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ANTIPARASITIC AND IMMUNOLOGICAL EFFECTS OF SPIRULINA OR PROBIOTICS LOADED ON METAL ORGANIC FRAMEWORK (MOFS) NANO PARTICLES IN MICE INFECTED WITH *CRYPTOSPORIDIUM*

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Abstract

One of the zoonotic health problem affecting is cryptosporidiosis. There is currently no approved treatment for cryptosporidiosis, apart from nitazoxanide (NTZ). This study evaluated the anti-protozoan and immune-modulating properties role of spirulina and probiotics, either alone or loaded on Cu-MOF, against *Cryptosporidium* in immunocompromised mice compared to NTZ. Seventy-eight male laboratory-bred pathogen-free, Swiss-albino mice were divided into seven groups each of 12 mice except negative control one with six mice as follows: GI: infected-untreated (positive control), GII: infected and Spirulina treated, GIII: infected and probiotics treated, GIV: infected and NTZ treated, GV: infected and Spirulina loaded on Cu-BTC MOF treated, GVI: infected and probiotics loaded on Cu-BTC MOF treated, GVII: neither infected nor treated (negative control). Drug efficacy was parasitological, biochemical and histopathological evaluated.

The results showed that all drugs significantly reduced oocysts' count and intestinal parasite burden as well as improved pathological squeals compared to corresponding infected mice. There was a decrease in amyloid and neopterin sera levels in treated mice compared to infected ones. NTZ gave the best treated results, followed by Spirulina nano formulation.

Keywords: Cryptosporidiosis, Mice, NTZ, Cu-BTC MOF, SAA, Neopterin.

Introduction

Cryptosporidiosis caused by *Cryptosporidium* sp. (Order: Eucoccidiorida), is a protozoan parasite infects all mammals (including man), reptiles, birds, and fish (El-Bahnasawy *et al,* 2018). Infection causes respiratory and gastrointestinal illness associated with risky diarrhea mainly among children, immunocompromised and malnourished people (Ryan *et al*, 2016). Cryptosporidiosis diarrheal severity global varied depending on species or subtypes and host's age, malnutrition, and/or absorption and immunity (Shirley *et al*, 2012). Cryptosporidiosis is one of the four main causes of linear growth faltering and second cause of watery diarrhea in children (Nasrin *et al*, 2021). Also, infection may be risky or fatal for persons with poor immunity, as organ transplant recipients (Ahmed *et al*, 2024), or HIV/AIDS ones (O'Connor *et al*, 2011). Infection is acquired from an infected person or animal or from a fecal contaminated food or water source as swimm ng pools or recreational facilities (CDC, 2008), scertain *Cryptosporidium* strains are chlorine-resistant (Walker, 2018).

Drugs as single, or combined ones were used to treat cryptosporidiosis, but with the variable efficacy (Sparks *et al*, 2015).

Nitazoxanide® (NTZ), is the FDA approved drug, showed low efficacy in the immunocompromised patients, such as those with HIV/AIDS and malnourished children (Checkley *et al*, 2015).

Spirulina is microalgae generally used as a dietary supplement with high nutritional value (Nicoletti, 2016). Besides, it has a medical usage, such as antioxidant, antiviral, and

anticancer (Pugh *et al*, 2015), anti-inflammatory (Ramez *et al*, 2021), and anti-protozoa effect (Al-Shuwaili *et al*, 2023). Also, it is safe without side effects even for long-term use (Chow and Deng, 2010).

