Project ID: 790

Competitive Research Grant

Sub-Project Completion Report

on

Development of probiotic feed supplement for calves

Project Duration

November 2017 to September 2018

Biotechnology Division
Bangladesh Livestock Research Institute
Savar, Dhaka-1341

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

September 2018

Competitive Research Grant (CRG)

Sub-Project Completion Report

on

Development of probiotic feed supplement for calves

Project Duration

November 2017 to September 2018

Biotechnology Division
Bangladesh Livestock Research Institute
Savar, Dhaka-1341

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

September 2018

Citation

Development of probiotic feed supplement for calves
Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

Edited and Published by:

Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

Acknowledgement

The execution of CRG sub-project has successfully been completed by Bangladesh Livestock Research Institute (BLRI) using the research grant of USAID Trust Fund and GoB through Ministry of Agriculture. We would like to thanks to the World Bank for arranging the grant fund and supervising the CRGs by BARC. It is worthwhile to mention the cooperation and quick responses of PIU-BARC, NATP 2, in respect of field implementation of the sub-project in multiple sites. Preparing the project completion report required to contact a number of persons for collection of information and processing of research data. Without the help of those persons, the preparation of this document could not be made possible. All of them, who made it possible, deserve thanks. Our thanks are due to the Director PIU-BARC, NATP 2 and his team who have given their whole hearted support to prepare this document. We hope this publication would be helpful to the agricultural scientists of the country for designing their future research projects in order to technology generation as well as increasing production and productivity for sustainable food and nutrition security in Bangladesh. It would also assist the policy makers of the agricultural sub-sectors for setting their future research directions.

Published in: September 2018

Printed by:

Acronyms

ADF	Acid detergent fiber
ADG	Average daily gain
BLRI	Bangladesh Livestock Research Institute
BS	*Bacillus subtilis*
BUN	Blood urea nitrogen
CCBDF	Central Cattle Breeding and Dairy Farm
CF	Crude fiber
CFU	Colony forming unit
CP	Crude protein
CPI	Crude protein intake
CRG	Competitive Research Grant
dl	Deciliter
DM	Dry matter
DMI	Dry matter intake
EE	Ether extract
FAO	Food and Agricultural Organization
FCR	Feed conversion ratio
g	Gram
HDL	High density lipoprotein
IgF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
L	Litter
LAB	Lactic acid bacteria
LDL	Low density lipoprotein
LoA	Letter of Agreement
LP	Lentil powder
LW	Live Weight
mg	Milligram
ml	Milliliter
mmol	Millimole
MRS	'De Man, Rogosa and Sharpe agar'
MYP	Mannitol Yolk Polymyxin
NDF	Neutral detergent fiber
NS	Not significant
OM	Organic matter
PCR	Project Completion Report
PDA	Potato dextrose agar
RCC	Red Chittagong Cattle
RP	Rice polish
SC	*Saccharomyces cerevisiae*
Tk.	Taka
vs	Versus
WB	Wheat bran
WHO	World Health Organization
µg	Microgram

Table of Contents

SL. No.	Subjects	Page No.
	Cover page	i
	Citation	ii
	Acronyms	iii
	Table of Contents	iv
	List of Tables and Figures	v
	Executive Summary	vi
A.	**Sub-project Description**	1
1	Title of the CRG sub-project	1
2	Implementing organization	1
3	Name and full address with phone, cell and E-mail of PI/Co-PI (s)	1
4	Sub-project budget	1
5	Duration of the sub-project	1
6	Justification of undertaking the sub-project	1
7	Sub-project goal	3
8	Sub-project objectives	3
9	Implementing locations	3
10	Methodology in brief	3
	10.1. Development of feed supplement for calves containing probiotic microbes	3
	10.2. Study the effects of developed feed supplement on growth performance and health of calves	6
11	Results and Discussions	7
	11.1. Development of feed supplement for calves containing probiotic microbes	7
	11.2. Effects of developed feed supplement on growth performance and health of calves	15
12	Research highlight/findings	20
B.	**Implementation Position**	20
	1. Procurement	20
	2. Establishment/renovation facilities	20
	3. Training/study tour/ seminar/workshop/conference organized	20
C.	**Financial and physical progress**	21
D.	**Achievement of Sub-project by objectives**	21
E.	**Materials Development/Publication made under the Sub-project**	22
F.	**Technology/Knowledge generation/Policy Support**	22
G.	**Information regarding Desk and Field Monitoring**	23
H.	**Lesson Learned**	23
I.	**Challenges**	24
	References	25

List of Tables

SL. No.	Title	Page No.
Table 1.	Formulation of probiotic feed for Experiment- 1	4
Table 2.	Probiotic feed formulation for Experiment- 2	5
Table 3.	Experimental layout for on-station animal trial	6
Table 4.	List of some commercial probiotic products available in local markets for livestock and poultry	7
Table 5	Live viable presence of desirable microorganism in different sources of probiotics	9
Table 6.	Dry matter content, pH and microbial count in probiotic feeds in Experiment- 1	9
Table 7.	Physical properties of feeds formulated in Experiment- 2	10
Table 8.	DM content of feeds at different stages	10
Table 9.	Effect of feeding probiotic feed supplement on intake and growth performance of calves	16
Table 10.	Effect of feeding probiotic feed supplement on physical properties of feces of calves	17
Table 11.	Effect of feeding probiotic feed supplement on fecal microbial load	18
Table 12.	Effect of feeding probiotic feed supplement on blood metabolic profile of milk-fed calves	19

List of Figures

SL. No.	Title	Page No.
Figure 1:	Changes in (a) pH, (b) NH3-N and (c) CP in probiotic feed supplements during shelf-life study	11
Figure 2:	Changes in (a) LAB, (b) BS and (c) SC contents in feeds at fresh, after incubation (Day 0) and at different time periods during shelf-life study	12
Figure 3:	Changes in DM and OM content in feed over time during shelf-life study.	13
Figure 4:	Changes in (a) pH, (b) NH3-N and (c) Crude protein content of feed at different time periods	14
Figure 5:	Changes in (a) LAB, (b) BS and (c) SC content in feed at different time periods	15

Executive Summary

The objectives of this research were to develop probiotic feed supplement for calves containing, preferably, of *Lactobacillus acidophilus* (LAB), *Bacillus subtilis* (BS) and *Saccharomyces cerevisiae* (SC); their evaluation in terms of chemical, microbial and shelf-life characteristics and finally testing the best selected probiotic supplement feed on health and growth performance of milk-fed calves. Above mentioned microbes were obtained from the known sources (commercial probiotic available in local market). Five feeds were developed based on five substrates. These were sole wheat bran (WB), rice polish (RP) and lentil powder (LP). The substrate WB and RP were fortified with LP at a ratio of 4:1 to produce (WB+LP) and (RP+LP). Each substrate was mixed with a solution containing water, molasses and mixed bacterial culture followed by incubation at 37°C for 3 days. Feeds were stirred two times daily for avoiding clump formation. After 3 days, feeds were found dried and clumpy in physical form and microbial count was very low, but high in pH. Therefore, a second formulation was made by changing composition of 'molasses-bacteria solution' and incubation time was increased to 6 days. Feed based on sole LP was discarded as color and odor of this feed was unpleasant. After 6 days of incubation, feeds were found in very good physical condition. Color was brown to deep brown, smell was sweet-sour as like silage, physical form was granular but not clumpy. The pH was down from 6.0 in fresh feed to 4.3 in probiotic feed on an average, and remained constant throughout the period of shelf-life study for 35 days. The concentrations of LAB, BS and SC were very high and sufficient to work as probiotic feed and remained constant as like pH remained. The dry matter (DM), organic matter (OM) crude protein (CP) and NH_3-N concentrations were good enough to keep probiotic feed quality in excellent form. After evaluation, all the four feeds were found potential to be used as probiotic feed; however, sole RP and WB based feeds were found to be the best. Therefore, these two feeds were further subjected to a different approach of shelf-life study for the determination of probable expire date after manufacturing.
The WB based probiotic feed was selected for evaluation on calf health, blood metabolites and growth performances. Twelve 2 weeks old RCC calves were selected and distributed equally in two groups maintaining gender and live weight balance between the groups. Calves were reared in individual pan and provided with *ad libitum* suckling, calf starter according to manufacturer's (ACI-Godrez) recommendation and *ad libitum* green grass (German grass). Mineral block was provided for licking and fresh clean drinking water was supplied for all the time. The probiotic fed group was supplied with developed probiotic feed, while the control/placebo group was supplied with same feed substrate without probiotic microbes. The trial was continued for 90 days and data were collected on growth performance, blood metabolic profile, immune status, fecal microbial load, morbidity etc. It was observed that milk intake, dry matter intake (DMI), daily gain and feed conversion ratio (FCR) did not differ ($P>0.05$) between the control and probiotic fed group. It was found that claves under probiotic fed group voided feces of better physical properties (color, odor and consistency) compared to the control. On an average, 13% cases of abnormal color of feces with bad odor in control compared to 6.6% in probiotic group were observed. The cases of diarrhea were observed to be 2.60% in the control group compared to 1.30% in the probiotic group. The Day-15 onward, up-to the end of the experiment, weekly *E. coli* count (log_{10} CFU/g) in feces was found significantly ($P<0.01$) lower (6.14) in probiotic group compared to the control (7.28). The plasma IgG (ng/ml) concentration was found significantly ($P<0.05$) higher (12.62) in probiotic group than in the control (8.38), and the total cholesterol level was tended to be high in the same ($P=0.071$). Among the four probiotic feed supplements developed, rice polish and wheat bran based feeds were found better considering their quality and shelf-life. Wheat bran based probiotic feed upon feeding to milk-fed calves, resulted improved fecal characteristics, lesser *E. coli* load, lowered diarrheal incidence and improved immunoglobulin status in calves. Further study however is needed, taking longer time, before recommending the wheat bran based probiotic feed formula for commercial production or for farmers for home production.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**

   Development of probiotic feed supplement for calves

2. **Implementing organization:**

   Bangladesh Livestock Research Institute (BLRI)

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**

Principal Investigator (PI):
Sardar Muhammad Amanullah, PhD
Senior Scientific Officer (Feed Biotechnology)
Biotechnology Division
Bangladesh Livestock Research Institute, Savar, Dhaka-1341
Cell: 01913715335, E-mail: amanullah@blri.gov.bd

Co-principal investigator (Co-PI):
Mohammed Abdus Samad, PhD
Senior Scientific Officer
Animal Health Research Division
Bangladesh Livestock Research Institute, Savar, Dhaka-1341
Cell: 01717047877, E-mail: samad_blri@yahoo.co.nz

4. **Sub-project budget (Tk):**
   4.1 Total: 2482144.00 BDT
   4.2 Revised (if any):
5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed): 06 November 2017
   5.2 End date : 30 September 2018

6. **Justification of undertaking the sub-project:**

Inadequate colostrum feeding and suckling followed by malnutrition is very much common in calf rearing system in Bangladesh. As a result, calf morbidity and mortality up to 12 months of age is much higher in the country. Hossain et al. (2014) reported average 5.6% calf mortality over 12 years in Central Cattle Breeding and Dairy Farm (CCBDF) with a range of 1.05 to 11.58% and about 70% of total mortality was reported up to 12 months of age. On the other hand, farmers have been extensively using antibiotics to increase disease resistance, reduce calf loss, and also as growth promoter. This may cause antibiotic resistance in both animal and human. Extensive and prolonged use of antibiotics may impair the intestinal flora ecosystem by gaining resistance to the antibiotics and increase susceptibility of calves to some pathogenic organisms, and consequently, increase the risk for diarrhea and malabsorption in intestines. Moreover, use of antibiotics and other growth stimulants in animal feeds causes the potential risk of antibiotic residues appearing in meat and milk. The need for a food supply that is perceived as safe by consumers has prompted livestock producers to explore alternative strategies to enhance the overall health conditions and performances of their herd. Probiotics, which defined as “live

microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002) are potential alternative to antibiotic for increasing feed intake and weight gain, earlier weaning, increased immunity, decreased scours and fecal coliform count in calves. The intestines of newly born calves are sterile at birth, but as the animals come in contact with the adult animals, suckling and feeding, they become infested with the microbes. The microbial environment of the gastro-intestinal tract influences the performance of the animal. Microbial feed additives facilitate the establishment and maintenance of suitable microbial flora in the gastrointestinal tract. The early establishment of large amount of beneficial microorganisms in the gut helps to combat the negative effects of unfavorable conditions or prevent the pathogenic organisms. The most commonly used microbial additive is *Lactobacillus* spp. These microbes have specific roles in the host's body, primarily responsible for the exclusion of enterotoxigenic bacteria (Fuller 1989).

