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# Synergistic Effects of Probiotics and Autogenous Bacterin Against Inositol Negative Motile Salmonella Species

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**Abstract**
Salmonellosis, a disease caused by pathogenic strains of *Salmonella*, has been reported to have an essential degree in morbidity and mortality of humans (especially farmers) and poultry resulting in low productivity. This study was undertaken to evaluate the synergistic effects of probiotics and autogenous bacterin against inositol negative motile *Salmonella* species isolated from different poultry feed collected from Orlu-west Local Government Area of Imo State. A total of 40 representative feed samples were aseptically collected and screened for the presence of inositol negative motile *Salmonella* species using pour plate method. The colonies generated from the primary isolation were sub-cultured, characterized and identified using their colony description, morphological and biochemical characteristics. The pathogenic potentials of the organism in broiler chicks were investigated by challenging the chicks orally using 0.5ml of the inoculum ($10^8$cells/ml).The protective effects of locally prepared autogenous bacterin (B), commercially prepared probiotics (P) and autogenous bacterin plus commercially prepared probiotics (BP) were investigated using *in vivo* technique. The study revealed the presence of inositol negative motile *Salmonella* species in the studied samples. There significant ($p<0.05$) pathological features and lesions in the liver and spleen of the infected chicks. The mean plate counts were significantly ($p<0.05$) recorded more in the liver than spleen. The *in vivo* study showed that P, B and BP showed pronounced activity against the tested isolates of which BP proved to be more effective.

## INTRODUCTION

*Salmonella* is a major microbial hazard in animal feed. *Salmonella* can persist for long periods in a wide range of materials. The lack of uniformity involved in *Salmonella* contamination and the large volumes of feed produced make accurate assessments of feed contamination rates difficult. *Salmonella* control principles maybe divided into three broad categories: effort to prevent contamination from entering the facility, work to reduce microbial multiplication within the plant, and procedures designed to kill the pathogen. Preventing contamination also involves controlling dust, managing the flow of equipment and humans, reducing Salmonella multiplication in feed manufacturing facilities involves discovering microbial growth niches and reducing conditions that lead to growth. Killing *Salmonella* may involve thermal processing or recontamination after thermal processing. Chemical additions to control *Salmonella* in feed primarily involve the use of products containing organic acid, formaldehyde, or a combination of such compounds. Use of autogenous bacterin is also another method used to control *Salmonella* infection in broiler chicks (Su and Chiu, 2007).

Antibiotics play very important roles in controlling and treatment of *salmonella* infections, in situation in which antibiotics are needed, ampicillin or amoxicillin are the best choices (Miller and Pegues, 2001). Ceftriaxone, cefotaxime, or flouroquinolones are effective option for antimicrobial resistant strains. Cephalosporin is recommended for animals at high risk of invasive disease. But misuse and frequent use of antibiotics have led to *Salmonella* drug resistant. Autogenous bacterin is also useful in protecting the animals from the infections, it is a killed bacterial vaccine created from the disease causing organism. This vaccine has served as a means of protecting the immune system of the animals against specific infections. It reduces the rate of diseases and death in animals (Sherrill *et al.,* 1999). In areas where there is lack of antibiotics or vaccines, may result in high rate of diseases and mass deaths.

Many researchers have studied different ways of synergistic effects of probiotics and autogenous bacterin against inositol negative motile *Salmonella* species such as Barrow *et al.,* (2007) stated that "Therefore the main form of controlling the presence of *Salmonella sp*ecies in poultry production is related to biosecurity measures and vaccination, associated with the right use of antibiotics, prebiotics and probiotics". Immerseel *et al.,* (2002) stated "because the level of protection offered by live vaccine strains depend on the administration route". In Nigeria, the importance of controlling moulds and mycotoxins in feeds is widely known and practiced, but the control of bacteria is less well understood and frequently overlooked (Maciorowski *et al.,* 2007). Though hygiene program with the use of a long acting chemical treatment on the poultry feed is the only way to minimize spread of the infections. This work has been designed to check the synergistic effects of probiotics and autogenous bacterin against inositol negative *Salmonella* that has been isolated from the chicken feed.