Probiotics are live microorganisms provide health benefit to hosts when given in adequate amounts (Hill *et al*, 2014). Lactobacilli are the most common used ones (Mombelli and Gismondo, 2000). Probiotics reduce pathogenicity, and stop causative agents' progression (Berrili *et al*, 2012). A good probiotic strain must be safe, resists to low pH and acids, so as to persist in intestine, and to adhere to epithelium (Gupta and Garg, 2009). Also, Khalifa, (2016) reported that probiotic definite role in *Cryptosporidium* reduction of shedding in infected mice. In nanomedicine, it was necessary to select a best particle for delivery mechanism to avoid medication's adverse effects (Abaza, 2016). Reason for choosing metal-organic framework type (MOF) is its high surface area, good stability in a water, its biocompatible components (iron and terephthalic acid) and low toxic to normal cells (Alavijeh and Akhbari, 2020). Adding bioactive molecules to pores, MOFs were functionalized as a drug delivery platform due to their special properties that prevented them from quick degrading (Horcajada *et al*, 2012). One kind of used MOF is copper-benzene tricarboxylic acid (Cu-BTC) composed of two Cu2+ ions joined by four carboxylate groups of BTC ligands (Soltani and Akhbari, 2022). Cu-BTC has outstand-surface-scattering effects, a high drug capacity load, and strong biocompatibility (Hang *et al*, 2021). Also, Cu-BTC combined with herbal extracts proved effective in chronic toxoplasmosis (Mohammad *et al*, 2023)

This study aimed to evaluate the anti-*Cryptosporidium* and immune-modulating properties of spirulina and probiotics, alone or loaded on Cu-MOF, against immunocompromised mice as compared to NTZ.

Materials and Methods

Experimental animals: Seventy-eight male laboratory-bred pathogen-free, Swiss-Albino mice, purchased from the Biology Supply Center at Theodor Bilharz Research Institute (TBRI), of 20-25g each and aged 6-8 weeks, were housed in a controlled room (22±2°C), allowed standard diet and water supplies.

Ethical consideration: The study protocol was approved by the Institutional Animal Care & Use Committee (IACUC), Menoufia University, No. MULI/F/Pa/ 1/24.

Parasite: Feces of naturally infected calves (1-2months old) with modified Ziehl-Neelsen (MZN) stained smears and microscopically examined to prove cryptosporidiosis. The positive samples were centrifuged 3 times at 1500rpm for 10 minutes in PBS, sieved via a stainless steel sieve, and materials in 2.5% Potassium dichromate were kept at 4ºC.

Mice infection: Each mouse was given 10^5 oocysts orally, by using a 25-gauge needle with a plastic tube at tip for gastric gavage.

Immunosuppression: Mice were orally immunosuppressed by synthetic dexamethasone (Dexazone 0.5mg, Kahira Pharmaceuticals and Chemical Industries Co., Cairo) by using an esophageal tube as 0.2µg/g/day for 14 days before infection, and maintained during the study.

Experimental design: All immunocompromised mice were divided into seven groups of each 12 mice each, but negative control was only six. GI: Mice infected, untreated as positive control, GII: Mice infected and spirulina treated, GIII: Mice infected and probiotics treated, GIV: Mice infected and NTZ treated, GV: Mice infected and spirulina loaded on Cu-BTC MOF treated, GVI: Mice infected and probiotics loaded on Cu-BTC MOF treated, & GVII: Mice neither infected nor treated as negative control.

Drugs were given 15th day post infection (dpi) for ten consecutive days.

Drugs: 1- Nitazoxanide (Cryptonaz® 60ml suspension of 100mg/5ml) purchased (Copad Pharma, Egypt for Trade & Pharmaceutical industries, Obour City, Cairo), and mice were given NTZ orally as 100m/ kg.

2- Spirulina: (800mg tablet), purchased from Now Foods Company, USA. Each tablet

was dissolved in distilled water and given to each mouse orally as 200mg/kg/day.

3- Probiotics: A human-derived probiotic in commercial form was supplied by Linex hard capsule (Sandoz, Novartis Group). Each capsule powder was dissolved in distilled water, and given orally as 15mg/kg/ day.

