サンザイム
Sanzyme Biologics

PrOme™ - Max

In-Vitro Studies

SANZYME BIOLOGICS (P) LTD
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CIN : U24110TG2016PTC112002

Factory Unit I: Door No: 7-4-115, Sy. No: 258 & 259, Gaganpahad, Bangalore Road, Hyderabad – 500052.,Telangana, India

Factory Unit II: Plot Nos. 19 to 22, Sy. No's. 321/1,11,12,13 & 276 & 277, Biotech Park, Phase - III, Genome Valley, Karkapatla (V), Markook (Mdl.), Siddipet (Dist.), Pin Code : 502 281,Telangana, India

サンザイム
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QUALITY CONTROL DEPERTMENT

Acid Tolerance Studies

Product: PROME MAX

Strain: *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL

Objective: To determine Acid tolerance assay of PROME MAX

Initial Potency for Study: 3000 CFU/ml

- **Acid Tolerance & Recovery Assay Protocol:**
  1. Prepare appropriate buffers as glycine HCl (Gly-HCl, pH 2.2 and 3.0) and Phosphate Buffered Saline (PBS, pH 7.0)
  2. Use overnight grown PROME MAX (~12 hour) culture, wash it thrice with PBS
  3. This washed and diluted culture is further diluted at 1:10 dilution in appropriate buffer at pH 2.0, 3.0 and 7. 0
  4. Measure Optical Density at 600 nm in a spectrophotometer at every hour for 6 hours starting from '0' hour
  5. At the same time, for each hour sample subject it for assay by taking 500 µl of sample, pour plate with Nutrient agar medium in triplicates, and incubate at 37^0C for 24 hours.
  6. Keep the culture sample prepared in normal saline without acid/buffer treatment as control.
  7. Below assay protocol can be adopted for pour plating.
  8. Plating:
     A. *Bacillus licheniformis* & *Bacillus subtilis*
        a) Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
        b) Incubate the solidified plates in an inverted position at 37 °C for 24 hours.
     B. *Bacillus coagulans*
        c) Set out sterile petri plates and add 1 ml of control and heat shocked (at 75 ^{0}C for 30 minutes) final dilution into each petri plate and then pour 15-20 ml molten

PNY medium into each of the petri plate and mix thoroughly (temperature around 45 °C)

d) Incubate the solidified plates in an inverted position at 37 °C for 48 hours.

9. Counting:

a) Count the number of CFU in each petri plate and calculate by taking the average number of CFU.

10. Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^9} = \text{Billion CFU /g}$$

*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
  - PNY Medium : Hi media Code: M835
- Preparation of sterile Isotonic Saline Solution
  - Weigh accurately about 9 g of Sodium Chloride in 1000 ml of distilled water. The solution shall be sterilized with steam at 1.5 kg/cm2 pressure at 121°C (or 15 lbs pressure) for 20 min and then cool.

Result:

Time (Hours)	pH 2.0 (CFU/ml)	% of Recovery	pH 3.0 (CFU/ml)	% of Recovery	pH 7.0 (CFU/ml)	% of Recovery
0	3000	100.00	3000	100.00	3000	100.00
1	2985	99.49	2979	99.29	2963	98.77
2	2981	99.35	2973	99.10	2954	98.46
3	2974	99.14	2970	99.00	2945	98.15
4	2969	98.96	2969	98.95	2939	97.95
5	2960	98.68	2962	98.74	2933	97.76
6	2940	98.00	2958	98.60	2925	97.50

Conclusion:

- PROME MAX was recovered 98.00% (2940 CFU/ml) at pH 2 and 6 Hours Incubation
- PROME MAX was recovered 98.60% (2958 CFU/ml) at pH 3 and 6 Hours Incubation
- PROME MAX was recovered 97.50% (2925 CFU/ml) at pH 7 and 6 Hours Incubation

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QUALITY CONTROL DEPERTMENT

Bile Tolerance Studies

Product: PROME MAX

Strain: *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL

Objective: To determine Bile tolerance of PROME MAX

Initial Potency for Study: 3000 CFU/ml

- **Bile Tolerance & Recovery Assay Protocol:**
  1. Prepare appropriate bile solutions at concentrations 0.15%, 0.3% and 0.6%
  2. Use overnight grown PROME MAX (~12 hour) culture, wash it thrice with PBS
  3. This washed and diluted culture is further diluted at 1:10 dilution in appropriate bile solution at 0.15%, 0.3% and 0.6% concentration
  4. Measure Optical Density at 600 nm in a spectrophotometer at every hour for 6 hours starting from '0' hour
  5. At the same time, for each hour sample subject it for assay by taking 500 µl of sample, pour plate with Nutrient agar medium in triplicates, and incubate at 37 ^{0}C for 24 hours.
  6. Keep the culture sample prepared in normal saline without bile solution treatment as control.
  7. Below assay protocol can be adopted for pour plating.
  8. Plating:
     a. *Bacillus licheniformis & Bacillus subtilis*
        a) Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
        b) Incubate the solidified plates in an inverted position at 37 °C for 24 hours.
     b. *Bacillus coagulans*
        c) Set out sterile petri plates and add 1 ml of control and heat shocked (at 75 ^{0}C for 30 minutes) final dilution into each petri plate and then pour 15-20 ml molten PNY medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
        d) Incubate the solidified plates in an inverted position at 37 °C for 48 hours.
  9. Counting:
        a) Count the number of CFU in each petri plate and calculate by taking the average number of CFU.

10. Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^9} = \text{Billion CFU /g}$$

*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
  - PNY Medium : Hi media Code: M835
- Preparation of sterile Isotonic Saline Solution
  - Weigh accurately about 9 g of Sodium Chloride in 1000 ml of distilled water. The solution shall be sterilized with steam at 1.5 kg/cm2 pressure at 121°C (or 15 lbs pressure) for 20 min and then cool.

Result:

Time (Hours)	Bile 0.15 % (CFU/ml)	% of Recovery	Bile 0.3 % (CFU/ml)	% of Recovery	Bile 0.6 % (CFU/ml)	% of Recovery
0	3000	100.00	3000	100.00	3000	100.00
1	2967	98.89	2969	98.96	2953	98.44
2	2953	98.43	2965	98.83	2947	98.24
3	2951	98.36	2962	98.72	2944	98.12
4	2935	97.82	2938	97.94	2938	97.93
5	2933	97.75	2927	97.55	2931	97.71
6	2928	97.60	2919	97.30	2928	97.60

Conclusion:

- PROME MAX was recovered 97.60% (2928 CFU/ml) at 0.15% Bile concentration and 6 Hours Incubation
- PROME MAX was recovered 97.30% (2919 CFU/ml) at 0.3% Bile concentration and 6 Hours Incubation
- PROME MAX was recovered 97.60% (2928 CFU/ml) at 0.6% Bile concentration and 6 Hours Incubation

SANZYME BIOLOGICS (P) LIMITED. QUALITY ASSURANCE

QUALITY CONTROL DEPERTMENT

Thermostability Studies

Product: PROME MAX

Strain: *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL

Objective: To determine the Thermostability activity of PROME MAX

Initial Potency for Study: 4 Billion CFU/g

Temperatures Exposed: 85 ºC

Time: Each sample was exposed to the said temperatures for 2 and 5 minutes

Heat Treatment & Recovery Assay Protocol:

1) Sample Preparation:
   a. Take 1.0 g of sample and transfer to 100 ml sterile standard volumetric flask
   b. Add approximately 60 ml of sterile saline solution and shake vigorously.
   c. Transfer 1 ml of this stock solution into 9.0 ml of saline solution in a sterile test tube and mix thoroughly.
   d. Continue serial dilution until the desired dilution series is obtained.
2) Heat Shock:
   a. Allow the final dilution to stand in the water bath at 85 °C for 2 & 5 minutes respectively
   b. Cool the test tubes to room temperature.
   c. Use final dilution without heat shock as control sample.
3) Plating:
   A. *Bacillus licheniformis* & *Bacillus subtilis*
      a) Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
      b) Incubate the solidified plates in an inverted position at 37 °C for 24 hours.
   B. *Bacillus coagulans*
      c) Set out sterile petri plates and add 1 ml of control and heat shocked (at 75 ^{0}C for 30 minutes) final dilution into each petri plate and then pour 15-20 ml molten PNY medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
      d) Incubate the solidified plates in an inverted position at 37 °C for 48 hours.
4) Counting:
   a) Count the number of CFU in each petri plate and calculate by taking the average number of CFU.

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5) Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^9} = \text{Billion CFU /g}$$

*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
  - PNY Medium : Hi media Code: M835
- Preparation of sterile Isotonic Saline Solution
  - Weigh accurately about 9 g of Sodium Chloride in 1000 ml of distilled water. The solution shall be sterilized with steam at 1.5 kg/cm2 pressure at 121°C (or 15 lbs pressure) for 20 min and then cool.

Results:

Sample No.	Initial Potency	Temp.	Time	Recovery Potency	
	Bn CFU/g	ºC	min.	Bn CFU/g	%
1	4.0	85	2	3.5	86.90%
2	4.0	85	5	3.4	84.30%

*Bn ➔ Billion
*Temp. ➔ Temperature

Conclusions:

- PROME MAX was recovered 86.90% (3.5 Bn CFU/g) when treated at 85 ºC for 2 minutes
- PROME MAX was recovered at 84.30% (3.4 Bn CFU/g) when treated at 85 ºC for 5 minutes

SANZYME BIOLOGICS (P) LIMITED. QUALITY ASSURANCE

QUALITY CONTROL DEPERTMENT

Antibiotic / AGP Compatibility Studies

Product: PROME MAX

Strain: *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL

Objective: To determine Antibiotic / AGP Compatibility of PROME MAX with commonly used Antibiotics / AGPs in Poultry Production Systems

Initial Potency for Study: 4 Billion Cells/g

- **Antibiotic Compatibility & Recovery Assay Protocol:**
  1) PROME MAX formulation was weighed upto100 g in sterile LDPE polythene bags.
  2) Appropriate antibiotic was added as per dosage pattern to the above bags containing PROME MAX formulation.
  3) The sample was blended thoroughly.
  4) PROME MAX and Antibiotic blended covers were kept at desired temperature (between 25 °C to 30 °C and RH 60% to 75%) for 15 days.
  5) At periodic time intervals, 1.0 g of samples from above pre-blended formulation bags at 0, 1, 3, 6 hours, were kept for total viable cells assay. Same samples stored at desired conditions were drawn and continued for assay counts after 24 hrs. 7days and 15 days.
  6) The total viable cells count were enumerated by maintaining antibiotic free formulation sample as control.
  7) Tenfold serial dilution technique was adopted in order to enumerate total viable cells.
  8) While conducting enumeration Nutrient agar (*Bacillus subtilis* & *Bacillus licheniformis*), PNY Medium (*Bacillus coagulans*) were used for plating.
  9) After incubation, colony forming units (CFU) were counted and compared against respective controls in order to find the survivability %
  10) Below assay protocol was adopted for pour plating.
  11) Plating:
      A. *Bacillus licheniformis* & *Bacillus subtilis*
         a) Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
         b) Incubate the solidified plates in an inverted position at 37 °C for 24 hours.
      B. *Bacillus coagulans*
         c) Set out sterile petri plates and add 1 ml of control and heat shocked (at 75 ^{0}C for 30 minutes) final dilution into each petri plate and then pour 15-20 ml molten

PNY medium into each of the petri plate and mix thoroughly (temperature around 45 °C)

d) Incubate the solidified plates in an inverted position at 37 °C for 48 hours.

12) Counting:

a) Count the number of CFU in each petri plate and calculate by taking the average number of CFU.

13) Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^9} = \text{Billion CFU /g}$$

*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
  - PNY Medium : Hi media Code: M835
- Preparation of sterile Isotonic Saline Solution
  - Weigh accurately about 9 g of Sodium Chloride in 1000 ml of distilled water. The solution shall be sterilized with steam at 1.5 kg/cm2 pressure at 121 °C (or 15 lbs pressure) for 20 min and then cool.

Results:

Time	Control	BMD	CTC	LIN	ZnB	MAD	NEO	TYL
	Potency Recovered (Bn CFU/g)							
0 Hr.	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
1 Hr.	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
3 Hrs.	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
6 Hrs.	4.00	4.00	3.96	3.95	3.94	3.98	3.89	3.96
1 Day	3.99	3.99	3.91	3.88	3.89	3.94	3.85	3.94
7 Days	3.99	3.98	3.84	3.87	3.89	3.93	3.84	3.91
15 Days	3.99	3.95	3.84	3.78	3.86	3.91	3.75	3.89

*BMD ➔ Bacitracin Methyl Disalicylate
*CTC ➔ Chlortetracycline
*LIN ➔ Lincomycin
*ZnB ➔ Zinc Bacitracin
*MAD ➔ Maduramicin
*NEO ➔ Neomycin
*TYL ➔ Tylosin
*Bn ➔ Billion

Conclusions:

- PROME MAX is compatible with Bacitracin Methylene Disalicylate and 98.8% (3.95 Billion CFU/g) PROME MAX was recovered after 15 days
- PROME MAX is compatible with Chlortetracycline and 95.9% (3.84 Billion CFU/g) PROME MAX was recovered after 15 days
- PROME MAX is compatible with Lincomycin and 94.5% (3.78 Billion CFU/g) PROME MAX was recovered after 15 days
- PROME MAX is compatible with Zinc Bacitracin and 96.4% (3.86 Billion CFU/g) PROME MAX was recovered after 15 days
- PROME MAX is compatible with Maduramycin and 97.8% (3.91 Billion CFU/g) PROME MAX was recovered after 15 days
- PROME MAX is compatible with Neomycin and 93.8% (3.75 Billion CFU/g) PROME MAX was recovered after 15 days
- PROME MAX is compatible with Tylosin and 97.3% (3.89 Billion CFU/g) PROME MAX was recovered after 15 days

    - PROME MAX is compatible with with commonly used Antibiotics / AGPs in Poultry Production Systems

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QUALITY CONTROL DEPERTMENT

Enzymatic Activity Studies

Product: SPORICH TOTAL

Strain: *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL + *Saccharomyces boulardii* SAN 158SB

Objective: To determine the Enzymatic Activity of SPORICH TOTAL

Potency for Study: 4 Billion CFU/g

- **Rapid plate assay for screening of enzyme activity:**
  1. Appropriate media are prepared, sterilized and kept for sterility for 24 hours at 37 °C.
  2. SPORICH TOTAL is inoculated & streaked directly on the surface of the suitable agar medium plates
     - for Protease activity – Skimmed Milk Agar (SMA)
     - for Amylase activity – Starch Agar (SA)
     - for Lipase activity – Tributyrin Agar (TA)
     - for Xylanase activity – Xylan Agar (XA)
     - for Cellulase activity – Carboxy Methyl Cellulose (CMC) Agar
  3. Agar medium plates are incubated at 37 °C for 24-48 hrs.
  4. After incubation, the agar medium plates are observed for clearence zone of different enzymes.

Observations:

Enzyme	Reagent/Indicator	Activity Observed
Protease	Direct	Halo/Clearance Zone around colony on SMA plates
Amylase	Iodine Solution	Clearance Zone around the colony on SA plates upon Iodine solution flooding
Lipase	Direct	Clearance Zone around the colony on TA plates
Xylanase	Congored solution	Clearance Zone around the colony on XA plates upon congo red solution washing
Cellulase	Congored solution	Clearance Zone around the colony on CMC agar plates upon congo red solution washing

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Protease Amylase Lipase Xylanase Cellulase

Conclusion:

- SPORICH TOTAL has Dominant Proteolytic, Amylolytic, Lipolytic, Xylanolytic & Cellulolytic activity

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QUALITY CONTROL DEPERTMENT

Antimicrobial Activity Studies

Product: PROME MAX

Strain: *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL

Objective: To determine Antimicrobial Activity of PROME MAX

Initial Potency for Study: 4 Billion Cells/g

Standard bacterial Cultures

The below mentioned bacteria were used along with test sample simultaneously to verify the susceptibility zones

S. No	Organism
1	*Salmonella enteritides* ATCC 13076
2	*Clostridium perfringens* ATCC 13124
3	*Escherichia coli* ATCC 8739

Protocol adopted for the experiment:

- One gram of PROME MAX was inoculated in to nurtient broth and incubated for 24 hours at 37 °C.
- Overnight grown listed standard cultures were taken and spreaded on pre-incubated Nutrient agar and Fluid thioglycolate agar plates
- Wells were made using cork borer, the wells were added with PROME MAX supernatant, pellet and culture, and plates were incubated for 24 hours at 37 °C.
- Plates were observed for presence/absence of zones around the wells followed by incubation.

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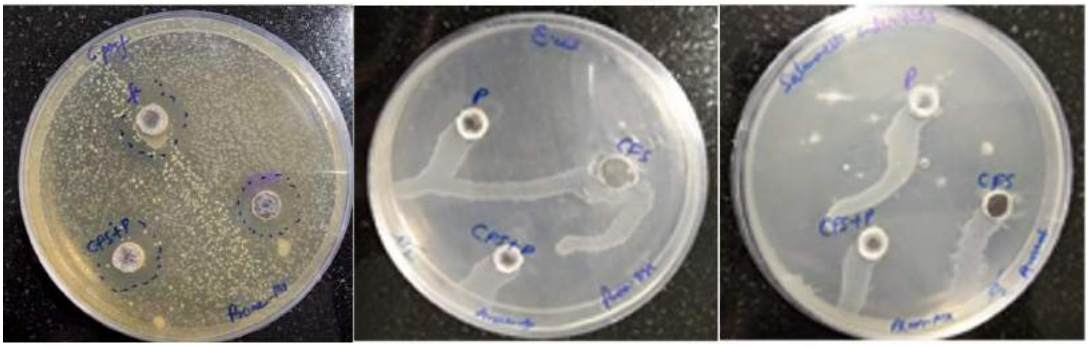
Observations and Results:

Antibacterial activity against	PROME MAX		
	Pellet	CFS	WB
Salmonella enteridis ATCC 13076	+	+	+
Clostridium perfringens ATCC 13124	++	++	++
Escherichia coli ATCC 8739	+	++	++

*CFS ➔ Cell free supernatant
*WB ➔ Whole broth
*+ ➔ Positive
*++ ➔ Moderate Zone

Zones of Inhibition against:



Clostridium perfringens *Escherichia coli* *Salmonella enteridis*

Conclusions:

- PROME MAX demonstrated significant Antimicrobial Activity against *Clostridium perfringens*, *Escherichia coli* & *Salmonella enteridis.*

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Room 542, Research Laboratory Unit, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Rd., Siriraj, Bangkoknoi, Bangkok 10700, Thailand

Antimicrobial Resistant Gene Detection Report

Date: 04.02.2020

Name & Address of customer		Sanzyme Biologics Plot No. 13, Sugar Society, Road No.2, Banjara Hills, Hyderabad-500 034	
Sample ID / type		*Bacillus subtilis* San144BS	
Date of sample receipt	22-01-2020	Method of test	Real-time PCR with specific probes
Date of sample testing	23-01-2020 to 03-02-2020	Condition of sample when receive	O.K.

Results

	Antimicrobial classes	Target gene	Results
1	β-lactams	CTX-M1	NF
2	(penicillin,	CTX-M2-M74	NF
3	amoxicillin,	CTX-M8-M25	NF
4	cephalosporin)	CTX-M9	NF
5		PER	NF
6		VEB	NF
7		CMY1-MOX	NF
8		CMY2-LAT	NF
9		DHA	NF
10		FOX	NF
11		ACT-MIR	NF
12		OXA-1	NF
13		OXA-9	NF
14	Carbapenems	KPC	NF
15		GES	NF
16		NDM	NF
17		VIM	NF
18		IMP	NF
19		OXA-48	NF
20	Folate pathway	*sul1*	NF
21	inhibitors	*sul2*	NF
22		*sul3*	NF
23		*dfrA1*	NF
24		*dfrA5-14*	NF
25		*dfrA12*	NF
26		*dfrA17*	NF

	Antimicrobial classes	Target gene	Results
27	Polymyxins	*mcr-1*	NF
28		*mcr-2*	NF
29	Tetracyclines	*tetA*	NF
30		*tetB*	NF
31	Phenicols	*cmlA*	NF
32		*floR*	NF
33		*catA1*	NF
34		*catB3*	NF
35	Aminoglycosides	*aacC1*	NF
36		*aacC2*	NF
37		*aacC4*	NF
38		*aphA1*	NF
39		*aphA6*	NF
40		*aadA1-2-17*	NF
41		*aadB*	NF
42		*armA*	NF
43		*rmtB*	NF
44	Macrolides	*ermB*	NF
45		*mphA*	NF
46	Quinolones	*qnrA*	NF
47		*qnrS*	NF
48		*qnrB1*	NF
49		*qnrB4*	NF
50		*Qep*A	NF

~ 3-8 X10^6 cells was tested. Positive grading criteria: 1+: ≥ 10^1-10^2, 2+: >10^2-10^3 and 3+ = >10^3 positive cells NF = Not found

Tested person: Rattapha Chinli
(Ms. Rattapha Chinli)

Authorized person: Suporn Foongladda
(Assoc.Prof.Dr. Suporn Foongladda)

Tel: 02-4199811, 0819390258 e-mail: suporn.foo@mahidol.ac.th

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ฝ่ายวิจัย สำนักงานคณบดี คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล โทร.0-24192995-7 โทรสาร.0-24183307
เลขประจำตัวผู้เสียภาษี 0994000158378

Room 542, Research Laboratory Unit, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Rd., Siriraj, Bangkoknoi, Bangkok 10700, Thailand

Antimicrobial Resistant Gene Detection Report

Date: 04.02.2020

Name & Address of customer		Sanzyme Biologics Plot No. 13, Sugar Society, Road No.2, Banjara Hills, Hyderabad-500 034	
Sample ID / type		*Bacillus coagulans* San135BC	
Date of sample receipt	22-01-2020	Method of test	Real-time PCR with specific probes
Date of sample testing	23-01-2020 to 03-02-2020	Condition of sample when receive	O.K.

