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Biochemical studies on the effect of probiotic in experimentally induced ulcerative colitis in rats

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ABSTRACT

The capacity of the colon to function properly is harmed by a variety of conditions. Some of these include Crohn's disease (CD), ulcerative colitis (UC) and inflammatory bowel disease (IBD). Ulcerative colitis which is the most common type of IBD. It's a relapsing and remitting disorder with a wide range of phenotypic and genotypic characteristics. In this study, potential protective and curative effect of probiotics on acetic acid-induced ulcerative colitis was evaluated. Ulcerative colitis was induced in rats by 2 ml of 3% glacial acetic acid (v/v) administered intracolonially for 3 successive days. Forty male albino rats were divided into five equal groups. Group I: (Control normal group) rats received no drugs. Group II: Early UC-induced group, Group III: Late UC-induced group, Group IV: (Early UC + probiotics protected) rats received probiotics (135 mg/kg b. wt./day) for 21 days prior to acetic acid administration. Group V: (Late UC + probiotics treated) rats first administered with acetic acid then after 3 days probiotics was received for 21 days. A significant increase in L-MDA and Myeloperoxidase (MOP) and up-regulation of IL-1β, caspase-3 with down-regulation in Bcl-2 gene expression as well as marked decrease in GSH and CAT were observed in colon tissue of UC-induced rats. However, administration of probiotics to UC-induced rats caused a marked improvement and reverses all previous parameters near the average range. In conclusion, probiotics protected colonic mucosa against UC and may be effective in enhancing the healing of ulcerated colon via anti-inflammatory and anti-apoptotic activities and regenerating endogenous antioxidant mechanism.

1. INTRODUCTION

Ulcerative colitis (UC) which is the most common type of inflammatory disease of the bowel (IBD). It is characterized by a chronic inflammation, which starts from rectal mucosa and spreads to its proximal parts. The peak age of onset for UC, between 30 and 40 years (Molodecky et al., 2012). The pathogenesis of ulcerative colitis is not well known. Several factors, such as the environment, genetic predisposition, and immunology, all play a role in the development of UC (Neuman and Nanau, 2012). Idiopathic diarrhea, abdominal pain, fever, rectal bleeding, and weight loss are common symptoms of UC, which can vary from mild to extreme depending on the degree and severity of the inflammation. In the colon, adipose tissue acts as an endocrine organ, secreting cytokines such as TNF- and IL-6 and modulating immune cell function. The altered balance of these cytokines results in inhibition of inflammatory cytokines or enhancement of the anti-cytokine defense system (Monteleone et al., 2013).

Acetic acid (AA) is a commonly used and easily inducible model for studying UC, and it is spread by administering AA intra-rectally to cause inflammation and ulceration in the rectum and colon. (Pereira *et al.,* 2015). Oxidative stress derived from excessive ROS development causes lipid peroxidation, damage to the intestinal mucosal barrier, bacterial translocation, and an inflammatory response. By causing lipid peroxidation, ROS cause cell death, and Malondialdehyde (MDA) is considered a toxic end product. MDA is a popular oxidative stress marker that increases in tissue biopsies from UC patients (Lambert, 2009).

Corticosteroids, immunosuppressant's, 5-aminosalicylates (5ASAs) and biological therapies are some of the medicines used to treat UC. Surgical care is reserved for patients who have experienced an acute complication or who have failed to respond to medical treatment. Antibiotics are rarely successful in the treatment of acute UC (Meek and Morton, 2016). Probiotic bacteria that bind with the host epithelium may be used as an alternative to treat inflammation (Andersson and Soderholm, 2009).

Clinical probiotics have been shown to enhance corrosive necrosis in rats with acetic acid ulcerative colitis and to modulate intestinal immunity. They will lower the pH of the colonic environment and thus inhibit the growth of potentially pathogenic microorganisms by developing short-chain fatty acids. They can inhibit excessive NFB pathway activation, reduce pro-inflammatory cytokine production (e.g., TNF, INF, and IL-8) and increase anti-inflammatory cytokine production and secretion (e.g., IL-10 and TGFβ) (Stephani et al., 2011). According to previous research findings, probiotics aid in the healing of the colon mucosa in acetic acid-induced colitis. Hence, the

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purpose of the present study was to explore the influence of probiotics against experimental model of ulcerative colitis in rats. Also, to see whether probiotics could reduce oxidative stress and inflammation in ulcerative colitis-induced rats, this would be beneficial for the prevention and treatment of colitis complications.

