Research Article

Combination of a Probiotic Mix and Diminazene Aceturate in Treatment of *Trypanosoma brucei* Infection in Sprague Dawley Rats

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Abstract | Panacean trypanocidal therapy remains elusive. The objective was to investigate how a probiotic mix affects treatment outcome in trypanosomosis. Thirty-six rats randomly assigned to six groups (A--F) were used for the experiment. Supplementation with multi-strain probiotics was started in groups A--C from day 0 post-supplementation (PS). On day 7 PS all experimental groups (A--E) were challenged with $1x10^6$ trypanosomes intraperitoneally except the uninfected control (F). When parasitaemia peaked, groups A and B received $7mgKg^{-1}$ and $3.5mgKg^{-1}$ of diminazene aceturate (DA) respectively alongside ongoing probiotics supplementation. Group C received only probiotics. Group D received $3.5mgKg^{-1}$DA only. Group E (infected control) received no treatment. Parasitaemia, haematobiochemical, and oxidative stress markers were determined. At day 13 PS, there were no significant ($p< 0.05$) variations in mean parasitaemia of groups A, B and D. At day 16 PS, the parasites had cleared from the peripheral blood of rats in groups A and B, but remained detectable in group D. Although group D had significantly higher total erythrocyte count and haemoglobin concentration compared to groups A--C, and E, no significant variation was seen in the total and differential leucocyte counts across all infected groups. By day 23 PS, groups A and B had similar serum alanine and aspartate aminotransferases, blood urea nitrogen, and serum creatinine levels as group D. The serum malondialdehyde and catalase levels were similar in groups A--D. Therefore, treatment with the probiotics, while enhancing clearance of trypanosomes did not improve the antioxidative and clinical outcome of the infection.

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Introduction

African Animal Trypanosomosis (AAT) is a group of diseases of domestic animals caused by *Trypanosoma* spp. Trypanosomosis remains a major setback to animal health and the livestock industry in sub-Saharan Africa (Yaro et al., 2016; Ishaku et al., 2019). Anaemia, leucopenia, serum biochemical derangements and oxidative damages are important pathologies in AAT (Akpa et al., 2008; Reddy et al., 2016; Eze et al., 2016; Nweze et al., 2017). Of the three major trypanocides in common use, homidium and

isometamidium are commonly used for prophylaxis, while diminazene aceturate is commonly used for therapeutic purposes (Giordani et al., 2016). Available trypanocides are over four decades old, and the problem of drug resistance is widespread (Delespaux et al., 2008; Mungunbe et al., 2012; Rathore et al., 2016); hence, emphasis is laid on fine and optimum use of the few available trypanocides (Giordani et al., 2016; Rathore et al., 2016).

Probiotics have been applied in management of several non-gut diseases. In mice suffering malaria, the administration of probiotics *Bifidobacterium* spp and *Lactobacillus* spp significantly reduced the *Plasmodium* burden (Villarino et al., 2016). Eze et al. (2012) reported improvement of immunosuppression and reduced parasitaemia in *Trypanosoma brucei* infected rats treated with the probiotic *Saccharomyces cerevisiae.* Several workers have documented the antioxidant, and serum biochemistry-normalizing effects of some probiotic strains (Lutgendorff et al., 2008; Majlesi et al., 2017). Several reports (Anukam et al., 2006; Truusalu et al., 2008; Venugopalan et al., 2010; Travers et al., 2011; Shukla et al., 2013; Choi et al., 2015) suggest that probiotics can complement classic chemotherapy available for some disease conditions. Therefore, we investigated the effect of a multi-strain probiotics alongside conventional trypanocidal therapy on recovery of *Trypanosoma brucei* infected rats from parasitaemia, anaemia, biochemical derangement, and oxidative stress.

Materials and Methods

Experimental Design and Experimental Animals

A randomized controlled experimental design was used for the study. Thirty (36) adult male Sprague Dawley rats weighing between 240 and 264 grams were used for the study. They were acclimatized for two weeks and then randomly assigned into six (6) experimental groups A, B, C, D, E and F, each having six (6) rats. They were housed in fly proof cages and fed proprietary rat feed and water *ad libitum*. Ethical considerations for the use of experimental animals were based on the procedures of the Animal Use and Care Committee of the Faculty of Veterinary Medicine University of Nigeria, which agrees with the NIH guidelines (NIH, 2011).