Results

	Antimicrobial classes	Target gene	Results
1	β-lactams	CTX-M1	NF
2	(penicillin,	CTX-M2-M74	NF
3	amoxicillin,	CTX-M8-M25	NF
4	cephalosporin)	CTX-M9	NF
5		PER	NF
6		VEB	NF
7		CMY1-MOX	NF
8		CMY2-LAT	NF
9		DHA	NF
10		FOX	NF
11		ACT-MIR	NF
12		OXA-1	NF
13		OXA-9	NF
14	Carbapenems	KPC	NF
15		GES	NF
16		NDM	NF
17		VIM	NF
18		IMP	NF
19		OXA-48	NF
20	Folate pathway	*sul1*	NF
21	inhibitors	*sul2*	NF
22		*sul3*	NF
23		*dfrA1*	NF
24		*dfrA5-14*	NF
25		*dfrA12*	NF
26		*dfrA17*	NF

	Antimicrobial classes	Target gene	Results
27	Polymyxins	*mcr-1*	NF
28		*mcr-2*	NF
29	Tetracyclines	*tetA*	NF
30		*tetB*	NF
31	Phenicols	*cmlA*	NF
32		*floR*	NF
33		*catA1*	NF
34		*catB3*	NF
35	Aminoglycosides	*aacC1*	NF
36		*aacC2*	NF
37		*aacC4*	NF
38		*aphA1*	NF
39		*aphA6*	NF
40		*aadA1-2-17*	NF
41		*aadB*	NF
42		*armA*	NF
43		*rmtB*	NF
44	Macrolides	*ermB*	NF
45		*mphA*	NF
46	Quinolones	*qnrA*	NF
47		*qnrS*	NF
48		*qnrB1*	NF
49		*qnrB4*	NF
50		*Qep*A	NF

~ 3-8 X10^6 cells was tested. Positive grading criteria: 1+: ≥ 10^1-10^2, 2+: >10^2-10^3 and 3+ = >10^3 positive cells NF = Not found

Tested person: Rattapha Chinli
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เลขประจำตัวผู้เสียภาษี 0994000158378

Room 542, Research Laboratory Unit, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Rd., Siriraj, Bangkoknoi, Bangkok 10700, Thailand

Antimicrobial Resistant Gene Detection Report

Date: 11.11.2021

Name & Address of customer		SANZYME BIOLOGICS (P) LTD Plot No.13. Sagar Society, Road No.2, Banjara Hills, Hyderabad-500 034., Telangana, India	
Sample ID / type		*Bacillus licheniformis* - SAN 136BL	PTA337
Date of sample receipt	01-11-2021	Method of test	Real-time PCR with specific probes
Date of sample testing	05-11-2021 to 10-11-2021	Condition of sample when receive	Pure colonies on slant agar

Results

	Antimicrobial classes	Target gene	Results
1	β-lactams	CTX-M1	NF
2	(penicillin,	CTX-M2-M74	NF
3	amoxicillin,	CTX-M8-M25	NF
4	cephalosporin)	CTX-M9	NF
5		PER	NF
6		VEB	NF
7		CMY1-MOX	NF
8		CMY2-LAT	NF
9		DHA	NF
10		FOX	NF
11		ACT-MIR	NF
12		OXA-1	NF
13		OXA-9	NF
14		GES	NF
15	Carbapenems	KPC	NF
16		NDM	NF
17		VIM	NF
18		IMP	NF
19		OXA-48	NF
20	Folate pathway	*sul1*	NF
21	inhibitors	*sul2*	NF
22		*sul3*	NF
23		*dfrA1*	NF
24		*dfrA5-14*	NF
25		*dfrA12*	NF
26		*dfrA17*	NF

	Antimicrobial classes	Target gene	Results
27	Polymyxins	*mcr-1*	NF
28		*mcr-2*	NF
29	Tetracyclines	*tetA*	NF
30		*tetB*	NF
31	Phenicols	*cmlA*	NF
32		*floR*	NF
33		*catA1*	NF
34		*catB3*	NF
35	Aminoglycosides	*aacC1*	NF
36		*aacC2*	NF
37		*aacC4*	NF
38		*aphA1*	NF
39		*aadA4-5*	NF
40		*aphA6*	NF
41		*aadA1-2-17*	NF
42		*aadB*	NF
43		*armA*	NF
44		*rmtB*	NF
45	Macrolides	*ermB*	NF
46		*mphA*	NF
47	Quinolones	*qnrA*	NF
48		*qnrS*	NF
49		*qnrB1*	NF
50		*qnrB4*	NF
51		*Qep*A	NF

~ 3-8 X10^6 cells was tested. Positive grading criteria: 1+: ≥ 10^1-10^2, 2+: >10^2-10^3 and 3+ = >10^3 positive cells NF = Not found

Tested person: Rattapha Chinli
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In-Vivo Studies

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CIN : U24110TG2016PTC112002

Factory Unit I: Door No: 7-4-115, Sy. No: 258 & 259, Gaganpahad, Bangalore Road, Hyderabad – 500052.,Telangana, India

Factory Unit II: Plot Nos. 19 to 22, Sy. No's. 321/1,11,12,13 & 276 & 277, Biotech Park, Phase - III, Genome Valley, Karkapatla (V), Markook (Mdl.), Siddipet (Dist.), Pin Code : 502 281,Telangana, India

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Effects of SANZYME BIOLOGIC's, **Prome™ - BS** & **Prome™ - Max** on intestinal integrity and immune response of broiler chickens challenged with *Clostridium perfringens*

Study **Location:** R&D Facility, Thailand

Study **Period:** July - Aug 2016 (42 days)

Experimental Design

Study Groups	**Description**	
T1	Practical Control (Positive control)	PC
T2	Positive Control + *Clostridium perfringens* (CP)	PC + CP
T3	PC + **Prome™-BS** (100g/T) + CP	PC + PBS + CP
T4	PC + **Prome™-Max** (100g/T) + CP	PC + PMX + CP
T5	PC + Competitor *Bacillus subtilis* (100g/T) + CP	PC + CBS + CP
T6	PC + Zinc Bacitracin (335g/T) + CP	PC + ZB + CP

Controlled Inputs

1	T2, T3, T4, T5 & T6 groups were infected with *Clostridium perfringens* (CP) $3x10^9$ CFU/g per bird per day on day 13, 14 & 15
2	T2, T3, T4, T5 & T6 groups were infected with oocysts of *Eimeria spp.* on day 7
3	All groups were induced with a heat stress through temperature scale by 3°C to 4°C
4	**Prome™-BS** is a proprietary strain of *Bacillus subtilis* SAN 144BS • Potency used for experiment = 1 Billion CFU/g
5	**Prome™-Max** is a proprietary blend of *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL • Potency used for experiment = 4 Billion CFU/g
6	Competitor product was a well-known brand of *Bacillus subtilis* • Potency used for experiment = 1 Billion CFU/g

Sanzyme Biologics Pvt Ltd, Regd. Office: Plot No.13. Sagar Society, Road No.2, Banjara Hills, Hyderabad-500 034, Telangana, India E-Mail: info@sanzymebiologics.com, Website: www.sanzymebiologics.com

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Growth Performance

FCR			
Groups	**Description**	0-18 day	0-42 day
T1	PC	1.275	1.759
T2	PC + CP	1.358	1.843
T3	PC + PBS + CP	1.313	1.610
T4	PC + PMX + CP	1.412	1.790
T5	PC + CBS + CP	1.330	1.650
T6	PC + ZB + CP	1.321	1.729

Serum Antibody Titers

Groups	**Description**	**Antibody Titers - ND**	**Antibody Titers - IBD**
		18^{th} day	42^{nd} day
T1	PC	2.33	514
T2	PC + CP	1.75	576
T3	PC + PBS + CP	2.20	644
T4	PC + PMX + CP	2.35	657
T5	PC + CBS + CP	1.83	521
T6	PC +ZB + CP	2.17	564

Intestinal Morphological Analysis (Histo-patholology Report)

Groups	**Description**	**V/C Ratio - Jejunum**	**V/C Ratio - Ileum**
		42^{nd} day	42^{nd} day
T1	PC	12.08	7.06
T2	PC + CP	7.99	5.37
T3	PC + PBS + CP	11.93	6.71
T4	PC + PMX + CP	11.98	6.75
T5	PC + CBS + CP	11.27	6.33
T6	PC +ZB + CP	10.45	6.42

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Bactericidal Effect on *Clostridium perfringens*
(Microbiological Assay Report)

Groups	Description	CP - Ileum (x 10^9 CFU/g)	CP - Caecum (x10^9 CFU/g)
		42nd day	42nd day
T1	PC	2.54	3.69
T2	PC + CP	6.23	6.29
T3	PC + PBS + CP	2.77	3.47
T4	PC + PMX + CP	2.65	3.32
T5	PC + CBS + CP	2.97	4.12
T6	PC + ZB + CP	3.88	5.63

Protein Retention Assessment in Gut
(Biochemical Assay Report)

Groups	Description	Protein Retention % (42nd day)
T1	PC	60.64
T2	PC + CP	57.73
T3	PC + PBS + CP	63.49
T4	PC + PMX + CP	64.01
T5	PC + CBS + CP	63.04
T6	PC + ZB + CP	63.24

Conclusions:

1. **Prome™-BS** & **Prome™-Max** have a strong activity against *Clostridium perfringens* as compared tocompetitor BS & AGP
2. **Prome™-BS** & **Prome™-Max** improved immunity significantly as compared to competitor BS
3. **Prome™-BS** & **Prome™-Max** improved overall zootechnical performance and gut integrity in challenged conditions as compared to competitor BS

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Factory Unit II: Plot Nos. 19 to 22, Sy. No's. 321/1,11,12,13 & 276 & 277, Biotech Park, Phase - III, Genome Valley, Karkapatla (V), Markook (Mdl.), Siddipet (Dist.), Pin Code : 502 281,Telangana, India

Test Result of Probiotics (Prome-Max) in Commercial Broilers

1、 Time & Date & Place

Start: Nov 5th 2016

End : Dec 20th 2016

Place: Shenyang Daxin Village Ying De Broiler Company.

2、 Test grouping

Our testing ground containing 35 broiler coops in total,4 floor cage-rearing, each coop raising AA broiler chicks for 12000, we have divided the 35 coops to 3 repeating groups(means 3 testing area)

Control group: Area 1 (9 coops in total: 13#, 14#, 17－23#)
Area 2 (11 coops in total: 1-9#, 11#, 12#)
Area 3 (10 coops in total: 24-29#, 31#, 33-35#)

Test group: Area 1 (2 coops in total: 15#, 16#)
Area 2 (1 coops in total: 10#)
Area 3 (2 coops in total: 30#, 32#)

Probiotic additive **“Prome-Max”** have been added in the drinking water of Test group, dosage : 100g / ton of feed(for the whole stage)

3、 Feeding Management：

3.1 The broilers in control groups and test groups are raising in the same factory area of the company. The temperature, moisture and illumination of the coops are in strict accordance with the feeding procedure. Regularly disinfection, moderate density. Both the two groups are raising in the same environment, feeding complete diet that provided by Ying De company, cafeteria feeding, plastic water fountain providing sufficient water.

3.2 Calculate the death amount, feed consumption of every coop separately. Regularly weighing every week, and calculate average weight separately.

3.3 During the whole stage, the control group broilers take medicines in accordance with broiler preventive medicine program

1-4d	Chubaobao	Amoxicillin	Application
1-4d	Gastritis capsule	Chinese medicine	Feed Premix
1-4d	Vitamin		
12-14d		Amoxicillin	Drinking water
22-24d		Gentamicin	Drinking water
22-24d		Chinese medicine	Feed Premix

32-35d		Neomycin	Drinking water
32-35d		Chinese medicine	Feed Premix

3.4 Vaccine immunization be proceed according to immune procedure, detail as below:

0d	Flu bursa of fabricius	Intramuscular
7d	New flow duplex	Intramuscular
7d	New branch of renal	Eye dropping
21d	New branch of renal	Drinking water

4、Test result

Table 1. Area 1 test result

Group	Coop	Start	Survival rate	Average Weight In marketing	FCR	Day	European efficiency
Control gruop	13	Nov 5	98.0	2.97	1.71	43	395.8
	14	Nov 5	97.0	2.97	1.69	43	396.4
	17	Nov 5	95.0	3.02	1.72	43	387.9
	18	Nov 5	96.0	2.99	1.68	43	397.3
	19	Nov 7	98.0	2.94	1.71	43	391.8
	20	Nov 7	96.0	2.91	1.72	42	386.7
	21	Nov 7	97.0	3.01	1.68	42	413.8
	22	Nov 7	96.0	2.93	1.72	43	380.3
	23	Nov 7	97.0	2.98	1.72	42	400.1
Average Value			96.7	2.97	1.71	42.7	394.5
Test group	15	Nov 5	96.0	3.07	1.71	43	400.8
	16	Nov 5	97.0	3.04	1.68	43	408.2
Average Value			96.5	3.06	1.70	43.0	404.5

Table 2. Area 2 test result

Group	Coop	Start	Survival rate	Average Weight In Marketing	FCR	Day	European efficiency
Control Group	1	Nov 9	96.7	2.86	1.72	42	382.8
	2	Nov 9	94.6	2.58	1.76	41	338.2
	3	Nov 9	97.2	2.78	1.69	42	380.7
	4	Nov 9	95.7	2.66	1.74	41	356.8
	5	Nov 11	97.5	3.02	1.73	44	386.8
	6	Nov 11	97.2	2.96	1.75	44	373.7
	7	Nov 11	97.6	2.97	1.70	43	396.5
	8	Nov 11	96.6	2.88	1.70	42	389.6
	9	Nov 11	96.4	2.98	1.72	42	397.7
	11	Nov 13	95.7	2.90	1.78	44	354.4
	12	Nov 13	95.5	2.80	1.73	43	359.5
Average Value			96.4	2.85	1.73	42.5	374.2
Test Group	10	Nov 13	97.0	2.92	1.73	42	389.8

Table 3. Area 3 test result

Group	Coop	Start	Survival Rate	Average Weight In marketing	FCR	Day	European efficiency
Control Group	24	Nov 13	96.0	2.97	1.72	42	394.7
	25	Nov 13	95.8	2.85	1.70	42	382.4
	26	Nov 15	95.0	2.94	1.72	43	377.6
	27	Nov 15	95.0	2.86	1.73	43	365.2
	28	Nov 15	95.8	2.85	1.70	42	382.4
	29	Nov 15	96.4	2.92	1.69	43	387.4
	31	Nov 15	93.6	2.92	1.74	42	374.0
	33	Nov 17	94.8	2.96	1.77	43	368.7
	34	Nov 17	98.0	3.15	1.71	43	419.8
	35	Nov 17	94.3	2.88	1.69	43	373.7
Average value			95.5	2.93	1.72	42.6	382.6
Test	30	11月17日	97.0	2.94	1.71	42	397.1

Group	32	11月17日	94.0	2.95	1.74	42	379.4
Average value			95.5	2.95	1.73	42.0	388.3

Table 4. Test Result Summary

	Broiler No.	Survival rate	Average Weight In marketing	FCR	Day	European Efficiency
Control group	360000	96.2	2.91	1.72	42.6	383.1
Test Group	60000	96.2	2.98	1.71	42.4	395.1

5、Conclusion

The probiotic additive **“Prome-Max”** can effectively increase the average weight in marketing of broilers, lower the FCR value and obviously improve the European efficiency, and improve the culture benefit.

英德集團
Ying De Group

F I N A L R E P O R T

A comparative study on the effects of dietary supplementation of a multi-strain probiotic (Prome-Max™) and a single strain probiotic (*Bacillus subtilis*) on growth performance, immune functions, histology of the small intestine and selected bacterial population in the caecal digesta of commercial broiler chickens

Sponsor

Sanzyme Biologics

Investigators from Agrivet Consultancy P Ltd

Dr Shivaji Dey (Director, Agrivet Consultancy Pvt, Ltd.), shivaji@agrivet.in
Dr Anirvid Sarkar (Director, Agrivet Consultancy Pvt Ltd.) anirvid@agrivet.in
Dr Amrita Kumar Dhara (Director, Agrivet Consultancy Pvt. Ltd.), amrita@agrivet.in
Dr Sudipto Haldar (Director, Agrivet Consultancy Pvt Ltd.) sudipto@agrivet.in

Study location

Research and Development Complex (Unit 1), Agrivet Consultancy P Ltd
714, Block A, Lake Town, Kolkata 700089

ACPL trial ID: 101/DGN/02/B/04-18

Objectives:

1. To study the effects of dietary supplementation of Bacillus subtilis (BS-4B) and Prome-Max (PM-4B) which is a multi-strain probiotic consisting of *Bacillus* spp. *Clostridium butyricum* and *Saccharomyces boulardii* on performance and gross carcass traits of commercial broiler chickens fed with a diet without any in-feed antibiotic growth promoter (AGP).
2. To evaluate the economics of broiler production by considering feed cost of the different experimental diets as the only variable.
3. To study the effects of dietary supplementation of BS-4B or PM-4B on mucosal layer thickness, villus height and crypt depth of the jejunum.
4. To study the effects of the said dietary treatments on population of selected bacterial population in the digesta of the experimental birds.
5. To estimate the vaccine titre against Newcastle disease (ND) and Infectious Bursal disease (IBD) in different groups of the experimental birds.

Materials and methods

General bird husbandry and measurement of performance traits

A straight run flock of 400 Cobb 430 broiler chickens was divided into four treatment groups according to the design described in Table 1. Each dietary group had 10 replicate pens (1.2 m x 1.2 m) and each pen housed 10 birds. The trial lasted for 42 days and the birds were fed with a starter (1-14 d), a grower (15-28 d) and a finisher (29-42 d) mash with ad libitum supply of drinking water. Standard vaccination and management practices were followed throughout the experiment for all the dietary groups. The vaccinations included the Infectious bronchitis administered at 0 d of age (IB Ma5) Newcastle Disease live vaccine (Clone 30) on the 5th and 20th d of age and Infectious Bursal Disease vaccine at 12 d of age (228E; Int plus). Lighting program included 24 h of light during the first week of age and 20 h of light thereafter till the end of the trial. Since the birds were reared in an open housing system so temperature and relative humidity was subjected to environmental variation and the daily records of these data are presented in Figures 1 and 2 respectively.

Body weight of the birds was recorded at weekly intervals on same time of the day without any fasting. The birds were weighed pen wise till 35 d of age and on 42 d individual body weight was recorded. A measured quantity of feed was offered to each of the pens every day in two equal divisions and cumulative feed intake was calculated at weekly intervals by subtracting the quantity of the orts left in each pen from the total quantity of the feed offered. Average daily body weight gain (ADG) and average daily feed intake (ADFI) were calculated for the respective feeding periods which corresponded to 1-14 d, 15-28 d, and 29-42 d and both the ADG and ADFI data during 1-14, 15-28, 1-28, 29-42, 15-42 and 1-42 d were reported. Feed conversion ratio (FCR) was calculated as a ratio between ADFI to ADG and the values for the periods mentioned above were reported. Additionally, ADG, ADFI and FCR were calculated during the periods of 36-42 d of age and 1-35 d of age as per the

suggestion of the study sponsor. Mortality was recorded as and when it happened, and the weight of the dead birds was recorded to adjust the data accordingly. Liveability was calculated and European Production Index (EPI) was calculated according to the following formula:

EPI = [(100 – mortality) x (mean live weight/age) x 100]/feed conversion ratios.

Experimental diets

The dietary treatments included feeding of a corn-soybean meal based negative control diet devoid of any in-feed antibiotic growth promoter (AGP) which was supplemented with the single strain probiotic supplement consisting of *Bacillus subtilis* BS-4B(250 mg/kg diet) and the multi-strain probiotic supplement, PM-4B at two different levels of inclusion (150 mg and 250 mg/kg diet). The details of the experimental design are given in Table 1. The ingredients and calculated chemical composition of the basal diet are presented in Table 2 while the proximate analysis of the experimental diets is presented in Table 3.

The economics of feeding different diets were calculated considering the existing market rates of the feed ingredients and that of live chicken at the time of harvest and feed cost per kg live weight was the sole determining factor.

Measurement of carcass traits

At 42 d of age one bird from each pen (10 birds in total from each of the dietary groups) was selected randomly. The selected birds were mechanically stunned, manually slaughtered and bled out, scalded (at 55^{o}C with intermittent dipping for 20 seconds at a time) and picked up using a de-feathering equipment. The carcasses were then manually eviscerated and rinsed. No chilling was performed, and all measurements were done in hot carcass only. Yield of dressed meat was calculated relative to the live weight. The carcass was then divided into commercial cuts which include the breast, thigh and legs and the weights of these cuts were expressed as a percentage of the live weight. The internal organs were also dissected out and weighed to express the values as percentage of live weight.

Table 1: *Description of dietary treatments*

Dietary treatments		Description of the treatments
T1: Negative control (NC)	:	The diet consisted of corn and soybean meal and was devoid of any in-feed AGP.
T2: NC+BS-4B 250	:	The NC diet was supplemented with *Bacillus subtilis* 4B containing probiotic supplement (250 mg/kg diet).
T3: NC + PM-4B 150	:	The NC diet was supplemented with Prome-Max 4B at the rate of 150 mg/kg diet.
T4: NC + PM-4B 250	:	The NC diet was supplemented with Prome-Max 4B at the rate of 250 mg/kg.

