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Therapeutic efficacy of probiotic *Alkalihalobacillus clausii* 088AE in antibiotic-associated diarrhea: a randomized controlled trial

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Methods

Method of Analysis

The investigational product (IP) containing active ingredient, *Alkalihalobacillus clausii* 088AE [2000 million spores per gram per sachet (2000×10^6 spore/g/sachet) which is equivalent to 2 billion spores per gram per sachet (2×10^9 spore/g/sachet)], was supplied by Advanced Enzyme Technologies Ltd., Thane, India. The viable spore count of *B. clausii* 088AE was determined by pour plate technique. Briefly, one sachet of IP powder (1.00 g) was suspended in tween peptone water (%, compositions: protease peptone, 1.0 %; sodium chloride, 0.5 %; disodium phosphate, 0.35 %; monosodium phosphate, 0.15; tween 80, 0.2 %) and kept under sonication (33±3 KHz) at 10°C. Sonicated samples were then serially diluted. The diluted samples were given heat shock in a water bath at 70°C for 15 min, followed immediate cooling to below 45°C. 1.0 ml of thus obtained treated spore suspension was dispensed in petriplate and presterilized molten brain-hreart infusion (BHI) agar (SM211, HiMedia, Mumbai, India) was added to the plates. The plates were allowed to solidify and invertedly incubated at 37 °C for 48–72 h. The activity was expressed in colony forming units of viable spores per gram (cfu/g) of powder by taking the average mean of results. The placebo contained only the excipient, maltodextrin. Both the investigational and placebo product had passed through other required specifications like physical appearance, microscopic appearance, strength, heavy metals and microbial limit [other aerobic microbial count, yeast & mold count, *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*]. Microbial limit tests were analysed following standard Indian Pharmacopeia guidelines or other international standard guidelines. All the analyses were performed in triplicate, for three times.

Details of Exclusion and Withdrawal Criteria

Exclusion criteria

The subjects who had following criteria were excluded for the study

- On antibiotics or laxatives within the preceding 6 weeks.
- Presence of inflammatory bowel disease
- Presence of acute GI tract infection
- Presence of fever, abdominal mass, signs of bowel obstruction
- History of colon cancer or diverticulitis
- Infection by human immunodeficiency virus (HIV), hepatitis B virus and hepatitis C virus
- Patients with celiac disease defined by biopsy of the duodenal mucosa.
- History of scleroderma and gastroparesis
- Hypothyroidism

Withdrawal criteria

The patient can be withdrawn from the study by the investigator for any of the following:

- Occurrence of an adverse event, associated with the administration of the IP and requiring its cancellation.
- Emergence of any diseases or conditions during the study that worsen the prognosis of the patient, as well as make it impossible for the patient to continue his/her participation in the clinical study.
- The need for a forbidden concomitant therapy.
- Pregnancy of the patient.
- Violation of the study protocol, like

  Improper inclusion of the patient who did not meet the inclusion criteria and/or met the relevant exclusion criteria; other violations of the protocol, which, according to the investigators, are significant.
- Withdrawal of the informed consent by the patient

Table S1 Assessment of vital signs of adolescent and adult subjects from both Test-AA and Placebo-AA arm suffered with antibiotic-associated diarrhea (AAD) and undergone treatment with *Alkalihalobacillus clausii* 088AE at different visits (visit 01 – visit 05). Values are expressed as mean ± SD and group mean differences (Test-AA to Placebo-AA) are calculated by Kruskal-Wallis one-way ANOVA.

Study arms	Parameters	Visit 01 (Day 1)	Visit 02 (Day 2)	Visit 03 (Day 3)	Visit 04 (Day 5)	Visit 05 (Day 8)
Test-AA	Pulse (per minute)	68.90±7.77	66.06±9.31	68.06±6.46	71.23±7.30	69.33±7.05
	Respiratory rate (per minute)	18.23±0.93	17.67±0.99	18.20±0.88	18.26±1.01	18.00±0.94
	Systolic blood pressure (mm/Hg)	121.06±8.75	121.46±9.17	120.53±6.70	121.67±7.31	122.60±8.29
	Diastolic blood pressure (mm/Hg)	78.20±6.50	78.26±5.55	77.13±5.51	79.93±5.88	79.13±5.67
	Temperature (°F)	98.98±0.89	98.46±0.12	98.47±0.10	98.48±0.11	98.48±0.07
Placebo-AA	Pulse (per minute)	68.16±7.78	65.46±8.92	69.13±5.91	70.96±7.21	70.13±5.77
	Respiratory rate (per minute)	17.90±1.37	17.83±1.02	18.10±1.03	18.10±1.02	18.13±1.16
	Systolic blood pressure (mm/Hg)	120.66±8.22	122.60±9.17	122.46±6.31	122.26±8.20	122.20±8.68
	Diastolic blood pressure (mm/Hg)	77.73±5.93	78.60±6.32	79.20±6.07	80.00±7.16	79.26±6.63
	Temperature (°F)	98.75±0.78	98.41±0.28	98.39±0.27	98.44±0.29	98.41±0.28

