

EFFECT OF *LACTOBACILLUS ACIDOPHILUS* ON *TRICHINELLA SPIRALIS* MUSCLE LARVAE IN EXPERIMENTALLY INFECTED MICE COMPARED TO ITS EFFECT WHEN COMBINED WITH ALBENDAZOLE AND/OR NITAZOXANIDE

By

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Abstract

This study evaluated the therapeutic and synergistic effects of *Lactobacillus acidophilus* (*L. acidophilus*), a probiotic, and nitazoxanide (NTZ) as compared to the used, albendazole (ABZ), as single therapy and in combination. ABZ/*L. acidophilus*-treated group showed the highest reduction of encysted larvae (92.5%) as compared to control and to ABZ-treated mice. Also, NTZ treatment showed a significant decrease in larval counts compared to control (40.95%), which was comparable to that produced by *L. acidophilus* single therapy. But, combined NTZ with *L. acidophilus* caused a significantly higher reduction of larvae (56.62%) highlighted synergistic abilities of both. Histopathological examination showed marked decrease in larval counts and inflammatory reactions in ABZ/*L. acidophilus*-treated group. Combined *L. acidophilus* with other therapeutics caused increased eosinophilia degeneration and larval replacement.

Key words: *Trichinella spiralis* larvae, *Lactobacillus acidophilus*, Nitazoxanide, Albendazole.

Introduction

Trichinellosis is a zoonotic nematode parasitic disease caused mainly by *Trichinella spiralis* represents a worldwide threat, despite intensive control efforts (Pozio, 2018), as a zoonotic foodborne parasitosis (Wang *et al*, 2020). In Egypt and other Arab Countries, human trichinellosis was rare, and stems mainly from religious practices and food habits (Youssef and Uga, 2014). Sylvatic trichinellosis is prevalent in the Mediterranean regions, and, domestic trichinellosis was reported in Egypt in slaughtered pigs (Morsy *et al*, 2000), and naturally infected rats (Morsy *et al*, 1989), rodents collected from and around abattoirs in Alexandria with a rate of 13.3% (Loutfy *et al*, 1999) and diaphragms of 2 stray dogs killed during a governmental anti-rabies campaign in Cairo (Mikhail *et al*, 1994).

Signs, symptoms, severity and duration of trichinellosis varied. Nausea, diarrhea, vomiting, fatigue, fever, and abdominal discomfort are often the first symptoms. Headaches, fevers, chills, cough, swelling of the face and eyes, aching joints and muscle pains, itchy skin, diarrhea, or constipation may

follow the first symptoms. If infection is heavy, patients may experience difficulty coordinating movements, and have heart and breathing problems. In severe cases, death can occur. For mild to moderate infections, most symptoms subside within a few months. Fatigue, weakness, muscle pain, and diarrhea may last for month. When a human or animal eats meat with infective *Trichinella* larvae, the acid in stomach dissolves the hard covering of the cyst around the larvae and releases the worms, which pass into the small intestine and, in 1-2 days, become mature. After mating, adult females lay eggs. Eggs develop into immature worms, travel via the arteries, and are transported to muscles, in the muscles, the worms curl into a ball and encyst; where they form intact capsules by the 30th day of infection (Othman *et al*, 2016). During muscular phase, larvae induced an inflammation responsible for the associating myositis (Bruschi and Chiumiento, 2011), and elicited a robust immunological and allergic response (Bucková *et al*, 2015).

Some of these infected myocytes develop into nurse cells that nourish the larval stage and shelter it from the immunological react-

ions of the host (Ock *et al*, 2013). To reach this target, the parasite induces new vascular formations (angiogenesis) around collagenous capsule (Park *et al*, 2015).

Albendazole (ABZ) is currently a preferred therapeutic agent for treatment of infection with *T. spiralis*. It is a benzimidazole derivative that works against intestinal and systemic parasite on a broad scale (Horton, 2002; Casulli *et al*, 2006). However, its effectiveness is hampered by its lower activity against encapsulated larvae, limited water solubility, the increase in developing resistance and being contraindicated in children and pregnant women (Yadav and Temjenmongla, 2012).

On considering the available drugs, their side effects and possible resistance development, a shortage in the addition of new drugs is obvious, mostly due to weak funding (Soliman *et al*, 2016). Repositioning already approved drugs, as an alternative, seems a more feasible approach (Panic *et al*, 2014).