Probiotics and Trypanosomes

The live multi-strain probiotics mix (CHR®Netherlands) was used. There were approximately 25x10^9 CFU of organisms per gram of the freeze dried culture. The mix contains the following five strains of probiotics organisms in equal proportions: *Bifidobacterium BB-12, Lactobacillus acidophilus LA-5, Lactobacillus delbrueckii LBY-27, Lactobacillus paracasei LC-01,* and *Streptococcus thermophilus STY-31*. The *Trypanosoma brucei* used in this study was isolated from a dog presented at the Veterinary Teaching Hospital, University of Nigeria Nsukka, and clinically diagnosed of trypanosomosis. The isolated trypanosome was identified at the Department of Veterinary Parasitology and Entomology, University of Nigeria.

Supplementation and Treatment

Supplementation with multi-strain probiotics (MP) in indicated groups started from day 0 and continued for 7 days before the rats were challenged with trypanosomes and till the end of the experiment. MP was delivered to rats in indicated groups as a suspension in 1ml of distilled water administered through gastric gavages. Treatment with diminazene aceturate (DA) (Pantec®, Netherlands) was given intraperitoneally on day 12 post supplementation (PS), by which time, the infected groups had peak parasitaemia. The treatments received by the rats according to their groups were as follows:

Group A: Infected+ 5x 10^9 CFU MP + Diminazene Aceturate (DA) 7mg/Kg
Group B: Infected+ 5x 10^9 CFU MP + DA 3.5mg/kg
Group C: Infected+ 5x 10^9 CFU MP only
Group D: Infected+ DA 3.5mg/kg only
Group E: Infected+ Untreated
Group F: Uninfected+ Untreated

Infection with Trypanosoma brucei

On day 7 post supplementation, groups A, B, C, D and E were infected intraperitoneally with approximately 1x 10^6 *Trypanosoma brucei* suspended in phosphate buffered saline (PBS).

Assays

Wet mount blood preparations from the tip of the tail of rats were prepared and used for detection and estimation of parasitaemia by the rapid matching technique (Herbert and Lumsden, 1976) starting from 48 hours post infection in experimental animals. The treated rats were monitored for relapse infection using the same technique for up to sixty days (60)

Table 1: *Mean parasitaemia (x 10^6 Trypanosomes/ml of Blood) of rats infected with Trypanosoma brucei and treated with multistrain probiotics and diminazene aceturate.*

Day post Supplementation	11	13	16	17	23
A	441.10 ± 109.63	22.25 ± 13.63^{a†}	0.25 ± 0.00^{**a}	0.25 ± 0.00^{a}	0.25 ± 0.00^{a}
B	141.52 ± 39.44	21.7 ± 1.92^{a†}	0.25± 0.00^{a}	0.25 ± 0.00^{a}	0.25 ± 0.00^{a}
C	457.75 ± 219.85	306.23 ± 167.13^{b†}	688.10 ±187.16^{b}	750.60 ± 143.99^{b}	***
D	204.59 ± 143.61	20.88 ± 14.81^{a†}	4.09 ± 2.22^{a}	0.25± 0.00^{a}	0.25 ± 0.00^{a}
E	46.44 ± 26.49	157.22 ± 31.33^{ab†}	481.92 ± 137.31^{b}	544.42 ± 114.66^{b}	***
P value	0.143	0.059	< 0.001	< 0.001	0.008

A: *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. Different superscripts (a, b, c) flag statistically significant ($p < 0.05$) variations down the groups. †= LSD Post hoc multiple comparison. ** = parasitaemia levels ≤ 0.25 X10^6represents clearance from peripheral circulation (Herbert and Lumsden, 1976). *** = surviving group members were euthanized.*

post treatment. About 1.5 ml of blood was collected from the retro-bulbar plexus of the median canthus of the eyes of rats. Out of this, 1ml of blood was collected into plain Eppendorf tubes, allowed to clot, and centrifuged at 3000 rpm for five minutes to separate the serum for biochemical assays, while the remaining 0.5ml of blood was collected into sample bottles treated with sodium EDTA for haematological studies. The activities of alanine and aspartate aminotransferases as well as the levels of blood urea nitrogen, and creatinine on days 16 and 23 post supplementation (corresponding to days 4 and 11 post treatment respectively) were determined spectrophotometrically using commercial kits (Randox®, United Kingdom). day 4 post treatment, serum catalase (CAT) activities were determined by the method of Sinha (1972), as modified by Hadwan (2016), while serum malondialdehyde (MDA) concentrations were determined by method described by Stocks and Dormandy (1971) and modified by Sicinska et al. (2017). The total and differential leucocyte counts, haemoglobin concentration and packed cell volumes were determined on days 16 and 23 post supplementation, using standard methods (Coles, 1986; Thrall and Weiser, 2002).