Group mean difference (Test-AA to Placebo-AA) by one-way ANOVA

	P value (p<0.05)
Pulse (per minute)	0.9168
Respiratory rate (per minute)	0.3472
Systolic blood pressure (mm/Hg)	0.2962
Diastolic blood pressure (mm/Hg)	0.3472
Temperature (°F)	0.0758

Table S2 Assessment of vital signs of pediatric subjects from both Test-PS and Placebo-PS arm suffered with antibiotic-associated diarrhea (AAD) and undergone treatment with *Alkalihalobacillus clausii* 088AE at different visits (visit 01 – visit 05). Values are expressed as mean ± SD and group mean differences (Test-PS to Placebo-PS) are calculated by Kruskal-Wallis one-way ANOVA.

Study arms	Parameters	Visit 01 (Day 1)	Visit 02 (Day 2)	Visit 03 (Day 3)	Visit 04 (Day 5)	Visit 05 (Day 8)
Test-PS (n=30)	Pulse (per minute)	85.70±6.44	85.07±6.64	85.53±6.66	86.63±5.37	86.70±6.09
	Respiratory rate (per minute)	20.63±3.06	20.73±3.05	20.90±2.68	20.50±2.82	21.43±2.91
	Temperature (°F)	97.02±0.87	97.12±0.71	97.26±0.54	97.12±0.55	97.35±0.75
Placebo-PS (n=30)	Pulse (per minute)	85.97±6.84	87.00±6.15	86.30±6.85	87.30±6.41	86.36±6.99
	Respiratory rate (per minute)	20.87±3.45	20.96±3.17	21.76±2.82	20.90±2.75	21.33±2.77
	Temperature (°F)	97.09±0.69	97.25±0.71	97.44±0.64	97.21±0.77	97.07±0.76

Group mean difference (Test-PS to Placebo-PS) by one-way ANOVA

	P value (p<0.05)
Pulse (per minute)	0.1745
Respiratory rate (per minute)	0.1436
Temperature (°F)	0.9168

Table S3 Assessment of hematological and biochemical parameters of adolescent and adult subjects from both Test-AA and Placebo-AA arm suffered with antibiotic-associated diarrhea (AAD) and undergone treatment with *Alkalihalobacillus clausii* 088AE at baseline and end of treatment (EOT). Values are expressed as mean ± SD and significance between mean difference (Test-AA to Placebo-AA) is tested at $p<0.05$.

