicity study	**100**
30.	Table.no.30 showing the statistical results of ANOVA test on food intake on 7th day	**102**
31.	Table.no.31 showing the statistical results of Dunnett's test on food intake on 7th da	**103**
32.	*Table.no.32 showing the statistical results of ANOVA test on food intake on 15th day*	**105**
33.	Table.no.33 showing the statistical results of Dunnett's test on food intake on 15th day	**106**
34.	*Table.no.34 showing the statistical results of ANOVA test on food intake on 21st day*	**108**
35.	Table.no.35 showing the statistical results of Dunnett's test on food intake on 21st day	**109**
36.	*Table.no.36 showing the statistical results of ANOVA on 30th day*	**111**
37.	Table.no.37 showing the statistical results of Dunnett's test on food intake on 30th day	**112**

38.	Table.no.38 showing the statistical results of ANOVA on water intake on 7th day	**114**
39.	Table.no.39 showing the statistical results of Dunnett's test on water intake on 7th day	**115**
40.	*Table.no.40 showing the statistical results of ANOVA on water intake on 15th day*	**116**
41.	Table.no.41 showing the statistical results of Dunnett's test on water intake on 15th day	**117**
42.	*Table.no.42 showing the statistical results of ANOVA test on water intake on 21st day*	**118**
43.	Table.no.43 showing the Statistical results of Dunnett's test on water intake on 21st day	**119**
44.	*Table.no.44 showing the statistical results of ANOVA test on water intake on 30th day*	**121**
45.	Table.no.45 showing the Statistical results of Dunnett's test on water intake on 30th day	**122**
46.	Table.no.46 showing the statistical results of ANOVA test on body weight on 7th day	**124**
47.	Table.no.47 showing the statistical results of Dunnett's test on on body weight on 7th day	**125**
48.	*Table.no.48 showing the statistical results of ANOVA test on body weight on 15th day*	**126**
49.	Table.no.49 showing the statistical results of Dunnett's test on body weight on 15th day	**127**
50.	*Table.no.50 showing the statistical results of ANOVA test on body weight on 21st day*	**129**
51.	Table.no.51 showing the statistical results of Dunnett's test on body weight on 21st day	**130**
52.	*Table.no.52 showing the statistical results of ANOVA test on body weight on 30th day*	**132**

53.	Table.no.53 showing the statistical results of Dunnett's test on body weight on 30th day	**133**
54.	Table.no.54 showing samples plates and their colony count	**136**
55.	Table.no.55 showing identification of Bacteria & their Morphology	**137**
56.	Table.no.56 showing Kruskal Wallis ANOVA test was used to compare eight groups with microbial colony numbers	**138**
57.	Table.no.57 showing pair wise comparisons of eight groups with microbial colony counts by Mann-Whitney U test	**139**
58.	Table.no.58 showing results of Clostridium difficile in stool samples	**141**
59.	Table.no.59 showing comparison of eight groups with status of C.Difficille	**143**
60.	Table.no.60 showing comparison of eight groups with status of test results	**144**
61.	Table.no.61 showing results of sample matching with respective strain no. & suspected organisms	**145**
62.	Table.no.62 showing comparison of eight groups with status of Congestion	**155**
63.	Table.no.63 showing comparison of eight groups with status of Congestion by Kruskal Wallis ANOVA	**156**
64.	Table.no.64 Mann-Whitney U test pairwise comparisons of eight groups with Congestion status	**157**
65.	Table.no.65 showing comparison of eight groups with status of Odema	**159**
66.	Table.no.66 Comparison of eight groups with status of Odema by Kruskal Wallis ANOVA	**159**
67.	Table.no.67 Mann-Whitney U test pairwise comparisons of eight groups with Odema status	**160**
68.	Table.no.68 showing comparison of eight groups with status of Haemorrhage	**162**

69.	Table.no.69 showing comparison of eight groups with status of Haemorrhage by Kruskal Wallis ANOVA	**162**
70.	Table.no.70 Mann-Whitney U test pairwise comparisons of eight groups with Haemorrhage status	**163**
71.	Table.no.71 showing comparison of eight groups with status of Inflammation	**165**
72.	Table.no.72 showing comparison of eight groups with status of Inflammation by Kruskal Wallis ANOVA	**165**
73.	Table.no.73 showing pair wise comparisons of eight groups with status of Inflammation by Mann-Whitney U test	**166**
74.	Table.no.74 showing comparison of eight groups with status of Loss of Intestinal glands	**168**
75.	Table.no.75 showing comparison of eight groups with status of Mucosal Ulcers	**168**
76.	Table.no.76 showing comparison of eight groups with status of Destruction of Villi	**169**
77.	Table.no.77 showing comparison of eight groups with status of Congestion	**169**
78.	Table.no.78 showing comparison of eight groups with status of Congestion by Kruskal Wallis ANOVA	**170**
79.	Table.no.79 Mann-Whitney U test pairwise comparisons of eight groups with Congestion status	**171**
80.	Table.no.80 showing comparison of eight groups with status of Odema	**173**
81.	Table.no.81showing comparison of eight groups with status of Odema by Kruskal Wallis ANOVA	**173**
82.	Table.no.82 Mann-Whitney U test was used to compare the status of eight groups with Odema.	**174**
83.	Table.no 83. showing comparison of eight groups with status of Haemorrhage	**176**

84.	Table.no.84 showing comparison of eight groups with status of Haemorrhage by Kruskal Wallis ANOVA	**176**
85.	Table.no.85 The Mann-Whitney U test for pairwise comparisons of eight groups with Haemorrhage status	**177**
86.	Table.no.86 showing comparison of eight groups with status of Inflammation	**179**
87.	Table.no.87 showing comparison of eight groups with status of Inflammation by Kruskal Wallis ANOVA	**179**
88.	Table.no.88 Mann-Whitney U test was used to compare the level of inflammation in eight groups	**180**
89.	Table.no.89 showing comparison of eight groups with status of Mucosal Ulcers	**182**
90.	Table.no.90 showing comparison of eight groups with status of Mucosal Ulcers by Kruskal Wallis ANOVA	**182**
91.	Table.no.91 Mann-Whitney U test was used to compare the status of eight groups with Mucosal Ulcers	**183**
92.	Table.no.92 showing comparison of eight groups with status of Loss of Intestinal glands	**185**
93.	Table.no.93 showing comparison of eight groups with status of Destruction of Villi	**185**
94.	Table.no.94 showing micro-organisms with claimed Probiotic Properties	**25**

LIST OF FIGURES

Sl.No	Content	Page No.
01	Fig.no.1 showing Pioneers of Probiotics	**18**
02	Fig.no.2 showing growth of probiotic organisms (LAB) in sample 1, 2 &3	**75**
03	Fig.no.3 showing growth of LAB organisms in 6 gm, 3 gm & 1.5 gm samples	**78**
04	Fig.no.4 showing Lyophilized product of the sample	**82**
05	*Fig.no.5 showing culture analysis of the Lyophilized sample:*	**82**
06	Fig.no.6 showing growth of LAB organisms in the Lyophilized culture analysis of Ayur-Probiotic	**83**
07	Figure.no.7 Photomicrograph of a section of wall of the Ano-Rectum & Caecum of the control group (Normal)	**186**
08	Figure.no.8 Photomicrograph of a section of wall of the Ano-Rectum and Caecal of the disease group	**186**
09	Figure.no.9 Photomicrograph of a section of wall of the Ano-Rectum & Caecum of the curd group	**187**
10	Figure.no.10 Photomicrograph of a section of wall of the Ano-Rectum of the BPM (Bilwaphala Majja) group	**187**
11	Figure.no.11 Photomicrograph of a section of wall of the Ano-Rectum of the Standard group	**187**
12	Figure.no.12 Photomicrograph of a section of wall of the Ano-Rectum of the test drug group	**188**
13	Figure.no.13 Photomicrograph of a section of wall of the Ano-Rectum of the Standard (Antibiotic + Sporlac) group	**188**
14	Figure.no.14 Photomicrograph of a section of wall of the Ano-Rectum of the Prophylactic (Antibiotic +Ayur-Probiotic) group	**188**

LIST OF GRAPH / BAR DIAGRAMS

Sl.No	Content	PageNo.
01	Graph no.1 showing cumulative food intake value for 30 days in experimental animals	**114**
02	Graph no.2 showing cumulative water intake value for 30 days in experimental animals	**124**
03	Graph no.3 showing cumulative Body weight value for 30 days in experimental animals	**134**
04	Bar Diagram.no.4 showing comparison of eight groups with microbial colony counts	**140**
05	Bar Diagram.no.5 showing comparison of eight groups with status of C.Difficille	**143**
06	Bar Diagram.no.6 showing comparison of eight groups with status of test results	**144**
07	Bar Diagram.no.7 showing comparison of eight groups with status of Congestion	**158**
08	Bar Diagram.no.8 showing comparison of eight groups with status of Odema	**161**
09	Bar Diagram.no.9 showing comparison of eight groups with status of Haemorrhage	**164**
10	Bar Diagram.no.10 showing comparison of eight groups with status of Inflammation	**167**
11	Bar Diagram.no.11 showing comparison of eight groups with status of Congestion	**172**
12	Bar Diagram.no.12 showing comparison of eight groups with status of Odema	**175**
13	Bar Diagram.no.13 showing comparison of eight groups with status of Haemorrhage	**178**
14	Bar Diagram.no.14 showing comparison of eight groups with status of Inflammation	**181**
15	Bar Diagram.no.15 showing comparison of eight groups with status of Mucosal Ulcers	**184**

INTRODUCTION

In the present era, the concept of "**PROBIOTICS**" is inspiring in food and pharmaceutical industry. Probiotics are live bacteria, if consumed in adequate amounts, provide the host with some health benefits. Probiotics like Lactobacillus and Bifidobacterium are most frequently utilized. Probiotics have been linked to anti-infection properties, a decrease in serum cholesterol, relief from lactose intolerance and an improved balance of the intestinal microbial flora, which can treat diarrhoea in all its forms, including antibiotic-associated diarrhoea (AAD), traveller's diarrhoea, diarrhoea brought on by infection (both bacterial and viral forms), constipation, the synthesis of vitamins and nutritional enhancement[1,2].

In treating the Gastro-Intestinal (GI) disorders especially the infection origin, antibiotics are the drugs of choices. Due to the growing use of wide spectrum antibiotics there was a rise in the frequency of diarrhoea. Some broad spectrum antibiotics such as Amoxicillin Clavulanate[20, 21] (β-lactum) produce an imbalance of intestinal bacterial flora leading to diarrhoea like conditions. The prevalence of Antibiotic Associated Diarrhoea[5] (AAD) is estimated at 5–39%[3, 21]. Therefore many clinicians are trying to prevent or cure intestinal infections with probiotics.

Ayurveda is gaining more importance, in treating Antibiotic Associated Diarrhoea (AAD) like conditions. It explains *dadhi* (curd) & *takra* (buttermilk) are the primary sources of probiotic. These can be found as dairy or non-dairy based products, as well as various fermented foods, medications and nutritional supplements[4,6,7]. These foods contain *Lactobacillus* and *Bifidobacterium* species at

10^9 viable cells per ml at the time of consumption[16,18]. Some plant based ingredients also have supportive effect when prescribed with curd or buttermilk.

So, one such drug in Ayurveda named Bilwa (**Aegle marmelos. Linn**), especially about its tender young fruit *(ie, apakwa bilwa phala)* which possesses *katu* (bitter), *tikta* (pungent), *kashaya* (astringent), *teekshna, snigdha* guna, *ushna* veerya and finest *samgrahika* (capable of absorbing fluids from the gastrointestinal tract), *deepaniya* (appetisers) and pacifies *kapha* and *vata dosha*[9,10,11]. *Apakwa bilwa phala* is having *marmelosin, luvangetin, aurapten, psoralen, marmelide & tannin* as an active ingradients[12,13].

Bi-products of milk such as curd, buttermilk contain various probiotics. The term "probiotics" has a modern definition that relates to friendly live microorganisms, whereas the Ayurvedic concept is more comprehensive[10]. Bacterial isolates from traditionally consumed foods, including Bifidobacterium and Lactic Acid Bacteria (LAB), have been reported to be harmless.

There is a direct reference of the concept of *sahaja krimi* ie, friendly micro-organisms which are located in the intestine explained in *Charaka* & *Sushruta Samhita* under the name "*Avaikaarika krimi"*[15] constitute "Probiotics" in contemporary medical science was well highlighted in the classical textbooks of *Ayurveda*. References of prescribing probiotic like formulations in *Ayurveda* classics in the form of medicated kanji, curd, buttermilk are well explained under *Atisara* and *Grahani chikitsa*[10,11,12,14] which implies that the importance of probiotics was recognized, if not precisely in a comparable way as it is today.

When curd is combined with various herbal drugs or plant extracts, its functionality like anti-oxidative, immuno-modulatory etc will be enhanced. Evidences are there for the Herbal based Probiotic preparations normalizing the composition of the intestinal micro-flora & regulate intestinal permeability & has no adverse effects[15,16,17,18,19,20]. Till date no research study related to herbal incorporated probiotic formulation / preparations are carried out in any Ayurveda institutions.

With considering all these supportive evidences, one such study was planned to develop "*Bilwaphala saara dadhi*" an Ayur-probiotic preparation which includes *apakwa bilwaphala majja in dadhi media.* And the same preparation will be used for evaluating its effect in Antibiotic Associated Diarrhoea (AAD) in the Wister rats.

BACKGROUND

Probiotics is currently one of the most significant terms associated with the goods sold in markets. The microorganisms known as probiotics are beneficial to our bodies and overall wellness. Our intestines contain a variety of bacteria that are crucial to keeping both our bodies and our intestines healthy.

Elli Metchnikoff is given the credit of introducing the term Probiotic a century ago. 5000 years, Acharya Charaka in his treatise Charaka Samhita, while explaining Krimi chiktisa highlighted the concept of friendly micro-organisms under the name “Sahaja krimi”.

Probiotics are the drug of choice in Antibiotic Associated Diarrhea (AAD). These beneficial bacteria, such as Lactobacillus acidophillus (LAB) and the Bifidobacterium genus, are supplied via substances known as probiotics.

Probiotics are essential bacteria that are produced in fermented food items such as curd, buttermilk, and pickles. No meal in India is complete without intake of yoghurt or buttermilk. According to Ayurvedic understanding dadhi (Curd) & takra (Buttermilk) are great probiotic sources. IBS and colitis-like intestinal illnesses are brought on by the repeated use of antibiotics and oxidative stress, which alters the balance of beneficial bacteria in the intestines and promotes the growth of bad bacteria and yeasts. These bacteria stop the growth of dangerous bacteria and a number of substances that are necessary for the body to stay in a healthy state. Therefore, according to Ayurvedic principles, consuming dadhi and takra as part of a diet is a straightforward and efficient treatment.

To make obvious the medicine combinations and dietary regimens described in Ayurveda for intestinal disorders, it is rational to clarify this notion. When we read through classical works like Charaka Samhita, Sushruta Samhita, Bhavaprakasha, Chakradatta, etc., we see that dadhi was used legally as medicine to treat Grahani and Arsha.

In Ayurveda, a pharmaceutical preparation such as Arishta and Asava obtained from poly-herbal formulations also contains probiotic bacteria are also known to contribute health benefits. Potential probiotic microorganisms may be found in traditional Ayurvedic remedies, both as a source & a reservoir. However, relatively little is being done to investigate the potential of probiotics derived from Ayurveda fermented sources.

Many dietetic preparations are mentioned in Ayurveda pharmaceutics which also includes fermentation products such as dadhi kalpana, takra kalpana, kanji, aranala preparations which also incorporates herbal preparations. These preparations have therapeutic effect and said to contain probiotic organisms.

In recent years, due to the increasing demand for plant based probiotic products which are said to have good therapeutic value in clinical practice are coming to limelight of medical practice.

Herbal supplemented curds were formulated with potential multi-functional health benefits to consumers.

When curd is combined with various herbal drugs or plant extracts, the viable probiotic organisms may incorporate in formulations.

"Bilwa"(Aegle marmalos) belonging to Rutacea family, locally known as, "Bael fruit" known to poses various medicinal properties. Ayurveda classical textbooks have mentioned Bilva in many formulations. Its parts like roots, bark, fruit, leaves, etc, have been indicated in conditions like Jwara, Medoroga, Rajayakshama, Arsha, Gulma, Shotha, Grahani and different types of Atisara. Charaka Samhita mentions the use of Phala majja (Fruit pulp) in different types of Atisara. It is known that the unripe fruit has anti-diarrheal qualities and is most useful in chronic diarrhoes. Besides, Aegle. marmelos has been proven effective in animal models of physiological diarrhoea and irritable bowel syndrome.

Apakwa bilwa phala (Un-ripened bael fruit)i.e., tender Aegle marmalos fruit possesses katu, tikta, kashaya rasa; teekshana, snighdha guna, ushna veerya and best among graahi.

Therefore, a formulation was prepared using the ingredients viz, unripe fruit pulp of A. marmalos and curd which are indicated to treat Atisara in Ayurveda.

Present study has been carried out to develop an Ayur-probiotic preparation & evaluating its effect in Antibiotic Associated Diarrhoea (AAD) in the Wistar rats.

AIM & OBJECTIVES

Aim:

- Development and efficacy study of an Ayur-Probiotic (Bilvaphalasaara dadhi*)* preparation

Objectives:

- To develop an Ayur-Probiotic preparation and assessment of its bacterial load
- To evaluate the efficacy of Ayur-Probiotic in Antibiotic Associated Diarrhoea (AAD) in experimental animals

REVIEW OF LITERATURE

PROBIOTICS

Probiotics:

Probiotics have long been ingested by humans in the custom of dairy products that have undergone fermentation and these probiotic bacteria are referred to as "friendly bacteria" meaning "good bacteria" found in the human gastro-intestinal tract.

Russian researcher **Elie Metchnikoff** works at the Pasteur Laboratory in France, first put forth the idea of "Modern Probiotics" in 1907. Due to the drinking of fermented milk containing lactobacilli, Metchnikoff noticed that the Bulgarian peasants lived longer lives. In his finding, came to the conclusion that the key to living a healthy, long life is to maintain a healthy gut microbiota.

In a related finding, a physician **Henry Tissier** noted in 1906 that sick children had fewer germs with the typical 'Y' shaped cell structure than did healthy kids. He also concluded that these bacteria are crucial based on his observations.

Probiotic products are widely accessible on the market nowadays. Although probiotics are often consumed as fermented foods, they are increasingly being introduced as supplements. Probiotics that have been isolated and are safe are added to food items to create functional meals. In the food sector, using probiotics during fermentation is not a new idea. Microbial fermentation is one of the first, most accessible and efficient techniques of food preservation. Probiotic meals, food additives and dietary supplements are just a few of the ways that probiotics are given

to the gastro-intestinal tract. Additionally, probiotics are offered as neutraceuticals in capsule form.

Various Definitions of "PROBIOTIC":

1. A microorganism that, when taken (as in food or a dietary supplement), maintains or replenishes the digestive tract's good bacteria[1].
2. The widely accepted meaning of probiotic is live bacteria that, when given in sufficient proportions, boost the host's health[2].
3. Occasionally, the word "probiotic" is incorrectly used to refer to apparently advantageous commensal microbiota members[2].
4. For regulatory purposes, the US FDA refers to live microbes by other names. Live bacteria used in livestock nutrition are referred to as "direct-fed microbials"[3, 4] and when they are meant to be used as human medications; they are categorized as "Live Bio-Therapeutics."
5. The World Health Organization (W.H.O) defined probiotics as "live microorganisms that, when given in sufficient quantities, bestow a health benefit on the host".

According to British Dictionary:

1. Usually dairy-based meal or supplement to the diet contains live bacteria that either augments or replaces the digestive tract's naturally occurring beneficial bacteria.
2. A bacteria found in the food and dietary supplement in question.
3. A harmless bacterium that helps to protect the body from harmful bacteria.
4. A substance that encourages the growth of natural healthy bacteria in the gut.

'Probiotics' was first used and defined in the following ways:

Probiotics were once thought of as antibiotics contrast. It is made up of the Greek terms "pro" with "biotos," which are both equivalent to the English word "life". He proposed giving malnourished patients food supplements to help them feel better. Kollath (1953) is credited with coining the phrase. Vergin proposed using probiotics in diets to balance out the microbial imbalance caused by antibiotic use in 1954. It's arguable that many people use this source as the way probiotics are now defined. Later, Lilly and Stillwell (1965), Parker (1974), and Fuller (1989) amended and finally defined the phrase.

The probiotics have been around since the dawn of human civilization. The information has been used once again for the greater good, with all of its positive health effects. Probiotic-based treatments in humans can only get better with appropriate research and knowledge sharing.

The idea that a healthy gut leads to a healthy body has been around since the beginning of time. Fermented milk has traditionally been consumed by people as a remedy for digestive issues. They were swallowing live bacteria that helped treat their digestive problems even though they were unaware of it at the time. They had probiotics in their system. Today's society is aware of the importance of probiotics, commonly referred to as good bacteria, in the stomach. The groups of hazardous bacteria that line the colon, intestines and stomach must be fought off by these helpful bacteria.

In addition to curing common digestive issues like bloating, diarrhoea and constipation, probiotics can prevent conditions such as IBS, Crohn's disease, and Ulcerative colitis.

Ancient masters were aware of the potent health advantages that probiotics like curd offer, but they had no idea what was causing these advantages. Our understanding of the microbiology of curd and other fermented milk products has improved throughout time. We are aware of which probiotic strains are most effective for various uses, how much of a particular strain is required and how to build healthy probiotic colonies in our stomach.

Probiotics have advanced significantly, yet their history dates all the way back to the dawn of civilization. Probiotics were probably first consumed through the ingestion of fermented dairy and other foods. Due to the positive impacts on their health, people in ancient civilizations developed the practice of consuming fermented food products.

Such habits were made possible by careful dietary observation and good information. Dairy products have been used as a source of probiotics since the earliest known civilizations. Even the Christian and Hindu sacred texts make reference to them. People all across the world regularly consumed a wide variety of traditional fermented foods. Even before the existence of bacteria was recognized, several of those foods were in use because of their potential therapeutic benefits for humans.

Evidence from the "Bible":

Probiotic use has been documented in the Bible. Pliny Elder, an ancient historian and naturalist, recommended drinking fermented milk to treat digestive

issues as early as 76 AD, and the Greeks have long been known to have used yogurt to maintain intestinal health. This practice dates back to the establishment of Athens. Even though the ancient world's inhabitants recognized that fermented milk was a potent treatment for digestive problems, it would take until the 19th century for humanity to comprehend why it worked.

Probiotics in Ancient Greece:

Hippocrates, a famous Greek physician, promoted the idea of functional foods long ago through his "LET FOOD BE YOUR MEDICINE" approach. One of the oldest techniques for preserving food for an extended length of time is the fermentation-based preparation of dairy products. The use of milk products that were fermented was previously prevalent in East and Middle Asia, but when the Mongols, Huns, and Tartars invaded these areas, they quickly brought it to Russia and Europe. Many fermented milk products, such as kefir and yoghurt, have since been traced back to Eastern Europe or Russian.

Lactobacillus bulgaricus and Thracian civilization:

Lactobacillus bulgaricus – the first all natural probiotic

Historical evidence suggests that the Thracians, who lived in Bulgaria, were aware of Lactobacillus bulgaricus. Lactic acid fermentation is one of the accomplishments of the Thracian civilization.

"**Yoghurt**" is a Thracian word. "Yoghu" denotes something solid and "rt" indicates milk. The mystery of fermentation of lactic acid has been studied by modern science as well as ancient mythology and philosophy. The Thracians are known for

living to be 100 years old because they eat primarily fermented dairy products, honey and bread, according to the ancient Greek thinker Thales (II century BC).

The Father of Civilization **Herodotus** (484–425 BC) left the earliest genuine written accounts of the lactic acid fermentation in human history. The Thracian healers combined honey, plant extracts and fermented milk. The soldiers of **Alexander** the Great's Thracian cavalry (356–323 BC) were tall men with blond hair and dark eyes who consumed fermented dairy products. Their medical staff bandaged wounds with the linen cloths.

The study of probiotics is built on the organism **Lactobacillus bulgaricus**. At the beginning of the 20th century, **Elie Metchnikoff**, the recipient of the Nobel Prize and the head of the Pasteur Laboratory in Paris, laid the foundation for current probiotic research. He claimed in his thesis "**Longevity and Revitalization of Man**" that, the daily ingestion of milk that has been fermented and other locally produced lactic acid products accounts for the longevity and good health of Bulgarian peasants. At that time, Bulgaria had 4% of the world's centenarians, as well as low rates of cancer and morbidity.

During the era of 18th Century:

Metchnikoff discovered that these people's diets were heavy on curd and other fermenting milk products. Metchnikoff investigated the chemicals and microbes in fermented milk under a microscope. What he discovered was surprising: the longevity of fermented dairy products like milk was due to organisms that played a role in the fermentation process. Even more remarkable was **Metchnikoff's** discovery of the precise microorganisms required for fermentation, which led to the processes

identified to support our gastrointestinal systems. **Metchnikoff** had long believed that bad bacteria in the stomach were mostly to blame for intestinal issues, but his research proved that the health of the gut was actually reliant on a healthy bacterial ecology in the digestive tract.

He was able to distinguish between the bacteria that benefit human gut health throughout his research, which led to the development of the genera **Bifidobacterium** and **Lactobacillus**. These two bacteria immediately rose to the top of nearly every probiotic's list of microorganisms. **Metchnikoff** would devote the remainder of his life to studying these bacteria, learning about their advantages for people and establishing probiotics as a practical means of enhancing quality of life. Metchnikoff discovered a way to permanently settle lactobacilli and suggested daily ingestion of lactic acid-based products to get rid of this detrimental effect.

Lactobacillus bulgaricus was first identified in these dairy products by the Bulgarian physician St.Grigorov in 1905. He made the claim that the **thermophilus & bulgaricus** are bacteria used to make **yogurt** have health benefits for the hosts who consume it because they can stop the growth and fermentation of the undesirable microflora in the intestines. He then went on to show how the good bacteria aid in enhancing immunological and digestive functions. Since the detrimental microorganisms that lived in the intestine depended on the host for nourishment, they can be altered and changed into advantageous versions. Metchnikoff's discoveries laid the groundwork for succeeding researchers to advance his study of probiotics, although the term "**probiotics**" wasn't developed until the middle of the 20th century, and its meaning has since undergone numerous changes.

The term "**probiotic**" (which in Greek literally translates as "for life") first entered the public domain in 1965. Although there is some disagreement about who came up with the word, St. John's University researchers **Daniel Lilly & Rosalie Stillwell** are frequently given credit. The term "substances produced by one microbe which encourage the growth of another" used to describe probiotics is technically accurate but is also deficient. The term changes in 1989. The word should be modified to "live microbial supplement that improve the microbial balance of the host animal," according to renowned intestinal microbiology researcher **Roy Fuller**. In this definition, the importance of live probiotics is emphasized, as well as the fact that probiotics must benefit the host in order to be effective. Probiotics must be provided externally to the host, according to this current definition, which adds another aspect to what they are. As opposed to natural sources like **yogurt**, this sparked interest in supplements, which resulted in an explosion of probiotics supplements.

In the 19th Century:

Despite the pre-existing evidence of the beneficial bacteria's health advantages, especially with regard to the industrial side, little was done to increase their popularity. The development of antibiotics throughout the world wars also did little to further the cause of probiotics acceptance. In fact, for a very long time, Metchnikoff theories were abandoned and between the years 1908 and 1964, little of note in the field was produced to support the growth of the Western probiotic sector.

Tissier showed that **bifidobacteria** have a significant role in preserving health in breastfed newborns in among the earliest research investigations relating to the probiotics' health effects. It was discovered that the helpful bacteria controlled the newborn’s unhealthy intestinal microbiota and shielded the intestines from infections. Individuals questioned whether L. bulgaricus strains delivered through yoghurt and

kindred food items might survive the transit through GI tract and colonization it to generate helpful effects at the time because there were so few individuals interested in the bacteria's health effects.

It was well known in 1920 that milk that has been fermented containing **L. acidophilus** may treat digestive problems. The ability of the beneficial bacteria to transmit health advantages was assumed to be dependent on their efficient development and colonization of the gut. As a result, **Shirota's** study in 1930 switched to the choice of intestinal strains of bacteria that could endure the harsh circumstances provided during passage through the gastrointestinal tract and he went on discovering **L. acidophilus Shirota**.

A 'Y'-shaped bacteria was discovered in the intestinal tract of breastfed children in 1899, according to **Henry Tissler**, an investigator at the Pasteur Institute. He gave the name "**Bifidobacteria**". According to **Tissler**, children with lactic acid bacteria in their gastrointestinal tracts experienced less digestive issues, as indicated by a decrease in diarrhoeal illnesses[5].

The prospective applications and advantages of probiotics excited German doctor and scientist **Alfred Nissle**. Since there were no antibiotics available at the time, methods for treating infections were required. In the stool of a World War I solder, who contracted shigella but not the diarrhoeal illness during an outbreak of shigellosis, Nissle found a totally new strain of **Eschericia coli**. A bacteria called Shigella produces extremely bad diarrhea. The newly discovered bacterium strain was given the name "Eschericia coli Nissle 1917." With tremendous effectiveness, Nissle treated intestinal illnesses including shigella and salmonella with the strain. The probiotic bearing Nissle's name continues to be used today[6]. It interacts strongly with the body's immune system. Probiotic research experienced setback in

the year 1920. Leo F. Rettger's experiments appeared to demonstrate that Metchnikoff's Lactobacillus bulgarius weren't able to in the gut and was actually wiped off by stomach acid.

Although probiotics can withstand stomach acid now, at the time it appeared to contradict Metchnikoff's hypothesis that those who fed his germs would live longer. The medicinal use of fermented foods quickly lost popularity. However, Rettger also showed that other gut-born bacteria might work as probiotics when added to the human digestive system, aiding in the restoration of regular bacterial colonization. **Lactobacillus acidophilus**, one of these bacteria, has been proven to be a successful treatment. Scientists discovered many types of probiotics, including Lactobacillus acidophilus, **Saccharomyces boulardi** & **Bifidobacterium infantis**; every one of and these have different properties and can have different effects on the body. One of these researchers was Japanese microbiologist **Minoru Shirota**[8], who established a new strain of bacteria called **Lactobacillus casei Shirota.**

The Prospects of Probiotics:

The advantages of probiotics for the gastrointestinal tract are already well established. These include improved digestion, easier bowel motions, less bloating and protection against serious conditions including IBS, colitis and Crohn's disease. Probiotics' effectiveness as a substance that reduces inflammation suggests they may be useful in avoiding diseases like cancer. There are correlations between probiotics and cardiovascular disease as well as brain health. **Clostridium difficile** can cause havoc in the intestines in a stomach that lacks probiotics, resulting in diarrhoea, bloating and even death. Antibiotics are the biggest contributory factor for Clostridium difficile. The role of probiotics in preserving a healthy body is one that cannot be overstated.

Probiotics today:

Probiotics are **"live organisms, which, when given in appropriate quantities, provide beneficial health effects on the host".** Antibiotics are known to alter the gut flora of the digestive and vaginal tracts, which can weaken the immune system and create digestive issues.

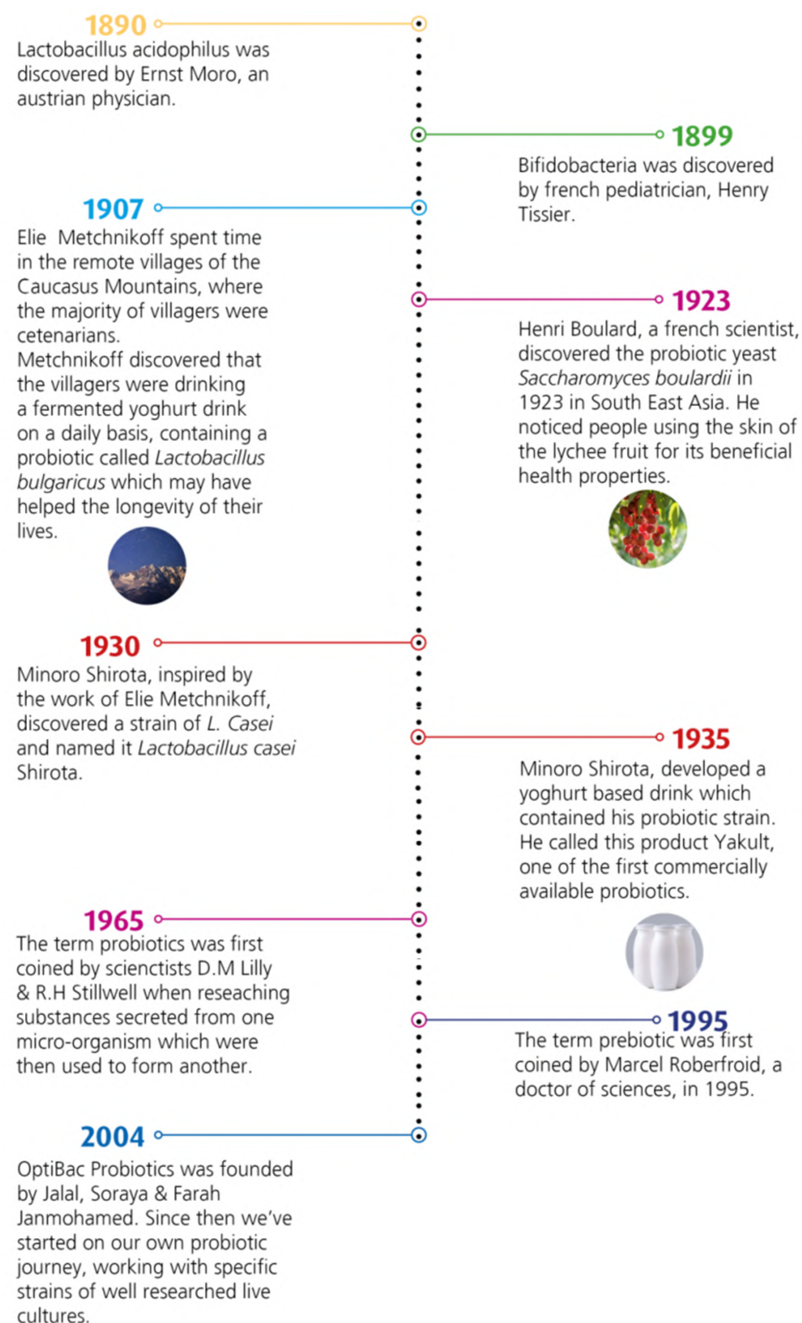


Figure.no.1 showing pioneers of Probiotics

These pioneers undoubtedly had a significant impact on the fascinating probiotic journey to date. Probiotics have positive benefits on human health by improving digestion, producing Vitamin B_{12} and Vitamin-K and boosting our immune system. By preserving an acidic environment in the stomach, they prevent disease-causing alkaline bacteria from doing their job. Probiotics can improve the digestion and absorption of nutrients, which increases the nutritional value of particular diets. Some are anti-carcinogenic and generate antibiotics. Some probiotics strengthen the immune system and aid in the prevention of IBS, vaginal infections & urinary tract infections. Although there are many more claims that have not yet received scientific validation, it is clear from the studies that certain types of probiotics do have beneficial benefits, particularly on the digestive tract[9, 10]. It should be underlined that the advantages of microbes can be negated by our lifestyle. It should be mentioned that our lifestyle choices have the potential to counteract the beneficial benefits of microorganisms. For instance, antibiotics found in pesticides or herbicide traces on fruits and vegetables, as well as chlorine in the water we consume, are some of the largest risks to probiotics. These can eliminate harmful bacteria, but they can also prevent our beneficial bacteria from growing[11].

Probiotic Viability:

A product's probiotic potential measures how long probiotics survive in the gastrointestinal tract. The strain characteristics, composition of nutrients, oxygen level, the pH level and activity in the water, other microbes' presence, temperature, preservation time and a few other factors can all affect how long probiotics can persist at different G.I. tract levels. Probiotics are transported to the gut by the meal, which provides nutrients for both consumers and probiotic microorganisms. Bioactive substances such as prebiotics, nutrients, dietary fibers & enzymes, flavors &

preservatives of food all promote the proliferation of probiotics in food matrices (Sanders and Marco, 2010). Probiotics' ability to survive and function within a food product depends on a number of elements. These include the added microorganism's physiological state, the quantity of live cells present at the time of consumption, as well as external factors like temperature variations, the product's chemical make-up and some interactions among beneficial bacteria and starter culture (Gallardo-Navarro and Rivera-Espinosa 2010).

Probiotics produce acid in the food matrixes due to their fermentative metabolism and a high level of acidity is harmful to their life. In order to create a superior nutritive framework with a higher pH, juices of fruits are therefore combined with dairy components. Studies on probiotics have shown that during the shelf life, the probiotic bacteria become more resistant to pH stress. Probiotics that have been given an induced tolerance to acidity are better able to withstand heat, oxygen and osmotic stressors than new culture.

Lactic Acid Bacteria (LAB)

The bacteria known as lactic acid bacteria or LAB, is made up of multiple species and several genera. Typically, these are coccobacilli that are Gram positive, non-mobile and do not create endospores. LAB develops in microaerophilic environments and lack catalase. The genus Lactobacillus is a member of the Firmicutes phylum, the Bacilli class, the Lactobacillales order, and the Lactobacillaceae family. The ability to tolerate NaCl, the ability to grow on various media, the ability to grow at particular temperatures, carbohydrate fermentation patterns and antibiotic resistance are all helpful for categorizing LAB into genera (Bonaparte et al.,1998). LAB are also chema-organotrophic. The two types of LAB

are **hetero-fermentative and homo-fermentative**, respectively, based on the structure of hexose metabolic (Corsetti et al., 2005; Ali, 2010). Three divisions within the "Lactobacillus" group are depending on how they process carbohydrates. They are facultative hetero-fermenters.

The **Bifidobacteria and Lactic Acid Bacteria** are the most common probiotic microorganisms. Commercial starter cultures frequently contain strains of Bifidobacterium, including Lactobacillus group of organisms. Along with **Streptococcus thermophillus**, other LAB species utilized as probiotics. From many different natural settings, LAB can be isolated.

Probiotic does a variety of health benefits that are related to the gut through their various modes of action. People are persuaded to prevent diseases by factors such an increase in longevity, a rise in diseases linked to modern lifestyles and the high expense of medical care. Throughout a person's lifetime, the GI tract's microflora undergoes periodic alterations. Bifidobacteria is an important component of the newborn flora. Bacteroides is the dominant flora in adults. Due to weakened immunity, a diet low in fiber and regular use of antibiotics, the microbiota has been altered.

Prevention of Diarrhoea:

Fermented milk is thought by the general public to prevent intestinal illnesses. According to **Lorens-Hattingh and Viljoen** (2001), the **LAB and Bifidobacteria found in fermented milk are responsible for this disease-preventing activity.** According to Lorens-Hattingh and Viljoen (2001), some genera of bacteria produce inhibitory compounds during their metabolism, including organic acid, hydrogen

peroxide, bacteriocins, and deconjugated bile salts. Probiotics improve immunity, preserve the mucosal barrier layer and lower the likelihood for diarrhoea by competitively excluding harmful bacteria. **Lactobacillus rhamnosus & Lactobacillus plantarum** do not contain Clostridium difficile, which causes diarrhoea brought on by antibiotics. Additionally, probiotic LAB has been shown to lessen the severity of traveler's diarrhoea (Shah, 2007). When probiotics are used in addition to the recommended treatment for irritable bowel syndrome, the symptoms have been shown to improve (Sanders & Marco, 2010).

The Performance of Probiotics

It is important to take into account biological characteristics including oxygen sensitiveness, stability during storage, resistance to GI tract enzymes, susceptibility to lysozymes and phenolic substances, antioxidant activity capacity and adherence to animal cells. One strain cannot reveal all the characteristics. Therefore, it would be advantageous to use several strains (Champagne & Gardner, 2005). The quantity of microorganisms in a product, their viability and their capacity to activate at the appropriate place in the alimentary tract are all crucial for obtaining the potential health benefit.

According to **Sanders** (1999), **100 ml or gm of food containing a therapeutic minimum** (10^6-10^8 CFU/ml or gm of the material) **would be sufficient to meet the daily requirement for beneficial bacteria, which is believed to be 10^9–10^{11} CFU.** Dairy products are suitable in this regard because product composition affects probiotic effectiveness significantly. As a result, dairy products such as curd, butter, cheese, cow's dairy products, ice cream and other dairy sweets are the best carriers of probiotics in various forms. Probiotic development & their ability to

overcome barriers inside the gastrointestinal tract may be impacted by the matrix of food itself as well as some added substances including sweets, salts, fragrance compounds and preservatives.

Probiotics are known for their safe usage in the food industry. **Bifidobacteria and Lactobacillus** are both "**Generally recognized as safe**" for a variety of causes. These two genera typically reside in human GI tract and are widely ingested in fermented foods without risk (von Wright, 2005). The infective dosage depends on the host and microbial variables, hence it cannot be expressed. Studies using animal models demonstrate that the presence of LAB in nutritional foods has no negative consequences (Salminen et al., 1998). For consumers to realize that an item is safe, proper labeling is crucial.

Need for "Prebiotics":

Prebiotics are essential for promoting probiotic growth. A substance needs to meet a number of requirements to qualify as a prebiotic. Short-chain fatty acids (SCFAs)including acetic, propionic and butyric acid are produced in greater quantities as a result of metabolism, which also raises bacterial biomass. These three types of fat each have a different purpose in the body in terms of providing energy. In muscle cells, acetic acid serves as an energy source. The liver uses propionic acid to make ATP, whereas colonocytes use butyrate as their energy source. The feces are voluminous because to the elevated bacterial biomass. Short Chain Fatty Acids inhibit growth of harmful bacteria by lowering the pH of the environment. Substances not only function as prebiotics but also hasten iron, magnesium and calcium absorption, increasing the amount of bone mineral content. Prebiotics are digested by short-chain fatty acids, which also act as energy sources. The primary source of energy for

colonocytes is butyric acid. Propionic acid and acetic acid reach the liver through the systemic blood circulation. Acetic acid & propionic acid are both converted in muscle tissues and via gluconeogenesis, respectively.

The duration of prebiotics' fermentation is influenced by their degree of polymerization (DP). According to the DP, the fermentation period for inulin could range from 5 to 15 hours. Additionally, giving 6 to 24 month old children 2 grams of oligo-fructose per day for 4 weeks enhanced Bifidobacteria development while lowering Clostridium development (de Vriese & Marteau, 2007). According to Forsten et al. (2011), **4g of prebiotics should be consumed per day**. According to de Wiele et al. (2004), **1-4gm of inulin should be consumed per day**. To achieve a bifidogenic effect, some recommend taking 2.5 to 5 grams of inulin-type prebiotics per day (Kelly, 2008). Over-indulgence may result in diarrhoea.

Non-Dairy Probiotics:

Dairy products are the ideal substrate for probiotic bacteria, as is well known. But due to various problems with dairy probiotics, a movement toward non-dairy probiotics is expanding. **Casein**, one of the potential allergens in dairy substrates, may be present; hence cold storage is necessary for the duration of the shelf-life. Dairy products contain a lot of cholesterol. As a result, vegetarianism is becoming more popular, and there is a growing need for novel tastes with non-dairy matrix (Ranadheera et al, 2010). Additionally, it is advised to produce probiotic products using everyday meals and drinks.

The most common probiotic microorganisms with claimed health benefits for humans from the most recent scientific literature are noted in the following Table.

Table.no.94 showing micro-organisms with claimed Probiotic Properties

Genus	**Species**	**Recently published health claims with references**
Lactobacillus	L. rhamnosus	Reduction of risk for developing allergic disease (*L. rhamnosus* GG) [58], (*L. rhamnosus* HN001 [59]; anti-diabetic potential (various strains from human infant faecal samples) [60]; prevention of necrotizing enterocolitis in newborns(*L. rhamnosus* GG) [61]; prevention or treatment of bacterial vaginosis (*L. rhamnosus* GR-1) [62]; aid in weight loss of obese women (*L. rhamnosus* CGMCC1.3724) [63]; treatment of acute gastroenteritis in children (*L. rhamnosus* GG) [64]; reduction of risk for rhinovirus infections in preterm infants (*L. rhamnosus* GG and *L. rhamnosus* ATCC 53103) [65]; protection of human colonic muscle fromlipo-polysaccharide-induced damage (*L. rhamnosus* GG) [66]
	L. acidophilus	Reduction of hospital stay of children with acute diarrhoea [67]; antifungal activity (*L. acidophilus* ATCC-4495)[68]; prevention or treatment of bacterial vaginosis [62]; treatment of *C. difficile*-associated diarrhoea[68]; reduction of incidence of febrile urinary tract infections in children [69]; reduction of irritable bowel syndrome symptoms [70].

	L. plantarum	Anti-fungal activity (*L. plantarum* NRRL B-4496) [68] reduction of irritable bowel syndrome symptoms [70].
	L. casei	treatment of *C. difficile*-associated diarrhoea [71]; restoration of vaginal flora of patient with bacterial vaginosis (*L. casei* Lcr35) [72]; reduction of irritable bowel syndrome symptoms [70]; reduction of diarrhoea duration of antibiotic-associated diarrhoea in geriatric patients (*L. casei* Shirota) [73]; immune-modulatory mechanisms (*L. casei* Shirota) [74]; improvement of rheumatoid arthritis status (*L. casei* 01) [75];protection against *Salmonella* infection (*L. casei* CRL-431) [76]; prevention of *Salmonella*-induced synovitis [77]; treatment of intravaginal staphylococcosis (*L. casei* IMV B-7280) [78].
	L. delbrueckii subsp.bulgaricus	Antibiotic resistance of yogurt starter culture[79]; enhancement of systemic immunity in elderly (*L. delbrueckii* subsp. *bulgaricus* 8481)[80]; antibacterial action against *E. coli* [81]; modulation of brain activity [82].
	L. brevis	Protective role in bile salt tolerance (*L.brevis* KB290) [83]; reduction in plague acidogenicity (*L. brevis*CD2) [84].
	L. johnsonii	Impact on adaptive immunity for protection against respiratory insults [85]; reduction of

		occurrence ofgastritis and risk of *H. pylori* infection (*L. johnsonii* MH-68) [86]; inhibition of *S. sonnei* activity (*L. johnsonii* F0421) [87]; treatment of perennial allergic rhinitis in children together with levocetirizine(*L. johnsonii* EM1) [88].
	L. fermentum	Prevention or treatment of bacterial vaginosis (*L. fermentum* RC-14) [62]; blockage of adherence of pathogenic microorganisms on vaginal epithelium [89]; anti-staphylococcal action (*L. fermentum* ATCC11739) [90]; potential for reduction of insulin resistance and hypercholesterolemia (*L. fermentum* NCIMB5221) [91].
	L. reuteri	treatment of acute gastroenteritis in children [64]; reduction of diarrhoea duration in children (*L. reuteri* ATCC 55730) [92]; management of infant colic (*L. reuteri* ATCC 55730 and *L. reuteri* DSM 17938)[93]; reduction of onset of gastrointestinal disorders in infants (*L. reuteri* DSM 17938) [94]; reduction of frequency of proven sepsis, feeding intolerance and duration of hospital stay in preterm infants (*L. reuteri* DSM 17938) [95].
Bifidobacterium	B. infantis	Reduction of irritable bowel syndrome symptoms [71]; reduction of necrotizing enterocolitis in preterm infants [96-98].
	B. animalis subsp. lactis	reduction of incidence of febrile urinary tract infections in children [70]; modulation of brain activity [82]; Reduction of necrotizing

		enterocolitis in preterm infants [96]; reduction of total microbial counts indental plaque (*B. animalis* subsp. *lactis* DN-173 010) [99]; reduction of total cholesterol (*B. animalis*subsp. *lactis* MB 202/DSMZ 23733) [100]; reduction of risk of upper respiratory illness (*B. animalis*subsp. *lactis* BI-04) [101].
	B. bifidum	Reduction of hospital stay of children with acute diarrhoea [67]; reduction of necrotizing enterocolitis inpreterm infants [97,98]; reduction of total cholesterol (*B. bifidum* MB 109/DSMZ 23731) [100].
	B. longum	Reduction of necrotizing enterocolitis with Bifidobacteria cocktail (*B. breve*, *B. infantis*, *B. bifidum*, *B. longum*) [98]; reduction of irritable bowel syndrome symptoms [71]; treatment of gastrointestinal diseases (*B. longum* CMCC P0001) [102]; perinatal intervention against onset of allergic sensitization (*B. longum* CCM 7952) [103].
	B. breve	reduction of necrotizing enterocolitis with Bifidobacteria cocktail (*B. breve*, *B. infantis*, *B. bifidum*, *B. longum*) [98]; reduction of cholesterol(*B. breve* MB 113/DSMZ 23732) [100].
Saccharomyces	S. boulardi	Treatment & reduction of diarrhoea duration regardless of cause[106], treatment of acute gastroenteritis in children [64].
Lactococcus	L. lactis subsp.	Modulation of brain activity [82]; antimicrobial activity against *C. difficile* [104]; antimicrobial and probiotic properties (*L. lactis* subsp. *lactis* ATCC 11454) [105].
Enterococcus	E. faecium	Treatment of antibiotic-associated diarrhoea[106]; efficient animal probiotic[107].

Streptococcus	S.hermophilus	Reduction of irritable bowel syndrome symptoms [71]; antibiotic resistance of yogurt starter culture [79]; reduction of necrotizing enterocolitis in preterm infants [96,97].
Pediococcus	P. acidilactici	Pediocin production with antimicrobial and probiotic properties (*P. acidilactici* UL5) [105]
Bacillus	B. subtilis	Efficient animal probiotic[108]; treatment of diarrhoea and aiding in *H. pylori* eradication (*B. subtilis* R0179)[109]
	B. cereus	Efficient animal probiotic (*B. cereus* NVH75/95) [110].

Some of the health claims of individual probiotic microorganisms noted based on *in-vitro* using cell cultures or animal models and others are based on *in vivo* studies. From the table we can see that health claims are very diverse and range from managing various gastrointestinal diseases or disorders to exhibiting antibiotic properties and reducing total cholesterol. Some authors even claim that probiotics reduce tumour growth, modulate brain activities and reduce allergies.

The literature overview shows that, although the vast majority of probiotics are generally regarded as safe and beneficial for healthy individuals, caution is needed in selecting and monitoring of probiotics when administering probiotics to patients with compromised immune systems, leaky gut or critical illnesses.

Drug Review

Bilwa: Aegle marmelos.Linn

Over 21000 plant species used for medicinal reasons are listed by the World Health Organization (W.H.O.). About 2500 species of plants from more than 1000 species are used in India's traditional medical practices[12]. With 16 agro-climatic zones and one of the world's 12 mega-biodiversity hotspots, India contains over 45000 plant species, 15000 of which are flowering plants with about 7000 of these having been classified as medicinal plants[15]. In the world of flowering plants, there are roughly 400 families, and India is represented by at least 315 of them[14].

The native Indian fruit known as Bael, which (Aegle marmelos Linn.) is a member of the Rutaceae family and is also referred to as the Bengali quince, Golden Apple, Sacred Fruits, Bael, Bilwa, Shripal and Stone Apple.

Origin:

This tree found in India's north-eastern Indo-Gangetic low-lands, Sub-Himalayan areas up to an elevation of 500 mtr, as well as arid and sub-tropical woods in the country's central and southern regions.

Description of the plant

Scientific Classification[13]:

- **Kingdom:** Plantae
- **Order:** Sapindales
- **Family:** Rutacae
- **Sub-Family:** Aurantiodae

- **Genus:** Aegle
- **Species:** Aegle marmelos
- **Botanical name:** Aegle marmelos

Vernacular names:

- Hindi : Bel, Bela
- Marathi : Bel
- Telugu : Maredu
- Gujarati : Bilvaphal
- Kannada : Bilva, Bilpatrae
- English : Bengal quince or bael

Synonyms and their meanings:

- Bilwa: Effective against bowel disorders, especially in diarrhoea
- Kantaki: A thorny tree
- Gandha garbha: Pulp with resinous odor
- Malura: effective against G.I disorders.
- Shantilya: provides relief from the disease
- Shilusha: Trees can be found even in steep locations
- Shriphala: The fruits are attractive
- Sadhaphala: The fruits are usually dangling from the tree
- Puthimarutha: Effective as a carminative and for eradicating odors
- Vathasara: Relieves abdominal flatulence and acts as a carminative
- Gandha patra: The leaves have a pleasant scent

Botanical views:

The eagle marmelos is a medium-sized, slow-growing tree that can grow to be up to 12 to 15 meters tall. It has a short body, heavy, tender, flaking the bark, and propagating, occasionally spiky branches, with the bottom ones drooping. A young sucker's spines are often stiff and straight. From broken branches, a clear, gooey sap that resembles gum Arabic drips down in long threads and gradually solidifies. When first tasted, it is delicious but soon becomes throat-irritating. Numerous bioactive chemicals have also been identified from the bael plant and are used extensively in traditional medicine.

Leaves & Flowers:

The deciduous, alternating leaves are borne alone or in groups of two or three and consist of three to five leaflets that are oval-shaped & shallowly toothed. When mature leaves are injured, an unpleasant stench is released.

Flowers have a sweet character and were greenish white in color. Possess a hypogynous, actinomorphic, bisexual stalk[6]. Fragrant flowers have four fleshy, recurved petals that are green on the outside and yellowish on the inside, and they are grouped in groups of four to seven along the juvenile branchlets (Orwa and colleagues, 2009).

Fruit and Seeds:

Fruit is spherical or oval in shape, with a diameter of 2 to 4 inches. Natural shell is hard, woody and as thin as paper. It is greenish when unripe and turns yellowish as it ripens. The pulp of the apple is segmented in 8 to 15 parts. The pulp is yellow, pasty, sweet and resinous, & fragrant. Fruition takes place in May and June. The pulp contains imbedded seeds. According to Lambole et al. (2010), the seeds are

tiny (less than 1 cm long), hard, flattened-oblong, covered in fuzzy hairs and wrapped in an adhesive sac.

Classification according to various Ayurveda Classics:

- **Charaka:** Shothahara, Arshoghna, Asthãpanopaga
- **Sushruta:** Varunãdi, Ambasthãdi, Brihat Panchamoola
- **Vagbhata:** Varunãdi, Ambasthãdi

Chemical constituents:

Several kinds of compounds has been extensively studied and its many parts have yielded the identification of **alkaloids, coumarins, terpenoids, & fatty acids**. The leaves of Aegle marmelos included **aegelin, lupeol, ruin, which marmesinin, sitosterol, flavones, glycosides, oisopentenyl halfordiol, marmeline, & phenylethyl cinnamamides**. The following are thorough analyses of isolated chemical classes.

Alkaloids:

Three new alkaloids, halfordino, ethylcinnamamide, and marmeline, were discovered in the leaves of Aegle marmelos. Anhydromarmeline, aegelinosides A and B and other novel compounds, were recently separated from Aegle marmelos' leaves and used as glucosidase inhibitors (Yadav and colleagues, 2009).

Phenylpropenoids:

Phenylpropenoids are phenolic chemicals that have a ring of aromatics with a three-carbon side chain attached. Hydroxylcoumarins, phenylpropenes and lignans are all phenylpropenoids.

Terpenoids:

It was found that the essential oils including leaves, twigs, as well as fruits frequently included phellandrene. According to reports, leaf oil included p-cymene (17%) & -phellandrene (56%). It has been established that limonene, which makes up 82.4% of the primary element of Aegle marmelos leaves, may be used to distinguish Aegle marmelos oils extracts.

Carotenoids:

Carotenoids are in charge of giving fruit its light color. Bael plant constituents marmelosin, skimmianine and umbellliferone have medicinal properties. Minor components include things like the antioxidant Vitamin-C, sitosterol, raw fibers, tannins, amyrin, carotenoids & crude peptides.

Tannins:

Fruit pulp can contain up to 9% tannin, however cultivated types have less. Skimmianine, a kind of tannin, can also be found in leaves.

Chemical / Active Ingradients of different parts of Bilwa

- **Moola:** furoqinoline, umbeliferone, marmesin, marmin, xanthotoxin, sitosterol, etc.
- **Pakwa phala:**Xanthotoxol, Marmesinin, and Sikmmin
- **Apakwa Phala:** Xanthotoxol, Marmeline, Aegeline, Imperatorin and Allomieperatorin

Table no.1 showing nutritional value of Bilwaphala (per 100 g)

(Source: *Purohit et al., 2004, Shankar, 1969 and Paricha, 2004*)

Components	Value (%)	Components	Value (%)
Water (moisture)	64.2	Potassium	0.6
Protein	1.8	Iron	0.3
Fat	0.2	Vitamin A (IU)	186
Mineral	1.5	Vitamin B1	0.01
Fiber	2.2	Nicotinic acid	0.9
Carbohydrate	30.6	Riboflavin	1.2
Calcium	0.09	Vitamin C	0.01

Parts used:

Fruit, Leaves & Root

Kaarmukata of different parts of bilwa:

- **Mula**: Mutrala, Sothagna, Sulaprasamana, Cardinigrahana
- **Phala**: Vidahi, Vishtambi, Anulomana, Dipana, Pacana, Grahi, Pittrakrt, Vatakaphahara.

Table.no.2 showing the properties of *pakwa and apakwa bilwa phala*:

Ripen fruit *(Pakwa bilwa phala)*	**Un-Ripen Fruit *(Apakwa bilwa phala)***
Sweet, aromatic, cooling, alterative, nutritive and laxative	Astringent, digestive, stomachic, little constipative stimulant, antipyretic, anti-scorbutic, bitter & pungent
Acrid, bitter, sweet, appetiser, binding tonic, febrifuge, causes biliousness and removes vata & kapha, good for heart	Oily, bitter, acrid, sour, tasty but difficult to digest, appetizer, binding, cures dysentery, removes pain

Table no.3 showing list of the properties of Bilva in Brihattrayi

Charak Samhita	***Sushrut Samhita***	***Ashtang Samgraha***
The best shamaka for the Kapha and Vata doshas, Deepaniya (appetizers), and Samgrahika (having the ability to absorb fluids from the gastro intestinal system) (Ch. Su. 25/40). The Vata pacifier is Bilva Patra (leaves). (Ch.Su. 27/107). Ripe fruit is Durjara (difficult to digest)	Bilwa's tender young fruit calms the vata and kapha energies. It has a hot potency and is unctuous, grahi, bitter, unpleasant and astringent in flavor. When it is mature, it has a sweet taste, Guru (which causes burning), is challenging to digest, causes unpleasant-smelling flatulence, Guna (which increases doshas), and has a good flavor (Su.Su.46/147). Bilva patra calms the Vata dosha since Shaka is astringent in Rasa and Ushna is acidic in Virya.(Su.Su. 46/254).	When mature, Bilwaphala is Durjara, Doshala, and Pooti-maruta (causes bad odor in the flatus); when unripe, Deepana, which balances Vata and Kapha, is present; both are Grahi. (Ast. Hur. Su. 6/125).