 

## MATERIALS AND METHODS

**Study Area:** The study was done in Mgbidi major market, Mgbidi in Orlu-west L.G.A., Imo State. Mgbidi community shares common borders with Uli town in Anambra State, Awo and Ugwuta in Imo State. Within the location of the market, the major activities are trading, domestic and farm works.

**Sample Collection:** A total of 40 representative samples of different types of poultry feeds were collected from different shops and open markets within Mgbidi Major Market using sterile polythene bags, and kept in priory disinfected cooler. The samples were brought to the laboratory in a cooler maintaining low temperature ($\leq 4^0$C) using ice blocks. The collected samples were processed within six hours of its collection. Sampling was performed normally from different bags such that the product was collected from different parts of the bags. The sample was pooled and mixed properly to form one cup of the feed sample, then 10 g of the mixture was taken for analysis.

**Isolation and Identification of Inositol Negative Motile *Salmonella Specie*s:**

Ten folds serial dilution was carried out on each different samples and 1.0ml was aseptically taken from the third test tube and pour plated into the *Salmonella Shigella* Agar and incubated at 37°C for 48 h. After 48 h incubation, the grown colonies were sub-cultured, characterized and identified using their colony descriptions, microscopic and biochemical characteristics (Iheukwumere and Umedum, 2013).

**Procurement of Chicks:** A total of twenty (20) day old chicks of mixed sex obtained from Ausonic farm at Ihiala, Anambra State were used for this study. The chicks were kept in separate, thoroughly cleaned and disinfected cages and provided with feeds and water frequently.

**Inoculum preparation:** This was carried out using the method of Wafaa *et al*. (2012). Broth culture of the isolate was centrifuged at 3000 r.p.m for 10 minutes. The sediment was diluted with sterile phosphate buffer saline (PBS) and adjusted to the $10^8$CFu/ml using 0.5 McFarland matching Standard (0.6ml of 1% $BaCl_2.2H_20$ + 99.4ml of 1% concentration of $H_2SO_4$).

**Preparation of Autogenous bacterin:** This was carried out by the method of Wafaa *et al.* (2012). The isolate was grown on nutrient broth at 37$^o$C for 24 h. The culture was centrifuged at 3000 r.p.m for ten (10) minutes and the supernatant was decanted. The sediment was washed with normal saline and suspended into 1% formal saline at room temperature for 24 hours. The sterile autogenous bacterin was obtained by adding equal volume of incomplete Freund's adjuvant to adjusted washed concentrate of inactivated bacterium and kept at refrigerator until when used.

**Quality control tests on the prepared Autogenous bacterin**

The prepared autogenous bacterin was tested for purity, complete inactivation and sterility.

- Purity: this test was done before inactivation of the isolate. It was done to confirm that the broth culture of the isolate was not contaminated by other bacteria before inactivation. This was done by sub-culturing the broth culture into Salmonella Shigella Agar and incubated at 37$^o$C for 24 hours. The colony was Gram stained, examined and finally confirmed using unique biochemical reactions.
- Complete inactivation test: This was carried out to ensure that the isolate was completely inactivated. Autogenous bacterin was inoculated into a *Salmonella Shigella* Agar and incubated at 37$^o$C for 48 hours. No visible growth of the isolate was seen.
- Sterility test: the prepared autogenous bacterin was confirmed to be free from any fungal contaminants by inoculating it into Sabouraud Dextrose Agar (SDA) plate and incubated at room temperature for 7 days.

**Experimental Design:** This was carried out using the modified method of Wafaa *et al.,* (2012). The chicks were grouped into five (5) groups which include group A, B, C, D and E. Each group contained four chicks each. The treatments to the group were as follows: Group A was intramuscularly administered autogenous bacterin; 0.2 ml/chick for the first dose and boostered on the 7$^{th}$ day with 0.5ml/chick and then challenged with 0.5ml of test organism after 14 days. Group B were giving commercially produced probiotics (This contained *Lactobacillus acidophilus* and *Streptococcus thermophilus*), this was giving 2 g/L of distilled water for 14 days and then infected with 0.5 ml of test organism. Group C were administered the autogenous bacterin and allowed to feed on commercially prepared probiotics for 14 days, and then infected with 0.5 ml of the test organism. Group D were only infected with 0.5 ml of the test organism without protection and Group E were giving only distilled water. The experimental chicks were carefully monitored for a period of 2 weeks for any obvious pathological signs.