Data Analysis

Data obtained were analysed using SPSS version 20 by the application of analysis of variance (ANOVA) statistics (Fisher, 1952). Means were separated using the least significant difference (LSD) at post hoc. Significance was accepted at $p < 0.05$. The results were presented as means ± standard errors of means using table and charts.

Results and Discussion

Parasitaemia

On day one post treatment with DA (day 13 PS), there was a sharp fall in mean parasitaemia across all infected groups except the infected untreated (group E), and infected supplemented control (group C) which had significantly higher ($p < 0.05$) parasitaemia when compared to other infected groups (Table 1). By day 16 PS (day 4 post treatment), trypanosomes had cleared from peripheral circulation in groups A and B, and then in group D (infected + DA 3.5 only) at day 17 PS (day 5 post treatment). No relapse of infection was noted over a period of sixty (60) days in groups A, B, and D. Following treatment of rats in groups A, B and D, and with continued increase in the parasitaemia of the untreated controls, they were humanely sacrificed on day 23 PS.

Haematological indices

At day 16 PS, all infected groups had significantly lower ($p < 0.05$) mean total erythrocyte counts (TEC), and haemoglobin concentrations (HBC) when compared to group D and the uninfected control (Figures 1 and 2). At day 23 PS (day 11 post treatment) groups A, B, and D had similar TEC and HBC while groups C and E (infected controls) had significantly lower values. At day 4 PT, there were no significant variations ($p > 0.05$) in mean total leucocyte counts (TLC) across all infected groups A, B, C, D, and E; however, the uninfected control (group F) had significantly higher ($p < 0.05$) values when compared to other groups (Figure 3). By day 23 PS, a general increase in the mean total white blood cell counts was seen in nearly all treated groups. Groups A and B (infected

+ DA and probiotics) had similar total leucocyte counts with group D (infected + DA only) which were significantly higher ($p < 0.05$) than values in the infected control. The mean absolute lymphocyte and neutrophil counts followed similar patterns as described for the TLC (Figures 4 and 5).

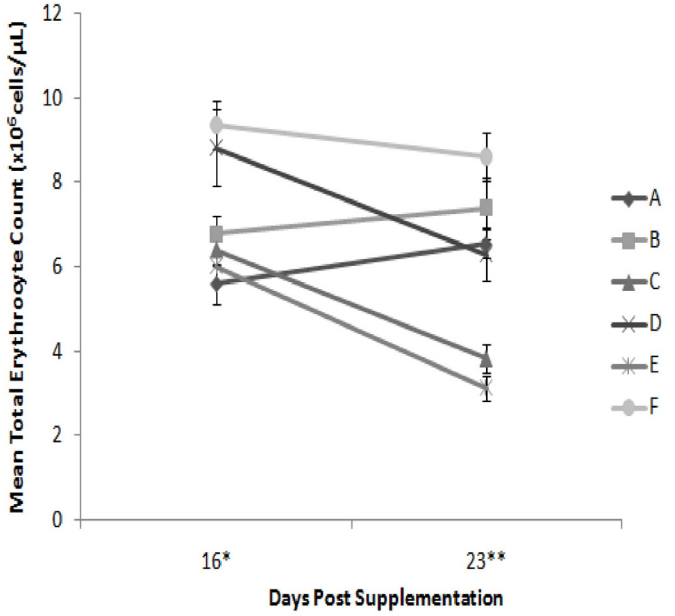

Figure 1: *Mean total erythrocyte counts (x10⁶cells/µL) of rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