Pharmaco-dynamics of un-ripened bael fruit (Apakva Bilwaphala):

- **रस** - कषाय, तिक्त
- **गुण** - लघु, रूक्ष्
- **वीर्य** - उष्ण
- **विपाक** - कटु
- **कर्म** - वात-कफहर, ग्राही, दीपन ऽ पाचन

Disease indications: Atisara, Grahani, Prameha, Shotha, Agnimandya

Matra (Dosage) of Bilwa:

- Decoction: 50-100 ml
- Powder: 3 to 5 grams
- Leaf juice: 10 to 20 ml

Therapeutic indications:

- ***Pittatisara***: Rice water is administered along with bilwa and madhuka fruit pulp that has been mixed with honey and sugar (Su.Su.40).
- ***Grahani***: Unripe Bilva fruit paste is administered along with shunti and Guda when a person is on a buttermilk diet (v.m.)
- In *atisaara*, *apakwa bilwaphala majja* is administered with dadhi
- In *amavata*, powdered unripe fruit pulp is administered with *guda*.
- In *sangrahani*, the ripened fruit is provided with *sharkara* in the early stages.
- Fruit pulp is thought to have digestive, laxative and fragrant properties.
- Leaf juice is used in Shillong & Ranchi to treat colds and coughs, coupled with honey.

Reported Pharmacological activity of fruit:

Glibenclamide was less efficient than the extract at restoring the levels of these parameters when given at a dosage of 250 mg kg-1. The findings of this investigation unequivocally demonstrated the fruit extract's hypoglycaemic action. Unripe fruit from Aegle marmelos was investigated for its anti-diarrhoeal properties by S. Brijesh et al.

Research Studies on bilwa fruit:

- Beal fruit's effect on amoebiasis is claimed to have **anti-diarrhoeal properties.** Bilwa powder is used in *atisara* & *pravahika* condition.

Folklore Practices of Bilwa:

- By removing toxic gases from the environment, bael species serve as climate purifiers. The fruit's juice provides relief from dyspepsia and constipation. The fruits are used to combat intestinal parasites and viral infections. Squashes and cold beverages are made with the fruits. After roasting, the unripe fruits might be consumed. It can be used to treat gynecological diseases and tuberculosis.

- Fruits provide a feeling of vigor and freshness. It serves as an astringent and carminative. It works well for disorders related to the thyroid. The other stated uses include coma, swollen joints, pregnancy problems, typhoid and heart stimulant. IBS is treated with this dry powder.

The various parts of the Bael fruit are used for a variety of therapeutic purposes, including the treatment of asthma, anemia, fractures, wound healing, enlarged joints and hypertension, jaundice, or diarrhoea, a healthy mind and brain,

typhoid issues during pregnancy and more (Sharma et al., 2011). Patients who are recovering from bacillary dysentery get a calming effect from drinking sweet beverages made from fruit pulp. Dyspepsia can be effectively and easily treated by eating ripe fruit (Parichha, 2004; Chowdhury and colleagues, 2008).

Products made from bael fruit:

Different processing techniques are used to create preserved goods with additional value. Fresh bael fruit may be kept at 30°C for 15 days when it is fully mature, 1 week when it is ripe, and 3 months when it is 9°C. The pulp from fruits can be kept for up to six months in heat-sealed jars.

Clinical Indication:

Almost every plant component of a tree, including the fruit, leaves, bark and root is utilized in various formulations to treat various ailments. Its various components have unique qualities and medicinal benefits. Their usages in nearly identical ailments were listed in all three Samhitas.

Charaka Samhita:

According to Charaka Samhita, ***bilva*** is used to treat conditions like *Atisara, Pravahika, Shotha, Gulma*, *Arsha, Grahani, Pandu, Shwasa, Hikka & Kasa*

Sushrut Samhita:

Sushrut suggested a number of diseseas to be treated with *apakwa bilwaphala majaa* such as *atisara, pravahika, gulma, anaha, agnimandya, grahani and mutravarodha*.

Ashtanga Hridaya:

Ashtanga Hridaya follows *Charaka Samhita* and *Sushruta Samhita* regarding to its text. In this treatise, *Bilva* is used to cure in the *Medoroga, Mandagni, Adhyavata, Shirahshula, Gulma, Vidradhi, Pakvatisara, Sandhaniya and Vrana ropana.*

Table no.4 showing Pharmacological Activity of Aegle marmelos Corr.

Sl.no.	**Drug-related activity**	**Research work**
1.	Anti-diarrheal and dysenteric qualities	Majja choorna of apakwa bilwa phala found to be effective in G.I infections when extracted with ethanol. The powerful antibacterial activity of the extract is demonstrated by a reduction of growth of bacteria at concentrations as small as 250 micrograms per milliliter. Shigella sonnei exhibits the greatest inhibition.
2.	Microbial resistance	1. The anti-biogram study revealed that the zone of inhibition for the ethyl acetate extract against S. aureus was 19 mm. The MIC value for S. aureus is 1.98 mg/ml with ethanol & ethyl acetate fruit extract. 2. A goat intestinal parasite was used to test the in vitro anti-helminthiasis.

COW MILK

Milk is a white liquid produced by the mammary glands of mammals. All mammals, including humans, will normally produce milk to feed their offspring until they are ready for solid food. Cow's milk has long been associated with good health, making it one of the most consumed beverages throughout the globe. It contains valuable nutrients, and it can offer a range of health benefits. However, some people are not able to digest lactose, the sugar in milk, after they are weaned, because they do not produce enough of an enzyme known as lactase. Lactase is needed to digest milk properly.

Appearance:

Both the fat globules and the smaller casein micelles, which are just large enough to deflect light, contribute to the opaque white color of milk. The fat globules contain some yellow-orange carotene, enough in some breeds (such as Guernsey and Jersey cattle) to impart a golden or "creamy" hue to a glass of milk. The riboflavin in the whey portion of milk has a greenish color, which sometimes can be discerned in skimmed milk or whey products[19]. Fat-free skimmed milk has only the casein micelles to scatter light, and they tend to scatter shorter-wavelength blue light more than they do red, giving skimmed milk a bluish tint.

Physical & Chemical attributes:

Milk is an emulsifier made up of fat particles, dissolved protein aggregates, minerals, and carbohydrates.[16] All of its components promote growth[17].

pH:

The pH value of milk varies over time and is between 6.4 and 6.8.

Lipids:

In the beginning, milk fat is produced as a fat globule encased in a membrane[18, 19].

Proteins:

Bovine milk is normally composed of 3.2% protein and comprises 30-35 grams protein per liter, around 80% of which is organized in casein micelles.

Caseins:

The largest structures in the fluid portion of the milk are "casein micelles": aggregates of several thousand protein molecules with superficial resemblance to a surfactant micelle, bonded with the help of nanometer-scale particles of calcium phosphate. Each casein micelle is roughly spherical and about a tenth of a micrometer across. There are four different types of casein proteins: αs1-, αs2-, β-, and κ-caseins. Collectively, they make up around 76–86%[17] of the protein in milk, by weight. Most of the casein proteins are bound into the micelles. There are several competing theories regarding the precise structure of the micelles, but they share one important

feature: the outermost layer consists of strands of one type of protein, k-casien, reaching out from the body of the micelle into the surrounding fluid. These kappa-casein molecules all have a negative electrical charge and therefore repel each other, keeping the micelles separated under normal conditions and in a stable colloidal suspension in the water-based surrounding fluid[19, 20].

Salts, minerals, and vitamins:

Minerals or milk salts are traditional names for a variety of cations and anions within bovine milk. Calcium, phosphate, magnesium, sodium, potassium, citrate, and chloride are all included as minerals and they typically occur at concentration of 5–40mM. The milk salts strongly interact with casein, most notably calcium phosphate. It is present in excess and often, much greater excess of solubility of solid calcium phosphate.[17] In addition to calcium, milk is a good source of many other vitamins. Vitamins-A, B_6, B_{12}, C, D, K, E, Thiamine, Niacin, Biotin, Riboflavin, Folates, and Pantothenic acid are all present in milk.

Sugars and Carbohydrates:

Milk contains several different carbohydrates including lactose, glucose, galactose and other oligosaccharides. The lactose gives milk its sweet taste and contributes approximately 40% of whole cow's milk's calories. Lactose is a disaccharide composite of two simple sugars, glucose and galactose. Bovine milk averages 4.8% anhydrous lactose, which amounts to about 50% of the total solids of skimmed milk. Levels of lactose are dependent upon the type of milk as other carbohydrates can be present at higher concentrations that lactose in milks.[17]

Health and nutrition:

Table.no.5 Showing nutritional value of cow milk (per 100 gm)

Constituents	**Value** (gm)
Water (gm)	87.8
Protein (gm)	3.2
Fat (gm)	3.9
Saturated fatty acids (gm)	2.4
Monounsaturated fatty acids (gm)	1.1
Polyunsaturated fatty acids (gm)	0.1
Carbohydrate (gm)	4.8
Cholesterol mg	14
Calcium mg	120
Moisture (gm)	81
Fibre (gm)	2
Phosphorus (mg)	13
Energy	66 K.cal

Milk is widely recognized as a healthy beverage since it is rich in a variety of nutrients. It is advised to take milk, meaning that 10% of calories should originate from it.

Milk and skeletal health:

Calcium, a mineral necessary for the growth and maintenance of strong bones and teeth, may be found in cow's milk. Because it provides an adequate amount of the mineral beneficial for the bones. According to several studies, children's bone health is not improved by milk consumption.[22] Despite this, milk and its derivatives are still regarded as being good for children's bone development.

Potassium is abundant in cow's milk. Additionally, cow's milk is heavy in cholesterol and saturated fat, both of which have been associated with a higher likelihood.

Milk & Cancer:

Vit-D may be involved in controlling cell growth and preventing cancer. The least-sunny regions of the world are associated with an increased likelihood of suffering from colorectal cancer, according to research. Vitamin D, which can provide comparable protection, is also present in milk. Additionally, the article cites certain studies that claim that more calcium & lactose from milk[24].

Increased milk consumption may increase the development and power of muscles during resistance training in both younger as well as older persons, according to a study of more than 20 clinical trials.[25] It doesn't appear that cow's milk considerably aids in weight loss. Higher amounts of milk from cows in the short term alongside calorie limitation exhibited no advantage for weight loss, while long-term research with a calorie restriction showed only small benefits[26]. Low-fat milk can offer milk's advantages while containing less fat.

Concerns and safety measures:

In addition, milk has been associated to an increased risk of breast cancer & prostate cancer, two malignancies of the reproductive system.[28,29,30] According to studies, type I diabetes is 30 % less common in infants who don't get exposed to protein from cow's milk in their initial three months of life.[31, 32, 33]

दधि (Curd)

Vyutpatti: -

दधातीति दधि (शब्दरत्नावली)

The word *dadhi* is derived from the *samskrit* root word “दधा”.

Nirukti:-

क्षीरोत्तरावस्थाभाव:।

तत्पर्याया: क्षीरजम्। मंगल्यम्। विरलम्। पस्यम्। (शब्दकल्पद्रुम/खंड.२/६७९)

A lacto-fermented milk product is referred to by the Samskrit word dadhi. This is commonly referred to curd in English and is known in India as dahi (Hindi). A tablespoon of the prior batch of dahi is added to heated milk to make dahi at home. At room temperature, it is then allowed to curdle.

Qualities and benefits of curd:

According to Charak Samhita –

रोचनंदीपनंवृष्यंस्नेहनंबलवर्धनम्।

पाकेऽम्लमुष्णंवातघ्नंमंगल्यंबृंहणं दधि॥

पीनसेचातिसारेचशीतकेविषमज्वरे |

अरुचौमूत्रकृच्छ्रेचकार्श्येचदधिशस्यते ||

शरद्ग्रीष्मवसन्तेषुप्रायशोदधिगर्हितम् |

रक्तपित्तकफोत्थेषुविकारेष्वहितंचतत् || [Cha.Su.27/225-227]

Curd is: रोचन, दीपन, वृष्य, स्नेहन, बलवर्धन, अम्ल-विपाक, उष्ण, वातघ्न, मांगल्य, बृंहण

Useful in - पीनस , अतिसार, शीतक ज्वर, विषमज्वर , अरुचि, मूत्रकृच्छ्र, कार्श्य

Typically, *dadhi sevana* in the fall, summer and spring seasons are detrimental to the health. It brought deterioration of Rakta, Pitta and Kapha in these seasons, so one should not consume it.

According to Ashtang Hrudayam:-

आम्लपाकरसंग्राहिगुरुउष्णम्दधिवातजित् |

मेदःशुक्रबलश्लेष्मपित्तरक्ताग्निशोफ़्कृत ||

रोचिष्णुशस्तमरुचौशीतकेविषमज्वरे |

पीनसेमूत्रकृच्छ्रेच, रूक्षंतुग्रहणीगदे ||

नऐवाद्य निषि नऐवोष्णम्वसन्तोष्ण शरत्सुन|

नामुद्गसूपं नाक्षौद्रं तन्नाघृतसिटोपलं||

न च आमलकं नापि नित्यम्नमन्दं अन्यथा|

ज्वरासृक्पित्त विसर्प कुष्टः पाण्डु भ्रम प्रदम् || [Ashtang Hrudayam.Su.5/29-32]

Dadhi is -

- आम्ल रस , आम्लपाक, ग्राही, गुरु, उष्ण, वातजित्

It enhances – मेद, शुक्र, बल, कफ-रक्तपित्त, अग्निकृत्, शोथकृत्, रोचिष्णु

Curd is useful in – अरुचि, विष्मज्वर, पीनस, मूत्रकृच्छ्र, ग्रहणी

There are following rules for consumtion of Dadhi:

Dadhi should not be consumed at night or regularly in diet or after heating orin *Vasanta, Greeshma* and *Sharad Ritu*. *Dadhi* should not be consumed

without *Ghrita, Sharkara, Mudga Yusha, Madhu, Amalaka* or *Lavana.* The people who consume *Dadhi* without above rules suffer
from *Jwara* (fever), *Raktapitta* (bleeding
disorders), *Visarpa* (erysipelas), *Kushtha* (skin disorders), *Pandu* (anemia), and *Bhrama* (dizziness).

The Sushruta Samhita states:

दधि तु मधुरमम्लमत्यम्लंच इति;

तत्कषायानुरसंस्निग्धमुष्णंपीनसविषमज्वरातिसार

अरोचकमूत्रकृच्छ्रकार्श्यापहंवृष्यंप्राणकरंमन्गल्यंच।

महाभिष्यन्दिमधुरंकफमेदोविवर्धनम्॥

कफपित्तकृद्म्लंस्यात्अत्म्लम्तक्तदूषणम्॥

विदाहिसृष्ट्विण्मूत्रंमन्दजातंत्रिदोषकृत्॥ [Su.Su.45/65-67]

Types of Dadhi: मधुर, आम्ल अति-आम्ल ऽ मंदक दधि

Guna-Karma of Dadhi: कषाय अनुरस, स्निग्ध, उष्णवीर्य

Indicated in – पीनस, विषमज्वर, अतीसार, अरुचि, मूत्रकृच्च्र, कृशता

In healthy individuals, dadhi has aphrodisiac, health-promoting and auspicious effects (Vrushya, Pranakarak).

Curd made from cow's milk:

स्निग्धंविपाकेमधुरंदीपनंबलवर्धनम्।

वातापहंपवित्रंचदधिगव्यंरुचिप्रदम्॥ [Su.su.45/67]

Qualities: स्निग्ध, मधुर विपाक, दीपन, बलवर्धन, वातापहम्, पवित्र

Table.no. 6 Showing the characteristics of dadhi as stated by Bruhatrayee

संहिता ग्रंथ	रस	वीर्य	विपाक	दोषघ्नता	गुण	रोगघ्नता	रोगकारकता
Charaka [Cha.Su. 27/225-227]		Ushna	Amla	Vatashamana	Rochana, Deepana, Vrushya, Snehana, Balavardhana, Mangalya, Brihmana	Peenasa, Atisara, Karshya, Sheeta jwara, Vishama jwara, Aruchi, Mootrakrucchra	Raktapitta, Kaphaja vikara
Sushrut [Su.su.45/65-67]	Types according to Rasa- 1) Madhur 2) Amla 3) Ati-Amla Kashaya Anurasa		Madhura	Vatashamana, Pitta-vardhana, Kapha-vardhana	Snigdha, Ushna, Vrushya, Prankara, Mangalya, Deepan, Balavardhan, Pavitra, Ruchiprada, Maha-abhishyandhi	Pinas, Vishamjwar, Atisara, Arochaka, Mutrakrucchra, Karshya	Kapha-Meda vivardhanam, Raktadushanam
Ashtanga Hrudaya [Ast.Hr.Su 5/29-32]	Amla		Amla	Vatashaman, Pittavardhan, Kaphavardhana	Grahi, Guru, Ushna, Meda-Shukra-Balakruta, Ruchikara	Aruchi, Shitajwara, Vishama Jwara, Pinasa, Mutrakrucchra, Grahani (by Ruksha Dadhi sevana)	Rakta vikar, Agni-dushti, Shotha, Jwara, Raktapitta, Visarpa, Kushtha, Pandu, Bhrama

Table.no.7 Showing dadhi as a Hetu (Aetiology)

Hetu	Reference
1. निजशोथ	च. सू.18/6, च.चि. 12/5, भा.प्र.म.42/1
2. रक्तदुष्टि	च .सू . 24/7, भा.प्र.म.(दधि)
3. शोफ	च .सू . 27/4, अष्ट. हृ. 5/30
4. रक्तपित्त	च .नि .2/4, अष्ट. हृ. 5/33
5. कफजगुल्म	च .नि . 3/10
6. प्रमेह	च .नि. 4/5, मा.नि.,भा.प्र.म.38/1
7. कुष्ट	च .चि .7/7, सु.चि.24/100, अष्ट. हृ.सू.5/33, भा.प्र.म. 54/4
8. श्लेष्मोदर	च चि .13/29
9. अर्श	च .चि .14/9
10. हिक्का, श्वास	च .चि . 17/15, भा.प्र. (दधि)
11. विसर्प	च .चि 21/16, सु.चि. 24/100, अष्ट.हृ.सू.5/33
12. उरुस्तम्भ	च चि .27/8
13. वातरक्त	च .चि .29/6, मा.नि, भा.प्र.म.29/2
14. ध्वजभंग	च .चि.30/164
15. प्रदर	च .चि .30/206
16. स्तन्यरोग	च .चि .30/234
17. पूयवर्धन	सु.सू . 19/16
18. पित्तप्रकोप	सु.सू .21/21
19. बहुगद्कर	सु.चि .24/100
20. कृमि	सु.उत्त्.54/4
21. कफजकृमि	सु.उत्त्. 54/17, अष्ट.हृ.नि.14/46, भा.प्र.म.25/36
22. ज्वर	अष्ट.हृ.सू .5/33
23. पांडु	अष्ट.हृ. सू .5/33
24. भ्रम	अष्ट.हृ. सू .5/33
25. मुखरोग	अष्ट.हृ. उत्त. 21/1, मा.नि., भा.म.66/4
26. विदाह	भा. प्र. पू. 4 (दधि)
27. कामला	भा. प्र. पू. 20(दधि)
28. कफ-प्रकोप	भा. प्र. पू. 47 (रोगि)

दधि **as a** **रोगघ्नता:** भा.प्र.पू.२ (दधि वर्ग). सु.सू.१५/३३; ४५/६५; अष्ट.हृ.सू.५/३०

Table.no.8 showing the presence of Guna and Karma of Dadhi in the disease prognosis

		Shopha	Agnivikara	Jwara	Raktapitta	Visarpa	Kushta	Pandu	Bhrama
Guna	गुरु	+	+	+	+	+	+	+	+
	उष्ण	+	+	+	+	+	+	+	+
	रूक्ष्	-	+	-	-	-	+	+	+
	स्निग्ध	+	+	+	+	+	+	-	-
Karma	ग्राही	+	-	+	-	+	+	+	-
	शोफकृत	+	-	-	+	+	+	-	-
	रोचन	-	-	-	-	-	-	-	-
	दीपन	-	-	-	-	-	-	-	-
	वृष्य	-	-	-	-	-	-	-	-
	स्नेहन	+	-	-	-	-	-	-	-
	बलवर्धन	-	-	-	-	-	-	-	-
	मांगल्य	-	-	-	-	-	-	-	-
	बृंहण	+	+	+	-	+	+	-	-
	पाणकर	-	-	-	-	-	-	-	-
	महाभिष्यन्धि	+	+	+	+	+	+	+	+
	रक्तदूषण	+	+	+	+	+	+	+	-
	विदाहि	+	+	+	+	+	+	+	+
	रुचिप्रदम	-	-	-	-	-	-	-	-

Table.no.9 Showing the relationship among Guna and Rogakaratva

गुण	**Affected Vyadhi number**
गुरु	८
उष्ण	८
रूक्ष्	४
स्निग्ध	६

Table.no.10 Showing relevance between Karma & Rogakaaratva

Karma	**No. of Vyadhi affected**
Graahi	05
Shophakrut	04
Rochana	00
Deepana	00
Vrushya	00
Snehana	01
Balvardhan	00
Mangalya	00
Bruhana	05
Pranakara	00
Maha-abhishyandi	08
Rakta dushana	07
Vidahi	08
Ruchiprada	00

AMOXYCILLIN

Description:

Penicillin G derivative amoxicillin, also known as BRL-2333, was originally mentioned in the scientific literature in 1972[34]. On January 18, 1974, the FDA approved amoxicillin[35].

Description of Chemical Taxonomy:

This substance is a member of the group of chemical substances known as penicillins.

Class: Lactams

Sub-class: Beta lactams

Kingdom: Organic Compounds

Super class: Organo-heterocyclic Compounds

Penicillins are the direct parents.

Molecular Framework: Aromatic heteropolycyclic compounds

External Descriptors: penicillin (CHEBI: 2676)

Synonyms: Amox, Amoxicilina, Amoxicillin, Amoxicilline, Amoxicillinum, Amoxycillin

Pharmacology:

Treatment:

Acute bacterial sinusitis, community-acquired pneumonia, infections of the lower respiratory tract, bacterial otitis media, skin and structure of the skin, skin diseases and urinary tract infections are all treated with amoxicillin and calvulanic acid[38, 39, 40]. Omeprazole and amoxicillin are administered together.

Associated Conditions:

- Community-acquired pneumonia (CAP), illnesses of the ears, nose and throat.

Pharmacodynamics:

Amoxicillin functions by competitively inhibiting penicillin binding proteins, which results in the upregulation of autolytic enzymes and subsequently inhibits the synthesis of the bacterial cell wall[43, 44,45]. Due to its extended duration of action, amoxicillin is typically administered twice daily[39]. Moreover, it boasts a broad therapeutic range, as even mild overdoses rarely cause significant toxicity [(38)]. Patients should receive counseling regarding potential risks, such as anaphylaxis, Clostridium difficile infections and the development of bacterial resistance[36].

Mechanism of action:

Amoxicillin competitively inhibits penicillin-binding protein 1 and other high molecular weight penicillin binding proteins[43, 44].

Metobolism:

When metabolized by human liver microsomes, amoxicillin produces 7 metabolites: M1 (hydroxylation), M2 (oxidative deamination), M3 to M5 (oxidation of the aliphatic chain), M6 (decarboxylation), and M7 (glucuronidation)[49].

Route of elimination:

Regarding elimination, 70-78% of amoxicillin doses ranging from 125 mg to 1g are excreted in the urine after 6 hours[34].

Half-Life

The half-life of amoxicillin is approximately 61.3 minutes.

Clearance:

Its mean clearance is about 21.3L/h[46, 47].

Toxicity:

In cases of overdose, patients may present with various symptoms, including hematuria, oliguria, abdominal pain, acute renal failure, vomiting, diarrhea, rash, hyperactivity, and drowsiness. Symptomatic and supportive treatment, such as emesis or hemodialysis, may be employed for managing overdose situations[38, 50].

Affected organism:

Amoxicillin exhibits effectiveness against a broad spectrum of organisms, including enteric bacteria.

Food Interactions:

Better to taken with food.

Lyophilization

Definition of lyophilization:

Lyophilization, also known as freeze-drying, is a process employed to remove water from perishable materials for the purpose of preservation, extending shelf life or facilitating transportation.

Working Mechanism:

Lyophilization involves freezing the material first, then reducing the pressure and applying heat to allow the frozen water in the material to sublimate, transforming it directly from a solid to a vapor state. Proper lyophilization can significantly reduce drying times, up to 30%.

Three Primary Stages of Lyophilization:

1. Freezing Phase:

- The material can be frozen using different methods, such as using a freezer, a chilled bath, or a shelf in the freeze dryer itself.
- Cooling the material below its triple point ensures sublimation occurs during the drying process rather than melting.
- To achieve the best results, large ice crystals are preferred, which can be obtained through slow freezing or annealing. Rapid freezing is employed for biological materials to prevent damage to cell walls. Annealing can be used for materials prone to precipitation, involving fast freezing followed by controlled crystal growth.

2. Primary Drying (Sublimation) Phase:

- In this phase, the pressure is lowered, and heat is applied to the material to promote water sublimation. Vacuum aids in speeding up sublimation.
- A cold condenser provides a surface for water vapor to solidify, protecting the vacuum pump from water vapor.
- About 95% of the water content is removed during primary drying, but it can be a slow process to avoid structural alterations in the material.

3. Secondary Drying (Adsorption) Phase:

- The final stage involves raising the temperature above that used in primary drying to break the bonds within the material & remaining water molecules.
- Freezed materials retain a porous structure after this phase.
- Soon after lyophilization process, the vacuum can be broken with an inert gas before sealing the material, leaving about 1-5% residual moisture.

Lyophilization Steps:

The process of lyophilization includes several steps:

1. Formulation
2. Packing (Bulk, Flask, Vials) into containers
3. Thermal treatment of freezing at atmospheric pressure
4. Vacuum-assisted primary drying (Sublimation)
5. Vacuum-assisted secondary drying (desorption)
6. Vial Backfill and Stoppering (under partial vacuum)
7. Taking dried goods out of the freeze dryer

Freeze Drying Equipment:

Freeze drying equipment consists of:

1. **Refrigeration System:** Cools the condenser and product chamber.
2. **Vacuum System:** Includes a vacuum pump, condenser, and product chamber.
3. **Control System:** Regulates temperature and pressure, offering options for programming the lyophilization process.
4. **Product Chamber:** Holds the material to be lyophilized, either in flasks or on shelves.
5. **Condenser:** Attracts vapors for sublimation, and its temperature must be lower than that of the product for effective condensation.

Based on the product chambers, freeze dryers can be divided into three groups: **shelf dryer** (with tray or direct shelf placements), **manifolds dryer** (for pre-frozen material in flasks), or **combination machines** that have both drying options.

Primary drying:

Main Drying and **Subsequent Drying** are the two basic phases of freeze drying. The majority of the product's water is removed during primary drying, which is accomplished by sublimating all of the free ice crystals. Organic solvents are also eliminated in this phase.

The process of primary drying, often referred to as sublimation, is slow and done at colder temperatures, safely below the product's critical collapse temperature. Heat energy is needed for sublimation in order to cause the phase transition from solids to gas. Convection, radiation, and conduction are the three heat transmission mechanisms that must be considered during freeze drying.

Heat is primarily transmitted to the flask/product in a straightforward manifold dryer through conduction as well as radiation from the surroundings. Managing the process gets more difficult because there is less control over heat flow. It could be required to cover or insulating the flask for items with low temperatures for collapse in order to slow down the transfer of heat and prevent collapse.

The majority of the heat is instead directed into the product in the shelf freeze dryer. Maximizing the surface interaction between the item, container, or tray & the shelf is essential. To maintain product homogeneity and process control, radiation and convection must also be taken into account.

The "edge effect" refers to how items on the edge of the shelf dry more quickly than those in the middle due to radiant heat from the wall cavities of the product chamber. Product in the front portion of the freeze dryers dry the quickest thanks to the action of the acrylic doors that are frequently used in pilots and research and development freeze dryers. Production freeze dryer are made with metal doors and tiny view ports because of this. Aluminum foil can sometimes be used as a barrier within a pilot freeze drier to block radiation, though doing so prevents product inspection while the operation is taking place.

Convective heat transfer might facilitate uniform product drying due to uneven shelf contact. System pressures between 100 mTorr and 300 mTorr typically promote sufficient convection. Because there are fewer gas molecules available to create convection at extremely low system pressures under 50 mTorr, drying is uneven and takes longer.

Primary drying is a controlled top-down process in freeze drying, characterized by a well-defined sublimation front moving through the product. Above the ice surface interface, the product forms a dried layer, often referred to as the "cake," while below this interface, There are still some ice crystals that need to melt. The product can seem entirely dried once all of the loose ice crystals undergo melting at the end of the initial drying. However, because there are water molecules that have been "sorbed" onto the product, the moisture content may still be between 5 and 10%.

The remaining factors to be controlled are the shelf temperatures and system vacuum level, which is normally, set several degrees cooler than the critical temperature. Setting the system pressure initially using the ice table's vapor pressure is a suggested strategy. Using thermocouples to measure the product temperature, the shelf temperatures set point is gradually raised until the product achieves the desired temperature. The shelf a temperature is held constant for the remainder of primary drying once the intended item's temperatures have been reached. To maintain the intended item's temperature and avoid collapse, it may be necessary in some circumstances to lower the shelf temperatures toward the end of primary air drying for products with significant vapor flow resistance in the dried region.

Determining when primary drying is complete:

There are numerous analytical techniques available to judge whether primary drying is finished. The simplest method is using a thermocouple, which is a probe to track the product's temperature. Since heat from the shelf is used for the melting phase shift, the product's temperature will be lower during active main drying than the shelf temperatures set point. The temperature of the product will rise until it reaches the

shelf temperature once the sublimation of the ice crystals is finished. The primary drying process is complete when the item's temperature reaches the shelf temperature.

Due to improved heat conduction, the particular vial containing the temperature sensor wire may dry sooner than other vial on the shelf. Similar to bulk drying, the thermocouple wire's immediate vicinity will dry the product tray quicker than other areas.

To ensure complete sublimation of all ice in the entire batch, it is advisable to allow some additional drying time (typically 30 minutes to 2 hours) after the product thermocouple temperature increases. The product dries top down during primary drying. The thermocouple's tip should be positioned in the middle and bottom of the container for reliable readings. The thermocouple touching the container's bottom is acceptable. When drying in vials, it's best to place the thermocouple in the center of the shelf because items or vials at the edge of the shelf may dry more quickly due to radiant heating effects.

Secondary drying:

The next stage after primary drying is secondary drying, during which water molecules that were bound to the product during primary drying are removed (desorbed). Desorption is accelerated by the higher temperatures employed in secondary drying, which are typically in the range of 30 to 50 oC. The product temperature affects secondary drying rates, and secondary drying typically doesn't require any more suction than original drying did.

Amorphous products may require careful control of the temperature increase from primary to secondary drying to avoid collapse. Secondary drying continues until the product reaches acceptable moisture content for long-term storage, which is typically between 0.5% and 3%. Certain intricate biological products may not be suitable for extreme dryness, in which case the secondary drying procedure should be modified.

In order to prevent rehydration due to atmospheric exposure, lyophilized items must be packed in airtight containers due to their high hygroscopicity after freeze drying. Some freeze dryers offer the "stoppering" capacity which seals the product while it's still within the machine under a partial vacuum. Before sealing, refilling with an inert gas, such as dry nitrogen, is another option.

Problems to avoid during lyophilisation:

To ensure a successful lyophilization process, certain problems should be avoided, such as overheating the product, causing melt-back or collapse, and overloading the condenser with excessive vapor due to factors like too much vapor creation, insufficient refrigeration, or improper vapor flow. Additionally, vapor choking, where the vapor is produced faster than it can escape through the vapor port, leading to an increase in chamber pressure, should be prevented.

Important terms related to Lyophilizer:

1. Eutectic Point or Eutectic Temperature:

The temperature at which the product exists solely in the solid phase, representing its minimum melting temperature. Some products may lack a eutectic point, or there could be multiple eutectic points.

2. Critical Temperature:

The maximum temperature during lyophilization at which the product's quality begins to degrade due to melt-back or collapse.

3. Crystalline:

Refers to a material that forms crystals when frozen. Crystalline substances have one or more eutectic points, and fast freezing can lead to small crystals that are challenging to dry. Annealing can facilitate the formation of larger crystals.

4. Amorphous:

Applies to multi-component mixtures that do not crystallize and lack a eutectic point. Instead, they transition into a glass-like state. For amorphous materials, freeze drying should be conducted below the glass transition temperature.

5. Collapse:

The point at which the product softens to the extent that it can no longer support its own structure. This can lead to various issues such as loss of physical structure, incomplete drying, decreased solubility, and formation of ablation (splat).

Culture Analysis Methods of the Lyophilized Sample:

Preparation of Medium: Establish the proper incubation conditions and prepare the suitable medium as well as any other reagents required for the growth and resurrection of the bacterial strain.

Starting Frozen Cultures:

1. Create a clean test tube and fill it with the product's suggested growing media.
2. Gently defrost the sample vial in a bath of water at the temperature the strain prefers for growth till all ice crystal have dissolved.
3. In a laminar flow chamber under rigorous aseptic conditions, clean the exterior of the vial with 70% ethanol.
4. Empty the full contents of the vial into the clean test tube containing the growth media. A part of the main culture can be transferred to additional test tubes to be injected.
5. Incubate the starter cultures at the suggested temperature and humidity level.
6. Examine cultures after the designated incubation period.

Initiating Lyophilized Cultures:

1. Aseptically add the recommended growth medium to the freeze-dried material using a Pasteur pipette and mix well.
2. Transfer the suspension to a test tube with the appropriate growth medium. Also, transfer drops of the suspension to an agar slant.
3. Incubate the cultures under the recommended conditions, and additional test tubes can be inoculated from the primary culture.
4. Examine cultures after the designated incubation period, noting that most freeze-dried cultures grow within a few days.

Bacterial Growth Conditions and Temperature:

Different bacterial species thrive under varying growth temperatures. Pathogenic or commensal strains typically grow well at body temperature (37°C),

while environmental strains prefer lower temperatures (25°C to 30°C). Bacterial species are categorized based on their growth temperature, such as psychrophiles (0°C to 20°C), mesophiles (25°C to 40°C), and thermophiles (45°C to 122°C).

Growth Media for Bacterial Culture:

Culture media can be defined, complex, selective, or enrichment medium. Defined media have a known chemical composition, while complex media contain biologically derived reagents with an unknown composition. Selective media inhibit specific bacterial species' growth or promote the growth of certain species. Enrichment media also allow for the growth of specific bacterial strains.

Bacterial Authentication:

Bacterial strains undergo phenotypic & genotypic examinations for accurate identification, purity & consistent biochemical results. Phenotypic characterization involves analyzing colony & bacterial morphology, sugar metabolism & antibiotic susceptibility. Genotypic characterization confirms species identity primarily through 16S ribosomal RNA (rRNA) sequencing.

JUSTIFICATION

- Ayurvedic understanding of the concept of probiotic is holistic[6].
- There is a direct reference of the concept of *sahaja krimi* (*Avaikaarika krimi*[24]) i.e friendly micro-organisms which are located in the intestine was also highlighted in *Charaka Samhita & Sushruta Samhita*. These *sahaja krimi* correlates as Lactic Acid Bacteria (LAB) and *Bifidobacteria in contemporary nutritional science under the name "Probiotics"*.
- As per Ayurveda classics, curd, buttermilk and other fermented foods[10, 11], drug & dietary supplements[27,28] which have been reported to be safe[9,10,11,12] are explained in management of *atisara* (diarrhoea) and *grahani* (IBS) like conditions.
- This is the principle in treating various conditions like *Atisaara, Grahani*, Antibiotic Associated Diarrhoea (AAD) and Irritable Bowel Syndrome (IBS). But this concept is hidden, un-explored & not scientifically analysed.
- Till date, no study related to herbal incorporated probiotic formulation / preparations are carried out in any Ayurveda institutions.

With considering all these aspects, this study was planned to evaluate the effect of Ayur-probiotic preparation with *apakwa bilvaphala (un-ripened bilva fruit) saara in dadhi* media and effect of the preparation was evaluated in Antibiotic Associated Diarrhea (AAD) in the Wister rats.

MATERIALS AND METHODS

The Research study was carried out in the following steps:

1. Development of Ayur-Probiotic under three Pilot Studies.
    - Pilot Study 1
    - Pilot Study 2
    - Pilot Study 3
2. Experimental Study of Ayur-Probiotic on Antibiotic Associated Diarrhoea (AAD) in experimental animals.

1. Preparation of Ayur-Probiotic:

Ayur-Probiotic was prepared by Lyophilization of *apakwa bilwaphala majja* with curd. This procedure was conducted in different ratio of combinations of *apakwa bilwaphala majja* with curd.

Materials & Methods:

Materials:

Ingredients of Ayur-Probiotic:

1. Cow Milk
2. Curd
3. *Apakwa bilwa phala majja* (un-ripened bael fruit pulp)
4. Amoxicillin trihydrate
5. Sporlac
6. Wistar rats

Procurement:

- Cow milk was procured by known milk vendor, Belagavi and Authentication of Cow milk sample was done at Divisional Food Laboratory, Belagavi.
- Curd was prepared by traditional method[1] at Pathyahara Unit, KLE BMK Ayurveda Hospital Shahapur, Belagavi. Quality Control (QC) of curd sample was done at Divisional Food Laboratory, Belagavi.
- Fresh un-ripened *bilwa* (*Aegle marmelos.* Linn) fruit was collected from the natural habitat, Herbal Garden, KAHER's Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi. Quality Control (QC) analysis was done in AYUSH approved Drug Testing Lab for ASU drugs, KAHER's Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya, Belagavi.
- Amoxicillin Trihydrate was procured from "Empree Pharmaceuticals" Belagavi.
- Sporlac was purchased from "APOLO Pharmacy", Belagavi.
- Wistar rats were purchased from "Shri. Raghavendra Enterprises", Breeders & Suppliers of Animals (Govt. of India Reg. 841/b/04/CPCSEA)Vijaya Nagar, Bengaluru.

Methodology:

Development of trial drug (Ayur-Probiotic) was done at CRF (Central Research Facility), KAHER's Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi and Dr.Prabhakar Kore Basic ScienceResearch Centre (BSRC) at V.K.I.D.S Campus, KAHER, Belagavi.

The parameter for authentication of cow milk was selected as per *FSSAI* Standards. The values obtained were within the normal range.

Preparation of Curd:

- Curd was prepared by traditional method[1] at Pathya Unit, KLE BMK Ayurveda Hospital Belagavi. Quality Control (QC) of curd sample was done at Divisional Food Laboratory, Belagavi.
- Cow milk was heated intensively to boil for 10 to 15 minutes and then it was cooled to room temperature. Cooled milk was added with previous day's cow curd, stirred and allowed to set undisturbed for overnight. Curd was stored at room temperature. Storage area was maintained clean and tidy to avoid any cross contamination.
- The parameter for assessment of curd was selected as per *FSSAI* Standards. The values obtained were within the normal range.

Extraction of un-ripened bilwaphala majja:

Method of extraction:

- Fresh un-ripened *bilwa* fruit (*apakwa bilwa phala*) was thoroughly washed in clean potable water to remove its physical impurities.
- The un-ripened fruit pulp of *bilwa* was extracted with the help of clean spatula & stored in clean vessel.

Development of Ayur-probiotic:

In the preliminary study, Ayur-probiotic was prepared in different ratio of curd with un-ripened *bilva* fruit pulp. Series of pilot studies have been conducted & assessed for count of Lactobacillus (LAB) & other probiotic organisms in the Ayur-probiotic. During screening of the organisms, the ratio showed sufficient colony count of Lactobacillus & other probiotic organisms and it was selected & approved for preparation of final Ayur-probiotic product.

Procedure of preparation of Ayur-probiotic:

- Freshly prepared curd was mixed with un-ripened *bilwa phala majja* and stirred thoroughly to mix homogeneously.
- Mixture was subjected to dryness by freeze drying technique (here mixture was kept for one day in deeper freezing compartment of refrigerator).
- Then, next day freeze dried material was kept in lyophilized bulbs of Lyophilizer.
- The temperature of lyophilizer was maintained up to -40°C[33] till the completion of lyophilization process. This procedure was known as lyophilization technique.
- The content was observed for amorphous dry powder form.
- The Ayur-Probiotic (lyophilized product) was stored in cold storage for further procedures.

Observation:

- -40°C for main drying & -20°C for final drying; *60-72 hrs* time duration required to get *Bilwaphala saara dadhi* in Lyophilized form.
- Lyophilized fine powder was obtained on 3rd day.
- Packing it in airtight container and stored in cold storage.

Precaution:

- Pulp should be complete devoid of seeds while collecting *phala majja*.
- *Phala majja* should not be washed with water.
- Clean vessels for collecting *phala majja* part & curd should be ascertained.

PILOT STUDY 1

In this study, lyophilization of curd in two quantities was prepared. Ayur-probiotic in two different ratio of curd and *Apakwa bilwa phala majja* was developed.

Table.no.11 Showing Lyophilized sample in different ratio of curd and *Bilva phala majja*

Name of Lyophilized sample	Curd quantity	*Apakwa Bilva phala majja* quantity
A	150 ml	-
B	200 ml	-
C	150 ml	50 gm
D	200 ml	50 gm

Results:

Table no.12 Showing the results of organoleptic characters of the Lyophilized samples

Name of the Lyophilized Sample	Form	Colour	Taste	Odour
A	Powder	White	Sour	Milky
B	Powder	White	Sour	Milky
C	Powder	Light brown	Sour & bitter	Characteristic
D	Powder	Light brown	Sour & bitter	Characteristic

Table no.13 Showing the results of Phyto-chemical analysis of the samples

Name of the Lyophilized Sample	Carbohydrate		Reducing sugar		Test for proteins		Alkaloids		Tannins		Glycosides	
	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol
Sample-A	+	+	+	-	-	-	+	+	-	-	-	-
Sample-B	+	+	-	-	-	-	-	-	-	-	-	-
Sample-C	+	+	+	-	+	-	-	-	+	+	+	-
Sample-D	+	+	+	-	+	+	-	-	+	+	+	-

Test for monosaccharides, pentose sugar, non-reducing sugar, hexose sugar, amino acids, steroids and flavonoids showed negative in all samples.

Table.no.14 Showing the results of Physico-Chemical properties of the Lyophilized samples

Name of the Lyophilized Sample	Loss on drying	Ash value	Acid insoluble ash	Water soluble extractive	Alcohol soluble extractive	pH value
Sample-A	11.706%	5.732%	0.797%	82.002%	53.302%	3.34
Sample-B	8.391%	6.972%	1.045%	93.884%	44.479%	3.74
Sample-C	8.449%	6.237%	0.948%	94.417%	45.772%	4.03
Sample-D	6.593%	5.932%	0.598%	55.980%	45.295%	4.34

Reason for rejection:

In this study, Curd lyophilization was carried out successfully in two selected quantity.

Ayur-probiotic was prepared in sample-C and D in the ratio of 150:50 and 200:50.

The colony forming units of lactobacillus was detected as "NO growth" in both the sample. This may be due to presence of large quantity of phyto-constituents like tannins which may act as anti-microbial and high acidic pH etc. So, the ratio in sample-C and D was rejected for further study.

Pilot study 2

In this study, lyophilization of 100 ml curd was prepared. By considering the results of Pilot I, the quantity of curd and *apakwa bilwa phala majja* was reduced and Ayur-probiotic was developed. Here, total two samples were lyophilized and assessed for count of Lactobacillus and other organisms.

Table.no.15 showing the ratio of curd and *apakwa bilva phala majja*

Name of Lyophilized sample	Curd quantity	*Apakwa Bilva phala majja* quantity
I	100 ml	12 gm
II	100 ml	24 gm
III	100 ml	--

In this study, 100 ml of curd, 12 gm & 24 gm un-ripened *bilwa phala majja* was selected.

1. ***Reasons for selection of 100 ml Curd:***

100 gm of curd is said to be the recommended daily allowance for consumption in order to get required amount of probiotic organisms for beneficial action over intestinal flora.

2. ***Reasons for selection of 12 gm Bilwaphala majja:***

Maatra (dose) of *Phala-majja* (fruit pulp) is explained as 1 *Karsha Pramaana* ie, 12 gm as per the standard dose in human, explained in the *Sharangadhar Samhita*.

Hypothesis for this:

- Culture test for the samples were undertaken. If growth occurs after keeping both the samples in the culture media, it confirms that samples contain LactoBacillus Acidophilus (LAB) & Bifidus organisms. Next to go for lyophilization.
- If we add 12 gm *apakwa bilwa phala majja* to the 100 ml curd, our assumption is to know & check whether probiotic organisms like Lacto Bacillus Acidophilus (LAB) & Bifidus organisms present in the curd sample will going to alter the formulation status in terms of survival & viability of probiotic organisms.
- If we add 24 gm (double the dose as recommended) *apakwa bilwaphala majja* to the 100 gm curd, our assumption is to know & check the survival & viability of the LactoBaccilus Acidophilus (LAB) & Bifidus organisms in this strength of the formulation.
- After confirmation of this aspect, then only we can plan lyophilization of both the samples.

- Then, again to check the state of survival & viability in the Lyophilized (powdered) samples.
- This will help us to know for calculation for proper dosage of the *Bilwaphala saara dadhi* (formulation) in experimental animals in the present study.

Figure.no. 2 showing growth of probiotic organisms (LAB) in sample 1, 2 & 3

Observation & Result:

Table.no.16 Showing observation & result of samples assessed

Sample	**I**	**II**	**III**
Ratio of Curd:*Bilwa phala majja*	100: 12	100: 24	100: 0
Growth of LAB organisms (seen in the culture test prior to lyophilization)	No growth	No growth	Growth
Conclusion of the pilot study	Rejected	Rejected	**Selected**

Conclusion:

- In plain curd sample III, culture test shows the presence of growth & viable of organisms.
- No growth was observed in both sample I and II
- The ratio selected in this both samples ie 100: 24 and 100: 12 has been rejected
- Culture test of both the samples were shown absence of growth of an organisms. Thus, it seems to be because of high dose of the *apakwa bilwaphala majja* either in 12 gm or 24 gm in curd media destroyed the probiotic organisms present in both the samples.
- One more assumption is that, *apakwa bilwaphala majja* being possessing *rooksha, ushna guna & graahi karma (drava shoshana* action) may destroyed / killed the probiotic organisms.
- Hence next pilot study has been planned and initiated.
- Because of non-availability of moisture content & nutrition to the probiotic organisms, they may not survive in both the samples.
  - ✓ No growth was observed in sample I and II of Pilot study I.
  - ✓ Thus, these two samples have been rejected.
  - ✓ Hence, next pilot study has been planned and initiated.

Pilot study 3

In this pilot study, plain curd (100 ml) sample was not selected for the culture test. Because, in the first pilot study only it reveals the growth of viable organisms when kept for culture test. By considering the results of Pilot study 2, the quantity of curd and a*pakwa bilwa phala majja* was reduced and Ayur-probiotic was developed. Here total three samples were lyophilized and assessed for Lactobacillus count.

Table.no.17 Showing the ratio of curd and apakwa Bilva phala majja

Name of Lyophilized sample	**Curd quantity**	***Apakwa Bilva phala majja* quantity**
IV	100 ml	6 gm
V	100 ml	3 gm
VI	100 ml	1.5gm

- After weighing 100 ml of curd was poured to the three clean sample vessels.
- 6 gm *apakwa bilwa phala majja* was weighed & mixed in 100 ml curd thoroughly with clean stirrer. Then appropriate labeling of the sample (Sample-IV) was done (ie, numbering of the sample, its name, quantity & date).
- 3 gm *apakwa bilwa phala majja* was weighed & mixed in 100 ml curd thoroughly with clean stirrer. Then appropriate labeling of the sample (Sample-V) was done (ie, numbering of the sample, its name, quantity & date).
- 1.5 gm *apakwa bilwa phala majja* was weighed & mixed in 100 ml curd thoroughly with clean stirrer. Then appropriate labeling of the sample (Sample-VI) was done (ie, numbering of the sample, its name, quantity & date).

Precaution: As same as the previous pilot study undertaken.

Culture test:

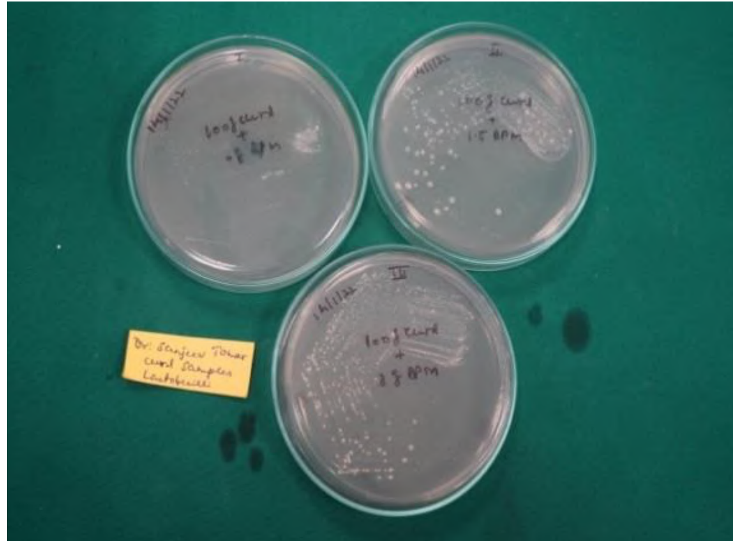


Figure.no. 3 showing growth of LAB organisms in 6 gm, 3 gm & 1.5 gm samples

Result of Culture test:

- In plain curd sample, culture test shows the presence of growth & viable of Probioticorganisms.
- Culture test of 3 gm of *apakwa bilwaphala majja* shown the presence of good growth of probiotic organisms in the curd media.
- It can be said that, *apakwa bilwaphala majja* in 3 gm dose allowed the survival of probiotic organisms.

Table.no.18 Showing observation & results of the samples assessed

Sample	**IV**	**V**	**VI**
Ratio of Curd: *Bilwa phala majja*	100: 6g	**100: 3g**	100: 1.5g
Growth of LAB organisms (seen in the culture test prior to lyophilization)	Mild growth ($1x10^3$CFU/ml)	Good growth & viable probiotics **($5x10^3$ CFU/ml)**	Good growth & viableprobiotics ($1x10^4$CFU/ml)
Conclusion of the pilot study	Rejected	**Selected for further study**	Rejected

***Conclusion**:*

- In the previous Pilot study, plain curd sample shown the presence of growth & viable of probiotic organisms. So, in this Pilot study, it is not carried out.
- Culture test of Sample-IV shown presence of growth of organisms. Thus, it seems to be because of low dose of the *bilwaphala-majja* in 6 gm in curd media.
- Culture test of Sample-V & Sample-VI were shown the presence of good growth of an probiotic organisms. Thus, it seems to be because of low dose of the *bilwa phala majja* in3 gm & 1.5 gm in curd media.
- It can be said that, *apakwa bilwa phala majja* in low dose allowed the survival of probioticorganisms.
- Because of proper availability of moisture content & nutrition to the probiotic organisms, they may have survived in all the three samples.

Development of Ayur-probiotic

The sample-III of Pilot study 3 was selected for development of Ayur-Probiotic. Preparation of Ayur-probiotic was prepared in CRF (Central Research Facility) KAHER's Shri. B.M.Kankanawadi Ayurveda College and BSRC, KAHER, Belagavi.

Preparation of Curd:

100 ml of milk was taken for the preparation of curd. Total quantity of curd after curdling was 120 ml.

Methodology of Ayur-Probiotic:

Table.no.19 Showing quantity of curd & *apakwa bilwaphala majja*

Ingredients	Quantity
Curd	100 ml
Apakwa Bilwa Phala Majja (un-ripened fruit pulp of bilwa)	3 gm

- After weighing, 100 ml of curd was poured to the clean sample vessels.
- 3 gm un-ripened *bilwaphala majja* was weighed & mixed in 100 ml curd thoroughly with clean stirrer. It was subjected to Lyophilization technique.
- 60 gm of White amorphous powder form of Ayur-Probiotic was obtained.

Precaution:

- Seeds should not be present while collecting *phala-majja* of the sample.
- *Phala-majja* should not be washed with water.
- Curd & *Phala-majja* part should be collected in clean vessels.

Standard Operating Procedure for freeze dryer of the product:

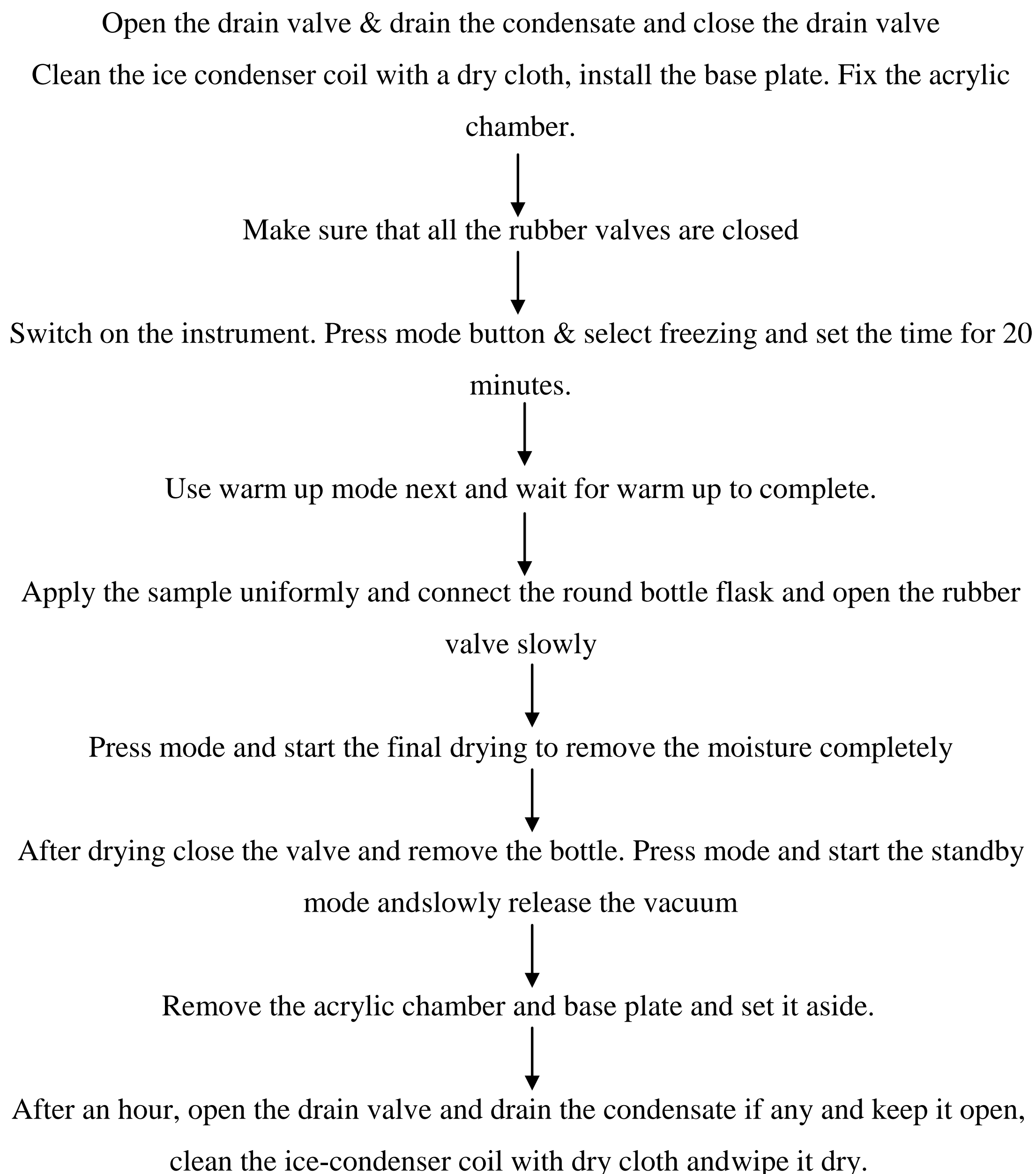


Figure.no. 4 Lyophilized product of the sample

Figure.no. 5 showing culture analysis of the Lyophilized sample

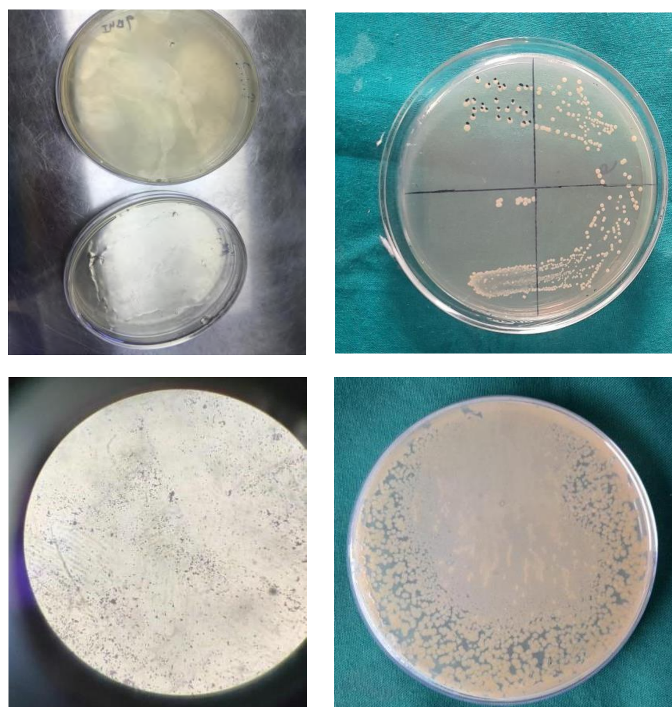


Figure.no. 6 showing growth of LAB organisms in the Lyophilized culture analysis of Ayur-Probiotic[32, 33]

The analysis of prepared Ayur-Probiotic was done at AYUSH approved Drug Testing Laboratory for ASU drugs and BSRC laboratory.

Following analytical parameters were assessed for prepared formulation.

- Organoleptic characteristics: Various organoleptic characters such as colour, taste, odor & texture of the sample were assessed.
- Physico-chemical characteristics: Various physico-chemical characters such as presence of loss on drying, ash value, acid insoluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive & pH value of the sample were assessed.
- Phyto-Chemical characteristics: Various phyto-chemical characters like presence of carbohydrate, sugar, non-reducing sugar, proteins, alkaloids, steroids, flavonoids, tannins, glycosides in water and alcohol extracts were assessed.
- Presence of Lactobacillus and Bifidobacterium species organisms were assessed.

Procedure of DNA Isolation & Sequencing for qPCR analysis of the Ayur-Probiotic product:

- Identification of specific probiotic bacteria & its strength:

Observation during blasting of the sequences in Mega software, following results were obtained.