**Detection of the Humoral Immune Response:** Just before the first dose of the autogenous bacterin (zero hour), the chicks were randomly selected and their blood were collected. Also just before the second booster dose, another blood sample was also collected on 14$^{th}$ day. The blood samples were allowed to separate. The separated sera were used against the isolate for agglutination reaction using micro agglutination titre techniques. The serum collected from the chicks was serial diluted using two-fold serial dilution. Then 0.1µL of the diluted serum ($^1/_{20}$, $1/_{40}$, 1/80, $^1/_{160}$ and$^1/_{320}$) was deposited on the wells of the micro filter and aseptically mixed with 0.1µL of the test isolate. This was incubated at 37$^0$C for 90 minutes. The agglutination results and titer value was recorded. This was repeated after 7 days (before booster dose) and 14 days (before challenge) and this is in

 

accordance with the methods of Wafaa *et al.* (2012).

**Examination of Protected Chicks:** The protected chicks were carefully observed for the obvious pathological signs of the administered test organism for period of 2 weeks, the protection rates of the inhibitory substances were determined, and the chicks were sacrificed and gross examination of the morphologies of internal organs and intestine were carried out. Also the internal organs were harvested and some portions of these organs were cultured on *Salmonella Shigella* agar, and incubated at 37$^{o}$C for 48 h. The counts were taken and the colonies were identified morphologically and biochemically (Wafaa *et al.,* 2012).

**Statistical Analysis:** The data generated from this study were represented as mean ±Standard deviation and then charts. The test for significance at 95% confidence interval was carried out using Student 'T' test and chi square (Iheukwumere and Umedum, 2013).

## RESULTS

Inositol negative motile *Salmonella species* was characterized and identified using its morphology, colony description and biochemical reactions (Table 1). The presence of the isolate in the chicken feed samples is shown in Table 2. Out of 40 (100%) chicken feed samples collected from different retailers at Mgbidi major market in Orlu – west Local Government Area of Imo State, 10 (25%) samples were positive to inositol Negative motile *Salmonella species*.

The prevalence of the isolate in the chicken feed samples is shown in Table 3. The study showed that isolate T1 was seen most in feed type A whereas isolate T2 was seen most in feed type B, and isolate T1 was seen more the studied samples.

Micro agglutination antibody titres generated from the sera of broiler chicks after vaccination with prepared autogenous bacterin and probiotics is shown in Table 4. On the first day (Before first vaccination dose), the antibody titre values (ATVs) of sera samples collected from the test and control chicks were zero. On the seventh day (before booster dose), ($^{1}/_{6}$) of the chicks vaccinated with the autogenous bacterin and probiotics had maximum ATVs $^{1}/_{320}$ whereas $^{3}/_{6}$ and $^{2}/_{6}$ of the remaining vaccinated chicks recorded $^{1}/_{80}$ and $^{1}/_{160}$ titre values respectively. On the 14$^{th}$ day (before challenge), $^{4}/_{6}$ and $^{3}/_{6}$ of the remaining vaccinated chicks recorded $^{1}/_{160}$ and $^{1}/_{320}$ respectively. There was no ATV value recorded from non-vaccinated chicks after 14$^{th}$ days. Isolate T2 had triggered more antibody than isolate T1

The obvious pathological signs of the challenged isolate in the infected and protected chicks are shown in Table 5. The chicks infected with the test organism without protection recorded series of obvious pathological signs of the test organism which was significantly ($p<0.05$) reduced in those chicks administered autogenous bacterin, probiotics and bacterin with probiotics. No obvious pathological sign was recorded among the control (non-infected chicks).

The gross pathological leisons of the internal organs of the infected and protected chicks is shown in Table 5. The chicks infected with the test organism without protection recorded series of gross pathological leisons which was significantly ($p<0.05$) reduced in those chicks administered autogenous bacterin, probiotics and bacterin with probiotics. No obvious pathological sign was recorded among the control (non-infected chicks).