Nevertheless, probiotic microorganisms for ruminants include species of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Bacillus* and *Propionibacterium* (Seo et al., 2010). These bacteria also are commonly used in probiotics for human and monogastric animals or as inocula for dairy product processing. Other distinctive bacterial species such as *Megasphaera elsdenii* and *Prevotella bryantii* have also been used as probiotic to stabilize or improve rumen function. These bacterial probiotic strains may be classified as lactic acid producing, lactic acid utilizing, or other microorganisms. Lactic acid production and utilization in the rumen is closely related to feed efficiency and animal health. Although bacterial probiotic are emphasized, fungal probiotic are also common feed additives to ruminant diets (Kung Jr, 2001). Most commercial yeast products contain species of *Saccharomyces* and *Aspergillus*. Yeast products based on *Saccharomyces cerevisiae* have been used as feed additives in dairy production system for more than two decades (Jiang et al., 2017). It is typically fed in dairy cattle rations to alter rumen fermentation in an attempt to improve nutrient digestion, N utilization, reduce the risk of rumen acidosis and improve animal performance (Seo et al., 2010). Recent multi-study analyses performed both in dairy and beef cattle have shown significant benefits with live yeast (*Saccharomyces cerevisiae*) on milk yield and feed efficiency (Jiang et al., 2017).

Tolerance of microorganisms to heat is also important for probiotic since they have to survive processing during feed production. In general, most yeast and lactic acid bacteria (LAB) are destroyed by heat during pelleting (Kung Jr, 2001). Spore forming bacteria have advantages as probiotics for humans and animals (Ripamonti et al., 2009). The ability to form spores also provides probiotics with higher resistance to stresses during production and storage processes (Hyronimus et al., 2000) and also higher resistance to gastric and intestinal environmental conditions (Hong *et al*., 2005). Bacillus species have specific mechanisms that inhibit gastrointestinal infection by pathogens or producing antimicrobials (Song et al., 2014). In addition to the practical advantages of spore forming bacteria, strong cellulolytic activity may support the potential of bacilli as probiotic for ruminant or non-ruminant animals by improving fiber digestion in the rumen and/or in the gastro-intestinal tract by supplying oligosaccharides to beneficial microorganisms. The probiotic culture for feeding ruminants may be single or mixed culture of more microorganisms. The combinations of probiotics strains could increase the beneficial health effects compared with individual strains, because of their synergetic adhesion effects (Collado et al., 2007).

The work on probiotic for cattle has increased in the recent years and positive effects have been found for feed intake, weight gain, milk yield and quality, early weaning, decrease of scouring and faecal coliform count and reduced demand for antibiotic treatment (Retta, 2016). Under an intestinal imbalance condition in calves due to feeding milk replacer and spray-dried whey powder, calves fed probiotic had higher daily gain, total feed intake, and starter diet intake as well as lower incidence of

diarrhea (Frizzo et al., 2010). Malik and Bandla (2010) reported higher average daily gain and feed efficiency in calves fed probiotic (*L. acidophilus* and *S. cerevisiae)* plus enzyme supplements. Improved feed intake and live weight gain, but reduced diarrhea incidence in calves by feeding probiotics were also reported by many scientists earlier (Abe et al., 1995; Jordan and Johnston, 1991; Tournut, 1989; Hughes, 1988). Microbial feed additives used in ruminant feeds are mainly for stabilization of the intestinal flora, enhancing the development of the adult rumen microflora, improving digestion and nitrogen flow towards lower digestive tract, and improving meat and milk production (Retta, 2016). Probiotics administration improves the health status of the animal by competing the nutrient utilization of the pathogenic microbes by having a positive influence on gut microflora. Furthermore, their anti-pathogenic activity may reduce the stress on animal (Seo et al., 2010).

Earlier, effort was made in Bangladesh Livestock Research Institute to develop *Lactobacilli* based probiotic for calves (Amanullah et al., 2009 a, b), where it was reported that probiotic feeding increased live weight gain in calves significantly up to 5 weeks of age, reduced *E. coli* attachment on intestinal wall, but increased attachment of *Lactobacillus spp.,* recued fecal shedding of *E. coli* and reduced incidence of diarrhea. Scopes are yet there to work with diversified microbes with more potential probiotic effects (e.g., *Bacillus subtilis* and *Saccharomyces cerevisiae*), find out efficient method of delivery to calf, product formulation, increasing shelf-life of products and finally make this cutting edge technology easily available at farmer's hand. The present research will help to develop probiotic feed supplement as alternative growth promoter and health booster for calves.

7. Sub-project goal

The goal of this research was to promote growth and health of calves through feeding beneficial microbes.

8. Sub-project objective (s)

i) To develop a feed supplement for calves containing a mixture of different probiotic microbes preferably of *Lactobacillus, Bacillus* and *Saccharomyces* species.

ii) To study the effects of developed feed supplement on growth performance and health of calves

9. Implementing location (s):

BLRI Headquarter, Savar, Dhaka

10. Methodology in brief:

This research was based on laboratory works and on-station animal trial. Primarily, the works of microbial enumeration, development of probiotic feed supplements and nutritional and microbial evaluation of feed were conducted in the laboratory. Then the developed feed was evaluated on animal performances through on-station feeding trial on calf at BLRI cattle farm.

10.1. Development of feed supplement for calves containing probiotic microbes:

Collection of commercial sources of probiotic microorganisms and their evaluation

Lactobacillus acidophilus, *Bacillus subtilis* and yeast (*Saccharomyces cerevisiae*) were collected as commercial probiotics from the local market and their live viable presence in the product was determined. The 'De Man, Rogosa and Sharpe agar' (MRS), Mannitol Yolk Polymyxin (MYP) Agar and Potato Dextrose Agar (PDA) were used for isolation of *Lactobacillus*, *Bacillus* and yeast, respectively. One

gram (1 g) of sample was taken in a screw-cap tube and diluted with 9.0 ml of 0.85% NaCl solution and thoroughly mixed with a vortex mixer. Then ten-fold serial dilutions of the suspension were prepared and 100 µl aliquots of three consecutive dilutions (10^{-5} to 10^{-7}) were plated in triplicate onto the selective agar medium for each microbe. Agar plates were placed in an incubator (Thermo Scientific, USA) at 37°C for 48h. Visible colonies were counted from the plates at appropriate dilutions and the number of colony forming units (CFU) was expressed per gram of sample. Isolated colonies were preserved in 30% glycerol solution in -70°C and reactivate by overnight activation in respective broth media for further use when necessary.

Development of probiotic feed and their evaluation

Experiment- 1: Initially, five probiotic feeds were formulated containing mixed culture of *Lctobacillus acidophilus*, *Bacillus subtilis* and *Saccharomyces cerevisiae,* according to the formula given in Table 1. Molasses, water and microbial culture were first mixed thoroughly in a solution and then sprayed on main substrate (rice polish, wheat bran, lentil powder and their mixture). The microbial concentration in solution was more than 6 $\log_{10}$ CFU/ml. All ingredients were autoclaved (except microbial culture) before mixing to kill existing organisms in the ingredients, if there is any. Then they all together were mixed homogenously, packed and incubated at 37°C for 3 days. Feeds were stirred 2 times daily for preventing clump formation and facilitating vigorous fermentation. After 3 days, feeds were harvested and evaluated for moisture, pH, viable count of each microbes, organic matter (OM), crude protein (CP) and ammonia-N content.

Table 1. Formulation of probiotic feed for Experiment- 1

Ingredients	Feed 1 (RP)	Feed 2 (WB)	Feed 3 (LP)	Feed 4 (RP+LP)	Feed 5 (WB+LP)
Rice polish	500 g	-	-	400 g	-
Wheat bran	-	500 g	-	-	400 g
Lentil powder	-	-	500 g	100 g	100 g
Molasses	70 g	70 g	70 g	70 g	70 g
Water	70 ml	70 ml	70 ml	70 ml	70 ml
LAB culture	7 ml	7 ml	7 ml	7 ml	7 ml
SC culture	7 ml	7 ml	7 ml	7 ml	7 ml
BS culture	7 ml	7 ml	7 ml	7 ml	7 ml

RP, rice polish; WB, wheat bran; LP, Lentil powder; LAB, Lactic acid bacteria; SC, Saccharomyces cerevisiae; BS, Bacillus subtilis

Experiment- 2: At harvest, the feeds used in Experiment-1 were found not good so far as the physical, chemical and microbial properties are concerned. So, in a second experiment, ingredient compositions and incubation time were changed according to the formula given in Table 2. After primary evaluation, the sole LP (Feed 3) feed was not considered in second formulation for its very unsatisfactory physical and microbial properties.

Table 2. Probiotic feed formulation for Experiment- 2

Ingredients	Feed 1 (RP)	Feed 2 (WB)	Feed 3 (RP+LP)	Feed 4 (WB+LP)
Rice polish	500 g	-	400 g	-
Wheat bran	-	500 g	-	400 g
Lentil powder	-	-	100 g	100 g
Molasses	100 g	100 g	100 g	100 g
Water	300 ml	300 ml	300 ml	300 ml
LAB culture	10 ml	10 ml	10 ml	10 ml
SC culture	10 ml	10 ml	10 ml	10 ml
BS culture	10 ml	10 ml	10 ml	10 ml

RP, rice polish; WB, wheat bran; LP, Lentil powder; LAB, Lactic acid bacteria; SC, Saccharomyces cerevisiae; BS, Bacillus subtilis

Newly formulated feeds as described in Table 2 were packed and incubated at 37°C for 6 days. All other procedures were similar as in Experiment-1.

The shelf-life of formulated feeds was evaluated in terms of concentrations of live viable microbes those were inoculated, pH, NH_3-N, DM, OM and CP in the feed over a time period of 35 days. The feed packet was kept at room temperature and sample was collected at 0, 5, 10, 15, 20 and 35 days. At each day of collection, after opening packet feed was mixed thoroughly to collect a homogenous sample and after collection the feed was re-packed air-tight. This kind of study allowed knowing the shelf-life of feed at practical condition at farms. Due to microbial growth in the feed pH will be reduced for higher lactic acid concentration and that will help to reduce the degradation of DM, OM and CP in career substrate. The concentration of NH_3-N will indicate the degradation of CP for microbial growth. Therefore, shelf-life study included the determination of microbial enumeration, pH, NH_3-N, DM, OM and CP in the probiotic feed.