Parameters	Visit	Test-AA	Placebo-AA	P value ($p<0.05$)	95% CI	Normal range
Hemoglobin (g/dL)	Baseline	13.71±1.56	13.55±1.50	0.687	-0.951, 0.631	13-16 (Male)
	EOT	13.73±1.43	13.78±1.25	0.886	-0.644, 0.744	12-15 (Female)
RBC count (million/mm^3)	Baseline	4.51±0.54	4.46±0.57	0.728	-0.337, 0.237	4.60-6.00 (Male)
	EOT	4.59±0.51	4.68±0.46	0.476	-0.161, 0.341	4.00-5.40 (Female)
Total leukocyte count (×10^3 cells/mm^3)	Baseline	6.84±1.72	6.78±1.58	0.889	-0.914, 0.793	4.00-11.00
	EOT	6.71±1.51	6.81±1.49	0.797	-0.675, 0.875	
Neutrophils (%)	Baseline	55.67±3.94	56.16±5.84	0.704	-2.084, 3.064	40-75
	EOT	55.03±3.32	56.46±5.38	0.220	-0.880, 3.740	
Lymphocytes (%)	Baseline	34.30±3.65	33.96±5.65	0.783	-2.798, 2.118	20-42
	EOT	35.10±3.13	33.53±5.09	0.155	-3.754, 0.613	
Eosinophils (%)	Baseline	4.86±0.86	4.38±1.09	0.063	-0.987, 0.027	1-7
	EOT	4.23±0.93	4.53±0.77	0.179	-0.141, 0.741	
Monocytes (%)	Baseline	5.16±2.10	5.56±1.97	0.449	-0.652, 1.452	2-10
	EOT	5.66±1.53	5.80±1.88	0.753	-0.745, 1.025	
Basophils (%)	Baseline	0.00	0.00	-	-	0-1
	EOT	0.00	0.00	-	-	
Hematocrit (PCV, %)	Baseline	40.01±3.78	39.40±3.81	0.536	-2.571, 1.351	40-50 (Male)
	EOT	40.04±3.01	40.09±2.63	0.945	-1.411, 1.510	36-46 (Female)
Platelet Count (lakh/mm^3)	Baseline	125.43±134.87	109.69±117.68	0.632	-81.155, 49.675	0.15-0.45
	EOT	129.75±135.88	117.13±121.46	0.706	-79.226, 53.986	
ESR (mm/hr)	Baseline	12.40±5.81	11.70±5.58	0.636	-3.644, 2.244	0-20 (Male)
	EOT	11.46±5.43	10.80±4.80	0.619	-3.308, 1.988	0-30 (Female)
SGOT (AST) (U/L)	Baseline	18.30±5.01	19.60±5.12	0.324	-1.318, 3.918	5-40
	EOT	19.83±3.71	20.66±3.69	0.388	-1.082, 2.742	
SGPT (ALT) (U/L)	Baseline	29.56±12.93	26.97±7.46	0.346	-8.045, 2.865	7-56
	EOT	25.67±5.56	27.66±7.71	0.256	-1.484, 5.464	
Creatinine (mg/dL)	Baseline	0.82±0.15	0.78±0.12	0.259	-0.110, 0.030	0.5-1.4
	EOT	0.79±0.11	0.80±0.08	0.689	-0.039, 0.059	
BUN (mg/dL)	Baseline	8.46±3.92	10.05±5.85	0.221	-0.984, 4.163	14-36
	EOT	9.76±2.69	9.09±2.42	0.315	-1.992, 0.652	
Albumin (g/dL)	Baseline	3.82±0.40	3.78±0.39	0.696	-0.244, 0.164	3.4-5.4
	EOT	3.82±0.36	3.86±0.41	0.689	-0.159, 0.239	
Serum sodium (mEq/L)	Baseline	138.03±2.75	137.58±2.33	0.497	-1.767, 0.867	135-145
	EOT	137.60±2.40	137.45±3.09	0.834	-1.579, 1.279	
Serum potassium (mmol/L)	Baseline	3.97±0.38	3.86±0.36	0.254	-0.301, 0.081	3.6-5.2
	EOT	4.02±0.38	3.94±0.39	0.424	-0.279, 0.119	
Total cholesterol (mg/dL)	Baseline	153.26±24.91	147.26±21.48	0.322	-18.020, 6.020	125-200
	EOT	148.96±19.39	142.36±17.73	0.174	-16.202, 3.002	
RBS (mg/dL)	Baseline	92.60±22.34	110.93±96.81	0.316	-17.980, 54.640	79-140
	EOT	93.46±15.60	108.93±84.45	0.328	-15.915, 46.855	

Table S4 Assessment of hematological and biochemical parameters of pediatric subjects from both Test-PS and Placebo-PS arm suffered with antibiotic-associated diarrhea (AAD) and undergone treatment with *Alkalihalobacillus clausii* 088AE at baseline and end of treatment (EOT). Values are expressed as mean ± SD and significance between mean difference (Test-PS to Placebo-PS) is tested at $p<0.05$.