The mean organ/body weight of experimental chicks administered autogenous bacterin and probiotics is shown in Table 7. The organ/body weights were more in the liver of the infected chicks. The organ/body weights of the experimental chicks administered autogenous bacterin and probiotics was more in the spleen and less in the liver. The spleen of the infected chicks without protection was not developed so the organ weight was not taken.

The total mean viable plate count of the studied isolates from the internal ortgans of the experimental chicks administered autogenous bacterin and probiotics is shown in Table 8. The counts were more in the liver and less in the spleen of the infected chicks without protection. The counts were significantly reduced in the protected chicks with autogenous bacterin and probiotics.

The protection rate of autogenous bacterin and probiotics against inositol negative motile *Salmonella species* is shown in Table 9. All the infected chicks protected with autogenous bacterin and probiotics were control positive and all of them survived. No death occurred. The experimental chicks was significantly ($p<0.05$) protected.

 

**Table 1: Characteristics and identities of inositol negative motile *Salmonella* species**

| Parameter | $T_1$ | $T_2$ |
|---|---|---|
| Appearance on S.S.A | Colorless with dark centered | Colorless with dark centered |
| Elevation | Slightly raised | slightly raised |
| Size (mm) | 1.50 | 1.30 |
| Motility | + | + |
| Gram Reaction | – | – |
| Cell morphology | Rod | Rod |
| Catalase | + | + |
| Citrate | + | + |
| $H_2S$ | + | + |
| Inositol | – | – |
| Sorbitol | + | + |
| Dulcitol | + | + |
| Xylitol | + | – |

S.S.A ——— Salmonella shigella agar, $H_2S$ Hydrogen sulphide

**Table 2: Presence of the isolate in chicken feed samples**

| Types of feed | Positive (%) | Negative (%) | Total (%) |
|---|---|---|---|
| A | 3 (40) | 7 (60) | 10 (25) |
| B | 2 (10) | 8 (70) | 10 (25) |
| C | 2 (20) | 8 (80) | 10 (25) |
| D | 3 (30) | 7 (70) | 10 (25) |
| Total | 10 (25) | 30 (75) | 40 (100) |

A ___ Sunchi feed, B Extra feed, C Top feed, D Vital feed

**Table 3: Prevalence of the isolates in the chicken feed samples**

| Feed Types | Isolate $T_1$ (%) | Isolate $T_2$ (%) | Total (%) |
|---|---|---|---|
| A | 3 (30) | 0 (0) | 3 (30) |
| B | 0 (0) | 2 (20) | 2 (20) |
| C | 1 (10) | 1 (10) | 2 (20) |
| D | 2 (20) | 1 (10) | 3 (30) |
| Total | 6 (60) | 4 (40) | 10 (100) |

A ___ Sunchi feed, B Extra feed, C Top feed, D Vital feed

 

**Table 4: Micro –agglutination antibody titres in the sera of the broiler chicks protected with autogenousbacterin and probiotics**

| Isolate | Day | Interval | Total | Antibody titres of the chicks serum at different dilutions | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 20 | 40 | 80 | 160 | 320 |
| $T_1$ | 0 | BFVD | 6 | 0 | 0 | 0 | 0 | 0 |
| | 7 | BBVD | 6 | 0 | 1 | 3 | 2 | 0 |
| | 14 | BC | 6 | 0 | 0 | 0 | 4 | 2 |
| $T_2$ | 0 | BFVD | 6 | 0 | 0 | 0 | 0 | 0 |
| | 7 | BBVD | 6 | 0 | 0 | 0 | 2 | 4 |
| | 14 | BC | 6 | 0 | 0 | 0 | 3 | 3 |
| Control | 0 | BFVD | 6 | 0 | 0 | 0 | 0 | 0 |
| | 7 | BBVD | 6 | 0 | 0 | 0 | 0 | 0 |
| | 14 | BC | 6 | 0 | 0 | 0 | 0 | 0 |

BFVD- Before First Vaccination Dose, BBVD- Before Booster Vaccination Dose, BC- Before Challenge

