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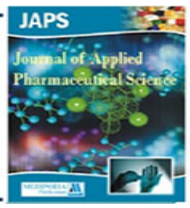

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Effect of Probiotics against Marine Pathogenic Bacteria on *Artemia franciscana*

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ABSTRACT

Presently an effort has been made to determine the effectiveness of probiotics against marine pathogenic bacterial load ingested by *Artemia franciscana* nauplii. In this experiment *Artemia franciscana* nauplii was allowed to ingest pathogenic bacterial strains, viz. *Escherichia coli, Salmonella typhi, Salmonella paratyphi, Vibrio cholerae and Shigella sp.* Probiotic organism (Bioremid) was used against the pathogenic strains on *Artemia franciscana* nauplii. On completion of the experiment it was observed that the use of Probiotic organism (Bioremid) reduced the pathogenic bacterial load, especially that of *Shigella sp.* on *Artemia franciscana.*

Keywords: Probiotics; marine pathogenic bacteria; *Artemia franciscana.*

INTRODUCTION

Artemia franciscana Kellogg, 1906 are branchiopod crustaceans that inhabit hypersaline habitats. They have been recorded in over 600 coastal and inland sites worldwide. *Artemia* have the ability to produce storable dormant embryos, or cysts that can hatch swiftly into live nauplii. For this reason *Artemia* is the most widely used live feed item in the larvi culture of fish and shellfish. The use of probiotics, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. The term probiotic (the opposite of the term antibiotic), meaning “for life” in the original Greek Language, was previously defined by Fuller (1989) as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.” At present, probiotics are well established for use in humans, poultry and cattle. The broad definition of probiotics in the field of aquaculture was also concerned with “organic wastes” and “pollutants”, as a result of incorporation of “bioremediation” and “biocontrol” when dealing with environmental problems. Juvenile and adult brine shrimp are used increasingly as suitable live diets for different aquaculture species.

The intensive culture of the brine shrimp *Artemia* has always suffered from unpredictable results due to incidental crashes in individual production tanks. Experimental infections of *Artemia* were done with *Vibrio proteolyticus* CW8T2, which has previously been shown to cause mortality in monoxenic *Artemia* cultures. The infection route was determined by means of transmission electron microscope observations. *In vivo* antagonism tests were performed to see whether the selected bacterial strains are able to protect *Artemia* from the pathogenic actions of *V. proteolyticus* CW8T2. In addition, filtrate experiments were done to verify whether extracellular compounds were involved in the protective action (Verschuere *et al.,* 2000). Researchers (Soto-Rodriguez *et al.,* 2003) studied from healthy and diseased penaeid shrimp from Asia and the Americas, 25 luminous and 2 non-luminous bacterial strains were isolated, and 14 were phenotypically identified as *Vibrio harveyi*; 9 isolates produced significant mortalities (45 to 80%) in *A. franciscana* nauplii at inoculation densities of 105 to 106 CFU ml–1 compared to the controls (unchallenged nauplii). Workers (Gomez-Gil B *et al.,* 2003) reported that the brine shrimp (*A. franciscana*) were enriched with different bacteria, and the dynamics of bacterial uptake by the nauplii were observed. The role of beneficial bacteria to limit and to control environmental pathogens has become particularly important in the future of aquaculture, especially with regard to increasing number of antibiotic resistant strains of bacteria (Dixon B., 1993). The potential of yeast as probiotics for fish was assessed in the past (Andlid *et al.,* 1994). Use of *Bacillus* sp. as an additive in rotifer medium improved the production rate (Lagos M *et al.,* 1994). A probiotic strain of *Vibrio alginolyticus* effective in reducing disease caused by *Aeromonas salmonicida, V. anguillarum* and *V.ordalii* was also reported (Dixon B., 1993). *A. salmonicida,* the causal agent of furanculosis in salmonid fish is the subject of interest for biotechnology, principally in terms of rapid diagnostic techniques and control measures, which included the use of disease resistant strains of fish vaccines, non-specific immunostimulants such as glucans and probiotics (Dixon B., 1993). The efficacy of formaldehyde was compared with that of antibiotics and the former was found to be very effective in controlling the bacteria of *Artemia* nauplii was reported (Griffith DRW., 1995). He also studied techniques for microbial control in the intensive rearing of marine larvae. The effect of different concentrations of formalin on cyst induction in *A. parthenogenetica* was studied as a function of rearing salinity and the survival of *A. parthenogenetica* was found to be influenced by both the concentration of formalin and also exposure duration. At the higher exposure duration of 6 hrs, mortality coincided with cyst induction (Griffith DRW., 1995). The aim of this study was to investigate whether pathogenic bacterial strains can also be active bio control agents against bacterial infections. Present attempt is to invigorate the pathogenic effect of *E. coli, S. typhi, S. paratyphi, V. cholarae,* and *Shigella* against commercial probiotics (Bioremid) on the *A. franciscana* nauplii.