Experiment- 3: Again, shelf-life of feed was studied in a different approach to know the tentative expire date of feed after production. In the previous approach, shelf-life was studied from a single bag from opening to the end (35 days). This approach considered the practical condition at farm level, i.e., after opening the bag how long the feed can be used by the farmer. In this approach, after incubation, feeds were packed differently for different time periods of 0, 7, 14, 21, 30 and 45 days. At the end of each time period, respective packets were opened for feed evaluation; to know how long the feed quality can be persisted in sealed condition. However, this study involved only two feeds (Feed 1 & Feed 2) those found best according to previous evaluation and shelf-life study.

Probiotic feeds in the bags for shelf-life study

10.2. Study the effects of developed feed supplement on growth performance and health of calves:

On-station animal trial

On-station calf trial was conducted to test the probiotic feed on calf production and health. On the basis of microbial concentration, shelf life, physical and chemical properties Feed 1 (RP) and Feed 2 (WB) were found very much potential. These two feeds were offered for a shorter time period (7 days) to the experimental calves to test calf's preference among these two feeds, as there was scope to go for animal trial with only one probiotic feed. It was found that Feed 2 (WB) was preferred by the animals. Therefore, Feed 2 (WB) was selected and tested on calf health and production.

Twelve Red Chittagong Cattle (RCC) calves of one week of age were selected and distributed into two treatments in a way that male: female ratio in each treatment group remained similar. Two treatment groups were supplied either with or without probiotic feed according to the layout given below. The calves irrespective of treatments were supplied with *ad libitum* suckling (roughly around 10% of their live weight) and commercial calf starter (ACI-Godrej) as basal diet. They were also provided with mineral blocks for licking. The trial was continued for 90 days. Data were collected on growth performance, blood metabolic profile, immune status, fecal microbial load and morbidity during the trial period. Live weight was measured weekly to calculate daily gain. Milk intake was measured fortnightly at 3 consecutive days by taking weight of animal before and after feeding. Calf starter, green grass, probiotic feed or placebo feed intake was recorded daily. Feces sample was collected fortnightly from each animal for *E. coli* and *Salmonella* enumeration and at 90 day for LAB, *B. subtilis* and *S. cerevisiae* enumeration. Feces were observed daily for physical property evaluation, which included color, odor and consistency. The consistency properties of feces, consistency scores and cases of diarrhea were recorded following the method of Amanullah et al. (2009a) with some modifications. Consistency was recorded as hard (constipation), normal, tended to be liquid and watery and a score value of 1, 2, 3 and 4 was given, respectively for each consistency class to each calf in each day. The average value was used to derive consistency score. Persistency of score 3 and 4 for consecutive 3 days was considered as the 'case of diarrhea'. At the end of the trial blood samples were collected from jugular vein at 3 consecutive days. Serum were separated and analyzed blood metabolic profile and other biochemical properties including blood glucose, blood urea nitrogen (BUN), insulin, total cholesterol, HDL, LDL, triglycerides, Immunoglobulin G (IgG), insulin-like growth factor-1 (IgF-1) and cortisol. Data were analyzed using paired sample *t*-test in computer package program SPSS. Significance were declared at $P<0.05$, while tendency was declared at $P<0.10$. The experimental layout is given in Table 3 below.

Table 3. Experimental layout for on-station animal trial

	T 1 (Control/Placebo)	**T 2 (Probiotic)**
Animal	RCC calves of around 2 weeks age	RCC calves of around 2 weeks age
Replication	6	6
Diet	*ad libitum* Suckling+ Calf starter + green Grass + Mineral blocks	*ad libitum* Suckling+ Calf starter + green Grass + Mineral blocks
Placebo/probiotic	Feed Substrate	Probiotic feed

11. Results and discussions:

This research was conducted through three major activities. Firstly, collection of commercial sources of probiotic microorganism and their evaluation; secondly, development of probiotic feed and their evaluation and thirdly, on-station calf feeding experiment for evaluating developed feed supplement on animal performances. The results from different research activities are described below along with discussion.

11.1. Development of feed supplement for calves containing probiotic microbes:

Collection of commercial sources of probiotic microorganisms and their evaluation

For collecting known source of desired probiotic microbes from the market, an informal market survey was conducted to know the available sources of commercial probiotic in the market. A total of eighteen products were listed along with their microbial content (Table 4).

Table 4: List of some commercial probiotic products available in local markets for livestock and poultry

Sl. No.	Trade name	Content name	Manufacture	Marketed by
1	AVI BAC, Powder	Dried *Bacillus subtilis* Dried *Bifidobacterium longum* Dried *Lactobacillus acidophilus*	Product from USA	Opsonin Pharma Ltd., Barisal, Bangladesh.
2	PROMIX-WSP Powder	*Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Lactobacillus casei* *Lactobacillus lactis* *Lactobacillus returi* *Streptococcus faecium* *Aspergillus oryzae* *Saccharomyces cerevisiae*	Indo American Technology, India.	Arena Agro, Bangladesh
3	NAVA PRO PLUS Bolus	*Saccharomyces cerevisiae* *Lactobacillus sporogense* *Bacillus subtilis* *Aspergillus niger*	BBS, India	Navan Pharma, Bangladesh.
4	PRO BOOST, Bolus	Live *Lactobacillus sporogense* Live Yeast culture	PVS Agrovet, India.	Bonafide Agrovet Ltd., Bangladesh.
5	ACTIVA, Bolus	*Lactobacillus sporogense* *Saccharomyces cerevisiae* (Live) Fermented Yeast culture *Saccharomyces boulardii*	Geevet Remidies, Gujrat, India.	
6	BIOLACT, Bolus	Live Yeast culture Activated dry yeast *Lactobacillus sporogense*	Baader Schulz Laboratorie, India	Square Pharmaceuticals Ltd. Bangladesh.
7	Prob WS, Powder	*Lactobacillus acidophilus* *Lactobacillus bifidum* *Lactobacillus reuteri* *Lactobacillus lactis* *Lactobacillus fermentum* *Lactobacillus faecium* *Aspergillus oryzae* *Streptococcus faecium*	Merlin Life Science, Singapore	Ample Animal Care, BD

Sl. No.	Trade name	Content name	Manufacture	Marketed by
8	Gutpro, Powder	*Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Lactobacillus plantarum* *Streptococcus faecium* *Bifidobacterium bifidus*	Ceva polchem private limited, India	Avon animal health,BD
9 (a)	Digestovet, Bolus	*Saccharomyces cerevisiae*	Vetsfarma Ltd, India	Oriental Pharma Agro Vets Ltd., BD
9(b)	Digestovet, powder	*Saccharomyces cerevisiae*	Vetsfarma Ltd, India	Oriental Pharma Agro Vets Ltd., BD
10	Probio-5, Powder	*Lactobacillus acidophilus* *Lactobacillus plantarum* *Bacillus subtilis* *Bacillus licheniformis* *Saccharomyces cerevisiae*	Soma Inc, Korea	Sky tech agro pharma
11	Probios, Powder	*Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Lactobacillus casei* *Lactobacillus plantarum* *Streptococcus Faecium* *Streptococcus themmophilus* Yeast: *Terulopsis spp.* *Aspergillus oryzae*	Stallen South Asia Pvt., India	Bengal oversees Ltd./ MSD Animal Health
12	Bio-top and Bio-top SC, Powder	*Bacillus subtilis* *Bacillus licheniformis* *Bacillus coagulans*	Shinil Biogen Co. ltd., Korea	Pharma and firm, Azad group, BD
13	Probio sol., powder	-	Soma inc, korea	Sun agro pharma
14	Progressive Hatchpro, powder	*Lactobacillus acidophilus* *Bacillus licheniformis* *Lactobacillus casei* *Lactobacillus plantarum* *Bacillus subtilis* *Enterococcus faecium*	EW nutrition, Germany	Nature care industry ltd.
15	SD prosol, powder	Live yeast cells *Lactobacillus sporogenes*	Saideep, India	Same
16	Vtc superior probiotic, powder	*Lactobacillus* *Bacillus* *Saccharomyces*	Soma inc, korea	SMG Animal health co. ltd.
17	Protexin	-	-	Elanco

Among these eighteen products identified, seven commercial sources of probiotic containing lactic acid bacteria (LAB) specifically *L. acidophilus,* Yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis* were collected and evaluated for the presence of respective live viable organism (CFU/g) specified by the manufacturer. The results are presented in Table 5. It was observed that products supposed to have LAB contained good count of LAB indeed. Only one product had contained neither LAB nor *Saccharomyces cerevisiae* as manufacturer specified. However, yeast/*S. cerevisiae* content was only observed in 2 products tested, while it was supposed to be present in the six products (Source 2 to 7). Presence of live viable organism in the final product might have been controlled by the preservation

and transportation condition in the market, especially temperature. As LAB, SC and BS were found in 5, 2 and 2 sources respectively, isolation was done from those sources for probiotic feed production.

Table 5: Live viable presence of desirable microorganism in different sources of probiotics

Commercial source	Live viable count (log_{10} CFU/g)		
	LAB (*Lactobacillus acidophilus*)	Yeast (*Saccharomyces cerevisiae*)	*Bacillus subtilis*
Source 1 (Powder)	10.94	-	9.93
Source 2 (Powder)	9.23	0	-
Source 3 (Bolus)	10.34	0	9.51
Source 4 (Bolus)	0	0	-
Source 5 (bolus)	9.31	0	-
Source 6 (Bolus)	9.17	7.0	0
Source 7 (Commercial yeast)	-	7.0	-

Development of probiotic feed and their evaluation

Evaluation of feeds manufactured in Experiment- 1

Feeds formulated at initial steps were evaluated for its physical, chemical and microbial properties and some important results are illustrated in Table 6. Results showed that all the five formulated feeds were low in moisture (high DM %), high in pH (>5.0) and low in microbial counts. The DM content varied from 74.63 to 19.05%, which was found not suitable for vigorous microbial growth. The pH of around 4.5 was considered as an indication of sufficient microbial growth during incubation, but pH irrespective of feeds remained above 5.40 in this formulation. It's an indication of poor microbial growth in the feed substrate. The physical forms of feeds were also not good. They were very much dried up but clumpy. So, in the second Experiment, ingredient ratios and incubation time were changed according to the formula given earlier in Table 2.

Table 6. Dry matter content, pH and microbial count in probiotic feeds in Experiment- 1

Feed	DM (%)		pH		LAB (log_{10} CFU/g)		SC (log_{10}CFU/g)		BS (log_{10} CFU/g)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Feed 1 (RP)	80.00	79.05	5.81	5.42	4.2	4.7	4.3	5.2	3.8	4.9
Feed 2 (WB)	78.60	77.85	6.64	6.36	4.0	4.7	3.8	4.8	3.8	5.0
Feed 3 (LP)	78.30	77.01	5.68	5.8	4.1	4.6	4.0	4.3	3.2	0.0
Feed 4 (RP+LP)	75.53	74.63	5.70	5.53	3.8	4.8	4.1	5.0	3.6	4.7
Feed 5 (WB+LP)	76.20	75.31	5.86	5.56	4.2	6.3	4.0	5.8	3.7	5.3

LAB, Lactic acid bacteria (L. acidophilus); SC, Saccharomyces cerevisiae; BS, Bacillus subtilis

Evaluation of feeds manufactured in Experiment- 2

Physical properties of feeds

Physical properties of feeds manufactured in Experiment-2 are illustrated in Table 7. It was found that feeds containing rice polish (RP) had brown color, silage-like sweet-sour smell and granular form without any clump. However, feed 3, which contained lentil powder (LP) along with RP had less pronounced smell. While, feeds manufactured with wheat bran (WB) were also of same physical quality except they looked little bit moisty.