Nitazoxanide (NTZ) is a broad-spectrum thiazolide compound that was initially used as an antiprotozoal and antibacterial agent (Gilles and Hoffman, 2002), but it was frequently explored for its antiviral and anti-helminthic potentials (Hemphill *et al*, 2006). NTZ proved effectiveness against some zoonotic nematodes as *Ascaris*, *Ancylostoma*, *Enterobius* and *Strongyloides* (Fonseca-Salamanca *et al*, 2003), with a higher efficacy against *T. spiralis* muscle larvae than adults (Ashour *et al*, 2016).

Probiotics are living microorganisms that have beneficial effects on human health (Hill *et al*, 2014) with proven safety and approved efficacy criteria (Gibson and Fuller, 2000). Probiotic bacteria anti-parasitic properties increasingly approached during years (El Temsahy *et al*, 2015). Probiotics have lowered the parasite burden and the related pathological alterations in experimental trichinellosis through the stimulation of local and systemic immune reactions (Bautista-Garfías *et al*, 1999, 2001; Martínez-Gómez *et al*, 2009, 2011; El Temsahy *et al*, 2015;

Dvorožňáková *et al*, 2016). Probiotics induce a non-specific stimulation of the immune system (Costamagna, 2005) with activation of various mediators as interleukin-2, interferon-gamma and nitric oxide production (Kato *et al*, 1999). Bacterial products as lactic acid, acetic acid, and others played vital role against parasites (Buckova *et al*, 2018).

This study aimed to assess the effects of ABZ, NTZ and a probiotic, *Lactobacillus acidophilus* (*L. acidophilus*) on *Trichinella spiralis* encapsulated muscle larvae, in experimentally infected mice, given alone or combined with *Lactobacillus* (ABZ/*L. acidophilus*) and (NTZ /*L. acidophilus*) by parasitic burden and histopathological changes in treated mice.

Materials and Methods

Experimental animals: A total of seventy laboratory-bred male Swiss albino mice, 8-week old and weighing approximately 22gm were used. They were obtained from and maintained at Theodore Bilharz Research Institute (TBRI) animal house in Giza Governorate. All animal handling and experimental procedures were conducted according to the national and institutional guidelines and ethics for the care and use of laboratory animals. They were kept under 12hrs light/dark regime and adjusted temperature and humidity conditions. They received standard pelleted diet and water *ad libitum*, and dwelt in well-ventilated, regularly cleaned customized housing-boxes.

Trichinella spiralis: *T. spiralis* isolate was maintained by serial passages in laboratory mice, with released from muscles by pepsin-hydrochloric acid (HCL) artificial digestion. After 12hrs fasting, each mouse was infected orally with 250 larvae in 250µl saline administered using an especially adapted tuberculin syringe with a blunt, ball-tipped, curved needle (Wassom *et al*, 1984).

Drugs: Albendazole (Alzental) suspension as 20mg/ml (EIPICO) was administered *per os* in a dose of 50mg/kg/day for 3 consecutive days; 35th day post-infection (Fahmy and Diab, 2021). Nitazoxanide (Nanazoxid) as

100mg/5ml suspension, product of Medizen, Utopia Pharmaceuticals, Egypt, was given at a daily oral dose of 50mg/kg for 14 consecutive days (Ashour *et al*, 2016). Probiotic bacteria; *Lactobacillus acidophilus* (*L. acidophilus*) Natrol LLC, United States were administered orally at a dosage of 10^9 CFU/ml/100 μ l daily for 5 consecutive days (Bucková *et al*, 2018).

Experimental design: Seventy clean laboratory bred male mice were divided into seven groups of 10 mice each and were infected with 250 *T. spiralis* larvae orally. They were G1: Negative control group (neither-infected nor treated); G2: Positive control (infected with *T. spiralis* larvae, non-treated); G3: ABZ-treated with 50 mg/kg/day for 3 consecutive days; G4: NTZ-treated with nitazoxanide 50mg/kg/day for 14 consecutive days; G5: *L. acidophilus*-treated with *L. acidophilus* 10^9 CFU/ml/100 μ l daily for 5 consecutive days; G6: ABZ/*L. acidophilus*-treated with albendazole 50mg/kg/day for 3 consecutive days + *L. acidophilus* 10^9 CFU/ml/100 μ l daily for 5 consecutive days; G7: NTZ/*L. acidophilus*-treated with nitazoxanide 50mg/kg/day for 14 consecutive days + *L. acidophilus* 10^9 CFU/ml/100 μ l daily for 5 consecutive days. Treatment started on 35th day P.I. and sacrificed on the 50th day.