MATERIALS AND METHOD

Experimental design and data analysis

A. franciscana, Kellogg, 1906 cysts (Unibest T M 0.0-73-0) were hatched under standard laboratory conditions (Sorgeloos., 1986).

Cysts of *A. franciscana* were decapsulated and subsequently incubated in 1 liter transparent cylinder at a concentration of 1.5 g/l of UV treated sterile seawater of 35 ppt. Hatching temperature was maintained at 28ºC, pH was adjusted to 8.0 throughout hatching and the container was supplied with strong aeration (saturated oxygen level more than 5 ppm). The light (2000 lux) was provided by a fluorescent lamp placed near the hatching cylinders. The photoperiod was maintained at 16**:**8 D/L. After 24 hrs of incubation, nauplii were observed and transferred into a 25 litre capacity epoxy coated rectangular tank. The experimental trial was carried out in six different tanks in duplicate. The entire experimental organisms were fed with the live feed viz., *Chaetoceros* sp. and *Skeletonema* sp. in twice per day at 10 am and 6 pm respectively.

Application procedure

The pathogenic bacteria isolates namely *1) Escherichia coli 2) Salmonella typhi 3) Salmonella paratyphi 4) Vibrio cholerae and 5) Shigella* sp were picked up from the departmental laboratory for the challenge test. Strains were first isolated and partially purified on thiosulphate citrate bile salt agar (TCBS), Nutrient agar *Salmonella Shigella* agar and TCBS agar supplemented with 2 % NaCl and incubated at 30 °C for 18 to 24 hours. All the pathogenic isolates were preserved I triovials at – 70 °C ultra low mechanic freezer according to the methodology proposed by pioneer researcher (Gherna, 1994).

Bacterial inoculums

Ten milliliters of a fresh bacterial culture were centrifuged at 5,000 rpm for 10 min at 10°C, the liquid supernatant was then discarded, and the pellet was suspended in sterile saline solution. This process was repeated again, and the cell concentration in the suspension was adjusted to an optical density of 1.00 at 610 nm in a spectrophotometer (model DR-2000; Hach, Loveland, Color). To estimate the bacterial concentration achieved, the suspension was serially diluted in sterile saline and spread plated in respected media. The plates were incubated overnight at 37ºC, and the colony forming units counted. The bacterial density used in all bath challenges was between 10^5 and 10^6 CFU ml^{-1}.

Bacterial characterization

Biochemical tests to characterize the isolates were performed following the scheme (Alsina *et al.,* 1994) and according to the methodologies (MacFaddin, 1990; Cowan *et al.,* 1993), except that NaCl was added to a final concentration of 2.5% to allow growth of the isolates.

Challenge with *A. franciscana* nauplii

The overall scheme for the challenge tests *A. franciscana* cysts from the Great Salt Lake (Prime *Artemia*) were employed in this study. Sterile *Artemia* nauplii were prepared as per protocol (Sahul Hameed *et al.*, 2002). Briefly, newly hatched nauplii were collected in a 120 µm sterile sieve and washed thoroughly with sterile seawater, before being placed in a petri dish with 18 ml of sterile seawater. For controls, nauplii were removed and placed (300 each) in 6 glass test tubes (18:150 mm) containing 20 ml of sterile seawater previously shaken for oxygenation. This protocol permitted the nauplii to incorporate bacteria as soon as their mouths opened . After 1 hr exposure, the nauplii were washed thoroughly with sterile seawater and then subdivided into 300 nauplii each in 5 replicate test tubes for each tested bacterial isolate. The experiment was aimed at detecting the pathogenic effect of (uniform concentration) *E. coli, S. typhi, S. paratyphi, V. cholarae*, and *Shigella* sp. were applied to the *Artemia* nauplii. Total 300 *Artemia* nauplii were stocked in each experimental tank with the capacity of 100 liter each and fed with *Skeletonema* and *Chaetoceros* at the 30,000 cells/ml. *Artemia* nauplii were starved for 24 hrs prior to administration of the pathogenic strain into the experimental tanks. However, the experimental organisms were treated with 0.1 ml of 1 x 10^5 – 10^7 CFU /ml of pathogenic strains viz., *E. coli, S. typhi, S. paratyphi, V. cholarae*, and *Shigella* sp. Permissible level of 1 g of commercial probiotics Bioremid is applied after 3hrs, 6hrs and 12hrs of post administration of pathogenic organisms into the experimental tanks to evaluate the efficacy of Bioremid against the pathogenic organisms on *A. franciscana* nauplii.