Table 7. Physical properties of feeds formulated in Experiment- 2

Feed	Color	Flavor	Physical Form
Feed 1 (RP)	Brown	Sweet-sour smell as like silage	Granular, no clump
Feed 2 (WB)	Deep brown	Sweet-sour smell as like silage	Granular but moisty, no clump
Feed 3 (RP+LP)	Brown	Sweet-sour smell, but in lesser extent	Granular, no clump
Feed 4 (WB+LP)	Deep brown	Sweet-sour smell, but in lesser extent	Granular but moisty, no clump

Chemical characteristics of feeds

The dry matter (DM) content of feeds at formulation, after incubation and during shelf-life study is described in Table 8. It was observed that the DM concentrations in different feed during fresh formulation were 58.6, 50.5, 61.5 and 53.7 in four feed, respectively and they remained almost similar after 6 days of incubation, as well as up to 35 days of shelf-life study period. The DM concentrations were found good enough to avoid dustiness as well as to prevent clump formation in feed.

Table 8. DM content of feeds at different stages

Feed		DM, %					
	Initial	Final/Day 0	Day 5	Day 10	Day 15	Day 20	Day-35
Feed 1 (RP)	58.6	56.8	58.3	58.0	56.97	56.43	57.01
Feed 2 (WB)	50.5	49.7	49.9	49.3	48.99	48.67	49.47
Feed 3 (RP+LP)	61.5	61.2	60.8	60.3	61.20	60.60	61.50
Feed 4 (WB+LP)	53.7	53.4	53.0	53.6	53.37	52.55	53.21

The pH, NH_3-N and crude protein (CP) content in different feeds at fresh, after 6 days of incubation (probiotic feed; day 0) and during shelf-life study period (Day 0 to 35) are shown in Figure 1. In all four feeds, after 6 days of incubation pH was dropped down to below 4.4 from around 6.0 in fresh feed. This indicated vigorous microbial growth during incubation that helped to produce sufficient lactic acid to reduce pH at such a low level. During shelf-life study, until 35 days, pH in all feeds remained below 5.0. The NH_3-N contents in fresh feed were observed 8.1, 28.0, 19.7 and 68.4 mg/100g in Feed 1, 2, 3 and 4, respectively. The NH_3-N contents were increased in feed 2 (42.5 mg/100g) and feed 4 (71.7 mg/100g) after 6 days of incubation, while decreased slightly in feed 1 (7.1 mg/100g) and feed 3 (18.3 mg/100g) at same day. This result indicated an increased amination in feeds containing wheat bran. However, the concentrations of CP in WB containing feeds were also remained high throughout the period (Figure 1). The CP contents of different feeds at fresh formulation were found 10.8, 14.3, 12.0 and 20.6% in Feed 1, 2, 3 and 4, respectively. After 6 days of incubation, CP contents of all feeds were reduced to 7.5, 14.1, 10.5 and 19.1%, respectively. However, from harvest day (Day 0) to the end of the shelf-life study (Day 35) CP contents in all feeds were reduced, excepting the feed 3, which showed almost constant CP concentration during shelf-life study. Usually CP contents in feeds are utilized by microbes for their multiplication and some might be degraded to ammonia by microbial enzyme. The range of pH, CP and

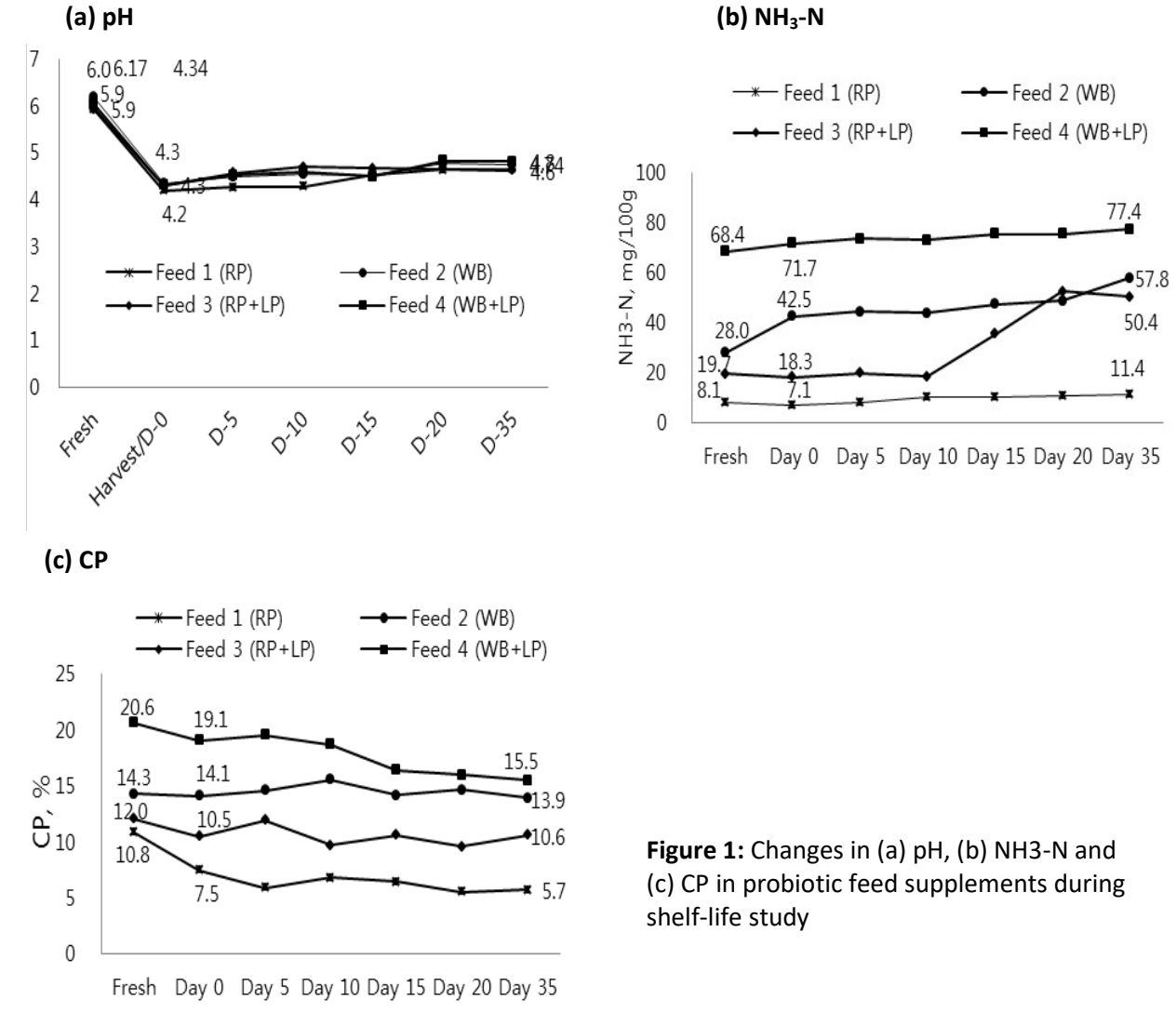


Figure 1: Changes in (a) pH, (b) NH3-N and (c) CP in probiotic feed supplements during shelf-life study

NH_3-N contents in feeds found god to maintain probiotic feed characteristics in all four feeds. However, lower pH, CP and NH3-N content in feed 1 supposed to provide more shelf-life quality of feed. High CP content in feed may increase NH3-N concentration, which may reduce keeping quality by increasing pH.

Microbial properties of feed

The live viable count of *L. acidophilus* (LAB), *Bacillus subtilis* (BS) and *Saccharomyces cerevisiae* (SC) in fresh feed, after 6 days of incubation (Day 0) and during shelf-life study are presented in Figure 2. It was observed that the concentration of LAB in fresh feed was from 4.9 to 5.2 log_{10} CFU/g, but after incubation that was increased to almost double in all feeds, ranging from 8.8 to 9.8 log_{10} CFU/g feed. In the cases of shelf-life, the microbial load was found to vary among the feeds and time periods and at the end (35 days) it was found to be 8.2, 8.1, 5.9 and 7.5 log_{10} CFU/g in feed 1, 2, 3 and 4, respectively. Similar results were obtained in the case of BS and SC. Initial concentrations of BS in fresh feeds were 4.5 to 5.3 log_{10} CFU/g, which increased to 7.5 to 9.4 log_{10} CFU/g after 6 days of incubation and persisted at a level of 6.1 to 7.3 log_{10} CFU/g at the end of 35 days. The concentrations of SC in fresh feeds were 5.2 to 5.8 log_{10} CFU/g, which increased to 9.0 to 11.0 log_{10} CFU/g after incubation and ended at 6.3 to 7.2 log_{10} CFU/g at 35 days. Feed 1 and 2 had higher concentrations of all three microbes at a level of >7.0 log_{10} CFU/g which considered sufficient to be a probiotic feed as most of the commercial sources contained probiotic around that concentration.