Parameters	Visit	Test-PS	Placebo-PS	P value (p<0.05)	95% CI	Normal range
Hemoglobin (g/dL)	Baseline	12.51±0.70	12.30±0.81	0.287	-0.601, 0.181	10.9-14.9
	EOT	12.50±0.72	12.31±0.80	0.337	-0.583, 0.203	
RBC count (million/mm^3)	Baseline	4.42±0.28	4.34±0.26	0.256	-0.219, 0.059	3.80-5.20
	EOT	4.39±0.22	4.35±0.23	0.494	-0.156, 0.076	
Total leukocyte count ($\times 10^3$ cells/mm^3)	Baseline	11.53±0.80	11.55±0.90	0.927	-0.420, 0.460	4.50-14.50
	EOT	9.93±0.81	10.06±1.18	0.620	-0.393, 0.653	
Neutrophils (%)	Baseline	65.22±3.53	65.37±4.28	0.882	-1.877, 2.177	38-68
	EOT	68.48±3.16	67.28±3.48	0.167	-2.917, 0.517	
Lymphocytes (%)	Baseline	43.48±7.28	44.14±4.52	0.674	-2.471, 3.791	25-54
	EOT	43.33±3.45	41.74±3.18	0.068	-3.304, 0.124	
Eosinophils (%)	Baseline	2.94±1.09	2.89±1.08	0.859	-0.610, 0.510	0-3
	EOT	2.95±0.79	3.05±0.75	0.617	-0.298, 0.498	
Monocytes (%)	Baseline	4.39±1.04	4.32±0.92	0.783	-0.577, 0.437	4-10
	EOT	4.61±0.80	4.70±0.76	0.656	-0.313, 0.493	
Basophils (%)	Baseline	0.31±0.57	0.31±0.25	1.000	-0.227, 0.227	0-1
	EOT	0.27±0.16	0.31±0.21	0.410	-0.056, 0.136	
Hematocrit (PCV, %)	Baseline	35.97±2.15	36.50±2.39	0.370	-0.644, 1.704	35-44
	EOT	35.84±1.87	35.99±2.08	0.770	-0.872, 1.172	
Platelet Count (lakh/mm^3)	Baseline	291.63±36.62	282.60±34.81	0.331	-27.494, 9.434	0.15-0.45
	EOT	283.76±29.74	283.53±25.79	0.974	-14.616, 14.156	
ESR (mm/hr)	Baseline	7.93±4.84	8.10±4.11	0.883	-2.150, 2.490	0-10
	EOT	8.16±4.01	8.43±3.67	0.786	-1.716, 2.256	
SGOT (AST) (U/L)	Baseline	39.25±3.51	39.11±2.85	0.865	-1.792, 1.512	5-40
	EOT	30.09±2.86	31.31±2.50	0.083	-0.168, 2.608	
SGPT (ALT) (U/L)	Baseline	40.21±3.11	40.09±2.86	0.876	-1.664, 1.424	7-56
	EOT	35.20±2.71	34.01±2.82	0.101	-2.619, 0.239	
Creatinine (mg/dL)	Baseline	0.64±0.06	0.66±0.07	0.239	-0.013, 0.053	0.5-1.0
	EOT	0.65±0.06	0.67±0.06	0.201	-0.011, 0.051	
BUN (mg/dL)	Baseline	14.30±5.88	16.32±3.97	0.124	-0.572, 4.612	5-18
	EOT	15.45±5.35	17.32±4.03	0.131	-0.577, 4.317	
Albumin (g/dL)	Baseline	3.93±0.35	3.89±0.46	0.706	-0.251, 0.171	3.4-5.4
	EOT	3.95±0.40	3.95±0.33	1.000	-0.189, 0.189	
Serum sodium (mEq/L)	Baseline	136.40±3.35	136.76±3.83	0.699	-1.499, 2.219	135-145
	EOT	137.93±2.55	139.60±4.85	0.100	-0.332, 3.672	
Serum potassium (mmol/L)	Baseline	4.10±0.67	4.34±0.56	0.137	-0.079, 0.559	3.4-4.7
	EOT	4.13±0.56	4.33±0.42	0.123	-0.056, 0.455	
Total cholesterol (mg/dL)	Baseline	156.50±19.22	150.23±18.83	0.206	-16.103, 3.563	125-200
	EOT	156.50±18.21	150.23±17.34	0.177	-15.459, 2.919	
RBS (mg/dL)	Baseline	98.30±14.94	95.73±14.44	0.501	-10.163, 5.023	79-140
	EOT	94.40±11.15	95.03±8.18	0.803	-4.423, 5.683	