**Table 5: Obvious pathological signs of challenged isolates in the infected and protected chicks**

| Pathological signs | N = 4 | | | | |
|---|---|---|---|---|---|
| | B | P | BP | $C_1$ | $C_2$ |
| Diarrhea | 0 | 0 | 0 | 3 | 0 |
| Respiratory distress | 0 | 1 | 0 | 3 | 0 |
| Weakness | 0 | 1 | 0 | 3 | 0 |
| Anorexia | 0 | 0 | 0 | 1 | 0 |
| Dysentery | 0 | 0 | 0 | 2 | 0 |
| Alopecia | 0 | 0 | 0 | 0 | 0 |
| Death | 0 | 0 | 0 | 0 | 0 |

N ▬ Total number of chicks, B Bacterin vaccination, $C_1$ Infected chicks without protection
$C_2$ ▬ Normal chicks, P Protection with probiotics, BP Protection with bacterin and probiotics

**Table 6: Gross pathological lesions of the internal organs of the infected and protected chicks**

| Gross lesion | N = 4 | | | | |
|---|---|---|---|---|---|
| | B | P | BP | $C_1$ | $C_2$ |
| Liver Oedema | 0 | 1 | 0 | 3 | 0 |
| Liver Haemorrhage | 0 | 0 | 0 | 3 | 0 |
| Perihepatitis | 0 | 1 | 0 | 3 | 0 |
| Congestion | 1 | 2 | 1 | 3 | 0 |
| FAI | 1 | 0 | 1 | 3 | 0 |
| Enterocolitis | 0 | 0 | 0 | 2 | 0 |
| Spleen deformation | 0 | 0 | 0 | ND | 0 |

N = Total number of chicks, B = Bacterin vaccinated chicks, $C_1$ = Infected chicks without protection
$C_2$ = Normal chicks, ND = Not developed, FAI = Fluid accumulation in the intestine P=Protection with probiotics
BP=Protection with bacterin and probiotics

**Table 7: Mean organ/body weights of the experimental chicks**

| O/B | L/B | S/B |
|---|---|---|
| B | 0.02 ±0.01 | 1.10±0.01 |
| P | 0.03±0.01 | 1.30±0.01 |
| BP | 0.02±0.01 | I.01±0.01 |
| $C_1$ | 0.04±0.02 | ND |
| $C_2$ | 0.02±0.01 | 1.00±0.02 |

O/B=Organ/Body weight, L/B=Liver/Body weight, S/B=Spleen/ Body weight, B=Bacterin protection before infection, P=Probiotics protection before infection, BP=Bacterin and probiotics protection before infection, $C_1$=Infection without protection, $C_2$=Normal chicks, ND=Not developed

 

**Table 8: Total mean viable plate counts of the studied isolates from the internal organs of infected nd protected chicks**

| Protection | Spleen (CFU/g) | Liver (CFU/g) |
|---|---|---|
| B | 7.00±5.70 | 11.00±9.86 |
| P | 11.00±0.00 | 14.00±0.00 |
| BP | 0.00±0.00 | 0.00±0.00 |
| $C_1$ | 19.00±7.30 | 25.00±6.50 |
| $C_2$ | 0.00±0.00 | 0.00±0.00 |

B=Bacterin vaccinated chicks, P=Probiotics protected chicks, BP=Bacterin and probiotics protected chicks
$C_1$=Infected chicks without protection, $C_2$=Normal chicks

**Table 9: Protection rate of autogenous bacterin and probiotics against inositol negative motile *Salmonella* Species**

| Protection | N | D | M (%) | S | P (%) |
|---|---|---|---|---|---|
| B | 4 | 0 | 0 | 4 | 100 |
| P | 4 | 0 | 0 | 4 | 100 |
| BP | 4 | 0 | 0 | 4 | 100 |
| $C_1$ | 4 | 0 | 0 | 4 | $0^d$ |
| $C_2$ | 4 | 0 | 0 | 4 | $100^a$ |

B=Bacterin vaccinated chicks, P=Probiotics protected chicks, BP=Bacterin and probiotics protected chicks
$C_1$=Infected chicks without protection, $C_2$=Normal chicks, N=Total number of chicks, D=Number of death
M=Mortality rate, S=Number survived, P=Protection rate, $100^a$=No protection, $0^d$=Control positive

## DISCUSSION

The presence of inositol negative (Ino$^-$) motile *Salmonella* species in the chicken feed samples could be linked to the management practices of the farm houses, dust, feed ingredients, as well as transportation of the feeds, poor handling and sanitary conditions attributed to the feed samples. Similar discoveries were reported by many researchers (Immerseel *et al.,* 2002; Jones and Richardson, 2004; Alshawabkeh, 2006; Maciorowski *et al.,* 2007).