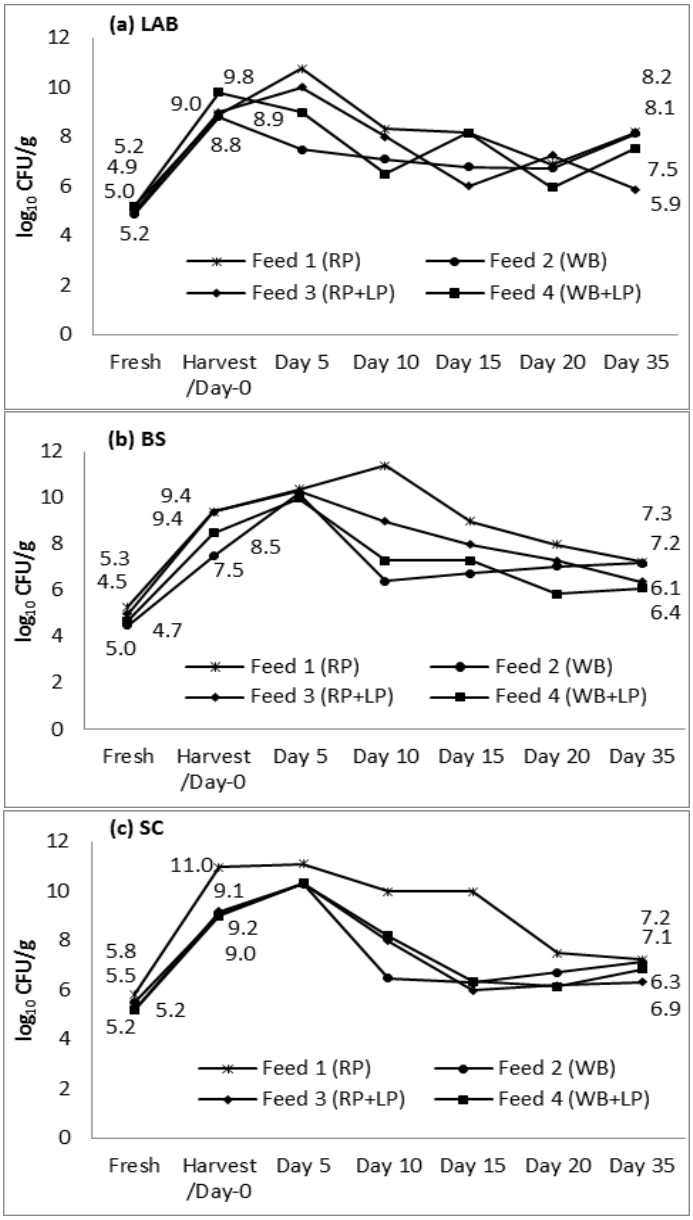


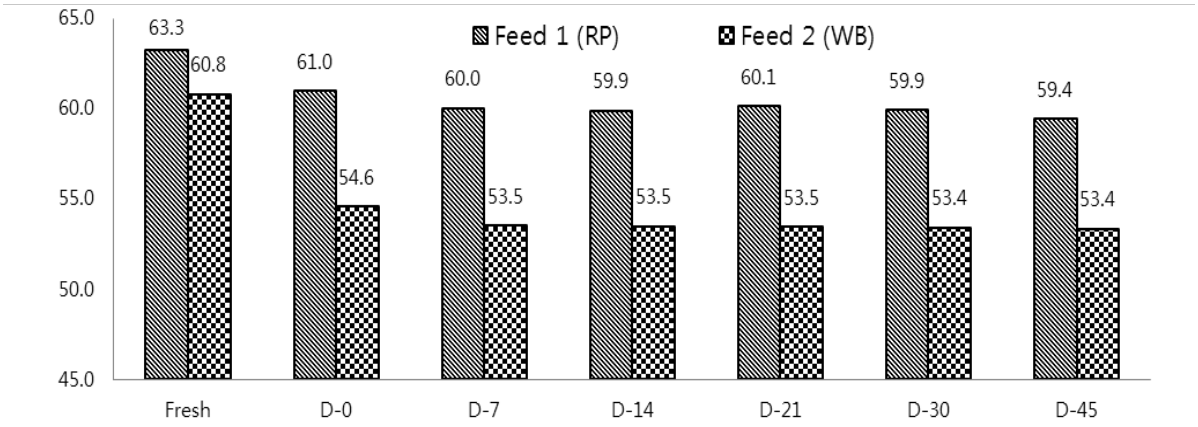
Figure 2: Changes in (a) LAB, (b) BS and (c) SC contents in feeds at fresh, after incubation (Day 0) and at different time periods during shelf-life study. (LAB, *Lactobacillus acidophilus;* BS, *Bacillus subtilis;* SC, *Saccharomyces cerevisiae*).

<u>Experiment- 3: Different approach in shelf-life study</u>

From the results of shelf-life study described above, all feeds were found potential to be used as probiotic feed. However, sole RP and WB based feeds (Feed 1 & 2) were found better than others considering pH, microbial counts, NH_3-N and CP contents and physical properties. Therefore, these feeds (Feed 1 & 2) were further subjected for a different approach of shelf-life study to know the tentative expire date after manufacturing and packing of feed. In this study, manufactured feeds were

packed separately for each period of 0, 7, 14, 21, 30 and 45 days. In the previous study, sampling were done from the single bag at different time periods and closed air-tight after each sampling, considering the utilization pattern at farm level. In later study, at the end of each period, bags for that particular period were opened, and feeds were sampled and analyzed for chemical and microbial characteristics. Contents of DM and organic matter (OM) in fresh feed, probiotic feed (Day 0) and during 45 days shelf-life study periods are illustrated in Figure 3.

(a) Dry matter



(b) Organic matter

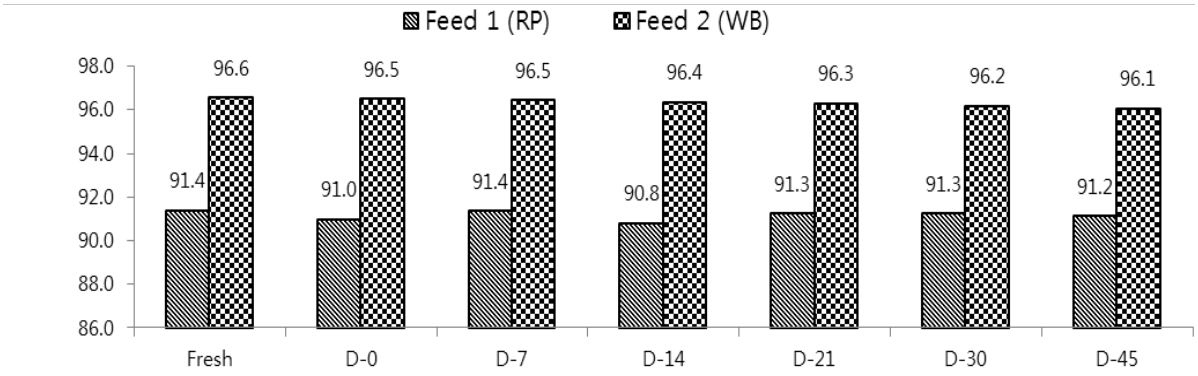


Figure 3: Changes in DM and OM content in feed over time during shelf-life study

DM contents in fresh RP and WB feed was found to be 63.3 and 60.8%, respectively, while after 6 days of incubation, in probiotic feed (Day 0) DM was dropped down to 61.0 and 54.6% in RP and WB feed, respectively. Thereafter, very little change in DM contents was observed in feeds until 45 days of study period. On the other hand, Initial OM content in RP and WB feed was observed to be 91.4 and 96.6%, respectively, which was not much changed in probiotic feed during the shelf-life study period.

The pH, NH_3-N and CP contents in RP and WB feeds are illustrated in Figure 4. It was found that the initial pH at fresh formulation were 6.22 and 6.38 in RP and WB feeds, respectively, which were dropped down to 4.61 and 4.90, respectively after 6 days of incubation. Until 45 days, the pH in feed remained almost similar, which were 4.75 and 4.74 in RP and WB feed respectively. The pattern of change in concentration of NH_3-N and CP usually found reverse. In fermentation process and during preservation usually, CP content reduced by microbial degradation, while, NH_3-N concentration increased. This feature was also found in this study with some variations. The initial CP contents were

10.88 and 14.51% in RP and WB feeds, respectively, while it reduced down to 7.88 and 13.73%, respectively after 6 days of incubation. This reduction in CP was continued during preservation time and reduced down to 5.70 and 12.75%, respectively at 45 days. On the other hand, NH_3-N content in WB feed was initially increased to 47.48 mg/100g after 6 days of incubation, which was 28.97 mg/100g in fresh feed. Then it remained almost constant up to day 14, while further increased to 61.32mg/100g at the end (Day 45). On the contrary, in RP feed, NH_3-N content was decreased from 8.73 to 6.88 during 6 days of incubation period and then remained constant up to 14 days, but gradually increased to 10.60 until day 45.

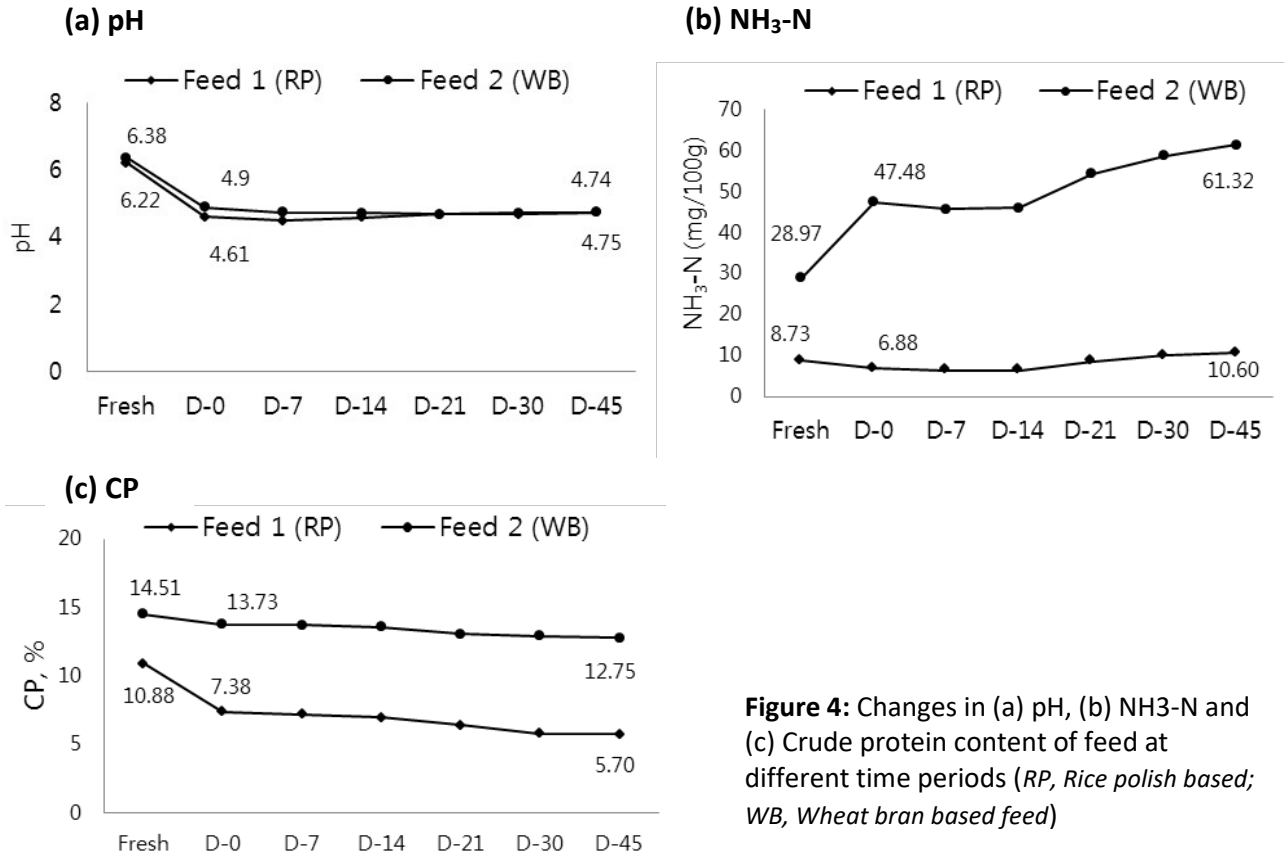


Figure 4: Changes in (a) pH, (b) NH3-N and (c) Crude protein content of feed at different time periods (*RP, Rice polish based; WB, Wheat bran based feed*)

Contents of *Lactic acid bacteria* (LAB), *Bacillus subtilis* (BS) and *Saccharomyces cerevisiae* (SC) in feeds at fresh, harvesting after 6 days of incubation and during shelf-life study (0 to 45 days) are illustrated in Figure 5. It was observed that, all the inoculated microbes were increased in both feed after incubation and they remained almost constant until the end of the study. The LAB count in fresh feed after inoculation of microbes was found to be 5.15 and 5.0 log_{10} CFU/g in RP and WB feed, respectively. Incubation helped to increase the colony number and reached to 7.32 and 6.94 log_{10} CFU/g in RP and WB feed, respectively. Similarly, BS and SC number were found 5.48 *vs* 4.91 (RP *vs* WB) and 6.14 *vs* 5.70 log_{10} CFU/g, respectively in fresh feed, which were increased to 7.37 *vs* 6.81 and 7.33 *vs* 6.30 log_{10} CFU/g, respectively after incubation and remained as high as 7.35 *vs* 7.15 and 7.36 *vs* 7.15 log_{10} CFU/g, respectively at 45 days. This indicated that, in air-tight packed condition, these feed could be maintained with higher number of probiotic microbes for longer period of at least 45 days.