2. MATERIAL AND METHODS

2.1. Experimental animals:
In this study, forty white male albino rats aged 12-16 weeks with an average body weight of 220-250 g were used. During the experiment, rats were kept in constant environmental and nutritional conditions, and clean drinking water was provided ad libitum. Before starting the experiment, the animals were given 14 days to acclimate.

2.2. Chemicals and drugs:
The chemicals and drugs used in the present study were:
Probiotic: This product is kindly supplied by Newdynovet Free Trade, El-montazah – Alexandria and manufactured by Multipharm. Co. USA. It is a powder added to drinking water at a dose of (0.0128×10^9) CFU per gram of rat body weight, which is equivalent to (135 mg/kg. wt.), ~~dissolved in drinking water~~ and received orally and daily for 21 days (Sanchez et al., 2015).
Glacial acetic acid: Glacial acetic acid is manufactured by Merck Millipore, Darmstadt, Germany.

2.3. Induction of ulcerative colitis:
Colitis was induced using the method described by (Millar et al., 1996). Where after 24 hours of fasting, the rats were anesthetized by sodium thiopental (500 mg dissolved in 12.5 ml of normal saline) injected at a dose of 0.2 ml/200g body weight (40 mg/kg b. wt./ i. p.). A polyethylene tube (2 mm in diameter) was inserted through the rectum into the colon to a distance of 8 cm and injected for 30s with three ml of acetic acid 3 % v/v). The acetic acid was held in the colon for another 30 minutes until the fluid was removed and animals' head were kept in a downward position for 30s sec to prevent leakage of the intracolonic instillation and then returned to cages (labib *et al*., 2016). The rats from the normal control group were respectively instilled with 0.5 ml physiologic saline via a cannula (Tanideh *et al*., 2014).

2.4. Experimental design:
Rats were randomly divided into five main equal groups as follow:
Group I: (Control normal group): Rats received balanced diets has no types of medication.
Group II: (Early ulcerative colitis-induced group): Rats administrated with glacial acetic acid (2 ml/rat) at 21stday of experiment for 3 successive days.
Group III:(Late ulcerative colitis - induced group): Rats first administered with glacial acetic acid (2 ml/rat) at the first day of experiment for 3 successive days, and all rats were sacrificed after 21 days from ulcerative colitis-induction.
Group IV: (Early UC + probiotics protected): rats received probiotics (135 mg/kg b. wt./day) for 21 days prior to acetic acid administration.
Group V: (Late UC + probiotics treated): rats first administered with acetic acid then after 3 days probiotics (135 mg/kg b. wt/day) was received for 21 days.

2.5. Sampling:
After sacrificing rats, colon tissue specimen was collected once after 21 days of treatment with probiotic for biochemical [Myeloperoxidase (MPO), L-Malondialdehyde(L-MDA), reduced glutathione (GSH) and Catalase (CAT)], molecular [IL-1β, Caspase-3 and Bcl-2 gene expression] analysis and histopathological examination.
2.5.1. Sample analysis:
2.5.2. Biochemical analysis:
Colonic tissue L-malondialdehyde (L-MDA), Catalase (CAT), reduced glutathione (GSH) and Myeloperoxidase (MPO) were determined according to the methods described by (Mesbah *et al.*, 2004), (Xu *et al.*, 1997), (Patterson and Lazarow, 1955) and (Shi *et al.*, 2005), respectively.
2.5.3. Molecular analysis:
The mRNA expressions content of Caspase-3, Bcl-2 and IL-1β in colon tissues of rats were determined using qPCR. B-actin was used as the load control. Total RNA was isolated using High Kit for pure RNA isolation (Thermo Scientific, Fermentas, #K0731), the produced cDNAs from the reverse transcribed template RNAsusing Revert Aid™ H Minus Reverse transcriptase kit (#EP0451, Thermo Scientific, Fermentas, USA) were amplified on Faststart Universal SYBR Green Master (Roche, GER). The target gene was normalized with β-actin by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Forward and reverse primers sequence for real time PCR.