4- Copper-benzene tricarboxylate metal organic framework (Cu-BTC MOF): 100ml of N, N-dimethylformamide (DMF, 98.8%, Sigma-Aldrich) were used to dissolve benzoene-1, 3, 5-tricarboxylic acid (BTC, 0.5g, 1.38mmol) and copper dinitrate trihydrate (Cu (II) (NO3)2, 0.5g, 2.53mmol). Mixture was heated in an open air for 3hrs at 60°C and 12hrs at 100°C to evaporate DMF. Residue was put in 50ml cold water, blue powder was filtered and dried at room temperature.

Spirulina and probiotics loaded on Cu-BTC MOF: Concentrations of both (ranged from 100 - 1000ppm) were dissolved in 100 ml of ethanol. A gram of Cu-BTC nanoparticles was added to the solution; mixture was kept undisturbed overnight, and then stirred by a magnetic stirrer at 600rpm for 90 minutes at room temperature. Suspension was centrifuged for five minutes at 5000rpm to separate the precipitate. Concentration of probiotics and spirulina before and after loading calculated loaded oil in supernatant. The following formula was used (El-Shafey *et al*, 2020) to determine loaded drug percent: A-B/A×100 (A= drug started concentration & B= final concentration). Spirulina and probiotics loaded on Cu-BTC MOF were given orally to mice as 200mg/kg & 15mg/kg daily respectively. All mice were sacrificed 10 days after treatment.

Blood samples by a cardiac puncture was withdrawn, sera were separated by centrifugation and kept at -80°C till needed to measure amyloid and neopterin levels.

Oocysts passed in feces: Fecal pellets were obtained from each mouse on the last day post infection, dissolved in a small amount of normal saline, and sieved to remove rough debris. MZN stained smears were microscopy examined for mean number of oocysts sheded in 10 high powers fields.

Oocysts in intestinal mucosa: Fragments of ileum were individually removed and fixed in 10% phosphate-buffered formalin (pH 7.4) and counting of oocysts.

Biochemical measurement: Amyloid level in sera was measured by the commercial sandwich ELISA kits (Quantikine R & D System, Inc., Minneapolis, MN, USA, Catalog No.: MSA-A00) and also in neopterin was measured by commercial ELISA kit (Amsbio Catalog No: AMS.E03-N0603).

Histopathological study: The ileocecal samples were fixed in a 10% buffered formalin solution, processed for paraffin blocks, sectioned, stained (H&E) and examined microscopically.

Statistical analysis: Data were collected, tabulated, and analyzed by an IBM personal computer with Statistical Package of Social Science version 20 (SPSS, Illinois, USA). Quantitative data were presented as mean ± SD, in numbers and percentages. Kruskal-Wallis's test compared between more than two quantitative nonparametric variables groups. Pearson coefficient (r) correlated between parametric quantitative variables. A P value <0.05 was considered significant.

Results

Oocyst in positive control was (98.0±5.78). Spirulina loaded on Cu-BTC MOF nanoparticles showed lowest significant oocysts in treated mice (28.5±2.31) compared to others ($P<0.001^{**}$), and spirulina alone was (61.1± 3.63). Probiotic loaded on Cu-BTC MOF nanoparticles was low (41.1±2.20) compared to others ($P <0.001^{**}$).

Positive control mean mucosal oocysts count was (62.8±5.40). Spirulina treated mice showed lower count (23.6±2.53), and lowest one was in Cu-BTC MOF nanoparticles loaded NTZ and spirulina mice (12.3±1.55& 15.3±1.55) respectively. Probiotic loaded on Cu-BTC MOF nanoparticles treated mice showed low count (24.8±2.51, $P <0.001^{**}$) compared to others.

Amyloid mean level in negative control was (12.2±0.45), and significantly increased

in treated ones (52.4±1.28) with (P <0.001). All mice showed significant reduction than positive control. Lowest one was in NTZ mice (28.6±0.81), loaded spirulina mice showed (36.1± 0.55), and loaded probiotic showed significant low (37.7±0.44, P <0.001**) compared to others.