Table 2: *Composition of the basal diets and their calculated chemical composition*

Raw materials	Starter	Grower	Finisher	*Nutrients*	*Starter*	*Grower*	*Finisher*
Maize	600.0	630.0	650.0	ME kcal/kg	2900	3000	3100
Soybean meal 50% CP	340.0	305.0	278.0	Digestible amino acids%			
Rice bran Oil	20.0	30.0	40.0	Lysine	1.26	1.15	1.05
Dicalcium phosphate	13.5	10.5	8.5	Methionine	0.46	0.44	0.42
Limestone powder	11.0	9.0	8.0	Met+Cys	0.90	0.84	0.80
Salt	2.5	2.5	2.5	Threonine	0.82	0.76	0.74
Sodium bi carbonate	2.0	2.0	2.0	Tryptophan	0.20	0.20	0.19
L- lysine HCl	2.3	2.3	2.3	Arginine	1.32	1.24	1.16
DL-methionine	2.5	2.5	2.5	Isoleucine	0.85	0.79	0.75
L-threonine	1.0	1.0	1.0	Valine	0.96	0.90	0.83
Toxin binder[1]	1.5	1.5	1.5	Protein%	23.0	21.7	20.0
Trace mineral premix[2]	1.0	1.0	1.0	Calcium%	0.90	0.80	0.80
Vitamin premix[3]	0.5	0.5	0.5	Available P%	0.50	0.45	0.42
Choline chloride 60%	1.5	1.5	1.5	Sodium%	0.22	0.20	0.20
Antioxidant	0.1	0.1	0.1	Potassium%	0.96	0.90	0.87
Phytase 5000 ftu/g[4]	0.1	0.1	0.1	Chloride%	0.24	0.20	0.20
Coccidiostat[5]	0.5	0.5	0.5	Choline ppm	2000	2000	2000

[1]Mycofix Secure (Biomin), [2]Yeast protein complex of copper, manganese, iron, zinc and selenium, [3]Rovimix Hy-D (DSM), [4]Escherichia coli phytase, [5]Maduramicin 0.75% and Nicarbazin 8% combination.
The test products were added over the top of the designated treatment diets.
All test products were supplied by the study sponsor, Sanzyme Biologics P Ltd.

Histology of small intestine

Histological study of the jejunum was performed at the end of the trial (42 d) taking 1 male bird from each pen (10 birds from each dietary group). The birds were mechanically stunned and killed by cervical dislocation. The carcass was opened, the small intestine and the caeca were dissected out, washed with sterile phosphate buffer saline and then the caeca were separated from the small intestine. The part of the small intestine measuring approximately 3 to 4 cm from the middle of the points preceding the Meckel's diverticulum and duodenal loop was further dissected out and the contents present therein was hand stripped carefully. The above-mentioned segment was repeated flushed with phosphate buffered saline and then fixed in Bouin's fluid for 24 h, dehydrated in ascending grade of alcohol (70%, 80%, 90%, 95% and 100%), cleared in xylene and embedded in paraffin blocks which were finally used for block preparation. Tissue sections measuring 2 µm were placed on glass slides and stained with haematoxylin and eosin. The stained sections were examined with a phase contrast microscope (Coslab Laboratory Equipment, India) coupled with an integrated digital imaging analysis system (FLIR Tools, Version 14, 2014). The variables measured were villus height and crypt depth. The villus length was measured from the villus tip to the villus-crypt junction, while crypt depth was defined as the depth of the invagination between two villi (Baurhoo

et al., 2007). Altogether 10 measurements were taken per bird for each variable; for purposes of statistical analysis, the average of these values was used.

Enumeration of selected bacterial population in caecal digesta

The digesta content present in the caeca of the killed birds mentioned above were used for enumeration of selected bacterial species viz., *Salmonella, Escherichia coli, Lactobacillus* spp. and *Clostridium perfringens.* Before dissecting out the caeca from the small intestine, ligatures were put at the ileo-caecal junction with the help of sterile twines. After repeated washing with sterile phosphate buffer saline the caeca were opened by giving an incision and the digesta contents in both the caeca were emptied by applying gentle pressure with the help of a spatula into already autoclaved polystyrene tubes. The tubes were stored at 4^{o}C immediately and cultured within 48 h in nutrient broth. The culture solution was decimally diluted and then further cultured in ready-to-use media plates specific for the said organisms. All the cultures were incubated at 37^{0}C for 36 to 48 h for development of visible counts. For clostridia, the plates were incubated anaerobically for 48 h in presence of carbon di oxide. All the visible colonies were counted in a colony counter and the values were expressed as $\log_{10}$ colony forming units (CFU) per g of caecal digesta.

Estimation of vaccine titre

Vaccine titre against ND and IBD were determined at 15 and 35 d of age. For this one male bird was selected from each of the pens (10 birds per dietary group) and these birds were kept marked for the subsequent collection as well. Since there was no mortality in the marked birds, therefore, in both the occasions blood could be collected from the same birds. Approximately 5 ml of whole blood was collected from the brachial vein of the birds into vacutainer tubes. Immediately after collection, the tubes were placed in ice for the blood to clot. The serum thus separated was harvested into polystyrene tubes and stored at -20^{o}C till analysed. Vaccine titre against ND and IBD was determined using an enzyme linked immune assay with the help of commercial ELISA kits obtained from IDEXX Laboratories Inc. and the values calculated by the machine were expressed as $\log_{10}$ titre and were reported directly as such.

Statistical analysis

The replicate pens were the experimental units, and all data obtained were pooled by replicates. The results were expressed as mean and pooled standard error of the mean. The data were statistically analysed by multi-variate ANOVA in the General Linear Model of SPSS (version 17.0). Whenever significant differences were found between treatments ($P < 0.05$), values were compared by Tukey's test. Probability values with $P < 0.1$ were considered as a trend.

Results

Chemical analyses of experimental diets

Proximate analysis of the experimental diets is presented in Table 3. The data indicated that the chemical composition of the experimental diets was within the normal range of variations. The

deviation observed regarding the available P values from the calculated values was due to the fact that the diets were formulated with the available P matrix of the phytase and the matrix contribution was not reflected in the chemical analysis.

Performance traits

Body weight and ADG (Table 4) of the birds improved numerically by probiotic supplementation and the effect was pronounced as the birds become older (28 d onwards). The differences did not reach the level of statistical significance at any given point of measurements ($P > 0.05$) but numerically, the effect was quite substantial especially at 35 and 42 d of age. At 42 d of age the birds in the NC+PM4B-150 group had almost 3% higher body weight as compared with the NC group. It may be inferred from the current data that in absence of any real intestinal challenge dietary supplementation of the probiotics in the form of BS or as PM-4B may be used as a growth promoting agent for broilers. Average daily gain in body weight data indicated that despite the statistical non-significance the birds supplemented with PM-4B either at 150 mg/kg or at 250 mg/kg gained greater body weight during 36 – 42 d as compared with the NC and the BS groups and this resulted in better ADG in both the BS-4B groups during 29-42 d and 15-42 d. Nevertheless, the data were not significant at any given point of measurement and hence any definite conclusion on the effect of the said dietary supplements on ADG could not be drawn.

Cumulative feed intake (Table 5) was not affected by the dietary treatments ($P > 0.05$). Numerically the birds in the BS-4B 250 and the PM-4B groups consumed more than 100 g feed in 42 d. Feed intake by probiotic supplementation increased especially during 36-42 d. The study indicated that dietary supplementation of the said probiotics improved feed intake particularly during the terminal phase of the trial and despite statistical insignificance the higher amount of feed intake explained the better body weight in the treated groups.

Feed conversion ratio (Table 6) during 1-14 d was improved by dietary supplementation of PM-4B at 150 mg/kg and 250 mg/kg as compared with the NC group ($P < 0.05$). During 1-28 d of age FCR was found to be superior in the BS-4B 250 group as compared with the NC group ($P < 0.05$). These variations were due to the numerical improvement in body weight in the probiotic supplemented groups without any substantial increment in feed intake during the corresponding growth periods. However, the effects of the dietary supplements were blunted as the experiment progressed and at the end of the experiment no statistically significant difference with regard to FCR was discernible between the groups. Liveability was numerically better in the BS-4B 250 and PM-4B 150 groups as compared with the NC and the PM-4B 250 groups and as a combined function of liveability, FCR and body weight, EPI was numerically superior in all the probiotic supplemented groups compared to the NC group (Table 6).

Carcass traits

The data related to different carcass traits of the experimental birds at 42 d of age is presented in table 7. The effects of the dietary treatments in this regard have been found to be variable. Live weight at the time of slaughter, eviscerated carcass weight and dressed meat yield was not affected by the dietary treatments and the differences observed in these cases were within the normal ranges of variations only. Weight of the heart and gizzard relative to live weight varied between the groups albeit without any specific trend. Yield of breast meat relative to body weight was higher in the NC group compared to that in the PM-4B 250 group and it is difficult to say if this happened due to any of the dietary treatments or an artefact.

Vaccine titre against Newcastle disease and Infectious bursal disease

The data related to vaccine titre against ND and IBD are presented in Table 8 and Figures 3 (a and b). Vaccine titre against ND was improved by dietary supplementation of PM-4B 150 as compared to BS-4B 250 on both 15 and 35 d of age and the effect was found to be significant ($P < 0.001$). Antibody response against IBD was found to be numerically superior in the BS-4B 250 group as compared with PM-4B groups on both 15 and 35 d of age and all the probiotic treated groups the titre was greater than that found in the NC group ($P < 0.01$) in both the occasions. In this study the maternal antibody levels against ND and IBD was not determined and this posed a problem in drawing a definite conclusion about the behaviour of the treated groups regarding the antibody responses. However, since the flock was obtained from a single hatch and from an apparently healthy parent stock, it may be assumed that the maternal antibody status remained similar across the dietary treatments. Having accepted this fact the data suggests that supplementation of both the single strain and multi-strain probiotics improved the half- life of the maternal antibodies against ND while the half-life of that against IBD was improved by BS-4B 250. The immune-stimulatory effects of the probiotic supplements were quite discernible since at 35 d antibody titre against both ND and IBD was significantly higher than that in the NC group. There are reports (Gill et al. 2000; Maassen et al. 2000; Talebi et al. 2008) that oral administration of probiotic organisms significantly enhanced specific immunoglobulin levels in animals. The antibody isotypes were not determined in the current study, but the data clearly suggested the probiotic supplements used in the study may be an effective tool to improve the generalised immune responses in broilers which ought to be an extremely important finding considering the extremes of the disease challenges the birds are exposed to in modern farming systems. Though the exact mechanism of action of immune-modulation by the dietary treatments cannot be fully explained by the set of data available with us, it is plausible that the organisms involved stimulated different subsets of the immune system present in the systemic and gut-associated immune cascades. This effect might be bolstered by the bio-active peptides produced as fermentation by-products of these organisms which by interacting

with the host cell and the pathogens or their structural components stimulated the T-cell and B-cell mediated immune responses as reported by Haghighi et al (2005).

Caecal bacterial counts

Data related to counts of *Salmonella, E. coli, Lactobacillus* and *Clostridium perfringens* in the caecal digesta are presented in Table 9 and figure 4. Dietary supplementation of PM-4B 250 decreased ($P < 0.001$) counts of *Salmonella* as compared to the NC group, BS-4B 250 and PM-4B 150. Counts of *E. coli* decreased when PM-4B (at both levels of inclusion) was supplemented to the diets ($P < 0.001$) as compared to the NC and BS-4B 250 supplemented groups. Counts of *Lactobacillus* increased by dietary supplementation of BS-4B 250 ($P < 0.001$) as compared to the NC and the PM-4B groups. Counts of *Clostridium perfringens* decreased ($P < 0.001$) by dietary supplementation of PM-4B (at both levels of inclusion) as compared to the NC and BS-4B 250 groups.

Histology of jejunum

Data related to villus height and crypt depth measured at 42 d of age in the proximal, middle and distal part of the jejunum is presented in Table 10 and Figure 6 (a and b). The photographs of the individual slides are presented in Figure 5 (a, b, c and d). There were subtle effects of the dietary treatments on these parameters and the differences observed between the groups were only numerical ($P > 0.05$). The statistical insignificance notwithstanding villus height in the proximal, middle and distal parts of the jejunum was numerically higher in the C+PM-4B 250 group as compared with the rest of the dietary treatments. When the individual slides were examined it was observed that the villi were arranged in a more compact manner especially in the distal part of the small intestine in the PM-4B 250 group. However, these differences were not translated to that extent into performance of the birds. Crypt depth in the respective treatment groups was found to be similar and even numerically the differences were just marginal. It is probable that there was not enough dietary challenge imposed on the birds which precluded the dietary treatments to elicit substantial effects in terms of protecting the intestinal mucosa which could be reflected through a taller villi and deeper crypt.

Conclusions

Based on the findings of the present study it was concluded that:

1. Dietary supplementation of BS-4B 250 and PM-4B (at 150 mg/kg and 250 mg/kg) numerically improved body weight of the experimental birds. The effect of PM-4B was superior to that of BS-4B 250 in this regard.
2. Supplementation of PM-4B at either level of inclusions improved FCR of the experimental flock as compared to the birds in the negative control and the BS supplemented group.
3. Liveability was better in the BS-4B 250 and PM-4B 150 supplemented groups as compared with the NC and the PM-4B 250 groups.

4. Probiotic supplementation improved antibody response against ND and IBD over the NC group. However, there were only subtle differences between the BS and PM-4B in this regard.
5. Supplementation of PM-4B against 3 pathogens as compared to BS group significantly reduced counts of *Salmonella, Escherichia coli* and *Clostridium perfringens* while having only subtle effect on *Lactobacillus* counts which was increased by supplementation of BS in this study.
6. Dietary treatments elicited only subtle effect on villus height and crypt depth in the jejunum of the experimental birds indicating little or no effect of the supplements in absence of any real enteric challenge.

It was finally concluded that in absence of any real enteric challenge, Multi strain probiotic Prome Max 4B has exhibited superior performance in terms of better pathogenic control, enhanced immune functions, better gut integrity and overall productivity as compared to single strain probiotic supplement BS.

Fig 1: *Daily temperature variations in the experimental house diring 1-42 d of age*

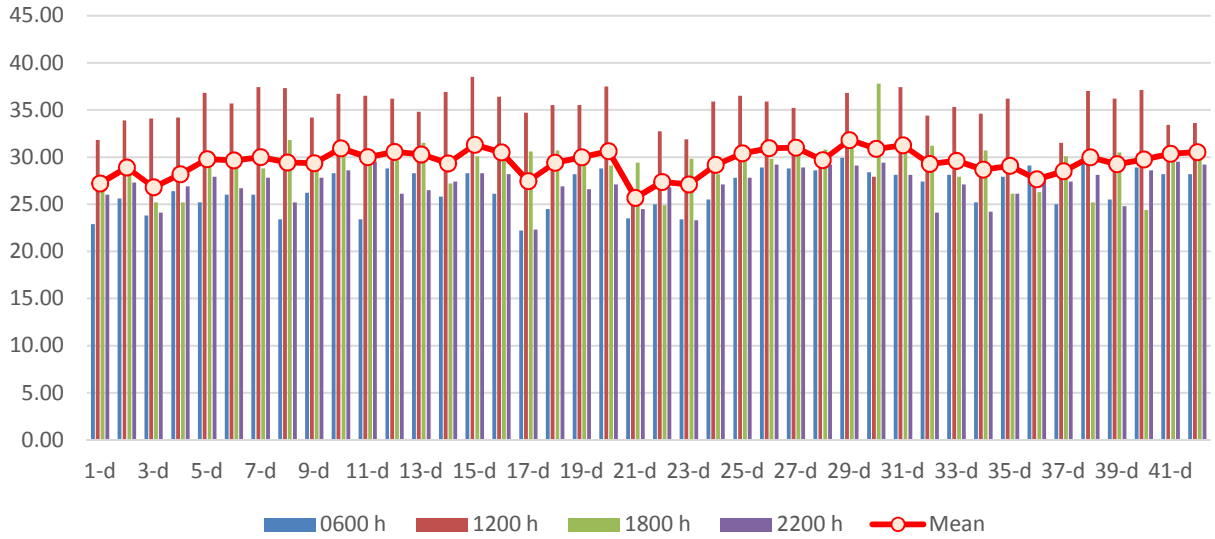


Fig 1 (b): Relative humidity (%) in the experimental house during 1-35 d of age

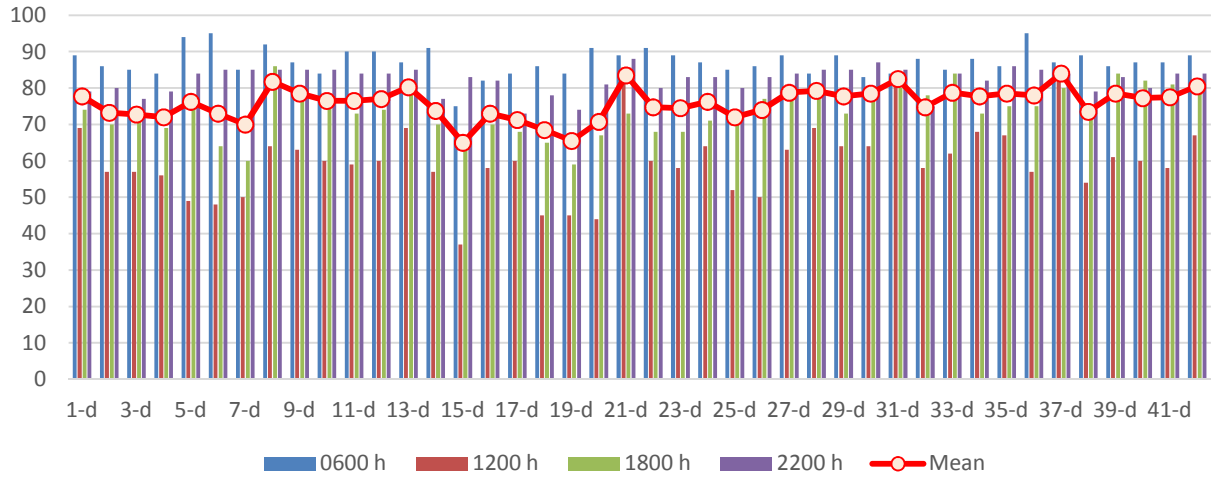


Table 3: *Chemical analysis of experimental diets (all values in g/100 g, unless stated otherwise)*

Diets	Moisture	Crude Protein	Crude fat	Crude fibre	Total Ash	Sand - Silica	Calcium	Total P	Particle size distribution			
									> 2 mm	> 1 mm	> 0.5 mm	< 0.5 mm
Starter												
NC	11.46	22.58	4.95	2.62	5.12	0.71	1.03	0.44	34	32	30	4
NC + BS-4B 250	11.23	22.78	4.68	2.43	5.18	0.75	1.02	0.43	29	34	32	5
NC + PM-4B 150	11.34	22.68	4.77	2.35	5.13	0.63	1.02	0.46	35	35	26	4
NC + PM-4B 250	11.49	22.63	4.89	2.48	5.35	0.51	1.99	0.44	34	33	28	5
Grower												
NC	12.37	21.72	4.96	2.82	4.03	0.23	0.82	0.38	40	32	24	4
NC + BS-4B 250	12.74	21.53	5.51	2.86	4.11	0.19	0.81	0.36	39	32	26	3
NC + PM-4B 150	12.76	21.43	5.41	2.63	4.22	0.18	0.81	0.36	38	32	28	2
NC + PM-4B 250	12.76	21.68	5.06	2.74	4.08	0.20	0.83	0.37	36	32	28	4
Finisher												
NC	12.23	19.98	6.23	2.62	4.08	0.15	0.73	0.34	58	25	14	3
NC + BS-4B 250	12.25	20.18	6.41	2.93	4.56	0.19	0.74	0.32	49	26	22	3
NC + PM-4B 150	12.18	20.09	6.37	2.91	4.15	0.13	0.75	0.33	45	28	25	2
NC + PM-4B 250	12.17	20.03	6.42	2.71	4.06	0.17	0.73	0.34	50	21	26	3

For details of the acronyms refer to Table 1.

Table 4: *Body weight and average daily body weight gain at different periods of the experiment (g)*

Diets	Body weight (g)						Average daily gain (g)							
	7-d	14-d	21-d	28-d	35-d	42-d	1-14 d	15-28 d	1-28 d	36-42 d	29-42 d	15-42 d	1-35 d	1-42 d
NC	211.4	512.1	930.2	1388.0	1898.8	2285.1	33.28	62.56	47.92	55.20	64.08	63.32	52.94	53.31
NC + BS-4B 250	213.9	517.1	940.5	1421.0	1943.2	2326.2	33.63	64.57	49.10	54.71	64.65	64.61	54.20	54.28
NC + PM-4B 150	209.9	512.5	941.7	1409.0	1951.2	2354.9	33.30	64.04	48.67	57.68	67.56	65.80	54.42	54.97
NC + PM-4B 250	211.7	516.5	939.7	1409.7	1945.6	2345.8	33.59	63.80	48.69	57.17	66.87	65.33	54.25	54.75
SEM	0.96	1.83	4.34	7.77	13.30	15.99	0.13	0.51	0.28	0.95	0.75	0.56	0.38	0.38
Pr>F	0.547	0.698	0.79	0.518	0.496	0.434	0.698	0.579	0.518	0.629	0.299	0.44	0.496	0.434

For details of the acronyms refer to Table 1.

Table 5: *Cumulative feed intake and average daily feed intake at different periods of the experiment (g)*

Diet	Cumulative Feed Intake (g)						Average daily feed intake (g)							
	7-d	14-d	21-d	28-d	35-d	42-d	1-14 d	15-28 d	1-28 d	36-42 d	29-42 d	15-42 d	1-35 d	1-42 d
NC	181.8	559.0	1147.1	1920.4	2783.0	3603.5	39.93	97.25	68.59	117.23	120.22	108.74	79.52	85.80
NC + BS-4B 250	181.7	557.9	1148.2	1929.8	2853.6	3718.4	39.85	97.99	68.92	123.55	127.76	112.88	81.52	88.53
NC + PM-4B 150	177.8	547.4	1144.3	1931.3	2846.3	3732.3	39.10	98.85	68.97	126.58	128.65	113.75	81.32	88.87
NC + PM-4B 250	177.2	550.0	1142.7	1926.2	2829.6	3708.7	39.29	98.30	68.79	125.58	127.33	112.81	80.84	88.30
SEM	0.918	2.390	4.770	9.388	17.160	26.427	0.171	0.601	0.335	1.48	1.518	0.933	0.493	0.629
Pr>F	0.143	0.234	0.978	0.98	0.479	0.299	0.234	0.832	0.98	0.52	0.179	0.23	0.48	0.299

For details of the acronyms refer to Table 1.