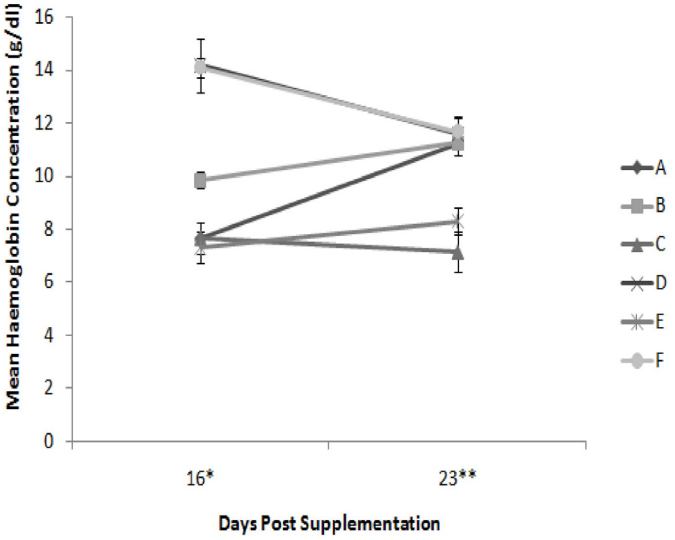

Figure 2: *Mean haemoglobin concentration (g/dl) of rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

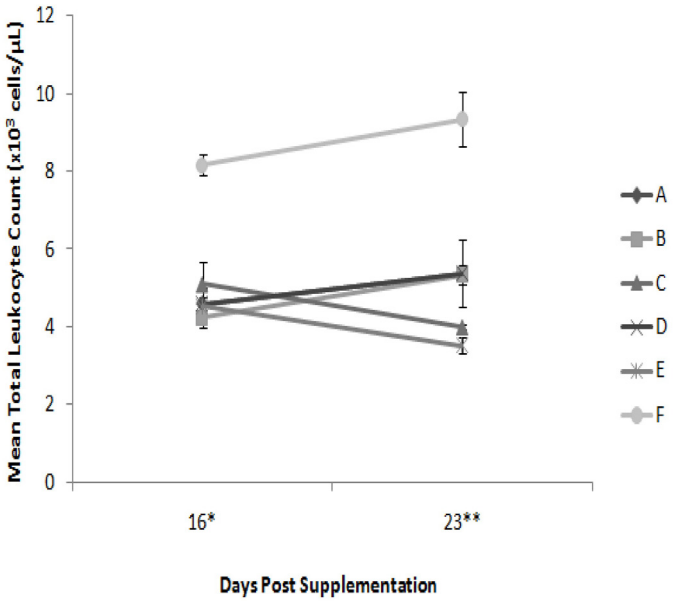

Figure 3: *Mean total leucocyte count (x10³ cells/µL) of rats infected with Trypanosoma brucei and treated with Multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

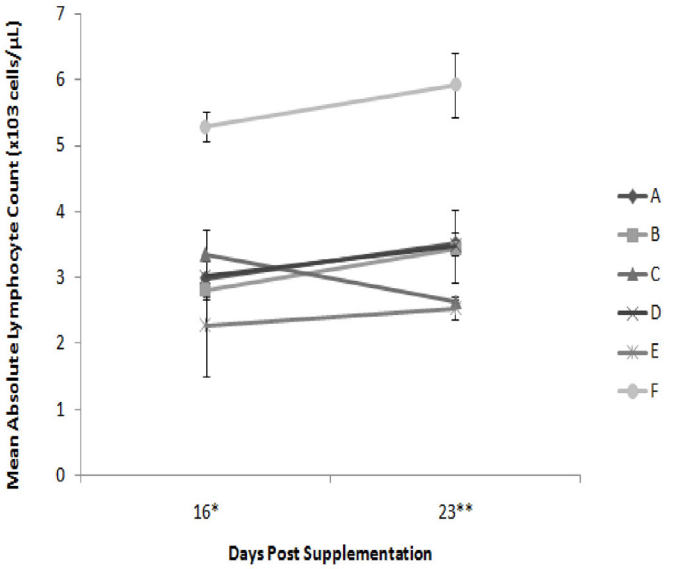

Figure 4: *Mean absolute lymphocyte counts (x10³ cells/µL) of rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

Serum biochemical indices and Oxidative Stress Markers

Despite the overall decline in the blood urea levels of all the experimental rats at day 23 PS, there was no significant difference ($p > 0.05$) in the levels of this parameter between the treatment groups (Figure 6). The serum creatinine concentrations were

similar across all the experimental groups by day 16 PS whereas by day 23 PS, groups C and E had significantly higher ($p < 0.05$) values (Figure 7). On day 16 PS, group C (infected + MP only) and group E (infected untreated) had significantly higher ($p < 0.05$) mean serum AST and ALT activities compared to all other experimental groups (Figures 8 and 9). By day 23 PS, group E (infected untreated) had a significantly higher ($p < 0.05$) mean serum AST and ALT activities compared to all other experimental groups. There was a significantly higher ($p < 0.05$) concentration of MDA in all the infected rats when compared to the uninfected control (Figure 10). The infected control (group E) had the least mean serum catalase activity which was significantly lower ($p < 0.05$) than values in group B (infected + MP + DA3.5) (Figure 11).