Neopterin mean serum level in negative control was (2.43±0.21), and significantly increased in treated ones (12.9±0.49) with (P <0.001). All mice showed a significant reduction level than positive control. Least was in NTZ treated (6.61±0.36) followed by probiotic loaded then spirulina loaded tones (8.40±0.15 & 9.37±0.17) respectively.

A significant negative relation was between oocyst counts in intestine and amyloid level (r= -0.713; P=0.009) in positive control. A significant positive relation was also, between fecal oocyst count and amyloid level (r= 0.687; P=0.014) in NTZ treated mice.

No significant difference was between mean neopterin levels and oocysts in either s stool or intestine in any group (P>0.05). Also, there was neither significant difference between eneopterin mean levels nor in amyloid (P>0.05).

Intestine of negative control was normal. Positive control showed distorted villous pattern with broadening of villi, ulceration, with infiltration of villous core by inflammatory cells and many oocysts in epithelial villous. NTZ treated mice showed some improvement as regular and regenerating villi with intact epithelial cells and brush borders. Probiotic treated mice showed distorted villous pattern with infiltration core by inflammatory cells and many oocysts. Probiotic loaded on Cu-BTC MOF nanoparticles treated ones showed light villous distortion with scanty oocysts. Spirulina either as single or loaded on Cu-BTC MOF nanoparticles treated mice showed nearly preserved villous pattern without any oocysts.

Details were given in tables (1, 2, 3, 4 & 5) and figures (1, 2, 3 & 4).

Table 1: Comparison of means *Cryptosporidium* oocysts/gm stool detected in groups at experimental end.

Studied groups	Oocyst/gm stool		Kruskal-Wallis's test	Post hoc test
	Mean ±SD	Range		
Positive (GI, n=12)	98.0±5.78	88.0 -105.0		P1 <0.001** P2 <0.001** P3 <0.001**
Spirulina (GII, n=12)	61.1±3.63	56.0- 66.0	74.5	P4 <0.001** P5 <0.001** P6 <0.001**
Probiotic (GIII, n=12)	68.8±2.51	65.0 -72.0		P7 <0.001** P8 <0.001** P9 <0.001**
NTZ (GIV, n=12)	32.5±2.31	29.0- 36.0		P10 <0.001** P11 <0.001** P12 <0.001**
Nano + Spirulina (GV, n=12)	28.5±2.31	25.0 -32.0		P13 <0.001** P14 <0.001** P15 <0.001**
Nano + Probiotic (GVI, n=12)	41.1±2.20	38.0 - 44.0		P16 0.004** P17 <0.001** P18 <0.001**
Negative control (GVII, n=6)	0.00±0.00	0.00	P <0.001**	P19 <0.001** P20 <0.001** P21 <0.001**

**Significant: Comparison of P1: GI vs. GII, P2: GI vs. GIII, P3: GI vs. GIV, P4: GI vs. GV, P5: GI vs. GVI, P6: GI vs. GVII P7: GII vs. GIII, P8: GII vs. GIV, P9: GII vs. GV, P10: GII vs. GVI, P11: GII vs. GVII P12: GIII vs. G IV, P13: GIII vs. GV, P14: GIII vs. GVI, P15: GIII vs. GVII, P16: GIV vs. GV, P17: GIV vs. GVI, P18: GIV vs. GVII, P19: GV vs. GVI, P20: GV vs. GVII &P21: GVI vs. GVII

Table -2: Comparison of means *Cryptosporidium* oocysts in intestinal mucosa in groups at experimental end.