ene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
Caspase3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
Bcl-2	AGTACCTGAACCGGCATCTG	CATGCTGGGGCCATATAGTT
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
β-actin	ACCCACACTGTGCCCATCTA	CGTCACACTTCATGATG

The specimens of colon tissue were stored in 10% buffered neutral formalin. The fixed tissue was rinsed in tap water before being dehydrated in a series of alcohols, cleared in xylene, and embedded in paraffin wax. Hematoxylin and eosin were used to stain 5 m thick sections (H&E) (Bancroft *et al., 1996).*

2.6. Statistical Analysis:
Results were expressed as mean ±SE using SPSS (13.0 software, 2009). Data were analyzed using one-way ANOVA followed by Duncan's test. Values were statistically significant at $p < 0.05$.

3. RESULTS

The obtained results in table (1) showed significant increase in L-MDA concentration and MPO activity with marked decrease in CAT activity and GSH concentrations in colonic tissue of UC–induced rats as compared to control group. However, a significant depletion of colon LMDA, MPO and marked increase in CAT and GSH were observed after probiotic administration in comparison with colitis untreated rats.
The obtained qPCR results (Table 2) revealed a significant up-regulation of IL-1β and Caspase-3 and down-regulation of Bcl-2 gene expression in colon of UC induced rats (G II and G IV) as compared to control normal group (G I). This expression was significantly down-regulated with significant up-regulation in Bcl-2 in colon tissues following administration of probiotics either before (G II) or after (G IV) induction of UC, with lower expression in preventive group (G III).

Table 1 Effect of probiotic administration on colon tissue MPO, L-MDA, GSH and CAT in ulcerative colitis - induced rats.

Animal groups	MPO (U/mg. tissue)	L-MDA (nmol/g. tissue)	GSH (ng/g. tissue)	CAT (mmol/min/g. tissue)
Group I: Normal control	0.07^{e}±0.001	23.86^{e}± 0.89	12.58^{a}± 0.64	111.5^{a}± 5.11
Group II: (Early UC-induced group)	0.50^{b} ±0.030	83.25^{b}± 2.430	1.00^{d}± 0.040	19.28^{d}± 1.010
Group III: (Late UC-induced group)	0.67^{a} ±0.03	95.36^{a}± 2.60	0.45^{e}± 0.02	12.61^{e}± 0.94
Group IV: (Early UC + probiotic protected group)	0.19^{d} ±0.02	46.19^{d}± 1.39	7.45^{b}± 0.45	53.13^{b}± 3.00
Group V: (late UC + probiotic treated group)	0.30^{c} ±0.02	64.09^{c}± 1.83	3.25^{c}± 0.20	30.62^{c}± 2.24

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table 2 Effect of probiotic administration on the relative expression of capase-3, Bcl-2 and IL-1β gene in colon tissue of ulcerative colitis - induced rats.

Animal groups	Caspase -3		Bcl-2		IL-1β	
	Fold change mean	SEM	Fold change mean	SEM	Fold change mean	SEM
Group I: Normal control	1.00^{e}	0.09	1.00^{a}	0.05	1.00^{d}	0.09
Group II: (Early UC-induced group)	8.57^{b}	0.47	0.20^{d}	0.01	8.75^{b}	0.38
Group III: (late UC-induced group)	10.70^{a}	0.52	0.18^{d}	0.01	10.48^{a}	0.42
Group IV: (early UC+ probiotic protected group)	3.56^{d}	0.19	0.69^{b}	0.04	3.36^{c}	0.22
Group V: (late UC+ probiotic treated group)	5.31^{c}	0.34	0.35^{c}	0.02	4.14^{c}	0.3

Means within the same column carrying different superscript letters are significantly different (P≤ 0.05).