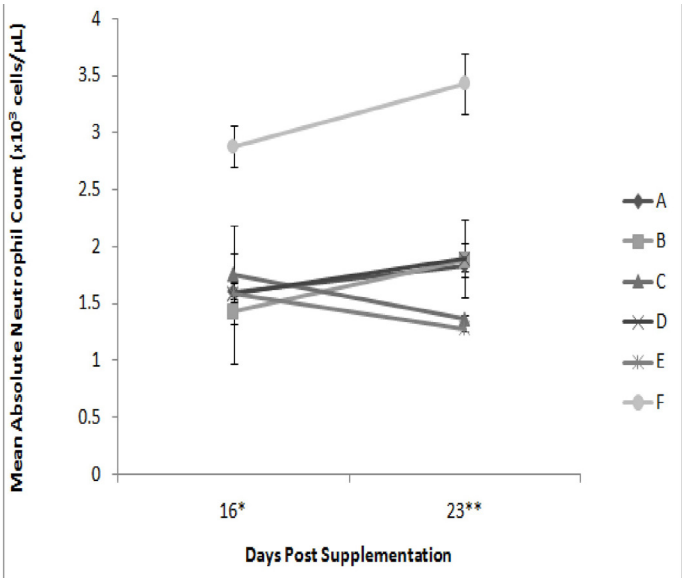

Figure 5: *Mean absolute neutrophils Counts (x10³ cells/µL) of rats infected with Trypanosoma brucei and treated with Multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

Infection of rats with *Trypanosoma brucei* was successful and the infected rats were parasitaemic as a result, reaching peak parasitaemia on day 12 post infection. Consequently, the rats were treated with diminazene aceturate at peak parasitaemia. The infection was gradual in onset in group E (infected control) but parasitaemia rose in the group till day 23 post supplementation when group C and the infected control animals were euthanized. At day 1 following treatment with diminazene aceturate, corresponding to day 13 post supplementation, the levels of parasitaemia fell to levels below 2.5×10^6 trypanosomes/ml of blood in all the treated groups with or without probiotics. These findings, together with the noted absence of relapse of infection following clearance of parasites in the blood, indicate that the DA used was a reasonably potent brand inspite of reports on the emergence of drug resistant trypanosomes. Similar claims on the effectiveness of DA have been reported elsewhere (Peregrine et al., 1991; Giordani et al., 2016).

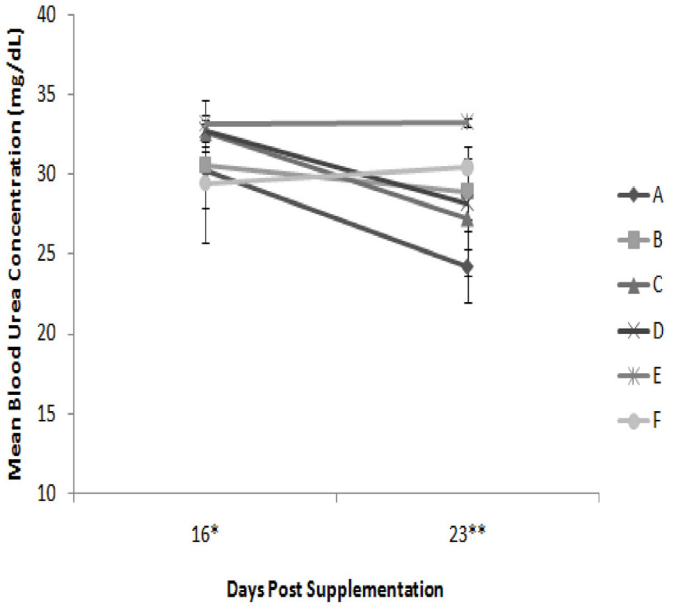

Figure 6: *Mean blood urea concentration (mg/dL) of rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

The earlier clearance of parasites from the blood in groups A and B at day 16 post supplementation (day 4 post treatment) than in group D where parasites cleared at day 17 post supplementation (day 5 post treatment) shows that the multi-strain probiotics mix may have enhanced the effectiveness of the drug. It may be argued however that the difference of one day may not be statistically significant (Greenland et al., 2016), but this may pose to be a very positive clinical outcome in cases of naturally infected hosts. Subject to Fisher's ANOVA (Fisher, 1952), there were no significant variations between the groups under comparison above; however, it is strongly thought that this finding may be of clinical significance. The level of parasitaemia which continued to increase in groups C, (received only probiotics), and the infected control (group E), clearly suggests that the probiotic strains,

on their own, were of no significant therapeutic value in experimental trypanosomosis but may serve as useful adjunct to conventional chemotherapy against trypanosomsosis.