Isolation of *T. spiralis* muscle larvae: After scarification, each mouse was dissected and muscle mass was digested with a mixture of 1% pepsin & 1% HCL in 1000ml distilled water and incubated at 37°C (Martinez-Gomez *et al*, 2009). Incubation was maintained for 2hrs with stirring on an electric stirrer. Digested mixture was strained through a sieve (50mesh/inch) to separate any coarse particles and re-strained using a sieve (200-mesh/inch) to collect larvae, which were washed with phosphate-buffered saline (PBS). This step was repeated to remove any acid residues, and then 200ml of PBS was added to re-suspend to collect larvae in a conical flask. After settling, the supernatant was discarded and larvae/ml in sediment was counted by conventional light mi-

croscope, using a hemocytometer (Denham, 1965).

Histopathological studies: Skeletal muscle samples were fixed in 10% neutral buffered-formalin and processed for paraffin embedding, sectioning at 5 μ m thickness and stained with Hematoxylin and Eosin (H&E). Ten low power microscopic fields (10x) were examined and scored for larval number as follows; score 1= < 5larvae/field, score 2= 5-10 larvae/field and score 3 =>10larvae/ field. Inflammatory reaction intensity was scored as mild = 1, moderate = 2 and intense reaction = 3 (Othman *et al*, 2016).

Statistical methods: Data were coded and analyzed using the Statistical Package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA), as mean and standard deviation. Comparisons between groups were done using ANOVA with multiple comparisons post hoc test for normally distributed quantitative variables while non-parametric Kruskal-Wallis test and Mann-Whitney test were used for abnormally distributed quantitative variables (Chan, 2003). P-values less than 0.05 were considered statistically significant.

Results

Trichinella larvae in muscles: All treated groups showed a significant reduction in *T. spiralis* muscle larvae load as compared to the infected non-treated control ones ($P < 0.001$) The NTZ and *L. acidophilus*-treated groups showed a significant decrease in muscle larvae numbers compared to infected control group ($P < 0.001$). ABZ alone displayed a high significant in number of larvae as compared to either drug alone ($P < 0.001$).

ABZ /*L. acidophilus* combination showed marked decrease in parasitic load ($P < 0.001$) with highest reduction of muscle larvae (92.5%) followed by ABZ (78.26%), NTZ/*L. acidophilus* combination (56.62%) and then NTZ and *L. acidophilus* alone (40.95%) and (40.76%) respectively.

Histopathological results: Skeletal muscle in positive control showed many encysted larvae diffusely embedded in muscles sarco-

plasm, with intense inflammatory reactions in form of lymphocytes, plasma cells and histiocytes infiltrating infected muscle fibers and pericapsular area of encysted larvae.

ABZ-treated group showed decreased encysted larvae numbers with degeneration and thinning of collagen capsule and decreased lymphocytic cellular infiltration. ABZ/ *L. acidophilus*-treated group showed a marked improvement with reduction in encysted larvae numbers, and destruction of collagen capsule, internal structure of larvae, and calcification of some degenerated parts. Fibrosis with chronic cellular infiltrates was in few skeletal muscles as a form of muscular healing, replacing degenerated and necrosis cyst.

In NTZ-treated mice, mild inflammatory

Table 1: Mean, standard deviation & reduction % of *T. spiralis* encysted muscle larvae in positive control and treated groups.

Encysted larvae load in muscles	Positive control	ABZ	ABZ & <i>L. acidophilus</i>	NTZ	NTZ & <i>L. acidophilus</i>	<i>L. acidophilus</i>	p-value
Mean	10720.00	2330.00*	800.00*	6330.00*	4650.00*	6350.00*	<0.001
SD	858.68	371.33	235.70	434.74	568.14	419.66	
Reduction		78.26%	92.5%	40.95%	56.62%	40.76%	

ABZ= Albendazole, NTZ= Nitazoxanide, *Significance at P < 0.05 compared to positive control

Discussion

None of the available classic treatments for human infection with *Trichinella* can be looked upon as a fully effective therapy (Patra and Sarkar, 2014). Benzimidazoles, especially ABZ and mebendazole were considered more effective if administered early in infection as they are lethal to adult worms and non-settled larvae but they have lower potency against encapsulated larvae in man (Pozio *et al*, 2001; Gottstein *et al*, 2009).

On the other hand, vaccine development is a costly, tedious process, slowed by the parasite's complicated antigenicity (Yang *et al*, 2010). With no effective management for late larval stages, emerged the demand to investigate effective alternatives (Bautista-Garfias *et al*, 2002).