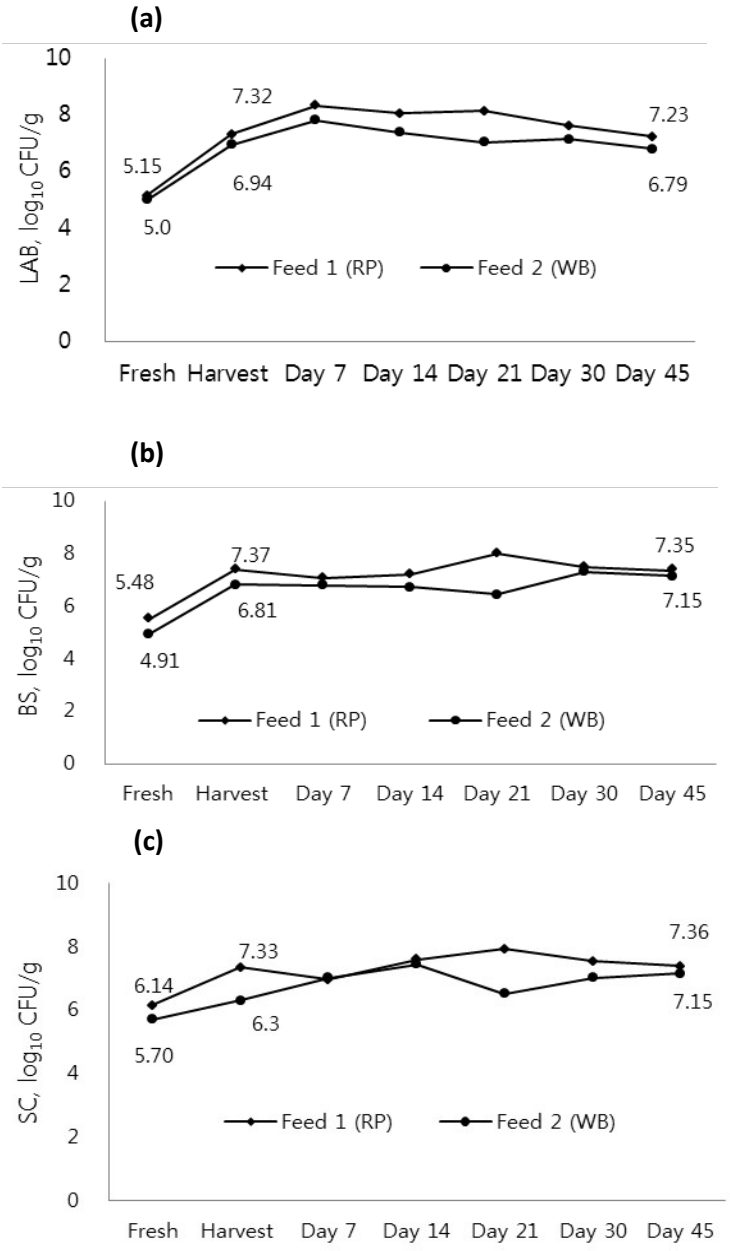


Figure 5: Changes in (a) LAB, (b) BS and (c) SC content in feed at different time periods (LAB, *Lactic acid bacteria*; BS, *Bacillus subtilis;* SC, *Saccharomyces cerevisiae*)

11.2. Effects of developed feed supplement on growth performance and health of calves:

On-station animal trial

Effects of feeding probiotic feed supplement on intake and growth performances of calves are described in Table 9. There were no differences (P>0.05) between the control *vs* probiotic treatment in initial live weight (20.68 *vs* 21.20 kg), final live weight (34.60 *vs* 36.12 kg), milk intake (1.0 *vs* 1.03 kg/day), total dry matter intake (DMI) (406.32 *vs* 423.36 g/d), total crude protein intake (94.70 *vs* 96.97 g/day), daily gain (152.97 *vs* 164.19 g/day) and feed conversion ratio (FCR) (2.66 *vs* 2.59), respectively. The difference in initial live weight was minimized during animal selection and distribution process. In agreement with the present study, Frizzo et al. (2011) reported no effect of probiotic supplement in feed intake, live weight gain and FCR in calves. Similar results were also observed by Saleem et al. (2017) on milk intake, average daily gain (ADG) and total gain in pre-weaning lamb and by Ataşoğlu et al. (2010) in the case of pre-weaning goat kids, where kefir was supplied as the source of probiotic. Better management and feeding systems irrespective of treatments might have dimed the effect of probiotic on intake of calves in this study. Earlier, Ruppert et al. (1994) stated that probiotic

supplementation in feed may affect calf's feed intake only when they were kept under stressful condition. Further, no differences in feed intake might be the underlying reason for unaffected daily gain of calves in this study.

Table 9. Effect of feeding probiotic feed supplement on intake and growth performance of calves

Parameters	Control	Probiotic	SEM	Significant or not
Initial LW, kg	20.68	21.20	0.718	NS
Milk intake, kg/d	1.00	1.03	0.033	NS
Total DMI, g/d	406.32	423.36	14.08	NS
Total CPI, g/d	94.70	96.97	3.36	NS
Final LW, kg	34.60	36.14	1.925	NS
Daily gain, g/d	152.97	164.19	14.32	NS
FCR	2.66	2.59	0.231	NS

LW, Live weight; DMI, Dry matter intake; CPI, Crude protein intake; FCR, Feed conversion ratio; NS, Not significant

Effects of feeding probiotic feed supplement on physical properties of feces and frequencies of diarrhea in calves are described in Table 10. The physical properties of feces such as, color, odor and consistency was observed better in probiotic group compared to the control. Results revealed that in probiotic feed supplemented calves 93.3% cases were found normal feces color, while it was 87.03% in the control. Yellowish to yellow and yellow green color of feces were considered as abnormal color, which was found 5.2 and 7.77% *vs* 2.03 and 4.63% in the control *vs* probiotic feed supplemented calves respectively. Similarly, normal and bad odor, which was practically relevant to color properties were found 87.03 and 12.96% *vs* 93.33 and 6.66% in the control *vs* probiotic fed calves, respectively. It was found that the percentage of hard, normal, tended to be liquid and watery feces in the control group was 1.11, 86.66, 7.97 and 4.26%, respectively and all together they derive a consistency score of 2.12. While in the probiotic feed supplemented group, they were 3.52, 90.0, 4.81 and 1.66%, respectively and they gave a consistency score of 2.04 in probiotic group. There were 14 cases (2.60%) of diarrhea in the control group compared to 7 cases (1.30%) in probiotic group. The decreased frequency of diarrhea in probiotic fed calves as observed in this study is in agreement with many previous findings (Isyk et al., 2004; Abe et al., 1995; Fox, 1988; Maeng et al., 1987 and Bechmen et al., 1977). A trend for reduced diarrhea in this study may be explained by an antagonistic action of probiotic microbes towards diarrhoeagenic *E. coli* and implantation of probiotic microorganisms in the intestinal tract (Amanullah et al., 2009b; Yamazaki et al., 1991; Namioka et al., 1991; Ozawa et al., 1983). Significantly increased serum IgG concentration in probiotic fed calves, as shown in Table 12, might also have contributed to prevent diarrhea in this study.

Table 10. Effect of feeding probiotic feed supplement on physical properties of feces of calves

Parameters	Control (n=540)	Probiotic (n=540)
Color		
Normal	470 (87.03%)	504 (93.33%)
Yellowish-Yellow	28 (5.2%)	11 (2.03%)
Yellow green	42 (7.77%)	25 (4.63%)
Odor		
Normal	470 (87.03%)	504 (93.33%)
Bad	70 (12.96%)	36 (6.66%)
Consistency		
Hard (+)	6 (1.11%)	19 (3.52%)
Normal (++)	468 (86.66%)	486 (90.0%)
Tended to be liquid (+++)	43 (7.96%)	26 (4.81%)
Watery (++++)	23 (4.26%)	9 (1.66%)
Consistency Score (1-4)	2.12	2.04
Cases of Diarrhoea	14 (2.60%)	7 (1.30%)

n= number of observations (no. of replications × days = 6 × 90 = 540)

The fecal count of *E. coli, Salmonella, Lactobacillus (LAB), Bacillus subtilis and Saccharomyces cerevisiae* at different days during the experimental period are illustrated in Table 11. It was found that the *E. coli* concentration (log_{10} CFU/g) at 0, 15, 30, 45, 60, 75 and 90 days in the feces from calves under the control *vs* probiotic group was 7.86 *vs* 8.16 (P>0.05), 8.22 *vs* 8.24 (P>0.05), 8.34 *vs* 7.44 (P<0.001), 8.45 *vs* 7.36 (P<0.001), 8.33 *vs* 7.46 (P<0.001), 8.27 *vs* 6.71(P<0.01), 7.28 *vs* 6.14 (P<0.001), respectively. No *Salmonella* were detected in feces of calves irrespective of treatments at any days. The feces of calves under probiotic treatment contained 9.11, 4.11 and 3.63 log_{10} CFU/g of *LAB, B. subtilis* and *S. cerevisiae,* respectively at 90 days. However, in control group, *B. subtilis* and *S. cerevisiae* was not detected in the feces of calves. Only LAB was found in the feces of calves under the control group, but at a significantly lower (P<0.01) concentration (6.96 log_{10} CFU/g) than that in probiotic fed group. In agreement with the present study, decreased fecal count of *E. coli* in female calves resulted from probiotic feeding was also reported by Mohamadi Roodposhti and Dabiri (2012). Earlier, few mechanisms were suggested by which probiotics may reduce harmful bacteria like *E. coli* in intestinal tract and feces in calves (FAO, 2016). Probiotic microorganisms produce inhibitory substances such as organic acids, hydrogen peroxide and bacteriocins, which acts as antimicrobial-like compounds. Secondly, probiotic bacteria may inhabit competitively by expelling harmful bacteria like *E. coli* on intestinal epithelial surfaces. Elam et al. (2003) also reported decreased fecal *E. coli* shedding in beef steers fed *Lactobacillus acidophilus.* The LAB as beneficial bacteria normally associated with a balanced normal in the gut flora (Bayatkoushar et al., 2013). Increases in numbers of *Lactobacilli* can show a normal occurrence in the development of intestinal flora of calves (Gilliland and Speck, 1977). Amanullah et al. (2009a) found significantly increased number of LAB, while significantly reduced *E. coli* on intestinal surfaces of calves fed with LAB probiotic compared to that in the control.