Fig 2 (a): Body weight at 42 d and ADG during 1-42 d (g)

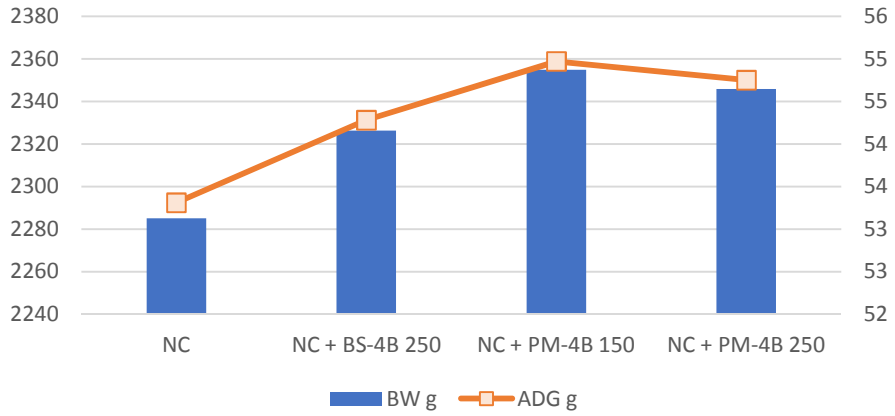


Fig 2 (b): Cumulative feed intake and ADFI during 1-42 d (g)

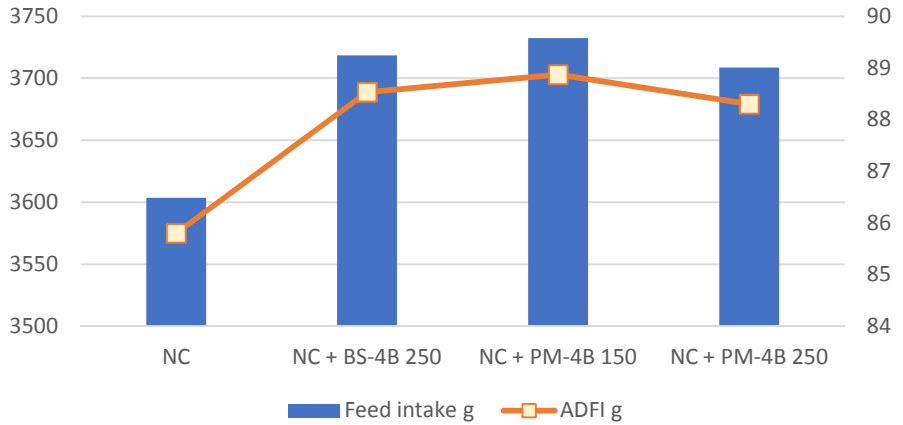


Fig 2 (c): Feed conversion ratio in different growth phases

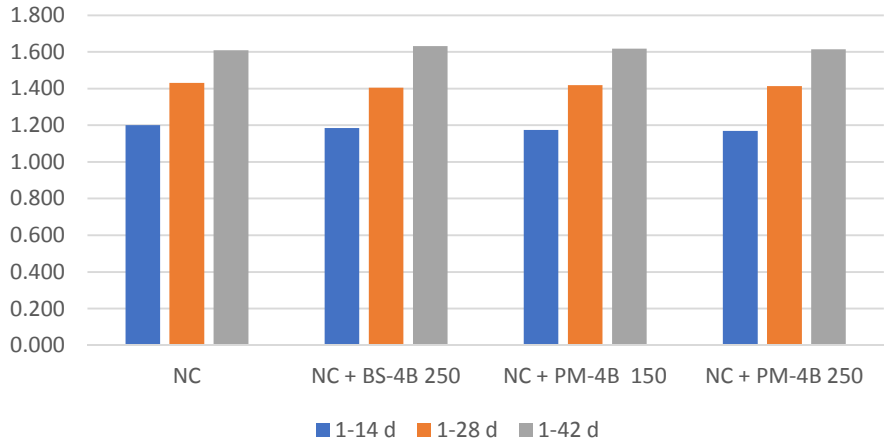
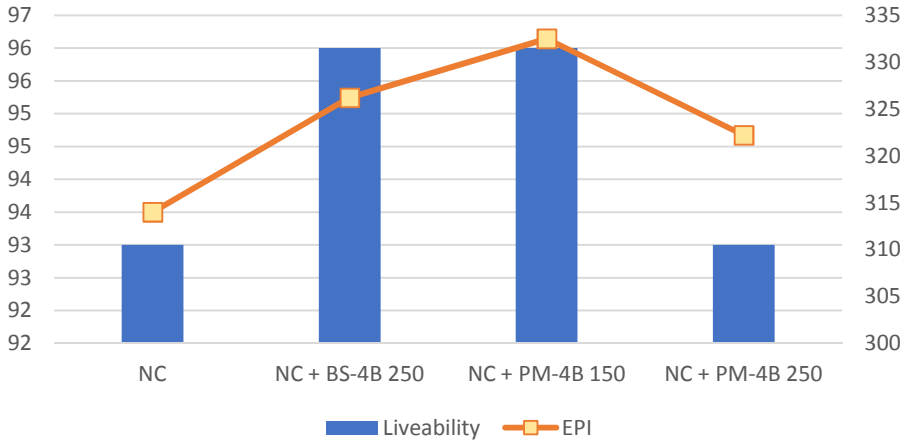


Fig 2 (d): Liveability and EPI during 1-42 d of age



For details of the acronyms refer to Table 1.

Table 6: *Feed conversion ratio, liveability and production efficiency factor at different periods of the experiment*

Diet	Feed Conversion Ratio								Liveability	EPI
	1-14 d	15-28 d	1-28 d	36-42 d	29-42 d	15-42 d	1-35 d	1-42 d	1-42 d	1-42 d
NC	1.200^{b}	1.555	1.431^{b}	2.133	1.876	1.717	1.502	1.609	93.0	314.0
NC + BS-4B 250	1.185^{ab}	1.519	1.404^{a}	2.274	1.981	1.749	1.505	1.632	96.0	326.2
NC + PM-4B 150	1.174^{a}	1.545	1.418^{ab}	2.208	1.906	1.729	1.495	1.617	96.0	332.5
NC + PM-4B 250	1.170^{a}	1.542	1.413^{ab}	2.193	1.908	1.729	1.490	1.614	93.0	322.2
SEM	0.003	0.006	0.003	0.042	0.018	0.010	0.006	0.007	0.534	5.040
Pr>F	0.003	0.141	0.038	0.706	0.223	0.712	0.790	0.749	0.634	0.598

Means with dissimilar superscripts in a column varied significantly. For details of the acronyms refer to Table 1.

Table 7: *Weight of hot and dressed carcass, internal organs and commercial cuts at 42 d of age (absolute weight* [*g*] *and relative to live weight* [*g/kg*]*).*

Diets	Weight g		Yield	Liver weight		Heart weight		Gizzard weight		Breast weight		Legs weight		Thigh weight	
	Live	Eviscerated	g/kg	g	g/kg	g	g/kg	g	g/kg	g	g/kg	g	g/kg	g	g/kg
NC	2377.1	1774.0	747.0	45.5	19.1	7.25^{a}	3.00^{a}	38.75^{ab}	16.3^{ab}	638.70	268.8^{b}	469.95	197.9	258.80	109.0
NC + BS-4B 250	2394.8	1755.8	733.0	47.4	19.8	8.45^{ab}	3.50^{ab}	41.70^{b}	17.4^{b}	605.05	252.7^{ab}	462.45	193.0	254.15	106.1
NC + PM-4B 150	2377.0	1742.8	733.0	42.3	17.8	8.35^{ab}	3.50^{ab}	36.40^{a}	15.3^{a}	609.15	256.4^{ab}	456.95	192.2	248.75	104.6
NC + PM-4B 250	2403.6	1754.4	730.0	47.0	19.6	9.25^{b}	3.90^{b}	39.20^{ab}	16.3^{ab}	594.50	247.3^{a}	471.30	196.0	260.20	108.2
SEM	8.087	7.671	0.254	1.010	0.041	0.227	0.009	0.593	0.025	6.177	0.268	4.630	0.185	3.283	0.133
Pr>F	0.584	0.566	0.088	0.280	0.331	0.014	0.017	0.012	0.020	0.064	0.027	0.680	0.683	0.618	0.652

Means with dissimilar superscripts in a column varied significantly. For details of the acronyms refer to Table 1.

Table 8: *Vaccine titre against Newcastle disease and Infectious bursal disease (log_{10})*

Diets	ND		IBD	
	15-d	35-d	15-d	35-d
NC	1220.4[a]	2675.5[a]	1770.6[a]	2205.3[a]
NC + BS-4B 250	1594.0[b]	3832.9[b]	2052.5[b]	3182.8[c]
NC + PM-4B 150	1711.9[b]	3897.5[b]	1704.4[a]	2623.8[b]
NC + PM-4B 250	1637.7[b]	3797.6[b]	1666.9[a]	2618.1[b]
SEM	43.6	126.0	45.9	92.3
Pr> F	<0.0001	<0.0001	0.008	0.001

Means with dissimilar superscripts in a column varied significantly.
For details of the acronyms refer to Table 1.

Fig 3 (a): Vaccine titer against Newcastle disease (log10)

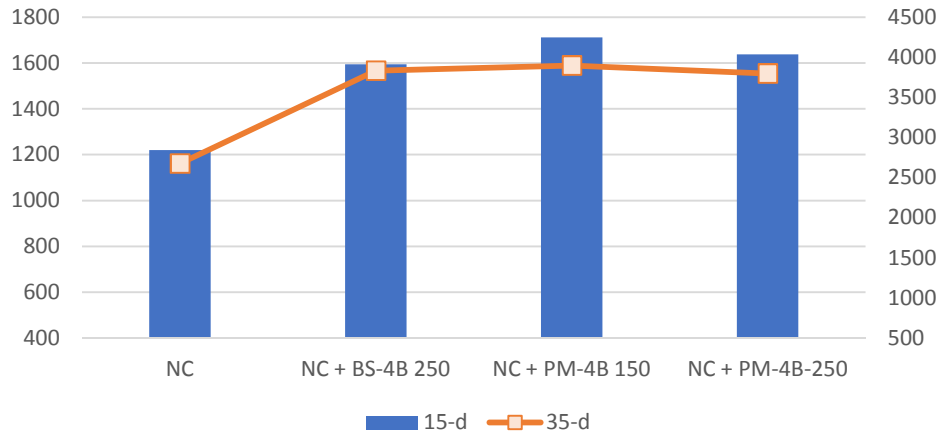


Fig 3 (b): Vaccine titer against Infectious bursal disease (log10)

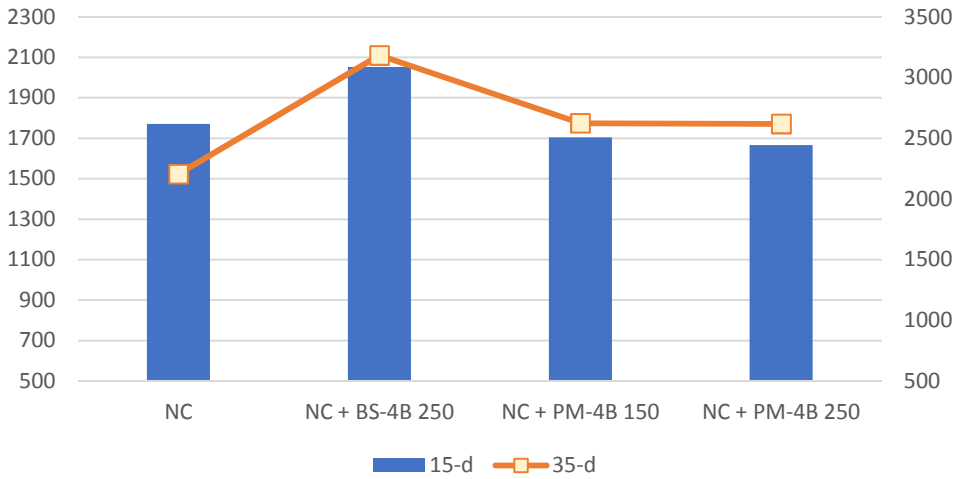
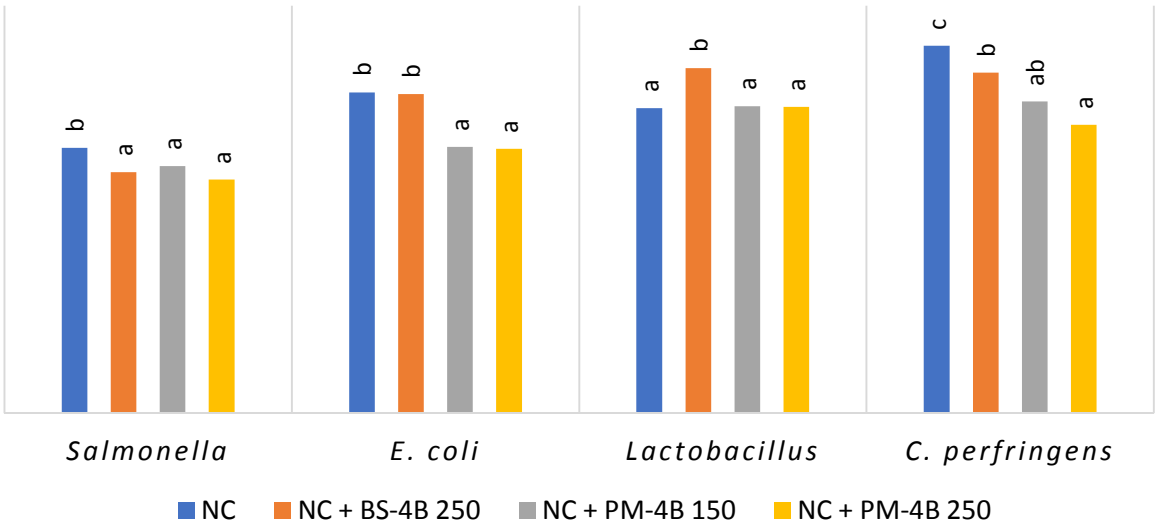


Table 9: *Numbers of selected bacterial species in caecal digesta of the experimental birds at 42 d*

Diets	*Salmonella* spp	*Escherichia coli*	*Lactobacillus* spp	*C. perfringens*
NC	6.254[b]	6.935[b]	6.741[a]	7.508[c]
NC + BS-4B 250	5.957[a]	6.916[b]	7.234[b]	7.177[b]
NC + PM-4B 150	6.032[a]	6.267[a]	6.765[a]	6.823[ab]
NC + PM-4B 250	5.865[a]	6.244[a]	6.757[a]	6.539[a]
SEM	0.038	0.059	0.039	0.067
Pr> F	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Fig 4: Counts of selected bacterial species in caecal digesta of experimental birds at 42 d of age



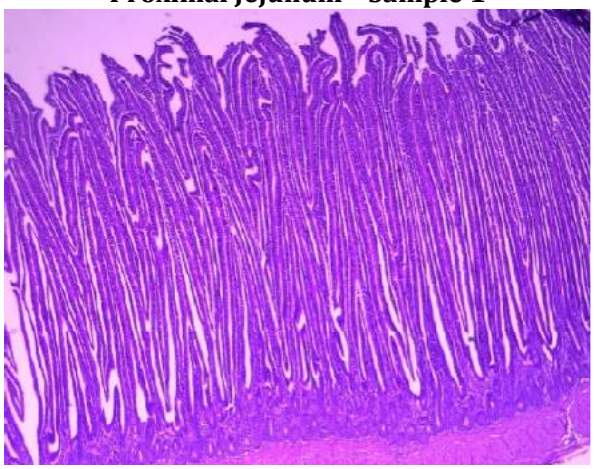
Bars with dissimilar letters varied significantly at P < 0.0001.

Table 10: *Villus height and crypt depth (µm) of the proximal, middle and distal part of the jejunum at 42 d of age in the experimental broilers*

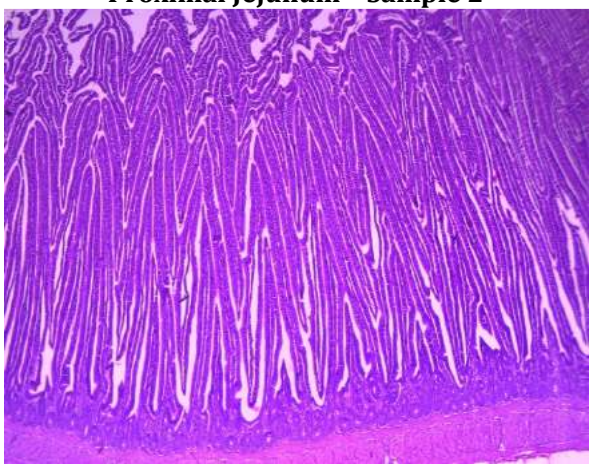
Diets	Villus height µm				Crypt depth µm			
	Proximal	Middle	Distal	Mean	Proximal	Middle	Distal	Mean
NC	676.71	534.06	405.05	538.61	134.40	101.55	74.20	103.38
NC + BS-4B 250	682.25	536.82	426.45	548.51	136.18	102.09	77.98	105.42
NC + PM-4B 150	688.33	535.51	430.12	551.32	131.24	101.16	86.20	106.20
NC + PM-4B 250	731.25	598.89	460.92	597.02	144.13	106.44	80.34	110.30
SEM	11.18	11.88	8.47	8.68	3.91	3.31	2.23	2.19
Pr > F	0.299	0.147	0.135	0.078	0.696	0.941	0.284	0.733

Fig 5 (a): *Histology of cross section of jejunum* – **SUBSAMPLE SET: NEGATIVE CONTROL**

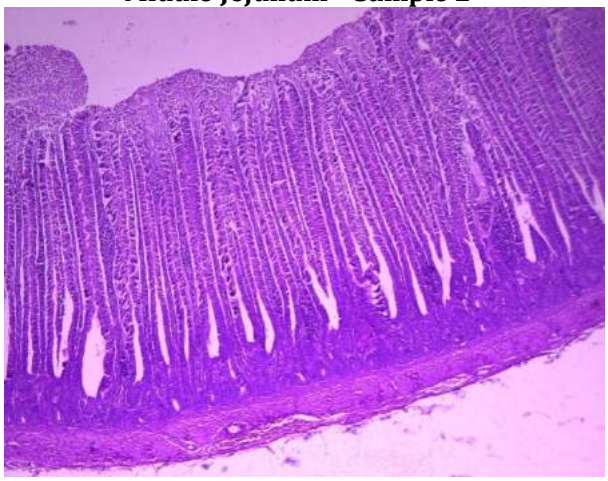
Proximal Jejunum – Sample 1



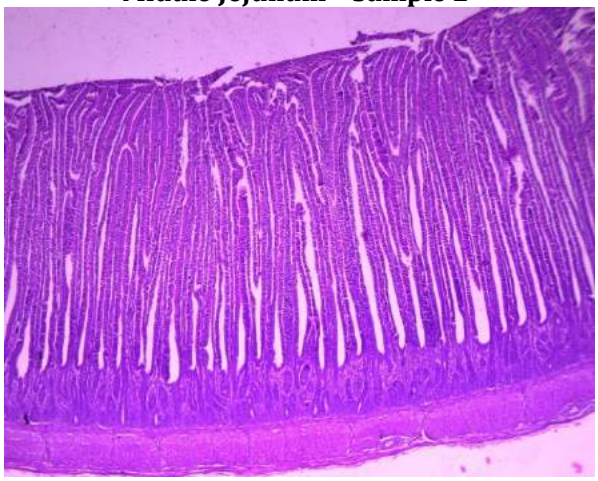
Proximal Jejunum – Sample 2



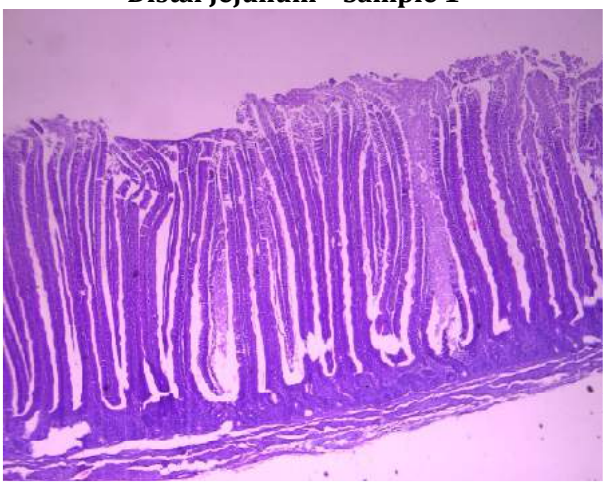
Middle Jejunum – Sample 2



Middle Jejunum – Sample 2



Distal Jejunum – Sample 1



Distal Jejunum – Sample 2

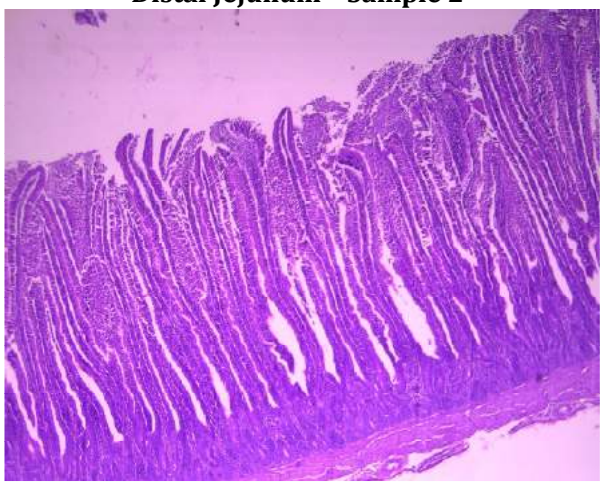
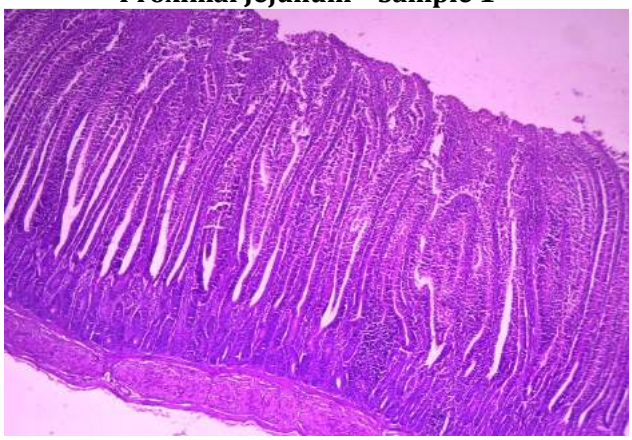
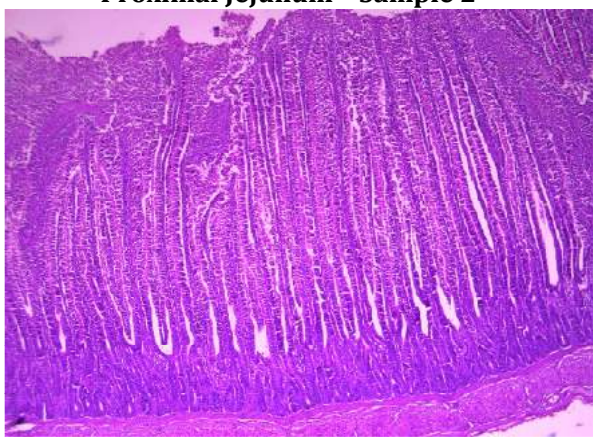