- Amplification was done by polymerase chain reaction (PCR) using Primer 1_F03.ab1.
- Sequencing PCR was performed for both of the strands using Primer 2_F03.ab1 andMega software.
- Purification and analysis of all sequencing reaction was done.
- Sequences were compared with the GenBank DNA database using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov) and identified as Staphylococcus epidermidis and Lysinibaccillus, Firmicutes bacteria & Braccillus species.
- Analysis of Organoleptic, Physico-chemical and Phyto-chemical parameters:[6,8,9,10] of the lyophilized sample were assessed & results obtained in the CRF of KAHER's Shri B M Kankanawadi Ayurveda Mahavidyalaya Shahapur, Belagavi.
- Aqueous and alcoholic extracts of Lyophilized sample were subjected for qualitative preliminary phyto-chemical screening[6,8,9,10].

EXPERIMENTAL STUDY

Experimental study was carried out at the Animal Experimental Laboratory of KAHERs' Shri. B.M.Kankanawadi Ayurveda Mahavidyalaya, Belagavi. The study protocol was approved by institutional Animal Ethical Committee of the College (BMK/IAEC/Res.No.-21/2021-09 dated 6th August 2021). The OECD test guidelines 420 was followed for acute toxicity study.

Materials:

1) Wistar rats
2) Ayur-Probiotic (formulation)
3) Amoxicillin trihydrate
4) Sporlac

Methodology:

Wistar rats were procured from "Shri.Raghavendra Enterprises", Breeders & Suppliers of Animals (Govt.of India Reg. 841/b/04/CPCSEA) Vijaya Nagar, Bengaluru and animals were kept at an ambient temperature 25 ± 5°C and relative humidity 45-55% with 12 hours light/dark cycles as per CPCSEA Guidelines.

Wistar rats were acclimatized in the laboratory for one week and provided to access standard commercial feed pellets and clean water *ad libitum* throughout the experimental periods.

Experimental Design:

It includes –

1. Acute Toxicity Study
2. Experimental study of Ayur-Probiotic in Antibiotic Associated Diarrhoea(AAD) in Wistar rats

1. Acute Toxicity Study:

The acute toxicity study included five female Wistar rats weighing 150 gm to 180 gm. Saturated picric acid was used to mark each animal for simple identification, as shown below. 1) The head 2) the neck 3) the body 4) the tail 5) the right limb.

Selection of Dose:

2000 mg/kg body weight

Route of administration:

The drug was administered by oral route with the help of a gastric catheter sleeved on to a tuberculin syringe.

Duration of drug administration:

The trial drug was given for a period of 14 days. 5 female rats weighing 150-180 gm were selected for the study.

Table.no.20 showing the dose schedule for – Acute toxicity study

Animal marked	Dose mg/kg	Duration in days
Head	2000 mg	14 days
Neck	2000 mg	14 days
Body	2000 mg	14 days
Tail	2000 mg	14 days
Right limb	2000 mg	14 days

Experimental study of Ayur-Probiotic in Antibiotic Associated Diarrhoea (AAD) in Wistar rats

Methodology:

Wistar rats were procured from “Shri. Raghavendra Enterprises”, Breeders & Suppliers of Animals (Govt. of India Reg. 841/b/04/CPCSEA) Vijaya Nagar, Bengaluru, kept at an ambient temperature 25 ± 5°C and relative humidity 45-55% with 12 hours light/dark cycles as per CPCSEA Guidelines.

Wistar rats were acclimatized in the laboratory for one week and provided to access standard commercial feed pellets and clean water *ad libitum* throughout the experiment periods.

Study design[34, 35] of Antibiotic Associated diarrhea in animals:

48 healthy, Wistar rats of either sex weighing 150-180 gm were selected for the study & divided into 8 groups with 6 rats in each group. Male & female rats were separated and each three rats were kept in polypropylene cages. All animals were marked as Head, Body & Tail for all the eight group animals. Initial weights of each rats were recorded. Potable drinking water & food pellets were provided daily.

Dose calculation:

Classical dose of *choorna* is *1 karsha pramana* ie, 12 gm and Animal dose was calculated by fallowing Paget and Barners' table.

Choorna adult dose **X** body surface area ratio convertibility factor.

= 12 gm (Max. therapeutic dose) X 0.018

= 0.216 mg

So, the dose is 0.216 mg/ 200 gm body weight of Wistar rats

Study Drug – Ayur-Probiotic (Lyophilized form)

Induction Drug – Amoxicillin trihydrate (Powder form)

Standard Drug – Sporlac (Powder form)

Instruments: Needle, sterilizer, cotton, surgical instruments, watch glass, pipette, refrigerator, mortar & pestle.

Duration of the study: 30 days

Grouping:

Table.no.21 showing experimental animal group details

Group name	Group Description	Intervention (30 days)
A	Control	Treated with Amoxicillin for 30 days: Normal food & water was given
B	Normal	No intervention (Un-treated): Normal food & water was given for 30 days
C	Curd	Treated first 15 days with Amoxicillin & later on curd as intervention for 15 days
D	*Apakwa bilwaphala majja* (BPM)	Treated first 15 days with Amoxicillin & later on bilwaphala majja (BPM) for 15 days
E	Sporlac	Treated first 15 days with Amoxicillin & later on Sporlac as proven probiotic for 15 days
F	Ayur-probiotic (Raw form)	Treated first 15 days with Amoxicillin & later on Ayur-Probiotic for 15 days
G	Amoxicillin + Sporlac	Treated all the 30 days with Amoxicillin
H	Amoxicillin + Ayur-Probiotic	Treated all the 30 days with Amoxicillin + Ayur-probiotic

- Wistar rats were kept in steel cages individually with absorbent paper and observed for watery wet stools.
- Respective drug solution gavage administered through oral route to each animal by insulin syringe.
- Syringes & other materials were autoclaved after completion of dosing to the animals on daily basis.
- Weight observation was noted weekly.
- Food and water observation was noted daily.
- According to the goals of each group, amoxicillin & Ayur-probiotic combinations were administered.
- Amoxicillin trihydrate was used to induce diarrhoea in the animals. According to the plan of the study, the antibiotic powders was dissolved in water and given daily by the gavage method. Consistency of stools was assessed as normal pelleted stools, soft-formed stools & watery stool or diarrhoea.
- After the induction of diarrhoea, all the Wistar rats were closely observed for their behavior and symptoms.

Observations:

- All relevant observations (signs of weakness, hunched posture, wet tail & weight loss during the experiment[35]) of animals were meticulously documented during the study. Changes in fecal consistency were recorded for each rat.
- During the commencement of the experiment & before administering the actual respective dose to the animals, weight of all the animals were noted & considered it as '0' day.

- Weight of each animal was measured & noted on every 7th day of the experimental animal group.
- The quantity and consistency of fecal output in rats confirmed that they were suffering from diarrhoea.
- After the experiment was complete, all 48 animals were sacrificed in accordance with the legal requirements and ethical standards.
- Samples of the caecum, rectum and anus (collected simultaneously) were taken. The histology slides were examined under Olympus BX-41 microscopy (Olympus, Japan) using 10% buffered formalin, which was used to treat all tissue samples.
- An integrated Olympus U-TV0.35XC-2 T8 camera (Olympus, Japan) was used to take the micro-photographs. Three fields/animal/intestinal segments were evaluated at a magnification of 40.
- The Microbiological parameters and Histo-Pathological assessments were assessed from intestinal samples.
- Organ samples collected & stored in sterilized container & sealed.
- Samples were sent for the microbiological analysis.

At the end of the 30th day, all the animals of respective groups were sacrificed & taken caecal with its contents (stool), anus & rectum organs (together) for histo-pathological study.

Statistical analysis:

- The starting body weight of the rat was compared using an ANOVA one-way test (p 0.05) to evaluate group uniformity and to ascertain whether changes in the body weights of the rat from the 8 groups were statistical significant.

- The inter-quartile range (IQR) was used to express the scores obtained for histopathology.
- The Kruskal-Wallis test was used to compare the scores of the caecum and ano-rectal sample specimens. The Mann-Whitney U test was used to analyze unpaired observations. P values under 0.05 were deemed statistically significant.
- Mean and SEM are used to express values. The Graph Pad Prism five software was used to analyze statistical data of the study.

OBSERVATION & RESULTS

The Results are obtained mainly under three headings:

- Analytical results
- Quality Control (QC) study of Ayur-probiotic results
- Experimental results

- **Analytical results:**

Table.no.22 showing analysis results of cow milk sample

Sl.no	Quality characteristics	Test References	Result	Standards
1	Milk fat	*FSSAI*	Detected	May be present
2	Milk solid not fat	*FSSAI*	3.8%	Not less than 3.2%
3	Test for sugar	*FSSAI*	10.08%	Not less than 8.3%
4	Test for starch	*FSSAI*	Not detected	Negative
5	Test for urea	*FSSAI*	Not detected	Negative
6	Test for carbonate	*FSSAI*	Not detected	Negative
8	Test for hydrogen peroxide	*FSSAI*	Not detected	Negative
9	Test for detergents	*FSSAI*	Not detected	Negative

Note: The sample confirms to the standards.

Table.no.23 showing analysis results of curd sample

Sl.no	Quality characteristics	Test reference	Result	Standards
1	Milk fat	*FSSAI*	Detected	May be present
2	Milk solid not fat	*FSSAI*	3.2%	Not less than 3.2%
3	Test for sugar	*FSSAI*	10.34%	Not less than 8.3%
4	Test for starch	*FSSAI*	Not detected	Negative
5	Test for urea	*FSSAI*	Not detected	Negative
6	Test for carbonate	*FSSAI*	Not detected	Negative
7	Test for detergents	*FSSAI*	Not detected	Negative
8	Test for hydrogen peroxide	*FSSAI*	Not detected	Negative

Note: The sample confirms to the standards.

Authentication of Apakwa Bilwa phala:

Table.no.24 showing authentication of Apakwa Bilwa phala

Sl.no	Sample name	Scientificname	Family	Part submitted	CRF Code	Authenticated as				
						Ayurvedic Name	Scientificname	Family	Part Authenticated	Remarks
01	Bilwa	Aegle marmelos(L.) Correa	Rutacae	Unripe fruit	CRF/Auth/ 2018/113	Bilwa	Aegle marmelos(L.) Correa	Rutaceae	Unripe fruit	**Authenticated**

Table.no.25 showing analytical study results of Apakwa Bilva Phala

Sl.no	Tests	Water Extract	Alcohol Extract
1.	Test for Carbohydrate	+	+
2.	Test for Reducing sugar	+	+
3.	Test for Monosaccharides	+	-
4.	Test for Pentose sugar	+	+
5.	Test for Hexose sugar	-	-
6.	Test for Non-Reducing sugar	+	+
7.	Test for Proteins	+	+
8.	Test for Aminoacids	+	+
9.	Test for Steroids	+	-
10.	Test for Flavonoids	+	+
11.	Test for Alkaloids	-	+
12.	Test for Tannins	+	+
	Test for Glycosides	+	+
A	Cardiac Glycosides	+	+
B	Anthraquinone glycosides	**-**	+
C	Saponin glycosides	+	--

+ = Presence - = Absence

❖ **Results of Quality Control (QC) study of Ayur-probiotic:**

- **Result of Phyto-chemical analysis of the Lyophilized sample:**

Table.no.26 showing the result of Phyto-chemical analysis of the Lyophilized sample

Sl.no	Name of the test	Water	Alcohol
1.	Test for carbohydrate	+	+
2.	Test for reducing sugar	+	-
3.	Test for monosaccharides	-	-
4.	Test for pentosesugar	-	-
5.	Test for non- reducing sugar	-	-
6.	Test for alkaloids	+	+
7.	Test fortannins	-	-
8.	Test for glycosides	-	-
9.	Test for hexosesugar	-	-
10.	Test for proteins	-	-
11.	Test for aminoacids	-	-
12.	Test forsteroids	-	-
13.	Test for flavonoids	-	-

+ = Presence - = Absence

- **Result of Physico-Chemical properties of the Lyophilized sample:**

Table.no.27 showing the result of Physico-Chemical properties of the Lyophilized sample

Sl.no	Name of the test	Water
1.	Loss on drying	11.706%
2.	Ash value	5.732%
3.	Acid insolubleash	0.797%
4.	Water soluble extractive	82.002%
5.	Alcohol soluble extractive	53.302%
6.	Ph	3.34

- **Result of Organoleptic characters of the Lyophilized sample:**

Table.no.28 showing the result of organoleptic characters of the Lyophilized sample

Sl.no	Name of the Characters	Result
1.	Form	Powder
2.	Colour	White
3.	Taste	Sour
4.	Odour	Milky

- **Result of Culture test:**

Good growth of viable probiotic organisms (>1x10^8 CFU/ml) was observed in the culture test. Further to know the maximum percentage of probiotic organisms present in the sample, sequencing methods for the characterization of Probiotic bacteria has been undertaken.

- **Identification of specific probiotic bacteria in Ayur-Probiotic product:**

Observation during blasting of the sequences in Mega software, following results were obtained.

- Amplification was done by polymerase chain reaction (PCR) using Primer 1_F03.ab1.
- Sequencing PCR was performed for both of the strands using Primer 2_F03.ab1 andMega software.
- Purification and analysis of all sequencing reaction was done.
- Sequences were compared with the GenBank DNA database using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov), and identified as Staphylococcus epidermidis and Lysinibaccillus, Firmicutes bacteria & Braccillus species.

Experimental results:

Acute toxicity:

- No mortality was seen at the dose of 2000 mg/ kg body weight in any rats during the study.
- No toxic effect of the Ayur-probiotic seen during the experimental animals.
- None of the animal showed any Signs of respiratory depression, narcosis, catatonia & other toxic signs during the experimental study.
- There was no loss of fur, change in colour of fur, skin colour of any rats.

Food consumption and water intake:

Food consumption and water intake was found normal (i.e 10-12 g/ rat and 15-20 ml/rat) throughout the study.

Body Weight:

Weight was increased in all the five animals around 5-8gm/rat during the experiment study.

Urine and stool:

No signs of diarrhoea, bloody stool, mucous in stool etc were observed. All the five experimental animals were found active throughout the study.

All five experimental animals were found active throughout the study.

Table no.29 showing observations of five animals throughout the acute toxicity study

Observations	Head	Body	Tail	Neck	Unmarked
Changes in skin:	None	None	None	None	None
Blanching	None	None	None	None	None
Cyanosis	None	None	None	None	None
Erythem	None	None	None	None	None
Itching	None	None	None	None	None
Changes in Fur:	None	None	None	None	None
Falling of fur	None	None	None	None	None
Piloerection	None	None	None	None	None
Discoloration	None	None	None	None	None
Changes in Eyes :	None	None	None	None	None
Exopthalamus	None	None	None	None	None
Redness	None	None	None	None	None
Ptosis	None	None	None	None	None
Lacrimation	None	None	None	None	None
Pupil constricted,	None	None	None	None	None
Pupil dilated	None	None	None	None	None
Behavioral pattern:	None	None	None	None	None
Restlessness	None	None	None	None	None
Grooming	None	None	None	None	None
Lying flat on belly	None	None	None	None	None
Lying flat on side,	None	None	None	None	None
Lying flat on back	None	None	None	None	None
Sleeping	None	None	None	None	None
Salivation:	None	None	None	None	None
Viscid	None	None	None	None	None
Watery	None	None	None	None	None
Respiration:	None	None	None	None	None

Depression / Stimulation	None	None	None	None	None
Failure	None	None	None	None	None
Increased motor activity / Decreased motor activity:	None	None	None	None	None
Muscle relaxation Analgesia	None	None	None	None	None
Arching	None	None	None	None	None
Rolling	None	None	None	None	None
Central nervous system	None	None	None	None	None
Defecation	None	None	None	None	None
Urination	None	None	None	None	None
Squatting	None	None	None	None	None
Ataxic gait	None	None	None	None	None
Tremors	None	None	None	None	None
Timidity	None	None	None	None	None
Writhing	None	None	None	None	None
Paresis of hind limbs	None	None	None	None	None
Paresis of forepaws	None	None	None	None	None
Twitches	None	None	None	None	None
Convulsions:-	None	None	None	None	None
Clonic	None	None	None	None	None
Tonic	None	None	None	None	None
Rolling	None	None	None	None	None

*None- not seen

Results on Food, Water intake & Body Weight of Experimental Animals

I. Food intake:

Table.no.30 showing the statistical results of ANOVA test on food intake on 7th day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	45	44.54	46.28	46.29	47.59	48.11	48.43	48.32
SEM	0.57	0.40	0.84	0.36	0.25	0.32	0.31	0.49
ANOVA (p–value)	F=25.04							
Significant	NS							

Table.no.31 showing the statistical results of Dunnett's test on food intake on 7th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	-0.4587	-7.441 TO 6.524	P>0.05	NS
Gr A-C	1.283	-5.699 TO 8.266	P>0.05	NS
Gr A-D	1.297	-5.686 TO 8.279	P>0.05	NS
Gr A-E	2.595	-4.388 TO 9.577	P>0.05	NS
Gr A-F	3.114	-3.8692 TO 10.10	P>0.05	NS
Gr A-G	3.431	-3.551 TO 10.41	P>0.05	NS
Gr A-H	3.321	-3.661 TO 10.30	P>0.05	NS
Gr B-C	1.742	-5.240 TO 8.724	P>0.05	NS
Gr B-D	1.755	-5.227 TO 8.738	P>0.05	NS
Gr B-E	3.053	-3.929 TO 10.04	P>0.05	NS
Gr B-F	3.572	-3.410 TO 10.55	P>0.05	NS
Gr B-G	3.890	-3.092 TO 10.87	P>0.05	NS
Gr B-H	3.780	-3.203 TO 10.57	P>0.05	NS
Gr C-D	0.0134	-6.969 TO 6.996	P>0.05	NS
Gr C-E	1.311	-5.671 TO 8.294	P>0.05	NS
Gr C-F	1.830	-5.152 TO 8.813	P>0.05	NS
Gr C-G	2.148	-4.834 TO 9.131	P>0.05	NS
Gr C-H	2.038	-4.945 TO 9.020	P>0.05	NS
Gr D-E	1.298	-5.684 TO 8.280	P>0.05	NS
Gr D-F	1.817	-5.166 TO 8.799	P>0.05	NS
Gr D-G	2.135	-4.848 TO 9.117	P>0.05	NS
Gr D-H	2.024	-4.958 TO 9.007	P>0.05	NS
Gr E-F	0.5188	-6.464 TO 7.501	P>0.05	NS
Gr E-G	0.8367	-6.146 TO 7.819	P>0.05	NS
Gr E-H	0.7263	-6.256 TO 7.709	P>0.05	NS
Gr F-G	0.3179	-6.665 TO 7.300	P>0.05	NS
Gr F-H	0.2075	-6.775 TO 7.190	P>0.05	NS
Gr G-H	-0.1104	-7.093 TO 6.872	P>0.05	NS

The mean value of cumulative food intake of group 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 4.

The mean value of cumulative food intake of group 4 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 1.

The mean value of cumulative food intake of group 1 was more than mean value of group 2

The difference in mean value of cumulative food intake in the inter group (ANOVA) was significant ($p< 0.0033$)

The difference in mean value of cumulative food intake of test group was non-significant ($p> 0.05$) when compared to other groups.

Table.no.32 showing the statistical results of ANOVA test on food intake on 15th day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	43.85	45.39	46.79	46.50	47.73	48.27	48.53	48.36
SEM	0.20	0.69	0.85	0.36	0.31	0.18	0.22	0.25
ANOVA (p – value)	F=25.04							
Significant	NS							

Table.no.33 showing the statistical results of Dunnett's test on food intake on 15th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	1.538	-5.444 TO 8.521	P>0.05	NS
Gr A-C	2.940	-4.042 TO 9.925	P>0.05	NS
Gr A-D	2.649	-4.333 TO 9.632	P>0.05	NS
Gr A-E	3.877	-3.105 TO 10.86	P>0.05	NS
Gr A-F	4.419	-2.564 TO 11.40	P>0.05	NS
Gr A-G	4.681	-2.301 TO 11.66	P>0.05	NS
Gr A-H	4.511	-2.471 TO 11.49	P>0.05	NS
Gr B-C	1.402	-5.580 TO 8.384	P>0.05	NS
Gr B-D	1.111	-5.871 TO 8.094	P>0.05	NS
Gr B-E	2.339	-4.643 TO 9.322	P>0.05	NS
Gr B-F	2.880	-4.102 TO 9.863	P>0.05	NS
Gr B-G	3.143	-3.839 TO 10.13	P>0.05	NS
Gr B-H	2.973	-4.009 TO 9.955	P>0.05	NS
Gr C-D	0.290	-7.273 TO 6.692	P>0.05	NS
Gr C-E	0.9372	-6.045 TO 7.920	P>0.05	NS
Gr C-F	1.478	-5.504 TO 8.461	P>0.05	NS
Gr C-G	1.741	-5.241 TO 8.724	P>0.05	NS
Gr C-H	1.571	-5.411 to 8.553	P>0.05	NS
Gr D-E	1.228	-5.754 to 8.210	P>0.05	NS
Gr D-F	1.769	-5.213 to 8.752	P>0.05	NS
Gr D-G	2.032	-4.950 to 9.014	P>0.05	NS
Gr D-H	1.862	-5.121 to 8.844	P>0.05	NS
Gr E-F	0.5412	-6.441 to 7.524	P>0.05	NS
Gr E-G	0.8041	-6.178 to 7.786	P>0.05	NS
Gr E-H	0.6339	-6.348 to 7.616	P>0.05	NS
Gr F-G	0.2628	-6.720 to 7.245	P>0.05	NS
Gr F-H	0.09267	-6.890 to 7.075	P>0.05	NS
Gr G-H	-0.1701	-7.153 to 6.812	P>0.05	NS

The mean value of cumulative food intake of group 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 4.

The mean value of cumulative food intake of group 4 was more than mean value of group 2.

The mean value of cumulative food intake of group 2 was more than mean value of group 1.

The difference in mean value of cumulative water intake in the inter group (ANOVA) was significant ($p < 0.0001$)

The difference in mean value of cumulative water intake of test group was non-significant ($p > 0.05$) when compared to other groups.

Table.no.34 showing the statistical results of ANOVA test on food intake on 21st day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	46	45.64	47.04	46.52	47.78	48.26	48.61	48.31
SEM	0.54	0.46	0.38	0.53	0.49	0.57	0.58	0.54
ANOVA (p – value)	P<0.001							
Significant	***							

Table.no.35 showing the statistical results of Dunnett's test on food intake on 21st day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	39.65	32.67 to 46.63	P<0.001	***
Gr A-C	41.04	34.06 to 48.03	P<0.001	***
Gr A-D	40.53	33.55 to 47.51	P<0.001	***
Gr A-E	41.78	34.80 to 48.76	P<0.001	***
Gr A-F	42.26	35.28 to 49.25	P<0.001	***
Gr A-G	42.62	35.63 to 49.60	P<0.001	***
Gr A-H	42.32	35.34 to 49.30	P<0.001	***
Gr B-C	1.395	-5.587 to 8.378	P > 0.05	ns
Gr B-D	0.879	-6.103 to 7.861	P > 0.05	ns
Gr B-E	2.132	-4.850 to 9.115	P>0.05	NS
Gr B-F	2.615	-4.367 to 9.597	P>0.05	NS
Gr B-G	2.967	-4.015 to 9.950	P>0.05	NS
Gr B-H	2.669	-4.313 to 9.652	P>0.05	NS
Gr C-D	-0.5162	-7.499 to 6.466	P>0.05	NS
Gr C-E	0.7371	-6.245 to 7.719	P>0.05	NS
Gr C-F	1.22	-5.763 to 8.202	P>0.05	NS
Gr C-G	1.572	-5.410 to 8.555	P>0.05	NS
Gr C-H	1.274	-5.708 to 8.257	P>0.05	NS
Gr D-E	1.253	-5.729 to 8.236	P>0.05	NS
Gr D-F	1.736	-5.246 to 8.718	P>0.05	NS
Gr D-G	2.088	-4.894 to 9.071	P>0.05	NS
Gr D-H	1.79	-5.192 to 8.773	P>0.05	NS
Gr E-F	0.4827	-6.500 to 7.465	P>0.05	NS
Gr E-G	0.8351	-6.147 to 7.818	P>0.05	NS
Gr E-H	0.5371	-6.445 to 7.520	P>0.05	NS
Gr F-G	0.3525	-6.630 to 7.335	P>0.05	NS
Gr F-H	0.05446	-6.928 to 7.037	P>0.05	NS
Gr G-H	-0.298	-7.280 to 6.684	P>0.05	NS

The mean value of cumulative food intake of group 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 4.

The mean value of cumulative food intake of group 4 was more than mean value of group 1.

The mean value of cumulative food intake of group 1 was more than mean value of group 2.

The difference in mean value of cumulative food intake in the inter group (ANOVA) was significant ($p< 0.0001$)

The difference in mean value of cumulative food intake of test group was significant ($p> 0.05$) when compared to control group.

Table.no.36 showing the statistical results of ANOVA on 30th day

Variables	Group-A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	44.55	49.66	45.11	56.55	53.77	52.55	49	50.11
SEM	0.50	0.57	0.36	0.43	0.42	0.37	0.31	0.15
ANOVA (p – value)	P<0.001							
Significant	***							

Table.no.37 showing the statistical results of Dunnett's test on food intake on 30th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	5.111	-1.871 to 12.09	P < 0.05	*
Gr A-C	0.5555	-6.427 to 7.538	P>0.05	NS
Gr A-D	12	5.018 to 18.98	P<0.001	***
Gr A-E	9.222	2.240 to 16.20	P<0.001	***
Gr A-F	8	1.018 to 14.98	P<0.001	***
Gr A-G	4.444	-2.538 to 11.43	P>0.05	NS
Gr A-H	5.556	-1.427 to 12.54	P < 0.05	*
Gr B-C	-4.556	-11.54 to 2.427	P>0.05	NS
Gr B-D	6.889	-0.09351 to 13.87	P<0.01	**
Gr B-E	4.111	-2.871 to 11.09	P>0.05	NS
Gr B-F	2.889	-4.094 to 9.871	P>0.05	NS
Gr B-G	-0.6667	-7.649 to 6.316	P>0.05	NS
Gr B-H	0.4444	-6.538 to 7.427	P>0.05	NS
Gr C-D	11.44	4.462 to 18.43	P<0.001	***
Gr C-E	8.667	1.684 to 15.65	P<0.001	***
Gr C-F	7.444	0.4620 to 14.43	P<0.001	***
Gr C-G	3.889	-3.094 to 10.87	P>0.05	NS
Gr C-H	5	-1.982 to 11.98	P < 0.05	*
Gr D-E	-2.778	-9.760 to 4.205	P>0.05	NS
Gr D-F	-4	-10.98 to 2.982	P>0.05	NS
Gr D-G	-7.556	-14.54 to -0.5732	P<0.001	***
Gr D-H	-6.444	-13.43 to 0.5380	P<0.01	**
Gr E-F	-1.222	-8.205 to 5.760	P>0.05	NS
Gr E-G	-4.778	-11.76 to 2.205	P>0.05	NS
Gr E-H	-3.667	-10.65 to 3.316	P>0.05	NS
Gr F-G	-3.556	-10.54 to 3.427	P>0.05	NS
Gr F-H	-2.444	-9.427 to 4.538	P>0.05	NS
Gr G-H	1.111	-5.871 to 8.094	P>0.05	NS

The mean value of cumulative food intake of group 4 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 2.

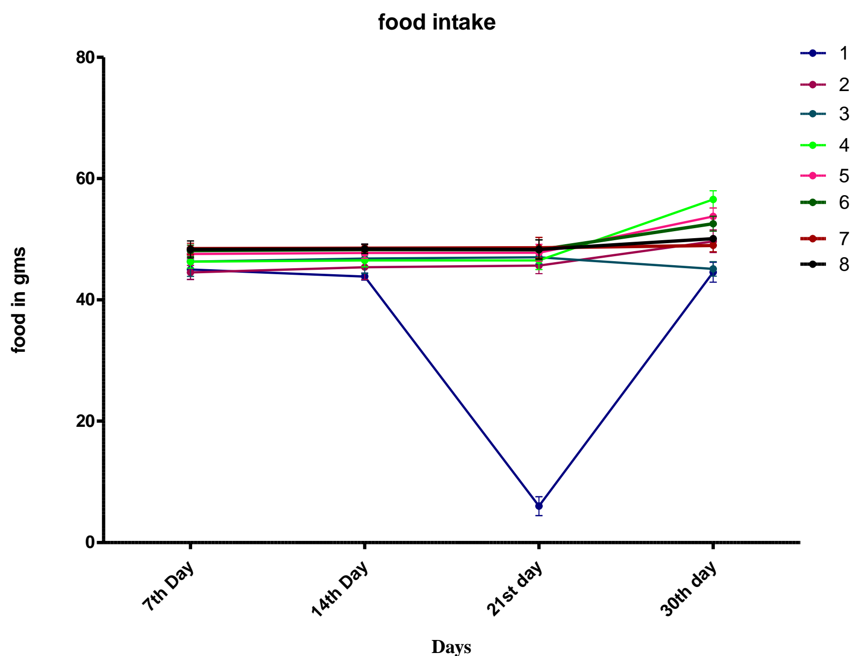
The mean value of cumulative food intake of group 2 was more than mean value of group 7.

The mean value of cumulative food intake of group 7 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 1.

The difference in mean value of cumulative food intake in the inter group (ANOVA) was significant ($p < 0.0001$)

The difference in mean value of cumulative food intake of test group was significant ($p > 0.05$) when compared to group A, C and D.



Graph Diagram no.1 showing cumulative food intake value for 30 days in experimental animals

II. WATER INTAKE:

Table.no.38 showing the statistical results of ANOVA on water intake on 7th day

Variables	Group -A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	122.71	112.14	117.71	117	115.57	117	117.14	115.42
SEM	0.6060	1.2817	1.1126	1.1180	1.4267	1.35400	0.89973	0.57217
ANOVA (p–value)	0.002							
Significant	***							

Table.no.39 showing the statistical results of Dunnett's test on water intake on 7th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	-10.57	-18.20 to -2.943	P<0.001	***
Gr A-C	-5	-12.63 to 2.628	P>0.05	NS
Gr A-D	-5.714	-13.34 to 1.914	P < 0.05	*
Gr A-E	-7.143	-14.77 to 0.4855	P<0.01	**
Gr A-F	-5.714	-13.34 to 1.914	P < 0.05	*
Gr A-G	-5.571	-13.20 to 2.057	P < 0.05	*
Gr A-H	-7.286	-14.91 to 0.3427	P<0.01	**
Gr B-C	5.571	-2.057 to 13.20	P < 0.05	*
Gr B-D	4.857	-2.771 to 12.49	P > 0.05	ns
Gr B-E	3.429	-4.200 to 11.06	P>0.05	NS
Gr B-F	4.857	-2.771 to 12.49	P>0.05	NS
Gr B-G	5	-2.628 to 12.63	P>0.05	NS
Gr B-H	3.286	-4.343 to 10.91	P>0.05	NS
Gr C-D	-0.7143	-8.343 to 6.914	P>0.05	NS
Gr C-E	-2.143	-9.771 to 5.486	P>0.05	NS
Gr C-F	-0.7143	-8.343 to 6.914	P>0.05	NS
Gr C-G	-0.5714	-8.200 to 7.057	P>0.05	NS
Gr C-H	-2.286	-9.914 to 5.343	P>0.05	NS
Gr D-E	-1.429	-9.057 to 6.200	P>0.05	NS
Gr D-F	0	-7.628 to 7.628	P>0.05	NS
Gr D-G	0.1429	-7.486 to 7.771	P>0.05	NS
Gr D-H	-1.571	-9.200 to 6.057	P>0.05	NS
Gr E-F	1.429	-6.200 to 9.057	P>0.05	NS
Gr E-G	1.571	-6.057 to 9.200	P>0.05	NS
Gr E-H	-0.1428	-7.771 to 7.486	P>0.05	NS
Gr F-G	0.1429	-7.486 to 7.771	P>0.05	NS
Gr F-H	-1.571	-9.200 to 6.057	P>0.05	NS
Gr G-H	-1.714	-9.343 to 5.914	P>0.05	NS

The mean value of cumulative food intake of group 1 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 7.

The mean value of cumulative food intake of group 7 was more than mean value of group 4 and 6.

The mean value of cumulative food intake of group 4 and 6 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 2.

The difference in mean value of cumulative water intake in the inter group (ANOVA) was significant ($p < 0.0001$)

The difference in mean value of cumulative water intake of test group was significant ($p > 0.05$) when compared to control group (group- B).

Table.no.40 showing the statistical results of ANOVA on water intake on 15th day

Variables	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**	**Group F**	**Group G**	**Group H**
Mean	121.28	111.42	119.71	120.57	115.57	112.57	110.57	111.42
SEM	1.2289	0.895	1.169	1.043	1.588	1.525	1.645	1.875
ANOVA(p–value)	0.002							
Significant	***							

Table.no.41 showing the statistical results of Dunnett's test on water intake on 15th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	-10.57	-18.20 to -2.943	P<0.001	***
Gr A-C	-5	-12.63 to 2.628	P>0.05	NS
Gr A-D	-5.714	-13.34 to 1.914	P < 0.05	*
Gr A-E	-7.143	-14.77 to 0.4855	P<0.01	**
Gr A-F	-5.714	-13.34 to 1.914	P < 0.05	*
Gr A-G	-5.571	-13.20 to 2.057	P < 0.05	*
Gr A-H	-7.286	-14.91 to 0.3427	P<0.01	**
Gr B-C	5.571	-2.057 to 13.20	P < 0.05	*
Gr B-D	4.857	-2.771 to 12.49	P > 0.05	Ns
Gr B-E	3.429	-4.200 to 11.06	P>0.05	NS
Gr B-F	4.857	-2.771 to 12.49	P>0.05	NS
Gr B-G	5	-2.628 to 12.63	P>0.05	NS
Gr B-H	3.286	-4.343 to 10.91	P>0.05	NS
Gr C-D	-0.7143	-8.343 to 6.914	P>0.05	NS
Gr C-E	-2.143	-9.771 to 5.486	P>0.05	NS
Gr C-F	-0.7143	-8.343 to 6.914	P>0.05	NS
Gr C-G	-0.5714	-8.200 to 7.057	P>0.05	NS
Gr C-H	-2.286	-9.914 to 5.343	P>0.05	NS
Gr D-E	-1.429	-9.057 to 6.200	P>0.05	NS
Gr D-F	0	-7.628 to 7.628	P>0.05	NS
Gr D-G	0.1429	-7.486 to 7.771	P>0.05	NS
Gr D-H	-1.571	-9.200 to 6.057	P>0.05	NS
Gr E-F	1.429	-6.200 to 9.057	P>0.05	NS
Gr E-G	1.571	-6.057 to 9.200	P>0.05	NS
Gr E-H	-0.1428	-7.771 to 7.486	P>0.05	NS
Gr F-G	0.1429	-7.486 to 7.771	P>0.05	NS
Gr F-H	-1.571	-9.200 to 6.057	P>0.05	NS
Gr G-H	-1.714	-9.343 to 5.914	P>0.05	NS

The mean value of cumulative food intake of group 1 was more than mean value of group 4.

The mean value of cumulative food intake of group 4 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 2 and 8.

The mean value of cumulative food intake of group 2 and 8 was more than mean value of group 7.

The difference in mean value of cumulative water intake in the inter group (ANOVA) was significant ($p < 0.0001$)

The difference in mean value of cumulative water intake of test group was significant ($p > 0.05$) when compared to control group.

Table.no.42 showing the statistical results of ANOVA test on water intake on 21st day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	121.571	112.1429	117.5714	115.7143	118.8571	118.5714	117.1429	114.428
SEM	1.0879	1.2616	0.3688	0.7781	0.7046	3.4216	2.5581	2.885
ANOVA (p–value)	0.01							
Significant	**							

Table.no.43 showing the Statistical results of Dunnett's test on water intake on 21st day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	-9.429	-17.06 to -1.800	P<0.001	***
Gr A-C	-4	-11.63 to 3.628	P>0.05	NS
Gr A-D	-5.857	-13.49 to 1.771	P < 0.05	*
Gr A-E	-2.714	-10.34 to 4.914	P > 0.05	Ns
Gr A-F	-3	-10.63 to 4.628	P>0.05	NS
Gr A-G	-4.429	-12.06 to 3.200	P>0.05	NS
Gr A-H	-7.143	-14.77 to 0.4856	P<0.01	**
Gr B-C	5.429	-2.200 to 13.06	P < 0.05	*
Gr B-D	3.571	-4.057 to 11.20	P>0.05	NS
Gr B-E	6.714	-0.9142 to 14.34	P<0.01	**
Gr B-F	6.429	-1.200 to 14.06	P < 0.05	*
Gr B-G	5	-2.628 to 12.63	P>0.05	NS
Gr B-H	2.286	-5.343 to 9.914	P>0.05	NS
Gr C-D	-1.857	-9.486 to 5.771	P>0.05	NS
Gr C-E	1.286	-6.343 to 8.914	P>0.05	NS
Gr C-F	1	-6.628 to 8.628	P>0.05	NS
Gr C-G	-0.4285	-8.057 to 7.200	P>0.05	NS
Gr C-H	-3.143	-10.77 to 4.486	P>0.05	NS
Gr D-E	3.143	-4.486 to 10.77	P>0.05	NS
Gr D-F	2.857	-4.771 to 10.49	P>0.05	NS
Gr D-G	1.429	-6.200 to 9.057	P>0.05	NS
Gr D-H	-1.286	-8.914 to 6.343	P>0.05	NS
Gr E-F	-0.2857	-7.914 to 7.343	P>0.05	NS
Gr E-G	-1.714	-9.343 to 5.914	P>0.05	NS
Gr E-H	-4.429	-12.06 to 3.200	P>0.05	NS
Gr F-G	-1.429	-9.057 to 6.200	P>0.05	NS
Gr F-H	-4.143	-11.77 to 3.486	P>0.05	NS
Gr G-H	-2.714	-10.34 to 4.914	P>0.05	NS

The mean value of cumulative food intake of group 1 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 7.

The mean value of cumulative food intake of group 7 was more than mean value of group 4.

The mean value of cumulative food intake of group 4 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 2.

The difference in mean value of cumulative water intake in the inter group (ANOVA) was significant ($p< 0.0001$)

The difference in mean value of cumulative water intake of test group was significant ($p> 0.05$) when compared to control group.

Table.no.44 showing the statistical results of ANOVA test on water intake on 30th day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	121.111	113.666	118.777	118.333	116.666	120.777	121.111	121
SEM	1.109	1.101	0.750	1.253	1.669	0.959	0.580	0.637
ANOVA (p–value)	0.05							
Significant	*							

Table.no.45 showing the Statistical results of Dunnett's test on water intake on 30th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	-7.444	-13.67 to -1.216	P<0.001	***
Gr A-C	-2.333	-8.562 to 3.895	P>0.05	NS
Gr A-D	-2.778	-9.006 to 3.451	P>0.05	NS
Gr A-E	-4.444	-10.67 to 1.784	P < 0.05	*
Gr A-F	-0.3333	-6.562 to 5.895	P>0.05	NS
Gr A-G	0	-6.229 to 6.229	P>0.05	NS
Gr A-H	-0.1111	-6.340 to 6.117	P>0.05	NS
Gr B-C	5.111	-1.117 to 11.34	P < 0.05	*
Gr B-D	4.667	-1.562 to 10.90	P < 0.05	*
Gr B-E	3	-3.229 to 9.229	P>0.05	NS
Gr B-F	7.111	0.8825 to 13.34	P<0.001	***
Gr B-G	7.444	1.216 to 13.67	P<0.001	***
Gr B-H	7.333	1.105 to 13.56	P<0.001	***
Gr C-D	-0.4445	-6.673 to 5.784	P>0.05	NS
Gr C-E	-2.111	-8.340 to 4.117	P>0.05	NS
Gr C-F	2	-4.229 to 8.229	P>0.05	NS
Gr C-G	2.333	-3.895 to 8.562	P>0.05	NS
Gr C-H	2.222	-4.006 to 8.451	P>0.05	NS
Gr D-E	-1.667	-7.895 to 4.562	P>0.05	NS
Gr D-F	2.445	-3.784 to 8.673	P>0.05	NS
Gr D-G	2.778	-3.451 to 9.006	P>0.05	NS
Gr D-H	2.667	-3.562 to 8.895	P>0.05	NS
Gr E-F	4.111	-2.117 to 10.34	P>0.05	NS
Gr E-G	4.444	-1.784 to 10.67	P < 0.05	*
Gr E-H	4.333	-1.895 to 10.56	P>0.05	NS
Gr F-G	0.3333	-5.895 to 6.562	P>0.05	NS
Gr F-H	0.2222	-6.006 to 6.451	P>0.05	NS
Gr G-H	-0.1111	-6.340 to 6.117	P>0.05	NS

The mean value of cumulative food intake of group 1 and 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 4.

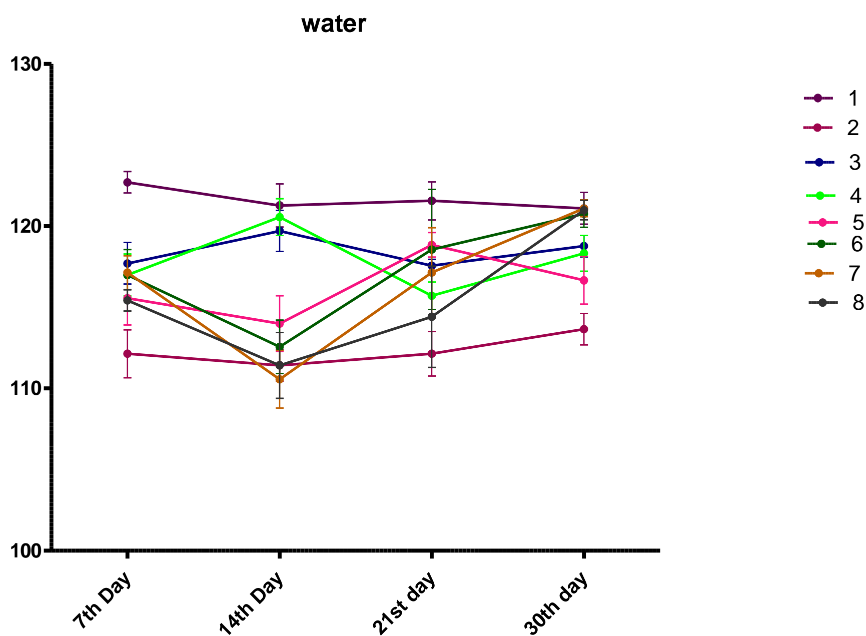
The mean value of cumulative food intake of group 4 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 2.

The difference in mean value of cumulative water intake in the inter group (ANOVA) was significant ($p < 0.0001$)

The difference in mean value of cumulative water intake of test group was non-significant ($p > 0.05$) when compared to control group.

The difference in mean value of cumulative water intake of test group was significant ($p > 0.05$) when compared to group-A.



Graph Diagram no.2 showing cumulative water intake value for 30 days in experimental animals

III. Body Weight:

Table.no.46 showing the statistical results of ANOVA test on body weight on 7th day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	GroupG	GroupH
Mean	176.5	217.33	225.33	225.33	207.5	214.166	243.5	243.16
SEM	5.90	17.20	13.42	6.52686	9.138	11.74568	9.301434	12.8748
ANOVA (p–value)	0.05							
Significant	*							

Table.no.47 showing the statistical results of Dunnett's test on on body weight on 7th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	217.3	40.83	P > 0.05	NS
Gr A-C	225.3	48.83	P>0.05	NS
Gr A-D	225.3	48.83	P < 0.05	*
Gr A-E	207.5	31	P > 0.05	NS
Gr A-F	214.2	37.67	P > 0.05	NS
Gr A-G	243.5	67	P<0.01	*
Gr A-H	243.2	66.67	P<0.01	**
Gr B-C	225.3	8	P < 0.05	*
Gr B-D	225.3	8	P > 0.05	NS
Gr B-E	207.5	-9.833	P>0.05	NS
Gr B-F	214.2	-3.167	P>0.05	NS
Gr B-G	243.5	26.17	P>0.05	NS
Gr B-H	243.2	25.83	P>0.05	NS
Gr C-D	225.3	0	P>0.05	NS
Gr C-E	207.5	-17.83	P>0.05	NS
Gr C-F	214.2	-11.17	P>0.05	NS
Gr C-G	243.5	18.17	P>0.05	NS
Gr C-H	243.2	17.83	P>0.05	NS
Gr D-E	207.5	-17.83	P>0.05	NS
Gr D-F	214.2	-11.17	P>0.05	NS
Gr D-G	243.5	18.17	P>0.05	NS
Gr D-H	243.2	17.83	P>0.05	NS
Gr E-F	214.2	6.667	P>0.05	NS
Gr E-G	243.5	36	P>0.05	NS
Gr E-H	243.2	35.67	P>0.05	NS
Gr F-G	243.5	29.33	P>0.05	NS
Gr F-H	243.2	29	P>0.05	NS
Gr G-H	243.2	-0.3333	P>0.05	NS

The mean value of cumulative food intake of group 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 3 & 4.

The mean value of cumulative food intake of group 3 & 4 was more than mean value of group 2.

The mean value of cumulative food intake of group 2 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 1.

The difference in mean value of the body weight in the inter group (ANOVA) was non-significant (p< 0.102)

The difference in mean value of the body weight of test group was significant (p> 0.05) when compared to control group.

Table.no.48 showing the statistical results of ANOVA test on body weight on 15th day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	194.333	240.1666	197.833	197.83	214.833	219.33	248.833	248.33
SEM	6.75113	25.413141	22.2731	11.697	9.0015	12.284	17.46	15.838
ANOVA (p–value)	0.05							
Significant	*							

Table.no.49 showing the statistical results of Dunnett's test on body weight on 15th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	240.2	45.83	P > 0.05	NS
Gr A-C	197.8	3.5	P>0.05	NS
Gr A-D	197.8	3.5	P < 0.05	*
Gr A-E	214.8	20.5	P > 0.05	NS
Gr A-F	219.3	25	P > 0.05	NS
Gr A-G	248.8	54.5	P < 0.01	*
Gr A-H	248.3	54	P<0.01	**
Gr B-C	197.8	-42.33	P < 0.05	*
Gr B-D	197.8	-42.33	P > 0.05	NS
Gr B-E	214.8	-25.33	P>0.05	NS
Gr B-F	219.3	-20.83	P>0.05	NS
Gr B-G	248.8	8.667	P>0.05	NS
Gr B-H	248.3	8.167	P>0.05	NS
Gr C-D	197.8	0	P>0.05	NS
Gr C-E	214.8	17	P>0.05	NS
Gr C-F	219.3	21.5	P>0.05	NS
Gr C-G	248.8	51	P>0.05	NS
Gr C-H	248.3	50.5	P>0.05	NS
Gr D-E	214.8	17	P>0.05	NS
Gr D-F	219.3	21.5	P>0.05	NS
Gr D-G	248.8	51	P>0.05	NS
Gr D-H	248.3	50.5	P>0.05	NS
Gr E-F	219.3	4.5	P>0.05	NS
Gr E-G	248.8	34	P>0.05	NS
Gr E-H	248.3	33.5	P>0.05	NS
Gr F-G	248.8	29.5	P>0.05	NS
Gr F-H	248.3	29	P>0.05	NS
Gr G-H	248.3	-0.5	P>0.05	NS

The mean value of cumulative food intake of group 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 2.

The mean value of cumulative food intake of group 2 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 3.

The mean value of cumulative food intake of group 3 was more than mean value of group 4.

The mean value of cumulative food intake of group 4 was more than mean value of group 1.

The difference in mean value of the body weight in the inter group (ANOVA) was non-significant ($p < 0.102$)

The difference in mean value of the body weight of test group was significant ($p > 0.05$) when compared to control group.

Table.no.50 showing the statistical results of ANOVA test on body weight on 21st day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	207	239.333	240.833	240.833	230.66	239.833	249.5	248.833
SEM	8.28251	16.7444	13.462	8.883	9.7456	11.771	19.191	15.258
ANOVA (p-value)	P>0.05							
Significant	NS							

Table.no.51 showing the statistical results of Dunnett's test on body weight on 21st day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	239.3	32.33	P > 0.05	NS
Gr A-C	240.8	33.83	P>0.05	NS
Gr A-D	240.8	33.83	P > 0.05	NS
Gr A-E	230.7	23.67	P > 0.05	NS
Gr A-F	239.8	32.83	P > 0.05	NS
Gr A-G	249.5	42.5	P > 0.05	NS
Gr A-H	248.8	41.83	P > 0.05	NS
Gr B-C	240.8	1.5	P > 0.05	NS
Gr B-D	240.8	1.5	P > 0.05	NS
Gr B-E	230.7	-8.667	P>0.05	NS
Gr B-F	239.8	0.5	P>0.05	NS
Gr B-G	249.5	10.17	P>0.05	NS
Gr B-H	248.8	9.5	P>0.05	NS
Gr C-D	240.8	0	P>0.05	NS
Gr C-E	230.7	-10.17	P>0.05	NS
Gr C-F	239.8	-1	P>0.05	NS
Gr C-G	249.5	8.667	P>0.05	NS
Gr C-H	248.8	8	P>0.05	NS
Gr D-E	230.7	-10.17	P>0.05	NS
Gr D-F	239.8	-1	P>0.05	NS
Gr D-G	249.5	8.667	P>0.05	NS
Gr D-H	248.8	8	P>0.05	NS
Gr E-F	239.8	9.167	P>0.05	NS
Gr E-G	249.5	18.83	P>0.05	NS
Gr E-H	248.8	18.17	P>0.05	NS
Gr F-G	249.5	9.667	P>0.05	NS
Gr F-H	248.8	9	P>0.05	NS
Gr G-H	248.8	-0.6667	P>0.05	NS

The mean value of cumulative food intake of group 7 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 3 & 4.

The mean value of cumulative food intake of group 3 & 4 was more than mean value of group 6.

The mean value of cumulative food intake of group 6 was more than mean value of group 2.

The mean value of cumulative food intake of group 2 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 1.

The difference in mean value of the body weight in the inter group (ANOVA) was significant ($p < 0.0337$)

The difference in mean value of the body weight of test group was non-significant ($p > 0.05$) when compared to control group.

Table.no.52 showing the statistical results of ANOVA test on body weight on 30th day

Variables	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
Mean	219.333	251.166	242.5	242.5	236.666	251.833	250.166	250.666
SEM	12.084	20.653	14.728	10.950	13.708	18.263	18.423	14.811
ANOVA (p–value)	P>0.05							
Significant	NS							

Table.no.53 showing the statistical results of Dunnett's test on body weight on 30th day

Groups	Mean Difference	95% CI of difference	The value of p	Summary
Gr A-B	251.2	31.83	P > 0.05	NS
Gr A-C	242.5	23.17	P>0.05	NS
Gr A-D	242.5	23.17	P > 0.05	NS
Gr A-E	236.7	17.33	P > 0.05	NS
Gr A-F	251.8	32.5	P > 0.05	NS
Gr A-G	250.2	30.83	P > 0.05	NS
Gr A-H	250.7	31.33	P > 0.05	NS
Gr B-C	242.5	-8.667	P > 0.05	NS
Gr B-D	242.5	-8.667	P > 0.05	NS
Gr B-E	236.7	-14.5	P>0.05	NS
Gr B-F	251.8	0.6666	P>0.05	NS
Gr B-G	250.2	-1	P>0.05	NS
Gr B-H	250.7	-0.5	P>0.05	NS
Gr C-D	242.5	0	P>0.05	NS
Gr C-E	236.7	-5.833	P>0.05	NS
Gr C-F	251.8	9.333	P>0.05	NS
Gr C-G	250.2	7.667	P>0.05	NS
Gr C-H	250.7	8.167	P>0.05	NS
Gr D-E	236.7	-5.833	P>0.05	NS
Gr D-F	251.8	9.333	P>0.05	NS
Gr D-G	250.2	7.667	P>0.05	NS
Gr D-H	250.7	8.167	P>0.05	NS
Gr E-F	251.8	15.17	P>0.05	NS
Gr E-G	250.2	13.5	P>0.05	NS
Gr E-H	250.7	14	P>0.05	NS
Gr F-G	250.2	-1.667	P>0.05	NS
Gr F-H	250.7	-1.167	P>0.05	NS
Gr G-H	250.7	0.5	P>0.05	NS

The mean value of cumulative food intake of group 6 was more than mean value of group 2.

The mean value of cumulative food intake of group 2 was more than mean value of group 8.

The mean value of cumulative food intake of group 8 was more than mean value of group 7.

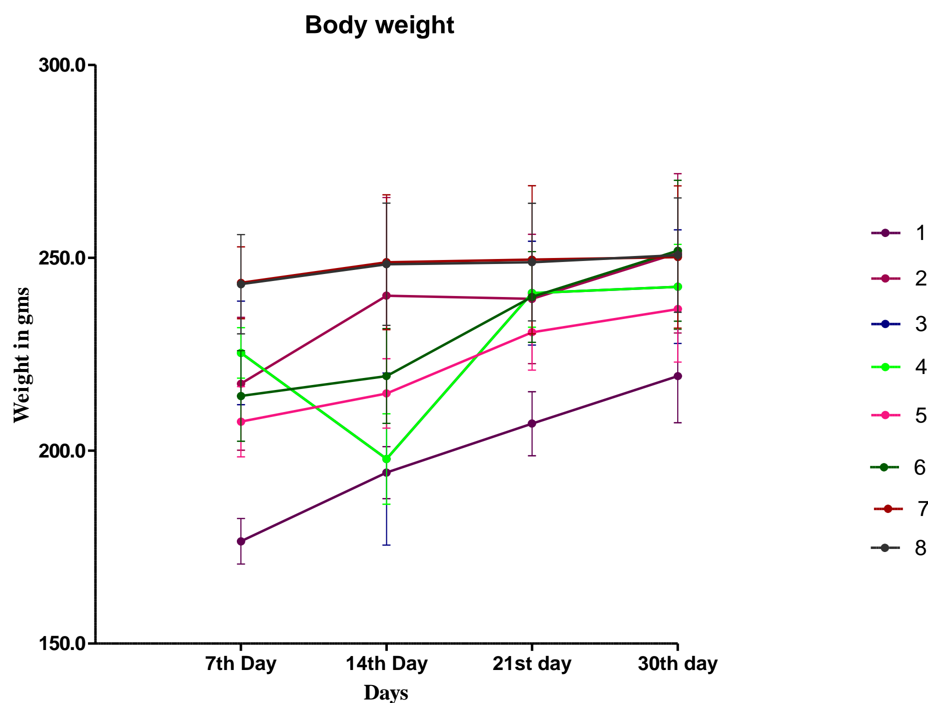
The mean value of cumulative food intake of group 7 was more than mean value of group 3 & 4.

The mean value of cumulative food intake of group 3 & 4 was more than mean value of group 5.

The mean value of cumulative food intake of group 5 was more than mean value of group 1.

The difference in mean value of the body weight in the inter group (ANOVA) was significant ($p < 0.0337$)

The difference in mean value of the body weight of test group was non-significant ($p > 0.05$) when compared to control group.



Graph Diagram no.3 showing cumulative Body weight value for 30 days in experimental animals

Stool observation:

- Stool examination of all rats except Group-B (Control) showed watery diarrhoea on administration of amoxicillin after 7th day onwards.
- On 31st day, during dissection of Group-C (Curd) animals, intestine of all the sacrificed animals shown more gaseous contents because of curd consumption.
- Intestine of sacrificed animals of Group-B & E, shown gaseous content because of Amoxycillin.
- Intestine of sacrificed animals of Group-H, shown hard and well-formed stool.

Experimental Study Results:

1. Results of Micro-biological findings of the specimens:

The results of the effects of Ayur-probiotic formulation (Bilwaphalasaara dadhi) on micro-biological parameters related with faecal contents in Wistar rats are presented here.

