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Sanzyme Biologics

SPORICH™-ResQ

In-Vitro Studies

SANZYME BIOLOGICS (P) LTD
Regd. Office : Plot No.13. Sagar Society, Road No.2,
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Website : www.sanzymebiologics.com |
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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

QUALITY CONTROL DEPERTMENT

Acid Tolerance Studies

Product: SPORICH RESQ

Strain: *Bacillus coagulans* SAN 135BC + *Saccharomyces boulardii* SAN 158SB

Objective: To determine Acid tolerance assay of SPORICH RESQ

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 SPORICH RESQ *(*~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 ^{0}C 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 coagulans*
        a) 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)
        b) Incubate the solidified plates in an inverted position at 37 °C for 48 hours.

     B. *Saccharomyces boulardii*
        c) Set out five sterile petri plates and add 1 ml of final dilution into each petri plate and then pour 15 ml of Sabouraud Chloramphenicol Agar into each of the petri plate and mix thoroughly.(temperature around 45°C)

d) The solidified plates are incubated in an inverted position at 25°C (± 2)°C for 5 days.

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:
  - PNY Medium : Hi media Code: M835
  - Sabouraud Chloramphenicol Agar Composition (SCA) (Himedia code: M1067)
- 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.
- SB Buffer Preparation and Composition:

Ingredients	Grams / Liter
Potassium Dihydrogen Phosphate (KH_2PO_4)	1.5
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	9
Mycological Peptone	10
Sodium Chloride	5
Dissolve the above ingredients in 1000 ml of distilled water.	
Sterilize by autoclaving at 121°C for 15 minutes. pH: 7.2 (± 0.2)	

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	2980	99.33	2977	99.23	2960	98.67
2	2976	99.20	2970	99.00	2950	98.33
3	2969	98.97	2964	98.80	2943	98.10
4	2967	98.90	2963	98.77	2934	97.80
5	2960	98..67	2960	98.67	2931	97.70
6	2938	97.93	2957	98.57	2920	97.33

Conclusion:

- SPORICH RESQ was recovered 97.93% (2938 CFU/ml) at pH 2 and 6 Hours Incubation
- SPORICH RESQ was recovered 98.57% (2957 CFU/ml) at pH 3 and 6 Hours Incubation
- SPORICH RESQ was recovered 97.33% (2920 CFU/ml) at pH 7 and 6 Hours Incubation

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QUALITY CONTROL DEPERTMENT

Bile Tolerance Studies

Product: SPORICH RESQ

Strain: *Bacillus coagulans* SAN 135BC + *Saccharomyces boulardii* SAN 158SB

Objective: To determine Bile tolerance of SPORICH RESQ

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 SPORICH RESQ (~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 coagulans*
       a) 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)
       b) Incubate the solidified plates in an inverted position at 37 °C for 48 hours.
     - *B. Saccharomyces boulardii*
       c) Set out five sterile petri plates and add 1 ml of final dilution into each petri plate and then pour 15 ml of Sabouraud Chloramphenicol Agar into each of the petri plate and mix thoroughly.(temperature around 45°C)

d) The solidified plates are incubated in an inverted position at 25°C (± 2)°C for 5 days.

9. Counting:
   - 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
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- Media Composition:
  - PNY Medium : Hi media Code: M835
  - Sabouraud Chloramphenicol Agar Composition (SCA) (Himedia code: M1067)
- 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.
- SB Buffer Preparation and Composition:

Ingredients	Grams / Liter
Potassium Dihydrogen Phosphate (KH_2PO_4)	1.5
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	9
Mycological Peptone	10
Sodium Chloride	5
Dissolve the above ingredients in 1000 ml of distilled water.	
Sterilize by autoclaving at 121°C for 15 minutes. pH: 7.2 (± 0.2)	

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	2961	98.70	2961	98.70	2943	98.44
2	2950	98.33	2951	98.37	2937	97.90
3	2945	98.17	2942	98.07	2934	97.80
4	2931	97.70	2929	97.63	2929	97.63
5	2927	97.57	2927	97.57	2922	97.40
6	2922	97.40	2909	96.97	2918	97.27

Conclusion:

- SPORICH RESQ was recovered 97.40% (2922 CFU/ml) at 0.15% Bile concentration and 6 Hours Incubation
- SPORICH RESQ was recovered 96.97% (2909 CFU/ml) at 0.3% Bile concentration and 6 Hours Incubation
- SPORICH RESQ was recovered 97.27% (2918 CFU/ml) at 0.6% Bile concentration and 6 Hours Incubation

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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
(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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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		*Saccharomyces boulardii* - SAN 158SB	PTA338
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		*QepA*	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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Comparative effect of SANZYME BIOLOGIC's, **SPORICH™-ResQ** on Fecal Score, Microbial Assay & V/C ratio of Broiler Chicken affected with *Clostridium perfringens*

Study **Location:** Broiler Farm, Hyderabad

Study **Period:** Aug 2018 (7 days)

Experimental Design

Study Groups	**Description**	
T1	Positive for *Clostridium perfringens*	PCP
T2	PCP + **SPORICH™-ResQ** (200g/T)	PCP + SPRQ
T3	PCP + Chlortetracycline (1500g/T)	PCP + CTC
T4	PCP + Bacitracin Methylene Disalicylate (200g/T)	PCP + BMD