RESULTS AND DISCUSSION

A. franciscana nauplii is an important live feed for a variety of finfishes and shellfishes and are given to over 85% of aquaculture species around the world. Careless use of this live food organism may be responsibly for the development of disease and mass mortality in larvae of fishes and shellfishes. *Artemia* nauplii carry a heavy bacterial load. In the present study, the total number of aerobic heterotrophic bacterial flora ranged from 3.8 ×10^3 to 8.1 × 10^3 CFU/nauplius on seawater nutrient agar and 9.4 × 10^2 to 4.3 × 10^3 CFU/nauplius on TCBS agar. Earlier studies have also reported that the heavy bacterial load associated with *Artemia* nauplii [4]. Similarly, the total aerobic heterotrophic bacteria of *Artemia* nauplii was determined on seawater nutrient agar and TCBS agar, and ranged from 3.8 x 10^3 to 8.1 x 10^3 and 9.4 x 10^2 to 4.3 x 10^3 Colony Forming Units (CFU) per nauplius on seawater nutrient agar and TCBS agar plates, respectively (Sahul Hameed *et al*., 2002). The bacterial loads of the *Artemia* nauplii used for the challenges were respectively: 2 x 10^6 *Vibrio anguillarum* cells per 6000 *Artemia* and 5 X 10^5 *V. anguillarum* cells per 6000 *Artemia.* Assuming that each *Artemia* nauplius contained the same number of *V. anguillarum* cells and that each turbot had eaten the same number of *Arternia* nauplii, the infection dose for each challenge would have been respectively 1 x 10^5 and 2.5 x 10^7 *V. anguillarum* cells per fish. However, it is unlikely that these assumptions are true, and variations in challenge dose between individual fish probably occurred. It is therefore necessary to control the bacterial population of *Artemia* nauplii to minimize the danger of bacterial infection before their use in culture systems. The effects of chemotherapeutants, ultraviolet irradiation treatments and freezing have been investigated to minimize the danger of bacterial infections associated with feeding live food (Muroga et al 1989). Use of antibiotics, a hypochlorite solution and formaldehyde have all been found to be effective in suppressing the bacterial flora of *Artemia* nauplii (Griffith DRW, 1995).

Water quality parameters

The water quality parameters recorded in control and experimental tanks viz., (1) *E. coli* (2) *S. typhi* (3) *S. paratyphi* (4) *V. cholerae and* (5) *Shigella* sp. challenged to *A. franciscana* nauplii, are presented as follows:

In both control and experimental tanks, temperature varied from 29 to 31ºC and there was no difference between the experimental and control tank. Similar trend was noticed with respect to salinity and DO levels which ranged from 34.11 to 34.45 ppt and 6.15 to 7.50 ppm respectively. The pH values fluctuated from 6.8 to 7.6. The pH levels decreased from 6.8 to 6.4 in the control. The levels are increased from 7.1 to 7.6 in the probiotics used tanks. A decreasing trend in ammonia was noticed in the experimental tanks where probiotics were used. But in control and other tanks, ammonia level was increased. It increased from 0.312 to 0.855 ppm in tanks where the shrimps were ablated and no probiotics added. However, in tanks having the probiotic medium, the level declined from 0.351 to 0.101 ppm. As in ammonia, variations in nitrite level were also recorded. Nitrite concentration in the experimental set up with probiotics increased in particular to *Shigella* sp treated tanks. The nitrite levels in *E.coli* and *S. typhi* experimental sets increased from 0.00156 to 0.00767 ppm and 0.00336 to 0.00567 ppm respectively and a decrease from 0.00121 to 0.00091 ppm and from 0.00113 to 0.00090 ppm was recorded in the *Salmonella paratyphi* and *E.coli* respectively. In control tanks and in other tanks where probiotics were not added a decreasing trend in nitrate concentration was observed. While the nitrate levels in *E.coli* and *Salmonella typhi* experimental sets increased from 0.00203 to 0.00497 ppm and from 0.00240 to 0.00324ppm respectively a decrease from 0.00124 to 0.00053 ppm and from 0.00225 to 0.00092 ppm was recorded in the *S. paratyphi* and *V. chlorae* respectively.Phosphate concentration is increased from 0.00142 to 0.00467 ppm in the tanks where only the probiotics were added and from 0.00120 to 00455 ppm in the tanks of ablated shrimps kept in probiotics medium. In the control tanks, the values decreased from 0.00316 to 0.00083 ppm and from 0.00128 to 0.00057 ppm respectively. A similar trend in silicate was noticed, in *S. typhi* and *S. paratyphi* tanks where the level increased from 0.00106 to 0.00791 ppm and from 0.00133 to 0.00405 ppm respectively. However, in the control and in the tanks where the *A. franciscana* probiotics not added, the levels decreased from 0.00123 to 0.00093 ppm and from 0.00139 to 0.00102 ppm. (Table.1).

Table. 1: Water quality parameters of both control and experimental tanks of *A. franciscana.*

Water quality parameters	Experimental Tanks					
	Control	***Escherichia coli***	***Salmonella typhi***	***Salmonella paratyphi***	***Vibrio cholerae***	***Shigella sp.***
Salinity (ppt)	34.11	34.32	34.40	34.45	34.41	34.33
pH	7.00	7.10	7.30	7.50	7.50	7.30
Temperature (ºC)	30.50	29.50	29.00	31.00	30.00	31.00
Dis.Oxygen (ppm)	6.50	6.15	7.00	7.10	7.20	7.50
Ammonia (mg/lit)	0.311	0.588	0.841	0.701	0.541	0.855
Nitrate (ppm)	0.00158	0.00166	0.00745	0.00761	0.00767	0.00658
Phosphate (ppm)	0.00442	0.00489	0.00456	0.00465	0.00567	0.00467
Silicate (ppm)	0.00106	0.00263	0.00564	0.00698	0.00754	0.00791

Table. 2: Effect of pathogenic bacteria in different hours after administration******A. franciscana* nauplii reared no addition of either pathogenic bacteria or probiotics

Application duration	Pathogenic bacteria (CFU/ml)					
	Number of colonies appeared after application of probiotics					
	Control	***E.coli***	***Salmonella typhi***	***Salmonella paratyphi***	***Vibrio chlorae***	***Shigella* sp.**
Just after application	45	118	122	128	120	136
3 hrs after application	44	19	22	21	17	25
6 hrs after application	47	8	11	10	6	13
24 hrs after application	51	2	3	3	2	4

Level of pathogenicity between isolates

Pathogenicity levels between pathogenic bacteria using was evaluated, initially zero hour after application of pathogenic bacteria the levels of colonies were also evaluated. Highest levels of 128 colonies were noticed in the tank administered with *Shigella* sp. and a least number of colonies of 118 found with the tank treated with *E.coli*. Both *Shigella* sp and *E.coli* were controlled towards probiotic application (Table. 2 & 6). Contrarily, in control tanks the stable trend, (microbial colonies) was observed without addition of either pathogenic bacteria applied nor probiotics treated.

Among five different isolates (1) *E. coli* (2) *S. typhi* (3) *S. paratyphi* (4) *V.cholerae and* (5) *Shigella* sp. studied, a significant probiotics effect was noticed with the only isolate *Shiegella* sp. A drastic trend was noticed immediately 3 hrs after application of bioremid probiotics into the experimental tank which treated with *Shigella* sp. A mean mortality of 146.11 only observed in the tank, which is comparatively lesser than other level of pathogenicity i.e., post application of probiotics on *A. franciscana* can be presented in the following ways:

After application of probiotics (Table.2.)