I. Microbial Colony Count by Serial Dilution and Spread Plate Culture Method:

Table.no.54 showing samples plates and their colony count

Main group	**Sub-group division**	**Dilution factor of plate**	**Colony count**(approximately)
A	A-3	10^{-1}	3
	A-2	10^{-1}	8
	A-2	10^{-4}	1
	A-4	10^{-5}	13
	A-4	10^{-3}	9
B	B-3	10^{-4}	1
	B-3	10^{-1}	11
	B-6	10^{-7}	10
	B-6	10^{-5}	10
C	C-1	10^{-1}	10
	C-1	10^{-7}	10
	C-5	10^{-1}	6
	C-4	10^{-4}	2
D	D-1	10^{-1}	10
	D-2	10^{-7}	12
	D-2	10^{-5}	18
E	E-4	10^{-7}	3
	E-4	10^{-4}	3
	E-4	10^{-1}	17
	E-5	10^{-4}	9
F	F-1	10^{-7}	1
	F-1	10^{-4}	1
	F-1	10^{-1}	6
G	G-3	10^{-7}	18
	G-3	10^{-4}	10
	G-3	10^{-1}	12
H	H-4	10^{-1}	13
	H-3	10^{-1}	10
	H-1	10^{-1}	15

List of bacteria identified from the selected samples:

Table.no.55 showing identification of Bacteria & their Morphology

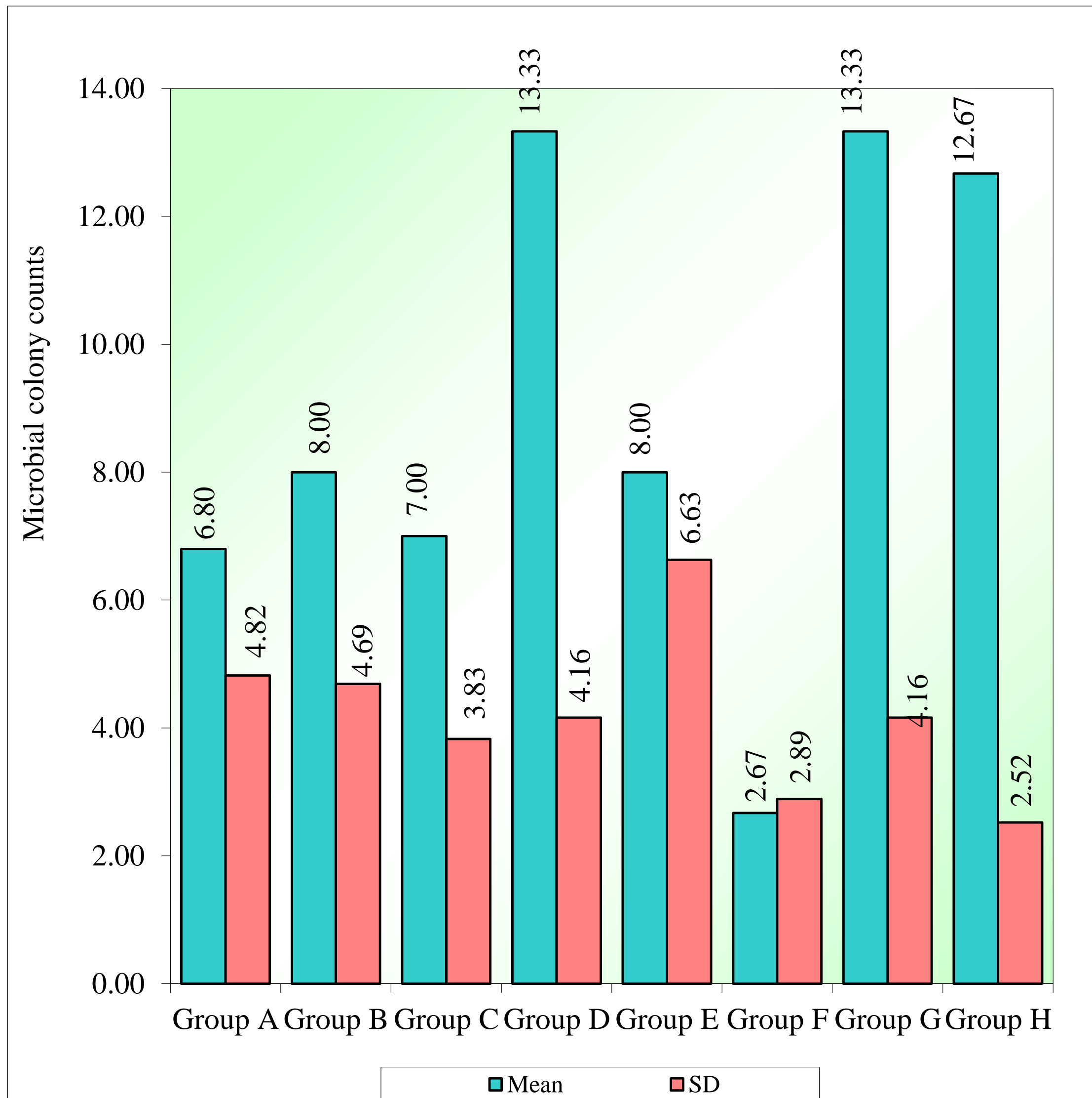
Main Group	Sub Division	Dilution Factor	Bacterial Name	Morphology
A	A-3	10^{-4}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	A-4	10^{-4}	*Proteus species (Enterobacteria strain)*	Gram negative, facultatively anaerobic, Rod shaped bacteria.
	A-5	10^{-5}	*Enterococcus faecalis*	***E. faecalis*** is a non-motile microbe and is a Gram-positive
B	B-6	10^{-5}	*Acetobacter indonesiensis*	Gram negative, Acetic acid bacteria, Pleomorphic.
C	C-4	10^{-1}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	C-5	10^{-1}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitishin colour
D	D-5	10^{-1}	*Bacillus tequilensis*	Gram positive, Motile, Rod shaped Spore forming bacteria
	D-1	10^{-1}	*Proteus vulgaris*	Gram negative, Actively motile, Non-capsulated, bacillus & shiny cream colony.
	D-5	10^{-4}	Bacillus subtilis	Gram positive, rod shaped whitish cream
E	E-2	10^{-7}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitishin colour
	E-5	10^{-4}	*Providencia spp.*	Gram negative, bacillus, motile by peritrichous flagella.
F	F-6 A	10^{-3}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative anaerobic.
	F-6 B	10^{-3}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative Anaerobic
	F-6 C	10^{-3}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated, whitish in colour
G	G-3	10^{-1}	*Enterococcus facium*	Gram positive, Bacillus, gamma-Hemolytic bacteria.
	G-3	10^{-7}	*Enterococcus facium*	Gram positive, Bacillus, gamma-hemolytic bacteria
H	H-1	10-1	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated, whitish in colour
	H-3	10^{-1}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative anaerobic

Table.no.56 showing Kruskal Wallis ANOVA test was used to compare eight groups with microbial colony numbers

Groups	Mean	SD	Mean rank	H-value	p-value
Group A	6.80	4.82	11.50	8.9094	0.2592
Group B	8.00	4.69	14.38		
Group C	7.00	3.83	12.13		
Group D	13.33	4.16	22.67		
Group E	8.00	6.63	13.38		
Group F	2.67	2.89	4.83		
Group G	13.33	4.16	22.67		
Group H	12.67	2.52	22.50		

Table.no.57 showing pair wise comparisons of eight groups with microbial colony counts by Mann-Whitney U test

Groups	Z-value	p-value
Grp A vs Grp B	-0.4899	0.6242
Grp A vs Grp C	-0.1225	0.9025
Grp A vs Grp D	-1.4907	0.1360
Grp A vs Grp E	-0.2449	0.8065
Grp A vs Grp F	1.1926	0.2330
Grp A vs Grp G	-1.4907	0.1360
Grp A vs Grp H	-1.6398	0.1011
Grp B vs Grp C	0.4330	0.6650
Grp B vs Grp D	-1.2374	0.2159
Grp B vs Grp E	0.1443	0.8852
Grp B vs Grp F	1.2374	0.2159
Grp B vs Grp G	-1.2374	0.2159
Grp B vs Grp H	-1.2374	0.2159
Grp C vs Grp D	-1.5910	0.1116
Grp C vs Grp E	0.0000	1.0000
Grp C vs Grp F	1.4142	0.1573
Grp C vs Grp G	-1.5910	0.1116
Grp C vs Grp H	-1.5910	0.1116
Grp D vs Grp E	1.2374	0.2159
Grp D vs Grp F	1.7457	0.0809
Grp D vs Grp G	0.0000	1.0000
Grp D vs Grp H	0.0000	1.0000
Grp E vs Grp F	1.2374	0.2159
Grp E vs Grp G	-1.2374	0.2159
Grp E vs Grp H	-0.8839	0.3768
Grp F vs Grp G	-1.7457	0.0809
Grp F vs Grp H	-1.7457	0.0809
Grp G vs Grp H	0.0000	1.0000



Bar diagram.no.4 showing comparison of eight groups with microbial colony counts

II. Test Results of Clostridium difficile:

Table.no.58 showing results of Clostridium difficile in stool samples

Testing Method	Name of the Sample group	*Sub-group*	Substance detected Toxin A or B	Test Results Presence (P) / Absence (A)	Performance characteristics	
					Reference method	Reference method
					Sensitivity*	Specificity**
Toxic Culture	A	A1	Toxin A	P	+	+
		A2	Toxin A	P	+	+
		A3	Toxin B	P	+	+
		A4	Toxin A	P	+	+
		A5	Toxin B	P	+	+
		A6	Toxin A	P	+	+
	B	B1	-	A	-	-
		B2	-	A	-	-
		B3	-	A	-	-
		B4	-	A	-	-
		B5	-	A	-	-
		B6	-	A	-	-
	C	C1	Toxin B	P	+	+
		C2	Toxin A	P	-	-
		C3	Toxin B	P	+	+
		C4	Toxin A	P	+	+
		C5	Toxin B	P	-	-
		C6	Toxin B	P	-	-
	D	D1	Toxin A	P	+	+
		D2	Toxin A	P	+	+
		D3	Toxin A	P	-	-
		D4	Toxin B	P	+	+
		D5	Toxin B	P	+	+
		D6	Toxin A	P	-	-
	E	E1	Toxin A	P	+	+
		E2	Toxin A	P	+	+

		E3	Toxin B	P	+	+
		E4	Toxin A	P	+	+
		E5	Toxin B	P	+	+
		E6	Toxin A	P	+	+
	F	F1	Toxin A	P	+	+
		F2	Toxin B	P	+	+
		F3	Toxin B	P	+	+
		F4	Toxin A	P	+	+
		F5	Toxin B	P	+	+
		F6	Toxin B	P	+	+
	G	G1	Toxin A	P	+	+
		G2	Toxin A	P	+	+
		G3	Toxin B	P	+	+
		G4	Toxin A	P	+	+
		G5	Toxin B	P	+	+
		G6	Toxin A	P	+	+
	H	H1	Toxin A	P	+	+
		H2	Toxin A	P	+	+
		A3	Toxin A	P	+	+
		H4	Toxin A	P	+	+
		H5	Toxin B	P	+	+
		H6	Toxin A	P	+	+

Abbreviations:

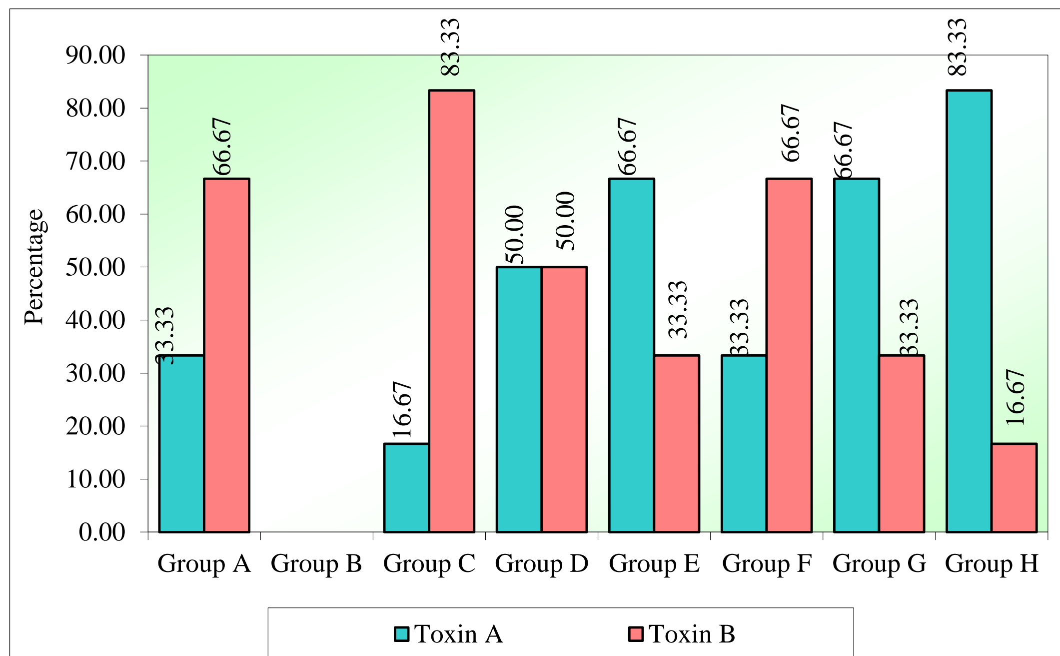
- ***Sensitivity** of a test indicates the probability of infection, the test will be positive
- ****Specificity** is the probability of not having the infection, the test will be negative.
- + indicates fair
- ++ good
- +++ very good
- ++++ excellent

Test Results:

Table.no.59 showing comparison of eight groups with status of C.Difficille

Groups	Toxin A	%	Toxin B	%	Total
Group A	2	33.33	4	66.67	6
Group B	-	-	-	-	-
Group C	1	16.67	5	83.33	6
Group D	3	50.00	3	50.00	6
Group E	4	66.67	2	33.33	6
Group F	2	33.33	4	66.67	6
Group G	4	66.67	2	33.33	6
Group H	5	83.33	1	16.67	6
Total	21	50.00	21	50.00	48
p=0.0001*					

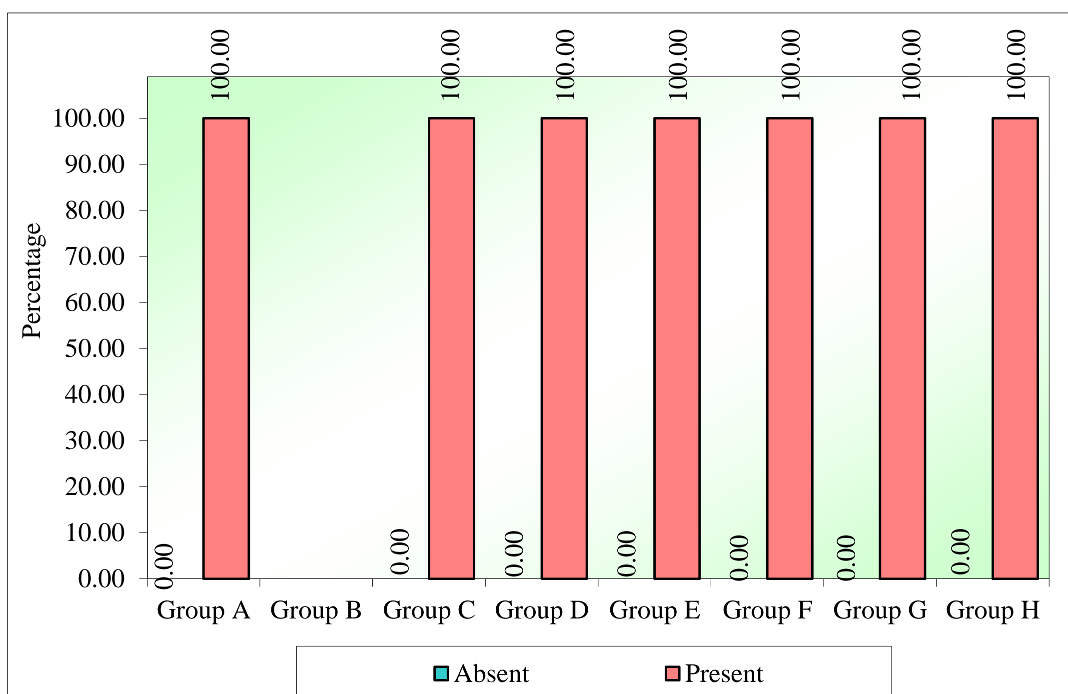
*p<0.05



Bar Diagram.no.5 showing comparison of eight groups with status of C.Difficille

Table.no.60 showing comparison of eight groups with status of test results

Groups	Absent	%	Present	%	Total
Group A	0	0.00	6	100.00	6
Group B	-	-	-	-	-
Group C	0	0.00	6	100.00	6
Group D	0	0.00	6	100.00	6
Group E	0	0.00	6	100.00	6
Group F	0	0.00	6	100.00	6
Group G	0	0.00	6	100.00	6
Group H	0	0.00	6	100.00	6
Total	6	12.50	42	87.50	48



Bar Diagram.no.6 showing comparison of eight groups with status of test results

III. Results of qPCR (Quantifying the different bacterial groups):

Table.no.61 showing results of sample matching with respective strain no. & suspected organisms

Group	Strain. No (Code name)	Sample is matching with suspected organisms
A	MN340241.1	Proteus mirabilis strain
	MT107058.1	Proteus sp. (enterobacteria) strain
	OQ607170.1	Enterococcus faecalis strain
B	OP741052.1	Acetobacter indonesiensis strain
C	KF051775.1	Proteus mirabilis strain
	OL629241.1	Proteus mirabilis strain
D	OP881561.1	Proteus mirabilis strain
	OQ405515.1	Bacillus subtilis strain
E	MK578252.1	Proteus mirabilis strain
	MN340241.1	Providencia sp. starin
F	MK571727.1	Enterococcus faecalis strain
G	MF369841.1	Enterococcus faecium strain
H	OQ405595.1	Enterococcus faecalis strain
	KX138516.1	Proteus mirabilis strain

Interpretation of the qPCR result:

Results of samples of all the groups are matching with respective strain no & suspected organisms.

Results of Histo-Pathological findings of the specimens:

The results of the effects of Ayur-probiotic formulation (Bilwaphalasaara dadhi) on histo-pathological parameters related with Caecum, Anus & Rectum in Antibiotic (Amoxycillin Trihydrate) induced diarrhoea in Wistar rats are presented here. On a scale from - to +++, where - stands for no reaction, + for a somewhat positive reaction, ++ for a definitely positive reaction, and +++ for a highly positive reaction, histopathological evaluations were categorized based on the degree of the reaction.

Group-A: Specimens of Ano-Rectum

Microscopy	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**
Congestion	+	+	-	+	+	+
Odema	-	+	-	+	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	+	+	-	+	+	+
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	+	-	-	-

Specimens of Colon:

Microscopy	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**
Congestion	+	+	-	+	+	+
Odema	-	-	-	-	-	+
Haemorrhage	-	-	-	-	-	-
Inflammation	+	+	-	+	++	++
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	+	-	+	+

Severe congestion is noted in caecum sample of all the specimens. Mild oedema & Haemorrhage is observed in caecum sample. Severe inflammation is seen in both the samples. Loss of Intestinal glands & Mucosal Ulcers were not observed in any sample.

Group-B: Specimens of Ano-Rectum

Microscopy	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**
Congestion	-	++	++	+++	++	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	-	++	+	++	+	+
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Specimens of Colon:

Microscopy	B1	B2	B3	B4	B5	B6
Congestion	+	+	+	+	+	+
Odema	+	-	-	+	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	++	++	++	++	++	+
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	+	-	-	-

Caecal and colonic mild congestion, oedema, ulceration and epithelial exfoliation in the sample specimens noted. Severe inflammation of the mucosal membrane of the colon samples is observed.

Group-C: Specimens of Ano-Rectum:

Microscopy	C1	C2	C3	C4	C5	C6
Congestion	+	+	+	+	+	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	+	++	-	-	-	+
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Specimens of Colon:

Microscopy	C1	C2	C3	C4	C5	C6
Congestion	+	+	+	+	+	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	+	+	+	+	-	-
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Moderate caecum & colonic pathology with congestion & inflammation within the submucosa and the lamina propria.

Group-D: Specimens of Ano-Rectum:

Microscopy	D1	D2	D3	D4	D5	D6
Congestion	+	+	++	+	+	-
Odema	-	+	-	-		-
Haemorrhage	-	-	-	-	-	-
Inflammation	-	+	-	-	-	-
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Specimens of Colon:

Microscopy	D1	D2	D3	D4	D5	D6
Congestion	-	+	+	+	+	-
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	-	+	+	-	+	-
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Moderate congestion, minimal haemorrhage and light oedema observed in both the sample specimens. No significant changes in histology in colon sample specimens.

Group-E : Specimens of Ano-Rectum:

Microscopy	E1	E2	E3	E4	E5	E6
Congestion	-	+	+	+	+	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	-	+	+	-	-	-
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Specimens of Colon:

Microscopy	E1	E2	E3	E4	E5	E6
Congestion	++	++	+	+	+	-
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	+	+	+	+	+	+
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Severe congestion and mild mild inflammation is observed in both the samples of specimens.

Group-F: Specimens of Ano-Rectum

Microscopy	F1	F2	F3	F4	F5	F6
Congestion	+	+	+	+	+	++
Odema			+		+	-
Haemorrhage						
Inflammation	+	+	+	+	+	+
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	+	+	+	+	+	+
Mucosal Ulcers	+	+	+	+	+	+

Specimens of Colon:

Microscopy	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Congestion	+	+	+	+	+	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	+	+	+		+	+
Destruction of Villi	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Mild to moderate congestion & inflammation is observed in both the samples. Mild oedema is seen in ano-rectal samples of the specimen.

Group-G: Specimens of Ano-Rectum:

Microscopy	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**
Congestion	++	+	+	+	+	-
Odema	-	-	-	-	-	
Haemorrhage	-	-	-	-	-	-
Inflammation	++	+	+	+	+	
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-		-	-	-

Specimens of Colon:

Microscopy	G1	G2	G3	G4	G5	G6
Congestion	+	+	+	+	+	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	-	-	-	-	-	-
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-

Mild to moderate congestion is observed in both the sample specimens. Mild inflammation is only observed in ano-rectal specimens of all samples.

Group-H: Specimens of Ano-Rectum:

Microscopy	H1	H2	H3	H4	H5	H6
Congestion	+	+	++	+	+	+
Odema	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-
Inflammation	+	+	-	-	-	+
Loss of Intestinal glands	-	-	-	-	-	
Mucosal Ulcers	-	-	-		-	-

Specimens of Colon:

Microscopy	H1	H2	H3	H4	H5	H6
Congestion	+	+	-	-	+	+
Odema	-	-	-	-	-	
Haemorrhage	-	-	-	-	-	-
Inflammation	+	-	-	-	+	+
Loss of Intestinal glands	-	-	-	-	-	-
Mucosal Ulcers	-	-	-		-	-

Mild to moderate congestion & inflammation is observed in both the samples.

No significant histo-pathological changes are seen in this group.

Results of Ano-Rectal Specimen samples of experimental animals:

Table.no.62 showing comparison of eight groups with status of Congestion

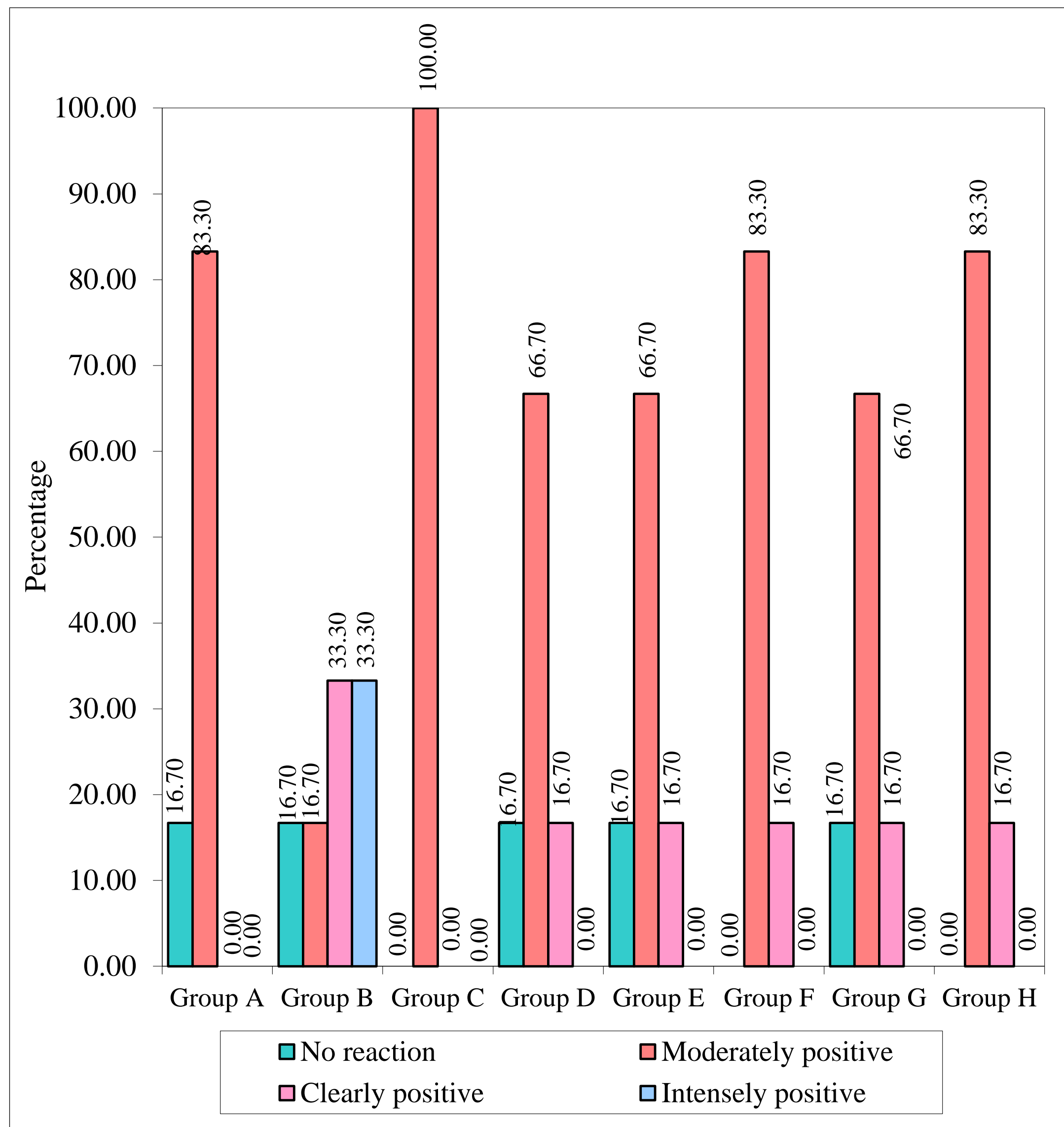
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Gr A	1	16.67	5	83.33	0	0.00	0	0.00	6
Gr B	1	16.67	1	16.67	2	33.33	2	33.33	6
Gr C	0	0.00	6	100.00	0	0.00	0	0.00	6
Gr D	1	16.67	4	66.67	1	16.67	0	0.00	6
Gr E	1	16.67	4	66.67	1	16.67	0	0.00	6
Gr F	0	0.00	5	83.33	1	16.67	0	0.00	6
Gr G	1	16.67	4	66.67	1	16.67	0	0.00	6
Gr H	0	0.00	5	83.33	1	16.67	0	0.00	6
Total	5	10.42	34	70.83	7	14.58	2	4.17	48

Table.no.63 showing comparison of eight groups with status of Congestion by Kruskal Wallis ANOVA

Group	Median	Mean rank	H-value	p-value
Gr A	1.00	19.25	6.8820	0.4410
Gr B	2.00	34.42		
Gr C	1.00	22.50		
Gr D	1.00	22.67		
Gr E	1.00	22.67		
Gr F	1.00	25.92		
Gr G	1.00	22.67		
Gr H	1.00	25.92		

Table.no.64 Mann-Whitney U test pairwise comparisons of eight groups with Congestion status

Groups	Z-value	p-value
Grp A vs Grp B	-1.5212	0.1282
Grp A vs Grp C	-0.4003	0.6889
Grp A vs Grp D	-0.3203	0.7488
Grp A vs Grp E	-0.3203	0.7488
Grp A vs Grp F	-0.8006	0.4233
Grp A vs Grp G	-0.3203	0.7488
Grp A vs Grp H	-0.8006	0.4233
Grp B vs Grp C	1.3611	0.1735
Grp B vs Grp D	1.2810	0.2002
Grp B vs Grp E	1.2810	0.2002
Grp B vs Grp F	1.1209	0.2623
Grp B vs Grp G	1.2810	0.2002
Grp B vs Grp H	1.1209	0.2623
Grp C vs Grp D	0.0000	1.0000
Grp C vs Grp E	0.0000	1.0000
Grp C vs Grp F	-0.4003	0.6889
Grp C vs Grp G	0.0000	1.0000
Grp C vs Grp H	-0.4003	0.6889
Grp D vs Grp E	0.0000	1.0000
Grp D vs Grp F	-0.3203	0.7488
Grp D vs Grp G	0.0000	1.0000
Grp D vs Grp H	-0.3203	0.7488
Grp E vs Grp F	-0.3203	0.7488
Grp E vs Grp G	0.0000	1.0000
Grp E vs Grp H	-0.3203	0.7488
Grp F vs Grp G	0.3203	0.7488
Grp F vs Grp H	0.0000	1.0000
Grp G vs Grp H	-0.3203	0.7488



Bar Diagram.no.7 showing comparison of eight groups with status of Congestion

Table.no.65 showing comparison of eight groups with status of Odema

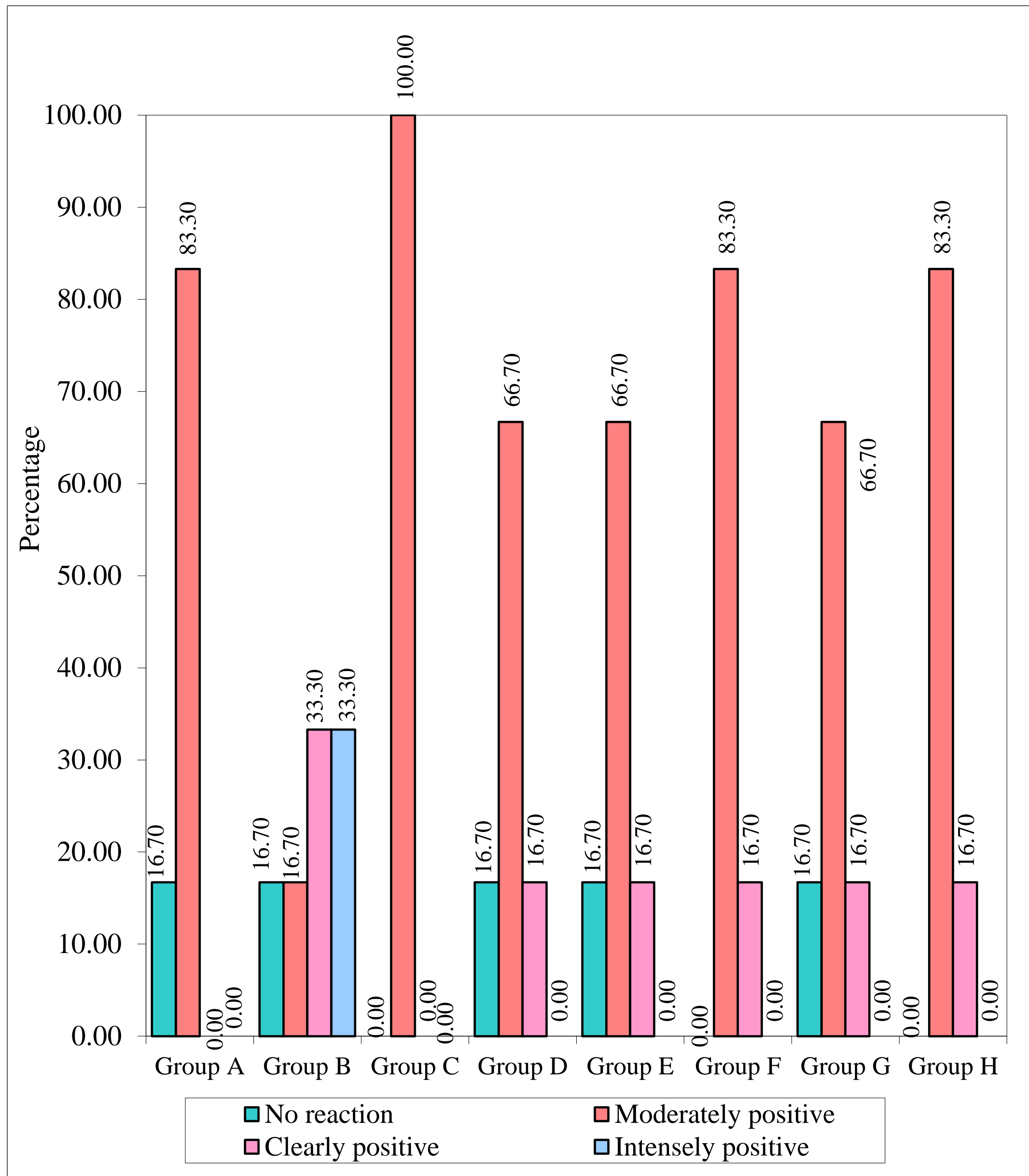
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Gr A	4	66.67	2	33.33	0	0.00	0	0.00	6
Gr B	5	83.33	1	16.67	0	0.00	0	0.00	6
Gr C	6	100.0	0	0.00	0	0.00	0	0.00	6
Gr D	5	83.33	1	16.67	0	0.00	0	0.00	6
Gr E	6	100.0	0	0.00	0	0.00	0	0.00	6
Gr F	4	66.67	2	33.33	0	0.00	0	0.00	6
Gr G	6	100.0	0	0.00	0	0.00	0	0.00	6
Gr H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	42	87.50	6	12.50	0	0.00	0	0.00	48

Table.no.66 Comparison of eight groups with status of Odema by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Gr A	0.00	29.50	8.2060	0.3150
Gr B	0.00	25.50		
Gr C	0.00	21.50		
Gr D	0.00	25.50		
Gr E	0.00	21.50		
Gr F	0.00	29.50		
Gr G	0.00	21.50		
Gr H	0.00	21.50		

Table.no.67 Mann-Whitney U test pairwise comparisons of eight groups with Odema status

Groups	Z-value	p-value
Grp A vs Grp B	0.4003	0.6889
Grp A vs Grp C	0.8807	0.3785
Grp A vs Grp D	0.4003	0.6889
Grp A vs Grp E	0.8807	0.3785
Grp A vs Grp F	0.0801	0.9362
Grp A vs Grp G	0.8807	0.3785
Grp A vs Grp H	0.8807	0.3785
Grp B vs Grp C	0.4003	0.6889
Grp B vs Grp D	-0.0801	0.9362
Grp B vs Grp E	0.4003	0.6889
Grp B vs Grp F	-0.4003	0.6889
Grp B vs Grp G	0.4003	0.6889
Grp B vs Grp H	0.4003	0.6889
Grp C vs Grp D	-0.4003	0.6889
Grp C vs Grp E	0.0000	1.0000
Grp C vs Grp F	-0.8807	0.3785
Grp C vs Grp G	0.0000	1.0000
Grp C vs Grp H	0.0801	0.9362
Grp D vs Grp E	0.4003	0.6889
Grp D vs Grp F	-0.4003	0.6889
Grp D vs Grp G	0.4003	0.6889
Grp D vs Grp H	0.4003	0.6889
Grp E vs Grp F	-0.8807	0.3785
Grp E vs Grp G	0.0000	1.0000
Grp E vs Grp H	0.0801	0.9362
Grp F vs Grp G	0.8807	0.3785
Grp F vs Grp H	0.8807	0.3785
Grp G vs Grp H	0.0801	0.9362



Bar Diagram.no.8 showing comparison of eight groups with status of Odema

Table.no.68 showing comparison of eight groups with status of Haemorrhage

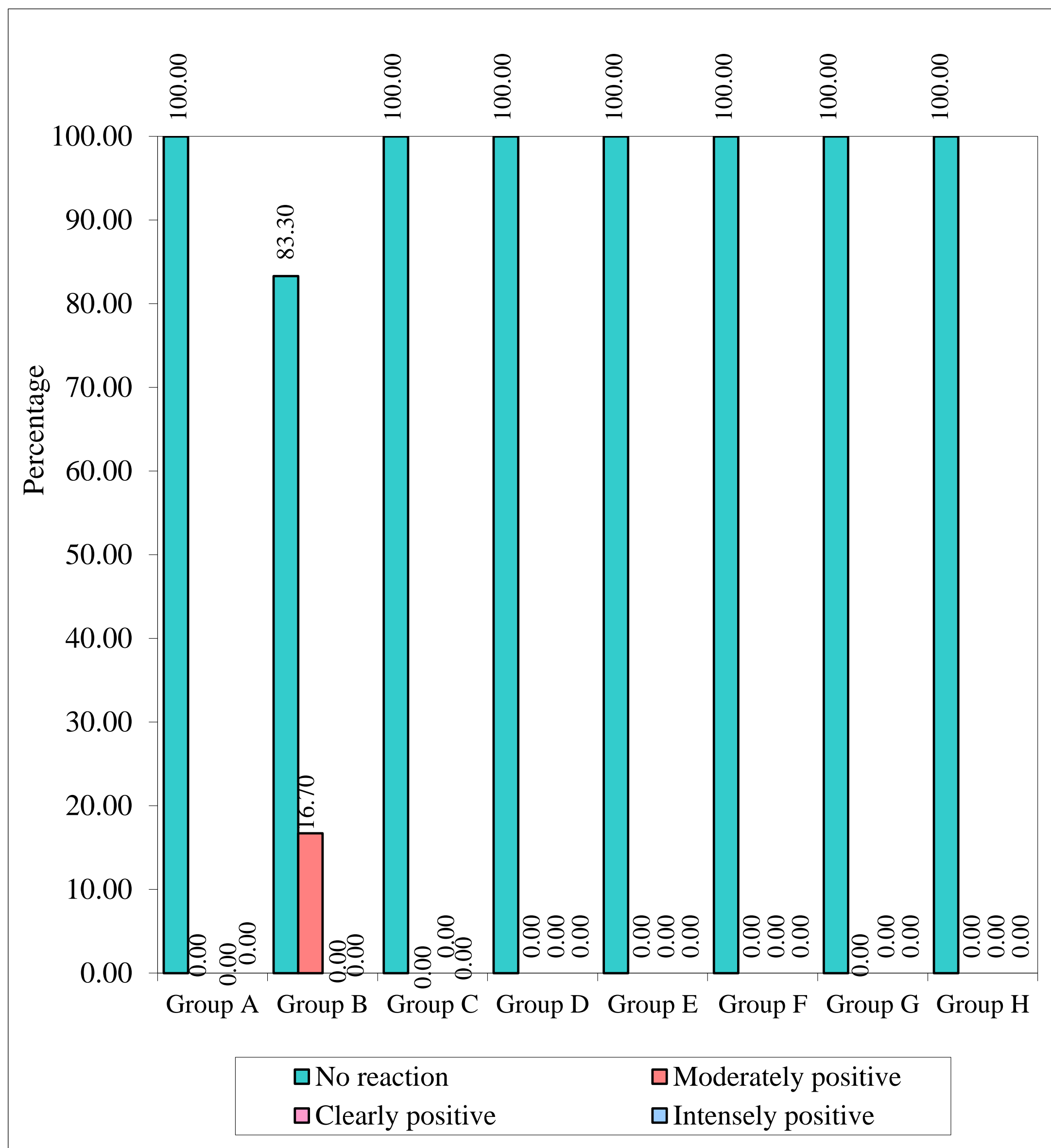
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	5	83.33	1	16.67	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	47	97.92	1	2.08	0	0.00	0	0.00	48

Table.no.69 showing comparison of eight groups with status of Haemorrhage by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Gr A	0.00	24.00	7.0000	0.4290
Gr B	0.00	28.00		
Gr C	0.00	24.00		
Gr D	0.00	24.00		
Gr E	0.00	24.00		
Gr F	0.00	24.00		
Gr G	0.00	24.00		
Gr H	0.00	24.00		

Table.no.70 Mann-Whitney U test pairwise comparisons of eight groups with Haemorrhage status

Groups	Z-value	p-value
Grp A vs Grp B	-0.4003	0.6889
Grp A vs Grp C	-0.0801	0.9362
Grp A vs Grp D	-0.0801	0.9362
Grp A vs Grp E	-0.0801	0.9362
Grp A vs Grp F	0.0801	0.9362
Grp A vs Grp G	-0.0801	0.9362
Grp A vs Grp H	-0.0801	0.9362
Grp B vs Grp C	0.4003	0.6889
Grp B vs Grp D	0.4003	0.6889
Grp B vs Grp E	0.4003	0.6889
Grp B vs Grp F	0.4003	0.6889
Grp B vs Grp G	0.4003	0.6889
Grp B vs Grp H	0.4003	0.6889
Grp C vs Grp D	0.0801	0.9362
Grp C vs Grp E	0.0000	1.0000
Grp C vs Grp F	0.0801	0.9362
Grp C vs Grp G	0.0000	1.0000
Grp C vs Grp H	0.0801	0.9362
Grp D vs Grp E	-0.0801	0.9362
Grp D vs Grp F	0.0801	0.9362
Grp D vs Grp G	-0.0801	0.9362
Grp D vs Grp H	-0.0801	0.9362
Grp E vs Grp F	0.0801	0.9362
Grp E vs Grp G	0.0000	1.0000
Grp E vs Grp H	0.0801	0.9362
Grp F vs Grp G	-0.0801	0.9362
Grp F vs Grp H	-0.0801	0.9362
Grp G vs Grp H	0.0801	0.9362



Bar Diagram.no.9 showing comparison of eight groups with status of Haemorrhage

Table.no.71 showing comparison of eight groups with status of Inflammation

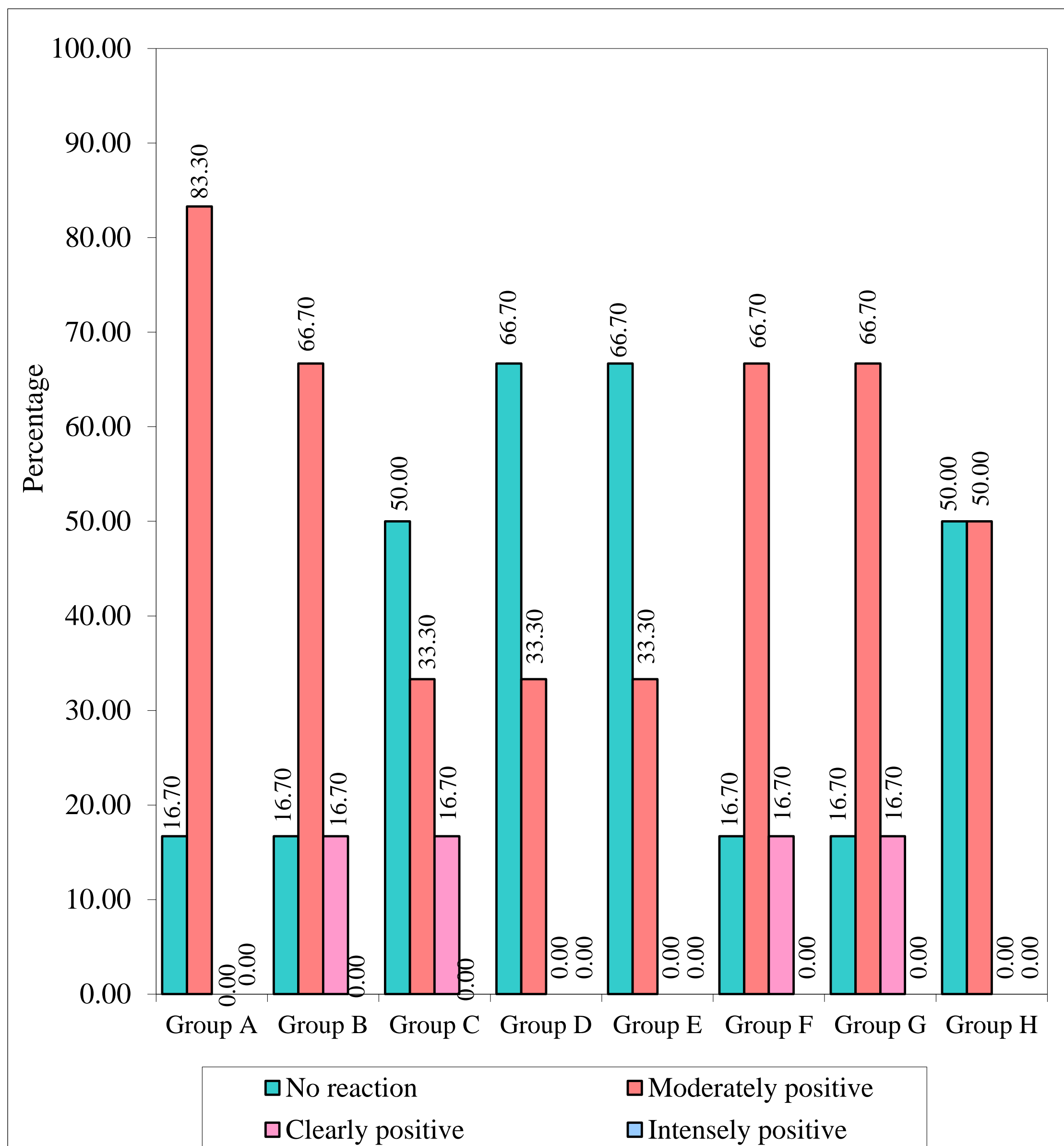
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	1	16.67	5	83.33	0	0.00	0	0.00	6
Group B	1	16.67	4	66.67	1	16.67	0	0.00	6
Group C	3	50.00	2	33.33	1	16.67	0	0.00	6
Group D	4	66.67	2	33.33	0	0.00	0	0.00	6
Group E	4	66.67	2	33.33	0	0.00	0	0.00	6
Group F	1	16.67	4	66.67	1	16.67	0	0.00	6
Group G	1	16.67	4	66.67	1	16.67	0	0.00	6
Group H	3	50.00	3	50.00	0	0.00	0	0.00	6
Total	18	37.50	26	54.17	4	8.33	0	0.00	48

Table.no.72 showing comparison of eight groups with status of Inflammation by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Group A	1.00	27.83	9.6720	0.2080
Group B	1.00	30.33		
Group C	0.50	23.00		
Group D	0.00	16.83		
Group E	0.00	16.83		
Group F	1.00	30.33		
Group G	1.00	30.33		
Group H	0.50	20.50		

Table.no.73 showing pair wise comparisons of eight groups with status of Inflammation by Mann-Whitney U test

Groups	Z-value	p-value
Grp A vs Grp B	-0.3203	0.7488
Grp A vs Grp C	0.4804	0.6310
Grp A vs Grp D	1.3611	0.1735
Grp A vs Grp E	1.3611	0.1735
Grp A vs Grp F	-0.3203	0.7488
Grp A vs Grp G	-0.3203	0.7488
Grp A vs Grp H	0.8807	0.3785
Grp B vs Grp C	0.7206	0.4712
Grp B vs Grp D	1.5212	0.1282
Grp B vs Grp E	1.5212	0.1282
Grp B vs Grp F	-0.0801	0.9362
Grp B vs Grp G	-0.0801	0.9362
Grp B vs Grp H	1.1209	0.2623
Grp C vs Grp D	0.5604	0.5752
Grp C vs Grp E	0.5604	0.5752
Grp C vs Grp F	-0.7206	0.4712
Grp C vs Grp G	-0.7206	0.4712
Grp C vs Grp H	0.1601	0.8728
Grp D vs Grp E	-0.0801	0.9362
Grp D vs Grp F	-1.5212	0.1282
Grp D vs Grp G	-1.5212	0.1282
Grp D vs Grp H	-0.4003	0.6889
Grp E vs Grp F	-1.5212	0.1282
Grp E vs Grp G	-1.5212	0.1282
Grp E vs Grp H	-0.4003	0.6889
Grp F vs Grp G	-0.0801	0.9362
Grp F vs Grp H	1.1209	0.2623
Grp G vs Grp H	1.1209	0.2623



Bar Diagram.no.10 showing comparison of eight groups with status of Inflammation

Table.no.74 showing comparison of eight groups with status of Loss of Intestinal glands

Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	6	100.0	0	0.00	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	48	100.0	0	0.00	0	0.00	0	0.00	48

Table.no.75 showing comparison of eight groups with status of Mucosal Ulcers

Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	6	100.0	0	0.00	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	48	100.0	0	0.00	0	0.00	0	0.00	48

Table.no.76 showing comparison of eight groups with status of Destruction of Villi

Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	6	100.0	0	0.00	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	48	100.0	0	0.00	0	0.00	0	0.00	48

Colon specimens:

Table.no.77 showing comparison of eight groups with status of Congestion

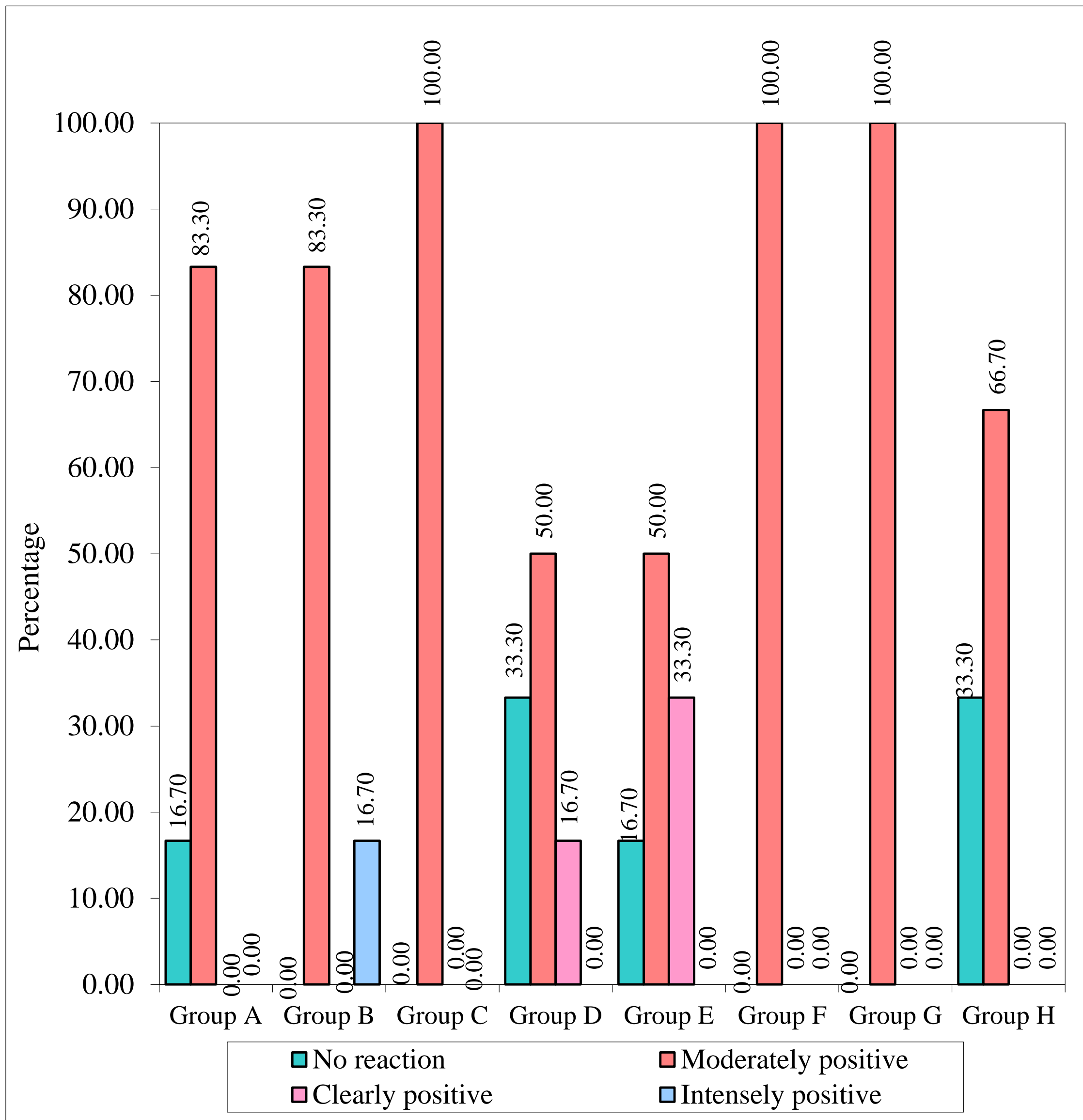
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	1	16.67	5	83.33	0	0.00	0	0.00	6
Group B	0	0.00	5	83.33	0	0.00	1	16.67	6
Group C	0	0.00	6	100.00	0	0.00	0	0.00	6
Group D	2	33.33	3	50.00	1	16.67	0	0.00	6
Group E	1	16.67	3	50.00	2	33.33	0	0.00	6
Group F	0	0.00	6	100.00	0	0.00	0	0.00	6
Group G	0	0.00	6	100.00	0	0.00	0	0.00	6
Group H	2	33.33	4	66.67	0	0.00	0	0.00	6
Total	6	12.50	38	79.17	3	6.25	1	2.08	48

Table.no.78 showing comparison of eight groups with status of Congestion by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Group A	1.00	21.83	6.0180	0.5380
Group B	1.00	29.25		
Group C	1.00	25.50		
Group D	1.00	21.58		
Group E	1.00	28.67		
Group F	1.00	25.50		
Group G	1.00	25.50		
Group H	1.00	18.17		

Table.no.79 Mann-Whitney U test pairwise comparisons of eight groups with Congestion status

Groups	Z-value	p-value
Grp A vs Grp B	-0.8006	0.4233
Grp A vs Grp C	-0.4003	0.6889
Grp A vs Grp D	0.0000	1.0000
Grp A vs Grp E	-0.7206	0.4712
Grp A vs Grp F	-0.4003	0.6889
Grp A vs Grp G	-0.4003	0.6889
Grp A vs Grp H	0.4003	0.6889
Grp B vs Grp C	0.4003	0.6889
Grp B vs Grp D	0.8006	0.4233
Grp B vs Grp E	0.0000	1.0000
Grp B vs Grp F	0.4003	0.6889
Grp B vs Grp G	0.4003	0.6889
Grp B vs Grp H	1.2010	0.2298
Grp C vs Grp D	0.4003	0.6889
Grp C vs Grp E	-0.4003	0.6889
Grp C vs Grp F	0.0801	0.9362
Grp C vs Grp G	0.0801	0.9362
Grp C vs Grp H	0.8807	0.3785
Grp D vs Grp E	-0.6405	0.5218
Grp D vs Grp F	-0.4003	0.6889
Grp D vs Grp G	-0.4003	0.6889
Grp D vs Grp H	0.2402	0.8102
Grp E vs Grp F	0.4003	0.6889
Grp E vs Grp G	0.4003	0.6889
Grp E vs Grp H	1.0408	0.2980
Grp F vs Grp G	-0.0801	0.9362
Grp F vs Grp H	0.8807	0.3785
Grp G vs Grp H	0.8807	0.3785



Bar Diagram.no.11 showing comparison of eight groups with status of Congestion

Table.no.80 showing comparison of eight groups with status of Odema

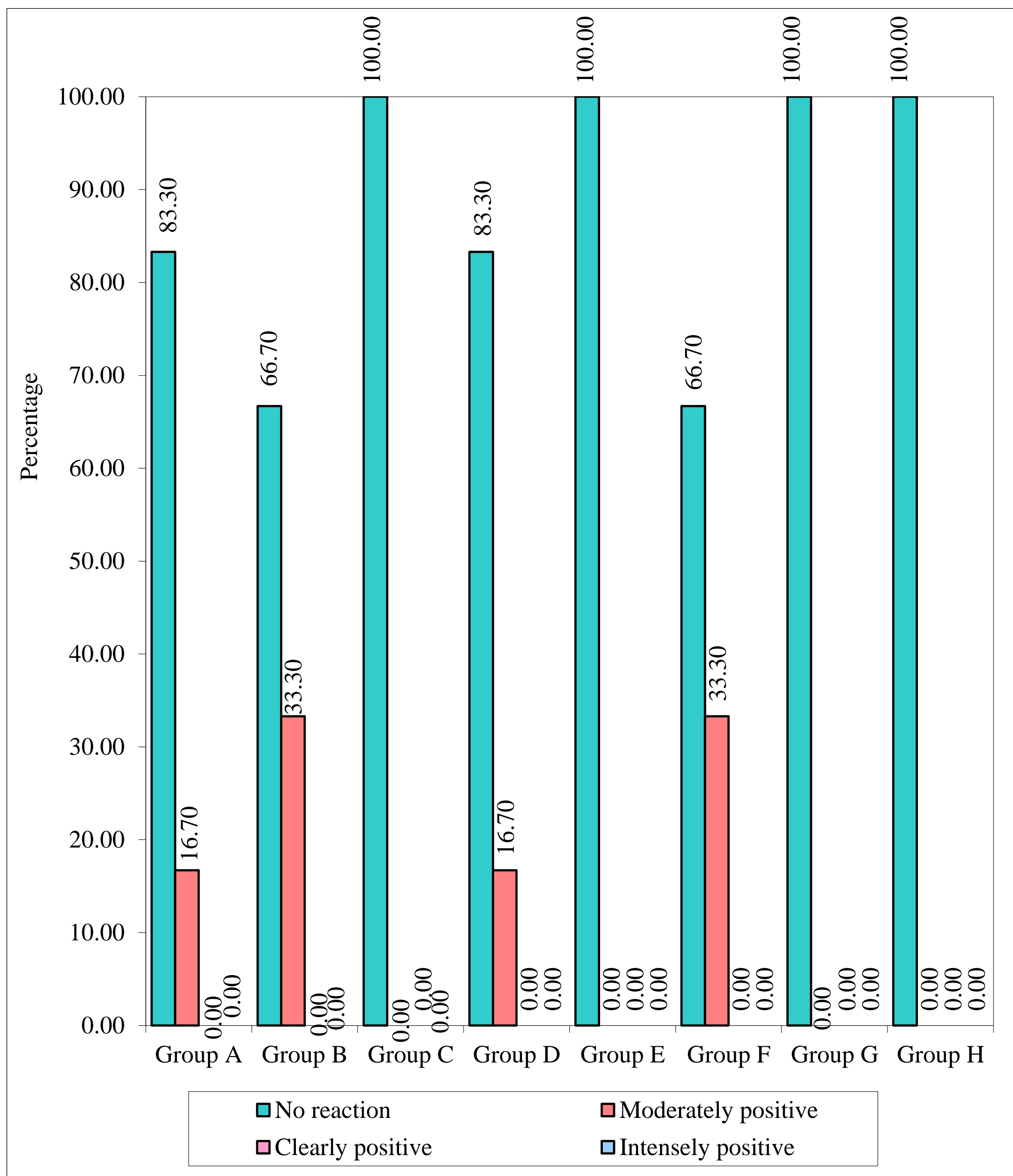
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	5	83.33	1	16.67	0	0.00	0	0.00	6
Group B	4	66.67	2	33.33	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	5	83.33	1	16.67	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	4	66.67	2	33.33	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	42	87.50	6	12.50	0	0.00	0	0.00	48

Table.no.81showing comparison of eight groups with status of Odema by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Gr A	0.00	25.50	8.2060	0.3150
Gr B	0.00	29.50		
Gr C	0.00	21.50		
Gr D	0.00	25.50		
Gr E	0.00	21.50		
Gr F	0.00	29.50		
Gr G	0.00	21.50		
Gr H	0.00	21.50		

Table.no.82 Mann-Whitney U test was used to compare the status of eight groups with Odema

Groups	Z-value	p-value
Grp A vs Grp B	-0.4003	0.6889
Grp A vs Grp C	0.4003	0.6889
Grp A vs Grp D	-0.0801	0.9362
Grp A vs Grp E	0.4003	0.6889
Grp A vs Grp F	-0.4003	0.6889
Grp A vs Grp G	0.4003	0.6889
Grp A vs Grp H	0.4003	0.6889
Grp B vs Grp C	0.8807	0.3785
Grp B vs Grp D	0.4003	0.6889
Grp B vs Grp E	0.8807	0.3785
Grp B vs Grp F	-0.0801	0.9362
Grp B vs Grp G	0.8807	0.3785
Grp B vs Grp H	0.8807	0.3785
Grp C vs Grp D	-0.4003	0.6889
Grp C vs Grp E	0.0801	0.9362
Grp C vs Grp F	-0.8807	0.3785
Grp C vs Grp G	0.0801	0.9362
Grp C vs Grp H	-0.0801	0.9362
Grp D vs Grp E	0.4003	0.6889
Grp D vs Grp F	-0.4003	0.6889
Grp D vs Grp G	0.4003	0.6889
Grp D vs Grp H	0.4003	0.6889
Grp E vs Grp F	-0.8807	0.3785
Grp E vs Grp G	-0.0801	0.9362
Grp E vs Grp H	-0.0801	0.9362
Grp F vs Grp G	0.8807	0.3785
Grp F vs Grp H	0.8807	0.3785
Grp G vs Grp H	-0.0801	0.9362