Table 11. Effect of feeding probiotic feed supplement on fecal microbial load

Name of organisms detected at different days	Control (log_{10} CFU/g)	Probiotic (log_{10} CFU/g)	SEM	Sig
Eschericia coli				
0 day	7.87	8.16	0.259	0.315
15 day	8.22	8.24	0.159	0.874
30 day	8.34	7.44	0.086	<0.001
45 day	8.45	7.36	0.052	<0.001
60 day	8.33	7.46	0.066	<0.001
75 day	8.27	6.71	0.306	<0.01
90 day	7.28	6.14	0.128	<0.001
Salmonella spp. (0, 15, 30, 45, 60, 75, 90 day)	nd	nd	-	-
Lactic acid bacteria (90 day only)	6.96	9.11	0.428	<0.01
Bacillus subtilis (90 day only)	nd	4.11	-	-
Saccharomyces cerevisiae (90 day only)	nd	3.63	-	-

CFU, colony forming unit; nd, not detected; Sig, Significance level

Effects of feeding probiotic feed supplement on plasma metabolic profiles in calves are described in Table 12. The contents of blood glucose and blood urea nitrogen (BUN), which usually considered as indicator of energy and protein status, in blood of calves under control *vs* probiotic feed supplemented group were found to be 3.7 *vs* 3.9 mmol/l, 31.78 *vs* 31.68 mg/dl, respectively, which did not differ significantly (P>0.05) between treatments. These results are in agreement with other previous findings (Noori et al., 2016 and Frizzo et al., 2010). Similarly, Antunovic et al. (2006) reported no change in blood glucose concentration in probiotic supplemented lambs.

The total cholesterol and low density lipoprotein (LDL) concentrations in control *vs* probiotic fed calves were observed to be 157.2 *vs* 171.52 mg/dl and 76.45 *vs* 82.26 mg/dl, respectively, and the differences were not significant (P>0.05). Usually, it is believed that serum cholesterol is decreased by probiotic feeding and there are two proposed mechanisms for the reduction of serum cholesterol level in animals fed on probiotics (Noori et al., 2016). Zarate et al. (2002) suggested an increase in degradation of cholesterol across the gastrointestinal tract, while Farnades et al. (1987) suggested simultaneous sediment of cholesterol and deconjugation of bile acids in animals fed with probiotic. However, in the present study, no such results were observed. Moreover, the HDL concentration in calves was tended (P<0.10) to be higher in probiotic fed group than that in the control (37.55 *vs* 25.8 mg/dl). This result was in agreement with Deroos and Katan (2000), who showed that dietary inclusion of probiotic, resulted in an increased serum HDL concentration. In contrast, some others reported no effects of probiotic on HDL concentration in animals (Noori et al., 2016 and Panda et al., 2000).

Table 12. Effect of feeding probiotic feed supplement on blood metabolic profile of milk-fed calves

Parameters	Control	Probiotic	SEM	Sig.
Blood glucose (mmol/l)	3.7	3.9	0.118	0.152
BUN (mg/dl)	31.78	31.68	1.566	0.952
Total Cholesterol (mg/dl)	157.2	171.52	23.857	0.575
HDL (mg/dl)	25.8	37.55	5.136	0.071
LDL (mg/dl)	76.45	82.26	7.562	0.477
Triglyceride (mg/dl)	8.02	10.82	3.733	0.489
Cortisol (µg/dl)	0.52	0.66	0.147	0.401
IgG (ng/ml)	8.38	12.62	1.353	0.026
IgF-1 (g/l)	0.62	0.76	0.419	0.738
Insulin (mIU/ml)	0.76	0.52	0.212	0.319

BUN, Blood urea nitrogen; HDL, High density lipoprotein; LDL, Low density lipoprotein; IgG, Immunoglobulin G; IgF-1, Insulin-like growth factor-1/Somatotropin-c; Sig, Significance level

The triglyceride concentrations in control *vs* probiotic fed calves were observed to be 8.02 *vs* 10.82 mg/dl, respectively, and the differences were not significant (P>0.05). The effects of probiotic feeding on serum triglyceride contents in animals were found variable. In agreement with our finding, Panda et al. (2000) reported no change in serum triglyceride in pig. Unlike this study, Noori et al. (2016) reported a significant increase in serum triglycerides of calves fed yogurt probiotic (pH 3.8). They suggested decrease in pathogenic bacteria resulted from probiotic feeding might reduce conversion of primary bile acids to secondary one, and in turn, fat metabolism was increased. However, effects also might come from probiotic career yogurt as they contained added fat. On the other hand, Chiofalo et al. (2004) observed significantly decreased serum triglycerides in kids as a result of feeding dietary probiotics.

The immunoglobulin G (IgG) content was significantly (P<0.05) increased in probiotic fed calves than that in the control (Control *vs* probiotic: 8.38 *vs* 12.62 ng/ml). Riddell et al. (2010) reported an increasing tendency of serum IgG1 in pre-ruminant calves at day 42 fed with *Bacillus* based probiotic. Previously, it was hypothesized that addition of a *Bacillus* based probiotic to the diet would stimulate an increase in IgG1 levels as an anti-spore immune response (Hong et al., 2005). Duc et al. (2004) indicated an increase in IgG1 level in mice dosed with *B. subtilis.* In contrast, some researchers reported no effect of probiotic on serum immunoglobulin (Mohamadi Roodposhti and Dabiri, 2012; Morill et al., 1995).

Results showed that probiotic feed supplements prepared based on wheat bran and molasses, containing lactic acid bacteria (*L. acidophilus*), *Bacilus subtilis* and *Saccharomyces cerevisiae* improved fecal physical properties, reduced *coliform* count but increased probiotic bacteria count in the feces of calves. This probiotic feed supplement also reduced diarrheal frequency and increased immunoglobulin status as indicated by serum IgG concentration in probiotic fed calves compared to the control. However, effects of probiotic feed supplement were not reflected in feed intake, daily gain or feed conversion ratio (FCR). Earlier studies suggested that, probiotics are most effective in times of stress. It is plausible that the lack of differences between treatments in growth performances may be due to lack of stress on experimental calves irrespective of treatments. The calves were housed in individual pan in well ventilated house and managed under very good hygienic condition. A higher level of induced stress may be necessary to observe greater benefits of feeding probiotic feed supplement to calves. On the other hand, the trial was continued only up to 115 days of calf's age. The beneficial effects from improved fecal health, reduced diarrheal frequency and improved immunoglobulin status on production performances might be achieved later in advanced age. Further research will be needed to get fine-tuned conclusion on the issue of feeding probiotic feed supplements to calves.

12. Research highlight/findings:

- Four probiotic feed supplements based on rice polish (RP), wheat bran (WB), RP+ lentil powder (LP) and (WB + LP) were evaluated. Probiotic feed supplements based on RP and WB (Rice polish or Wheat bran 500g; Molasses 100g; Water 300ml; LAB, SC and BS culture 10ml each at a concentration of 6 $\log_{10}$ CFU/ml) could be recommended for feeding calves considering their physical, microbial and chemical characteristics and shelf-life.

- Among the RP and WB based probiotic feed supplements, WB based feed was found to be well accepted (voluntary intake) by calves and considered for evaluation through feeding trial.

- Upon feeding WB based probiotic feed supplement to milk-fed calves, as estimated at 90 days after birth, resulted in improved fecal characteristics, lesser fecal *E. coli* load ($\log_{10}$ CFU/g was found to be 7.28 in control compared to 6.14 in probiotic fed calves), on the contrary there were higher probiotic microbes in the feces (calves under probiotic treatment contained 9.11, 4.11 and 3.63 $\log_{10}$ CFU/g of *LAB, B. subtilis* and *S. cerevisiae,* respectively. However, in control group, *B. subtilis* and *S. cerevisiae* was not detected in the feces of calves), lower diarrheal incidence (2.60% in the control group compared to 1.30% in the probiotic group) and improved immunoglobulin status of calves (8.38ng/ml in control compared to 12.62ng/ml in probiotic fed group of calves).

- Further study, taking longer time, is needed before recommending the studied feed formula as probiotic feed supplement for feed manufacturer for producing and marketing or for farmers for home production of feed from known sources of probiotic bacteria.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	2 (Two)	20000.00	2 (Two)	19500.00	
(b) Lab &field equipment	2 (Two)	520000.00	2 (Two)	519000.00	
(c) Other capital items (Chemicals, Glassware & apparatus)	2 (Two)	743107.00	2 (Two)	743107.00	

2. Establishment/renovation facilities: Not applicable.

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: Not applicable.

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	355551.00	355551.00	310166.00	45385.00	87.24	Co-PI did not receive honorarium
B. Field research/lab expenses and supplies	1377404.00	1377404.00	1376975.00	429.00	99.97	
C. Operating expenses	59689.00	59689.00	59689.00	-	100.0	
D. Vehicle hire and fuel, oil & maintenance	0.00	0.00				
E. Training/workshop/seminar etc.	0.00	0.00				
F. Publications and printing	107200.00	35848.00	0.0	107200.00	0.0	Returned to BARC.
G. Miscellaneous	42300.00	42300.00	42300.00	-	100.0	
H. Capital expenses	540000.00	540000.00	538950.00	1050.0	99.81	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	**Major technical activities performed in respect of the set objectives**	**Output (i.e. product obtained, visible, measurable)**	**Outcome (short term effect of the research)**
1. To develop a feed supplement for calves containing a mixture of different probiotic microbes.	- Collection and evaluation of commercial sources of probiotic microorganisms - Formulation, evaluation and shelf-life study of probiotic feeds	Two (02) probiotic feed supplements, rice polish (RP) and wheat bran (WB) based, for calves developed.	
2. To study the effects of developed feed supplement on growth performance and health of calves.	- On station animal trial - Data collection, sample collection and laboratory analysis - Statistical analysis.	WB based Probiotic feed supplement was efficient in decreasing *E. coli* load (log_{10} CFU/g was found to be 7.28 in control compared to 6.14 in probiotic fed calves) and increasing probiotic microbes in the feces (calves under probiotic treatment contained 9.11, 4.11 and 3.63 log_{10} CFU/g of *LAB, B. subtilis* and *S. cerevisiae,* respectively. WB based probiotic feed also helped decreasing diarrhea (2.60% in the control group compared to 1.30% in the probiotic group) and increasing immunoglobulin status (8.38ng/ml in control compared to 12.62ng/ml in probiotic fed group) of calves.	

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.	01		
Journal publication	02		Under submission process
Information development			
Other publications, if any		01	Presented and 'Executive Summary' published in the proceedings of Annual Research Review Workshop (2018) of Bangladesh Livestock Research Institute held during 09-10 December, 2018

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

None

ii. **Generation of new knowledge that help in developing more technology in future**

The existing form of probiotic in market resembles to pharmaceutical products and marketed as bolus/powder/liquid. The knowledge generated from this research will enable to produce probiotic feed by the feed manufacturer. Even enthusiastic farmers will be enabling to make probiotic feed supplement at farm from commercial probiotic sources or from their own bacterial culture (as traditional yogurt makers). This will reduce cost and improve probiotic feeding management. However, extensive research is necessary to isolate probiotic bacterial species from natural sources followed by their probiotic characteristic study and use in feed.

iii. Technology transferred that help increased agricultural productivity and farmers' income

None

iv. Policy Support

None

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring:

During the project implementation period, monthly monitoring was done by Bangladesh Livestock Research Institute in every month through monthly Annual Development Program (ADP) Meeting headed by the Director General. All Divisional Heads, Section Heads, Project Directors and Principal Investigators (PIs) of different donor/other funded projects were participants of those meetings. In this meeting, the PI of CRG Sub-project had to present monthly physical, financial and technical progress of the sub-project and suggestions/recommendations were made where necessary.

The Project implementation unit of BARC (PIU-BARC-NATP-2) organized following workshops to evaluate the project activities and results.