Shigella sp. 136 > *S. paratyphi 128 > S. typhi 122 > V. chlorae 120 > E.coli 118*

3 hours after application

Shigella sp. 25 > *S. typhi 22 > S. paratyphi 21 > E.coli 19 > V. chlorae 17*

6 hours after application

Shigella sp. 13 > *S. typhi 11 > S. paratyphi 10 > E.coli 8 >V. chlorae 6*

12 hours after application

Shigella sp. 4 > *S. typhi 3= S. paratyphi 3 > E.coli 2 =V. chlorae2.*

The level of pathogenicity of against pathogenic bacteria with the five different isolates significantly at 5 % level. (Table 3-5). Highest level of mortality is noticed in the tanks treated with *V. chlorae* with 93.40 %, a least level is recorded 10.35 % with *E.coli*. Similar to experimental trial, a highest level of mortality 85.60 % and 49.99 % is recorded in both duplicate and triplicate respectively (Table.6). Moreover, in all the control tanks the pathogenic bacteria were not added, survival of *A. franciscana* is remains significant and found there is not much mortality (fig1). High naupliar mortalities up to 100% have been observed with 2 strains of *V. parahaemolyticus* and *V. alginolyticus* (Rico-Mora *et al.*, 1995) and 1 strain of *V. Proteolyticus.* In the present study, the maximum mortality observed was 82% with Strain Z2. Such differences have also been found in bath challenges with penaeid shrimps. Variability is possibly connected with shrimp species tested (Vera *et al*.,1992), doses used, time of exposure, age of the shrimp (Jun LI *et al.*, 1998) or pathogenic factors of the strains employed . No correlation was observed between isolation source and percent mortality, and pathogenicity is not guaranteed when strains are isolated from diseased crustaceans. It would be interesting to challenge shrimp larvae with this set of strains, but a reproducible challenge protocol for penaeid larvae is still not available. Results of the present study are closely correlates / supporting the percent mortality and pathogenicity finding of previous researchers.

Table. 3: ANOVA (2 ways) for the differences in density of pathogenic bacteria bioencapsulated with *A.franciscana* mortality in experimental tanks**.**

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	2398.639	5	479.7277	0.985616	< 0.05	5.050329
Columns	13079.88	1	10947.5	25.86647		6.607891
Error	10947.5	5	423.2313			
Total	15150.41	11				

Table. 4: ANOVA (2 ways) for the differences in density of pathogenic bacteria bioencapsulated with *A.franciscana* mortality in duplicate tanks.

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	2086.75	5	417.3501	0.986104	< 0.05	5.050329
Columns	10947.5	1	10947.5	25.86647		6.607891
Error	2116.156	5	423.2313			
Total	15150.41	11				

Table. 5: ANOVA (2 way) for the differences in density of pathogenic bacteria bioencapsulated with *A.franciscana* mortality in triplicate tanks.

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	695.4191	5	139.0838	0.91181	< 0.05	5.050329
Columns	2904.43	1	2904.43	19.04096		6.607891
Error	762.6795	5	152.5359			
Total	4362.528	11				

Overall performance of probiotic organism (Bioremid) namely against *Shigella* sp. was found more effective (46.11 % of mortality) than other pathogenic bacteria. Standard Error (SE) results also showed 2.15, 3.2 and 3.20 in experimental, duplicate and triplicate found significant. Many scientists (Makridis *et al.*, 2000; Roque et al ., 2000) suggested that *A. franciscana* nauplii have a maximum capacity for bioencapsulation (ingestion) of bacterial cells ranging from 10^2 to 10^4 CFU nauplius–1, independent of the bacterial density to which they are exposed (10^6 to 10^8 CFU ml–1) but dependent on the bacterial strain employed.

CONCLUSION

This present study proves that the use of probiotic organism (Bioremid) can reduce the bacterial load of pathogenic bacterial strains viz. *E. coli, S. typhi, S. paratyphi, V. cholerae and Shigella* sp. ingested by *Artemia franciscana* nauplii. The probiotic organism showed more effectiveness against *Shigella sp.* There is a significant control was found with *Shiegella* sp. and a least control of *E.coli* was noticed using commercial probiotics Bioremid against pathogenic bacteria on *A. franciscana* nauplli developed in captivity. A drastic trend was observed immediately 3hours after the application of Bioremid in the experimental tanks that was treated with *Shigella* sp. A mean mortality of 46.11 % was encountered in all the pathogenic bacteria inoculated tanks and a stable trend of microbial colonies found in all the control tanks without addition of either pathogenic bacteria or bioremid applied (Table.2). Pathogenic level is significantly declined from 136, 25, 13 and 4 with *Shigella* sp. with respect to appearance of colonies and identified that this bacterial isolate could be restricted in the aquaculture practices in particular in the commercial hatchery operations while using *A. franciscana* in captive conditions. Probiotic control using Bioremid towards bacterial isolates such as *E. coli* and *Vibrio cholerae* strains are very merely less effective on *A. franciscana.*