Bar Diagram.no.12 showing comparison of eight groups with status of Odema

Table.no 83. showing comparison of eight groups with status of Haemorrhage

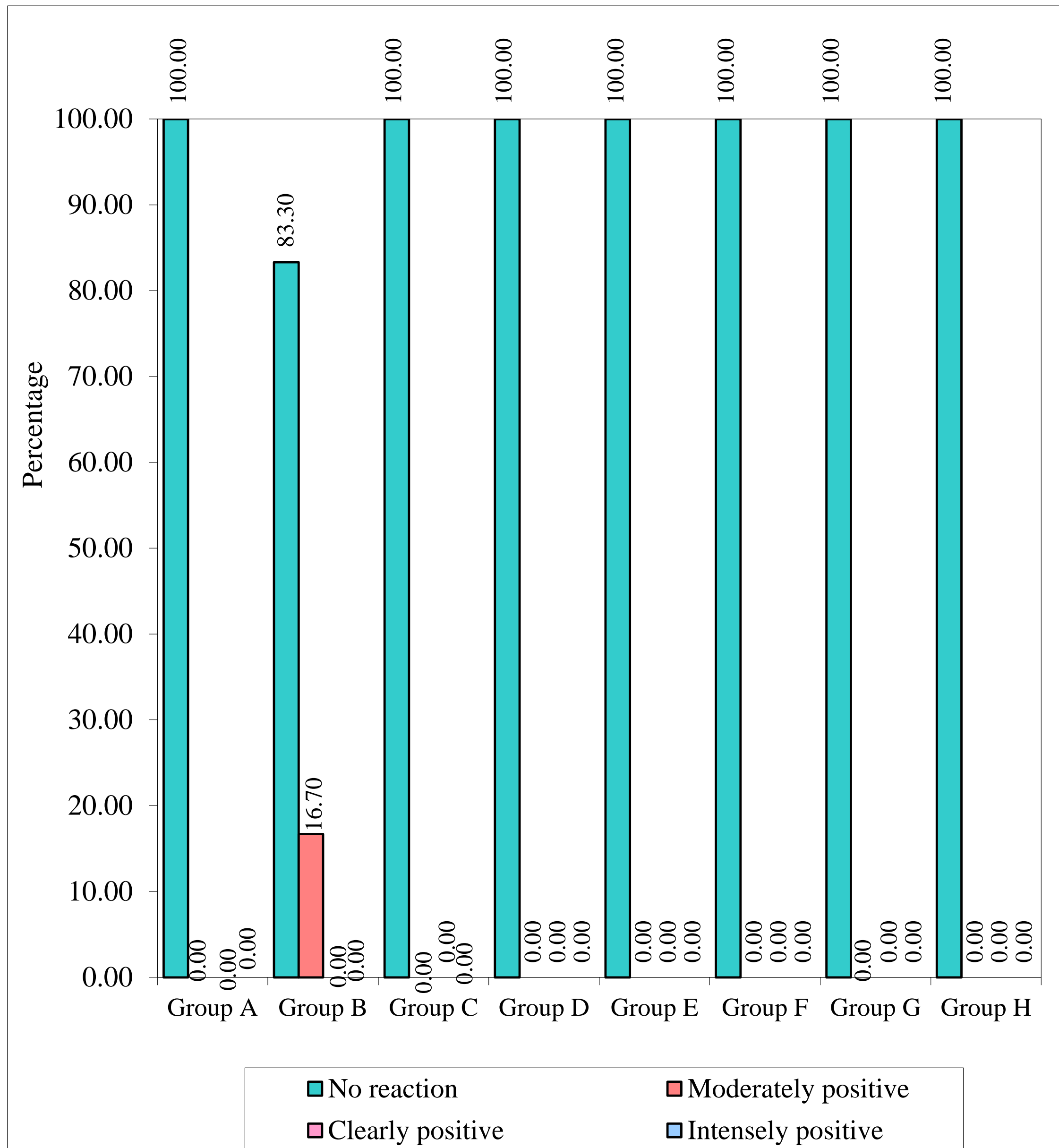
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	5	83.33	1	16.67	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	47	97.92	1	2.08	0	0.00	0	0.00	48

Table.no.84 showing comparison of eight groups with status of Haemorrhage by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Gr A	0.00	24.00	7.0000	0.4290
Gr B	0.00	28.00		
Gr C	0.00	24.00		
Gr D	0.00	24.00		
Gr E	0.00	24.00		
Gr F	0.00	24.00		
Gr G	0.00	24.00		
Gr H	0.00	24.00		

Table.no.85 The Mann-Whitney U test for pairwise comparisons of eight groups with Haemorrhage status

Groups	Z-value	p-value
Grp A vs Grp B	-0.4003	0.6889
Grp A vs Grp C	-0.0801	0.9362
Grp A vs Grp D	-0.0801	0.9362
Grp A vs Grp E	-0.0801	0.9362
Grp A vs Grp F	0.0801	0.9362
Grp A vs Grp G	-0.0801	0.9362
Grp A vs Grp H	-0.0801	0.9362
Grp B vs Grp C	0.4003	0.6889
Grp B vs Grp D	0.4003	0.6889
Grp B vs Grp E	0.4003	0.6889
Grp B vs Grp F	0.4003	0.6889
Grp B vs Grp G	0.4003	0.6889
Grp B vs Grp H	0.4003	0.6889
Grp C vs Grp D	0.0801	0.9362
Grp C vs Grp E	0.0801	0.9362
Grp C vs Grp F	0.0801	0.9362
Grp C vs Grp G	0.0801	0.9362
Grp C vs Grp H	-0.0801	0.9362
Grp D vs Grp E	-0.0801	0.9362
Grp D vs Grp F	0.0801	0.9362
Grp D vs Grp G	-0.0801	0.9362
Grp D vs Grp H	-0.0801	0.9362
Grp E vs Grp F	0.0801	0.9362
Grp E vs Grp G	-0.0801	0.9362
Grp E vs Grp H	-0.0801	0.9362
Grp F vs Grp G	-0.0801	0.9362
Grp F vs Grp H	-0.0801	0.9362
Grp G vs Grp H	-0.0801	0.9362



Bar Diagram.no.13 showing comparison of eight groups with status of Haemorrhage

Table.no.86 showing comparison of eight groups with status of Inflammation

Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	1	16.67	3	50.00	2	33.33	0	0.00	6
Group B	0	0.00	1	16.67	5	83.33	0	0.00	6
Group C	2	33.33	4	66.67	0	0.00	0	0.00	6
Group D	3	50.00	3	50.00	0	0.00	0	0.00	6
Group E	0	0.00	6	100.00	0	0.00	0	0.00	6
Group F	1	16.67	5	83.33	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	3	50.00	3	50.00	0	0.00	0	0.00	6
Total	16	33.33	25	52.08	7	14.58	0	0.00	48

Table.no.87 showing comparison of eight groups with status of Inflammation by Kruskal Wallis ANOVA

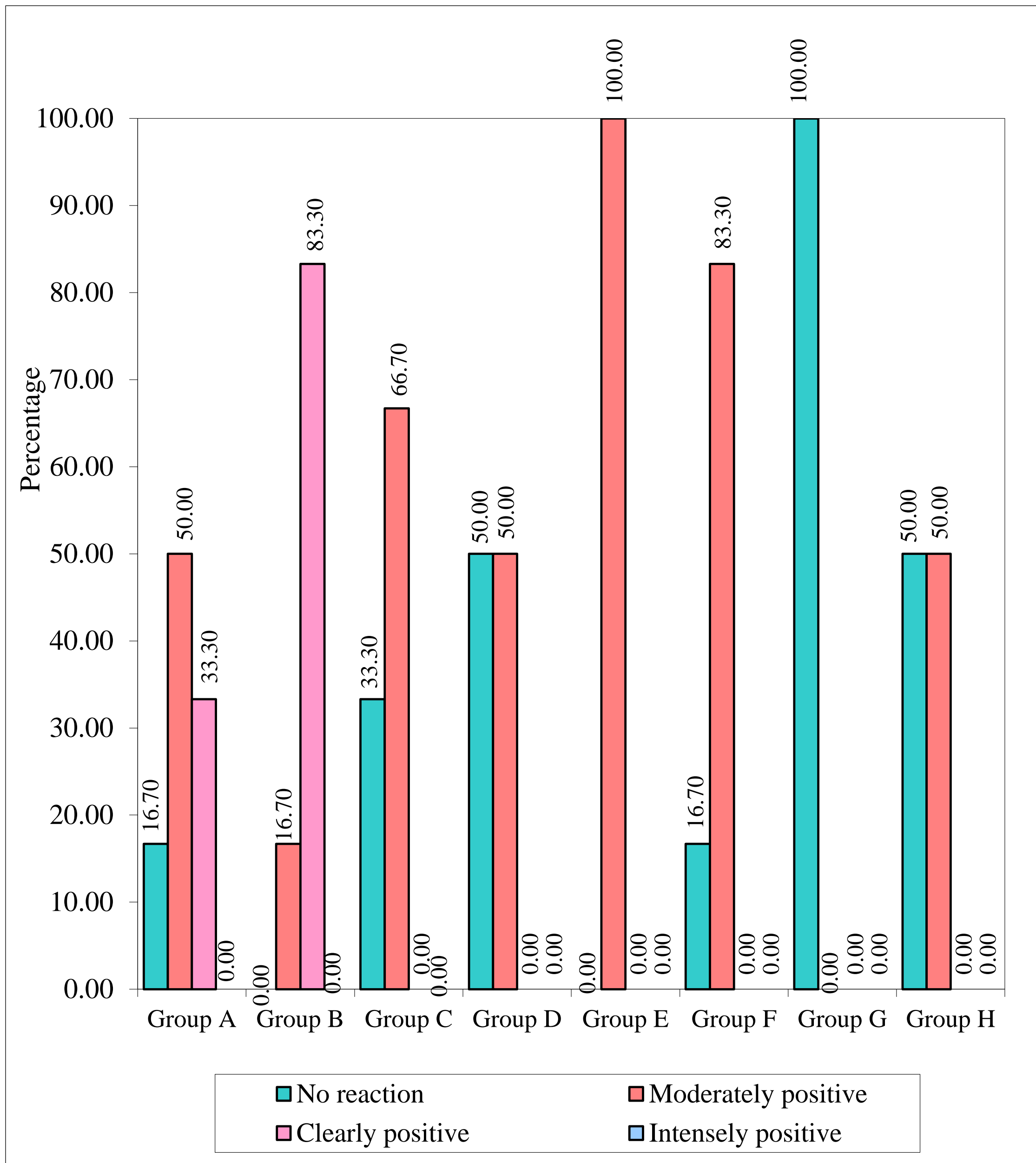
Groups	Median	Mean rank	H-value	p-value
Group A	1.00	30.92	26.4730	0.0001*
Group B	2.00	42.33		
Group C	1.00	22.17		
Group D	0.50	18.75		
Group E	1.00	29.00		
Group F	1.00	25.58		
Group G	0.00	8.50		
Group H	0.50	18.75		

*P<0.05

Table.no.88 Mann-Whitney U test was used to compare the level of inflammation in eight groups

Groups	Z-value	p-value
Grp A vs Grp B	-1.4412	0.1495
Grp A vs Grp C	1.0408	0.2980
Grp A vs Grp D	1.3611	0.1735
Grp A vs Grp E	0.4003	0.6889
Grp A vs Grp F	0.7206	0.4712
Grp A vs Grp G	2.3219	0.0202*
Grp A vs Grp H	1.3611	0.1735
Grp B vs Grp C	2.4820	0.0131*
Grp B vs Grp D	2.5621	0.0104*
Grp B vs Grp E	2.3219	0.0202*
Grp B vs Grp F	2.4019	0.0163*
Grp B vs Grp G	2.8022	0.0051*
Grp B vs Grp H	2.5621	0.0104*
Grp C vs Grp D	0.4003	0.6889
Grp C vs Grp E	-0.8807	0.3785
Grp C vs Grp F	-0.4003	0.6889
Grp C vs Grp G	1.8415	0.0656
Grp C vs Grp H	0.4003	0.6889
Grp D vs Grp E	-1.3611	0.1735
Grp D vs Grp F	-0.8807	0.3785
Grp D vs Grp G	1.3611	0.1735
Grp D vs Grp H	-0.0801	0.9362
Grp E vs Grp F	0.4003	0.6889
Grp E vs Grp G	2.8022	0.0051*
Grp E vs Grp H	1.3611	0.1735
Grp F vs Grp G	2.3219	0.0202*
Grp F vs Grp H	0.8807	0.3785
Grp G vs Grp H	-1.3611	0.1735

*P<0.05



Bar Diagram.no.14 showing comparison of eight groups with status of Inflammation

Table.no.89 showing comparison of eight groups with status of Mucosal Ulcers

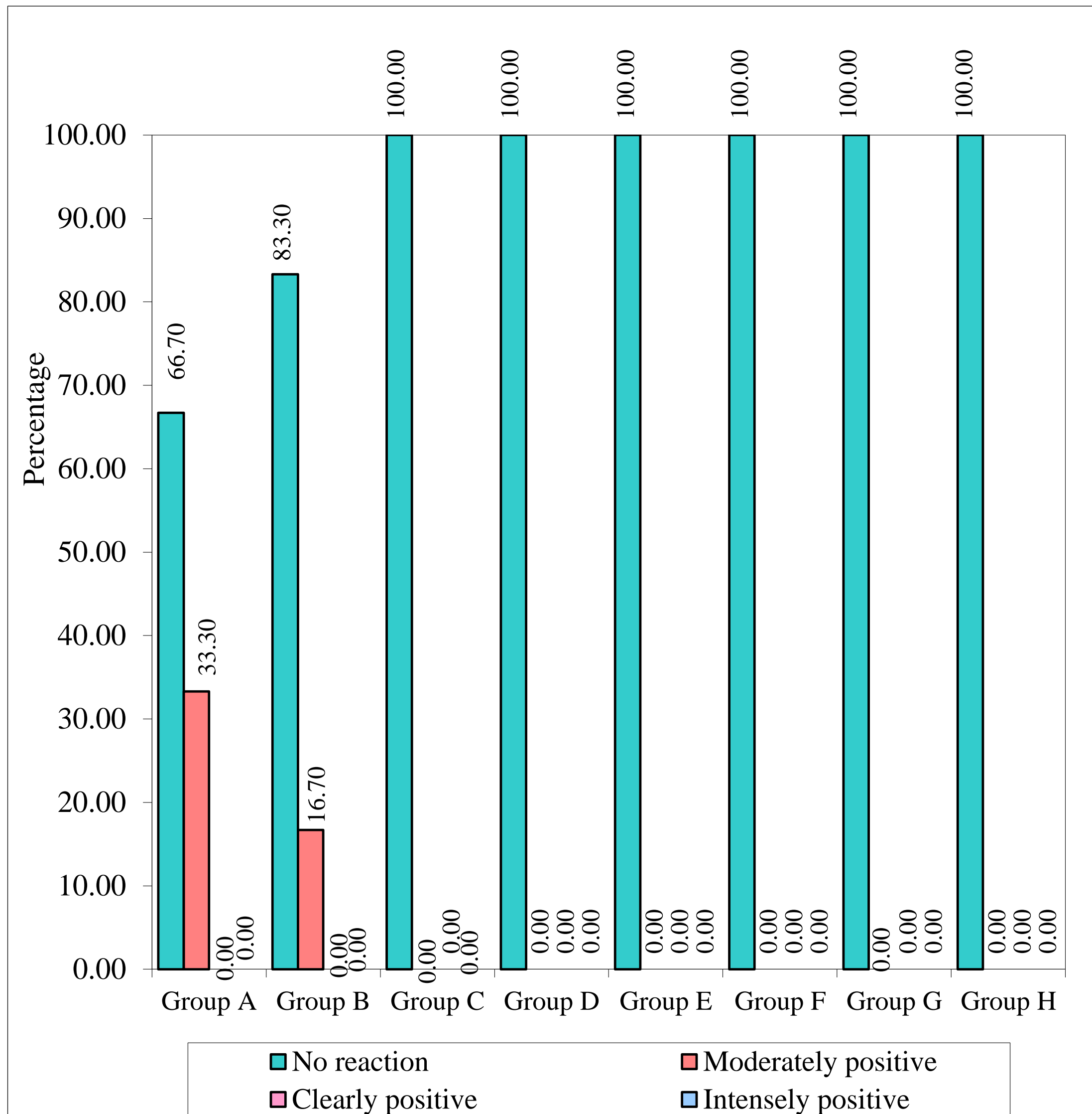
Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	4	66.67	2	33.33	0	0.00	0	0.00	6
Group B	5	83.33	1	16.67	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	45	93.75	3	6.25	0	0.00	0	0.00	48

Table.no.90 showing comparison of eight groups with status of Mucosal Ulcers by Kruskal Wallis ANOVA

Groups	Median	Mean rank	H-value	p-value
Gr A	0.00	31.00	10.7930	0.1480
Gr B	0.00	27.00		
Gr C	0.00	23.00		
Gr D	0.00	23.00		
Gr E	0.00	23.00		
Gr F	0.00	23.00		
Gr G	0.00	23.00		
Gr H	0.00	23.00		

Table.no.91 Mann-Whitney U test was used to compare the status of eight groups with Mucosal Ulcers

Groups	Z-value	p-value
Grp A vs Grp B	0.4003	0.6889
Grp A vs Grp C	0.8807	0.3785
Grp A vs Grp D	0.8807	0.3785
Grp A vs Grp E	0.8807	0.3785
Grp A vs Grp F	0.8807	0.3785
Grp A vs Grp G	0.8807	0.3785
Grp A vs Grp H	0.8807	0.3785
Grp B vs Grp C	0.4003	0.6889
Grp B vs Grp D	0.4003	0.6889
Grp B vs Grp E	0.4003	0.6889
Grp B vs Grp F	0.4003	0.6889
Grp B vs Grp G	0.4003	0.6889
Grp B vs Grp H	0.4003	0.6889
Grp C vs Grp D	-0.0801	0.9362
Grp C vs Grp E	0.0801	0.9362
Grp C vs Grp F	0.0801	0.9362
Grp C vs Grp G	-0.0801	0.9362
Grp C vs Grp H	-0.0801	0.9362
Grp D vs Grp E	0.0801	0.9362
Grp D vs Grp F	0.0801	0.9362
Grp D vs Grp G	-0.0801	0.9362
Grp D vs Grp H	-0.0801	0.9362
Grp E vs Grp F	-0.0801	0.9362
Grp E vs Grp G	-0.0801	0.9362
Grp E vs Grp H	-0.0801	0.9362
Grp F vs Grp G	-0.0801	0.9362
Grp F vs Grp H	-0.0801	0.9362
Grp G vs Grp H	0.0801	0.9362



Bar Diagram.no.15 showing comparison of eight groups with status of Mucosal Ulcers

Table.no.92 showing comparison of eight groups with status of Loss of Intestinal glands

Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	6	100.0	0	0.00	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	48	100.0	0	0.00	0	0.00	0	0.00	48

Table.no.93 showing comparison of eight groups with status of Destruction of Villi

Groups	No reaction	%	Moderately positive	%	Clearly positive	%	Intensely positive	%	Total
Group A	6	100.0	0	0.00	0	0.00	0	0.00	6
Group B	6	100.0	0	0.00	0	0.00	0	0.00	6
Group C	6	100.0	0	0.00	0	0.00	0	0.00	6
Group D	6	100.0	0	0.00	0	0.00	0	0.00	6
Group E	6	100.0	0	0.00	0	0.00	0	0.00	6
Group F	6	100.0	0	0.00	0	0.00	0	0.00	6
Group G	6	100.0	0	0.00	0	0.00	0	0.00	6
Group H	6	100.0	0	0.00	0	0.00	0	0.00	6
Total	48	100.0	0	0.00	0	0.00	0	0.00	48

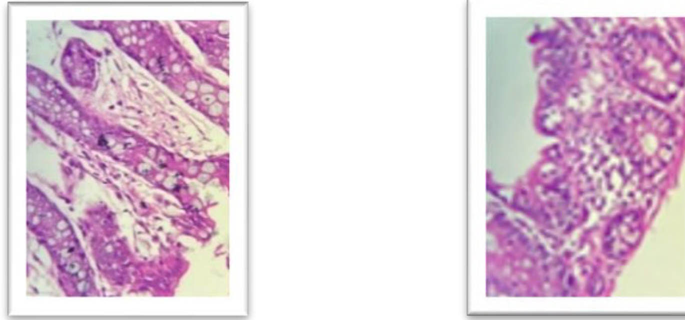


Figure.no.7 Photomicrograph of a section of wall of the Ano-Rectum & Caecum of the control group (Normal), showing normal sub-mucosa with Brunner's gland, mucosa & tall cylindrical willi with goblet cells. Severe congestion is noted in caecum sample of all the specimens. Mild oedema & Haemorrhage is observed in caecum sample. Severe inflammation is seen in both the samples. Loss of Intestinal glands & Mucosal Ulcers were not observed in any sample.

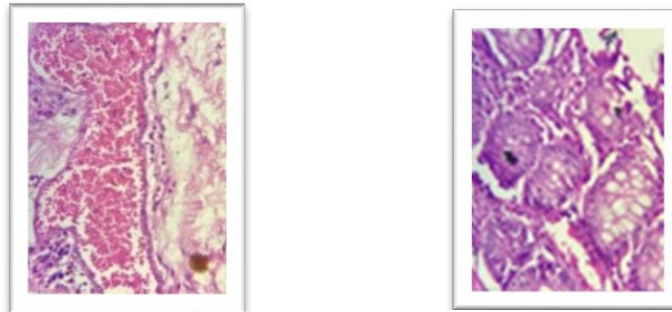


Figure.no.8 Photomicrograph of a section of wall of the Ano-Rectum and Caecal of the disease group, showing colonic mild congestion, oedema, ulceration and epithelial exfoliation in the sample specimens noted. Severe inflammation of the mucosal membrane of the colon samples is observed.

Figure.no.9 Photomicrograph of a section of wall of the Ano-Rectum & Caecum of the curd group, showing moderate caecum & colonic pathology with congestion & inflammation within the sub mucosa and the lamina

Figure.no.10 Photomicrograph of a section of wall of the Ano-Rectum of the BPM (Bilwaphala majja) group, showing moderate congestion, minimal haemorrhage and light oedema observed in both the sample specimens. No significant changes in histology in colon sample specimens.

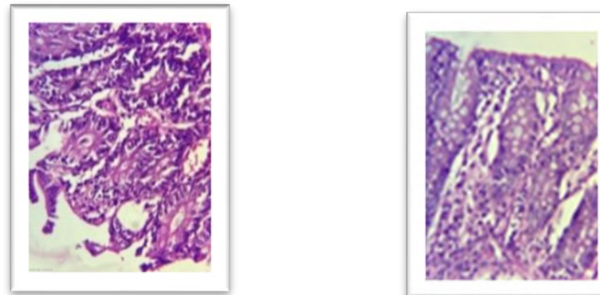


Figure.no.11 Photomicrograph of a section of wall of the Ano-Rectum of the Standard group, showing severe congestion & mild inflammation is observed in both the samples of specimens.

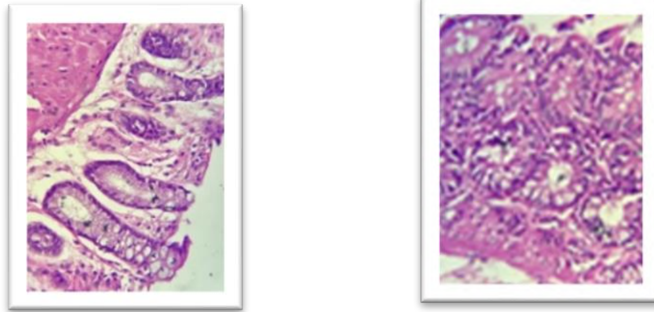


Figure.no.12 Photomicrograph of a section of wall of the Ano-Rectum of the test drug group, showing mild to moderate congestion & inflammation is observed in both the samples. Mild oedema is seen in ano-rectal samples of the specimen.

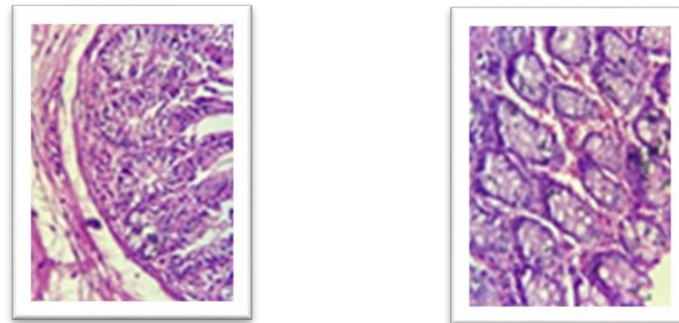


Figure.no.13 Photomicrograph of a section of wall of the Ano-Rectum of the Standard (Antibiotic + Sporlac) group, showing mild to moderate congestion is observed in both the sample specimens. Mild inflammation is only observed in ano-rectal specimens of all samples.

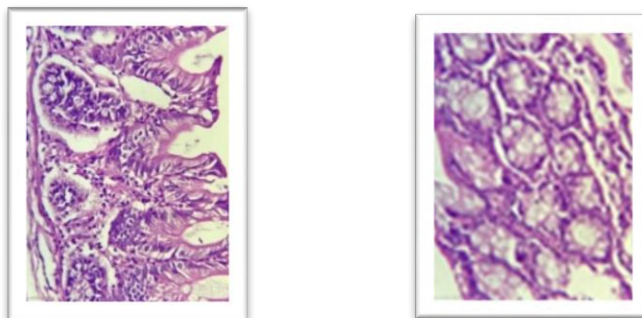


Figure.no.14 Photomicrograph of a section of wall of the Ano-Rectum of the Prophylactic (Antibiotic +Ayur-Probiotic) group, showing mild to moderate congestion & inflammation is observed in both the samples. No significant histo-pathological changes are seen in this group.

DISCUSSION

- About 5–30% of individuals get antibiotic-associated diarrhoea (AAD), either at the beginning of their antibiotic medication or for as long as two months after it has ended[189, 190, 191].

- Amoxicillin is known to induce dys-microbism by altering the microbial flora of the gut, which leads to observable intestinal inflammation[212].

- It is well recognized that antibiotics can change the delicate balance of the gut microbiota, allowing harmful organisms like Clostridium difficile to colonize.

- In the present study, the Ayur-probiotic's ability to counteract the effects of amoxicillin directly affected the degree of dys-microbism.

- *Apakwa bilwaphala majja* (un-ripened bael fruit pulp) is one of the popular ingredients used in many Ayurvedic formulations with wide range of indications. It possesses a substantial amount of mucilage, which acts as a demulcent and is effective in treating chronic diarrhoeal illnesses. Furthermore, fruit's role in treating diarrhoea and dysentery is mentioned in the British Pharmacopoeia[146].

- The pharmacological activities of *apakwa bilwaphala majja* are attributed to its wide range of phyto-constituents which include polyphenols, coumarins, tannins, alkaloids, phenolic acids, organic acids, flavonoids, tocopherols and carotenes[154].

- The raw form (*Bilwa pahalasaara dadhi*) is prone to spoil quickly in its fresh state. Therefore, the formulation was freeze dried (lyophilized).

- Phyto-chemical properties of lyophilized *bilwaphala saara dadhi* with water and alcohol extract shown the presence of carbohydrate, reducing sugar, alkaloids and tannins. Physico-chemical properties of lyophilized *bilwaphala saara dadhi* of the sample with 0.5% aqueous extract shows the highest percentage of loss on drying of 11.706 % & ash of 5.732%.

- The combination of curd and *apakwa bilwaphala majja* (un-ripened bael fruit pulp) was subjected for lyophilization technique. Presence of viable probiotic organisms depends on the quantity of *apakwa bilwaphala majja* in Ayur-probiotic.

- The quantity selected from 50gm to 6gm by reducing half every time and assessment of viable organisms showed "No Growth". This may be due to presence of optimum quantity of phyto-chemicals and *Bilwa* has *Krimighna* and *graahi* in nature as per Ayurvedic classical text.

- A maximum viable probiotic organism seen in 100: 3 ratios is $5x10^4$ CFU (Colony Forming Units).

- The lyophilized sample was creamy white with milky odour, sour and was coarse in texture. Presence of carbohydrates, proteins, alkaloids and tannins in Ayur-probiotic formulation indicate the thermo-liable compounds are seen after lyophilization process.

On Water Intake:

- Group-B animals has shown significantly less in water intake when compared to Group-D, E, F, G & H ($P<0.001$). There was a relative decrease in water intake till 14th day ($p<0.05$) in all the groups observed but after the 21st day gradual increase was seen. Group-D (ie, Bilwa phala majja) showed increase in water intake when compared to Group- E, F, G & H ($p<0.001$)

On Food Intake:

- All the eight animal groups have shown relative food intake till 30th day ($P<0.01$). Significant reduction in food intake was seen till 21st day later gradual increase in food intake was found in Group-A ($P<0.05$).

On Body Weight:

- The Group-A (ie, Amoxicillin) Showed less increase in relative body weight when compared to other Groups, may be due to antibiotic induced diarrhoea till 30th day.
- Group-B, C, G & H did not exhibit significant body weight gain till 30th day. However, the Group-D (ie, Bilwa phala majja) showed drastic reduction of weight (27.47%) from 7th to 14th day and gradual increase in weight seen till 30th day. Group-E & F shows no significant difference from 7th to 14th day but after 14th day relative increase was seen.

Body weight results revealed that antibiotic induced diarrhoea significantly ($p<0.05$) decreased in body weight in Wistar rats of Group-B as compared with other groups. In Group-F, body weight increased

significantly ($p<0.05$) that received Ayur-Probiotics & Group-H as compared with Control Group-B & Group-A.

The non-significant due to the efficacy of the two provided products, values can be explained. ie, curd & *apakwa bilwaphala majja* can alter the microbiome of the intestines and subtly affect how quickly nutrients are absorbed. Thus, in spite of eating an average quantity of food, the animals lost weight, just like the control group. It is possible to link the experimental intervention directly to this result. In comparison to the Control Group-B, Groups-F had a higher feed conversion ratio. Additionally, the quantity of food that was consumed was equivalent among groups and within physiological criteria.

- The FCR (Food Consumption Rate) for Group-E was adverse. It may be said that the probiotic given after the antibiotic therapy was stopped, did not have the desired impact when compared to other groups, who consumed roughly the same amount of food. Changing the gut flora might cause digestion issues in experimental animals.

On stool observation of animals with special reference to diarrhoea:

❖ Stool examination of all rats except Group-B (Control) showed watery diarrhoea on administration of amoxicillin after 7th day onwards.

❖ On 31st day, during dissection of Group-C (Curd) animals, intestine of all the sacrificed animals shown more gaseous contents because of curd consumption.

- ❖ Intestine of sacrificed animals of Group-B & E, shown gaseous content because of Amoxicillin.

- ❖ Intestine of sacrificed animals of Group-H (Ayur-probiotic), shown hard and well-formed stool.

On Microbiological Parameters:

- **On Microbial Colony Count:**

  - ❖ Microbial colony count of probiotic organisms was done by serial dilution method. In 10^{-1} dilution, the control Group (B), Group-D (*apakwa bilwaphala majja*) and Group-G (Amoxicillin+Sporlac) showed less count of probiotic organisms.

  - ❖ Group-C (Curd) showed moderate count but Group-H (Ayur-probiotic) showed optimal count of probiotic organisms. This protects the microbial flora of the intestine by its classical properties.

- **On Clostridium difficile(CD)Toxin assessment:**

  - ❖ All the groups, except Group-B (Control) (P<0.0001) showed presence of C.difficile toxin in stool specimens. It may be due to the presence of amoxicillin induced diarrhoea in other groups.
  - ❖ A positive finding of C. difficile bacteria toxin denotes the presence of the bacteria in the gastrointestinal tract. Negative test findings for the toxin and bacteria could indicate that a bacterium besides C. difficile is to blame for the symptoms of diarrhoea and other ailments.

- **On the qPCR result:**

  - ❖ qPCR showed quantifying the different bacterial groups in all experimental groups such as Enterococcus faecalis, Bacillus subtilis & other organisms.

  - ❖ Results of samples of all the groups are matching with respective strain no & suspected organisms.

- **On Histo-Pathological parameters:**

On Ano-Rectal samples:

- ❖ There was no significant improvement observed between the groups in the histo-pathological results of the ano-rectal samples of animals in relation to congestion, oedema, haemorrhage, inflammation and loss of intestinal glands.

On Colon (Caecum) samples:

- ❖ There was no significant improvement observed between the groups in the histo-pathological results of the caecum samples of animals in relation to congestion, oedema, haemorrhage, destruction of villi and mucosal ulcers except inflammation ($P<0.0001$).
- ❖ There was significant decrease in inflammation of caecum samples seen in the groups-C, D, E, F, G & H when compared to Group-B (control) ($P<0.01$) and Group-G (ie, Amoxicillin + Sporlac).
- ❖ Inflammation is reduced when compared to Group-E (Sporlac) and Group-H (Ayur-Probiotic) ($P<0.005$).

- The analysis of histo-pathological scoring in caecum, anus & rectum shown, Ayur-Probiotic formulation administered Group-F & H reported improvement in villus height compared with the Group-A.

- No remarkable changes seen in histo-pathological report of Ayur-Probiotic treated Group-F & H compared to those groups either treated with Group-C (curd) or with Group-D (*Apakwa bilwaphala majja*) which indicate that, effect of Ayur-Probiotic formulation alone in promoting mucosal healing to normal mucosa.

In Groups-A, B & E, the experimental interventions induced intestinal dys-microbism which subtly altered how well they metabolised meals. Values indicate that despite eating enough, weight loss may occur. Poor duodenal absorption may provide an explanation for these phenomena. This idea is supported by the histological features that were seen at this level. Probiotics are thought to have the potential to affect how well nutrients are absorbed in the intestine. The mechanism underpinning this theory is the induction of advantageous bacterial populations, which would keep the digestive system healthy[131]. On the other hand, dys-microbism has an impact on nutrition in the small intestine[132,133]. Probiotic administration's potential to cause dys-microbiasis as well as the duodenal histological features. The colonic mucosa responds well to the application of probiotic based on bacterium spores during inflammation, according to additional studies[136].

Due to their ability to develop spores and hence increase their survivability in challenging settings, like low pH levels within G.I.T[137,138,139,140] and the presence of digestion enzyme[141,142], several bacteria within the Bacillus genus are thought to provide a good probiotic potential. Probiotics can, however, affect the intestine in a way known as a trophic impact. Thus, using probiotics can boost the pace at which enterocytes proliferate, strengthening the intestinal wall and enhancing the body's ability to fight off pathogens. The pace of enterocyte growth, on the other hand, can be slowed down by pathogenic bacteria, which results in mucosal atrophy and a weakened immune system in the intestine. When the intestinal mucosa is compromised by dys-microbism, the treatment of probiotics entails eliminating the cells hypo-proliferative condition and enhancing intestinal absorption capacity[143]. Nevertheless, the epithelial barrier may be breached by bacteria or their by-products. This is brought on by either mucosal lesions or certain mechanisms whose main objective is to stimulate the immune system in the gut mucosa. These cellular responses are demonstrated by the creation of several inflammatory mediators, which play a part in triggering the immunological response to the mucosa[144]. Therefore, assuming that intestine dys-microbism will trigger an inflammatory response and result in alterations in the intestine, one of the consequences may be the denudation of the wall of the basement membrane. Additionally, the above-mentioned characteristic and the presence of sub-epithelial clefts were seen at the caecum levels in those groups that were indicative of the dys-microbism (rats from Groups A, C and E).

- **Mode of Action of Ayur-Probiotic Formulation on AAD (Antibiotic Associated Diarrhoea) in experimental animals:**

  - Ayur-probiotic reduced permeability in the intestine, increase goblet cell mucin secretion, increase defencins that inhibit pathogen colonization, produce short-chain fats (SCF) and stimulate IgA secretion, lower pH and increase immune cell tolerance to commensal microorganisms. It is thought that Ayur-Probiotic colonizes the gut, creating bactericidal acids & peptides while contending with other microorganisms for resources and epithelial adherence.

  - Ayur-Probiotic had positive benefits on its host without altering the long-term microbiome, which was made feasible by the intestine's temporary colonization. As a result, using an Ayur-probiotic during the use of antibiotics may safeguard gut homeostasis[207].

  - The main outcome of this study is that giving experimental animals an Ayur-probiotic formulation dramatically reduces their probability of developing AAD (Antibiotic Associated Diarrhea).

In the present study, Ayur-Probiotic formulation treatment shown significant gastro-intestinal protective effect in AAD (Antibiotic Associated Diarrhoea) in experimental animals.

According to this study, Ayur-Probiotic has a good preventive effect on preventing AAD (Antibiotic Induced Diarrhoea).

CONCLUSION

- Ayur-probiotic can be developed by lyophilization technique, by combining Curd and apakwa bilwaphala majja (un-ripened bael fruit pulp).

- In the ratio of 100 ml curd and 3gm apakwa bilwaphala majja, Ayur-probiotic formulation showed required count of Probiotic organisms in the range of 10^1 to 10^{15} Colony Forming Units (CFU) of viable organisms which include Enterococcus faecalis, Bacillus subtilis & other organisms.

- The microbial colony count of Group-G (Amoxicillin with Ayur probiotic) animals showed optimum count of micro-organisms in 10^{-1} dilution. The most of the identified organism are Proteus mirabilis and Enterococcus faecalis.

- The present study, revealed the maintenance of microbiome of the intestinal flora & histological texture of the gut.

- The formulated Ayur-probiotic treatment showed significant protection in Antibiotic Associated Diarrhoea (AAD) in experimental animals.

- The study proved the efficacy of Ayur-probiotic formulation in reduction of Antibiotic Associated Diarrhoea (AAD) in experimental animals.

Hence, it could be novel therapeutic formulation in clinical practice & antibiotic associated damage can be prevented by using this formulation.

SUMMARY

Bilwa ie, Aegle marmelos (L) Cor., a species belonging to the Rutacea family, is extensively used as an ingredient in most of the Ayurvedic formulations for a range of illnesses, including *grahani* and several types of *atisara*. It is also known as "Bael" and belongs to the Rutacea family. Studies have supported the ancient folks claiming un-ripened fruit can treat diarrhoea, which is described in the *Charaka Samhita* as a remedy for a number of kinds of *atisara*. The activities of *apakwa bilwaphala majja* are attributed to its wide range of phyto-constituents which include polyphenols, coumarins, tannins, alkaloids, phenolic acids, organic acids, flavonoids, tocopherols and carotenes.

Probiotics are live bacteria that, if consumed in adequate amounts, provide the host with some health benefits. In addition to preserving gut microbiota, curd contains a large number of probiotic bacteria that are helpful in treating diarrhoea. The ICMR recommendations state that 100 ml of curd per day is the suggested dosage for creating the necessary quantity of probiotic bacteria for favourable action over gut flora.

The raw form (*bilwapahala saara dadhi*) is prone to spoil quickly in its fresh state. Therefore, the formulation was freeze dried (lyophilized). Phyto-chemical properties of lyophilized bilwaphala saara dadhi with water and alcohol extract shown the presence of carbohydrate, reducing sugar, alkaloids and tannins. Physico-chemical properties of lyophilized bilwaphala saara dadhi of the sample with 0.5% aqueous extract shows the highest percentage of loss on drying of 11.706 % & ash of 5.732%.

The combination of curd and un-ripened Bilva-fruit pulp was subjected for lyophilization technique.

With considering all these supportive evidences, one such study is planned to develop "*Bilwaphala saara dadhi*" an Ayur-probiotic preparation which includes *apakwa bilwaphala majja in dadhi media.* And the same preparation will be used for evaluating its effect in Antibiotic Associated Diarrhoea (AAD) in the Wister rats.

Group-B has shown significantly less in water intake when compared to D, E, F, G & H groups ($P<0.001$). There was a relative decrease in water intake till 14th day ($p<0.05$) in all the groups observed but after the 21st day gradual increase was seen. Group-D (*bilwa phala majja*) showed increase in water intake when compared to Group- E, F, G & H ($p<0.001$). In comparison to Group-A, all the groups had relative food intake through day 30 (P 0.01). Up to the 21st day, there was a significant decrease in food consumption; after that, there was a gradual rise in food intake in Group-A ($P< 0.05$).

The Group-A (Amoxycillin) Showed less increase in relative body weight when compared to other Groups may be due to Antibiotic induced diarrhoea till 30 days. Group-B, C, G & H did not exhibit significant body weight gain till 30th day. However the Group-D (*bilwa phala majja*) showed drastic reduction of weight (27.47%) from 7th to 14th day and gradual increase in weight seen till 30th day. Group E & F shows no significant difference from 7th to 14th day but after 14th day relative increase can be seen.

Stool examination of all rats except Group-B (Control) showed watery diarrhoea on administration of amoxicillin after 7th day onwards. On 31st day, during dissection of Group-C(Curd) animals, intestine of all the sacrificed animals shown more gaseous contents because of curd consumption. Intestine of sacrificed animals of Group-B & E, shown gaseous content because of Amoxicillin. Intestine of sacrificed animals of Group-H, shown hard and well-formed stool.

Microbial colony count of organisms was done by serial dilution method. In 10^{-1} dilution, the control group, Bilwaphala majja and Ayur-probiotic group showed less count of microorganisms. Group-C (Curd), group-G (Amoxicillin+sporlac) showed moderate count but group-H (Amoxicillin+ Ayur-probiotic) showed optimum count of microorganism. This protects the microbial flora of the intestine by its classical properties.

Stool specimens were examined for C. difficile toxin presence, found that, all the groups except Control Group ie, Group-B ($P<0.0001$). It may be due to the presence of amoxicillin induced diarrhoea in other groups.

qPCR showed Quantifying the different bacterial groups in all experimental groups such as Enterococcus faecalis, Bacillus subtilis & other organisms. Results of samples of all the groups are matching with respective strain no & suspected organisms.

There was no significant improvement observed between the groups in the histo-pathological results of the ano-rectal samples of animals in relation to Congestion, Oedema, Haemorrhage and Inflammation and loss of intestinal glands. There was no significant improvement observed between the groups in the histo-

pathological results of the Caecum samples of animals in relation to Congestion, Oedema, Haemorrhage, destruction of villi and mucosal ulcers except inflammation ($P<0.0001$). There was significant decrease in inflammation of caecum samples seen in the groups-C, D, E, F, G & H when compared to control group(B) ($P<0.01$) and group-G (Amoxicillin + Sporlac). Inflammation is reduced when compared to Group-E (Sporlac) and Group-H (Ayur-Probiotic) ($P<0.005$). The analysis of histo-pathological scoring in caecum, anus & rectum shown, Ayur-Probiotic formulation administered group-F & H reported improvement in villus height compared with the group-A. No remarkable changes seen in histo-pathological report of Ayur-Probiotic treated group-F & H group compared to those groups either treated with group-C (curd) or with group-D (a*pakwa bilwaphala majja*) which indicate that, effect of Ayur-Probiotic formulation alone in promoting mucosal healing to normal mucosa.

Ayur-Probiotic had positive benefits on its host without altering the long-term microbiome, which was made feasible by the intestine's temporary colonization.

As a result, using an Ayur-probiotic during the use of antibiotics may safeguard gut homeostasis. The main outcome of this study is that giving experimental animals an Ayur-probiotic formulation dramatically reduces their probability of developing AAD (Antibiotic Associated Diarrhea).

In the present study, Ayur-Probiotic formulation treatment shown significant gastro-intestinal protective effect in AAD (Antibiotic Associated Diarrhoea) in experimental animals.

Hence, it could be novel therapeutic formulation in clinical practice & Antibiotic associated damage can be well prevented by using this formulation.

LIMITATIONS OF THE STUDY

- Incidence of diarrhoea was the only outcome assessed in this study.
- Examination of the quality-of-life indicators.

RECOMMENDATIONS FOR FURTHER STUDY

- Research study on efficacy of Ayur-probiotic on different antibiotics.
- Clinical Trial on Ayur-probiotic.

BIBLIOGRAPHY

1. Hurley BW, Nguyen C C. The spectrum of pseudomembranous enterocolitis and antibiotic- associated diarrhea. Arch Intern Med 2002; 162: 2177-2184 [PMID: 12390059].
2. Heller K J; Probiotic bacteria in fermented foods: product characteristics and starter organisms. Am J ClinNutr 2001; 73:374S-379S.
3. Bartlett JG. Clinical Practice. Antibiotic- Associated Diarrhea. N Engl J Med 2002; 346: 334-339 [PMID: 11821511]
4. McFarland LV. Epidemiology, risk factors and treatments for antibiotic-associated diarrhea. Dig Dis 1998; 16: 292-307 [PMID: 9892789]
5. Barbut F, Meynard JL. Managing antibiotic associated diarrhea. BMJ 2002; 324: 1345-1346 [PMID: 12052785]
6. Szajewska H, Mrukowicz JZ. Probiotics in prevention of antibiotic associated diarrhea: meta-analysis. J Pediatr2003; 142: 85 [PMID: 12569905].
7. Hogenauer, C., Hammer, H.F., Krejs, G.J. and Reisinger, E.C. (1998) Mechanisms and management of antibiotic-associated diarrhea. Clin Infect dis 27: 702-710.
8. Maria Kechagia, DimitriosBasoulis, StavroulaKonstantopoulou, Dimitra Dimitriadi, Konstantina Gyftopoulou, Nikoletta Skarmoutsou, and Eleni Maria Fakiri: Health Benefits of Probiotics. A Review ISRN Nutrition Volume 2013, Article ID 481651, 7 pages; http://dx.doi.org/10:5402/2013/481651.
9. Lab. Manual l; Manual of Methods of Analysis of foods, Food safety and standards authority of India (fssai); Ministry of health and family welfare Government of India. New Delhi 2015.

10. Kavitha. P, Banumathi. M, Sindhuja. D; Isolation of Lactobacillus Sps. From Yoghurt and its Application on Probiotic Chocolate. International Journal of Science and Research (IJSR) : 2319-7064, Volume 5 Issue 2, February 2016; pg.no:1696-1697.
11. Amal Bakr Shori, Ahmad S. Baba; Viability of lactic acid bacteria and sensory evaluation in Cinnamomum verum and Allium sativum-bio-yoghurts made from camel and cow milk. Journal of the Association of Arab Universities for Basic And Applied Sciences (2012) 11, pg.no:50-55.
12. Khandelwal KR; Practical Pharmacognosy, 13th edition, Nirali Prakashan, 2005; Pg.no. 143-53:
13. McFarland LV. Epidemiology, risk factors and treatments for antibiotic associated diarrhea. Dig Dis 1998; 16: 292-307.
14. Rajeshwari et al: Probiotics In Ayurveda; International Ayurvedic Medical Journal IAMJ: Volume 1; Issue 2; March- April 2013.
15. C. J. Ziemer and G. R. Gibson, "An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies," International Dairy Journal, vol. 8, no. 5-6, pp. 473-479, 1998.
16. Abedi, D.; Feizizadeh, S.; Akbari, V.; Jafarian-Dehkordi, A. *In vitro* anti-bacterial andanti-adherence effects of *Lactobacillus delbrueckii*subsp*bulgaricus*on *Escherichia coli.Res.Pharm. Sci*. **2013**, *8*, 260–268.
17. Tillisch, K.; Labus, J.; Kilpatrick, L.; Jiang, Z.; Stains, J.; Ebrat, B.; Guyonnet, D.;Legrain-Raspaud, S.; Trotin, B.; Naliboff, B.; Mayer, E.A. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology* **2013**, *144*, 1394–1401.

18. Suzuki, S.; Yakabe, T.; Suganuma, H.; Fukao, M.; Saito, T.; Yajima, N. Cell-bound exopolysaccharides of *Lactobacillus brevis*KB290: protective role and monosaccharide composition. *Can. J. Microbiol.***2013**, *59*, 549–555.

19. Pagnini C, et al. Probiotics promote gut health through stimulation of epithelial innate immunity. Proc Natl AcadSci USA 2009 Dec 14 Epub

20. Campus, G.; Cocco, F.; Carta, G.; Cagetti, M.G.; Simark-Mattson, C.; Strohmenger, L.;Lingström, P. Effect of a daily dose of *Lactobacillus brevis* CD2 lozenges in high caries riskschoolchildren. *Clin.Oral Investig*.**2014**, *18*, 555–561

21. Bordoni, A.; Amaretti, A.; Leonardi, A.; Boschetti, E.; Danesi, F.; Matteuzzi, D.; Roncaglia, L.;Raimondi, S.; Rossi, M. Cholesterol-lowering probiotics: *In vitro* selection and *in vivo* testing ofbifidobacteria. *Appl. Microbiol. Biotechnol.***2013**, *9*, 8273–8281.

22. Lanou, A.J., Berkow, S.E., Barnard, N.D. (2005) Calcium, dairy products, and bone health in children and young adults: a reevaluation of the evidence. *Pediatrics*115:736–743.

23. Antinoro, L. (n.d.). Increasing Dietary Potassium - Find Out Why Most People Need to Consume More of This Mineral. *Today's Dietitian 14*, 12, 50. Retrieved from http://www.todaysdietitian.com/newarchives/121112p50.shtml

24. Calcium and Cancer Prevention: Strengths and Limits of the Evidence. (2009, May). *National Cancer Institute*.

25. Giles-Smith, K. (2013, March). Milk proteins: Packing a powerful nutritional punch. *Today's Dietitian 15*3: 26.

26. Chen, M., Pan, A., Malik, V.S., Hu, F.B. (2012, October). Effects of dairy intake on body weight and fat: a meta-analysis of randomized controlled trials.

American Journal of Clinical Nutrition 96(4):735-47. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/pmid/22932282/3

27. Choline. (2008, December). *Nutrition 411*. Retrieved from http://www.nutrition411.com/component/k2/item/438-choline

28. Qin, L., Xu, J., Wang, P., Tong, J., Hoshi, K. (2007). Milk consumption is a risk factor for prostate cancer in Western countries: Evidence from cohort studies. *Asia Pacific Journal of Clinical Nutrition* 2007;16:467–476.

29. Song, Y., Chavarro, J.E., & Cao, Y. (2013). Whole milk intake is associated with prostate cancer-specific mortality among U.S. male physicians. *Journal of Nutrition*; 143:189-196. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23256145

30. Kroenke, C.H., Kwan, M.L., Sweeney, C., Castillo, A., Caan Bette, J. (2013). High-and low-fat dairy intake, recurrence, and mortality after breast cancer diagnosis. *Journal of the National Cancer Institute* 2013;105:616-623. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23492346

31. Saukkonen, T., Virtanen, S.M., &Karppinen, M. (1998). Significance of cow's milk protein antibodies as risk factor for childhood IDDM: Interaction with dietary cow's milk intake and HLA-DQB1 genotype. Childhood Diabetes in Finland Study Group. *Dibetologia*. 1998; 41:72–78.

32. Kimpimaki, T., Erkkola, M., & Korhonen, S. (2001). Short-term exclusive breastfeeding predisposes young children with increased genetic risk of type I diabetes to progressive beta-cell autoimmunity. *Diabetologia*. 2001; 44: 63–69.

33. Eidelman, A.I., Schanler, R.J. (2012). Policy statement: Breastfeeding and the use of human milk. *Pediatrics*. 2012;129:827–841.

34. Bodey GP, Nance J: Amoxicillin: in vitro and pharmacological studies. Antimicrob Agents Chemother. 1972 Apr;1(4):358-62. doi: 10.1128/aac.1.4.358. [PubMed:4598841]

35. FDA Approved Drug Products: Amoxil Amoxicillin Oral Capsules (Discontinued) [Link]

36. 3FDA Approved Drug Products: Amoxicillin Oral Suspension [Link]

37. FDA Approved Drug Products: Amoxicillin Oral Tablets

38. FDA Approved Drug Products: Augmentin XR Amoxicillin and Clavulanate Oral Extended Release Tablets

39. FDA Approved Drug Products: Augmentin Amoxicillin and Clavulanate Oral Tablets and Suspension

40. FDA Approved Drug Products: Amoxicillin and Clavulanate Oral Suspension

41. FDA Approved Drug Products: Talicia Amoxicillin, Omeprazole, and Rifabutin Oral Delayed Release Capsules

42. FDA Approved Drug Products: Omeclamox-Pak Oral Capsules and Tablets

43. Okamoto T, Yoshiyama H, Nakazawa T, Park ID, Chang MW, Yanai H, Okita K, Shirai M: A change in PBP1 is involved in amoxicillin resistance of clinical isolates of Helicobacter pylori. J Antimicrob Chemother. 2002 Dec;50(6):849-56. [PubMed:12461003]

44. Sauvage E, Terrak M: Glycosyltransferases and Transpeptidases/Penicillin-Binding Proteins: Valuable Targets for New Antibacterials. Antibiotics (Basel). 2016 Feb 17;5(1). pii: antibiotics5010012. doi: 10.3390/antibiotics5010012. [PubMed:27025527]

45. Akhavan BJ, Vijhani P: Amoxicillin . [PubMed:29489203]

46. Gordon C, Regamey C, Kirby WM: Comparative clinical pharmacology of amoxicillin and ampicillin administered orally. Antimicrob Agents Chemother. 1972 Jun;1(6):504-7. doi: 10.1128/aac.1.6.504. [PubMed:4680813]

47. de Velde F, de Winter BC, Koch BC, van Gelder T, Mouton JW: Non-linear absorption pharmacokinetics of amoxicillin: consequences for dosing regimens and clinical breakpoints. J Antimicrob Chemother. 2016 Oct;71(10):2909-17. doi: 10.1093/jac/dkw226. Epub 2016 Jun 20. [PubMed:27330071]

48. Carlier M, Noe M, De Waele JJ, Stove V, Verstraete AG, Lipman J, Roberts JA: Population pharmacokinetics and dosing simulations of amoxicillin/clavulanic acid in critically ill patients. J Antimicrob Chemother. 2013 Nov;68(11):2600-8. doi: 10.1093/jac/dkt240. Epub 2013 Jun 25. [PubMed:23800901]

49. Szultka M, Krzeminski R, Jackowski M, Buszewski B: Identification of In Vitro Metabolites of Amoxicillin in Human Liver Microsomes by LC-ESI/MS. Chromatographia. 2014;77:1027-1035. doi: 10.1007/s10337-014-2648-2. Epub 2014 Mar 22. [PubMed:25089048]

50. Belko J, Urueta G, Emre U: Amoxicillin overdose manifested by hematuria and acute renal failure. Pediatr Infect Dis J. 1995 Oct;14(10):917-9. [PubMed:8584329]

51. Todar K. Todar's Online Textbook of Bacteriology. Copyright; Madison, WI, 2008-2012.

52. Janda J.M., Abbot S.L. Bacterial identification for publication: when is enough enough? J Clin Microbiol 40(6): 1887-1891, 2002.

53. Shetty N., Hill G., Ridgway G.L. The Vitek analyser for routine bacterial identification and susceptibility testing: protocols, problems, and pitfalls. J Clin Pathol 51(4): 316-323, 1998.

54. Clarridge III J. Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clin Microbiol Rev 17(4): 840-862, 2004.

55. Janda J.M., Abbott S.L. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 45(9): 2761-2761, 2007.

56. Bruce J. Automated system rapidly identifies and characterizes micro-organisms in food. Food Technology 50: 77-78, 1996.

57. Woese C. Bacterial evolution. Microbiology Review 51: 221-271, 1987

58. Licciardi, P.V.; Ismail, I.H.; Balloch, A.; Mui, M.; Hoe, E.; Lamb, K.; Tang, M.L. Maternal supplementation with LGG reduces vaccine-specific immune responses in infants at high-risk of developing allergic disease. *Front. Immunol.***2013**, *4*, doi:10.3389/fimmu.2013.00381.

59. Wickens, K.; Stanley, T.V.; Mitchell, E.A.; Barthow, C.; Fitzharris, P.; Purdie, G.; Siebers, R.;Black, P.N.; Crane, J. Early supplementation with *Lactobacillus rhamnosus*HN001 reduceseczema prevalence to 6 years: Does it also reduce atopic sensitization? *Clin. Exp. Allergy*. **2013**,*43*, 1048–1057.

60. Panwar, H.; Calderwood, D.; Grant, I.R.; Grover, S.; Green, B.D. *Lactobacillus* strains isolatedfrom infant faeces possess potent inhibitory activity against intestinal alpha- and beta-glucosidases suggesting anti-diabetic potential. *Eur. J. Nutr.* **2014**, doi: 10.1007/s00394-013-0649-9.

61. Jakaitis, B.M.; Denning, P.W. Commensal and probiotic bacteria may prevent NEC by maturingintestinal host defenses. *Pathophysiology* **2014**, *21*, 47–54.

62. Homayouni, A.; Bastani, P.; Ziyadi, S.; Mohammad-Alizadeh-Charandabi, S.; Ghalibaf, M.;Mortazavian, A.M.; Mehrabany, E.V. Effects of probiotics on the recurrence of bacterialvaginosis: A review. *J. Low Genit. Tract. Dis*. **2014**, *18*, 79–86.

63. Sanchez, M.; Darimont, C.; Drapeau, V.; Emady-Azar, S.; Lepage, M.; Rezzonico, E.; Ngom-Bru, C.;Berger, B.; Philippe, L.; Ammon-Zuffrey, C.; *et al.* Effect of *Lactobacillus rhamnosus*CGMCC1.3724 supplementation on weight loss and maintenance in obese men and women. *Br. J.Nutr*. **2013**, *111*, 1507–1519.

64. Ciccarelli, S.; Stolfi, I.; Caramia, G. Management strategies in the treatment of neonatal and pediatric gastroenteritis. *Infect. Drug Resist*. **2013**, *6*, 133–161.

65. Luoto, R.; Ruuskanen, O.; Waris, M.; Kalliomäki, M.; Salminen, S.; Isolauri, E. Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: A randomized, placebo-controlled trial. *J. Allergy Clin. Immunol*.**2013**, *133*, 405–413.

66. Ammoscato, F.; Scirocco, A.; Altomare, A.; Matarrese, P.; Petitta, C.; Ascione, B.; Caronna, R.;Guarino, M.; Marignani, M.; Cicala, M.; *et al. Lactobacillus rhamnosus*protects human colonic muscle from pathogen lipopolysaccharide-induced damage. *Neurogastroenterol.Motil*.**2013**,doi:10.1111/nmo.12232.

67. Phavichitr, N.; Puwdee, P.; Tantibhaedhyangkul, R. Cost-benefit analysis of the probiotic treatment of children hospitalized for acute diarrhea in Bangkok, Thailand. *Southeast Asian J.Trop. Med. Public Health* **2013**, *44*, 1065–1071.

68. Cortés-Zavaleta, O.; López-Malo, A.; Hernández-Mendoza, A.; García, H.S. Antifungal activityof*Lactobacilli* and its relationship with 3-phenyllactic acid production. *Int. J. Food. Microbiol.***2014**, *173*, 30–35.

69. Mohseni, M.J.; Aryan, Z.; Emamzadeh-Fard, S.; Paydary, K.; Mofid, V.; Joudaki, H.;Kajbafzadeh, A.M. Combination of probiotics and antibiotics in the prevention of recurrent urinary tract infection in children. *Iran. J. Pediatr*. **2013**, *23*, 430–438.

70. Ortiz, L.; Ruiz, F.; Pascual, L.; Barberis, L. Effect of two probiotic strains of *Lactobacillus* on *in-vitro* adherence of *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Staphylococcus aureus*to vaginal epithelial cells. *Curr.Microbiol.***2014**, doi:10.1007/s00284-014-0524-9.

71. Wu, Z.J.; DU, X.; Zheng, J. Role of *Lactobacillus* in the prevention of *Clostridium difficile*-associated diarrhea: A meta-analysis of randomized controlled trials. *Chin. Med. J.(Engl.)* **2013**, *126*, 4154–4161.

72. Kovachev, S.; Dobrevski-Vacheva, R. [Effect of *Lactobacillus casei*var. *rhamnosus*(Gynophilus)in restoring the vaginal flora by female patients with bacterial vaginosis—Randomized, openclinical trial]. *Akush.Ginekol. (Sofiia)* **2013**, *52* (Suppl. 1), 48–53.

73. Wright, K.; Wright, H.; Murray, M. Probiotic treatment for the prevention of antibiotic-associated diarrhoea in geriatric patients: A multicentrerandomised controlled pilotstudy. *Australas. J. Ageing* **2014**, doi:10.1111/ajag.12116.