In the present study, combination of *L. acidophilus* with drugs resulted in an interestingly significant decrease in encysted larval load in muscles that was highest with the ABZ/*L. acidophilus* combination (92.5%) & with NTZ/*L. acidophilus* (56.62%) compared with control group. It also showed a sig-

cellular infiltrates were around intact collagen capsule with degeneration of encysted larvae. Capsule thinning was in NTZ/*L. acidophilus*-treated ones with fragmentation of encysted larvae and pericapsular lymphocytic cellular infiltration. Viable larvae with moderate inflammatory reaction was in *L. acidophilus*-treated mice.

Score of encysted larval number and inflammatory intensity significantly decreased in all groups as compared to control. Reduction was more in ABZ/*L. acidophilus*-treated mice with improvement degree in ABZ treated mice and NTZ/*L. acidophilus*-treated as compared to NTZ &/or *L. acidophilus* alone. Details were given in table (1) and figures (1, 2, 3 & 4)

nificant difference compared to the observed decrease with single drug administration of either ABZ (78.26%) or NTZ (40.95%) therapy. On the same accord, a similar study by Fathy *et al*. (2021), documented that combined probiotics with ivermectin for treatment of *Trichinella* in mice resulted in an 88.1% reduction in larval load compared to probiotics (38.2%), and ivermectin (79.6%) separately. This suggested that *L. acidophilus* use was beneficial in drug combinations therapies. In addition, this effect of oral bacterial intake on distant tissue larval stages indicates an immune response modulation.

In agreement, recent studies as to the regulation of host immune response as a promising approach to control *T. spiralis* infection in man (Wang *et al*, 2020). Actually, during the last decade, probiotics were studied as a non-chemical therapeutic for the treatment of various intestinal and extra-intestinal pathogens. They have a direct beneficial effect on the intestinal environment. Other studies pointed to their immune modulation abilities and the possible potency of

some of their secreted active molecules that can alter the parasites' pathogenicity (Travers *et al.*, 2011). Probiotics immunomodulatory effects were not limited to the intestinal zone but extend to affect distant organs (Frederich *et al.*, 2017). *Lactobacilli* are reported to promote T-cell proliferation in muscle phase of *T. spiralis* infection (Dvorožňáková *et al.*, 2016). Moreover, probiotics have a stimulatory effect on polymorphonuclear leukocytes (PMNL) respiratory burst activity and can increase phagocytosis. But, this may be maintained for only 2 to 3 weeks after parasitic infection (Dvorožňáková *et al.*, 2019).

In the current study, *L. acidophilus* was administered orally for 5 days starting on the 35th day P.I, to study its effect on encysted larvae in muscle tissue. On scarification, a significant decrease in larval load compared to control group was observed (40.76%); though it was not as high as in the mentioned combinations. This agreed with Bautista-Garfías *et al.* (1999) who recorded that *Lactobacillus casei* induced larval load reduction starting from 46.6% and also Fathy *et al.* (2021) who found a comparable reduction of 38%.

On the contrary, oral administration of *L. acidophilus* was reported by El Temsahy *et al.* (2015) to produce a 60% decrease in larval load on the 28th day of infection. Also, Farrag *et al.* (2021) reported a 73.5% larval reduction on oral administration of *L. acidophilus*. However, both studies administrated the treatment a week before infection, thus assessing *L. acidophilus* effect as a prophylactic rather than a therapeutic agent. Their higher effect may be attributed to affecting adult worms' survival or fertility, and destroying newborn larvae, rather than a direct action on the encysted larval stage.

In the current study, NTZ against *T. spiralis* caused decrease in muscle larvae load (40.95%) compared to control group. In the same context, Ashour *et al.* (2016) and Hassan *et al.* (2021) reported encysted larval count decrease after the NTZ therapy with a reduction 76.7% and 73.7% respectively.

Results were reported against other nematode larvae like *Toxocara canis* *in vivo* with reduction 61.2% (Delgado *et al.*, 2008), even cestodal larvae like hydatid protoscolices showed reduced motility *in vitro* (Soliman *et al.*, 2016). But, neither Fonseca-Salamanca *et al.* (2003), nor Speich *et al.* (2012) reported NTZ activity against pre-adult stages of *T. spiralis* *in vitro*, or on *Trichuris trichura* in children.