Fig 5 (b): *Histology of cross section of jejunum* – **SUBSAMPLE SET: NC + BS-4B 250**

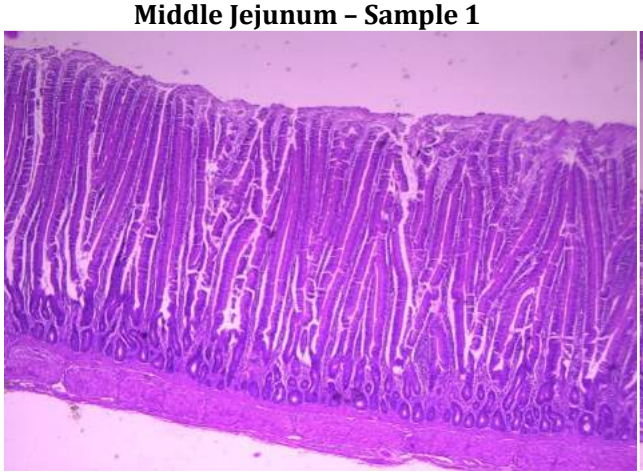
Proximal Jejunum – Sample 1



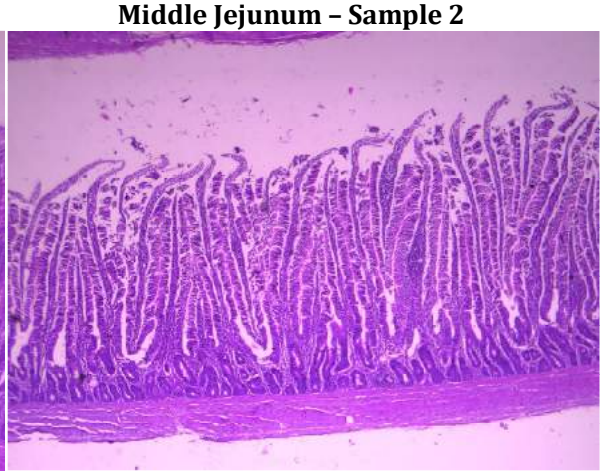
Proximal Jejunum – Sample 2



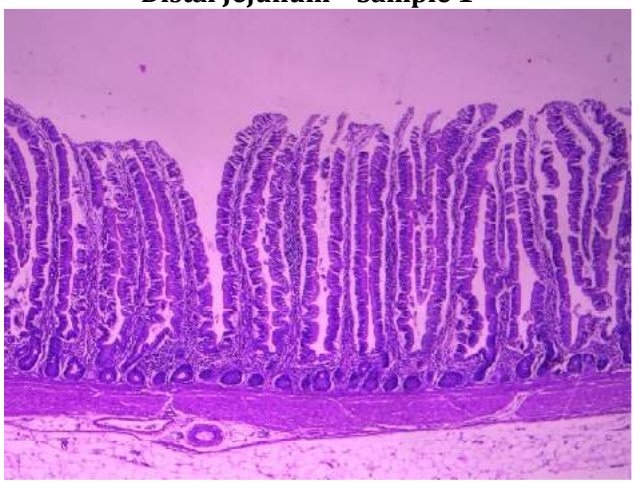
Middle Jejunum – Sample 1



Middle Jejunum – Sample 2



Distal Jejunum – Sample 1



Distal Jejunum – Sample 2

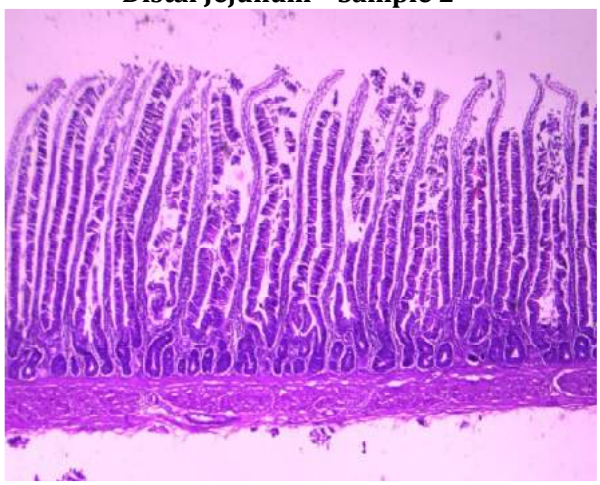
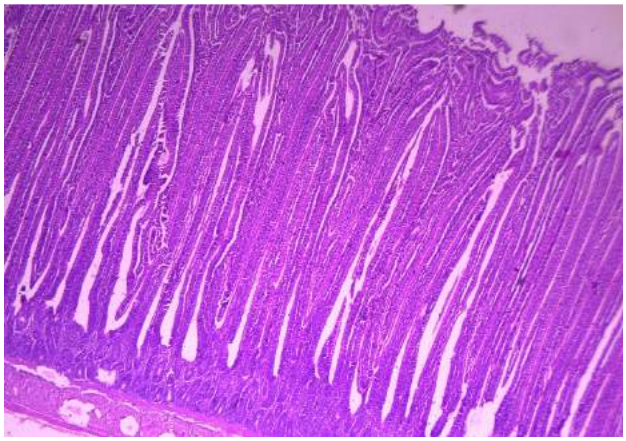
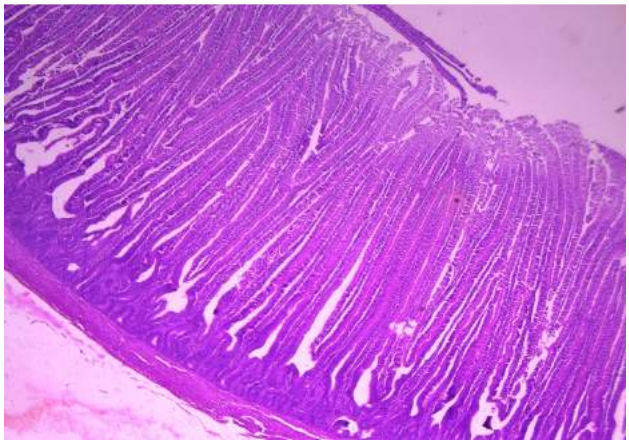


Fig 5 (c): *Histology of cross section of jejunum* – **SUBSAMPLE SET: NC + PM-4B 150**

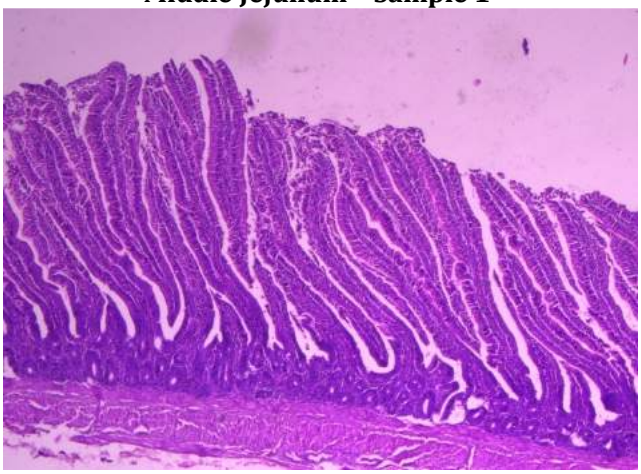
Proximal Jejunum – Sample 1



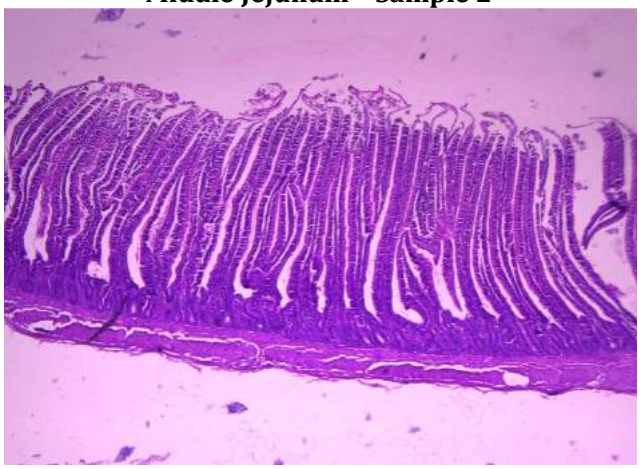
Proximal Jejunum – Sample 2



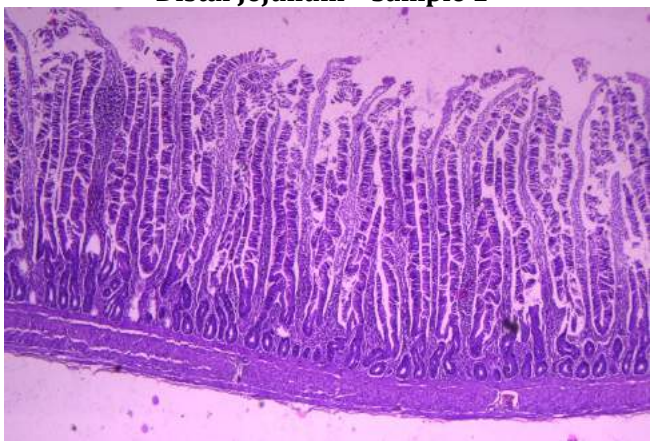
Middle Jejunum – Sample 1



Middle Jejunum – Sample 2



Distal Jejunum – Sample 1



Distal Jejunum – Sample 2

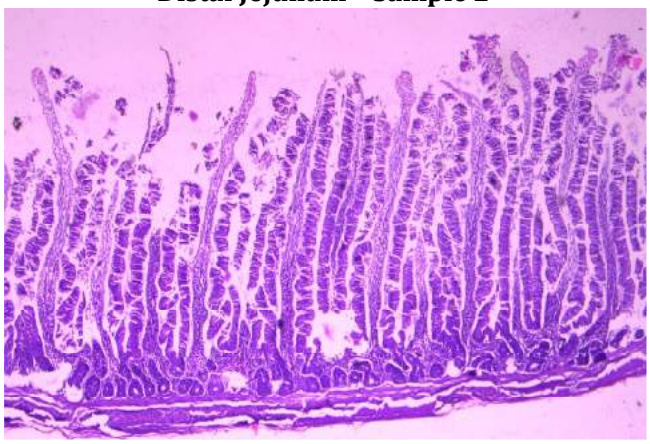
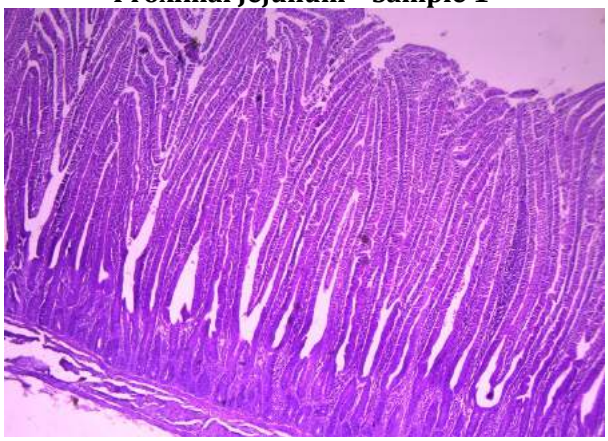
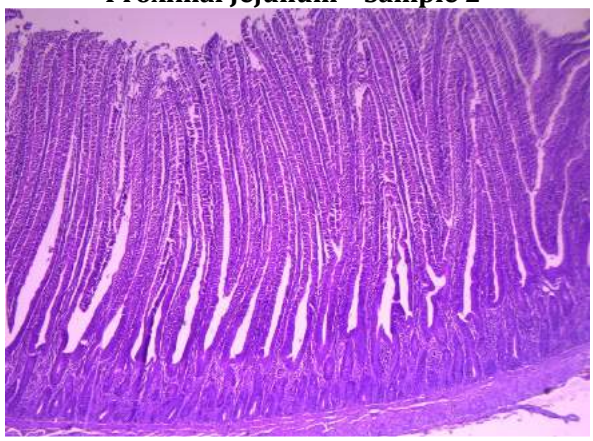


Fig 5 (d): *Histology of cross section of jejunum* – **SUBSAMPLE SET: NC + PM-4B 250**

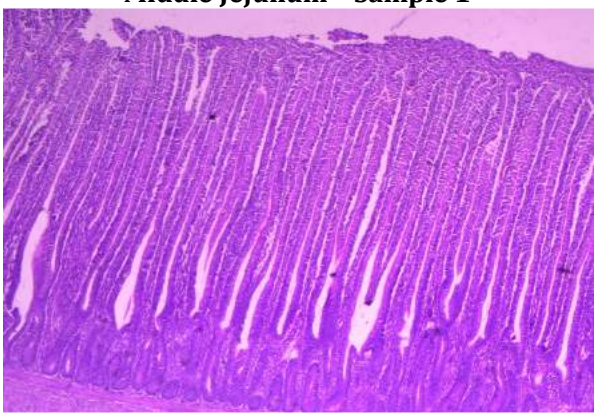
Proximal Jejunum – Sample 1



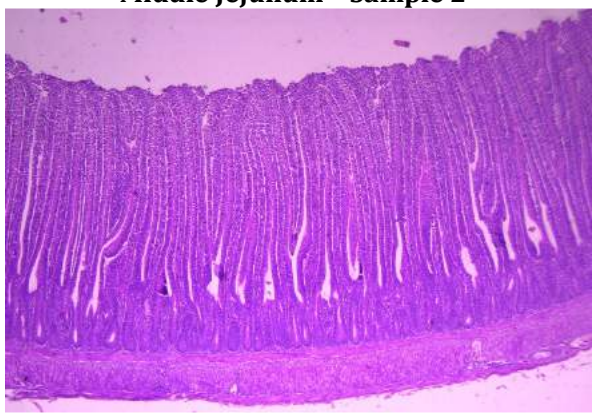
Proximal Jejunum – Sample 2



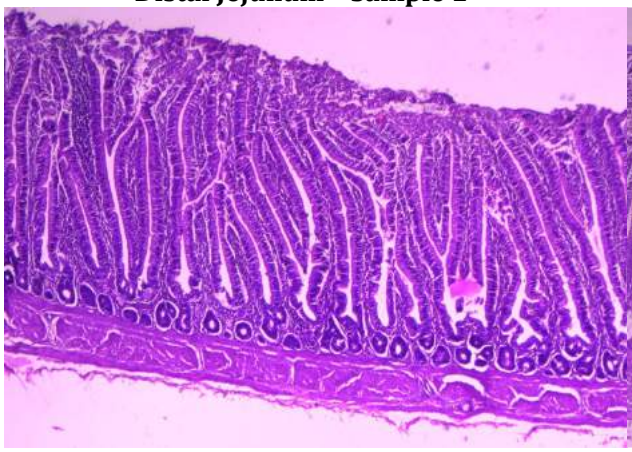
Middle Jejunum – Sample 1



Middle Jejunum – Sample 2



Distal Jejunum – Sample 1



Distal Jejunum – Sample 2

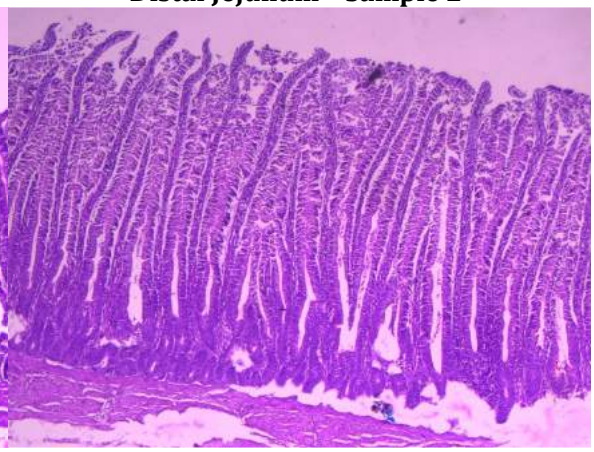


Fig 6 (a): *Villus height (µm) at the proximal, middle and distal part of the jejunum measured in the experimental birds at 42 d of age*

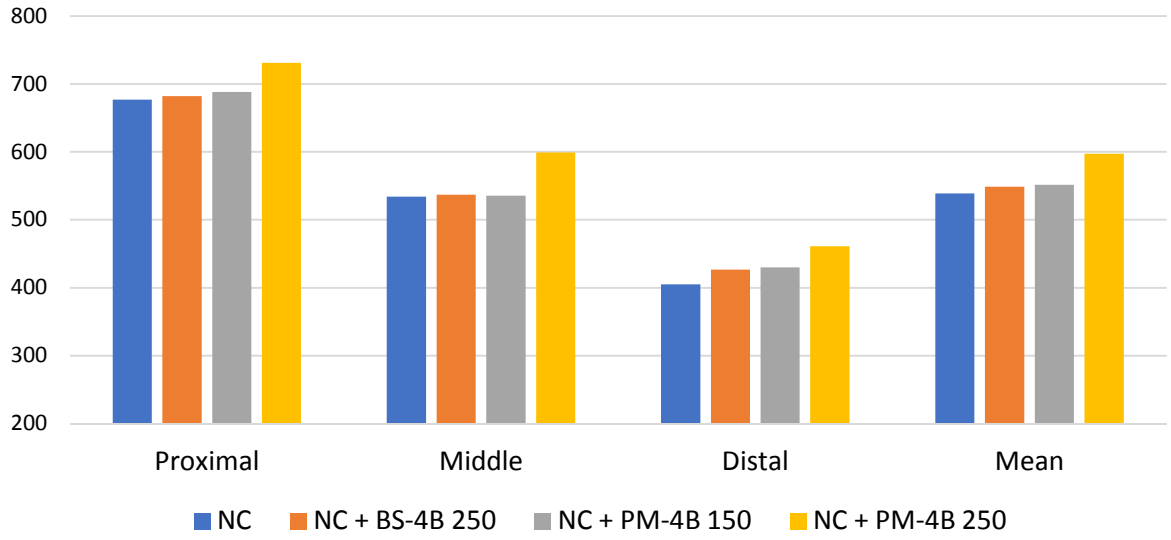
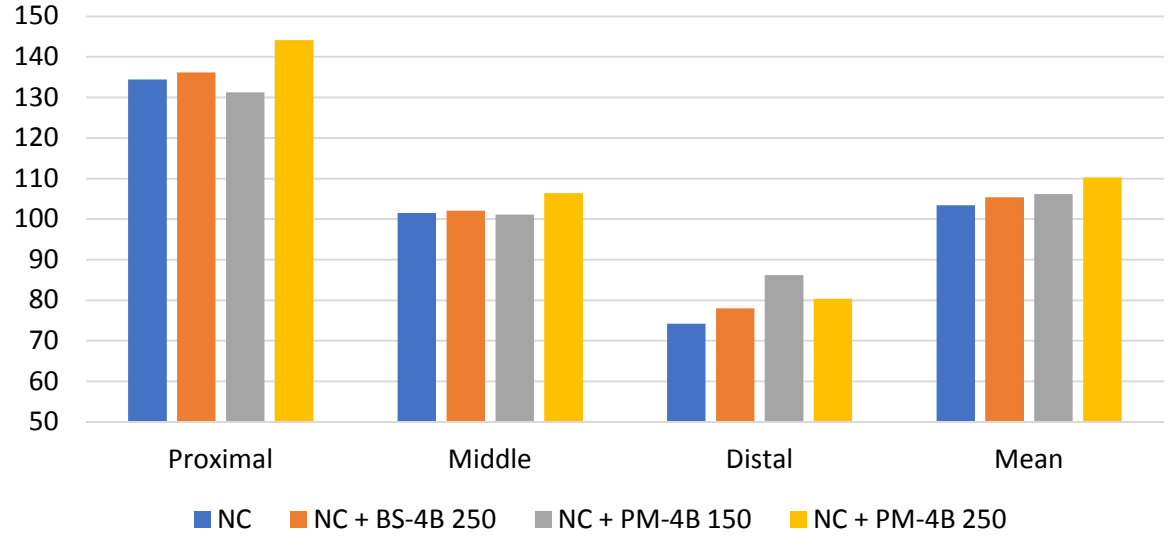


Fig 6 (b): *Crypt depth (µm) at the proximal, middle and distal part of the jejunum measured in the experimental birds at 42 d of age*