74. Shida, K.; Nomoto, K. Probiotics as efficient immune-potentiators: Translational role in cancer prevention. *Indian J. Med. Res*. **2013**, *138*, 808–814.

75. Vaghef-Mehrabany, E.; Alipour, B.; Homayouni-Rad, A.; Sharif, S.K.; Asghari-Jafarabadi, M.;Zavvari, S. Probiotic supplementation improves inflammatory status in patients with rheumatoidarthritis. *Nutrition* **2013**, *30*, 430–435.

76. Castillo, N.A.; de Moreno de LeBlanc, A.; Galdeano, C.M.; Perdigón, G. Comparative study ofthe protective capacity against *Salmonella* infection between probiotic and non-probiotic*Lactobacilli*. *J. Appl. Microbiol*. **2013**, *114*, 861–876.

77. NotoLlana, M.; Sarnacki, S.H.; AyaCastañedaMdel, R.; Bernal, M.I.; Giacomodonato, M.N.;Cerquetti, M.C. Consumption of *Lactobacillus casei*fermented milk prevents*Salmonella*reactive arthritis by modulating IL-23/IL-17 expression. *PLoS One* **2013**, *10*,doi:10.1371/journal.pone.0082588.

78. Babenko, L.R.; Lazarenko, L.M.; Shynkarenko, L.M.; Mokrozub, V.V.; Pidgorskyi, V.S.;Spivak, M.J. The effect of Lacto- and Bifidobacteria in monoculture on the vaginal microflorainnorm and in cases of intravaginalstaphylococcosis.*Mikrobiol. Z.* **2013**, *75*, 46–55.

79. Qin, Y.; Li, J.; Wang, Q.; Gao, K.; Zhu, B.; Lv, N. [Identification of lactic acid bacteria incommercial yogurt and their antibiotic resistance]. *Acta Microbiol.Sin.***2013**, *53*, 889–897.

80. Moro-García, M.A.; Alonso-Arias, R.; Baltadjieva, M.; FernándezBenítez, C.;Fernández Barrial, M.A.; DíazRuisánchez, E.; Alonso Santos, R.; Alvarez Sánchez, M.;Saavedra Miján, J.; López-Larrea, C. Oral supplementation with *Lactobacillus delbrueckii*subsp.*bulgaricus*8481 enhances systemic immunity in elderly subjects. *Age (Dordr)* **2013**, *35*,1311–1326.

81. Abedi, D.; Feizizadeh, S.; Akbari, V.; Jafarian-Dehkordi, A. *In vitro* anti-bacterial andanti-adherence effects of *Lactobacillus delbrueckii* subsp *bulgaricus* on *Escherichia coli.Res.Pharm. Sci*. **2013**, *8*, 260–268.

82. Tillisch, K.; Labus, J.; Kilpatrick, L.; Jiang, Z.; Stains, J.; Ebrat, B.; Guyonnet, D.;Legrain-Raspaud, S.; Trotin, B.; Naliboff, B.; Mayer, E.A. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology* **2013**, *144*, 1394–1401.

83. Suzuki, S.; Yakabe, T.; Suganuma, H.; Fukao, M.; Saito, T.; Yajima, N. Cell-boundexopolysaccharides of *Lactobacillus brevis*KB290: protective role and monosaccharide composition. *Can. J. Microbiol*.**2013**, *59*, 549–555.

84. Campus, G.; Cocco, F.; Carta, G.; Cagetti, M.G.; Simark-Mattson, C.; Strohmenger, L.;Lingström, P. Effect of a daily dose of *Lactobacillus brevis* CD2 lozenges in high caries risk school children. *Clin.Oral Investig*.**2014**, *18*, 555–561,

85. Fujimura, K.E.; Demoor, T.; Rauch, M.; Faruqi, A.A.; Jang, S.; Johnson, C.C.; Boushey, H.A.;Zoratti, E.; Ownby, D.; Lukacs, N.W.; *et al.* House dust exposure mediates gut microbiome *Lactobacillus* enrichment and airway immune defense against allergens and virus infection. *Proc.Natl. Acad. Sci. USA* **2014**, *111*, 805–810.

86. Hsieh, P.S.; Tsai, Y.C.; Chen, Y.C.; The, S.F.; Ou, C.M.; King, V.A. Eradication of *Helicobacterpylori* infection by the probiotic strains *Lactobacillus johnsonii*MH-68 and *L. salivarius*ssp.*salicinius*AP-32. *Helicobacter* **2012**, *17*, 466–477.

87. Zhang, Y.C.; Zhang, L.W.; Ma, W.; Yi, H.X.; Yang, X.; Du, M.; Shan, Y.J.; Han, X.; Zhang, L.L.Screening of probiotic *Lactobacilli* for inhibition of

Shigellasonnei and the macromolecules involved in inhibition. *Anaerobe* **2012**, *18*, 498–503.

88. Lue, K.H.; Sun, H.L.; Lu, K.H.; Ku, M.S.; Sheu, J.N.; Chan, C.H.; Wang, Y.H. A trial of adding*Lactobacillusjohnsonii*EM1 to levocetirizine for treatment of perennial allergic rhinitis in children aged 7–12 years. *Int. J. Pediatr. Otorhinolaryngol.***2012**, *76*, 994–1001.

89. Ortiz-Lucas, M.; Tobías, A.; Saz, P.; Sebastián, J.J. Effect of probiotic species on irritable bowel syndrome symptoms: A bring up to date meta-analysis. *Rev. Esp. Enferm. Dig*. **2013**, *105*, 19–36.

90. Chen, P.W.; Jheng, T.T.; Shyu, C.L.; Mao, F.C. Synergistic antibacterial efficacies of the combination of bovine lactoferrin or its hydrolysate with probiotic secretion in curbing the growth of meticillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol*. **2013**, *62*, 1845–1851.

91. Tomaro-Duchesneau, C.; Saha, S.; Malhotra, M.; Jones, M.L.; Labbé, A.; Rodes, L.; Kahouli, I.;Prakash, S. Effect of orally administered *L. fermentum* NCIMB 5221 on markers of metabolic syndrome: An *in vivo* analysis using ZDF rats. *Appl. Microbiol. Biotechnol.***2014**, *98*, 115–126.

92. Szajewska, H.; Urbańska, M.; Chmielewska, A.; Weizman, Z.; Shamir, R. Meta-analysis: *Lactobacillus reuteri* strain DSM 17938 (and the original strain ATCC 55730) for treating acute gastroenteritis in children. *Benef.Microbes*.**2014**, doi:10.3920/BM2013.0056.

93. Anabrees, J.; Indrio, F.; Paes, B.; AlFaleh, K. Probiotics for infantile colic: A systematic review.*BMC Pediatr*.**2013**, *13*, 186, doi:10.1186/1471-2431-13-186.

94. Indrio, F.; Di Mauro, A.; Riezzo, G.; Civardi, E.; Intini, C.; Corvaglia, L.; Ballardini, E.;Bisceglia, M.; Cinquetti, M.; Brazzoduro, E.; *et al.* Prophylactic

use of a probiotic in the prevention of colic, regurgitation, and functional constipation: A randomized clinical trial. *JAMAPediatr*.**2014**, 168, 228–233.

95. Oncel, M.Y.; Sari, F.N.; Arayici, S.; Guzoglu, N.; Erdeve, O.; Uras, N.; Oguz, S.S.; Dilmen, U. *Lactobacillus reuteri* for the prevention of necrotising entero-colitis in very low birthweight infants: A randomised controlled trial. *Arch. Dis. Child. Fetal Neonatal.Ed*.**2013**, *99*,F110–F115.

96. Jacobs, S.E.; Tobin, J.M.; Opie, G.F.; Donath, S.; Tabrizi, S.N.; Pirotta, M.; Morley, C.J.;Garland, S.M; ProPrems Study Group. Probiotic effects on late-onset sepsis in very preterminfants: A randomized controlled trial. *Pediatrics*.**2013**, *132*, 1055–1062.

97. Li, D.; Rosito, G.; Slagle, T. Probiotics for the prevention of necrotizing enterocolitis in neonates:An 8-year retrospective cohort study. *J. Clin. Pharm. Ther*. **2013**, *38*, 445–449.

98. Janvier, A.; Malo, J.; Barrington, K.J. Cohort study of probiotics in a North American neonatalintensive care unit. *J. Pediatr*. **2014**, doi:10.1016/j.jpeds.2013.11.025.

99. Pinto, G.S.; Cenci, M.S.; Azevedo, M.S.; Epifanio, M.; Jones, M.H. Effect of yogurt Containing *Bifidobacterium animalis* subsp. *lactis* DN-173010 probiotic on dental plaque and saliva inorthodontic patients. *Caries Res*. **2014**, *48*, 63–68.

100. Bordoni, A.; Amaretti, A.; Leonardi, A.; Boschetti, E.; Danesi, F.; Matteuzzi, D.; Roncaglia, L.;Raimondi, S.; Rossi, M. Cholesterol-lowering probiotics: *In vitro* selection and *in vivo* testing of bifido bacteria. *Appl. Microbiol. Biotechnol*.**2013**, *9*, 8273–8281.

101. West, N.P.; Horn, P.L.; Pyne, D.B.; Gebski, V.J.; Lahtinen, S.J.; Fricker, P.A.; Cripps, A.W.Probiotic supplementation for respiratory and gastrointestinal

illness symptoms in healthy physically active individuals. *Clin.Nutr*.**2013**, doi:10.1016/j.clnu.2013.10.002.

102. Yu, H.; Liu, L.; Chang, Z.; Wang, S.; Wen, B.; Yin, P.; Liu, D.; Chen, B.; Zhang, J. Genome sequence of the bacterium *Bifidobacterium longum* strain CMCC P0001, a probiotic strain used for treating gastrointestinal disease. *Genome Announc*.**2013**, doi:10.1128/genomeA.00716-13.

103. Schwarzer, M.; Srutkova, D.; Schabussova, I.; Hudcovic, T.; Akgün, J.; Wiedermann, U.;Kozakova, H. Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to major birch pollen allergen Bet v 1. *Vaccine* **2013**, *31*, 5405–5412.

104. Lee, J.S.; Chung, M.J.; Seo, J.G. *In vitro* evaluation of antimicrobial activity of lactic acidbacteria against *Clostridium difficile*.*Toxicol. Res*. **2013**, 29, 99–106.

105. Fernandez, B.; Hammami, R.; Savard, P.; Jean, J.; Fliss, I. *Pediococcusacidilactici* UL5 and *Lactococcuslactis* ATCC 11454 are able to survive and express their bacteriocin genes undersimulated gastrointestinal conditions. *J. Appl. Microbiol.* **2013**, doi: 10.1111/jam.12391.

106. Choi, C.H.; Jo, S.Y.; Park, H.J.; Chang, S.K.; Byeon, J.S.; Myung, S.J. A randomized, double-blind, placebo-controlled multi-center trial of *Saccharomyces boulardii*in irritable bowel syndrome: Effect on quality of life. *J. Clin. Gastroenterol*.**2011**, *45*, 679–683.

107. Hempel, S.; Newberry, S.J.; Maher, A.R.; Wang, Z.; Miles, J.N.; Shanman, R.; Johnsen, B.; Shekelle, P.G. Probiotics for the prevention and treatment of antibiotic- associated diarrhea: A systematic review and meta-analysis. JAMA 2012, 307, 1959–1969.

108. Cao, G.T.; Zeng, X.F.; Chen, A.G.; Zhou, L.; Zhang, L.; Xiao, Y.P.; Yang, C.M. Effects of a probiotic, Enterococcus faecium, on growth performance, intestinal morphology, immune response, and cecal microflora in broiler chickens challenged with Escherichia coli K88. Poult. Sci. 2013, 92, 2949–2955.
109. Larsen, N.; Thorsen, L.; Kpikpi, E.N.; Stuer-Lauridsen, B.; Cantor, M.D.; Nielsen, B.; Brockmann, E.; Derkx, P.M.; Jespersen, L. Characterization of Bacillus spp. strains for use as probiotic additives in pig feed. Appl. Microbiol. Biotechnol. 2013, doi:10.1007/s00253-013-5343-6.
110. Tompkins, T.A.; Xu, X.; Ahmarani, J.A. comprehensive review of post-market clinical studies performed in adults with an Asian probiotic formulation. Benef. Microbes. 2010, 1, 93–106.
111. IS 1479 (Part II) – 1961 (Reaffirmed 1997) Methods of test for Dairy Industry - Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi).
112. IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi.
113. Manual Methods of Analysis for Adulterants & Contaminants in Foods. I.C.M.R 1990, page 27
114. IS.1479 (Part I) – 1960 (Reaffirmed 2003) Methods of test for Dairy Industry, Part – I Rapid examination of Milk.Bureau of Indian Standards, New Delhi.
115. Journal of Food Science and Technology, Vol 22 (1985) page 207-208
116. *FSSAI;* Manual of methods of Analysis of foods *Milk and milk products* Food Safety and Standards Authority of India, Ministry of Health and Family Welfare, Government of India, New Delhi; 2015
117. *A.O.A.C, 17th edn, 2000 Official method 905.02 Fat in milk*

118. Modified IS 12333 – 1997 / I.S.O 6731-1989 Milk, Cream and Evaporated Milk -Determination of Total Solid Content – Reference method. Bureau of Indian Standards, New Delhi; Pearson's Composition and Analysis of Foods, 9thedn. Page 570

119. IDF (2005) IDF Standard 151, Yoghurt. Determination of total solid content, International DairyFederation: Brussels.

120. Crobach MJT, Planche T, Eckert C, et al. European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection. Clin Microbiol Infect 2016;22(Suppl 4):S63–S81

121. McDonald LC, Gerding DN, Johnson S, et al. Clinical practice guidelines for Clostridium difficile infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis 2018;66(07):e1–e48

122. Burnham CAD, Carroll KC. Diagnosis of Clostridium difficile infection: an ongoing conundrum for clinicians and for clinical laboratories. Clin Microbiol Rev 2013;26(03):604–630.

123. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB. Antibiotic-associated pseudomembranous colitis due to toxinproducing clostridia. N Engl J Med 1978;298(10):531–534

124. Chang TW, Lauermann M, Bartlett JG. Cytotoxicity assay in antibiotic-associated colitis. J Infect Dis 1979;140(05):765–770.

125. Chang TW, Lauermann M, Bartlett JG. Cytotoxicity assay in antibiotic-associated colitis. J Infect Dis 1979;140(05):765–770

126. Tichota-Lee J, Jaqua-Stewart MJ, Benfield D, Simmons JL, Jaqua RA. Effect of age on the sensitivity of cell cultures to Clostridium difficile toxin. Diagn Microbiol Infect Dis 1987;8(04):203–214

127. Fang FC, Polage CR, Wilcox MH. Point-counterpoint: what is the optimal approach for detection of Clostridium difficile infection? J Clin Microbiol 2017;55(03):670–680

128. Planche TD, Davies KA, Coen PG, et al. Differences in outcome according to Clostridium difficile testing method: a prospective multicentre diagnostic validation study of C difficile infection. Lancet Infect Dis 2013;13 (11):936–945.

129. Riegler M, Sedivy R, Pothoulakis C, Hamilton G, Zacherl J, Bischof G, et al. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. *J Clin Invest.* 1995;95:2004–11.

130. PeriniWilson KH, Perini F. Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. *Infect Immun.* 1988;56:2610–4.

131. Mazkour, S.; Shekarforoush, S.S.; Basiri, S. The effects of supplementation of Bacillus subtilis and Bacillus coagulans spores on the intestinal microflora and growth performance in rat. Iran. J. Microbiol. 2019, 11, 260–266.

132. Tomasello, G.; Tralongo, P.; Damiani, P.; Sinagra, E.; Di Trapani, B.; Zeenny, M.N.; Hussein, I.H.; Jurjus, A.; Leone, A. Dismicrobism in inflammatory bowel disease and colorectal, cancer: Changes in response of colocytes. World J. Gastroenterol. 2014, 20, 18121–18130.

133. Al-Qayim, A.J.M.; Ebraheem Abass, D. Effects of probiotics (Lactobacillus acidophilus) on some intestinal physiological aspects in experimental colitis in rats. Appl. Sci. Rep. 2015, 9, 27–33.

134. Al-Qayim, A.J.M.; Ebraheem Abass, D. Effects of probiotics (Lactobacillus acidophilus) on some intestinal physiological aspects in experimental colitis in rats. Appl. Sci. Rep. 2015, 9, 27–33

135. Dheer, R.; Santaolalla, R.; Davies, J.M.; Lang, J.K.; Phillips, M.C.; Pastorini, C.; Vazquez-Pertejo, M.T.; Abreu, M.T. Intestinal epithelial Toll-like receptor 4 signaling affects epithelial function and colonic microbiota and promotes a risk for transmissible colitis. Infect. Immun. 2016, 84, 798–810.

136. Catinean, A.; Neag, M.A.; Krishnan, K.; Muntean, D.M.; Bocsan, C.I.; Pop, R.M.; Mitre, A.O.; Stanca Melincovici, C.; Buzoianu, A.D. Probiotic Bacillus Spores Together with Amino Acids and Immunoglobulins Exert Protective Effects on a Rat Model of Ulcerative Colitis. Nutrients 2020, 12, 3607.

137. [Schmitz, S.; Suchodolski, J. Understanding the canine intestinal microbiota and its modification by pro-, pre- and synbioticswhat is the evidence? J. Vet. Med. Sci. 2016, 2, 71–94.

138. Penaloza-Vazquez, A.; Li, M.M.; Rayas-Duarte, P. Isolation and characterization of Bacillus spp. strains as potential probiotics for poultry. Can. J. Microbiol. 2019, 65, 762–774.

139. Mainville, I.; Arcand, Y.; Farnworth, E. A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. Int. J. Food Microbiol. 2005, 99, 287–296.

140. Matei-Lat,iu, M.C.; Buza, V.; Chirilă, F.; Boros, Z.; Lat,iu, C.; Szakacs, A.R.; S, tefãnut, , L.C. In Vitro Qualitative Assessment of Tolerance to Simulated

Gastric Juice, Bile, Fructose, Glucose and Lactose for Different Probiotic Bacteria. Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Vet. Med. 2022, 79, 71–76.

141. Bagci, U.; Sine, O.T.; Ayhan, T.; Mustafa, A. Probiotic characteristics of bacteriocin-producing Enterococcus faecium strains isolated from human milk and colostrum. Folia Microbiol. 2019, 64, 735–750.

142. Nithya, V.; Halami, P.M. Evaluation of the probiotic characteristics of Bacillus species isolated from different food sources. Ann. Microbiol. 2013, 63, 129–137.

143. Ichikawa, H.; Kuroiwa, T.; Inagaki, A.; Shineha, R.; Nishihira, T.; Satomi, S.; Sakata, T. Probiotic bacteria stimulate gut epithelial cell proliferation in rat. Dig. Dis. Sci. 1999, 44, 2119–2123.

144. Cario, E.; Podolsky, D.K. Differential Alteration in Intestinal Epithelial Cell Expression of Toll-Like Receptor 3 (TLR3) and TLR4 in Inflammatory Bowel Disease. Infect. Immun. 2000, 68, 7010–7017. [CrossRef]

145. Ahirrao HA, Sangoram AM. Bilva (Aegle marmelos L.Correa) - A Review from Ancient literature. World Journal of Pharmacy and Pharmaceutical Sciences. 2017; 6(3):501-522.

146. Chopra R. Indigenous drugs of India. Academic Publishers. Calcutta. 1982.

147. Nadkarni AK Indian Materia Medica, Popular Prakashan, Mumbai 1954, pp 1-3.

148. Satyavati GV, Gupta AK, Tandon N. Medicinal Plants of India, Indian Council of Medical Research, New Delhi 1976.

149. Shoba FG, Thomas M. Study of antidiarrhoeal activity of four medicinal plants in castor-oil induced diarrhoea. J. Ethnopharmacol, 2001; 76:73-76.

150. Jagtap AG, Shirke SS, Phadke AS. Effect of a polyherbal formulation on experimental models of inflammatory bowel diseases. J. Ethnopharmacol. 2004; 90:195-204.

151. Dhuley JN. Investigation on the gastroprotective and antidiarrhoeal properties of *Aegle marmelos* unripe fruit extract. Hind Antibiot Bull. 2003; 45-46:41-46.

152. Roy SK, Saran S, Kitinoja L. Bael [*Aegle marmelos* (L.) Corr. Serr.] In Postharvest Biology and Technology of Tropical and Subtropical Fruits, Woodhead Publishing, Sawston, UK 2011, pp 186–216.

153. Patkar AN, Desai NV, Ranage AA, Kalekar AS. A review on *Aegle marmelos*: A potential medicinal tree. International Research Journal of Pharmacy. 2012; 3(8):86-91

154. Sharma N, Radha KM, Zhang B, et al. *Aegle marmelos* (L.) Correa: An Underutilized Fruit with High Nutraceutical Values: A Review. Int. J. Mol. Sci. 2022; 23(10889):2-18.

155. Kaur A, Kalia M. Physico chemical analysis of bael (*Aegle Marmelos*) fruit pulp, seed and pericarp. Chem. Sci. Rev. Lett. 2017; 6:1213–1218.

156. Singh R. Characteristics and technology of traditional and cultural dairy products. Bulletin of the Dairy International Federation. 2007; 415:11-20.

157. Shekhar S, Joe J, Kumar R, et al. Heat treatment of milk on the sensory and rheological quality of dahi prepared from cow milk. Research and Reviews. J. of Food and Dairy Technol. 2013; 1(1):8-14.

158. Nyanzi R, Jooste PJ, Buys EM. Invited review: Probiotic yogurt quality criteria, regulatory framework, clinical evidence, and analytical aspects. Journal of Dairy Science. 2021; 104(1):1-19.

159. Anonymous. ICMR-DBT Guidelines for Evaluation of Probiotics in Food. Indian J Med Res. 2011; 134:22-25.

160. Paneliya AM, Patgiri B, Galib R, Bedarkar P, Prajapati PK. Pharmaceutical development of granules of Vasa Avaleha. Ann Ayurvedic Med. 2013; 2:16-21.

161. Khemuka N, Galib R, Patgiri BJ, Prajapati PK. Pharmaceutical standardization of Kamsaharitaki granules. Ayu. 2015; 36:416-20.

162. Balamurugan R, Chandragunasekaran AS, Chellappan G, Rajaram K, Ramamoorthi G, Ramakrishna BS. Probiotic potential of lactic acid bacteria present in homemade curdin southern India. Indian Journal of Medical Research. 2014; 140(3):345-355.

163. https://www.nin.res.in/downloads/DietaryGuidelinesforNINwebsite.pdf

164. Nireesha G, Divya L, Sowmya C, Divya L. Venkatesan N, Lavakumar V. Lyophilization/freeze drying-a review. Int J Nov Trends Pharm Sci 2013; 3:87-98.

165. Gaidhani KA, Harwalkar M, Bhambere D, Nirgude PS. Lyophilization / Freeze Drying – A Review. World Journal of Pharmaceutical Research. 2015; 4(8):516-143.

166. Coskun ZK, Kerem M, Gurbuz N, Omeroglu H, Demirtas C, Lortlar N, Salman B, Pasaoglu OT, Turgut HB.2011. "the study of biochemical and histological effects of spirulina in rats with TNB-induced colitis " Bratisl. Lek. Listy.,2011, 112, 5, 235-243.

167. Kuo SM, Merhige PM, Hagey LR.2013. The Effect of Dietary Prebiotics and Probiotics on Body Weight, Large Intestine Indices, and Fecal Bile Acid Profile in Wild Type and IL10−/− Mice. PLOS|ONE, 2013, 21.

168. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, et al.2009. Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am. J. Physiol. Gastro-intest. Liver Physiol.,2009, 296, G1140–G9.

169. (A.J. al-Qayim M, Ebraheem Abass D. 2015. Effects of probiotics (Lactobacillus acidophilus) on some intestinal physiological aspects in experimental colitis in rats. Applied Science Reports, 9 (1), 27-33.)

170. (Marteau PR, de Vrese M, Cellier CJ, Schrezenmeir J.2001. Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr, 2001, 73, 2 , 430S-436S.).

171. (Heyman M, Ménard S.2002. Probiotic microorganisms: how they affect intestinal Pathophysiology., C.M.L.S. Cell. Mol. Life Sci.J., 2002, 59, 1001–1015.)

172. (Sood A, Midha V, Makharia GK, Ahuja V, Singal D, et al.2009. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. Clin. Gastroenterol. Hepatol.,2009, 7, 1202–1209.).

173. (Madsen K, Cornish A, Soper P, Mckaigney C, Jijon H, Yachimec C, Doyle J, Jewell L, de Simone C.2001. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology, 2001, 121, 580–591.).

174. (Perez Chaia A, Zárate G.2005. Dairy propionibacteria from milk or cheese diets remain viable and enhance propionic acid production in the mouse caecum. Le. Lait. J,2005, 85 , 1–2 85–98.)

175. (Barbara G, Stanghellini V, Brandi G, Cremon C, Di Nardo G, Giorgio R, Corinaldesi R.2005.Interactions between commensal bacteria and gut

sensorimotor function inhealth and disease. Am. J. Gastroenterol, 2005, 100, 11, 2560-2568);

176. (Delcenserie V, Martel M, Lamoureux D, Amiot J, Boutin Y, Roy D.2009. Immunomodulatory Effects of Probiotics in the Intestinal Tract. Curr. Issues Mol. Biol.,2009, 10, 37-54.).
177. (Castillo M, Martin-Orue SM, Taylor-Pickard JA, Perez JF, Gasa J.2008. Use of mannan-oligosacharides and zinc chelate as growth promoters and diarrhea pre-ventative in weaning pigs: Effects on microbiota and gut function. Journal of Animal Science,2008, 86, 94–101.).
178. (Iji PA, Saki AA, Tivey DR.2001. International structure and function of broil chickens on diets supplemented with a mannan oligosaccharide. J. Sci. Food Agric.,2001, 81, 1186-1192.).
179. (Caspary WF.1992. Physiology and pathophysiology of intestinal absorption. American Journal of Clinical Nutrition,1992, 55, 299 .).
180. (Willing BP, Van Kessel AG. 2007. Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria,New Advances inthe Basic and Clinical Gastroenterology74, in the neonatal gnotobiotic pig. Journal Of Animal Science,2007, 85, 12, 3256-66)
181. (Awad WA, Ghareeb K, Abdel-Raheem S, Böhm J.2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci., 2009, 88,1, .49-56).
182. Elia SCC, Souza HSP.2001. Imunologia da mucosa intestinal: da bancada ao leito. Editora. Atheneu., Rio de Janeiro,2001, 1-147.) .

183. (Chiou PWS, Lu TW, Hsu JC, Yu B.1996. Effect of different sources of fiber on the intestinal morphology of domestic geese. Asian Australas. Journal of Animal Science, 1996, 4, 539–550 .) .

184. (Chiou PWS, Lu TW, Hsu JC, Yu B.1996. Effect of different sources of fiber on the intestinal morphology of domestic geese. Asian Australas. Journal of Animal Science, 1996, 4, 539–550 .) .

185. Gauffin CP, Agüero G, Perdigon G.2002. Adjuvant effects of Lactobacillus casei added to a renutrition diet in a malnourished mouse model. Biocell, 2002, 26, 1, 35-48

186. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, et al.2009. Probiotic mixture VSL#3 protects the epithelial barrier bymaintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am. J. Physiol. Gastrointest. Liver Physiol.,2009, 296, G1140–G9.

187. Lee YK, Phuong KY, Ouwehand AC, Salminen S.2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J. Med. Microbiol., 2003, 52, 925–930.

188. Ng SC, MRCP AL, Hart Ph.D, Kamm MA, et al.2009. Mechanisms of Action of Probiotics: Recent Advances.,2009, 15, 163-310.

189. 1 Wiström J, Norrby SR, Myhre EB, Eriksonn S, Grandström G, Lagergren L, et al. Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. J Antimicrob Chemother 2001;47:43-50.

190. McFarland LV. Epidemiology, risk factors and treatments for antibioticassociated diarrhea. Dig Dis 1998;16:292-307.

191. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB. Antibiotic-associated pseudomembranous colitis due to toxin producing Clostridia. N Engl J Med 1978;298:531-4

192. Wiström J, Norrby SR, Myhre EB, Eriksonn S, Grandström G, Lagergren L, et al. Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. J Antimicrob Chemother 2001;47:43-50

193. Barbut F, Meynard JL, Guiguet M, Avesani V, Bochet MV, Meyohas MC, et al. Clostridium difficile-associated diarrhea in HIV infected patients: epidemiology and risk factors. J Acq Immun Def Synd 1997;16:176-81.

194. McFarland LV, Surawicz CM, Stamm WE. Risk factors for Clostridium difficile carriage and C. difficile-associated diarrhea in a cohort of hospitalized patients. J Infect Dis 1990;162:678-84

195. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB. Antibiotic-associated pseudomembranous colitis due to toxin producing Clostridia. N Engl J Med 1978;298:531-4

196. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB. Antibiotic-associated pseudomembranous colitis due to toxin producing Clostridia. N Engl J Med 1978;298:531-4

197. Teasley DG, Gerding DN, Olson MM, Peterson LR, Gebhard RL, Schwartz MJ, et al. Prospective randomized trial of metronidazole vs vancomycin for Clostridium difficile-associated diarrhea and colitis. Lancet 1983;2:1043-6.

198. Marteau PR, de Vrese M, Cellier CJ, Schrezenmeir J. Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nut 2001;73 (suppl):4306-S.

199. Wilcox MH, Cunniffe JG, Trundle C, et al. Financial burden of hospital-acquired Clostridium difficile infection. J Hosp Infect 1996; 34:23–30.

200. Walk ST, Young VB. Emerging insights into antibiotic-associated diarrhea and Clostridium difficile infection through the lens of microbial ecology. Interdiscip Perspect Infect Dis 2008; 2008:125081.

201. Goldenberg JZ, Yap C, Lytvyn L, et al. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. Cochrane Database Syst Rev 2017;382

202. Hurley BW, Nguyen CC. The spectrum of pseudomembranous enterocolitis and antibiotic-associated diarrhea. Arch Intern Med 2002; 162: 2177.

203. Horosheva T, Sorokulova I, Vodyanoy V. Efficacy of Bacillus probiotics in prevention of antibiotic-associated diarrhoea: a randomized, double-blind, placebo-controlled clinical trial. JMM Case Reports;1:1–6

204. Goldenberg JZ, Yap C, Lytvyn L, et al. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. Cochrane Database Syst Rev 2017;382

205. Goldenberg JZ, Yap C, Lytvyn L, et al. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. Cochrane Database Syst Rev 2017;382

206. Barko, P.C.; Michael, M.A.; Swanson, K.S.; Williams, D. A The gastrointestinal microbiome: A review. J. Vet. Intern. Med. 2018, 32, 9–25.

207. Barko, P.C.; Michael, M.A.; Swanson, K.S.; Williams, D. A. The gastrointestinal microbiome: A review. J. Vet. Intern. Med. 2018, 32, 9–25.

208. Desbonnet, L.; Garrett, L.; Clarke, G.; Bienenstock, J.; Dinan, T.G. The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. J. Psychiatr. Res. 2009, 43, 164–174.

209. Graversen, K.B.; Bahl, M.I.; Larsen, J.M.; Ballegaard, A.-S.R.; Licht, T.R.; Bøgh, K.L. Short-Term Amoxicillin-Induced Perturbation of the Gut Microbiota Promotes Acute Intestinal Immune Regulation in Brown Norway Rats. Front. Microbiol. 2020, 11, 496.

210. Graversen, K.B.; Bahl, M.I.; Larsen, J.M.; Ballegaard, A.-S.R.; Licht, T.R.; Bøgh, K.L. Short-Term Amoxicillin-Induced Perturbation of the Gut Microbiota Promotes Acute Intestinal Immune Regulation in Brown Norway Rats. Front. Microbiol. 2020, 11, 496.

211. Cario, E.; Podolsky, D.K. Differential Alteration in Intestinal Epithelial Cell Expression of Toll-Like Receptor 3 (TLR3) and TLR4 in Inflammatory Bowel Disease. Infect. Immun. 2000, 68, 7010–7017.

212. Graversen, K.B.; Bahl, M.I.; Larsen, J.M.; Ballegaard, A.-S.R.; Licht, T.R.; Bøgh, K.L. Short-Term Amoxicillin-Induced Perturbation of the Gut Microbiota Promotes Acute Intestinal Immune Regulation in Brown Norway Rats. Front. Microbiol. 2020, 11, 496.

213. Wang, Y.; Wang, C.; Huang, J.; Xie, M.; Li, X.; Fu, L. Butyricicoccus plays a key role in mediating the antagonism between probiotic and antibiotic on food allergy. Food Agric. Immunol. 2019, 30, 446–461.

214. Dieleman, L.A.; Goerres, M.S.; Arends, A.; Sprengers, D.; Torrice, C.; Hoentjen, F.; Grenther, W.B.; Sartor, R.B. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. Gut 2003, 52, 370–376.

215. Konieczna, P.; Groeger, D.; Ziegler, M.; Frei, R.; Ferstl, R.; Shanahan, F.; Quigley, E.M.; Kiely, B.; Akdis, C.A.; O'Mahony, L. Bifidobacterium infantis 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: Potential role for myeloid and plasmacytoid dendritic cells. Gut 2012, 61, 354–366.

216. Chaiyawan, N.; Taveeteptaikul, P.; Wannissorn, B.; Ruengsomwong, S.; Klungsupya, P.; Buaban, W.; Itsaranuwat, P. Characterization and probiotic properties of Bacillus strains isolated from broiler. Thai J. Vet. Med. 2010, 40, 207–214. 31.

217. Simon, O.; Vahjen, W.; Scharek, L. Micro-organisms as feed additives-probiotics. Adv. Pork Prod. 2005, 16, 161–167. 32. Hong, H.A.; Duc, L.H.; Cutting, S.M. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 2005, 29, 813–835.

218. Schultz, M.; Sartor, R.B. Probiotics and inflammatory bowel diseases. Am. J. Gastroenterol. 2000, 95, S19–S21.

219. Madsen, K.L.; Doyle, J.S.; Jewell, L.D.; Tavernini, M.M.; Fedorak, R.N. Lactobacillus species prevents colitis in interleukin 10 gene–deficient mice. Gastroenterology 1999, 116, 1107–1114.

ANNEXURES

SHRI B.M.K. AYURVEDA MAHAVIDYALAYA
A constituent unit KLE Academy of Higher Education & Research
Deemed–to-be-University
Central Research Facility
DRUG AUTHENTICATION REPORT

Submitted By : Dr. Sanjeev Tonni

Submitted Date : 20/07/2018

Date of Issue :21/07/2018

Sl.	Sample Name	Scientific Name	Family	Part submitted	CRF Code	Authenticated as				
						Ayurvedic Name	Scientific Name	Family	Part Authenticated	Remarks
01.	Bilwa	*Aegle marmelos* (L.) Corrêa	Rutaceae	Unripe Fruit	CRF/Auth /2018/113	Bilwa	*Aegle marmelos* (L.) Corrêa	Rutaceae	Unripe Fruit	Authenticated

Signature:

Authentication Expert Name : Mr. Ajit Lingayat

Date:21/07/2018

Signature of Coordinator
ASU Drug Testing Laboratory

Office of the Chief Food Analys
Divisional Food Laboratory
Belgaum Division, Vaccine
Institute Compound,
Belagavi-590006.

No: DFL/BGM/NFSSA/2901-2902/2018 **Date: 28/07/2018**

To
Dr.Sanjeev S Tonni MD(Ayu)
Reader & Ph.D Scholar,
Dept of Swasthavritta
KAHER Shri.B.M.Kankanawadi
Ayurveda Mahavidyalaya,
Shahapur, Belagavi.

Sub: Analysis report of food Sample– regarding.

Ref No: Nil, dated: 20/07/2018

Received samples of Cow Milk & Curd for analysis on 21/07/2018.

2901. Cow Milk

Sl. No	Quality Characteristics	Name of the Method of the test used	Result	Prescribed Standards as per FSS (FOOD PRODUCTS STANDARDS & FOOD ADDITIVES) Rules-2011
1	Test for formalin	FSSAI	Detected	May be present
2	Milk Fat	FSSAI	3.8 %	Not less than 3.2 %
3	Milk Solids not fat	FSSAI	10.08 %	Not less than 8.3 %
4	Test for starch	FSSAI	Not Detected	Shall not be present
5	Test for Sugar	FSSAI	Not Detected	Shall not be present
6	Test for urea	FSSAI	Not Detected	Shall not be present
7	Test for carbonates	FSSAI	Not Detected	Shall not be present
8	Test for H_2O_2	FSSAI	Not Detected	Shall not be present
9	Test for Detergents	FSSAI	Not Detected	Shall not be present

Opinion: The Sample conforms to the Standards.

2902. Curd [Prepared from Cow Milk]

Sl. No	Quality Characteristics	Name of the Method of the test used	Result	Prescribed Standards as per FSS (FOOD PRODUCTS STANDARDS & FOOD ADDITIVES) Rules-2011
1	Test for formalin	FSSAI	Detected	May be present
2	Milk fat	FSSAI	3.2 %	Not less than 3.2 %
3	Milk solid not fat	FSSAI	10.34 %	Not less than 8.3 %
4	Test for Sugar	FSSAI	Not detected	Shall be negative
5	Test for Starch	FSSAI	Not detected	Shall be negative
6	Test for urea	FSSAI	Not detected	Shall be negative
7	Test for carbonate	FSSAI	Not detected	Shall be negative
8	Test for detergents	FSSAI	Not detected	Shall be negative
9	Test for Hydrogen peroxide	FSSAI	Not detected	Shall be negative

Opinion: The Sample conforms to the Standards.

Chief Food Analyst

CHIEF FOOD ANALYST
Divisional Food Laboratory
Belagavi Division, Belagavi

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA

A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)

CENTRAL RESEARCH FACILITY

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/212/18-19
Submited by :Dr. Sanjeev Tonni
Sample : Bilwa Majja
Product : PLANT

Batch No. : NA
Sample Qty :50gm

Registration Dt.:31/07/2018
Requstion no: --
Part/Form :Powder
Report Date : 14/08/2018

TEST REPORT

Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

TESTS	WATER EXTRACT	ALCOHOL EXTRACT
Test for Carbohydrates	Positive	Positive
Test for Reducing sugar	Positive	Positive
Test for Monosaccharides	Positive	Negative
Test for Pentose Sugar	Positive	Positive
Test for Hexose Sugar	Negative	Negative
Test for Non-Reducing sugar	Positive	Positive
Test for Protiens	Positive	Positive
Test for Amino Acids	Positive	Positive
Test for Steroids	Positive	Negative
Test for Flavonoids	Positive	Positive
Test for Alkaloids	Negative	Positive
Test for Tannins	Positive	Positive
Test for Glycosides:		
A.Cardiac Glycosides	Positive	Positive
B.Anthraquinone glycosides	Negative	Positive
C.Saponin glycosides	Positive	---

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA
A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)
CENTRAL RESEARCH FACILITY
(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/25/2020-21
Submitted by:Dr.Sanjeev Tonni
Sample : Plain curd-Lyophilized sample-A
Ref : Prop
(* N/A - Not Available)

Batch No.: NA
Sample Qty: 25gm
Mfg Date: NA

Registration Dt:-16/01/2021
Requisition no :---
Part/Form: Powder
Report Date: 27/01/2021
Exp. Date: NA

TEST REPORT

Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

Description Macroscopic :

TESTS	RESULTS
FORM	: Powder
COLOUR	: White
TASTE	: Sour
ODOUR	: Milky Odour

Physico Chemical Standards :

TESTS	RESULTS
Loss on Drying at 105 C	:11.706 %
Ash Value	:5.732 %
Acid insoluble Ash	:0.797 %
Water soluble extractive	:82.002 %
Alcohol soluble extractive	:53.302 %
pH Value (In 5% solution)	: 3.34

Note : **API** Standards are not Available.Given results are of the submitted sample.

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA

A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)

CENTRAL RESEARCH FACILITY

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/25/2020-21
Submitted by:Dr.Sanjeev Tonni
Sample :Plane curd -Lyophilized sample A Batch No. : I
Ref : Prop
(* N/A - Not Available)

Sample Qty:25gm
Mfg Date: NA

Registration Dt:-16/01/2021
Requisition no :---
Part/Form :Powder
Report Date :27/01/2021
Exp. Date: NA

TEST REPORT

Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

Preliminary Phytochemical Screening:

TESTS	WATER	ALCOHOL
Test for Carbohydrates	Positive	Positive
Test for Reducing sugar	Positive	Negative
Test for Monosaccharides	Negative	Negative
Test for Pentose Sugar	Negative	Negative
Test for Non reducing sugar	Negative	Negative
Test for Hexose Sugar	Negative	Negative
Test for Proteins	Negative	Negative
Test for Amino Acids	Negative	Negative
Test for Steroids	Negative	Negative
Test for Flavonoids	Negative	Negative
Test for Alkaloids	Positive	Positive
Test for Tannins	Negative	Negative
Test for Glycosides:		
A.Cardiac Glycosides	Negative	Negative
B.Anthraquinone glycosides	Negative	Negative
C.Saponin glycosides	Negative	Negative

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA
A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)
CENTRAL RESEARCH FACILITY
(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/26/2020-21
Submitted by:Dr.Sanjeev Tonni
Sample : Plane curd -Lyophilized sample B Batch No.:NA
Ref : prop
(* N/A - Not Available)
Sample Qty:25gm
Mfg Date: NA

Registration Dt:-16/01/2021
Requisition no :---
Part/Form: Powder
Report Date: 27/01/2021
Exp. Date: NA

TEST REPORT

Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

Description Macroscopic :

TESTS	RESULTS
FORM	: Powder
COLOUR	: White
TASTE	: Sour
ODOUR	: Milky Odour

Physico Chemical Standards :

TESTS	RESULTS
Loss on Drying at 105 C	:8.391 %
Ash Value	:6.972 %
Acid insoluble Ash	:1.045 %
Water soluble extractive	:93.884 %
Alcohol soluble extractive	:44.479 %
PH Value (In 5% solution)	:3.74

Note : **API** Standards are not Available.Given results are of the submitted sample.

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA
A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)
CENTRAL RESEARCH FACILITY
(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/26/2020-21 | Registration Dt:-16/01/2021
Submitted by:Dr.Sanjeev Tonni | Requisition no :---
Sample : Plane curd -Lyophilized sample B | Batch No. :NA | Part/Form :Powder
Ref : Prop | Sample Qty:25gm | Report Date :27/01/2021
(* N/A - Not Available) | Mfg Date: NA | Exp. Date:NA

TEST REPORT

Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

Preliminary Phytochemical Screening:

TESTS	WATER	ALCOHOL
Test for Carbohydrates	Positive	Positive
Test for Reducing sugar	Negative	Negative
Test for Monosaccharides	Negative	Negative
Test for Pentose Sugar	Negative	Negative
Test for Non reducing sugar	Negative	Negative
Test for Hexose Sugar	Negative	Negative
Test for Protiens	Negative	Negative
Test for Amino Acids	Negative	Negative
Test for Steroids	Negative	Negative
Test for Flavonoids	Negative	Negative
Test for Alkaloids	Negative	Negative
Test for Tannins	Negative	Negative
Test for Glycosides:		
A.Cardiac Glycosides	Negative	Negative
B.Anthraquinone glycosides	Negative	Negative
C.Saponin glycosides	Negative	Negative

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA

A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)

(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)

CENTRAL RESEARCH FACILITY

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/27/2020-21 | Registration Dt:-16/01/2021
Submitted by:Dr.Sanjeev Tonni | Requisition no :---
Sample:Bilwa phalasaara dadhi sample C Batch No.:NA | Part/Form: Powder
Ref: Prop | Sample Qty: 25gm | Report Date: 27/01/2021
(* N/A - Not Available) | Mfg Date: NA | Exp. Date: NA

TEST REPORT

Form-50 [See Rule 160-D (f)]

(The Drugs & Cosmetic Act 1940 and the rules there under)

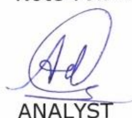
Description Macroscopic :

TESTS	RESULTS
FORM	: Powder
COLOUR	: Light brown
TASTE	: Sour & Bitter
ODOUR	: Characteristic

Physico Chemical Standards :

TESTS	RESULTS
Loss on Drying at 105 C	:8.449 %
Ash Value	:6.237 %
Acid insoluble Ash	:0.948 %
Water soluble extractive	:94.417 %
Alcohol soluble extractive	:45.772 %
PH Value (In 5% solution)	:4.03

Note : **API** Standards are not Available.Given results are of the submitted sample.



ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA

A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)

(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)

CENTRAL RESEARCH FACILITY

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/27/2020-21 | Registration Dt:-16/01/2021
Submitted by:Dr.Sanjeev Tonni | Requisition no :---
Sample :Bilwa phalasaara dadhi sample C | Batch No.:NA | Part/Form :Powder
Ref: Prop | Sample Qty:25gm | Report Date :27/01/2021
(* N/A - Not Available) | Mfg Date: NA | Exp. Date: NA

TEST REPORT

Form-50 [See Rule 160-D (f)]

(The Drugs & Cosmetic Act 1940 and the rules there under)

Preliminary Phytochemical Screening:

TESTS	WATER	ALCOHOL
Test for Carbohydrates	Positive	Positive
Test for Reducing sugar	Positive	Negative
Test for Monosaccharides	Negative	Negative
Test for Pentose Sugar	Negative	Negative
Test for Non reducing sugar	Negative	Negative
Test for Hexose Sugar	Negative	Negative
Test for Protiens	Positive	Negative
Test for Amino Acids	Negative	Negative
Test for Steroids	Negative	Negative
Test for Flavonoids	Negative	Negative
Test for Alkaloids	Negative	Negative
Test for Tannins	Positive	Positive
Test for Glycosides:		
A.Cardiac Glycosides	Negative	Negative
B.Anthraquinone glycosides	Negative	Negative
C.Saponin glycosides	Positive	Negative

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA
A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)

CENTRAL RESEARCH FACILITY

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/28/2020-21
Submitted by:Dr.Sanjeev Tonni
Sample:Bilwa phalasaara dadhi sample D
Ref : Prop
(* N/A - Not Available)

Batch No.:NA
Sample Qty: 25gm
Mfg Date: NA

Registration Dt:-16/01/2021
Requisition no :---
Part/Form: Powder
Report Date: 27/01/2021
Exp. Date: NA

TEST REPORT

Form-50 [See Rule 160-D (f)]

(The Drugs & Cosmetic Act 1940 and the rules there under)

Description Macroscopic :

TESTS	RESULTS
FORM	: Powder
COLOUR	: Light brown
TASTE	: Sour & Bitter
ODOUR	: Characteristic

Physico Chemical Standards :

TESTS	RESULTS
Loss on Drying at 105 C	:6.593 %
Ash Value	:5.932 %
Acid insoluble Ash	:0.598 %
Water soluble extractive	:55.980 %
Alcohol soluble extractive	:45.295 %
PH Value (In 5% solution)	: 4.34

Note : **API** Standards are not Available.Given results are of the submitted sample.

ANALYST

AUTHORISED SIGNATORY

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA
A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)
CENTRAL RESEARCH FACILITY
(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No:CRF/FG/28/2020-21
Submitted by:Dr.Sanjeev Tonni
Sample : Bilwa phalasaara dadhi sample D Batch No. :NA
Ref :Prop Sample Qty:25gm
(* N/A - Not Available) Mfg Date:NA

Registration Dt:-16/01/2021
Requisition no :---
Part/Form :Powder
Report Date :27/01/2020
Exp. Date:NA

TEST REPORT

Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

Preliminary Phytochemical Screening:

TESTS	WATER	ALCOHOL
Test for Carbohydrates	Positive	Positive
Test for Reducing sugar	Positive	Negative
Test for Monosaccharides	Negative	Negative
Test for Pentose Sugar	Negative	Negative
Test for Non reducing sugar	Negative	Negative
Test for Hexose Sugar	Negative	Negative
Test for Protiens	Positive	Positive
Test for Amino Acids	Positive	Positive
Test for Steroids	Negative	Negative
Test for Flavonoids	Negative	Negative
Test for Alkaloids	Negative	Negative
Test for Tannins	Positive	Positive
Test for Glycosides:		
A.Cardiac Glycosides	Negative	Negative
B.Anthraquinone glycosides	Positive	Negative
C.Saponin glycosides	Positive	Negative

ANALYST

AUTHORISED SIGNATORY

Ref.No. KAHER/BSRC/22-23/90 Date: 28/07/2022

Dr. Prabhakar Kore Basic Science Research Center, KLE Academy of Higher Education and Research

Report

Title of Research: **"Development of New Ayur-Probiotic formulation and its evaluation in experimental animals"**

Ph.D Scholar: Dr. Tonni Sanjeev

Guide: Dr. R. R. Hiremath

Objective Parameters:

1. Measurement of Colony count and culturing report

Laboratory Methods for assessing Antimicrobial susceptibility tests:

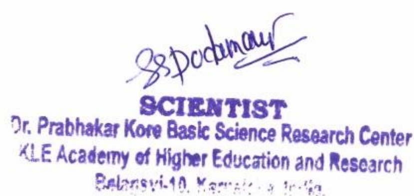
1. Bacterial Culture identification
2. Method used: Serial dilution techniques and Agar lawn method
3. Sample: Lyophilized probiotic sample

Lab Investigations done in BSRC

Sample	Concentration	Bacterial load [CFU/ml]	
		Raw sample	**Lyophilized Sample**
	100gm Curd + 3 gm [ABPM]	$5x10^3$ CFU/ml	$3x10^4$ CFU/ml

ABPM- ***Apakwa, Bilwa, Phala, Majja***

Reference: Bergeys manual of bacteriology, Microbiology Practical Manual by K R Aneja



SHRI BM KANKANAWADI AYURVED MAHAVIDYALAYA
Post Graduate Studies & Reseach Centre
(Approved by Central Council of Indial Medicine, New Delhi & M/o AYUSH, GoI)
A Constituent Unit of
KLE ACADEMY OF HIGHER EDUCATION & RESEARCH
(DEEMED-TO-BE- UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)

First AYUSH Institution having NAAC & NABH Accreditation

File No.25/369/2010-AWD Government of India Ministry of Environment & Forests & Climate Change (Animal Welfare Division)
1017/PO/Re/S/06/CPCSEA Dated: 24/11/2016

IMPARTING AYURVEDA EDUCATION SINCE 1933

Certificate

This is to certify that the project entitled:

"Development of a new Ayur-Probiotic formulation and its evaluation in experimental animals".

Submitted by: **Dr.Tonni Sanjeev Shivappa,** dated **6th August 2021**

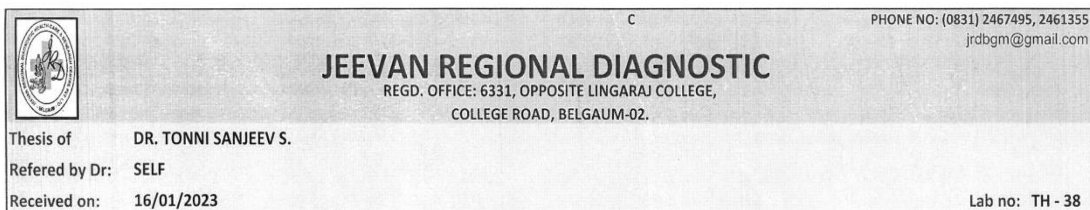
Has been approved by the IAEC having IAEC approval No **BMK/IAEC/Res.No.-21/2021- 09.**

Dr.Rudramma R. Hiremath
MEMBER SECRETARY
Institutional Animal Ethics Committee
KAHER Shri B.M. Kankanwadi Ayurveda
Mahavidyalaya Shahapur, Belagavi.

Mr. Mallikarjun Kolhar
CPCSEA Nominee
Institutional Animal Ethics Committee
KAHER Shri B.M. Kankanwadi Ayurveda
Mahavidyalaya Shahapur, Belagavi.

Note:- Meeting was conducted online due to Covid-19 lockdown Reference letterF.No25/2/2020-CPCSEA-DADF dated 13th April 2020. The proposal has been approved and noted in meeting minutes

Shahapur, Belagavi – 590 003, Karnataka, India
Phone: +91 831 2486286; 7204969289 Fax: +91 831 2424157
Website: www. kleayurworld.edu.in, Email: bmkprincipal.kaher@kleayurworld.edu.in



C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC
REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.
Refered by Dr: SELF
Received on: 16/01/2023 Lab no: TH - 38

HISTOPATHOLOGY REPORT

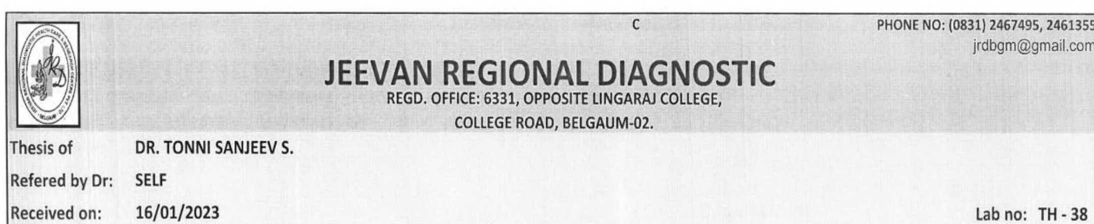
SPECIMEN:
Received specimens of anorectum labelled as A1, A2, A3, A4,A5, A6, B1, B2, B3, B4, B5, B6, C1, C2, C3, C4, C5, C6, D1, D2, D3, D4, D5, E1, E2, E3, E4, E5, E6, F1, F2, F3, F4, F5 and F6.

MICROSCOPY	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
Congestion	-	++	++	+++	++	+	+	+	-	+	+	+
Odema	-	-	-	+	-	-	-	+	-	+	-	-
Haemorrhage	-	-	-	+	-	-	-	-	-	-	-	-
Inflammation	-	++	+	++	+	+	+	+	-	+	+	+
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST



C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC
REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.
Refered by Dr: SELF
Received on: 16/01/2023 Lab no: TH - 38

HISTOPATHOLOGY REPORT

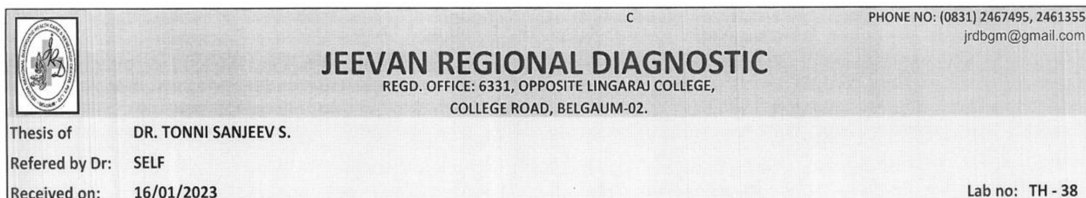
SPECIMEN:
Received specimens of anorectum labelled as A1, A2, A3, A4,A5, A6, B1, B2, B3, B4, B5, B6, C1, C2, C3, C4, C5, C6, D1, D2, D3, D4, D5, E1, E2, E3, E4, E5, E6, F1, F2, F3, F4, F5 and F6.

MICROSCOPY	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	D5
Congestion	+	+	+	+	+	+	+	+	++	+	+
Odema	-	-	-	-	-	-	-	+	-	-	-
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-
Inflammation	+	++	-	-	-	+	-	+	-	-	+
Destruction of villi	-	-	-	-	-	-	-	-	-	-	-
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST



C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC

REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.

Refered by Dr: SELF

Received on: 16/01/2023 Lab no: TH - 38

HISTOPATHOLOGY REPORT

SPECIMEN:

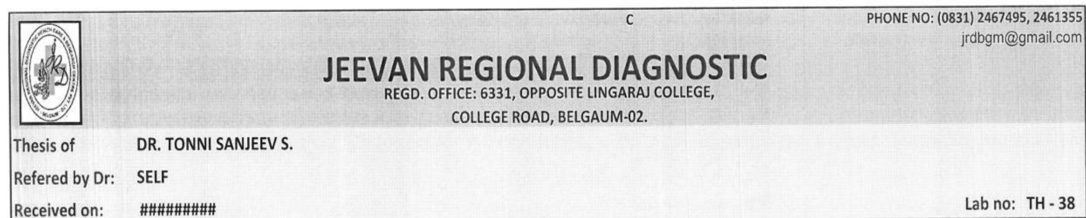
Received specimens of colon labelled as A1, A2, A3, A4,A5, A6, B1, B2, B3, B4, B5, B6, C1, C2, C3, C4, C5, C6, D1, D2, D3, D4, D5, E1, E2, E3, E4, E5, E6, F1, F2, F3, F4, F5 and F6.

MICROSCOPY	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	D5
Congestion	+	+	+	+	+	+	-	+	+	+	+
Odema	-	-	-	-	-	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-
Inflammation	+	+	+	+	-	-	-	+	+	-	+
Destruction of villi	-	-	-	-	-	-	-	-	-	-	-
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST



C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC

REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.

Refered by Dr: SELF

Received on: ######### Lab no: TH - 38

HISTOPATHOLOGY REPORT

SPECIMEN:

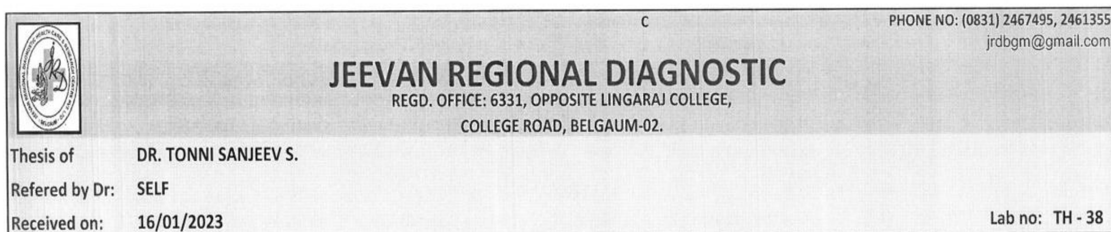
Received specimens of colon labelled as A1, A2, A3, A4,A5, A6, B1, B2, B3, B4, B5, B6, C1, C2, C3, C4, C5, C6, D1, D2, D3, D4, D5, E1, E2, E3, E4, E5, E6, F1, F2, F3, F4, F5 and F6.

MICROSCOPY	E1	E2	E3	E4	E5	E6	F1	F2	F3	F4	F5	F6
Congestion	++	++	+	+	+	-	+	+	+	+	+	+
Odema	-	-	-	-	-	-	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-	-
Inflammation	+	+	+	+	+	+	+	+	+	-	+	+
Destruction of villi	-	-	-	-	-	-	-	-	-	-	-	-
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST



C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC

REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.