Controlled Inputs

1	T1, T2, T3 & T4 were grouped after finding positive for *Clostridium perfringens* on Day'15
2	T3 was supplemented Chlortetracycline of a reputed brand through mash feed from Day'16
3	T4 was supplemented Bacitracin Methylene Disalicylate of a reputed brand through mash feed from Day'16
4	T2 was supplemented **SPORICH™-ResQ** - a proprietary blend of *Bacillus coagulans* SAN 135BC + *Saccharomyces boulardii* SAN 158SB from SANZYME BIOLOGICS - through mash feed from Day'16 • Potency used for experiment = 5 Billion CFU/g

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Fecal Score

Groups	Description	Day'16	Day'17	Day'18	Day'19	Day'20
T1	PCP	+++++	+++++	+++++	+++++	+++++
T2	PCP + SPRQ	+++++	+	+	+	-
T3	PCP + CTC	+++++	++++	++++	+++	++
T4	PCP + BMD	+++++	+++++	++++	++++	+++

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

Groups	Description	CP - Jejunum (x 10^9 CFU/g)	CP - Ileum (x 10^9 CFU/g)
		Day'21	Day'21
T1	PCP	5.50	5.50
T2	PCP + SPRQ	2.00	1.50
T3	PCP + CTC	3.50	3.00
T4	PCP + BMD	4.50	4.00

Intestinal Morphological Analysis
(Histopathology Report)

Groups	Description	V/C Ratio - Jejunum	V/C Ratio - Ileum
		Day'21	Day'21
T1	PCP	3.80	3.25
T2	PCP + SPRQ	4.03	3.44
T3	PCP + CTC	3.93	3.40
T4	PCP + BMD	3.88	3.36

Conclusions:

1. **SPORICH™-ResQ** reduced the Fecal Score from 5 to 1 within 24 hours as compared to antibiotics
2. **SPORICH™-ResQ** effectively reduced the microbial counts of *Clostridium perfirengenes* in both Jejunum & Ileum
3. **SPORICH™-ResQ** optimized the Villi Height & Crypt Depth

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Effects of SANZYME BIOLOGIC's, **SPORICH™- ResQ** on Litter Condition of Layer Breeder Hens

Study **Location:** Layer Breeder Farm – Namakkal, TN

Study **Period:** Jan – Feb'23 (4 Weeks)

Controlled Inputs

1	Severe Loose Droppings in Layer Breeder Hens of 42nd Week • Condition started @ 26th Week and gradually worsened
2	**SPORICH™-ResQ** supplemented through mash feed for 4 Weeks @ 250 g / Ton of feed
3	**SPORICH™-ResQ** - a proprietary blend of *Bacillus coagulans* SAN 135BC + *Saccharomyces boulardii* SAN 158SB from SANZYME BIOLOGICS • Potency used for experiment = 5 Billion CFU/g
4	No change in Feeding & Management Practices apart from the supplementation of **SPORICH™-ResQ**

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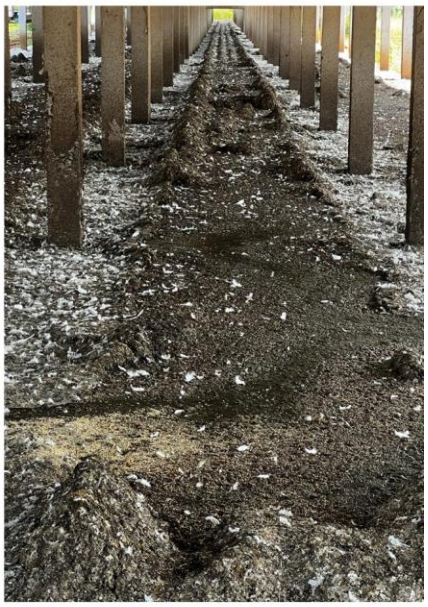
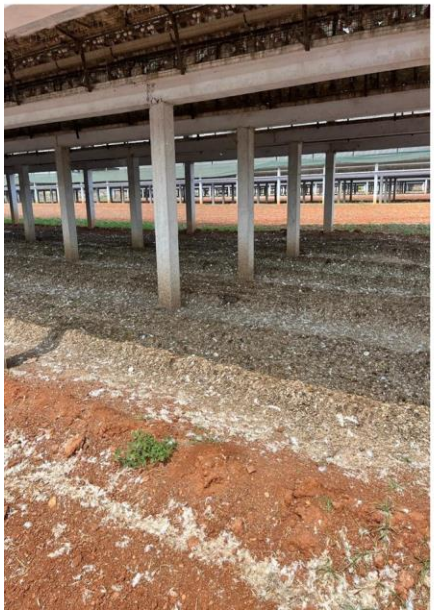
Condition at 42nd Week



Condition at end of 45th Week



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Effects of SANZYME BIOLOGIC's, **SPORICH™- ResQ** on Litter Condition of Layer Hens

Study **Location:** Layer Farm – Hyderabad,
Study **Period:** Feb – Mar'23 (4 Weeks)

Controlled Inputs

1	Severe Loose Droppings in Layers of 46th Week • Condition started @ 27th Week and gradually worsened
2	**SPORICH™-ResQ** supplemented through mash feed for 4 Weeks @ 250 g / Ton of feed
3	**SPORICH™-ResQ** - a proprietary blend of *Bacillus coagulans* SAN 135BC + *Saccharomyces boulardii* SAN 158SB from SANZYME BIOLOGICS • Potency used = 5 Billion CFU/g
4	No change in Feeding & Management Practices apart from the supplementation of **SPORICH™-ResQ**

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Condition at 46th Week

Condition at end of 50th Week

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