Appendix: Calculation of cost of productions and return on investment

Table A-I: *Feed cost and cost of feed intake during different growth phases*

Diets	Feed cost per kg (Rs)			Cost of feed intake (Rs)			
	Starter	Grower	Finisher	Starter	Grower	Finisher	Total
NC	25.67	25.25	25.07	14.35	34.38	42.19	90.92
NC + BS-4B 250	25.80	25.38	25.19	14.39	34.82	45.06	94.27
NC + PM-4B 150	25.75	25.34	25.15	14.10	35.06	45.30	94.46
NC + PM-4B 250	25.81	25.39	25.20	14.20	34.94	44.93	94.07

Fig A-I: Cost of Feed (RS/kg) for different growth phases

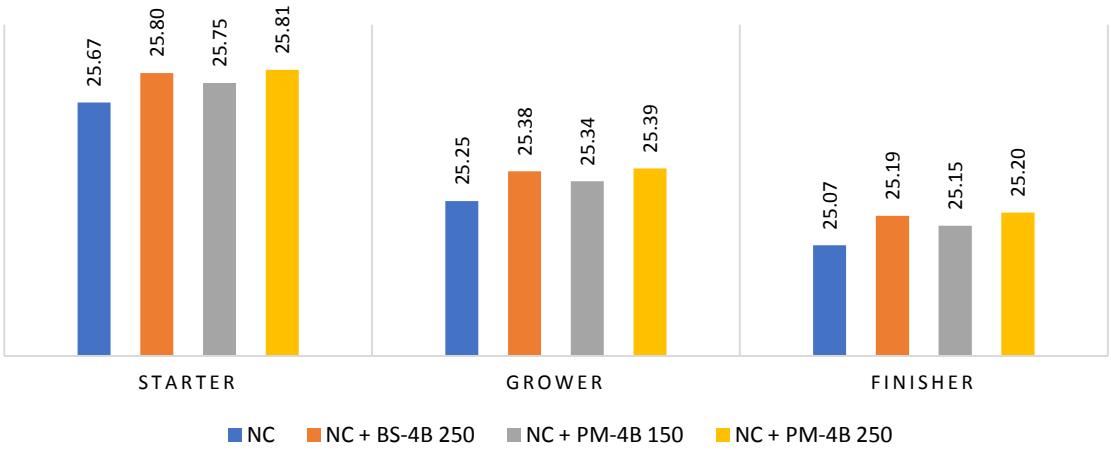


Fig A-II: Feed Intake during different periods of the experiment (kg)

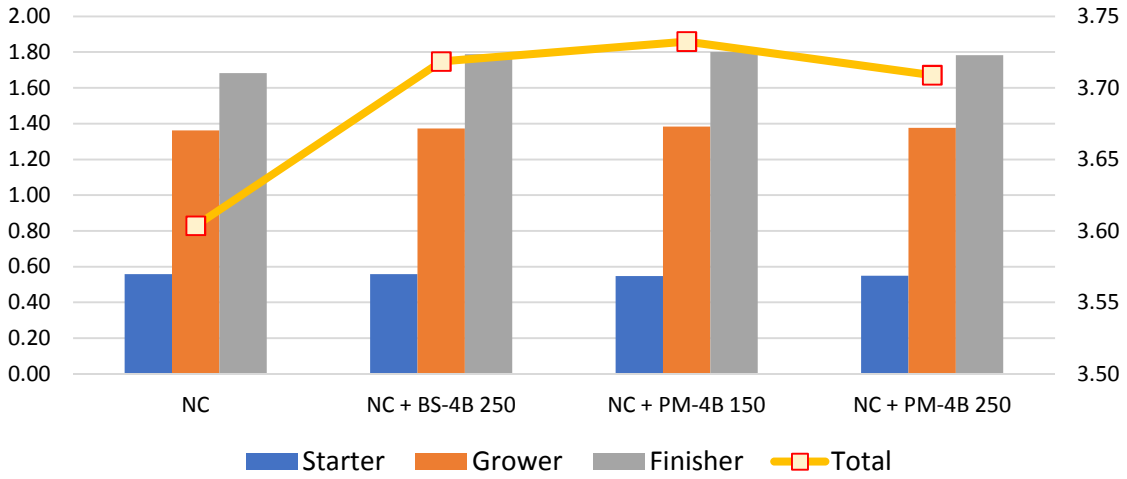


Table A-II: *Feed intake and cost of feed intake at different growth periods*

Diets	Feed intake kg				Cost of feed intake Rs			
	Starter	Grower	Finisher	Total	Starter	Grower	Finisher	Total
NC	0.559	1.362	1.683	3.604	14.35	34.38	42.19	90.92
NC + BS-4B 250	0.558	1.372	1.789	3.718	14.39	34.82	45.06	94.27
NC + PM-4B 150	0.547	1.384	1.801	3.732	14.10	35.06	45.30	94.46
NC + PM-4B 250	0.550	1.376	1.783	3.709	14.20	34.94	44.93	94.07

Fig A-III: Feed Intake durng different periods of the experiment (kg)

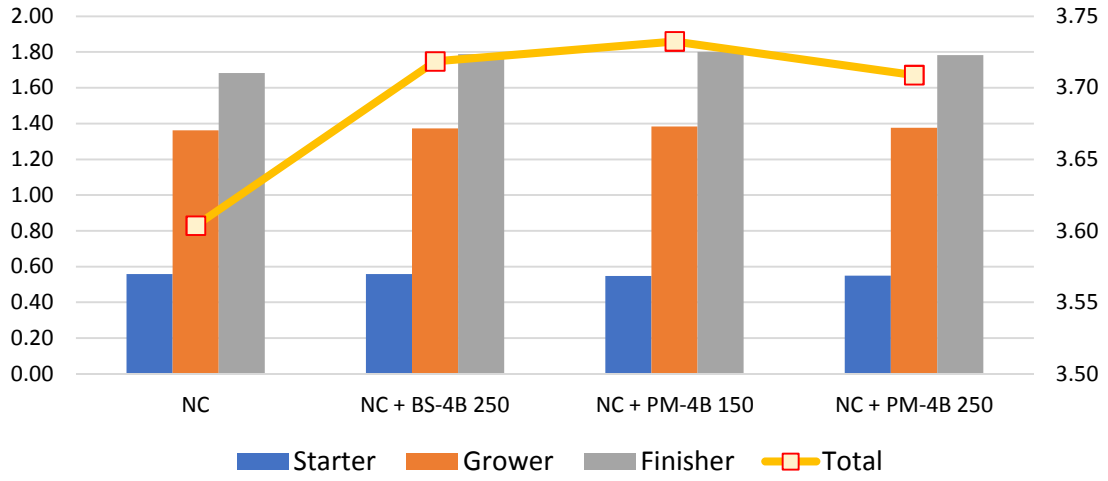


Fig A-IV: Cost of feed intake during different periods of the experiment (RS)

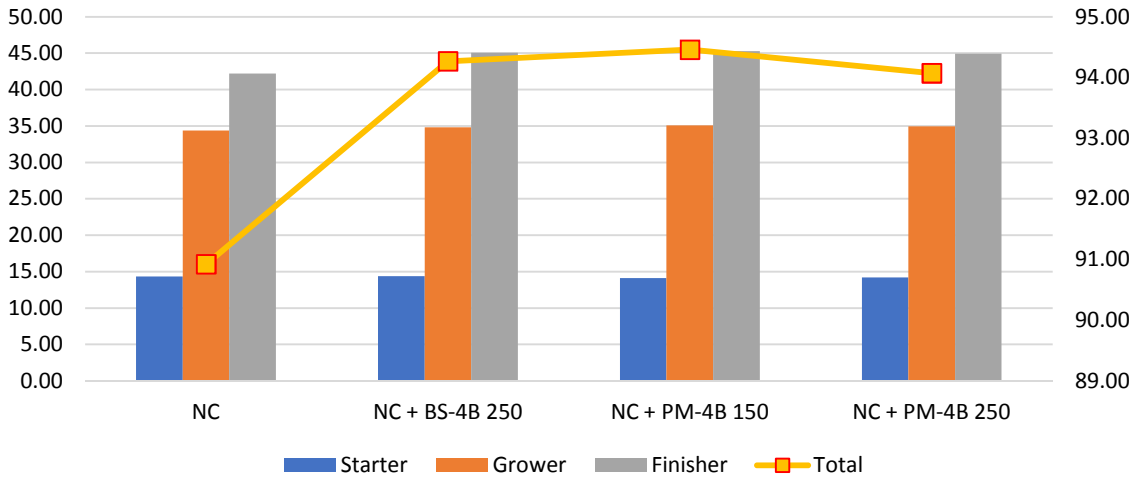


Fig A-V: Body weight, feed consumed per kg body weight and Feed cost per kg live weight (Rs)

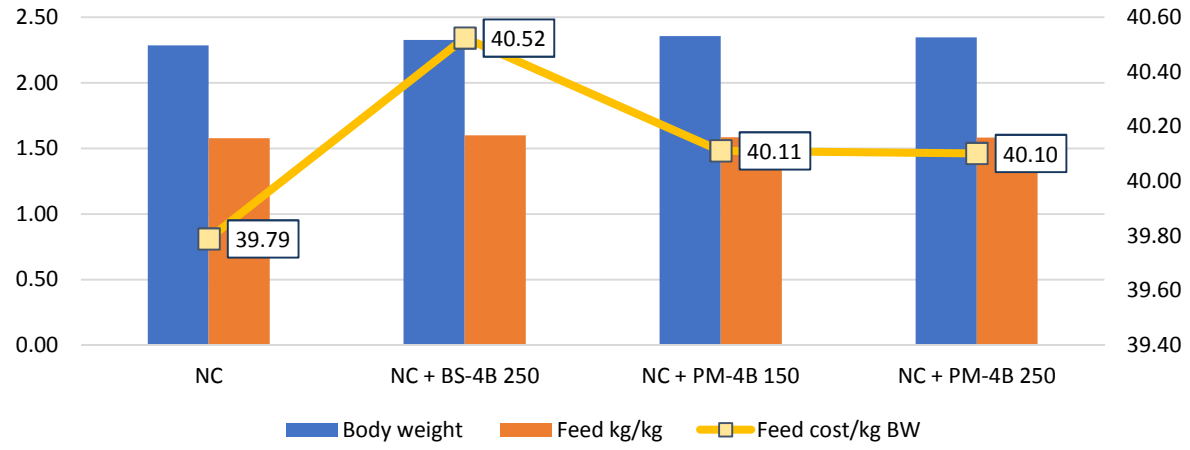
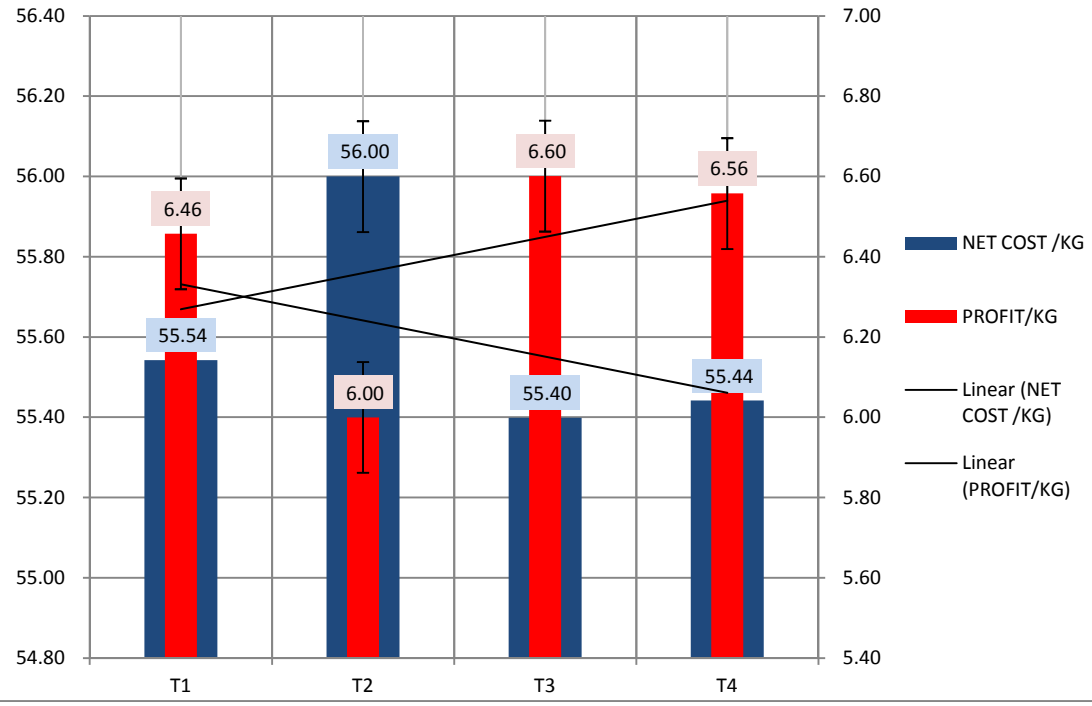
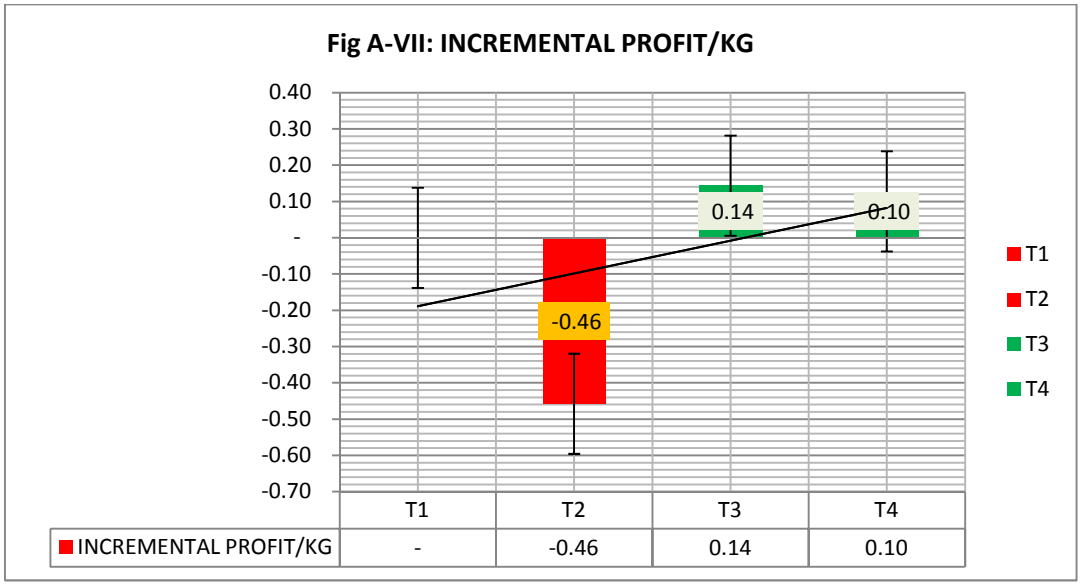


Fig A-VI: Net cost/Kg and Profit/Kg of live weight (Rs)





ECONOMICS CALCULATION				
	NC	**NC + BS-250**	**NC + PM-4B 150**	**NC + PM-4B-250**
PARAMETERS	**T1**	**T2**	**T3**	**T4**
CHICK COST	21.00	21.00	21.00	21.00
FEED INTAKE				
STARTER	559.00	557.90	547.40	550.00
GROWER	1,361.40	1,371.90	1,383.90	1,376.20
FINISHER	1,683.10	1,788.60	1,801.00	1,782.50
TOTAL	3,603.50	3,718.40	3,732.30	3,708.70
FEED COST				
STARTER	25.67	25.80	25.75	25.81
GROWER	25.25	25.38	25.34	25.39
FINISHER	25.07	25.19	25.15	25.20
FEED COST/BIRD				
STARTER	14.35	14.39	14.10	14.20
GROWER	34.38	34.82	35.07	34.94
FINISHER	42.20	45.05	45.30	44.92
TOTAL	90.92	94.27	94.46	94.06
MISCELLANEOUS COST	15.00	15.00	15.00	15.00
NET COST /BIRD	126.92	130.27	130.46	130.06
FINAL BODY WEIGHT	2,285.10	2,326.20	2,354.90	2,345.80
NET COST /KG	55.54	56.00	55.40	55.44
SELLING PRICE	62.00	62.00	62.00	62.00
PROFIT/KG	6.46	6.00	6.60	6.56
INCREMENTAL PROFIT/KG	-	-0.46	0.14	0.10

Disclaimer

Agrivet Consultancy Pvt. Ltd has taken every care to ensure that the contents of this report provide a correct reflection of its current understanding of the results and that the information presented here is accurate. Agrivet Consultancy Pvt. Ltd cannot, however, accept responsibility for any inadvertent errors in the information presented. No responsibility is accepted for any interpretation made from the information provided. Agrivet Consultancy P Ltd acted as the executor of the project only which has been funded by the sponsoring agency and shall, in no circumstances, be held responsible for any legal consequences which may arise out of the findings of the present trial.

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"EFFECT OF PROBIOTIC FEED SUPPLEMENT IN COMMERCIAL LAYER CHICKENS"

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ABSTRACT

The study was conducted to assess the three types of Competitor-BS 1 Billion CFU/g, Sporich-Total 4 Billion CFU/g (100 & 200 g/ton of feed), Prome-Max 4 Billion CFU/g (100 & 200 g/ton of feed) (SANZYME BIOLOGICS PVT. LTD.) and Bacitracin Methylene Disalicylate (50 g/ton of feed) supplemented in diet on *isocaloric* and *isonitrogenous* basis fed to a total of 288 White Leghorn layers (BV300), allocated to each of the eight treatments in a replicated manner, six replicates/treatment from 16 weeks of age (six birds/replicate) and evaluated production performance from 22 to 37 week of age.

The trial results revealed that, the per cent hen day egg production for four laying periods in White Leghorn layers from 22 to 37 weeks of age was significantly ($P<0.05$) influenced by supplementation of Competitor-BS, Sporich-Total and Prome-Max and Bacitracin Methylene Disalicylate (BMD). The higher Percent Egg Production was noticed in Sporich-Total @ 200 g/ton of feed (89.37%) compared with control and remaining treatments.

The mean Feed Intake and Feed Conversion Ratio per Dozen Eggs were significantly ($P<0.05$) influenced by supplementation of Competitor-BS, Sporich-Total & Prome-Max and BMD. The feed intake was significantly ($P<0.05$) lower in Control group (112.16g) and highest in Prome-Max @ 200 g/ton of feed (118.79g). The better Feed Conversion Ratio per Dozen Eggs was noticed in Sporich-Total @ 100 g/ton of feed (1.563).

Egg weight was significantly difference ($P<0.05$) was observed. The higher egg weight in Sporich-Total @ 100g/ton of feed (58.85g). The internal and external egg quality parameters of Haugh Unit, Yolk Index, Shell Thickness, Shell Percentage and Shell Strength were significantly ($P<0.05$) influenced by supplementation of Probiotic and BMD at different graded

levels. Whereas Albumen Index, Shell Weight and Density were not influenced by all the dietary supplementation groups. The mortality was within the limitation during 22-37 weeks of age.

The Competitor-BS, Sporich-Total and Prome-Max supplemented in diet influenced Net Profit per Dozen Eggs is highest in Sporich-Total @ 100 g/ton of feed (Rs.9.43) compared to other treatment groups during the 22-37 weeks of age.

It can be concluded that, supplementation of Multi Strain Probiotic Sporich-Total @ 100 g/ton of feed and Prome-Max @ 100 g/ton of feed as an alternative to antibiotic (BMD) and has better performance compared to the single strain Competitor-BS @ 100 g/ton of feed and Control.

Sporich-Total @ 100g/ton of feed has exhibited better production performance of commercial White Leghorn layer birds during the 22-37 weeks of age.

INTRODUCTION

Probiotics are defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance and there mode of action is by "competitive exclusion of harmful pathogens" in the gut. The use of sub-therapeutic levels of antibiotics as routine feed additive has been banned in many countries because of public concern, over possible antibiotic residual effects and the development of drug resistant bacteria. This has led to the development and application of many non-antibiotic substances performance enhancers. Initially Probiotics were introduced as an alternative to antibiotics and subsequently became an area of great research interest. Several studies in layers indicated that supplementation of Probiotics can serve as alternatives to antibiotics for increasing performance and disease resistance in poultry (Patterson and Burkholder, 2003). The addition of Probiotics could improve egg production, egg weight, and egg quality (Mohan *et al*., 1995; Ramasamy *et al.,* 2009) in laying hens.

MATERIALS AND METHODS

HOUSING AND MANAGEMENT

The experiment was conducted during August to December 2018. A total of 288 White Leghorn pullets (BV300) at the age of 16 weeks were procured from private agency then, leg banded and weighed individually & housed in California type cages (16"Hx 12"Wx18"D) having provision of feeders and nipple watering system. The birds housed in cages are given feed and water *ad lib* and the birds are raised under identical management conditions.

Before the commencement of the actual experiment, the cages, feeders and nipple drinking system were thoroughly cleaned, disinfected, and sprayed against external parasites. Other health precautions and sanitary measures were also taken throughout the study period. Diets were offered in separate feeder for different treatments with clear demarcation between replicates. Fluorescent lamp was placed for the lighting system to increase the lighting period to 16 h per day in order to increase feed intake and laying (Yasmeen *et al.,* 2008). Birds were adapted to respective treatment diet for a week before the commencement of the actual data collection.

EXPERIMENTAL DESIGN

The White Leghorn pullets (16weeks of age) were distributed randomly in to 8 different treatments with 6 replicates having 6 birds/replicate. Prior to experiment, the pre layer ration was fed to the birds until 21 weeks and layer ration from 22 weeks onwards.

EXPERIMENTAL DIETS

Three types of Probiotic supplementation at graded levels of 100 & 200 g/ton and Bacitracin Methylene Disalicylate (BMD) at 50 g/ton feeding fed to commercial layer chickens.