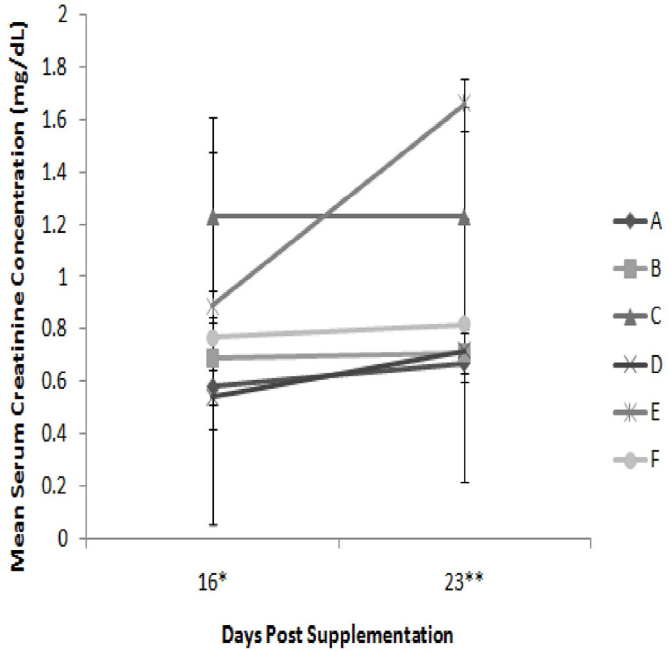

Figure 7: *Mean serum creatinine concentration (mg/dL) of rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

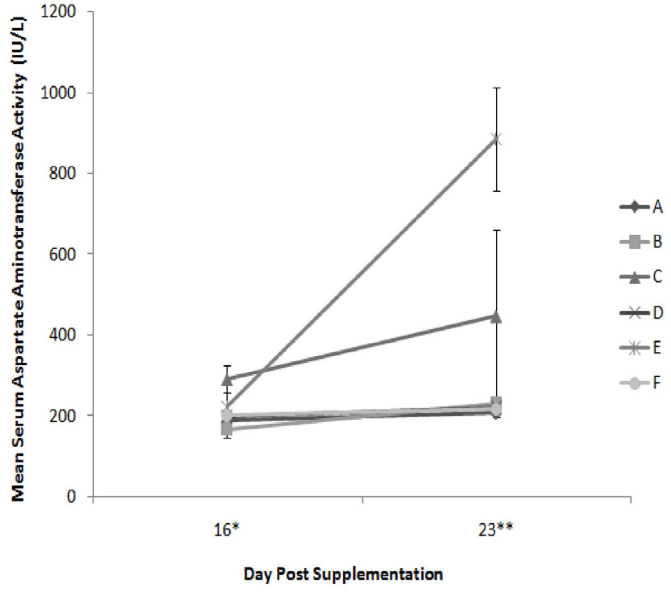

Figure 8: *Mean serum aspartate aminotransferase (AST) (IU/L) of rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