Studied groups	oocyst in intestinal mucosa		Kruskal-Wallis's test	*Post hoc test*
	Mean ±SD	Range		
Positive	62.8±5.40	55.0- 71.0		P1 <0.001** P2 <0.001** P3 <0.001**
Spirulina	23.6±2.53	19.0- 27.0	73.4	P4 <0.001** P5:<0.001** P6:<0.001**
Probiotic)	37.1±2.75	33.0- 41.0		P7 <0.001** P8:<0.001** P9:<0.001**
NTZ	12.3±1.55	10.0- 14.0		P10 < 0.328** P11 <0.001** P12 <0.001**
Nano + Spirulina	15.3±1.55	13.0- 17.0		P13 <0.001** P14 <0.001** P15 <0.001**
Nano + Probiotic	24.8±2.51	20.0- 27.0	P <0.001**	P16 < 0.013* P17:<0.001** P18:<0.001**
Negative control	0.00±0.00	0.00		P19 <0.001** P20 <0.001** P21 <0.001*

Table 3: Correlation between amyloid level and number of oocysts in stool and intestine ingroups:

Variations		Number of oocysts in stool	Oocysts in intestinal mucosa
Positive	r	0.257	-0.713
	P value	0.421	0.009**
Spirulina	r	-0.143	0.058
	P value	0.658	0.858
Probiotic	r	-0.014	-0.099
	P value	0.965	0.759
NTZ	r	0.687	-0.097
	P value	0.014*	0.764
Nano + Spirulina	r	-0.240	0.108
	P value	0.452	0.739
Nano + Probiotic	r	0.339	-0.108
	P value	0.281	0.739

Table 4: Correlation between neopterin level and number of oocysts in stool and intestine in groups:

Variations		Serum Neopterin level	
		Number of oocysts in stool	Oocysts in intestinal mucosa
Positive	r	0.325	0.014
	P value	0.302	0.965
Spirulina	r	-0.364	-0.054
	P value	0.244	0.867
Probiotic	r	-0.506	0.00
	P value	0.093	1.00
NTZ	r	0.227	-0.158
	P value	0.479	0.624
Nano + Spirulina	r	-0.022	-0.247
	P value	0.947	0.438
Nano + Probiotic	r	0.144	-0.394
	P value	0.655	0.205

Table 5: Correlation between mean serum levels of amyloid and neopterin in groups:

Neopterin level		Amyloid level
Positive	r	0.007
	P value	0.983
Spirulina	r	-0.321
	P value	0.308
Probiotic	r	-0.135
	P value	0.676
NTZ	r	0.244
	P value	0.445
Nano + Spirulina	r	-0.071
	P value	0.827
Nano + Probiotic	r	-0.007

Discussion

Generally, the nano-drug delivery systems enhance therapeutic efficacy by prolonging drug action, increasing surface area, faster dissolution, and smaller particle size (Fahmy *et al*, 2021).

In the present study, treatment efficacy depended on oocysts shedding in stool and decreasing. The NTZ treated mice showed oocysts shedding (32.5±2.31) with mucosal clearance (12.3±1.55). This agreed with Ollivett *et al*. (2009), Sadek *et al*. (2020), and Matar *et al*. (2023), they reported various NTZ efficacy degrees in treating cryptosporidiosis infected mice. NTZ and/or spirulina loaded on Cu-BTC MOF nanoparticles gave more efficacies. This agreed with Guitard *et al.* (2006); Khalifa (2016); Metawae *et al*. (2021); Moawad *et al*. (2021) and Soliman *et al*. (2023). No doubt, non-pathogenic probiotics prevented and treated many human diseases, by modifying acquired and innate immunity at systemic and mucosal enhanced CDs differentiation into Th1/Th2 response, inducing cytokines (IL10, IL12, & IFNγ), and increasing IgA (Travers *et al*, 2011). But, Salazar-Lindo *et al*. (2004) reported that infant with acute *C. parvum* diarrhea was not treated with milk Lactobacillus.

Generally, the acute phase reaction (APR)

effects and acute phase proteins (APPs) alteres infections (Razavi *et al,* 2023). The cellular, and molecular interactions for SAA improved knowledge of the diseases pathogenesis, treatment and prevention (Sack, 2018).