Workshop	Date
Progress workshop	24-25 April, 2018
Monitoring workshop	15-16 May, 2018
Annual workshop	22-23 September, 2018

ii) Field Monitoring (time& No. of visit, Team visit and output):

Monitoring team	Date(s) of visit	Total visit till date (No.)	Output
Livestock Division, BARC	20 February, 2018	01	Valuable suggestions and recommendations were made
Internal team monitoring, BLRI	30 July, 2018	01	Valuable suggestions and recommendations were made

H. Lesson Learned (if any)

i) For very short time duration of the project, isolation of microbes from natural sources, their probiotic characteristics study and using in feed formulation, as planned in submitted project proposal, was found impossible to complete in time and therefore, livestock division/technical expert suggested to use commercial sources of known microbes for probiotic feed formulation. However, it would have been better, if microbes from natural sources were used for the study.

ii) For time and resource limitations, especially limitation of finding sufficient number of calves of same age, the animal trial was conducted considering only two treatments. More treatments with commercial probiotic supplement or antibiotic may provide more realistic data, especially when 'probitoic' is said as alternative to antibiotic growth promoter.

iii) More blood parameters like CBC and liver function test would be better but was not considered in this study due to constraints of time.

I. Challenges (if any)

i) Project duration was too short (only 11 months) to complete all the activities smoothly.

ii) All required calves of same age and sex were not available at a time; they entered into the trial when available. Therefore, total length of animal trial was lengthened which was 'daily labor' and other 'input' intensive.

iii) Recruited scientist resigned at the middle of animal trial. Without the support of scientific staff, it was a great challenge to finish all tasks within time.

Signature of the Principal Investigator
Date …….
Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

References:

Abe, F., Ishibashi, N. and Shimamura. S., 1995. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 78:2838–2846.

Amanullah, S. M., Alam, M. S., Subarna, R. N., Bateen, R., Huque, K. S. and Sultana, A. 2009b. Feeding *Lactobacilli* as probiotic and proportion of *Escherichia coli* in the intestine of calves. The Bang. Vet., 26(1): 17-22

Amanullah, S. M., Huque, K. S., Bateen, R., Subarna, R. N. and Alam, M. S. 2009a. Study of feeding Lactobacillus on growth performances of calves. Annual Report (2008-09), Animal Production Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka

Antunovic, Z., Speranda, M., Amidzic, D., et al. 2006. Probiotic application in lambs nutrition. Krmiva 4:175-80.

Ataşoğlu C, Akbağ HI, Tölü C, et al. 2010. Effects of kefir as a probiotic source on the performance of goat kids. South Afri. J. Anim. Sci. 4:363-70.

Bayatkouhsar, J., Tahmasebi, A. M., Naserian, A. A., Mokarram, R. R. and Valizadeh, R. 2013. Effects of supplementation of lactic acid bacteria on growth performance, blood metabolites and fecal coliform and lactobacilli of young dairy calves. Anim. Feed Sci. Technol. 186: 1-11.

Bechman, T. J., Chambers, J.V. and Cunningham, M.D. 1977. Influence of Lactobacillus acidophilus on the performance of young calves. J. Dairy Sci. Gl (Supp1. 1):74.

Chiofalo, V., Liotta, L. and Chiofalo, B. 2004. Effects of the administration of Lactobacilli on body growth and on the metabolic profile in growing Maltese goat kids. Reprod. Nutr. Dev. 44:449–457.

Collado MC, Meriluoto J, Salminen S (2007). Measurement of aggregation properties between probiotics and pathogens: In vitro evaluation of different methods. J. Microbiol. Meth. 71:71-74. PMID: 17719109.

Deroos, N. and Katan, M. B. 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and arcinogenesis: a review. Am. J. Clin. Nutr. 71:405–411.

Duc, L. H., Hong, H. A., Uyen, N. Q., and Cutting, S. M. 2004. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine. 22:1873-1885.

Effect of Feeding *Bacillus subtilis natto* on Hindgut Fermentation and Microbiota of Holstein Dairy Cows. Asian Australas. J. Anim. Sci. 27:495-502.

Elam, N. A., J. F. Gleghorn, J. D. Rivera, M. L. Galyean, P. J. Defoor, M. M. Brashears and S. M. Younts-dahl. 2003. Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacteroum freudenreichii* on performance, carcass, and intestinal characteristics and *Escherichia coli* strain 0157 shedding of finishing beef steers. J. Anim. Sci. 81: 2686-2698.

Fernades, C., Shahani, F. K. M. and Amer, M. A. 1987. Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. FEMS Microbiol. Lett. 46:343–356.

Food and Agriculture Organization of the United Nations/World Health Organization FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics in Food. Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food; London, Ontario, Canada: 2002.

Fox, S. M. 1988. Probiotics intestinal inoculants for production animals. Vet. Med. 83:806–830.

Frizzo, L. S., Bertozzi, E., Soto, L. P., Sequeira, G., Rodríguez Armesto, R. and Rosmini, M. R. 2010. Studies on translocation, acute oral toxicity and intestinal colonization pf potentially probiotic lactic acid bacteria administered during calf rearing. Lives. Sci. 128: 28-35

Frizzo, L. S., Soto, L. P., Zbrun, M. V., Signorini, M. L., Bertozzi, E., Sequeira, G., Rodríguez Armesto, R. and Rosmini, M. R. 2011. Effect of lactic acid bacteria and lactose on growth performance and intestinal microbial balance of artificially reared calves. Lives. Sci. 140: 246-252.

Fuller, R. 1989. Probiotics in man and animal: A review. Appl. Bacteriol. 66: 365-378.

Gilliland, S. E., and Speck, M.L. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and food borne pathogens in associative cultures. J. Food Prot. 40: 820–823.

Hong, H. A., L. H. Duc and S. M. Cutting. 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev.29:813-835.

Hossain, M. M., Islam, M. S., Kamal, A. H. M., Rahman, A. K. M. A. and Cho, H. S. 2014. Dairy cattle mortality in an organized herd in Bangladesh. Vet. World. 7:331-336.

Hughes, J.1988. The effect of a high strength yeast culture in the diet of early weaned calves. Anim. Prod. 46:526.

Hyronimus, B., C. Le Marrec, A. Hadj Sassi and A. Deschamps.2000. Acid and bile tolerance of spore-forming lactic acid bacteria. Int. J. Food Microbiol. 61:193-197.

Isyk, M., Ekimler, F., Ozen N. and Firat, M.Z. 2004. Effect of using probiotics on the growth performance and health of dairy calves. Turk J. Vet. Anim. Sci., 28: 63-69.

Jiang,Y., Ogunade,I. M., Arriola,K. G., Qi,M., Vyas,D., Staples,C. R. and Adesogan, A. T. 2017. Effects of the dose and viability of *Saccharomyces cerevisiae*. 2. Ruminal fermentation, performance of lactating dairy cows, and correlations between ruminal bacteria abundance and performance measures. J. Dairy Sci. 100:1-17.

Jordan, R. M. and Johnston, L. 1990. Yeast culture supplemented lamb diets. J. Anim. Sci. 68(Suppl 1):493.

Kung Jr, L. 2001. Direct-fed microbials for dairy cows and enzymes for lactating dairy cows: New theories and applications. In: 2001 Pennsylvania State Dairy Cattle Nutrition Workshop, Grantville, PA. pp. 86-102.

Maeng, W.J., Kim, C.W. and Shin, H.T 1987. Effect of a lactic acid bacteria concentrate (*Streptococcus faecium* Cernelle 68) on growth rate and scouring prevention in dairy calves. J. Dairy Sci. 9:204–210.

Malik, R. and Bandla, S. 2010. Effect of source and dose of probiotics and exogenous fibrolytic enzymes (EFE) on intake, feed efficiency, and growth of male buffalo (*Bubalus bubalis*) calves. Trop. Anim. Health Prod. 42:1263-1269.

Mohamadi Roodposhti, P. and Dabiri, N. 2012. Effects of probiotic and prebiotic on average daily gain, fecal shedding of *Escherichia Coli*, and immune system status in newborn female calves. Asian-Aust. J. Anim. Sci. 25: 1255-1261.

Morrill, J. L., M. Morrill and A. M. Feyerherm. 1995. Plasma protein and probiotic as ingredients in milk replacer. J. Dairy Sci. 78:902-907.

Namioka, S. Sasaki, T. and Maeda, Y. 1991. Immunopotentiation of the small intestine of weaning piglets by peptidoglycan derived from Bifidobacten'um thermophilum. Bifidobacteria Microflora 10: 1.

Noori, M., Alikhani, M and jahanian, R. 2016. Effect of partial substitution of milk with probiotic yogurt of different pH on performance, body conformation and blood biochemical parameters of Holstein calves. J. Appl. Anim. Res. 44: 221-229.

Ozawa. K., Yabu-uchi, K., Yamanaka, K., Ymashita, Y., Nomura,S**.** and Oku, I. 1983. Effect of *Streptococcus faecalis* BIO-4R on intestinal flora of weanling piglets and calves. Appl. Environ. Microbiol. 45: 15 13.

Panda, A. K., Reddy, M. R., Rama Rao, S. V., Raju, M. V. L. N. and Paraharaj, N. K. 2000. Growth, carcass characteristics, immunocompetence, and response to *Escherichia coli* of broiler fed diets with various level of probiotic. Archive für Geflugelkunde. 64:152–156.

Retta, K. S. 2016. Role of probiotics in rumen fermentation and animal performance: A review. I. J. Livest. Prod., 7(5): 24-32.

Riddell, J. B., Gallegos, A. J., Harmon, D. L., and McLeod, K. R. 2010. Addition of a *Bacillus* based probiotic to the diet of preruminant claves: influence on growth, health, and blood parameters. Intern. J. Appl. Vet. Med. 8: 78-85.

Ripamonti, B., A. Agazzi, A. Baldi, C. Balzaretti, C. Bersani, S.Pirani, R. Rebucci, G. Savoini, S. Stella, A. Stenico and C. Domeneghini. 2009. Administration of *Bacilluscoagulans* in calves: Recovery from faecal samples and evaluation of functional aspects of spores. Vet. Res. Commun. 33:991-1001.

Ruppert, L. D., McCoy, G.C. and Hutjens, M.F. 1994. Feeding of probiotics to calves. J. Dairy Sci. 77(Suppl. 1):296.

Saleem, A. M., Zanouny, A. I. and Singer, A. M. 2017. Growth performance, nutrient digestibility, and blood metabolites of lambs fed diets supplemented with probiotics during pre- and post-weaning period. Asian-Australian. J. Anim. Sci. 30: 523-533.

Seo, J. K., Kim, S. W., Kim, M. H., Upadhaya, S. D., Kam., D. K. and Ha, J. K. 2010. Direct-fed microbials for ruminant animals. Asian-Aust. J. Anim. Sci. 23(12): 1657-1667.

Song,D. J., Kang,H. Y., Wang,J. Q., Peng, H. and Bu, D. P.2014.

Yamazaki, S., Tsuyuki, S*.,* Akashina, H., Kamimura, H., Kimura, M.,Kawashma, T., and Ueda, K. 1991. Immune response of Bifidobacterium-monoassociated mice. Bifidobacteria Microflora 10: 19.

Zarate, G., Chaia, A. P. and Oliver, G. 2002. Some characteristics of practical relevance of the β-Galactosidase from potential probiotic strains of *Propionibacterium acidipropionici*. Anaerobe. 8:259–267.