The maximum titre value attained by inositol negative (Ino$^-$) motile *Salmonella* species bacterin was $^{1}/_{320}$ respectively while there was no antibody titre recorded from non-vaccinated chicks after the 14$^{th}$ day. Similar reports was given by other researchers that there is enhancement of immune response against inositol negative (Ino$^-$) motile *Salmonella* species infected chicks through vaccination using locally prepared autogenous bacterin (Davies and Breslin, 2004; Okamura *et al.,* 2004).

The *in vivo* study was carried out to determine the protection rate of locally prepared autogenous bacterin (B), commercially prepared probiotics (P) and locally prepared autogenous bacterin plus commercially prepared probiotics (BP). The absence of growth observed in the internal organs administered BP supports the findings of Wafaa *et al.* (2012). Several other researchers have documented that the frequency of (Ino$^-$) *Salmonella* species re-isolated from the internal organs was significantly reduced in the protected chickens (Khan *et al.,* 2003; Radwan, 2007). The significant decrease in TMPCs showed by the internal organs from those chickens administered BP corrorated to the findings of other researchers (Barbour *et al.,* 2003; Wafaa *et al.,* 2012). The competitive exclusion mechanism exhibited by probiotics against the pathogen (Ino$^-$) *Salmonella* species was comprehensively studied by several researchers. From this result, it was reported that probiotics maintained or increased the normal intestinal flora which are normally found in the intestinal tracks of hatched chicken and these flora can exclude *Salmonella* species colonization (Mead, 2000; Wafaa *et al.,* 2012). The absence of visible growth of *Salmonella* species observed in non- infected (normal) day- old chicks supports the finding of Magdelena *et al.* (2011), who reported that during the first 3 days of life, chicken was protected from incoming antigens by increased expression of $\beta$-defensins (gallinacins 1,2,4 and 6), which made the chicks germ-free.

The maximum protection achieved by vaccinating those chicks fed with diet supplemented with commercially prepared probiotics could be attributed to the synergistic effects of the two substances. The bacterin activated and boosted the humoral and cellular components of immune response (Wafaa *et al.,* 2012) whereas the probiotics produced lactic acid that created unfavourable $P^H$ for the growth of the *Salmonella* species pathogens (Alkoms *et al.,* 2000; Johasen *et al.,* 2004). The probiotics also compete with the pathogens (Wafaa *et al.,* 2012) and produced bacteriocin that was toxic to the enteric bacteria (Pascual *et al.,* 2009). The positive effect of feeding diet containing probiotic on the immune response indicates the enhancement of the formulating bacteria on the acquired immune response exerted by T and B lymphocytes. The direct effect might be related to the stimulation of lymphatic tissue, whereas the indirect effect may occur via changing the microbial population of the lumen of gastrointestinal tract or through the reduction of *Salmonella* species pathogen colonization. Shoeib *et al.* (2007) reported that the bursa of probiotic treated chickens showed an

 

increase in the number of follicles with high plasma cell reaction in the medulla. Christensen *et al.* (2002) suggested that some of these effects were mediated by cytokines secreted by immune cells stimulated with vaccination and probiotic bacteria. On the other hand, vaccinating chickens fed with diet supplemented with probiotics has beneficial effects for chicks, particularly during the first days of life.

## CONCLUSION

This study has shown the presence of inositol negative (Ino$^{-}$) *Salmonella* species in the chicken feed samples collected from different shops at Mgbidi major market in Oru-West Local Government Area of Imo State. The *in vivo* study of the susceptibility pattern of this organism to the used antimicrobial agents, showed safe and pronounced activities with locally prepared autogenousbacterin (B) and commercially prepared probiotics (P), but locally prepared autogenous bacterin plus commercially prepared probiotics (BP) proved to be more effective.

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