In the present study, anthelmintic activity of NTZ (40.95% reduction) was exceeded by that of ABZ (78.26%) as compared to controls. However, Reuter *et al.* (2006) reported a faster effect of NTZ on multilocular hydatid cysts. Deep tissue larvae need high drug bioavailability which is limited by poor absorption of benzimidazoles (Petri, 2011). Also, NTZ administration needed a repeated dosing, through a long period of time, to give an effective anthelmintic threshold in blood (Hu *et al.*, 2013; Ashour *et al.*, 2016). Thus, in the current study, NTZ was administered for two weeks.

In the present study, NTZ showed a significantly higher efficacy when combined with probiotics compared to single administration, 56.62% versus 40.95% reduction respectively. This agreed with the NTZ synergistic activity in combination with other anthelmintic drugs like ABZ, pyrantel[®] (Somvanshi *et al.*, 2014) and ivermectin[®] (Hassan *et al.*, 2021).

The present histopathological treated mice showed a significant decrease in encysted larvae and inflammatory reactions compared to control. ABZ/*L. acidophilus* treated mice showed the least number of muscle larvae with minimal inflammatory cellular infiltrates. This agreed with Fahmy and Diab (2021), who reported that lymphocytic and fibroblastic cellular infiltrations were signs of improvement and elimination of degenerated encysted larvae, by albendazole/mefloquine group. *Lactobacillus* has a marked effect on the vitality of larvae, and contribute to their demise, especially by hydrogen peroxide reduction in larval load (Buckova *et*

al, 2018).

Also, NTZ-treated group showed larval fragmentation with a significant decrease in their numbers and moderate inflammation compared to control group. Lower inflammatory reactions and larval counts were seen with NTZ/*L. acidophilus* treated group with thinning of the cyst capsule and eosinophilic material deposition. This agreed with Ashour *et al.* (2016) and Hassan *et al.* (2021) reported similar NTZ results with encysted larvae (late infection). The latter also acknowledged a better reduction in larvae, inflammation and infiltration of destroyed capsule using NTZ-drug combination with ivermectin. This also advocated that NTZ synergistic effect with other anthelmintic drugs.

In the current study, marked degenerative changes; larval obliteration and intracapsular eosinophilic amorphous material deposition were more noticed on using drug combinations rather than single therapy. This might indicate a synergistic potency of probiotics-drug combinations reported as histopathological changes rather than a parasitic load decrease (unviable encysted larvae). Fathy *et al.* (2021) found that probiotics caused fair histopathological improvement, but, including them in a combined therapy lead to a significantly higher decrease of larvae and inflammatory reactions, with recognizable replacement of larvae with homogenous degenerative material.

Conclusion

Generally, trichinosis is serious zoonotic infectious parasite particularly in pigs rearing countries

L. acidophilus significantly improved the albendazole treatment of *T. spiralis* encysted larvae. Also, *L. acidophilus* and NTZ combination showed synergistic effect in treating encysted larvae. *L. acidophilus* has anthelmintic action particularly when combined with albendazole

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Explanation of figures

- Fig.1: Comparison of *T. spiralis* muscle larvae load in positive control and treated groups. *significant compared to positive control group.
- Fig.2: Skeletal muscle H&E-stained sections: (A) Negative control group showed normal skeletal muscles fibers, (B) Infected untreated (positive control) group showed numerous *T. spiralis* encysted larvae embedded in skeletal muscle with intense inflammatory cellular infiltration (asterisk), each encyst larva consisted of a nurse cell (1), with thick collagen capsule (2) and intersected muscle larvae inside (3), (C) ABZ-treated group showing pericapsular histo-lymphocytic cellular infiltration (black arrow) with degenerated capsule of encysted larva (red arrow) in-between necrosed muscles, (D) ABZ/*L.acidophilus*-treated group showed thinning of cyst collagen capsule (arrow) containing eosinophilic amorphous debris with calcification of some parts (asterisk), (E) NTZ-treated group showed fragmentation of encysted larva (asterisk) with intact capsule and mild pericapsular histo-lymphocytic cellular infiltrates, (F) NTZ/*L.acidophilus*-treated group showed thin walled cyst containing eosinophilic degraded larva (arrow) with minimal cellular inflammation, (G) Viable larva (arrow) with moderate inflammatory cells infiltration (asterisk) in group treated with *L. acidophilus*.
- Fig.3: Lesion score of encysted larvae numbers in different groups (M \pm SD). *Significant at p -value<0.05.compared to positive control group

Fig.4: Lesion score of inflammatory reaction in different groups (M±SD). *Significant p -value<0.05, compared to positive control group.