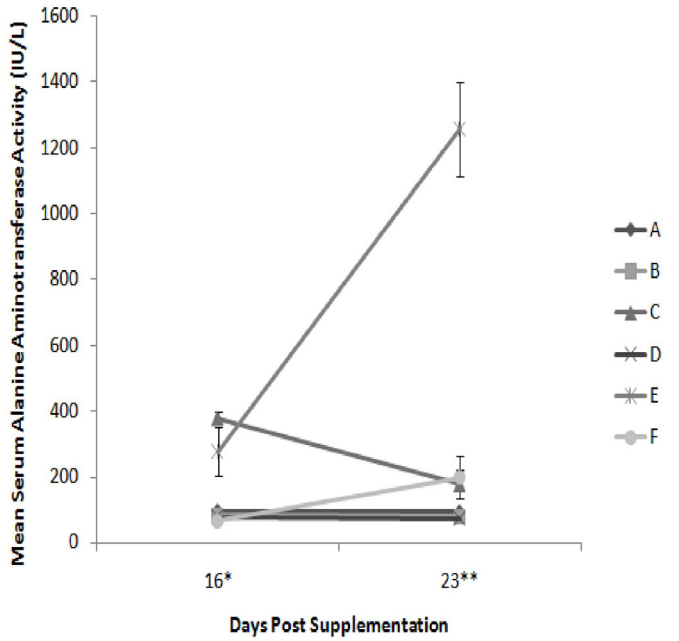

Figure 9: *Mean serum alanine aminotransferase (ALT) (IU/L) activity of rats infected with Trypanosoma brucei and treated with multistrain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated. *corresponds to day 4 post treatment; **corresponds to day 11 post treatment.*

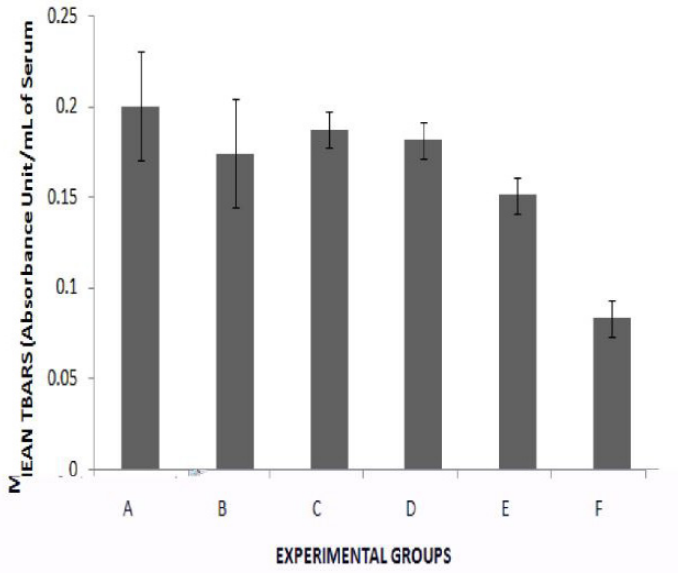

Figure 10: *Mean serum malondialdehyde (MDA) concentration on Day 16 post supplementation (day 4 post treatment) in Rats infected with Trypanosoma brucei and treated with multi-strain Probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated; F: Uninfected + untreated.*

At day 16 post supplementation (day 4 post treatment), the total erythrocyte counts and haemoglobin concentration of group D (DA only) were significantly higher than those of groups A

and B (probiotics and DA); however, at day 23 post supplementation (day 11 post treatment), groups A, B and D, had made full recovery from anaemia, and their mean erythrocyte counts and haemoglobin concentrations were comparable to those of the uninfected control. Furthermore, the group treated with only DA showed an early recovery from trypanosomosis induced anaemia when compared to groups treated with combination of probiotics and DA. At day 16 PS, both the groups treated with DA only and with probiotics and DA had similar total leucocyte counts (TLC) that continued to increase through day 23 PS. This showed a gradual recovery from the initial leucopoenia induced by trypanosomosis. This finding agrees with previous reports that treatment with trypanocides, improves the overall picture of the leucocytes within few days in trypanosomosis (Anosa, 1988). Remarkably, the TLC remained significantly lower in the group treated with only probiotics as well as in the infected control. Anaemia and leucopenia, as identified and reported in this study, have been noted as cardinal features of African animal trypanosomosis (Reddy et al., 2016; Nweze et al., 2017; Onyiliagha et al., 2018). The results indicate that the use of the multi-strain probiotic mix did not improve the haematological indices in trypanosomosis. Earlier reports have similarly shown that treatment with certain probiotic strains did not result in significant variations of haematological parameters of broilers (Alkhalf et al., 2010) and rats (Anukam et al., 2004).