In the present study, infected mice showed highest SAA levels (52.4±1.28) with immune system well responded to *C. parvum* infections, but levels decreased in treated mice. This agreed with El-Deeb *et al*. (2022), who found higher serum HP and SAA levels in *C. parvum*-infected calves. Also, Kabu (2023) showed that SAA concentration in cryptosporidiosis infected calves was much higher before treatment. Dinler *et al*. (2017) found higher SAA concentration was in neonatal lambs than in control ones. Kabu *et al*. (2023) reported that SAA concentrations were high in cryptosporidial diarrheic lambs. But, Censiz *et al*. (2017) reported that SAA levels in *C. parvum* infected calves were the same before and after treatment.

In the present study, there was significant correlation between SAA concentration and oocyst shedding were in NTZ treated mice. This agreed with Dinler *et al*. (2017), they found that in infected mice SAA concentration significantly correlated with intestinal oocysts.

In the present study, NPT high levels were (12.9±0.49) in positive control, but decreased in NTZ (6.61±0.36). This agreed with El-Deeb *et al*. (2022), who found that NPT a component of cell-mediated immune system of monocytes and macrophages has adventages over APP in rapid declining in sera of diseased animals and short half-life.

Also, Eisenhut (2013) reported that NPT was a helpful indicator for disease response to treatment, severity and prognosis.

In the present study, cryptosporidiosis intestinal histopathology showed the best results with spirulina loaded treatment. This agreed with Chandrarathna *et al*. (2020), who reported that spirulina improved intestinal picture. Also, it agreed with Gaber *et al.* (2022), who reported non-specific histological changes, such as intestinal crypt cell hyperplasia, flattening of villi, and infiltration of inflammatory cells into the luminal propria.

Generally, spirulina is one of the nutritious alga with antioxidant, antiviral, anti-genotoxic, anti-hypertensive and immunostimulant effects (Finamore *et al*, 2017). Abdel-Dayem *et al*. (2015) and Ma *et al*. (2019) reported that spirulina regenerated damaged gastrointestinal villi by polysaccharides activity. Also, its extract preserved damaged tissues by its anti-inflammatory activities and lowering phagocytic infiltration (Ożarowski and Karpiński, 2021). Besides, its C-phycocyanin promotes tissue regeneration and proliferation prevented cell death by eliminating free radicals aiding in healing (Mohammadi-Gouraji *et al*, 2019), and igamma-linolenic acid altered immune system (Ramez *et al*, 2021).

In the present study, probiotic-Cu-MOF treated mice showed good intestinal improve ment. This agreed with Qin *et al*. (2005), they reported that *Lactobacilli* completely restored the intestinal integrity.

Conclusion

Both spirulina and probiotics loaded on the Cu-MOF successfully treated mice, which survived infection. Intestinal histopathology was more or less normal, altered by immunomodulatory of the SAA and NPT levels.

Studies on Cu-MOF side effects on man, animals and environment are ongoing and will be published in due time else-where.

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Explanation of figures

Fig. 1: Mean serum amyloid level in groups.

Fig. 2: Mean serum neopterin level in groups.

Fig. 3: Histopathological sections of ileuocecal region from groups (H&E): (A&B): positive control (GI) showed distorted villous pattern with infiltration of villous core by inflammatory cells (black arrows) and recognition of many cryptospores (red arrows)(C&D) spirulina treated (GII) showed nearly preserved villous pattern without recognition of any cryptospores (E&F) probiotic treated (GIII) showed distorted villous pattern (black arrows) and recognition of many cryptospores (red arrows) (X200 & X1000).

Fig. 4: Histopathological sections of ileuocecal region from groups (H&E): (G&H) NTZ treated (GIV) showed regular and regenerating villi (black arrows) with intact epithelial cells and brush borders (blue arrow) (I&J) spirulina loaded on Cu-BTC MOF treated (GV) showed nearly preserved villous pattern with no recognition of any cryptospores (K&L) probiotics loaded on Cu-BTC MOF treated (GVI) showed mildly distorted villous pattern with infiltration of the villous core by inflammatory cells (black arrows) and recognition of few cryptospores ((red arrow) (X200 & X1000).