An experimental trial was conducted in layer chickens by feeding diets incorporated with various levels of Single Strain Probiotic - **COMPETITOR-BS 1 Billion CFU/g** and Multi Strain Probiotics of **SANZYME BIOLOGICS: SPORICH -TOTAL 4 Billion CFU/g and PROME-MAX 4 Billion CFU/g** in mash feed and to evaluate production performance in four laying periods (each period consist of 28 days).

The experimental layer diets were formulated (*isonitrogenous* and *isocaloric).*

- (T1 / Control) – The Basal Diet consisted of corn and soybean meal.
- (T2) – Basal Diet + 1×10^{9} CFU/g of Competitor-BS contain (*Bacillus subtilis*) @ 100 g/ton of feed
- (T3) – Basal Diet + 1×10^{9} CFU/g of Competitor-BS (*Bacillus subtilis*) @ 200 g/ton of feed;
- (T4) – Basal Diet + 4×10^{9} CFU/g of Sporich-Total contain (*Bacillus subtilis, Bacillus coagulans, Bacillus licheniformis & Saccharomyces boulardii*) @ 100 g/ton of feed
- (T5) – Basal Diet + 4×10^{9} CFU/g of Sporich-Total (*Bacillus subtilis, Bacillus coagulans, Bacillus licheniformis & Saccharomyces boulardii*) @ 200 g/ton of feed
- (T6) – Basal Diet + 4×10^{9} CFU/g of Prome-Max containing (*Bacillus subtilis, Bacillus coagulans & Bacillus licheniformis*) @ 100 g/ton of feed
- (T7) – Basal Diet + 4×10^{9} CFU/g of Prome-Max (*Bacillus subtilis, Bacillus coagulans & Bacillus licheniformis*) @ 200 g/ton of feed
- (T8) – Basal Diet + of AGP Bacitracin Methylene Disalicylate (BMD) 50 g/ton of feed
    - Production Performance of Hen Day Egg Production, Feed Intake, Feed Conversion Ratio per Dozen Eggs, Egg Weight, Egg Quality Parameters, Livability and Relative Economics were evaluated & studied for a total of four laying periods of 28 days each from 22 to 37 week of age.

Table 1. Ingredient Composition of Basal Diets (in Kg.) fed to the commercial White Leghorn layer birds from 22 to 37 week of age

Ingredients	Control / T1	T2	T3	T4	T5	T6	T7	T8
Maize	56	56	56	56	56	56	56	56
Soya bean meal	23	23	23	23	23	23	23	23
DORB	8.63	8.62	8.61	8.62	8.61	8.62	8.61	8.58
Shell grit	10.2	10.2	10.2	10.2	10.2	10.2	10.2	10.2
DCP	1.1	1.1	1.1	1.1	1.1	1.1	1.1	2
Salt	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Lysine	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Methionine	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vit. mix*	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Trace Mineral Mixture**	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Choline chloride (50%)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Toxin binder	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Sodium bicarbonate	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Probiotic	0.0	0.01	0.02	0.01	0.02	0.01	0.02	0.05
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

* Vitamin premix provided per Kg diet: Vitamin A 200000 IU, Vitamin B2 25 mg, Vitamin D3 3000IU, Vitamin K 2mg.

Riboflavin 25mg, Vitamin B1 1mg, Vitamin B6 2mg, Vitamin B12 40mg and Niacin 15mg.

** Trace mineral provided per Kg diet: Manganese 120mg, Zinc 80mg, Iron 25mg, Copper 10mg, Iodine 1mg and Selenium 0.1mg.

Nutrient Composition								
CP (%)	16.97	16.97	16.97	16.97	16.97	16.97	16.97	16.97
ME(kcal/Kg)	2620	2620	2620	2620	2620	2620	2620	2620
Calcium (%)	3.45	3.45	3.45	3.45	3.45	3.45	3.45	3.45
Available (%) Phosphorus	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Lysine (%)	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Methionine (%)	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38

RESULTS

1. PRODUCTION PERFORMANCE IN WHITE LEGHORN LAYERS DURING 22-37 WEEKS OF AGE

1.1 Percent Hen- Day Egg Production

The data on Percent Hen Day Egg Production as influenced by two types of Competitor-BS, Sporich-Total and Prome-Max at graded levels as shown in the Table 2. The Per Cent Hen Day Egg Production in White Leghorn layers were significantly (P<0.05) influenced during period-P1 (22-25wks.), period-P2 (26-29wks.), period-P3 (30-33wks.), period-P4 (34-37wks.) and over all period (22-37 wks.) of age.

It is observed that in overall period (22-37wks.) was significant (P<0.05) difference in Percent Hen Day Egg Production among different levels of Probiotic fed groups. Highest Percent Hen Day Egg Production in Sporich-Total @ 200 g/ton of feed (89.37%) followed by Sporich-Total @ 100 g/ton of feed (89.02%), Prome-Max @ 200 g/ton of feed (88.90%), Competitor-BS @ 100 g/ton of feed (88.40%), Competitor-BS @ 200 g/ton of feed (87.95%), Prome-Max @ 100 g/ton of feed (86.64%), Control (86.09%) and BMD @ 50 g/ton of feed (85.33%)

Table 2. Effect of dietary supplementation of Probiotic at graded levels on Percent Hen Day Egg Production in White Leghorn Layers during 22-37 weeks of age

Treatment		g /ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	73.61^{d}	87.34^{f}	92.20^{b}	91.20^{cd}	**86.09^{d}**
T2	Competitor-BS	100	74.26^{cd}	93.10^{ab}	94.11^{ab}	92.11^{cd}	**88.40^{c}**
T3	Competitor-BS	200	72.50^{cd}	93.85^{ab}	92.87^{b}	92.56^{c}	**87.95^{c}**
T4	Sporich-Total	100	80.56^{a}	91.01^{a}	91.45^{a}	93.04^{a}	**89.02^{a}**
T5	Sporich-Total	200	80.27^{c}	92.09^{e}	91.09^{c}	94.04^{d}	**89.37^{d}**
T6	Prome-Max	100	72.40^{cd}	91.26^{cd}	91.60^{a}	91.28^{b}	**86.64^{bc}**
T7	Prome-Max	200	76.65^{c}	92.05^{bc}	93.53^{a}	93.35^{b}	**88.90^{b}**
T8	BMD	50	70.78^{b}	89.38^{de}	90.97^{ab}	90.18^{b}	**85.33^{bc}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.001	0.001	0.001	**0.001**

Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate

1.2 Feed intake

The present study shows that the data on Feed Intake in commercial White Leghorn layers as influenced by different dietary treatments is presented in Table 3. The feed consumption was significant (P<0.05) difference during in first (22-25wks.), second (26-29 wks.), third (30-33 wks.) and fourth (34-37 wks.) periods and overall period (22-37 wks.) was significantly (P<0.05) influenced by supplementation with Competitor-BS, Sporich-Total & Prome-Max and BMD.

It is observed that in overall period (22-37wks.) was significant (P<0.05) difference in Feed Intake among different levels of Probiotic fed groups. Highest feed intake was with Prome-Max @ 200 g/ton of feed (118.79 g) followed by Sporich-Total @ 200 g/ton of feed (117.45 g), Sporich-Total @ 100 g/ton of feed (116.43 g), BMD @ 50 g/ton of feed (116.14 g), Prome-Max @ 100 g/ton of feed (115.55 g), Competitor-BS @ 100 g/ton of feed (115.36 g), Competitor-BS @ 200 g/ton of feed (114.98 g) and least feed intake in Control (112.16 g)

Table3. Effect of dietary supplementation of Probiotic at graded levels on Feed Intake (g/hen/day) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	110.89^{bc}	111.15^{d}	109.87^{c}	116.83^{h}	**112.16^{e}**
T2	Competitor-BS	100	108.54^{de}	116.86^{ab}	116.32^{a}	119.67^{g}	**115.36^{cd}**
T3	Competitor-BS	200	107.24^{e}	116.50^{ab}	115.03^{ab}	121.17^{f}	**114.98^{d}**
T4	Sporich-Total	100	109.48^{c}	115.44^{bc}	116.15^{a}	124.50^{e}	**116.43^{c}**
T5	Sporich-Total	200	111.85^{ab}	117.74^{a}	113.93^{ab}	126.17^{d}	**117.45^{b}**
T6	Prome-Max	100	110.32^{c}	114.24^{c}	110.20^{c}	127.33^{c}	**115.55^{cd}**
T7	Prome-Max	200	112.92^{a}	117.50^{ab}	116.27^{a}	128.33^{b}	**118.79^{a}**
T8	BMD	50	107.11^{e}	114.15c	113.37^{b}	129.83^{a}	**116.14^{c}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.001	0.001	0.001	**0.001**

Means with different superscripts in a column differ significantly (P<0.05),
BMD= Bacitracin methylene disalicylate

1.3 Feed conversion ratio per dozen eggs

The data on Feed Conversion Ratio per Dozen Eggs (Kg. of Feed consumed for every Dozen Eggs Produced) is presented in the Table 4. The results revealed that, there was significantly (P<0.05) difference in Feed Conversion Ratio per Dozen Eggs during period-P1 (22-25 wks.), period-P2 (26-29 wks.), period-P3 (30-33 wks.), period-P4 (34-37 wks.) and over all period (22-37 wks.) of age.

During the overall period (22-37 wks..) the best Feed Conversion Ratio per Dozen Eggs in commercial White Leghorn layers was observed when fed with supplementation of Sporich-Total @ 100 g/ton of feed (1.563) followed by Sporich-Total @ 200 g/ton of feed (1.566), Prome-Max @ 100 & 200 g/ton of feed (1.569), Competitor-BS @ 100 & 200 g/ton of feed (1.577 & 1.600), BMD @ 50 gton of feed (1.603) and poor in and Control (1.633).

Table 4. Effect of dietary supplementation of Probiotic at graded levels on Feed Conversion Ratio (Kg Feed Consumed for Dozen Eggs) in White Leghorn Layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	1.773^{ab}	1.569^{a}	1.530^{a}	1.661^{a}	**1.633^{a}**
T2	Competitor-BS	100	1.631^{d}	1.558^{c}	1.555^{bc}	1.564^{c}	**1.577^{d}**
T3	Competitor-BS	200	1.790^{a}	1.515^{b}	1.461^{bc}	1.634^{b}	**1.600^{b}**
T4	Sporich-Total	100	1.794^{a}	1.512^{b}	1.428^{c}	1.519^{d}	**1.563^{c}**
T5	Sporich-Total	200	1.661^{d}	1.499^{b}	1.448^{bc}	1.656^{a}	**1.566^{c}**
T6	Prome-Max	100	1.739^{c}	1.496^{b}	1.482^{b}	1.560^{c}	**1.569^{c}**
T7	Prome-Max	200	1.728^{bc}	1.491^{bc}	1.486^{b}	1.571^{c}	**1.569^{c}**
T8	BMD	50	1.776^{ab}	1.525^{bc}	1.487^{d}	1.624^{b}	**1.603^{c}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.001	0.001	0.001	**0.001**

Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate

2. EGG WEIGHT

The data on Egg Weight (g) is presented in the Table 5. The Egg Weight was significant (P<0.05) influenced by Competitor-BS, Sporich-Total, Prome-Max and BMD supplementation.

The overall Egg Weight was significantly (P<0.05) higher in Sporich-Total @ 100 g/ton of feed (58.85 g) followed by Competitor-BS @ 200 g/ton of feed (58.18 g), Prome-Max @ 200 g/ton of feed (58.12 g), Sporich-Total @ 200 g/ton of feed (58.09 g), Competitor-BS @ 100 g/ton of feed (57.90 g), BMD @ 50 g/ton of feed (57.74 g), Prome-Max @ 100 g/ton of feed (57.39 g) and Control (57.00 g).

Table 5. Effect of dietary supplementation of Probiotic at graded levels on Egg Weight (g) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	55.17^{bc}	55.65^{c}	57.12^{c}	60.07^{c}	**57.00^{d}**
T2	Competitor-BS	100	55.77^{b}	57.72^{ab}	57.83^{c}	60.26^{bc}	**57.90^{bc}**
T3	Competitor-BS	200	55.41^{b}	57.61^{ab}	58.34^{bc}	61.35^{ab}	**58.18^{b}**
T4	Sporich-Total	100	57.00^{a}	58.44^{a}	59.83^{a}	60.12^{c}	**58.85^{a}**
T5	Sporich-Total	200	56.32^{ab}	57.06^{b}	58.01^{bc}	60.97^{abc}	**58.09^{b}**
T6	Prome-Max	100	54.14^{cd}	57.78^{ab}	59.21^{ab}	58.43^{d}	**57.39^{cd}**
T7	Prome-Max	200	55.29^{bc}	55.71^{c}	59.91^{a}	61.58^{a}	**58.12^{b}**
T8	BMD	50	53.75^{d}	57.61^{ab}	58.17^{bc}	61.42^{ab}	**57.74^{bc}**
n			6	6	6	6	**6**
P-Value			0.001	0.001	0.001	0.001	**0.001**

Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate

3. INTERNAL EGG QUALITY PARAMETERS

3.1 Haugh Unit

Haugh Unit (HU) was significantly (P<0.05) influenced during first (22-25wks.), second (26-29 wks.), third (30-33 wks.) and fourth (34-37 wks.) and overall period (22-37 wks.) by supplemented with Competitor-BS, Sporich-Total & Prome-Max and BMD at graded levels.

The higher Haugh Unit value was observed in Prome Max @ 200 g/ton of feed (87.68) indicating the quality of albumen was better and lower Haugh Unit value observed in BMD @ 50 g/ton of feed (84.95) as shown in the (Table 6).

Table 6. Effect of dietary supplementation of Probiotic at graded levels on Haugh Unit in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	87.61^{a}	78.50^{d}	88.78^{a}	86.95^{cd}	**85.46^{cd}**
T2	Competitor-BS	100	87.77^{a}	80.98^{c}	86.84^{b}	84.43^{c}	**85.01^{d}**
T3	Competitor-BS	200	87.53^{a}	81.72^{c}	88.67^{a}	84.22^{e}	**85.53^{cd}**
T4	Sporich-Total	100	84.89^{b}	84.92^{a}	89.72^{a}	87.67^{bcd}	**86.80^{b}**
T5	Sporich-Total	200	86.41^{a}	85.20^{a}	86.56^{b}	86.34^{d}	**86.12^{bc}**
T6	Prome-Max	100	86.29^{a}	83.17^{b}	86.97^{b}	88.81^{ab}	**86.31^{b}**
T7	Prome-Max	200	87.56^{a}	84.65^{a}	90.28^{a}	88.26^{abc}	**87.68^{a}**
T8	BMD	50	83.97^{b}	79.28^{d}	87.05^{b}	89.51^{a}	**84.95^{d}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.001	0.001	0.001	**0.001**

Means with different superscripts in a column differ significantly (P<0.05),

BMD= Bacitracin methylene disalicylate

3.2 Albumen index

The present study shows that the data on Albumen Index as influenced by different dietary treatments is presented in Table 7. Albumen Index was significantly (P<0.05) influenced by supplementation of Probiotic during fourth period (34-37 wks.) of age.

The higher Albumen Index value for overall period (22-37 wks.) was noticed in Sporich-Total @ 100 g/ton of feed (0.085) followed by Competitor BS @ 100 g/ton of feed, Sporich-Total @ 200 g/ton of feed and Prome-Max @ 100 & 200 g/ton of feed (0.082), Control, Competitor BS @ 200 g/ton of feed and BMD @ 50 g/ton of feed (0.080) respectively.

Table7. Effect of dietary supplementation of Probiotic at graded levels on Albumen Index in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	0.084	0.084	0.084	0.083^{b}	**0.080**
T2	Competitor-BS	100	0.084	0.084	0.084	0.084^{b}	**0.082**
T3	Competitor-BS	200	0.085	0.083	0.083	0.083^{b}	**0.080**
T4	Sporich-Total	100	0.085	0.085	0.083	0.087^{a}	**0.085**
T5	Sporich-Total	200	0.083	0.084	0.085	0.083^{b}	**0.082**
T6	Prome-Max	100	0.085	0.083	0.083	0.084^{b}	**0.082**
T7	Prome-Max	200	0.086	0.085	0.085	0.084^{b}	**0.082**
T8	BMD	50	0.086	0.084	0.083	0.083^{b}	**0.080**
	n		6	6	6	6	**6**
	P-Value		0.078	0.078	0.122	0.016	**0.251**

Means with different superscripts in a column differ significantly (P<0.05),
BMD= Bacitracin methylene disalicylate

3.3 Yolk Index

It is observed that in period-P1 (22-25wks.), period-P2 (26-29wks.), period-P4 (34-37wks.) and over all period (22-37 wks.) was significant (P<0.05) difference in Yolk Index among different levels of Probiotic fed groups.

The overall period Yolk Index was better in the Sporich-Total @ 100 g/ton of feed, Prome-Max @ 100 & 200 g/ton of feed (0.473), followed by Competitor-BS @ 200 g/ton of feed (0.467), Competitor-BS @ 100 g/ton of feed & Sporich-Total @ 200 g/ton of feed (0.465), BMD @ 50 g/ton of feed (0.463) and Control (0.462).

Table 8. Effect of dietary supplementation of Probiotic at graded levels on Yolk Index in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	0.453^{d}	0.455^{c}	0.472	0.458^{a}	**0.462^{a}**
T2	Competitor-BS	100	0.493^{a}	0.467^{bc}	0.463	0.447^{ab}	**0.465^{ab}**
T3	Competitor-BS	200	0.487^{ab}	0.467^{bc}	0.465	0.452^{ab}	**0.467^{ab}**
T4	Sporich-Total	100	0.490^{ab}	0.492^{a}	0.463	0.453^{ab}	**0.473^{a}**
T5	Sporich-Total	200	0.463^{cd}	0.468^{bc}	0.470	0.460^{a}	**0.465^{ab}**
T6	Prome-Max	100	0.475^{abc}	0.485^{ab}	0.462	0.462^{a}	**0.473^{a}**
T7	Prome-Max	200	0.487^{ab}	0.492^{a}	0.462	0.440^{b}	**0.473^{a}**
T8	BMD	50	0.472^{bcd}	0.475^{abc}	0.458	0.447^{ab}	**0.463^{ab}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.002	0.669	0.036	**0.043**

Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate

4. EXTERNAL EEGG QUALITY PARAMETERS

4.1 Egg Shell Weight

The present experiment revealed that there is no significant difference was observed statically between control and various experimental groups of Egg Shell Weight. However, the values ranged between 5.581 to 5.712 g (Table 9).

Table 9. Effect of dietary supplementation of Probiotic at graded levels on Egg Shell Weight (g) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	5.438	5.473	5.778	6.161	**5.712**
T2	Competitor-BS	100	5.333	5.622	5.841	5.969	**5.692**
T3	Competitor-BS	200	5.21	5.433	5.624	6.091	**5.589**
T4	Sporich-Total	100	5.257	5.472	5.743	6.053	**5.631**
T5	Sporich-Total	200	5.163	5.411	5.807	6.122	**5.626**
T6	Prome-Max	100	5.121	5.534	5.635	6.032	**5.581**
T7	Prome-Max	200	5.415	5.336	5.682	6.172	**5.652**
T8	BMD	50	5.114	5.608	5.834	6.116	**5.668**
	n		6	6	6	6	**6**
	P-Value		0.227	0.496	0.887	0.846	**0.804**

Means with different superscripts in a column differ significantly (P<0.05),
BMD= Bacitracin methylene disalicylate

4.2 Shell Thickness

The Shell Thickens was significant (P<0.05) difference in first and second period between 22-25 and 26-29 weeks of age where as third and fourth period between 30-33 and 34-37 weeks of age there was non-significant. However, during the overall period there was significant (P<0.05) difference among the treatments (Table 10).

Table 10. Effect of dietary supplementation of Probiotic at graded levels on Egg Shell Thickness (mm) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	0.406^{bc}	0.397^{b}	0.41	0.407	**0.404^{bc}**
T2	Competitor-BS	100	0.396^{e}	0.396^{b}	0.397	0.4	**0.397^{d}**
T3	Competitor-BS	200	0.401^{cde}	0.396^{b}	0.402	0.406	**0.401^{bc}**
T4	Sporich-Total	100	0.412^{ab}	0.387^{c}	0.407	0.406	**0.404^{bc}**
T5	Sporich-Total	200	0.394^{e}	0.386^{c}	0.405	0.41	**0.399^{cd}**
T6	Prome-Max	100	0.403^{cd}	0.407^{a}	0.404	0.404	**0.405^{a}**
T7	Prome-Max	200	0.415^{a}	0.398^{b}	0.405	0.407	**0.406^{a}**
T8	BMD	50	0.402^{cde}	0.399^{b}	0.404	0.411	**0.404^{bc}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.001	0.061	0.425	**0.001**

Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate

4.3. Shell Percentage

The data on Shell Percentage was evaluated in layer chicken as influenced by different dietary treatments with Probiotic fed diets are presented in Table 11. During the first period between 22-25 wks. of age the Shell Percentage was significant (P<0.05) difference reported. However, the remaining periods were no significant difference among the treatments. During over all period (22-37wks.) the supplementation of Probiotic significantly (P<0.05) improve the Shell Percentage. The higher Shell Percentage was in the Sporich Total @ 100 g/tom (10.02) when compared with treatment groups.