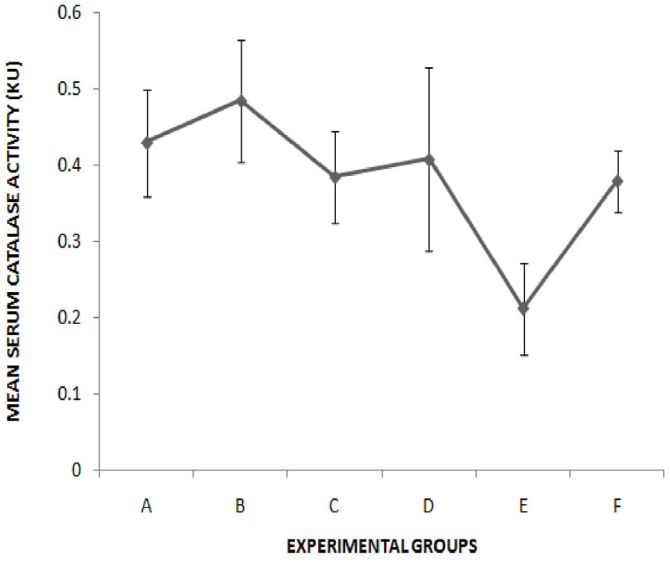

Figure 11: *Mean serum catalase activity on day 16 post supplementation (day 4 post treatment) in rats infected with Trypanosoma brucei and treated with multi-strain probiotics and diminazene aceturate.* **A:** *Infected + Probiotics + 7mg/Kg Diminazene;* **B:** *Infected + Probiotics + 3.5mg/Kg Diminazene;* **C:** *Infected + Probiotics only;* **D:** *Infected + Diminazene 3.5mg/kg only;* **E:** *Infected + untreated;* **F:** *Uninfected + untreated.*

Careful interpretation of serum biochemical parameters of patients are of essential diagnostic and prognostic importance (Bush, 1991). Generally, at day 16 PS groups A and B which received probiotics and diminazene, had similar serum ALT, AST, BUN and creatinine levels as group D which received diminazene only; and these values were lower than levels seen in the infected control. At day 23 PS the results showed a continued biochemical derangement in the experimental group treated with only probiotics (group C), and the infected and control (group E). These findings indicate that combination of the multistrain probiotics mix and diminazene did not improve the clinical pathological picture in terms of serum ALT, AST, BUN, and creatinine during early stages of recovery from trypanosomosis.

Oxidative injuries are important in the pathogenesis of trypanosomosis, and the administration of antioxidant substances can ameliorate the pathology of AAT (Umar et al., 2010; Eghianruwa and Anika, 2011; Eze et al., 2016). At day 16 post supplementation (day 4 post treatment), all the infected groups had significantly higher mean serum malondialdehyde (MDA) concentration than the uninfected control. Increase in serum MDA following *Trypanosoma* spp infection were similarly reported in rats (Eze et al., 2016) and camel (Saleh et al., 2009). Groups that received probiotics and DA had comparable mean MDA concentration with the group that received only diminazene aceturate. These findings indicate that oxidative injury persisted even for few days following of trypanocidal therapy, and treatment with the multistrain probiotic mix in this study did not necessarily prevent infection nor hasten recovery from trypanosomosis-induced lipid peroxidation. Although some strains of lactic acid producing bacteria have long been shown to possess antioxidant abilities (Lutgendorff et al., 2008), the effects are generally strain specific. The mean serum catalase activity of group B (infected + Probiotics + DA3.5) was comparable to the values obtained in all other experimental groups except the infected control which had significantly lower catalase activity probably due to active parasitaemia that persisted in the group. This agrees with reports that following the establishment of trypanosomosis, most antioxidant enzymes such as superoxide dismutase, catalase, and glutathione show reduced activity (Saleh et al., 2009; Eze et al., 2016). Between the probiotics treated groups and group that received diminazene aceturate only, there

were no significant variations in their mean catalase activities; hence, the probiotic strains did not improve the antioxidative capacity of trypanosomic rats.

Conclusions and Recommendations

In conclusion, despite the enhanced early clearance of blood stream trypanosomes following DA treatment, the mutistrain probiotics mix treatment did not improve the clinical pathological picture with respect to serum ALT, AST, BUN, creatinine levels, haematological indices, or the antioxidative capacity of rats infected with trypanosomosis. It is known that the bio-activities of probiotics are strain specific (Travers et al., 2011; Gogineni et al., 2013; Majlesi et al., 2017); the use of multistrain probiotic mix may be re-evaluated in further studies.

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Author's Contributions

Chukwuemekeka Calistus Okolo: Design, collection of data, data analysis and drafting of manuscript; Ikenna Onyemah Ezeh: Design, collection of data, data analysis and review of manuscript; Chinelo Nnenna Uju: Design, collection of data, and review of manuscript; Nwakaego Ernestina Nweze: Design, collection of data, and review of manuscript.

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