Refered by Dr: SELF

Received on: 16/01/2023

Lab no: TH - 38

HISTOPATHOLOGY REPORT

SPECIMEN:

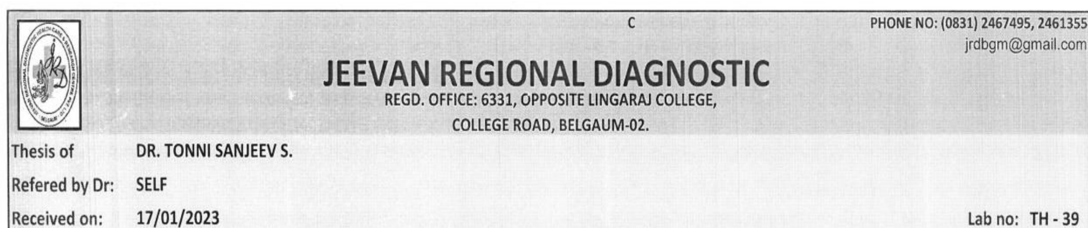
Received specimens of anorectum labelled as A1, A2, A3, A4,A5, A6, B1, B2, B3, B4, B5, B6, C1, C2, C3, C4, C5, C6, D1, D2, D3, D4, D5, E1, E2, E3, E4, E5, E6, F1, F2, F3, F4, F5 and F6.

MICROSCOPY	E1	E2	E3	E4	E5	E6	F1	F2	F3	F4	F5	F6
Congestion	-	+	+	++	+	+	+	+	+	+	+	++
Odema	-	-	-	-	-	-	-	-	+	-	+	-
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-	-
Inflammation	-	+	+	-	-	-	+	+	+	+	-	++
Destruction of villi	-	-	-	-	-	-	-	-	-	-	-	-
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST



C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC

REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.

Refered by Dr: SELF

Received on: 17/01/2023

Lab no: TH - 39

HISTOPATHOLOGY REPORT

SPECIMEN:

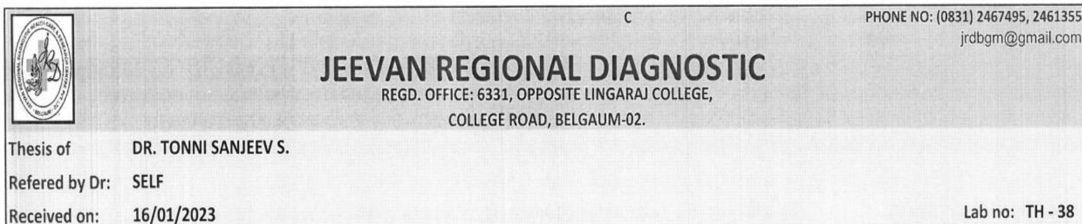
Received specimens of anorectum labelled as G1, G2, G3, G4, G5, G6, H1, H2, H3, H4, H5 and H6.

MICROSCOPY	G1	G2	G3	G4	G5	G6	H1	H2	H3	H4	H5	H6
Congestion	++	+	+	+	+	-	+	+	++	+	+	+
Odema	-	-	-	-	-	-	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-	-
Inflammation	++	+	+	+	+	-	+	+	-	-	-	+
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST


C

PHONE NO: (0831) 2467495, 2461355
jrdbgm@gmail.com

JEEVAN REGIONAL DIAGNOSTIC

REGD. OFFICE: 6331, OPPOSITE LINGARAJ COLLEGE,
COLLEGE ROAD, BELGAUM-02.

Thesis of DR. TONNI SANJEEV S.

Refered by Dr: SELF

Received on: 16/01/2023 Lab no: TH - 38

HISTOPATHOLOGY REPORT

SPECIMEN:

Received specimens of colon labelled as G1, G2, G3, G4, G5, G6, H1, H2, H3, H4, H5 and H6.

MICROSCOPY	G1	G2	G3	G4	G5	G6	H1	H2	H3	H4	H5	H6
Congestion	+	+	+	+	+	+	+	+	-	-	+	+
Odema	-	-	-	-	-	-	-	-	-	-	-	-
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-	-
Inflammation	-	-	-	-	-	-	+	-	-	-	+	+
Loss of intestinal glands	-	-	-	-	-	-	-	-	-	-	-	-
Mucosal Ulcers	-	-	-	-	-	-	-	-	-	-	-	-

THANKS FOR YOUR REFERENCE

DR. V. V. YENNI MD
CONSULTANT PATHOLOGIST

DR.A.S.AMMANAGI MD DNB MNAMS MSSC
CONSULTANT PATHOLOGIST

Maratha Mandal's Central Research Laboratory
Maratha Mandal's NGH Institute of Dental Sciences and Research Centre
R.S. No. 47A/2, Bauxite Road, BELAGAVI - 590 010.

Mrs. RAJSHREE NAGARAJU
Chairperson

Results

Sr. No	Test group	Colony forming units
1	Mixture 12gms drug + 100gms curds	NG
2	Mixture 24gms drug + 100gms curds	NG
3	Plain curds	> 1X 10^5CFU/ml

Note:

NG- No Growth
Drug – Un-ripened Bilwphalamajja

Date: 07.01.2022
Place: Belgaum

Dr. Kishore G. Bhat
MD, (Microbiology)

☎ 0831-2477682, Fax : 0831-2479323
E-mail : mmnghids@gmail.com / mmcrl01@gmail.com / drkgbhat@yahoo.com

Maratha Mandal's Central Research Laboratory
Maratha Mandal's NGH Institute of Dental Sciences and Research Centre
R.S. No. 47A/2, Bauxite Road, BELAGAVI - 590 010.

Mrs. RAJSHREE NAGARAJU
Chairperson

Results

Sr. No	Test group	Colony forming units
1	6gms drug + 100gms curds	1×10^{3} CFU/ml
2	3gms drug + 100gms curds	5×10^{3} CFU/ml
3	Mixture 1.5gms drug + 100gms curds	1×10^{4} CFU/ml

Note:

Drug – Un-ripened Bilwphalamajja

Date: 17.01.2022
Place: Belgaum

Dr. Kishore G. Bhat
MD, (Microbiology)

☎ 0831-2477682, Fax : 0831-2479323
E-mail : mmnghids@gmail.com / mmcrl01@gmail.com / drkgbhat@yahoo.com

Maratha Mandal's Central Research Laboratory
Maratha Mandal's NGH Institute of Dental Sciences and Research Centre,
R.S.No. 47A/2, Bauxite Road, Belgaum-590010
Ph. No. 0831-2477682 E-mail: mmnghids@gmail.com/ mmcrl01@gmail.com

Results:

		Report		Report
Raw sample	100 gm curd + 3 gm ABPM)	($5x10^3$ CFU/ml)	Lyophilized sample	$3X 10^4$ CFU/ml

Date: 27.07.2022
Place: Belgaum

Dr. Kishore G. Bhat
MD, (Microbiology)

Director: Dr. Kishore G. Bhat, MD
E-mail id: drkgbhat@yahoo.com

Results of qPCR (Quantifying the different bacterial groups)

Results of sample matching with respective strain no. & suspected organisms

Group	Strain. No (Code name)	Sample is matching with suspected organisms
A	MN340241.1	Proteus mirabilis strain
	MT107058.1	Proteus sp. (enterobacteria) strain
	OQ607170.1	Enterococcus faecalis strain
B	OP741052.1	Acetobacter indonesiensis strain
C	KF051775.1	Proteus mirabilis strain
	OL629241.1	Proteus mirabilis strain
D	OP881561.1	Proteus mirabilis strain
	OQ405515.1	Bacillus subtilis strain
E	MK578252.1	Proteus mirabilis strain
	MN340241.1	Providencia sp. starin
F	MK571727.1	Enterococcus faecalis strain
G	MF369841.1	Enterococcus faecium strain
H	OQ405595.1	Enterococcus faecalis strain
	KX138516.1	Proteus mirabilis strain

Interpretation of the qPCR result:

Results of samples of all the groups are matching with respective strain no &suspected organisms.

Number of samples taken:

There are 8 fecal main samples are there in which each main group has 6 subgroups. From each 8 main samples we had taken 3 subgroups which had sample s nearly 1 gm. And we had performed serial dilution and spread plate culture for those 3 subgroups.

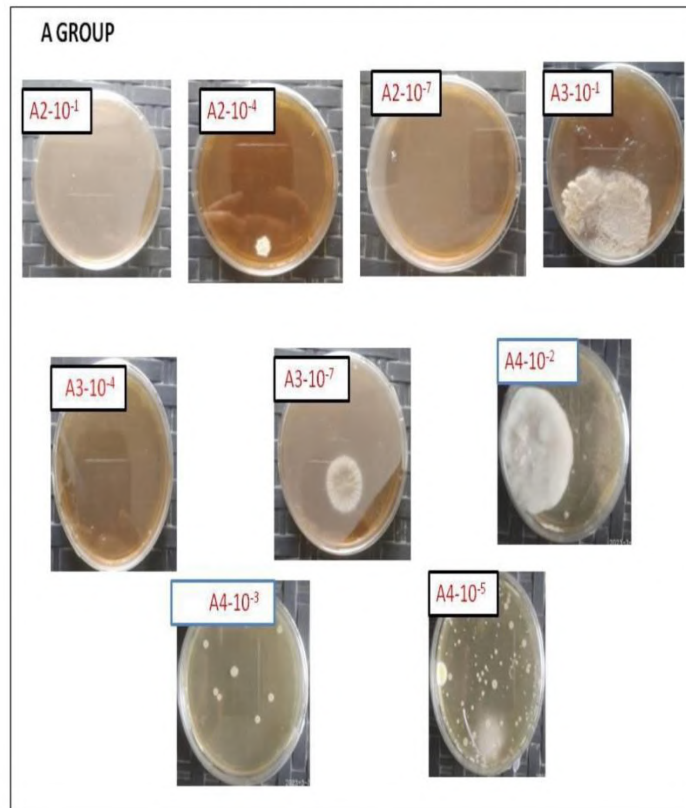
Samples plates and their colony count

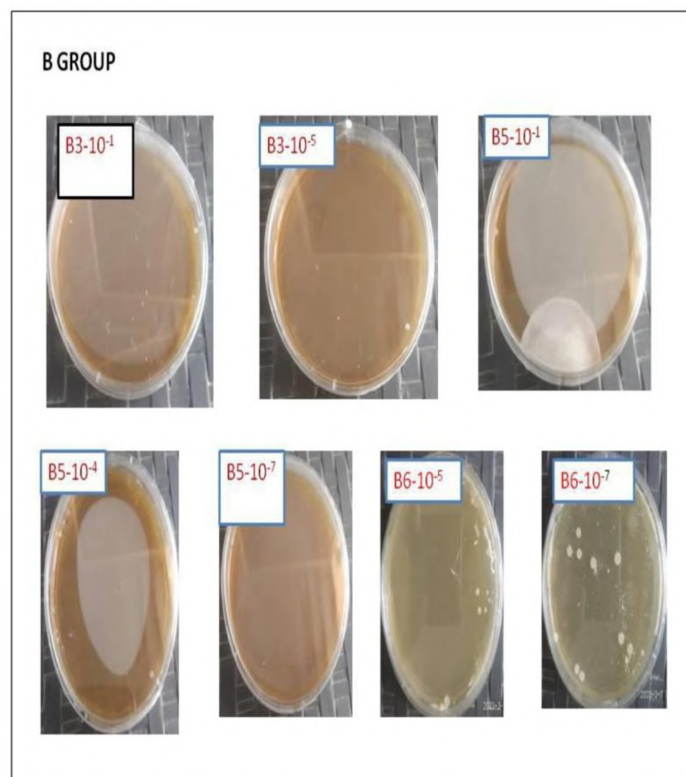
Main group	Sub group division	Dilution factor of plate	Colony count (approximately)
A	A-3	10^{-1}	3
	A-2	10^{-1}	8
	A-2	10^{-4}	1
	A-4	10^{-5}	13
	A-4	10^{-3}	9
B	B-3	10^{-4}	1
	B-3	10^{-1}	11
	B-6	10^{-7}	10
	B-6	10^{-5}	10
C	C-1	10^{-1}	10
	C-1	10^{-7}	10
	C-5	10^{-1}	6
	C-4	10^{-4}	2
D	D-1	10^{-1}	10
	D-2	10^{-7}	12
	D-2	10^{-5}	18
E	E-4	10^{-7}	3
	E-4	10^{-4}	3
	E-4	10^{-1}	17
	E-5	10^{-4}	9
F	F-1	10^{-7}	1
	F-1	10^{-4}	1
	F-1	10^{-1}	6
G	G-3	10^{-7}	18
	G-3	10^{-4}	10
	G-3	10^{-1}	12
H	H-4	10^{-1}	13
	H-3	10^{-1}	10
	H-1	10^{-1}	15

List of bacteria identified from the selected samples

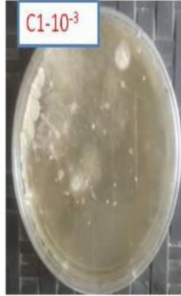
Main Group	Sub Division	Dilution Factor	Bacterial Name	Colour And Morphology
A	A-3	10^{-4}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	A-4	10^{-4}	*Proteus species(Enterobacteria strain)*	Gram negative, facultatively anaerobic, Rod shaped bacteria.
	A-5	10^{-5}	*Enterococcus faecalis*	*E. faecalis* is a non-motile microbe and is a Gram-positive
B	B-6	10^{-5}	*Acetobacter indonesiensis*	Gram negative, Acetic acid bacteria, Pleomorphic.
	C-4	10^{-1}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	C-5	10^{-1}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
D	D-5	10^{-1}	*Bacillus tequilensis*	Gram positive, Motile, Rod shaped Spore forming bacteria
	D-1	10^{-1}	*Proteus vulgaris*	Gram negative, Actively motile, non-capsulated, bacillus and shiny cream colony.
	D-5	10^{-4}	Bacillus subtilis	Gram positive, rod shaped whitish cream.
E	E-2	10^{-7}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	E-5	10^{-4}	*Providencia spp.*	Gram negative, bacillus, motile by peritrichous flagella.
F	F-6 A	10^{-3}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative anaerobic.
	F-6 B	10^{-3}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative anaerobic
	F-6 C	10^{-3}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	G-3	10^{-1}	*Enterococcus facium*	Gram positive, Bacillus, gamma-Hemolytic bacteria.
G	G-3	10^{-7}	*Enterococcus facium*	Gram positive, Bacillus, gamma-hemolytic bacteria
H	H-1	10-1	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	H-3	10^{-1}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative anaerobic

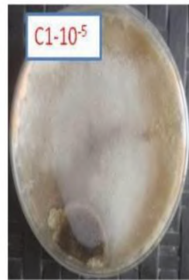
Photographs of bacterial colonies based on selected groups

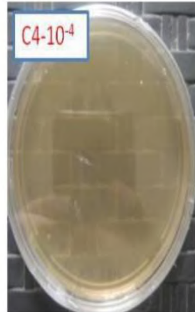


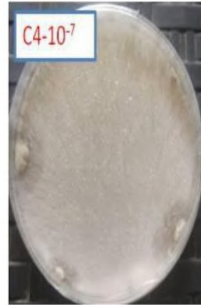


C GROUP

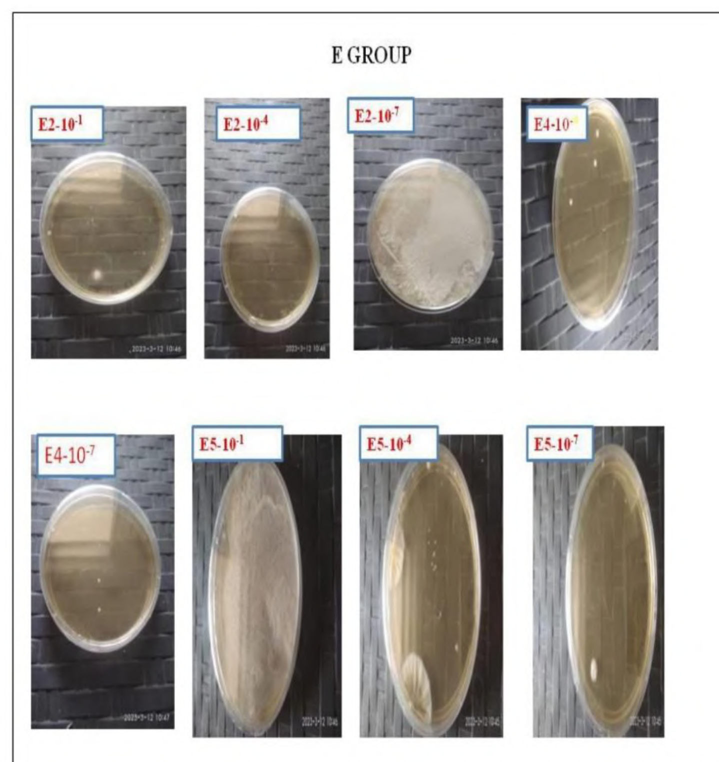


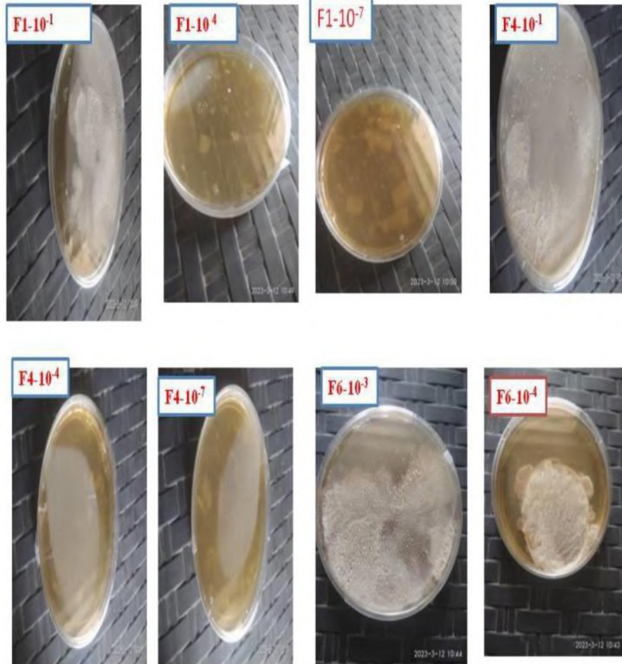


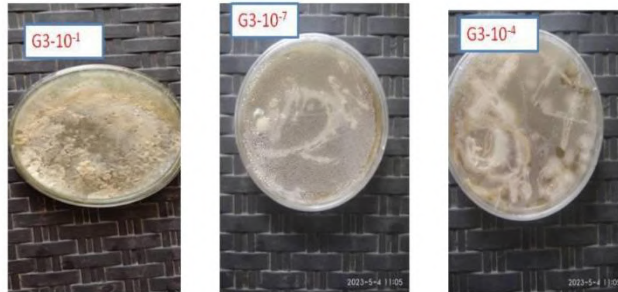


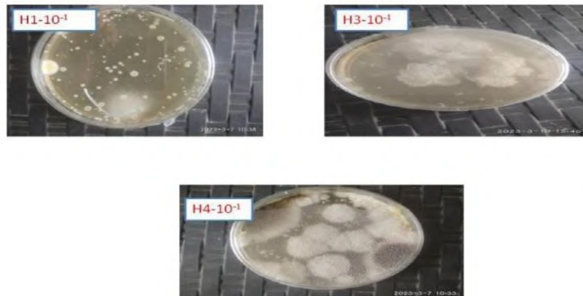


D GROUP
D1-10^{-}
D1-10^{-4}
D1-10^{-7}
D2-10^{-1}
D2-10^{-2}
D2-10^{-3}
D2-10^{-4}
D2-10^{-6}
D2-10^{-7}
D3-10^{-7}
D5-10^{-1}
D5-10^{-4}

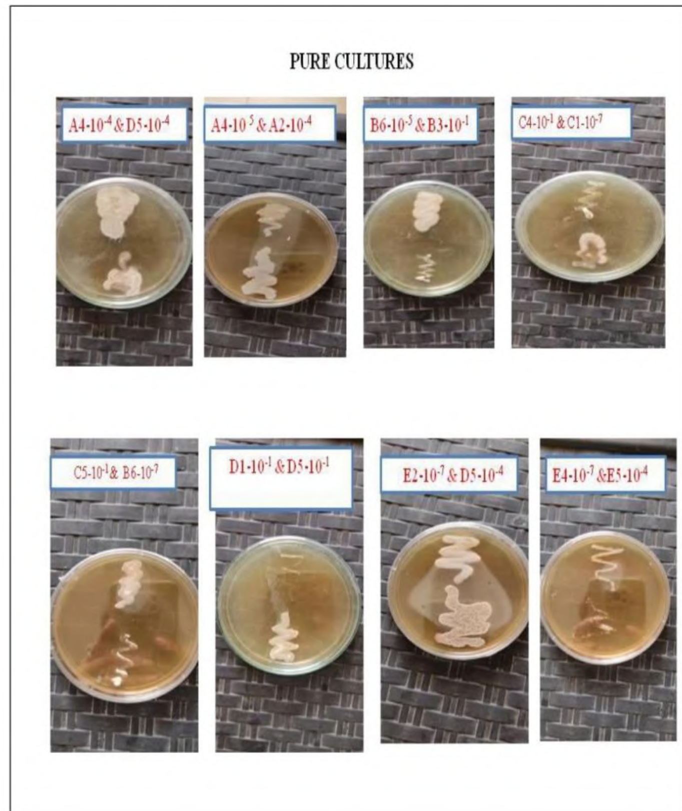

E GROUP
E2-10^{-1}
E2-10^{-4}
E2-10^{-7}
E4-10^{-}
E4-10^{-7}
E5-10^{-1}
E5-10^{-4}
E5-10^{-7}

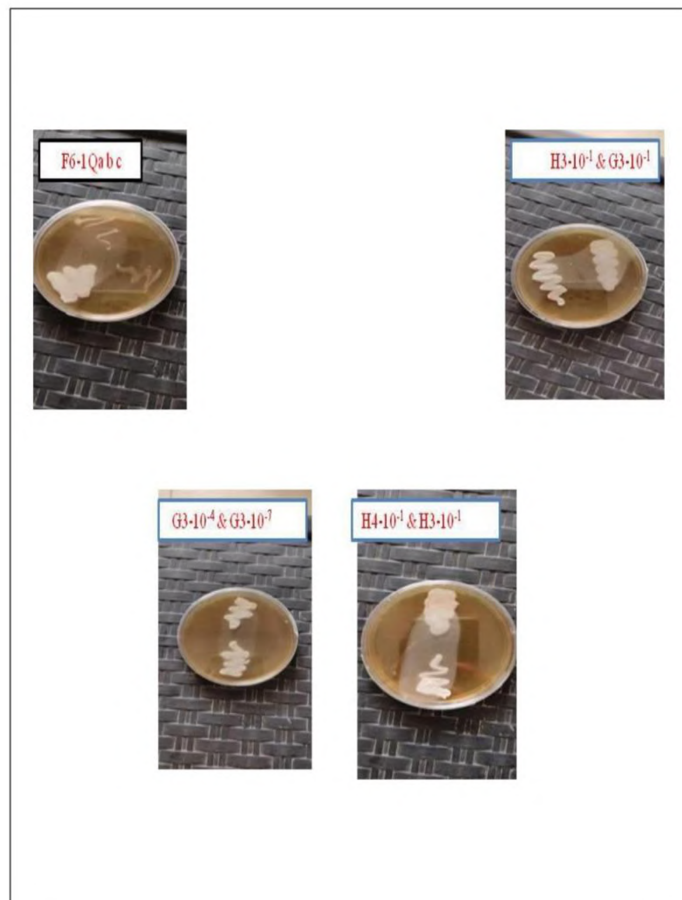

F GROUP
F1-10-1
F1-10-4
F1-10-7
F4-10-1
F4-10-4
F4-10-7
F6-10-3
F6-10-4


G GROUP
G3-10-1
G3-10-7
G3-10-4


H-GROUP
H1-10-1
H3-10-1
H4-10-1

Photographs of Pure culture selected for molecular identification





Test Results of Clostridium difficile

Results of Clostridium difficile in stool samples

Testing Method	Name of the Sample group	*Sub-group*	Substance detected Toxin A or B	Test Results Presence (P) / Absence (A)	Performance characteristics	
					Reference method	Reference method
					Sensitivity*	Specificity**
Toxic Culture	A	A1	Toxin A	P	+	+
		A2	Toxin A	P	+	+
		A3	Toxin B	P	+	+
		A4	Toxin A	P	+	+
		A5	Toxin B	P	+	+
		A6	Toxin A	P	+	+
	B	B1	-	A	-	-
		B2	-	A	-	-
		B3	-	A	-	-
		B4	-	A	-	-
		B5	-	A	-	-
		B6	-	A	-	-
	C	C1	Toxin B	P	+	+
		C2	Toxin A	P	-	-
		C3	Toxin B	P	+	+
		C4	Toxin A	P	+	+
		C5	Toxin B	P	-	-
		C6	Toxin B	P	-	-
	D	D1	Toxin A	P	+	+
		D2	Toxin A	P	+	+
		D3	Toxin A	P	-	-
		D4	Toxin B	P	+	+
		D5	Toxin B	P	+	+
		D6	Toxin A	P	-	-
	E	E1	Toxin A	P	+	+
		E2	Toxin A	P	+	+
		E3	Toxin B	P	+	+
		E4	Toxin A	P	+	+
		E5	Toxin B	P	+	+
		E6	Toxin A	P	+	+
	F	F1	Toxin A	P	+	+
		F2	Toxin B	P	+	+
		F3	Toxin B	P	+	+
		F4	Toxin A	P	+	+
		F5	Toxin B	P	+	+
		F6	Toxin B	P	+	+
	G	G1	Toxin A	P	+	+
		G2	Toxin A	P	+	+
		G3	Toxin B	P	+	+
		G4	Toxin A	P	+	+
		G5	Toxin B	P	+	+
		G6	Toxin A	P	+	+
	H	H1	Toxin A	P	+	+
		H2	Toxin A	P	+	+
		A3	Toxin A	P	+	+
		H4	Toxin A	P	+	+
		H5	Toxin B	P	+	+
		H6	Toxin A	P	+	+

*__Sensitivity__ of a test indicates the probability of infection, the test will be positive
**__Specificity__ is the probability of not having the infection, the test will be negative.
+ indicates fair
++ good
+++ very good
++++ excellent

Microbial Colony Count by Serial Dilution and Spread Plate Culture Method

----

Sample plates and their colony count:

Main group	Sub-group division	Dilution factor of plate	Colony count (approximately)
A	A-3	10^{-1}	3
	A-2	10^{-1}	8
	A-2	10^{-4}	1
	A-4	10^{-5}	13
	A-4	10^{-3}	9
B	B-3	10^{-4}	1
	B-3	10^{-1}	11
	B-6	10^{-7}	10
	B-6	10^{-5}	10
C	C-1	10^{-1}	10
	C-1	10^{-7}	10
	C-5	10^{-1}	6
	C-4	10^{-4}	2
D	D-1	10^{-1}	10
	D-2	10^{-7}	12
	D-2	10^{-5}	18
E	E-4	10^{-7}	3
	E-4	10^{-4}	3
	E-4	10^{-1}	17
	E-5	10^{-4}	9
F	F-1	10^{-7}	1
	F-1	10^{-4}	1
	F-1	10^{-1}	6
G	G-3	10^{-7}	18
	G-3	10^{-4}	10
	G-3	10^{-1}	12
H	H-4	10^{-1}	13
	H-3	10^{-1}	10
	H-1	10^{-1}	15

List of bacteria identified from the selected samples

Identification of Bacteria & their Morphology

Main Group	Sub Division	Dilution Factor	Bacterial Name	Morphology
A	A-3	10^{-4}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	A-4	10^{-4}	*Proteus species (Enterobacteria strain)*	Gram negative, facultatively anaerobic, Rod shaped bacteria.
	A-5	10^{-5}	*Enterococcus faecalis*	***E. faecalis*** is a non-motile microbe and is a Gram-positive
B	B-6	10^{-5}	*Acetobacter indonesiensis*	Gram negative, Acetic acid bacteria, Pleomorphic.
C	C-4	10^{-1}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitish in colour
	C-5	10^{-1}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitishin colour
D	D-5	10^{-1}	*Bacillus tequilensis*	Gram positive, Motile, Rod shaped Spore forming bacteria
	D-1	10^{-1}	*Proteus vulgaris*	Gram negative, Actively motile, Non-capsulated, bacillus & shiny cream colony.
	D-5	10^{-4}	Bacillus subtilis	Gram positive, rod shaped whitish cream
E	E-2	10^{-7}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated whitishin colour
	E-5	10^{-4}	*Providencia spp.*	Gram negative, bacillus, motile by peritrichous flagella.
F	F-6 A	10^{-3}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative anaerobic.
	F-6 B	10^{-3}	*Enterococcus faecalis*	Gram positive, Bacillus, facultative Anaerobic
	F-6 C	10^{-3}	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated, whitishin colour
G	G-3	10^{-1}	*Enterococcus facium*	Gram positive, Bacillus, gamma-Hemolytic bacteria.
	G-3	10^{-7}	*Enterococcus facium*	Gram positive, Bacillus, gamma-hemolytic bacteria
H	H-1	10-1	*Proteus mirabilis*	Gram negative, Bacillus, Actively motile and non-capsulated, whitish in colour
	H-3	10^{-1}	*Enterococcus faecalis*	Gram positive, Bacillus, facultativeanaerobic

Standard Operative Procedure (SoP) for analysis of Cow milk sample:

Preparation of Sample of Cow Milk:

- 0.4% formalin was added to the cow milk sample soon after its collection.
- Warming the sample to 37- 40°C by transferring it to the beaker and keeping it in a waterbath maintained at 40 - 45°C.
- Stirring it slowly for proper homogenization.
- Mixing sample thoroughly by pouring back into the bottle, mixing to dislodge any residual fat sticking to the sides and poured it back in the beaker.
- During mixing do not shake the bottle vigorously.
- Allowing the sample to come to room temperature (26- 28°C) and withdrawing immediately for analysis.

Note: If small clots or lumps are observed in the sample which cannot be dispersed, a few drops of liquor ammonia may be used during homogenization. If even after homogenization the sample shows lumps or clots or droplets of oil are visible suggestive of curdling /splitting of milk, the sample shouldbe deemed unfit for analysis and rejected.

Detection of Adulterants in Milk[112]:

Detection and Quantification of Cane Sugar in Milk:

Sucrose is absent in milk and its presence in milk indicate adulteration. Presence of sucrose in milk can be determined by the following method.

Qualitative Method:

Modified Seliwanoff's Method:

Fructose in cane sugar (sucrose) reacts with resorcinol in HCl to give red colour.

Reagents used:

A. Resorcinol Solution (0.5%): Weighing 0.5 g of resorcinol in about 40 ml of distilledwater.

Adding 35 ml of concentrated HCl (12 N) to it and make up the volume to 100 ml usingdistilled water.

Note: The resorcinol flakes should be white in colour.

Procedure:

- Take 1 ml of milk in a test tube.
- Add 1 ml of Resorcinol Solution and mix.
- Place the tube in boiling water bath for 5 min.
- Withdraw the tube and observe the colour.
- Appearance of deep red colour indicates presence of sucrose, or a ketose sugar.
- In pure milk samples, no such red colour is developed and sample remains white innature.
- The limit of detection of method is 0.1%.

Detection of Cellulose in Milk[113]:

Cellulose in milk gives blue colour with Iodine – Zinc Chloride reagent.

Reagents used:

A. Iodine–Zinc Chloride reagent: Dissolve 20 g $ZnCl_2$ in 8.5 ml water and when cool, introduce the iodine solution (3 g potassium iodide and 1.5 g iodine in 60 ml water) drop by drop until iodine begins to precipitate.

Procedure:

- Take about 10 g of milk in a 100 ml beaker.
- Add 50 ml of hot water and stir thoroughly for about 2 min.
- Pour the mixture on a nylon cloth and wash the residue with 50 ml of hot watertwice.
- Scrape the residue with a spatula and place it in a spotting plate.
- Stain a part of residue with Iodine-Zinc Chloride reagent and another part with iodinesolution (see Reagent 1.2.2.2.1.).
- Development of blue colour in Iodine-Zinc Chloride reagent and absence of bluecolour in Iodine Solution confirms presence of cellulose.

Detection of Added Urea in Milk:

- Urea is a natural constituent of milk and it forms a major part of the non-proteinnitrogen of milk.
- Urea concentration in milk is variable within herd.
- Urea content in natural milk varies from 20 mg/100 ml to 70 mg/100 ml.
- However, urea content above 70 mg/100 ml in milk indicates milk containing 'addedurea'.
- The addition of urea to milk can be detected by using para-dimethyl-amino-benzaldehyde (DMAB).

This method is based on the principle that urea forms a yellow complex with DMAB in alow acidic solution at room temperature.

Qualitative Method:

This method is based on the principle that urea forms a yellow complex with DMAB in a low acidic solution at room temperature.

Reagents used:

A. DMAB reagent (1.6%, w/v): Dissolve 1.6 g DMAB in 100 ml ethyl alcohol and add 10ml concentrate HCl.

Procedure:

- Mix 1 ml of milk with 1 ml of 1.6% DMAB reagent.
- Distinct yellow colour is observed in milk containing added urea.
- The control (normal milk) shows a slight yellow colour due to presence of naturalurea.
- The limit of detection of method is 0.2%.

Detection of Presence of Foreign Fat in Milk:

For isolation of milk fat from milk, modified Gerber butyrometer can be used where both ends of the butyrometer are open. Stem side opening of the butyrometer (which is generally closed) is closed with a good quality removable silicon stopper. After the milk fat test, silicon stopper is removed and milk fat is removed with the help of a syringe and same is subjected to B.R. at 40°C.Since Gerber sulfuric acid causes some hydrolysis of fatty acids/triglycerides, the B.R is multiplied by a factor to obtain corrected B.R.The fat in suspected milk sample can also be isolated by solvent extraction method in which case correction of B.R. is not required.

Method.1

Using modified Gerber Method[114]:

Reagents used: Gerber sulphuric acid, Iso-amyl alcohol.

Procedure:

- Isolate the fat from milk by Gerber method using specially designed milk butyrometer, which is open at both ends. Close the stem side opening with a good quality acid resistant silicon stopper. Add 10 ml of Gerber sulphuric acid, 10.75 ml milk and 1 ml iso-amyl alcohol. Close the neck side with lock stopper; mix the content and centrifuge at 1200 rpm, 5 min to get a clear fat column. Remove the silicon stopper from the stem side and take out the fat from the stem of the butyrometer using a capillary or a syringe.
- For taking B.R. reading of the milk fat, clean the prism of the Butyro-Refractometer with diethyl ether. Allow the ether to evaporate to dryness. Maintain the temperature of the prism at 40°C by circulating water using a thermostatically controlled water- bath. Calibrate the Butyro-Refractometer by applying standard liquid solution of known B.R. reading. Again clean the prism with diethyl ether; apply 1-2 drops of clear, extracted fat between the prism. Wait for 2 min before taking the reading so that sample should attain temperature of 40°C. A correction of 0.55 is added to the observed B.R. reading for each degree above 40°C or subtracted for each degree below40°C to get corrected B.R. reading of the sample.

Calculation:

- Calculate the Corrected B.R. reading of isolated fat as follows:
- Corrected B.R. = Observed B.R. x 1.08

Interpretation: If the BR reading differs from the prescribed limit of variability (not more than 42 in case of non-cotton tract area and not more than 45 in case of cotton tract area), presence of foreign fat in the milk may be suspected.

Note: Further check for presence of extraneous fat can be done by checking the fatty acid profile of the extracted fatby GLC (See Manual on Oils and Fats for determination of fatty acid composition of oils and fats).

Test for Presence of Skimmed milk Powder in Natural milk (Cow, buffalo, goat, sheep)[115]

As per the law, use of skimmed milk powder (SMP) is not allowed for adjustment of SNF in case of sale of cow/buffalo or mixed milk. A method has been developed for

the detection of presence of SMP in liquid milk. The method is based on the fact that the coagulum obtained from reconstituted skim milk powder by addition of acetic acid, gives intense blue colour on boiling with phosphomolybdic acid due to certain reducing groups present in the proteins of milk powder which are able to cause reduction of molybdenum blueresulting in formation of blue colour.

Reagents used:

A. Acetic acid 4%.

B. Phosphomolybdic acid: 1% solution in water.

Procedure:

- Take 50 ml of milk in a 60 ml centrifuge tube.
- Place the tube in the centrifuge and balance it properly. Centrifuge at 5000 rpm for 15minutes. Decant the supernatant creamy layer carefully.
- Add 0.5 ml of 4% acetic acid to skim milk portion for coagulation of protein. Centrifuge the tubes at 5000 rpm for 5 min.
- Decant the supernatant and wash the precipitate with distilled water twice. Discard the washings. Then, add 2 ml of 1% phosphomolybdic acid to the washed precipitates.
- Mix the contents thoroughly and heat in a water bath at boiling temperature for 15minutes and then cool.
- The curd obtained from pure milk shall be greenish in colour whereas the curd ofsample containing skimmed milk powder shall be bluish in colour.
- The intensity of bluish colour depends on the amount of the skim milk powder present in the sample *(**Ref**:- Journal of Food Science and Technology, Vol 22 (1985) page 207-208).*

Tests for Chemical Analysis of milk[116]

Alkaline Phosphatase Test for Checking Efficiency of Pasteurization in Liquid Milk:

Alkaline phosphatase is an indigenous milk enzyme. The enzyme activity is destroyed at pasteurization temperature and has been adopted as an index of the efficiency of pasteurization. Since milk is a proven vector for a number of pathogenic bacteria, including *Salmonella*, *Campylobactor* and *Listeria*, the test is of very great significance to the dairy industry as a means of policing the thoroughness of heat treatments or the addition of raw milk to heated or unheated products. In the following method, a solution of disodium *p*- nitrophenyl phosphate in a buffer of pH 10.2 is used as substrate. This compound, colourless in solution, is hydrolyzed by alkaline phosphatase of milk to liberate *p*-nitrophenol, which under alkaline condition gives an intense yellow colouration to the solution. The liberated *p*- nitrophenol is measured by direct comparison with standard colour discs in a Lovibond comparator.

The test does not apply to sour milk and milk preserved with chemical preservatives.

Reagents/Apparatus used:

All reagents should be of analytical grade.

- **Buffer solution:** 1.5 g of sodium bicarbonate and 3.5 g of anhydrous sodium

carbonate dissolved in water and made up to one litre. Store in a refrigerator and discard after 1 month.

- **Disodium *p*- nitrophenylphosphate.** The solid substrate must be kept in the refrigerator.
- **Buffer-substrate solution** - Weigh accurately 0.15 g of substrate (disodium *p*--nitrophenyl phosphate) into a 100 ml measuring cylinder and make up to 100 ml with buffer solution. The solution should be stored in refrigerator and protected from light. The solution should give a reading of less than the standard marked 10 on comparator disc APTW or APTW 7 when viewed through a 25 mm cell (distilled water is used asa blank). The solution must be discarded after one week.
- A Lovibond Comparator with stand for work in reflected light.
- A lovibomd comparator disc APTW or APTW 7.
- Two Fused glass cells of 25 mm depth.
- A water bath or incubator capable of being maintained at 37.5±0.5°C.
- 1 ml pipette and 5 ml pipette.
- *1 litre graduated flask.*
- **100 ml measuring cylinder**.
- **Test tubes**, nominal size 150/16 mm with rubber stoppers.

Procedure:

- Into a test tube pipette 5 ml of buffer substrate solution, stopper and bring the temperature to 37°C.
- Add 1 ml of test milk to it shake and replace stopper, incubate at 37°C for 2 hrs.
- Incubate one blank prepared from boiled milk of the same type as that undergoing the test with each series of sample. Remove the tubes after 2 h and the content should be well mixed.
- Place the boiled milk blank on left hand side of the comparator stand and test sample on the right.
- Take reading in reflected light by revolving the disc until the test sample is matched.
- Record readings falling between two standards by affixing a plus or minus sign to the figure for the nearest standard.

Interpretation:- The test is considered satisfactory if it gives a reading of 10 µg or less of *p*-nitrophenyl per ml of milk. Properly pasteurized milk gives no discernible colour.

Precautions

- All glassware must be cleaned before use. Cleaning should be done by soaking in Chromic acid solution prepared by slowly adding 4 volumes of concentrated H2SO4 to 5 volumes of 8% potassium dichromate. After cleaning in chromic acid glassware must be rinsed in warm water and distilled water and finally dried. Glassware used for the test must not be used for any other purpose and must be kept apart from other apparatus in the laboratory.
- A fresh pipette must be used for each sample of milk. Pipettes must not be contaminated withsaliva.

- The sample of milk should be examined as soon as possible after arrival at the laboratory. If not examined immediately it must be kept at a temperature between 3°C and 5°C until examined. The sample must be brought to room temperature immediately before being tested.

Standard operative Procedure (SoP) for analysis of CURD sample[117]:

1. Preparation of Sample of curd:

- To get a representative sample particularly in case of a thick-set product it may be necessary to pour out the whole of the contents in a mortar and mix it thoroughly until the mass is homogeneous. Aliquots are weighed out from this well mixed sample.

2. Determination of Fat in curd:

Rose-Gottlieb method:

- The milk sample is treated with ammonia and ethyl alcohol; the former to dissolvethe protein and the latter to help precipitate the proteins.
- Fat is extracted with diethyl ether and petroleum ether.
- Mixed ethers are evaporated and the residue weighed.
- This method is considered suitable for reference purposes.
- Strict adherence to details is essential in order to obtain reliable results.

Reagents /Apparatus used:

- Ammonia solution, containing approximately 25% (m/m) of NH3, sp.gr ($\rho 20$) ≈910 g/l
- Ethyl alcohol (95%).
- Diethyl ether, peroxide-free.
- Petroleum ether, boiling range 40-60°C.
- Mojonnier fat extraction flask or any other suitable extraction tube (as per IS specification).
- Cork or stopper of synthetic rubber unaffected by usual fat solvents.
- 100 ml flat bottom flask with G/G joint or stainless steel or aluminium dishes of 5.5 cmheight and 9 cm diameter or glass bowl.

Procedure:

- Weigh accurately about 10 g of sample (liquid milk), transfer to extraction tube.
- Add 1.25 ml of ammonia sp. gr. 0.91 (or an equivalent volume of a more concentratedammonia solution may be used), mix and shake thoroughly.
- Add 10 ml ethyl alcohol and mix again.
- Add 25 ml of diethyl ether (peroxide free) stopper and shake vigorously for about aminute.
- Then add 25 ml petroleum ether (boiling range 40 – 60°C and shake again vigorouslyfor about half a minute.
- Let it stand until the upper ethereal layer has separated completely and is clear. (Alternatively use low r.p.m. Mojonnier centrifuge).
- If there is a tendency to form emulsion, a little alcohol may be added to help separationof the layers.

- Decant off the clear ethereal layer into a suitable vessel (flask, glass bowl, aluminiumdish, etc.).
- Wash the delivery end of the extraction tube with a little ether and add the washings tothe flask.
- Repeat twice extraction of the liquid remaining in the extraction tube using 15 ml ofeach solvent every time.
- Add the ethereal extract to the same container and evaporate off completely.
- Dry the flask in an air oven at 102 ± 2°C for two hours, cool in a desiccator and weigh.
- Heat the flask again in the oven for 30 min. Cool in a desiccator and weigh.
- Repeat the process of heating and cooling and weighing until the difference betweentwo successive weights does not exceed 1 mg.
- Wash out the fat from the flask with petroleum ether carefully leaving any insolubleresidue in the flask.
- Dry the flask in the oven and reweigh.
- The difference in weights represents the weight of fat extracted from the milk.
- Correct weight of extracted fat by blank determination on reagents used.
- If reagent blank is more than 0.5 mg purify or replace reagents.
- Difference between duplicate determinations obtained simultaneously by the same analyst should not be more than 0.03 g fat /100g product.

Calculation:

Fat % (w/w) = Weight of Extracted
Fat X 100Weight of milk

3. Test for the Presence of Starch in curd:

If test for starch is positive, quantitative estimation of starch is to be carried out for determination of SNF in milk sample.

- The sample of milk is curdled with alcohol, and made free from lactose which is naturally present in milk. The precipitated starch is washed with 50% alcohol to free it from lactose.
- The precipitated starch is hydrolysed to convert it into reducing sugars.

Reagents used:

- Ethanol (98%).
- 10% sodium hydroxide.
- Sodium carbonate.

Procedure:

- Weigh approximately 25 g sample in a 250 ml beaker.
- Add 20 ml of ethanol to curdle the milk.
- Filter the precipitate on a filter paper and wash the precipitate with 50% ethanol till theprecipitate is free from lactose/sugar i.e. when the washings give a negative test with resorcinol.
- Transfer the precipitate to a 500 ml flask with about 200 ml water and add 10 ml concentrate HCl to hydrolyse the starch by refluxing in a boiling water bath for 2.5 hours.
- Cool and neutralise with 10% sodium hydroxide and sodium carbonate towards theend using litmus paper.
- Make up to 500 ml with water.
- Shake well and filter if necessary. Determine reducing sugar by Lane and Eynonmethod (Section 9.4).
- Calculate starch as follows:

% starch = % reducing sugar x 0.9

4. Determination of Total Solids in curd[118]:

The total solids are determined after neutralization of developed acidity with alkali.

Methods adopted:

1. **Sodium Hydroxide Method:**

In this procedure (IS 12333: 1997), NaOH is used for neutralization of developed acidityfollowed by determination of total solids by evaporation at 100 ± 2°C.

Reagents used:

- Sodium hydroxide
- Phenolphthalein Indicator Solution:
  - Dissolve 1g of phenolphthalein in 100 ml of ethanol (95%, v/v).
  - Add 0.1N NaOH solutions until one drop gives a faint pink coloration.
  - Dilute with distilled water to 200 ml.

Apparatus used:

- Shallow flat-bottom dishes of aluminium, nickel, stainless steel, porcelain or silica, 7-8 cm diameter, about 1.5 cm in height and provided with easily removable but closely fitting lids.

Procedure:

- Heat the clean dry empty dish and lid in oven maintained at 100 ± 2°C for onehour,cool in a desiccator and weigh.
- Quickly weigh to the nearest 0.1 mg 4-5 g of prepared sample of curd or dahi, replacethe lid and weigh again.
- Add 1-2 drops of phenolphthalein solution to the sample in the dish and neutralise with

  0.1 N sodium hydroxide solution to a faint pink colour.
- Note the volume of 0.1N sodium hydroxide required to neutralise the sample.
- Place the dish without lid on a boiling water bath until the water is removed from thesample.
- Wipe the under-surface of the dish and place in the oven maintained at 100 ± 2°C, for3 hrs.
- Remove the dish along with the lid and cool in a desiccator and weigh.
- Continue heating and re-weighing at hourly intervals until successive weighing do notvary by more than 0.5 mg.
- Deduct half weight of the 0.1N sodium hydroxide added to neutralize the sample fromthe residue after drying and calculate total solids as for whole milk.

Calculation:
a = N x T.V x 401000 x 2
Total solids percent w/w = 100 (W2−a)1
Where -

- N = Normality of NaOH
- T. V = Titre value
- W2 = Weight in g of residue left after drying
- W1= Weight in g of the prepared sample taken
- a = Half of the volume of 0.1 N Sodium hydroxide added

Method.2:

Zinc oxide Method[119]:

- In the following procedure (IDF 151: 1991), the water is evaporated from a test portionof the sample in the presence of zinc oxide at a temperature of 100 ± 2°C in drying oven.
- Lactic acid (determined by titration method) content is determined separately in the sample and is added to the moisture determined in the drying step to compensate forthe loss of water as a result of neutralizing the sample by means of zinc oxide.

Reagent used:

Zinc oxide

Apparatus used:

- Analytical balance
- Desiccator: provided with efficient drying agent.
- Drying oven: ventilated, maintained thermostatically at 100 ± 2°C.
- Flat bottom dishes: of height 20 to 25 mm, diameter 50 to 75 mm, and made of appropriate material (for example stainless steel, nickel or aluminium), provided withwell-fitting, readily removable lids.
- Boiling water-bath.
- Spoon or spatula.
- Homogenizer for homogenizing fruit yogurts.

Procedure:

- Bring the sample to temperature of 20-25°C. Mix the sample carefully by means of spatula using a rotatory motion which passes from the lower layers to the surface layer of the sample so as to displace and mix them well.
- Heat an open dish containing approximately 2 g of zinc oxide, together with lid and a stirring rod for 1 h in the oven maintained at 100 ± 2°C.
- Transfer the dish along with lid and stirring rod to the desiccator, allow it to cool to room temperature (at least 45 min) and weigh the dish with the stirring rod and lid to the nearest 0.1mg.
- Move the zinc oxide to one side of the prepared dish by tilting. Place on the clearspace approximately 1.0 g of prepared test sample, replace the lid on the dish with the stirring rod on top and weigh the dish to the nearest 0.1 mg.
- Add 5 ml of water to the test portion and thoroughly mix the diluted test portion and zinc oxide. Spread the mixture evenly over the bottom surface of the dish. Heat thedish on the boiling water bath and continue heating for approximately 30 min with frequent mixing of the contents of the dish during the early stages of the drying so asto obtain maximum evaporation of liquid.
- Remove the dish from the water bath and wipe its base to remove any water. Leave the stirring rod in the dish and then place it together with lid by its side, in the drying ovenfor 3 h.
- After drying, cover the dish with its lid and immediately transfer to the desiccator. Allow the dish and contents to cool in the desiccator to room temperature (at least 45 min) and weigh to the nearest 0.1 mg.
- Again heat the dish and contents together with its lid for a further 1h, cover the dish and transfer to the desiccator. Allow to cool and weigh to the nearest 0.1 mg. repeatthe process of reheating and weighing until the difference in mass between two successive weighing does not exceed 1 mg. Record the lowest weight.

- In order to compensate for the loss of water as a result of neutralizing the yoghurt by means of zinc oxide, determine the titratable acidity (expressed as g of lactic acid per 100 g of product) of the sample.

Calculation:

The total solids contents, expressed as percentage by mass, is equal to:

= $M2{-}M0M1{-}M0\ x$ 100 +0.1 a

Where,

- M0= is the mass, in g of the dish (including zinc oxide), lid and stirring rod.
- M1 = is the mass, in g, of the dish (including zinc oxide), lid, stirring rod & test portion.
- M2 = is the mass, in g, of the dish, lid stirring rod and dried test portion (including zinc oxide) a = is the mass, in g, of lactic acid as obtained in Step I. it is equal to 0.1 g per g of lactic acid content. (**Ref***: IDF (2005) IDF Standard 151, Yoghurt. Determination of total solid content, International* Federation: Brussels).

Methodology of Microbial Study of Curd sample[3]

Cell Counting: Spread plate method used for counting the number of cells in the curd

1. Pipette curd 1ml. Dilute with 9ml of distilled water, and make serial dilution.
2. Pipette 0.1ml of the dilution to culture medium, *Lactobacillus bulgaricus* agar and *Streptococcus thermophiles* agar.
3. Dip triangle glass rod in alcohol, then fire and wait to cool. Use glass rod to spread dilution on surface of medium until dry.
4. Incubate medium at 40-45°C for 48 hours in anaerobic jar for recording colony numbers.

Methodology of Phyto-chemical & Physico-chemical Analysis of the Ayur-probiotic preparation

I) TEST OF CARBOHYDRATES:

a) Molisch's Test (General Test): 2 – 3 ml aq. Extract + few drops of alpha naphthol solution in alcohol shake and add concentrated H_2SO_4 from sides of test tube.
- Violet ring is formed at the junction of 2 liquids.

1) Test for reducing Sugars

a) **Fehling's Test**: Mix 1 ml Fehling's A and 1 ml Fehling's B solution boil for 1 minute.

Add equal volume of test solution. Heat in boiling water for 5 –10 minutes.

- 1st a yellow, then brick red precipitate is observed.

b) **Benedict's test:** Mix equal volume of Benedict's reagent and test solution in the testtube. Heat in boiling water bat for 5 minutes.

- Solution appears green, yellow or red depending on amount of reducing sugar presentin test solution.

2) Test for Monosaccharides:

a) **Barfoed's Test:** Mix equal volume of Barfoed's reagent and test solution.Heat for 1 –2 minutes in boiling water bath and cool.

- Red precipitate is observed

3) Tests for Hexose Sugars:

a) **Selwinoff's Test (for Ketohexose like fructose);** Heat 3 ml Selwinoff's reagent and 1 ml test solution in boiling water bath for 1 to 2 minutes.

- Red colour is found.

b) **Tollenphloroglucinaol test for galactose:** Mix 2.5 ml concentrated HCl and 1ml 0.5% phloroglucinol. Add 1 – 2 ml test solution.

- Yellow to red colour appears.

c) **Cobalt Chloride Test:** Mix 3 ml test solution with 2 ml Cobalt chloride. Boil and cool. Add few drops of NaOH solution.

- Solution appears greenish blue (glucose) or purplish (fructose) or upper layergreenish blue and lower layer purplish (mixture of glucose and fructose)

4) Test for Non-reducing sugars:

a) Test solution does not give response to Fehling's & Benedict's Tests.

b) Hydrolyse Test solution; Fehling's & Benedict's test are positive.

5) Test for Non-reducing Polysaccharides (Starch):

a) **Iodine Test:** Mix 3 ml test solution and few drops of dilute Iodine solution. Bluecolour appears, it disappears on boiling and reappears on cooling.

b) **Tannic acid test for starch:** With 20% tannic acid test solution gives precipitate.

II) TEST FOR PROTEINS:

a) Biuret Test (General Test): To 3 ml T. S., add 4% NaOH and few drops of 1% $CuSO_4$ solution.

- Violet or pink colour appears.

b) Million's Test for Proteins: Mix 3 ml. T. S. with 5 ml. Millions Reagent gives whiteprecipitate. Warm precipitate turns brick red or the precipitate dissolves giving red coloured solution.

c) Xanthoprotein Test (for Protein containing tyrosine or tryptophan): *Mix 3 ml*

T.S. with 1 ml concentrated H_2SO_4

- White precipitate is formed. Boil
- Precipitate turns yellow, Add NH_4OH
- Precipitate turns orange.

d) Test for Proteins containing Sulphur: Mix 5 ml T. S. with 2 ml 40% NaOH and 2 drops of 10% lead acetate solution. Boil, solution turns black or brownish due to PbS formation.

e) Precipitation Test:

The Test solution gives white colloidal precipitate with following reagents:

a) Absolute alcohol.

b) 5% $HgCl_2$ solution.

c) 5% Lead Acetate

d) 5% Ammonium sulphate

III) TESTS FOR AMINO ACIDS:

a) Ninhydrin Test (General Test): Heat 3 ml T.S. and 3 drops 5% Ninhydrin solutionin boil water bath 10 minutes

- Purple or bluish colour appears.

b) Test for Tyrosine: Heat 3 ml T. S. and 3 drops Million's reagent solution.

- Solution shows dark red colour.

c) Test for Tryptophan: To 3 ml T.S. and few drops of glyoxalic acid and concentrated H_2SO_4

- Reddish Violet ring appears at junction of the two layers.

d) Test for Cysteine: To 5 ml T.S. and add few drops of 40% NaOH and 10% leadacetate solution. Boil it. Black precipitate of lead sulphate is formed

IV) TESTS FOR TANNINS AND PHENOLIC COMPOUNDS:

To 2–3 ml of aqueous or alcoholic extracts, add few drops of following reagents:

a) 5% $FeCl_3$ solution: Deep blue-black color.

b) Lead acetate solution: - White precipitate

c) Gelatin solution:- White precipitate

d) Bromine water:- Discoloration of bromine water

e) Acetic acid solution:- Red color solution

f) Potassium dichromate: Red precipitate

g) Dilute iodine solution: Transient red color

h) Dilute HNO_3: Reddish to yellow color

i) Dilute $KMNO_4$ solution: Discoloration.

V) TESTS FOR STEROIDS:

a) Salkowski reaction: To 2 ml of extract, add 2 ml of chloroform and 2 ml concentrated H_2SO_4. Shake well.

- Chloroform layer appears. Red and acid layer shows greenish yellow fluorescence.

b) Liebermann –Burchard reaction: Mix 2 ml extract with chloroform. Add 1–2 ml acetic anhydride and 2 drops concentrated H_2SO_4 from the side of test tube. First red, then blue and finally green colour appears.

c) Liebermann's Reaction: Mix 3 ml extract with 3 ml acetic anhydride. Heat and cool. Add few drops of concentrated H_2SO_4. Blue colour appears.

VI) TESTS FOR GLYCOSIDES:

Tests for Cardiac Glycosides:

a) Test for Deoxysugars (Keller – Killani Test):

To 2 ml extract, add glacial acetic acid, one drop 5% $FeCl_3$ and concentrated H_2SO4. Reddish brown colour appears at junction of the two liquid layers and upper layer appears bluish green.

Test for saponin glycosides:

b) Foam Test: Shake the drug extract or dry powder vigorously with water persistent.

METHODOLOGIES FOR THE LAB INVESTIGATIONS DONE

I. Methodology (SoP) for Microbiological Tests:

1. Isolation of Lactic Acid Bacteria (LAB) from fecal samples:

For isolation, 1g of feces from each animal was removed under aseptic conditions, then suspended in 9 ml sterile saline and homogenized for 3 min. Bacterial isolation was performed by preparing serial dilutions of the samples with sterilized maximum recovery diluents (pH -7); 0.1 ml of the dilution was spread on the MRS agar for LAB isolation. In current study Faecal samples of various groups (A, B, C, D, E, F, G and H) were diluted serially from 10^{-1} to 10^{-7}, then 0.1 ml aliquot of the higher dilutions were spread on to MRS plates and incubated at 37^0C for 24-48 h (De Man et al., 1960). White and creamy colonies were picked up randomly and purified by three successive transfers on MRS medium. The cultures were routinely checked. The pure cultures were maintained by sub culturing twice in MRS broth before use. Further Pure cultures were stored at cold conditions for longer storage. At the end of incubation (37°C for 48 h), the LAB were selected based on their morphology in their selective media such as colony character. Selected bacterial isolates will be screened for molecular 16s RNA gene sequencing for identification of bacteria.

2. Extraction of bacterial DNA:

The DNA extraction and purification from bacterial isolates were carried out according to the procedure described by Cheng and Jiang, 2006. Overnight bacterial

cultures were centrifuged individually at 15,000g for 10 min. Pellets were washed with 400 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5), then centrifuged at 10,000g for 10 min. The pellets were resuspended in TE buffer (Tris-EDTA buffer, pH 8.0), 100 µl tris-saturated phenol (pH 8.0) was added and the suspensions were centrifuged at 10,000g for 10 min at 4 ^{0}C. Mixtures composed each of 160 µl of the obtained aqueous phase, 40 µl TE buffer and100µl chloroform were centrifuged at 10,000g for 10 min. The resulting supernatant (100 µl) was mixed with 40 µl TE buffer and 5 µl RNase (10 mg/ml) and incubated at 37 ^{0}C for 10 min. Then 100 µl of chloroform was added and the mixture was centrifuged at 15,000g for 10min at 4^{0}C. The isolated DNA was subjected for 16s RNA gene sequencing for molecular identification.