Table 11. Effect of dietary supplementation of Probiotic at graded levels on Egg Shell Percentage in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	9.248^{cd}	9.542	9.681	10.02	**9.623^{b}**
T2	Competitor-BS	100	9.805^{ab}	9.332	9.736	10.05	**9.732^{b}**
T3	Competitor-BS	200	9.362^{bcd}	9.568	9.777	9.933	**9.658^{b}**
T4	Sporich-Total	100	9.869^{a}	9.838	10.09	10.27	**10.02^{a}**
T5	Sporich-Total	200	9.712^{abc}	9.473	9.728	10.2	**9.775^{ab}**
T6	Prome-Max	100	9.343^{bcd}	9.723	9.686	9.983	**9.680^{b}**
T7	Prome-Max	200	9.536^{abcd}	9.738	10	9.983	**9.818^{ab}**
T8	BMD	50	9.187^{d}	9.593	9.763	10.03	**9.638^{b}**
	n		6	6	6	6	**6**
	P-Value		0.01	0.299	0.55	0.526	**0.019**

Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate

4.4. Shell strength

The Shell Strength was significant (P<0.05) in supplementation with Competitor-BS, Sporich-Total & Prome-Max and BMD at different graded levels during the period and overall period. The Shell Strength was significantly (P<0.05) higher value in Sporich Total @ 100 g/tom (21.15 N) compared with other treatment groups (Table 12).

Table 12. Effect of dietary supplementation of Probiotic at graded levels on Egg Shell Strength (N*) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	21.02^{b}	17.95^{e}	18.92^{b}	19.33^{d}	**19.30^{de}**
T2	Competitor-BS	100	20.02^{bc}	20.50^{ab}	17.69^{c}	21.01^{c}	**19.81^{cd}**
T3	Competitor-BS	200	24.54^{a}	19.67^{abcd}	18.72^{bc}	19.57^{d}	**20.63^{ab}**
T4	Sporich-Total	100	19.24^{c}	20.10^{abc}	19.55^{b}	25.71^{a}	**21.15^{a}**
T5	Sporich-Total	200	19.27^{c}	19.39^{bcd}	18.76^{bc}	22.84^{b}	**20.06^{bc}**
T6	Prome-Max	100	19.50^{c}	19.05^{cde}	21.35^{a}	23.75^{b}	**20.91^{a}**
T7	Prome-Max	200	19.81^{bc}	20.83^{a}	21.68^{a}	21.33^{c}	**20.92^{a}**
T8	BMD	50	20.22^{bc}	18.53^{de}	17.73^{c}	18.34^{d}	**18.71^{e}**
	n		6	6	6	6	**6**
	P-Value		0.001	0.001	0.001	0.001	**0.001**

.Means with different superscripts in a column differ significantly (P<0.05)
BMD= Bacitracin methylene disalicylate
*Newton's

4.5 Density

The density (g/cm^3) of egg was non-significant in all the treatment groups during the period wise and overall period (Table 13).

Table 13. Effect of dietary supplementation of Probiotic at graded levels on Density (g/cm3) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	1.09	1.09	1.09	1.09	**1.09**
T2	Competitor-BS	100	1.09	1.09	1.09	1.09	**1.087**
T3	Competitor-BS	200	1.09	1.09	1.09	1.09	**1.087**
T4	Sporich-Total	100	1.09	1.09	1.09	1.09	**1.086**
T5	Sporich-Total	200	1.08	1.08	1.09	1.09	**1.085**
T6	Prome-Max	100	1.08	1.08	1.09	1.09	**1.085**
T7	Prome-Max	200	1.09	1.08	1.09	1.09	**1.086**
T8	BMD	50	1.08	1.09	1.09	1.09	**1.087**
	n		6	6	6	6	**6**
	P-Value		0.07	0.06	0.73	0.94	**0.18**

Means with different superscripts in a column differ significantly (P<0.05),
BMD= Bacitracin methylene disalicylate

5. LIVEABILITY

Between the 22-37 weeks of age, the mortality was within the limitation. During first (22-25wks.) second (26-29wks.) and fourth (34-37wks.) period there was no mortality of birds but whereas in third period (30-33 wks.) there was only one bird mortality was recorded in Competitor-BS @ 100 g/ton of feed (Table 14).

Table 14. Effect of dietary supplementation of Probiotic at graded levels on Liveability (%) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	100	100	100	100	**100**
T2	Competitor-BS	100	100	100	97	100	**99.25**
T3	Competitor-BS	200	100	100	100	100	**100**
T4	Sporich-Total	100	100	100	100	100	**100**
T5	Sporich-Total	200	100	100	100	100	**100**
T6	Prome-Max	100	100	100	100	100	**100**
T7	Prome-Max	200	100	100	100	100	**100**
T8	BMD	50	100	100	100	100	**100**

6. RELATIVE ECONOMICS IN WHITE LEGHORN LAYERS DURING 22-37 WEEKS OF AGE

The supplementation of Probiotic at different graded levels influenced Net Profit per Dozen Eggs is highest in Sporich-Total @ 100 g/ton of feed (Rs.9.43) compared with control (Rs.8.67) and also with Competitor-BS and Prome-Max (100 & 200 g/ton) and BMD (50 g/ton) of feed during the 22-37 weeks of age (Table 15).

Table 15. Effect of dietary supplementation of Probiotic at graded levels on Relative Economics in White Leghorn layers during 22-37 weeks of age

S.No	Details	Cntrl	Comp.BS	Comp.BS	SPT	SPT	PMX	PMX	BMD
Supplemented @		T1	T2	T3	T4	T5	T6	T7	T8
			100 g/ton	200 g/ton	100 g/ton	200 g/ton	100 g/ton	200 g/ton	50 g/ton
1	Cost of feed per Kg (Rs.)	23.25	23.3	23.3	23.3	23.3	23.3	23.3	23.3
2	Feed consumption/dozen eggs (Kg)	1.563	1.569	1.569	1.527	1.633	1.568	1.6	1.566
3	Feed cost/dozen eggs (Rs.)	36.33	36.55	36.55	35.57	38.97	36.53	37.28	36.48
4	Selling price of dozen eggs (Rs.)	45	45	45	45	45	45	45	45
5	Net profit per dozen eggs (Rs.)	8.67	8.45	8.45	9.43	6.03	8.47	7.72	8.52
6	Net profit over control (Rs.)		-0.22	-0.22	0.76	-2.64	-0.2	-0.95	-0.15

Selling price of egg Rs: 3.75, Avg. Probiotic cost Rs: 500/Kg
Cntrl = Control
Comp.BS = Competitor BS
SPT = Sporich Total
PMX = Prome Max
BMD = Bacitracin methylene disalicylate

7. BROKEN EGGS AND SOILED EGGS:

Broken Eggs

Broken Eggs was low in Sporich-Total @ 100 g per ton of feed and Prome-Max @ 100g per ton of feed followed by Competitor-BS @ 100 & 200 g per ton of feed per ton compared to Control and BMD treatments.

Table 14. Effect of dietary supplementation of Probiotics at graded levels on Broken Eggs (No.) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	2	2	1	2	7
T2	Competitor-BS	100	1	1	1	2	5
T3	Competitor-BS	200	2	1	1	1	5
T4	Sporich-Total	100	2	1	1	0	4
T5	Sporich-Total	200	1	2	2	2	7
T6	Prome-Max	100	1	2	0	1	4
T7	Prome-Max	200	2	2	1	2	7
T8	BMD	200	2	1	2	2	7

BMD= Bacitracin methylene

Soiled Eggs

Soiled Eggs were low in Sporich-Total @ 100g and Competitor-BS @ 100g per ton of feed compared to control and BMD.

Table 15. Effect of dietary supplementation of Probiotics at graded levels on Soiled Eggs (No.) in White Leghorn layers during 22-37 weeks of age

Treatment		g / ton	Age (weeks)				Mean
			22-25 (P1)	26-29 (P2)	30-33 (P3)	34-37 (P4)	22-37 wks.
T1	Control	0	1	1	1	1	4
T2	Competitor-BS	100	1	1	1	0	3
T3	Competitor-BS	200	0	2	1	1	4
T4	Sporich-Total	100	1	0	1	1	3
T5	Sporich-Total	200	1	2	1	1	5
T6	Prome-Max	100	1	2	2	0	5
T7	Prome-Max	200	2	1	0	2	5
T8	BMD	200	1	0	2	1	4

BMD= Bacitracin methylene

DISCUSSION

1 PRODUCTION PERFORMANCE OF COMMERCIAL LAYERS

1.1 Hen-Day Egg Production

In this experiment the dietary supplementation of graded levels of Probiotic in White Leghorn (BV 300) layers had shown significant effect ($P<0.05$) on Percent Hen Day Egg Production. The Percent Hen Day Egg Production was significantly ($P<0.05$) higher in Sporich Total (200 g/ton) when compared with control and this trend has continued from 22-37 weeks.

The above findings are in agreement with (Tortuero and Fernandez, 1995; Nahashon *et al*., 1996; Zhang *et al*., 2012; Yalcin *et al*., 2010; Panda *et al*., 2006; Abdelqader *et al*., 2013) reported that Percent Hen Day Egg Production was increased and improved in commercial layer birds and also Berrin (2011) & Wei Fen Li *et al*. (2011) reported in Japanese quail and Shaoxing Ducks.

Contrary to the findings of the present study, reported by Davis and Anderson 2002, Mahdavi *et al*., 2005,Yousefi and Karkoodi 2007, Balevi*et al*., 2009, Kalavathy Ramasamy *et al*., 2009, Mikulski *et al*., 2012, Shalaei *et al*., 2014, Sobczak*et et al*., (2015), Forte *et al*., 2016, Upadhaya *et al*., 2016, Sheoran *et al*., 2018, Fathi *et al*., 2018 observed did not show any significant variation of percent hen day egg production in commercial layer birds .

Different opinions were expressed by Sattar Bageri Dizaji and Rasoul Pirmohammadi 2009, Moorthy *et al*., 2010 observed that significantly ($P<0.05$) decrease the per cent hen day egg production by supplemented with Probiotic in White Leghorn layers.

Variability in response to the use of Probiotic giving good results in terms of Percent Hen Day Egg Production in White Leghorn layers with statistically significant, which may be reasonably due to the bacterial sensitivity, health and hygiene of birds used in the trials as well as the environmental factors.

1.2 Feed intake

In the experiment it is observed that the commercial layer birds fed with Probiotic supplemented diet throughout the experimental period had consumed significantly ($P<0.05$) more in Prome Max (200 g/ton) compared with supplemented Probiotic groups & control.

The results were in accordance with the earlier reports of Nahashon *et al*. (1996), Falaki *et al*. (2010) and Sheoran *et al.* (2018) found that significantly ($P<0.05$) increase in feed consumption in White Leg horn layers.

Contrary to the above results Tortuero and Fernandez 1995, Panda *et al*., 2003, Yoruk *et al*., 2004, Mahdavi *et al*., 2005, Panda *et al*., 2006, Yousefi and Karkoodi 2007, Panda *et al*., 2008 , Kalavathy Ramasamy *et al*., 2009, Moorthy *et al*., 2010, Yalcin *et al*., 2010, Mikulski *et*

al., 2012, Zhang *et al*., 2012, Abdelqader *et al*., 2013,Sobczak *et al*., 2015 and Fathi *et al*.,2018 were reported that no significant difference of feed intake in commercial White Leghorn birds supplemented with Probiotic and yeast culture.

The treatment groups feed consumption increased compare with control it may be Probiotic increasing the occurrence of naturally occurring beneficial bacteria in the intestinal tract of birds, which may result in the improvement of feed consumed.

1.3 Feed Conversion Ratio per Dozen Eggs

The Feed Conversion Ratio per Dozen Eggs was significantly ($P<0.05$) reduced observed when supplemented with Sporich-Total (100 g/ton) of feed in White Leghorn between 22-37 weeks of age.

Better feed efficiency observed in this study with supplementation of Probiotic is in accordance with the earlier findings of Nahashon *et al*., 1996, Yoruk *et al*., 2004, Panda *et al*., 2006 & 2008, Balevi *et al*., 2009, Yalcin *et al*., 2010, Shivani Katoch *et al*., 2011, Mikulski *et al*., 2012, Zhang *et al*., 2012 Abdelqader *et al*., 2013, Shalaei *et al*., 2014 and Sheoran *et al*., 2018 observed that significant ($P<0.05$) improvement in feed conversion ratio in commercial layer birds fed with Probiotic supplementation at different levels.

In contrast to the results of the present study reported by Tortuero and Fernandez 1995, Panda *et al*., 2003, Yoruk *et al*., 2004, Mahdavi *et al*., 2005, Yousefi and Karkoodi 2007, Yalcin *et al*., 2008, Moorthy *et al*., 2010,, Sobczak *et al*., 2015, Forte *et al*., 2016 and Fathi *et al*., 2018) were reported no significant difference in feed conversion ratio per dozen eggs in White Leghorn supplementation with Probiotic.

The effect of Probiotic might be attributable to the probable production of natural antibiotic like acidophil in which is active against pathogenic microbes like *E.coli* and *Salmonella*. Further, the Probiotic not only check the growth of pathogenic microorganisms but also could improve the feed utilization with neutralization of toxins, apparently increased the absorption of nutrients and alteration of microbial metabolism.

2. EGG WEIGHT

The supplementation with Probiotic and BMD in White Leghorn showed significantly ($P<0.05$) increase Egg Weight. Similar to these findings with the Tortuero and Fernandez 1995 & Davis and Anderson 2002, Balevi *et al*., 2009, Kalavathy Ramasamy *et al*., 2009; Yalcin *et al*., 2010, Mikulski *et al*., 2012, Zhang *et al*., 2012, Abdelqader *et al*., 2013 and Shalaei *et al*., 2014.

Contrary to the above results (Nahashon *et al*., 1996, Mahdavi *et al*., 2005, Panda*et al*., 2006 &2008, Yousefi and Karkoodi 2007, Yalcin *et al*., 2008, Sattar Bageri Dizaji and Rasoul Pirmohammadi 2009, Balevi *et al*., 2009, Sobczak *et al*., 2015, Forte *et al*., 2016 and Sheoran *et al*., 2018 found that the White Leghorn layer birds egg weight was non-significant in all treatment groups.

The improved egg weight observed in the present and earlier studies is probably due to the ability of Probiotic cultures to perform well under stressful conditions as egg weight increments in egg-type hens have been reported to be largely affected by environmental factors, although egg weight is a highly heritable trait in chickens. Furthermore, supplementation of Probiotic in animal feed may improve digestion and absorption of nutrients in the host.

The improvement in egg size was associated with the higher calcium and nitrogen retentions in the Probiotic fed hens. Davis reported similar result and Anderson 2002, in which hens supplemented with PrimaLac exhibited a higher percentage of extra-large eggs than control.

3 INTERNAL EGG QUALITY

3.1 Haugh Unit

Albumen quality evaluated as Haugh Unit Score was significantly ($P<0.05$) influenced supplemented Probiotic and BMD during 22-37 weeks of age. Similar to the above findings agreement with , Tortuero and Fernandez 1995, Zhang *et al*., 2012 and Sobczak *et al*., 2015.

Contrary to the observations several others, Nahashon *et al*.,1996, Mahdavi *et al*., 2005, Panda *et al*., 2008, Yalcin *et al*., 2008, Berrin 2011, Mikulski *et al*.,2012 and Fathi *et al*.,2018 results revealed no significant difference among the treatment groups.

3.2 Albumin Index

The effect of different periods was not significant of albumen index. However, there was significant differences of albumen index during third period without following any specific trend. This might be due to biological variation. These findings were in accordance with Yoruk *et al*., 2004, Valavan *et al*., 2006, Yalcin *et al*., 2008 and Berrin 2011.

3.3 Yolk Index

The effect of Yolk Index was significantly ($P<0.05$) influenced during the first (22-25wks.) second (25-29wks.) and fourth period (33-37wks.) and overall period (22-37 wks.) However, during the third period (30-33wks.) non-significant difference was recorded.

Different opinions were expressed by various workers Yoruk *et al*., 2004 Valavan *et al*., 2006, Yalcin *et al*., 2008, Berrin2011 and Mikulski *et al*., 2012 found that no significant difference in yolk index .

4. EXTERNAL EGG QUALITY

4.1 Shell Weight

The effect of different periods was non-significant on Egg Shell Weight. Similar to these findings, Forte *et al*., 2016.

Contrary to the above results, Yousefi and Karkoodi 2007, Panda *et al*., 2008 and Fathi*et al*.,2018 reported that there was significant (P<0.05) difference in Shell Weight among the treatment groups.

4.2 Shell Thickness

The effect of first, second and overall periods were significantly effect on Shell Thickness. However, the Shell Thickness was not affected during third and fourth periods.

These results are in agreement with Panda *et al*., 2006, Yousefi and Karkoodi 2007, Panda *et al*., 2008, Mikulski *et al*., 2012, Zhang *et al*., 2012 Abdelqader *et al*.,2013, Shalaei *et al*., 2014, Sobczak *et al*.,2015 and Fathi *et al*.,2018.

Contrary to the findings of the present study Mahdavi *et al*., 2005 and Yalcin *et al*., 2008 showed that shell thickness was no significant difference among the treatment groups in layers birds.

This beneficial effect may be attributed to a favourable environment in the gastrointestinal tract resulting from the administration of Probiotic to birds (Mohan *et al*., 1995; Panda *et al*., 2008; Mikulski *et al*., 2012). Probiotic bacteria increase the rate of fermentation and the production of short-chain fatty acids (SCFAs), which reduces the luminal pH (Scholz-Ahrens *et al*., 2007). Low luminal pH increases calcium solubility and absorption (Van den Heuvel *et al*., 1999). SCFAs stimulate intestinal epithelial cell proliferation and villus height (Garcia *et al*., 2007), which increases absorption efficiency (Scholz-Ahrens *et al*., 2007). As a result, more nutrients, including calcium, can be assimilated, thus improving eggshell quality

4.3. Shell Percentage

The mean Shell Percentage was significantly (P<0.05) influenced by three types of Probiotic and BMD supplementation. However, differences in Shell Percentage was recorded during the first period.

The findings of the present study are in agreement with those of Mikulski *et al*., 2012 and Sobczak *et al*., 2015.

In contrast to the results of the present study, various workers Yousefi and Karkoodi (2007), Forte *et al*. (2016) Shalaei *et al*. (2014).

4.4. Shell Strength

The Shell Strength was significant differences in White Leghorn eggs during 22-37 weeks of age without following any specific trend.

. The above results are in agreement with Panda *et al*. 2008, Sobczak*et al*., 2015, Upadhaya *et al*., 2016 and Fathi *et al*., 2018 resulted in a significantly (P<0.05) improved Shell Strength in White Leghorn layers.

Shell Breaking Strength was significantly higher in the Probiotic-fed groups. This could be attributed to the higher Shell Thickness, which might have created greater resistance resulting in higher breaking strength

Concomitant to the findings of the present study, Mahdavi *et al*., 2005 and Shalaei *et al*., 2014 found that did not observe any difference in the Shell Strength.

4.5 Density

The Density (g/cm^3) of egg was non-significant in all the treatment groups during the period wise and overall period.

5. Livability

There was no mortality in layer birds. Compared to the findings of the present study, Panda *et al.,* 2006 and Balevi *et al.,* 2009 observed mortality was not effected in White Leghorn chicks supplemented with Probiotic. However, Yoruk *et al*., 2004 and Vicente *et al*., 2007 reported a significant (P<0.01) reduction in mortality.

6. RELATIVE ECONOMICS

The supplementation of Probiotic at different graded levels influenced Net Profit per Dozen Eggs is highest in Sporich-Total @ 100 g/ton (Rs. 9.43) compared with Control (Rs 8.67) and also with Competitor-BS and Prome-Max (100 & 200 g/ton) and BMD (50g/ton), during the 22-37 weeks of age.

Davis and Anderson (2002) noticed that feeding of direct fed microbial, Probiotic supplement “Primalac” to Single Comb White leghorn, Hy-Line W-36 and DeKalb XL laying hens significantly (P<0.002) reduced the feed cost.

Sabiha *et al*. (2005) observed that the cost of production in broilers was lower in 0.025 and 0.05% Probiotic (Yea-sacc1026, *Lactobacillus acidophilus, Streptococcus faecium*etc.) supplemented groups at six and eight weeks of age, respectively.

Moorthy *et al*. (2010) reported that diets supplemented with Probiotic and prebiotic supplements did not affect the return over feed cost per bird in White Leghorn layers aged 21-52 weeks of age.

7. Broken Eggs

Multi strain Probiotics such as Sporich-Total & Prome-Max @ 100g per ton of feed has lower number of Broken Eggs compared to control, BMD and Competitor-BS.

8. Soiled Eggs

Soiled Eggs are lower in Sporich-Total & Competitor-BS @100g per ton of feed.

CONCLUSION

Based on the findings of this study the following conclusions were drawn:

- The supplementation of Sporich-Total @ 100 g per ton of feed during 22-37 weeks of age to the commercial White Leghorn layers has influenced significantly ($P<0.05$) and more effective among all the dietary treatments in terms of major parameters such as mean Percent Hen Day Egg Production, Feed Conversion Ratio (feed consumed per Kg egg mass and per dozen eggs), Yolk Index, Broken Eggs and Soiled Eggs.
- The supplementation of Prome Max @ 100 g per ton of feed during 22-37 weeks of age to the commercial White Leghorn layers has also influenced significantly ($P<0.05$) and effective among the dietary treatments in terms of parameters such as Haugh Unit, Albumen Index, Yolk Index, Shell Percentage, Shell Strength & Net Profit.
- The supplementation of Probiotic at different inclusion rates influenced Net Profit per Dozen Eggs and is highest in Sporich-Total @ 100 g per ton of feed (Rs. 9.43) compared with control (Rs.8.67) during the 22-37 weeks of age.
- During the laying between 22-37 weeks of age, the broken eggs and soiled eggs production were within in the limitation and lowest in Sporich-Total @ 100g per ton of feed.
- Mortality rate was within the limits & one, only mortality recorded in Competitor-BS @ 100g per ton of feed, and no specific disease was recorded in all the treatments.
- It can be concluded that, supplementation of Multi Strain Probiotic Sporich-Total @ 100 g per ton of feed & Prome Max @ 100 g per ton of feed can be used as an alternative to AGP / Antibiotics (BMD) and have significantly better performance compared to the Single Strain Probiotic (Competitor-BS) and Control.
- Sporich-Total @ 100 g per ton of feed has exhibited better production performance of commercial White Leghorn layer birds during the 22-37 weeks of age.