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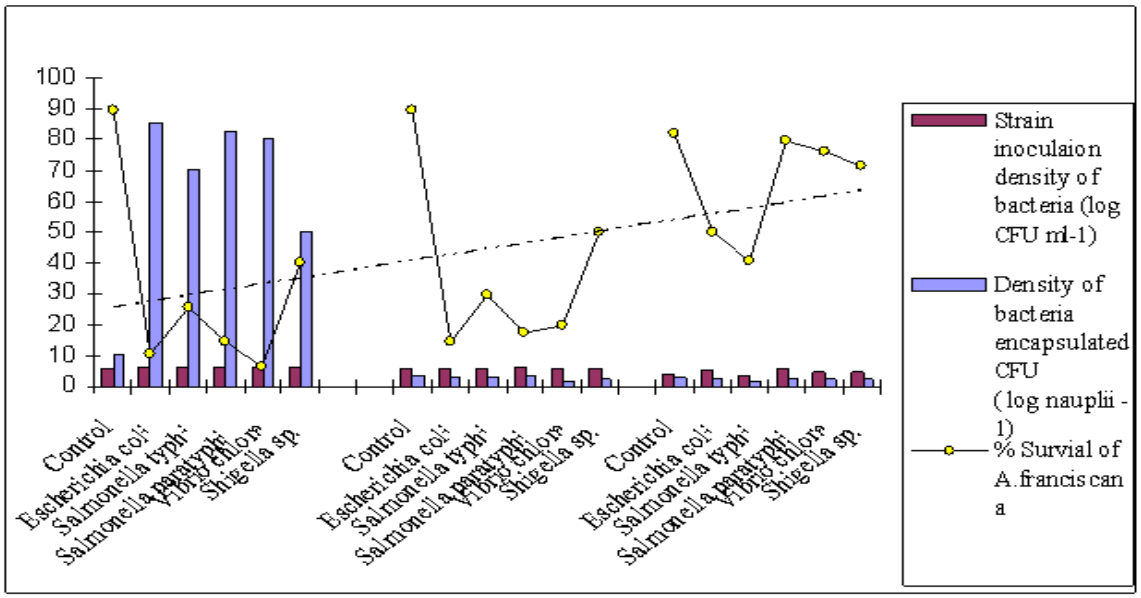

Fig. 1: *A. franciscana* challenge density of different bacteria, number ingested and percent mortality of challenged nauplii in triplicate.

Table.6: *A. franciscana* challenge density of different bacteria, number ingested and percent mortality of challenged nauplii in triplicate.

Different Strains	Strain Inoculation density of bacteria (log CFU ml^{-1})	Density of bacteria Bioencapsulated (log CFU $nauplius^{-1}$)	Mean mortality (%)	SE
Experiment				
Control	6.06	3.10	10.35	2.50
E. coli	6.28	3.03	89.50	5.92
S. typhi	6.37	3.20	74.57	4.90
S. paratyphi	6.24	3.32	85.23	5.54
V. chlorae	6.26	1.97	93.40	5.43
Shigella sp.	6.23	2.25	60.00	3.33
Duplicate				
Control	5.55	3.25	10.20	2.15
E.coli	5.89	3.00	85.60	5.85
S. typhi	6.06	3.12	70.57	4.30
S. paratyphi	6.13	3.29	82.30	5.43
V. chlorae	5.91	1.87	80.43	5.23
Shigella sp.	5.55	2.12	50.00	2.35
Triplicate				
Control	3.92	2.91	18.00	5.34
E.coli	5.43	2.14	49.99	5.43
S. typhi	3.35	1.46	59.20	3.51
S. paratyphi	5.55	2.21	20.50	5.59
V. chlorae	4.90	2.44	24.08	4.63
Shigella sp.	4.67	2.25	28.33	3.20

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