3. Polymerase Chain Reaction (PCR) amplification:

DNA was isolated from the provided culture. Quality was evaluated on 1.8% Agarose Gel; a single band of high-molecular weight DNA has been observed. Isolated DNA was amplified with *16s rRNA* Specific Primer (8Fand 1492R) using Veriti® 96 well Thermal Cycler. A single discrete PCR amplicon band of ~1500 bp was observed (Figure.1). The PCR amplicon was bead purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3500Dx Genetic Analyzer. Consensus sequence of 1500bp of *16s region* was generated from forward and reverse sequencedata using aligner software.

II. Methodology (SoP): Histological Samples Preparation:

All tissue samples were fixed in 10% buffered formalin for 5 days, followed by a progressive dehydration procedure using several alcoholic baths of different percentages (70%, 96%, and absolute ethanol). Afterwards, the clarification of the samples was realized using three repeated baths of 1-butanol (one hour each). The samples thus prepared were embedded in paraffin and sectioned using the Leica microtome (RM2125, Leica, Nussloch, Germany) into 5 µm sections. After displaying the sections on the slides, they were stained using Goldner's trichrome technique.

III. Laboratory Tests for the Clostridium difficile:

Clostridium (re-classified as "Clostridioides") difficile is an anaerobic, gram-positive bacterium that causes significant disease through elaboration of two potent toxins in patients whose normal gut microbiota has been altered through antimicrobial or chemotherapeutic agents (dysbiosis).

A variety of methods exist for testing patients at risk for C. difficile. These can be broadly categorized into two groups:

(1) those tests that detect the organism itself (or a component of the organism), such as anaerobic bacterial culture, glutamate dehydrogenase (GDH), the common antigen present in both toxigenic and nontoxigenic strains, and nucleic acid tests that detect the genes that encode toxins A and B.

(2) Tests that detect free toxin, such as cell culture cytotoxicity neutralization assays (CCCNA) and enzyme immunoassays (EIAs). Bacterial culture with toxin testing of recovered isolates (toxigenic culture [TC]) and CCCNA are considered reference methods and the standards against which other methods are compared[120, 121, 122].

Cell Culture Cytotoxicity Neutralization Assays (CCCNA):

The development of a CCCNA by Bartlett et al and Chang et al using initially primary human amnion cell cultures, facilitated the discovery that cytotoxins elaborated by C. difficile were responsible for pseudomembranous colitis, and this became the first diagnostic test for detection of C. difficile in stool[123,124].

Cell Culture Cytotoxicity Neutralization Assay (CNA) and Toxigenic Culture (TC) are considered as gold standard reference tests for detection of C. difficile. (Gold standard tests)

Methodology:

This test method requires multiple steps that include placing an aliquot of stool in a buffer solution, centrifuging it, and filtering the supernatant. The filtrate is diluted and then inoculated to a cell culture monolayer. A variety of different cell types can be used and human fibroblasts are considered the most sensitive[125, 126]. After incubation at 37°C for 24 hours, the plates are assessed for cytopathic effect (CPE) that is characterized by rounding and spiculation of the cells. CPE must be neutralized by C. difficile or Clostridium sordellii antitoxin to prove that CPE is not related to nonspecific substances in stool. Although CCCNA is considered as a reference method, and is a sensitive method for the detection of toxin B, clinical sensitivity is <90%.

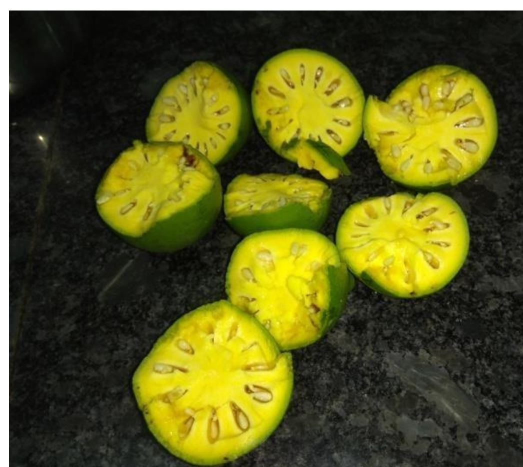
Detection of Cytotoxin in stool samples by CCCNA is a more accurate reflection of active disease than other methods[127, 128].

Methodology:

An extract of the fecal flora contained a substance important for the colonization of the normal ceca flora. Fluid secretion in the small intestine was caused by all toxins, but only toxins A and C caused shedding of epithelial cells from the villi without visible damage to the crypt cells. Toxin B, as well toxin A induced intestinal epithelial cell damage, increased mucosal permeability and caused an acute inflammatory response[129]. Wilson and[130] did not use a mouse model but studied the effects of using the entire cecal flora from mice for elimination of *C. difficile* from the mouse cecum. They suggested that as yet unidentified commensal organisms competed more efficiently than *C. difficile* for the available nutrients, and that substances produced by indigenous flora may decrease the growth rate even when a nutritionally rich medium was available.

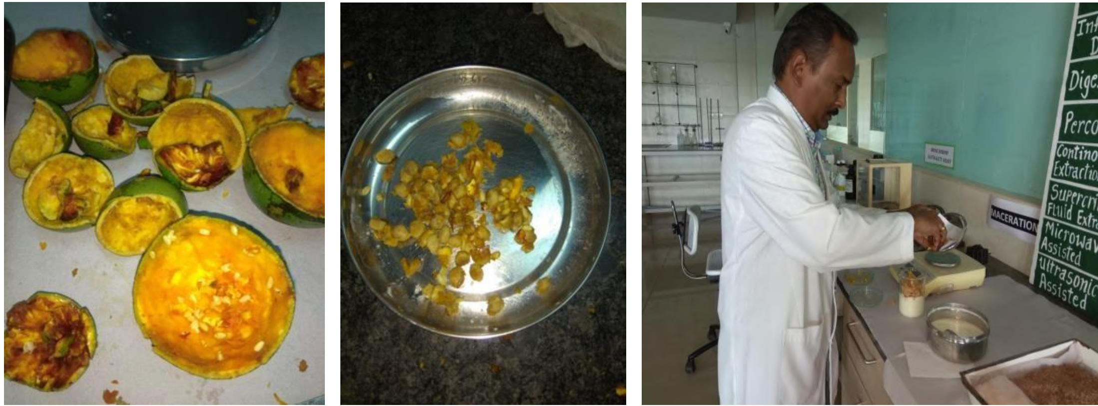
IMAGES

COW MILK SAMPLE **COW MILK CURD SAMPLE**



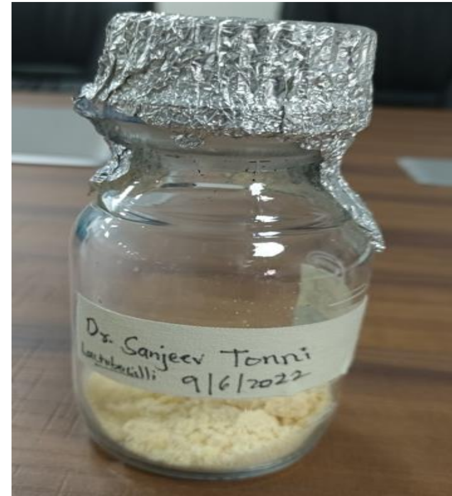
APAKWA BILWA PHALA **(Un-ripened Bael fruit)**

Deseeded unripened Bael fruit)



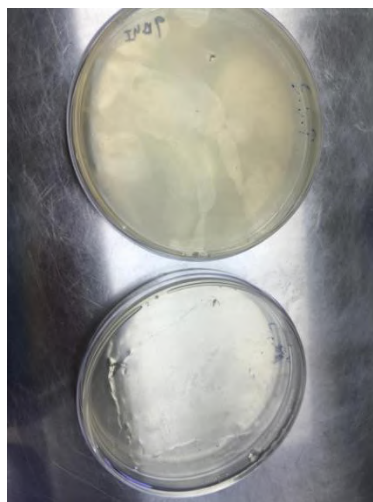
Measurement of curd & apakwa bilwaphala majja

Transferring formulation into Lyophilizer bulbs

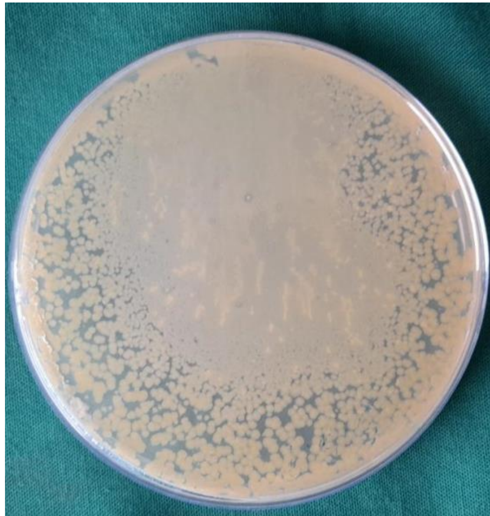


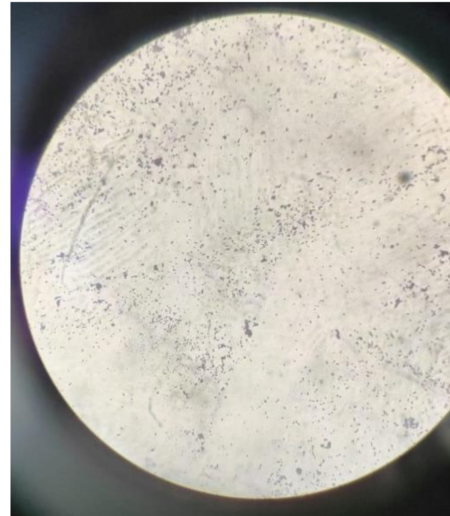
Lyophilized sample of plain curd

Bilwaphalasaara dadhi in Lyophilized form



Culture test



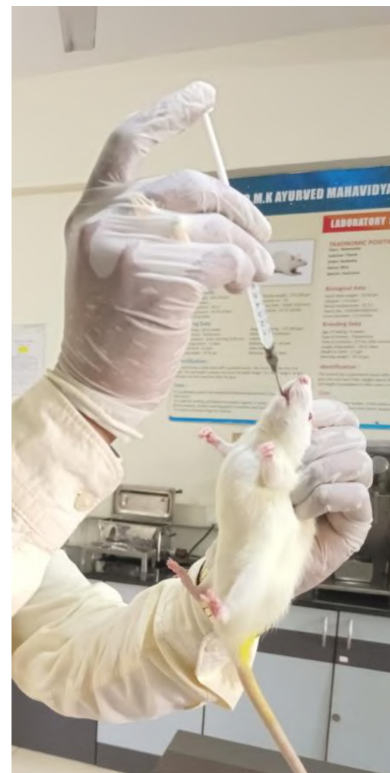


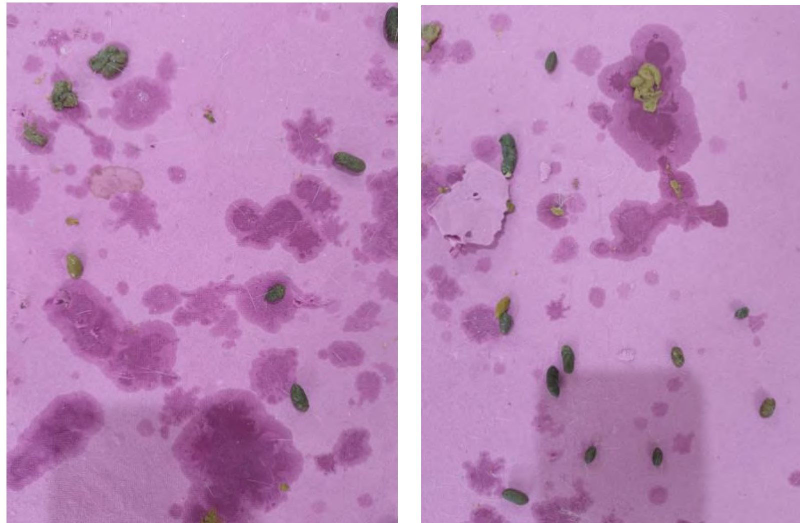
Showing growth of probiotic organisms (LAB)

Gram staining

Animal Grouping & Marking details

Therapeutic dose was converted according to Paget's Table for each group intervention





Stool observation

Animal Experimentation

PUBLICATIONS

ISSN: 2455-6211
www.ijaresm.com

International Journal of All Research Education & Scientific Methods

(International UGC & Peer Reviewed Referred Journal)

Volume 10, Issue 5
May-2022
(Part-13)

Published By:

IJARESM Publication, India
450/1, Bhagwan Mahavir Marg, Opposite Main Bus Stand, Rithala, Rohini, New Delhi - 110085, India
Mobile: +91-8607698989, +91-8684930049, E-Mail Id: editor.ijaresm@gmail.com

International Journal of All Research Education and Scientific Methods (IJARESM), ISSN: 2455-6211
Volume 10, Issue 5, May-2022, Impact Factor: 7.429, Available online at: www.ijaresm.com

Development of Lyophilized Bilwaphalasaara dahi -A Novel Formulation

Dr. Tonni Sanjeev Shivappa[1], Dr. Rudramma R Hiremath[2], Dr. Khatib Nayeem[3]

[1]Ph.D Scholar, Department of Swasthavritta, KAHER's Shri.B M K Ayurveda Mahavidyalaya Shahapur Belagavi, Karnataka India
[2]Professor and Head, Department of Agadatantra, KAHER's Shri.B M K Ayurveda Mahavidyalaya Shahapur Belagavi, Karnataka India
[3]Professor and Head, Department of Pharmacology KLE's College of Pharmacy KAHER, Belagavi, Karnataka India

---*****************---

ABSTRACT

Introduction: Recently there has been an increasing interest in the use of natural food additives and incorporation of health promoting substances into the diet. A study was undertaken to assess the effect of lyophilized Bilwaphalasaara dahi on the organoleptic characters, physico-chemical and phyto-chemical properties. An objective of the study is to develop bilwaphala saara dahi in a lyophilized form with different strengths and to assess the quality control analysis of all the lyophilized samples of bilwaphala saara dahi.

Materials & Methods: Here apakwa-bilwa phala majja (Un-ripened Bael fruit pulp) is mixed with dahi(curd). The product was analyzed for variour parameters.

Result: Presence of reducing sugar was identified in Sample-A, C & D. Presence of protein was identified in sample-C of water extract and sample-D in both the extract. Sample-D showed the presence of amino acids in both the extract. Presence of tannins was identified in Sample-C and D in both the extract. Highest percentage of loss on drying of 11.706 % of Sample-A was recorded. Highest percentage ash of 6.972 % was recorded in Sample-B. Highest percentage of acid insoluble ash of 1.045 % was recorded in Sample-B. Highest percentage of water soluble extracts of 94.417 % was recorded in Sample-C. Highest percentage of alcohol soluble extracts of 53.302 % was recorded in Sample-A. Highest pH of 4.34 was recorded in Sample-D followed by 4.03 (Sample-C), 3.74 (Sample-B) and 3.34 (Sample-A).

Discussion: Dahi is a semi-solid fermented milk product made by fermentation process of fresh milk and considered as a functional food because of its lactic acid bacteria (LAB) that provide significant therapeutic value. When curd is combined with various herbal drugs or plant extracts, its functionality like anti-oxidative, immuno-modulatory etc will be enhanced.

Conclusion: Study shown that apakwa-bilwaphala saara (fruit pulp extract) added dahi is superior to any other herbal incorporated dahi extract in the lyophilized form.

Key words: Bilwa, Dahi, Beal pulp, Lyophilization, Phytochemical, Organoleptic

INTRODUCTION

Milk is very nutritious and perhaps an indispensable food for human being. But in this era of industrialization, food habit of common people is changing. It is preferable as it is healthy, delicious food to fresh raw foods. Hence, milk is converted to various milk products including dahi. About 9% of the total milk produced in India is converted to fermented milk products[1]. Dahi is the most popular fermented dairy product in the Indian sub-continent and consumed by most of the people with everyday meal and it is also used to prepare several culinary dishes[2]. Dahi is highly nutritious, delicious and contain lots of gut health promoting factors in it[3] thereby increasing the longevity of human life. Nowadays, natural way of preservation with the help of erbal extract is gaining popularity. One such Ayurveda drug is bilwa (bael fruit), because of its antimicrobial and antioxidant profile, known to be widely prescribed drug[4]. Moreover, bael fruit is easily available at all seasons, very cheap and utilized by people for different purposes. The present study was aimed to produce bilwaphala saara dahi in a lyophilized form and its organoleptic, physico-chemical and phyto-chemical properties was investigated. The study was carried out with the following objectives:

1. To develop bilwaphala saara dahi in a lyophilized form with different strengths.
2. To assess the quality control analysis of all the lyophilized samples of bilwaphala saara dahi.

International Journal of All Research Education and Scientific Methods (IJARESM), ISSN: 2455-6211
Volume 10, Issue 5, May-2022, Impact Factor: 7.429, Available online at: www.ijaresm.com

MATERIALS AND METHODS

Collection and Authentication of Cow milk & curd[5]:

- Cow milk sample was procured by known milk vendor.
- Curd was prepared by traditional method.
- Authentication of Cow milk & curd sample was done at Divisional Food Laboratory, Vaccine Depo, Belagavi.

Collection and Authentication of Plant Material:

- The fresh un-ripened bilwa (*Aegle marmelos.* **Linn***)* fruit was collected from the natural habitat, herbal garden of KLE Academy of Higher Education and Research (KAHER) Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi.
- Authentication was done in AYUSH approved Drug Testing laboratory, KAHER's Shri. B. M. Kankanawadi Ayurveda Mahavidyalaya.

Phyto-chemical Screening[6]:

Qualitative Analysis of tannins, phenols, flavonoids, saponins, alkaloids, steroids, terpenoids was carried out in AYUSH approved Drug Testing Lab, KAHER's Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya.

Methodology of preparation of Bilva phala saara dahi:

Preparation of Bilva phala sara dadhi was done in Dr.Prabhakar Kore Basic Science Research Centre (BSRC) laboratory, KAHER, Belagavi. Fresh un-ripened Bael fruit was thoroughly washed in clean water to remove its physical impurities. The washed un-ripened Bael fruits were broken and the pulp along with the seeds and fibre were scooped out. Then seeds were separated from the pulp. The pulp was collected in clean vessel. 100ml of curd was poured to the clean vessel from the sample. 50gm pulp ie, *apakwa phala majja* (pulp of un-ripened bael fruit) was weighed. Then, pulp in the curd will be mixed thoroughly with clean stirrer. Squeezed and blended the curd with blender for uniform size of the pulp. Labeled the sample (Name, quantity & date). The sample was kept it for one day in deeper part of the freezing compartment for further process ie, lyophilization. Next day, the sample was taken from the storage & the contents were transfer to lyophilized bulbs of lyophilizer. Adjusting the temperature at 40*c for 3 days, the sample became lyophilized with fine powder. Then the lyophilized product was transferred to clean airtight container. The lyophilized product sample was stored in cold storage for further assessing analytical parameters of the study[7].

- **Organoleptic characters, Physico-chemical and Phyto-chemical analysis**[6,8,9,10] of the Lyophilized samples (A, B, C & D ie, two curd samples of different quantity & two Bilwaphalasaara dadhi samples of different proportions) were assessed & results obtained in the CRF of KAHER's Shri B M K Ayurveda College, Belagavi.
- Aqueous and alcoholic extracts of Lyophilized samples A, B, C & D were subjected for qualitative preliminary phyto-chemical screening[6,8,9,10].

RESULTS

Table: 1. Showing the results of Organoleptic characters of the Lyophilized samples

Name of the Lyophilized Sample	Form	Colour	Taste	Odour
Sample-A	Powder	White	Sour	Milky
Sample-B	Powder	White	Sour	Milky
Sample-C	Powder	Light brown	Sour & bitter	Characteristic
Sample-D	Powder	Light brown	Sour & bitter	Characteristic

Table: 2. Showing the results of Phytochemical analysis of the samples

Name of the Lyophilized Sample	Test for carbohydrate		Test for reducing sugar		Test for monosaccharides		Test for pentose sugar		Test for non-reducing sugar	
	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol
Sample-A	+	+	+	-	-	-	-	-	-	-
Sample-B	+	+	-	-	-	-	-	-	-	-

International Journal of All Research Education and Scientific Methods (IJARESM), ISSN: 2455-6211
Volume 10, Issue 5, May-2022, Impact Factor: 7.429, Available online at: www.ijaresm.com

Sample-C	+	+	+	-	-	-	-	-	-	-
Sample-D	+	+	+	-	-	-	-	-	-	-

Table: 3. Showing the results of Phytochemical analysis of the samples

Name of the Lyophilized Sample	Test for hexose sugar		Test for proteins		Test for amino acids		Test for steroids		Test for flavonoids	
	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol
Sample-A	-	-	-	-	-	-	-	-	-	-
Sample-B	-	-	-	-	-	-	-	-	-	-
Sample-C	-	-	+	-	-	-	-	-	-	-
Sample-D	-	-	+	+	+	+	-	-	-	-

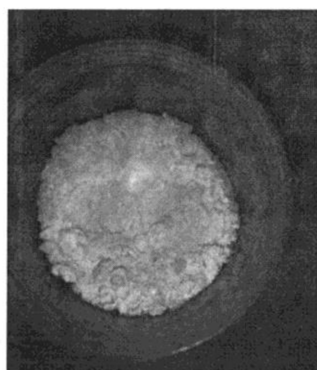
Table: 2. showing the results of Phytochemical analysis of the samples

Name of the Lyophilized Sample	Test for alkaloids		Test for tannins		Test for glycosides	
	Water	Alcohol	Water	Alcohol	Water	Alcohol
Sample-A	+	+	-	-	-	-
Sample-B	-	-	-	-	-	-
Sample-C	-	-	+	+	+	-
Sample-D	-	-	+	+	+	-

+ = Presence - = Absence

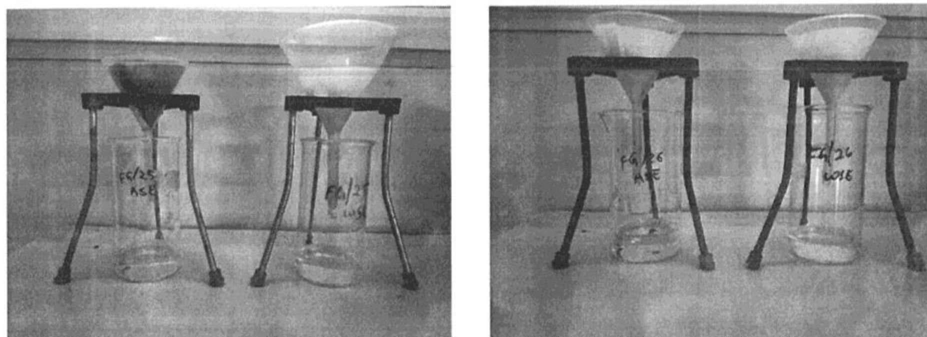
Table: 4. Showing the results of Physico-Chemical properties of the Lyophilized samples

Name of the Lyophilized Sample	Loss on drying	Ash value	Acid insoluble ash	Water soluble extractive	Alcohol soluble extractive	Ph value (5%)
Sample-A	11.706%	5.732%	0.797%	82.002%	53.302%	3.34
Sample-B	8.391%	6.972%	1.045%	93.884%	44.479%	3.74
Sample-C	8.449%	6.237%	0.948%	94.417%	45.772%	4.03
Sample-D	6.593%	5.932%	0.598%	55.980%	45.295%	4.34



Lyophilized samples of Bilwaphalasaara dahi

International Journal of All Research Education and Scientific Methods (IJARESM), ISSN: 2455-6211
Volume 10, Issue 5, May-2022, Impact Factor: 7.429, Available online at: www.ijaresm.com



Alcohol soluble & water soluble extracts of the lyophilized samples

DISCUSSION

Phyto-chemical analysis of Bilwaphalasaara dahi:

Table.1 shows the phyto-chemical properties of lyophilized bilwaphala saara dahi with water and alcohol extract stored at refrigerated temperature. Presence of carbohydrate was identified in all the four samples of water & alcohol extract. Presence of reducing sugar was identified in Sample-A, C & D but it was absent in Sample-B of water extract whereas all are shown absent in alcohol extract.

Presence of monosaccharide, pentose sugar, non-reducing sugar, hexose sugar, steroids and flavonoids was not identified in both the extracts in all the four samples. Presence of protein was identified in sample-C of water extract and sample-D in both the extract. Absence of protein was seen in Sample-A, B of water extract and same was also not seen in Sample-a, B and C of alcohol extract.

Presence of amaino acids was not identified in Sample A, B and C in both the extract, whereas Sample-D shown the presence of amino acids in both the extract. Presence of alkaloids was identified in Sample-A in both the extract, whereas absence was noted in rest of the samples of both the extract.

Presence of tannins was identified in Sample-C and D in both the extract, whereas absence was noted in the Sample-A and B of both the extract. Presence of glycosoides was identified in in Sample-C and D of water extract but shown absence in Sample-A and B, whereas glycosides are seen absent in all the four samples of alcohol extract.

Physico-chemical analysis of Bilwaphalasaara dahi:
Table.2 shows the physico-chemical properties of lyophilized bilwaphala saara dahi of all the four samples with 0.5% aqueous extract stored at refrigerated temperature.

Highest percentage of loss on drying of 11.706 % of Sample-A was recorded followed by 8.391%, 8.449 % and 6.593 % of Sample-B, C and D respectively. Ash content of herbal incorporated dahi differed significantly in most of the treatment combinations. Ash variation was attributed due to the ratio variation of *apakwa bilwaphala majja* (Pulp of un-ripened bael fruit) used in different proportions.

Highest percentage ash of 6.972 % was recorded in Sample-B followed by 6.237 % of Sample-C, 5.932 % of Sample-D and 5.732 % in Sample-A. Ash content of herbal incorporated dahi differed significantly in most of the treatment combinations. Ash variation was attributed due to the ratio variation of apakwa biwaphala majja used in different proportions.

Highest percentage of acid insoluble ash of 1.045 % was recorded in Sample-B followed by 0.948 % of Sample-C, 0.797 % of Sample-A and 0.598 % of Sample-D. Acid insoluble ash variation was attributed due to the ratio variation of apakwa biwaphala majja used in different proportions.

Highest percentage of water soluble extracts of 94.417 % was recorded in Sample-C followed by 93.884 % of Sample-B, 82.002 % of Sample-A and 55.980 % in Sample-D. Variations in water soluble extracts were attributed due to the ratio variation of apakwa biwaphala majja used in different proportions.

International Journal of All Research Education and Scientific Methods (IJARESM), ISSN: 2455-6211
Volume 10, Issue 5, May-2022, Impact Factor: 7.429, Available online at: www.ijaresm.com

Highest percentage of alcohol soluble extracts of 53.302 % was recorded in Sample-A followed by 45.772 % of Sample-C. 45.295 % of Sample-D and 44.479 % of Sample-B. Variations in alcohol soluble extracts were attributed due to the ratio variation of apakwa biwaphala majja used in different proportions.

Highest pH of 4.34 was recorded in Sample-D followed by 4.03 (Sample-C), 3.74 (Sample-B) and 3.34 (Sample-A). pH content of herbal incorporated dahi differed significantly in most of the proportionated combinations.

Organo-leptic analysis of Bilwaphalasaaradahi:

All the four samples were in the powdered (lyophilized) form. Sample-A and B are seen white in colour and tasted sour whereas Sample-C and D are seen in light brown colour and tasted sour and bitter. This is because of *apakwa biwaphala majja* (unripened bael fruit pulp) incorporated in different proportions. Sample-A and B perceived milky odour whereas Sample-C and D shown of its characteristic odour. The difference in the odour and taste noted in different combinations was due to the ratio variation of *apakwa biwaphala majja* incorporated in different proportions.

Lyophilization technique has many advantages, especially for heat sensitive products. The lyophilized products can be stored at ambient temperature over a 2 years shelf life and it can enhance product stability in a dry state. Therefore, in this study, lyophilized technique was used for preparing the sample[11]. Functional food provides biological and therapeutic properties beyond their basic nutritional value[12] (Hasler, 2002) which incorporate readily into diet food and proposed to reduce disease risk[13] (Buckley et al., 2011). Functional foods can be regarded as foods with a health benefit beyond satisfying traditional nutritional requirements[14] (Sanders, 1998). Curd (Dahi) is considered as a functional food because of its lactic acid bacteria (LAB) that provide significant therapeutic value during milk fermentation including the highly digestible nutrients[15] (Deeth and Tamine, 1981) as well as the ability to produce various antimicrobial compounds. reduce serum cholesterol, alleviate lactose intolerance ,stimulate immune system and stabilize guts microflora[16](Gibson et al.,1997).

CONCLUSION

The current study was designed to develop a novel Ayurvedic probiotic and determine the phytochemical, physico-chemical and organoleptic qualities of lyophilized bilwaphalasaara dahi samples of different proportions. Standard method was followed to prepare dahi and apakwa bilwaphala majja extract. Prepared lyophilized samples were stored under refrigerated condition and subjected to various phytochemical, physico-chemical and organoleptic quality analysis at periodic interval. The presence of high amount of carbohydrates, proteins, alkaloids, and tannins in lyophilized Samples indicates that the thermo-liable compounds are seen after lyophilisation process. Therefore, lyophilized extract can also be used to prepare supplementary products for gastro-intestinal tract involving conditions such as mal-absorption syndrome, in bacterial and viral origin diarrhoea, irritable bowel syndrome and recently it can also be utilized in antibiotic associated diarrhoea.

Hence Study may be concluded that apakwa bilwaphalasaara (fruit pulp extract) extract added dahi is superior to any other herbal incorporated dahi extract in the lyophilized form.

Conflicts of Interest: There are no conflicts of interest

Funding: None

Acknowledgement: We thank to **Dr.Giridhar Vedantam** Unit Head, CRF (Central Research Facility), Dr.Mahadev G. Coordinator, Phytochemical Analysis Wing KAHER's Shri.B.M.K Ayurveda Mahavidyalaya Shahapur Belagavi Karnataka India for their timely help and support.

REFERENCES

[1] Singh, R. (2007). Characteristics and technology of traditional and cultural dairy products. Bulletin of the Dairy International Federation. 415: 11-20.

[2] Shekhar,S., Joe,J Kumar,R Jyothi,J Kumar,R.M.K, Priya,Y.A Rao,K.J and Pagote,C.N. (2013) Heat treatment of milk on the sensory and rheological quality of dahi prepared from cow milk. Research and Reviews.J. of Fd.and Dairy Technol.,1(1):8-14.

[3] Hosono, A., Kashina,T and Kada,T. (1986) Anti-mutagenic properties of lactic acid cultured milk on chemical and fecal mutagens. J. Dairy Sci. 69:2237-2242.

[4] Chakraborty, D and Shah, B. (2011) Antimicrobial, anti-oxidative and anti-hemolytic activity of Piper betel leaf extracts. Int. J. of Pharmacy and Pharmaceutical sciences, 3(3):193-199.

[5] *FSSAI*: Manual of methods of analysis of foods *Milk and milk products*. Food Safety and Standards Authority of India, Ministry of Health and Family Welfare, Government of India, New Delhi; (2015).

International Journal of All Research Education and Scientific Methods (IJARESM), ISSN: 2455-6211
Volume 10, Issue 5, May-2022, Impact Factor: 7.429, Available online at: www.ijaresm.com

[6] Khandelwal KR; Practical Pharmacognosy, 13th Edition, Nirali Prakashan, Pg.no.143-53. (2005).

[7] Nireesha G, Divya L, Sowmya C, Venkateshan N, Babu MN, Lavakumar V. Lyophilization/freeze drying-an review. Int J Nov Trends Pharm Sci 2013; 3:87-98.

[8] Savithramma N., Linga Rao M., Suhrulatha D., Screening of Medicinal Plants for Secondary Metabolites. MiddleEast J. Sci. Res., 8 (3): 579-584, (2011).

[9] Soni A., Sosa S., Phytochemical Analysis and Free Radical Scavenging Potential of Herbal and Medicinal Plant Extracts. J Pharma. Phytochem.,2 (4): 22-29 , (2013).

[10] Shah P., Modi H. A., Shukla M. D., Lahiri S. K., Preliminary Phytochemical Analysis and Antibacterial Activity of Ganoderma lucidum collected from Dang District of Gujarat, India. Int. J. Curr. Microbiol. App. Sci., 3(3): 246-255, (2014).

[11] Nireesha G, Divya L, Sowmya C, Venkateshan N, Babu MN, Lavakumar V. Lyophilization/freeze drying-an review. Int J Nov Trends Pharm Sci 2013; 3:87-98.

[12] Hasler.C.M, (2002). Functional foods: benefits, concerns and challenges-a position paper from the American council on Science and Health , Journal of Nutrition 132 ,3772-3781.

[13] Buckley.N.D,C.P.Champagne,A.I.Mastti,L.E.Wagar,T.a.Tompkins,J.M.Green Johnson (2011) Harnessing functional food strategies for the health challenges of space ravelFermented soy for astronaut nutrition. Acta Astronautica 68, 731-73.

[14] Sanders, M.E. (1998). Probiotics Food Technology, 53:67-77.

[15] Deeth.H.C and A.Y.Tamine (1981) - Yogurt and therapeutic aspect. Journal of Food Protection 44 -48-86.p

[16] Gibson. G.R,J.M.Saveedra, S.Mac-Farlane,G.T.Mac-Farlane (1997)-Probiotic and intestinal infection: R.fuller (Ed.),Probiotic.2:Application and practical Aspect, Chapman & Hall, New York pp,10-39

JOURNAL OF AYURVEDA

An Official Publication of the National Institute of Ayurveda Deemed to be University (De-novo), Jaipur (Rajasthan)

Wolters Kluwer

Medknow

https://journals.lww.com/joay

joa Manuscript Accepted for Publication: joa_42_23

From: Journal of Ayurveda (ja@journalonweb.com)

To: sanjeevtonni@yahoo.com

Date: Sunday, 30 April, 2023 at 05:44 pm IST

Dear Dr Tonni,

The Editorial team of Journal of Ayurveda is pleased to inform you that your manuscript joa_42_23 entitled "Quality control parameters of Ayurvedic probiotic formulation 'Apakva Bilvaphalasara Dadhi'", is accepted for publication in the journal.

We will be sending you page proofs of the manuscript in about 2-3 weeks for final checking and corrections if any., through the manuscript management site before the publication of the manuscript.

Please note that the journal reserves the right to make changes in the language, grammar, presentation, etc. to suit the journal's requirements.

We thank you for submitting your valuable research work to Journal of Ayurveda.

With warm personal regards,

Yours sincerely,

The Editorial Team

Journal of Ayurveda

Journal Ethics

Wolters Kluwer and Journal/Association are committed to meeting and upholding standards of ethical behavior at all stages of the publication process. We follow closely the industry associations, such as the Committee on Publication Ethics (COPE), International Committee of Medical Journal Editors (ICMJE) and World Association of Medical Editors (WAME), that set standards and provide guidelines for best practices in order to meet these requirements. For a summary of our specific policies regarding duplicate publication, conflicts of interest, patient consent, etc., please visit www.Medknow.com/EthicalGuidelines.asp

Open Access Publication and Creative Commons Licensing

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

Digital Archiving

Wolters Kluwer Medknow provides for long-term digital preservation through two primary partnerships, Portico and CLOCKSS.

Portico is a leading digital preservation service worldwide. The content is preserved as an archival version and is not publically accessible via Portico, but is provided when required under specific conditions, such as discontinuation of the collection or catastrophic failure of the website.

CLOCKSS will enable any library to maintain their own archive of content from Wolters Kluwer Medknow and other publishers, with minimal technical effort and using cheaply available hardware.

Ahead of Print policy

Articles published online under the Ahead of Print model are considered published and can be cited and quoted using the DOI as the reference source. Wolters Kluwer Medknow has a policy that changes will not be made after publication of an article without following accepted procedures for making corrections to the scientific record.

Advertisements

While advertisements are crucial to this journal to be able to keep all content free for everyone, ethical considerations are in place to ensure the integrity of the journal and its content:

- "Pop-up" and "banner" ads appear on a random, rotating basis. The advertiser has no control or input over the pages where their ads appear.
- The Editorial Board has full and final approval over the content of all advertisements.
- Advertisers will never be shown any manuscripts or other content prior to publication.

Scope of the journal

JOURNAL OF AYURVEDA (JOAY) (Online ISSN: 2582-7693 and Print ISSN: 2321-0435), published on behalf of National Institute of Ayurveda, Deemed to be University (De - novo), is a peer-reviewed print and online (Open Access) Quarterly journal. The journal aims at continuous contribution for research, education and propagation of Ayush and interdisciplinary Sciences. JOAY includes articles related to health, ethical and social issues in the field of Literary, Pre-Clinical and Clinical Research. Articles with clinical interest and implications are given preference. The Journal publish several types of articles like editorial, letter to editor, preclinical study, clinical study, survey study, analytical study, review, case report etc. The journal does not charge for submission, processing or publication of manuscripts and online access.

Medknow Publications

joa Manuscript for Final Proof Uploaded: joa_42_23

From: Journal of Ayurveda (ja@journalonweb.com)

To: sanjeevtonni@yahoo.com

Date: Friday, 1 September, 2023 at 04:53 pm IST

Dear Dr Tonni

Thank you for completing proofing Journal of Ayurveda joa_42_23 Quality control parameters of Ayurvedic probiotic formulation 'Apakva Bilvaphalasara Dadhi' through the manuscript management site before publication of the manuscript.

We shall get back to you soon for any further corrections.

Please note that the journal reserves the right to make changes in the language, grammar, presentation, etc. to suit the journal's requirements.

We thank you for submitting your valuable research work to Journal of Ayurveda.

With warm personal regards,

Yours sincerely,
The Editorial Team
Journal of Ayurveda

Original Article

Quality Control Parameters of Ayurvedic Probiotic Formulation "*Apakva Bilvaphalasara Dadhi*"

Abstract

Background: *Aegle marmelos* known as "*Bilva*" and curd (*dadhi*) are widely used in Ayurvedic formulations with various indications. **Objectives:** A study was undertaken with the objective to develop quality control (QC) parameters for lyophilized Ayurvedic probiotic formulation prepared using a combination of *Apakva Bilva Phalasara* (unripe fruit pulp) and *Dadhi* (curd). **Methods:** The QC parameters studied were organoleptic characters, physicochemical properties, micro and macroelements, nutritive contents, preliminary phytochemical, and culture tests. **Results:** The lyophilized sample was creamy white with milky odor, sour, and coarse in texture. The pH was 3.57 ± 0.23. The macroelements, namely, nitrogen potassium, phosphorus, magnesium, calcium, and sodium, were 0.87% ± 0.06%, 0.17% ± 0.02%, 1.44% ± 0.40%, 0.54% ± 0.02%, 0.22% ± 0.01%, and 0.24% ± 0.02%, respectively. The preliminary phytochemical study found saponins, phenols, tannins, glycosides, alkaloids, terpenoids, flavonoids, and steroids. The culture test revealed the presence of probiotic organisms in lyophilized samples. **Conclusion:** Probiotic organisms were found in lyophilized samples demonstrating that it can be preserved for longer duration. Furthermore, the study forms QC parameters for the Ayurvedic probiotic formulation *Apakva Bilvaphalasara Dadhi*.

Keywords: *Apakva Bilva Phalasara, lyophilization, probiotic, quality control parameters, traditional*

Sanjeev Shivappa Tonni, Rudramma Rachayya Hiremath[1], Nayeem Khatib[2]

Departments of Swasthavritta and [1]Agadatantra, KAHER's Shri B M K Ayurveda Mahavidyalaya, [2]Department of Pharmacology, KLE's College of Pharmacy, KAHER, Belagavi, Karnataka, India

Introduction

Aegle marmelos (L) Corr. belonging to Rutacea locally known as "*Bael*" or "*Bilva*" known to possess various medicinal properties in traditional systems of medicine. It is one of the popular ingredients used in many Ayurvedic formulations with wide range of indications. Ayurveda classical texts such as *Charak Samhita*, *Sushruta Samhita*, *Ashtanga Sangraha*, *Astanga Hridaya*, and *Bhavasprakasha* have mentioned *Bilva* in many formulations, its parts such as root, bark, fruit, and leaves have been indicated in conditions such as *Jwara*, *Medoroga*, *Rajayakshma*, *Arsha*, *Gulma*, *Shotha*, *Grahani,* and different types of *Atisara* (watery or loose stools with or without blood or mucous). The *Charaka Samhita* mentions the use of fruit pulp or *Phalamajja* in different types of *Atisara* (Ch.Su. 2/19, 20; Ch.Chi. 3/182, 8/127, 14/230, 19/20, 19/26, 19/52, 19/104, 19/109, 19/110, 19/114).[1] The fruit is known to possess therapeutic qualities, since *A. marmelos* possesses a substantial amount of mucilage, which acts as a demulcent and is effective in treating chronic diarrheal illnesses, few researches have supported the traditional claim that unripe fruit may treat diarrhea. Furthermore, fruit's role in treating diarrhea and dysentery is mentioned in the British Pharmacopoeia.[2] It is known that the unripe fruit has anti-diarrheal qualities and is most useful in chronic diarrheas.[3,4] Besides, *A. marmelos* has been proven effective in animal models of physiological diarrhea and irritable bowel syndrome.[5-7] Ripe fruits are restorative, tonic, astringent, laxative, and beneficial to the heart and brain; Due to its digestive, astringent, and stomachic properties, the unripe fruits are utilized for the management of dysentery and diarrhea.[8] In addition to being used as an astringent in dysentery and a stomachic in diarrhea, fine powder of dried unripe fruit was also efficient against *Entamoeba histolytica* and *Ascaris lumbricoides*.[9] The activities of *A. marmelos are* attributed to its wide range of phytoconstituents

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-Share Alike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Tonni SS, Hiremath RR, Khatib N. Quality control parameters of Ayurvedic probiotic formulation "*Apakva Bilvaphalasara Dadhi.*" J Ayurveda 2023;

Submitted: 17-Feb-2023 **Revised:** 26-Apr-2023 **Accepted:** 30-Apr-2023 **Published:**

Address for correspondence:
Dr. Sanjeev Shivappa Tonni, Department of Swasthavritta, KAHER's Shri B M K Ayurveda Mahavidyalaya, Belagavi, Karnataka, India. E-mail: sanjeevtonni@yahoo.com

Access this article online

Website: https://journals.lww.com/joay/pages/default.aspx

DOI: 10.4103/joa.joa_42_23

Quick Response Code:

which include, polyphenols, coumarins, tannins, alkaloids, phenolic acids, organic acids, flavonoids, tocopherols, and carotenes.[10] The fruit has high nutritional composition which includes minerals (K, P, Fe, Cu, Zn, Ca, Cr, and Mg), fat, fiber (pectin, lignin, cellulose, and hemicellulose), carbohydrate, protein, vitamins (B_1, B_2, B_3, and C), amino acids, and fatty acids.[11]

The human gut microbiota plays a major role in nutritional development. Probiotics are "live microorganisms when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Consumption of food preparations containing probiotic bacteria, such as curd and yogurt, has increased significantly as a result of the probiotics concept.

Most individuals include curd in their regular meals as it is a well-known fermented milk product. Curd is nourishing, delicious, and full of components that support gut flora.[12,13] The potential health benefits of probiotic microbes such as *Lactobacillus johnsonii* La1, *Lactobacillus salivarius*, *Lactobacillus acidophilus,* as a result of lacto bacilli's improved lactose tolerance and digestion, the pathogen's ability to grow and activity of urease enzyme to survive in the stomach's acidic environment is inhibited. *Lactobacillus rhamnosus* GG, *Lactobacillus casei*, *Bifidobacterium lactis* BB-12, *Bifidobacterium bifidum*, and *Streptococcus thermophiles* improves intestinal function and prevents or treats acute diarrhea caused by bacterial infections.[14]

It is evident from the traditional literature and research studies that *A. marmelos* plays a significant role in treating diarrhea, also curd with plenty of probiotic microorganisms is useful in treating diarrhea besides maintaining gut microbiota. Therefore, a formulation was prepared using the ingredients *namely* unripe fruit pulp of *A. marmelos* and curd which are indicated to treat *Atisara* in Ayurveda. The formulation must be prepared afresh whenever it is required, and since it is unstable and prone to spoil quickly in its fresh state, it is also likely to have a limited shelf life. Therefore, these flaws remained to be of concern. Hence, the formulation was freeze-dried (lyophilized) assumed to give it a longer shelf life and similar efficacy. The study was carried with the objectives to develop a formulation in the combination of unripe fruit pulp of *A. marmelos* (*Bilva phalasara*) and curd (*Dadhi*) in lyophilized form and to assess the quality of lyophilized sample of *Apakva Bilvaphalasara Dadhi* (ABD).

Materials and Methods

Collection of raw materials

The fresh unripe *Bilva* fruits were collected from identified *A. marmelos*. (L.) Corr. The plant is located in the herbal garden of BMK Ayurveda College, Belagavi. The milk required for the preparation of curd was procured locally from native cow breeds [Figures 1 and 2].

Figure 1: Unripe fruit

Extraction of fruit pulp

The fruits were collected in the month of May, which varied in size and shape from nearly spherical and weighed 100–150 g were properly cleaned under running water to get rid of physical impurities. The unripe fruit pulp along with seeds was scooped out using sterile scoop, and the extracted fruit pulp was stored in a sterile container at room temperature till further use.

Preparation of curd (curdling)

A liter of milk collected was boiled in a clean stainless steel vessel and cooled to room temperature. About 10 g of inoculum of previously prepared curd was added to the vessel containing milk and kept undisturbed for a minimum of 6 h and the formed curd was used.

Preparation of *bilva phalasara dadhi*

The seeds were separated from fruit pulp. Three gram of fruit pulp was accurately weighed and collected and 100 mL of curd was accurately weighed and added to the vessel containing fruit pulp. The contents of the vessel were mixed thoroughly using stirrer until a homogenous mixture was formed. Three separate batches of mixtures were prepared and used separately for further analysis. The prepared mixtures were freeze-dried using lyophilizer (Christ), and the lyophilized samples were stored in dried container till further studies.

Organoleptic characters

The lyophilized sample of ABD was studied for organoleptic characters, namely color, texture, odor, and taste, following the standard methods.[15]

Physicochemical properties, assessment of micro and macroelements, and nutritive contents

Physicochemical parameters, namely loss on drying (at 105°C), total ash value, acid insoluble ash, alcohol soluble extractive, water-soluble extractive refereeing to

Figure 2: Curd

Figure 3: Lyophilized form of

standard reference and expressed in terms of % w/w, and pH, were observed as per standard methods.[15-17] Macro and microelements were assessed by atomic absorption techniques, nitrogen content was estimated by Kjeldahl method, nutritive contents, namely protein, fat, carbohydrates, and sugars, were studied as per standard procedures, and results are expressed in terms of percentage.[18-20]

Preliminary phytochemical analysis

Lyophilized sample was used to prepare aqueous and ethanol extracts, 10% of both the extracts were prepared and stored in 4°C till further use. To confirm the secondary metabolites, each extracts were subjected to preliminary phytochemical analysis using various reagents: Dragendorff's reagent for the detection of alkaloids, NaOH was used for the detection of flavonoids, ferric chloride was used for the detection of phenolic compounds and tannins, glacial acetic acid and ferric chloride were used for detection of glycosides.[19]

Culture test

The culture test was performed as explained by Mackie *et al.*[21]

- Two grams of each of samples of freshly prepared ABD and lyophilized sample of *Bilva phalasara dadhi* were taken separately in a flask containing MRS Broth as enrichment media and distilled water was topped up to 100 ml
- Both the samples were inoculated in blood agar media and incubated at 37°C in anaerobic jar for 48–72 h
- After 72 h of incubation, plates were removed and colonies were counted
- It was identified by classic microbiology examination including Gram-staining for detecting morphology, catalase tests, motility, and carbohydrates fermentation tests.

Results

On the basis of the pilot study conducted, 3 g of fresh unripe fruit pulp maintains the viability of probiotic activity of curd, and hence, 3 g of fresh unripe fruit pulp was mixed with 100 mL of curd. The yield of the lyophilized sample obtained after freeze-drying was 23.65 ± 0.33 g (n = 3).

Organoleptic characters

Organoleptic character refers to sensory evaluation wherein the methods of analysis such as color, odor, taste, and texture are performed using senses. The powder obtained by freeze-drying is depicted in Figure 3 and was used to evaluate the organoleptic characters, results are mentioned in Table 1.

Physicochemical properties

The results of physicochemical properties are mentioned in Table 2. All the parameters were studied in triplicates and the values are presented as mean ± standard deviation. The results revealed loss on drying, total ash, acid-insoluble ash, water-soluble extractive, and alcohol-soluble extractive were 11.55% ± 0.14%, 5.65% ± 0.08%, 27.22% ± 45.71%, 81.97% ± 0.15%, and 53.43% ± 0.13% w/w. The values of the pH were found to be 3.57 ± 0.23.

Estimation of macro and microelements

The results macro and microelements estimation are mentioned in Table 3. The macroelements, namely, nitrogen potassium, phosphorus, magnesium, calcium, and sodium, were 0.87% ± 0.06%, 0.17% ± 0.02%, 1.44% ± 0.40%, 0.54% ± 0.02%, 0.22% ± 0.01%, and 0.24% ± 0.02%. Similarly, microelements, namely, iron, zinc, copper, and manganese, were 21.73 ± 0.44, 12.60 ± 0.33, 11.75 ± 0.36, and 24.94 ± 0.34 PPM.

Estimation of nutritive composition

The nutritive composition of protein, fat, carbohydrates, and sugars was 8.97% ± 0.15%, 4.83% ± 0.15%, 5.77% ± 0.15%, and 4.17% ± 0.06%. The results are presented in Table 4.

Preliminary phytochemical analysis

The results of tests conducted for the detection of phytochemicals are mentioned in Table 5. The tests

Table 1: Organoleptic characters

Characters	Result
Color	Creamy white
Odor	Milky
Taste	Sour
Texture	Coarse powder

Table 2: Physicochemical properties

Parameters	Values (n=3)
Loss on drying	11.55±0.14*
Total ash value	5.65±0.08*
Acid insoluble ash	27.22±45.71*
Water-soluble extractive	81.97±0.15*
Alcohol-soluble extractive	53.43±0.13*
pH	3.57±0.23

*% w/w

Table 3: Estimation of macro and microelements

Elements	Values
Macroelements (%)	
Nitrogen	0.87±0.06
Potassium	0.17±0.02
Phosphorus	1.44±0.40
Magnesium	0.54±0.02
Calcium	0.22±0.01
Sodium	0.24±0.02
Microelements (ppm)	
Iron	21.73±0.44
Zinc	12.60±0.33
Copper	11.75±0.36
Manganese	24.94±0.34

revealed the presence of flavonoids, saponins, phenols, tannins, alkaloids, steroids, terpenoids, and glycosides in both extracts.

Culture test

By using a microscope, the isolated bacteria were examined. It is evident that the bacteria was rod-shaped, Gram-positive coccobacilli that occur single or in chains. The Gram staining indicated that the isolated bacteria were lactobacilli. It was evident from the result that the lactobacilli were observed in both freshly prepared ABD and also in its lyophilized sample. However, the colonies of lactobacilli varied quantitatively. The results are presented in Table 6.

Discussion

Transforming classical formulations into different dosage forms to gain numerous advantages besides retaining the therapeutic properties has become increasingly important in recent years.

Over the decade, probiotic products have become increasingly prevalent in the Indian market. However,

Table 4: Estimation of nutritive composition

Nutritive composition	Values (%)
Protein	8.97±0.15
Fat	4.83±0.15
Carbohydrates	5.77±0.15
Sugars	4.17±0.06

Table 5: Preliminary phytochemical analysis

Phytochemicals	Aqueous	Ethanol
Flavonoid	+	+
Saponin	+	+
Phenol	+	+
Tannin	+	+
Alkaloid	+	+
Steroids	+	+
Terpenoids	+	+
Glycosides	+	+

Table 6: Culture test

Sample	Result
Fresh *Bilva phalasara dadhi*	5×10^3 CFU/mL
Lyophilized sample	3×10^4 CFU/mL

CFU: Colony-forming unit

there has not been an organized investigation of probiotics in food to validate their efficacy and safety.[22] Standardization, quality assurance, and product shelf life are further major concerns. The formulation's shelf life is always determined by the pharmaceutical process and its composition. There are couple of reports, in which classical Ayurvedic formulations have been transformed into convenient dosage forms.[23,24] Fresh curd is a good source of bioavailable vitamins, minerals, and folate. Consumption of curd may enhance the immune response in the elderly. These health benefits have been linked to the presence of live bacteria.[25] According to the guidelines, 100 mL of curd is said to be the recommended dosage for a day consumption which is capable of producing the required amount of probiotic organisms for beneficial action over intestinal flora.[26] The lyophilization process offers various benefits, especially for products that are sensitive to heat. The lyophilized products may be transported and stored at room temperature, and in addition to having a longer shelf life, it can enhance the stability of the product in a dry state and ensure that the constituents are homogenously distributed.[27,28] Therefore, in the present study lyophilized technique was applied for preparing the sample. The presence of nutrients such as carbohydrates, proteins, and fats and secondary metabolites such as flavonoids, saponins, alkaloids, and tannins in lyophilized samples indicate that the thermolabile compounds are found even after the lyophilization process. Therefore, lyophilized extract can also be used to prepare supplementary products for the gastrointestinal tract involving conditions such

as malabsorption syndrome, bacterial and viral infested diarrhea, irritable bowel syndrome, and recently it can also be utilized in antibiotic-associated diarrhea.

The study forms quality control parameters for the traditional probiotic formulation ABD prepared using unripe fruit pulp of *Bilva* and curd. It is also evident from the result that probiotic organisms are viable in lyophilized samples, which is a clear indication that the formulation can be stored for a longer duration in its dry form. Further studies are in the pipeline to evaluate its efficacy on animals and humans.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Ahirrao HA, Sangoram AM. Bilva (*Aegle marmelos* L.Correa) – A review from ancient literature. World J Pharm Pharm Sci 2017;6:501-22.
2. Chopra R. Indigenous Drugs of India. Calcutta: Academic Publishers; 1982.
3. Nadkarni AK. Indian Materia Medica. Mumbai: Popular Prakashan; 1954. p.1-3.
4. Satyavati GV, Gupta AK, Tandon N. Medicinal Plants of India. New Delhi: Indian Council of Medical Research; 1976.
5. Shoba FG, Thomas M. Study of antidiarrhoeal activity of four medicinal plants in castor-oil induced diarrhoea. J Ethnopharmacol 2001;76:73-6.
6. Jagtap AG, Shirke SS, Phadke AS. Effect of polyherbal formulation on experimental models of inflammatory bowel diseases. J Ethnopharmacol 2004;90:195-204.
7. Dhuley JN. Investigation on the gastroprotective and antidiarrhoeal properties of *Aegle marmelos* unripe fruit extract. Hindustan Antibiot Bull 2003;45-46:41-6.
8. Roy SK, Saran S, Kitinoja L. Bael [*Aegle marmelos* (L.) Corr. Serr.]. In: Postharvest Biology and Technology of Tropical and Subtropical Fruits. Sawston, UK: Woodhead Publishing; 2011. p.186-216.
9. Patkar AN, Desai NV, Ranage AA, Kalekar AS. A review on *Aegle marmelos*: A potential medicinal tree. Int Res J Pharm 2012;3:86-91.
10. Sharma N, Radha, Kumar M, Zhang B, Kumari N, Singh D, *et al. Aegle marmelos* (L.) Correa: An underutilized fruit with high nutraceutical values: A review. Int J Mol Sci 2022;23:10889.
11. Kaur A, Kalia M. Physico chemical analysis of bael (*Aegle Marmelos*) fruit pulp, seed and pericarp. Chem Sci Rev Lett 2017;6:1213-8.
12. Singh R. Characteristics and technology of traditional and cultural dairy products. Bull Dairy Int Fed 2007;415:11-20.
13. Shekhar S, Joe J, Kumar R, *et al.* Heat treatment of milk on the sensory and rheological quality of dahi prepared from cow milk. Res Rev J Food Dairy Technol 2013;1:8-14.
14. Nyanzi R, Jooste PJ, Buys EM. Invited review: Probiotic yogurt quality criteria, regulatory framework, clinical evidence, and analytical aspects. J Dairy Sci 2021;104:1-19.
15. Ankad GM, Konakeri RT, Hegde HV, Roy S. Variation in pharmacognostic characters and polyphenolic contents among four species of medicinal plants from the genus *Spermacoce* (Rubiaceae). Indian J Pharm Sci 2015;77:446-52.
16. Ankad GM, Pai SR, Upadhya V, Hurkadale PJ, Hegde HV. Pharmacognostic evaluation of *Achyranthes coynei*: Leaf. Egypt J Basic Appl Sci 2014;2:25-31.
17. Upadhya V, Pai SR, Ankad GM, Hegde HV. Pharmacognostic screening of *Piper trichostachyon* fruits and its comparative analysis with *Piper nigrum* using chromatographic techniques. Pharmacogn Mag 2016;12:S152-8.
18. Upadhya V, Ankad GM, Pai SR, Hegde HV. Preliminary pharmacognostic screening of *Achyranthes coynei* stem. J Ayu Integr Med 2015;6:134-8.
19. Ankad GM, Pai SR, Upadhya V, Hurkadale PJ, Hegde HV. Pharmacognostic evaluation of *Achyranthes coynei*: Leaf. Egypt J Basic Appl Sci 2015;2:25-31.
20. Ankad GM, Hiremath J, Patil RT, Pramod HJ, Hegde HV. Nutrient analysis of Kunapa jala and Pancha gavya and their evaluation on germination of Ashwagandha (*Withania somnifera* Dunal) and Kalamegha (*Andrographis paniculata* Nees.) seeds: A comparative study. J Ayurveda Integr Med 2018;9:13-9.
21. Mackie TJ, Collee JG, McCartney JE. Mackie and McCartney practical medical microbiology. Edinburgh: Churchill Livingstone; 1978.
22. Indian Council of Medical Research Task Force, Co-ordinating Unit ICMR, Co-ordinating Unit DBT. ICMR-DBT guidelines for evaluation of probiotics in food. Indian J Med Res 2011;134:22-5.
23. Paneliya AM, Patgiri B, Galib R, Bedarkar P, Prajapati PK. Pharmaceutical development of granules of Vasa Avaleha. Ann Ayurvedic Med 2013;2:16-2:1
24. Khemuka N, Galib R, Patgiri BJ, Prajapati PK. Pharmaceutical standardization of *Kamsaharitaki* granules. Ayu2015;36:416-20.
25. Balamurugan R, Chandragunasekaran AS, Chellappan G, Rajaram K, Ramamoorthi G, Ramakrishna BS. Probiotic potential of lactic acid bacteria present in home made curd in Southern India. Indian J Med Res 2014;140:345-55.
26. Available from: https://www.nin.res.in/downloads/Dietary Guidelines for NINwebsite.pdf.
27. Nireesha G, Divya L, Sowmya C, Divya L, Venkatesan N, Lavakumar V. Lyophilization/freeze drying-a review. Int J Nov Trends Pharm Sci 2013;3:87-98.
28. Gaidhani KA, Harwalkar M, Bhambere D, Nirgude PS. Lyophilization/Freeze drying – A review. World J Pharm Res 2015;4:516-143.