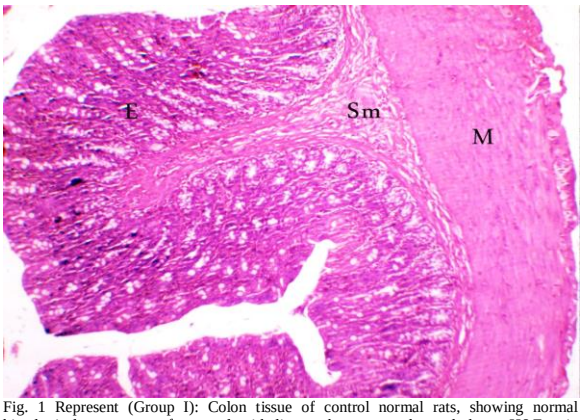

Fig. 1 Represent (Group I): Colon tissue of control normal rats, showing normal histological appearance of mucosal epithelium, submucosa and muscle layer. H&E stain x 100. (simple columnar epithelium lined the mucosa, crypt of Lieberkühn lined by mucous cells, columnar cells and paneth cells. The submucosa consisted of connective tissues. The tunica musclosa consisted of inner circular and outer longitudinal smooth muscle fibers. The tunica serosa consisted of loose connective tissues and covered by serous membrane)

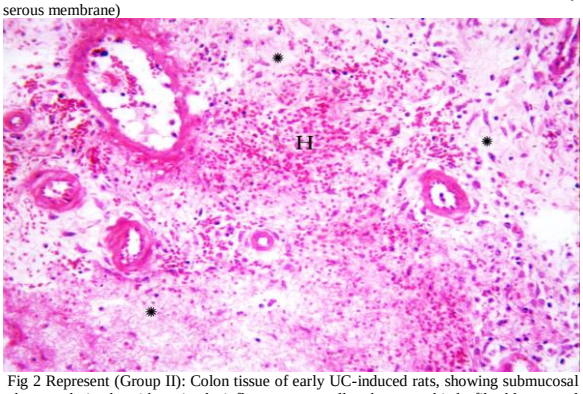

Fig 2 Represent (Group II): Colon tissue of early UC-induced rats, showing submucosal edema admixed with mixed inflammatory cells, hypertrophied fibroblasts and hemorrhage. Note also loss of the endothelium of submucosal blood vessels H&E stain x 200.

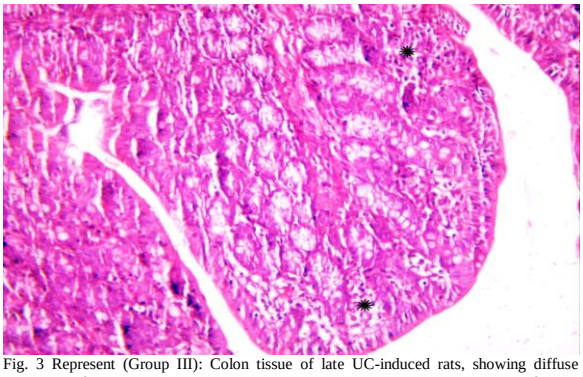

Fig. 3 Represent (Group III): Colon tissue of late UC-induced rats, showing diffuse expansion of the lamina propria by edema admixed with a lymphoplasmocytic infiltrate. H&E stain x 200.

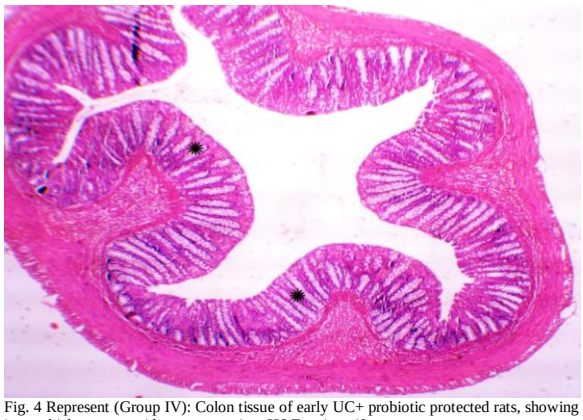

Fig. 4 Represent (Group IV): Colon tissue of early UC+ probiotic protected rats, showing intact thick mucosa without any erosion. H&E stain x 40.

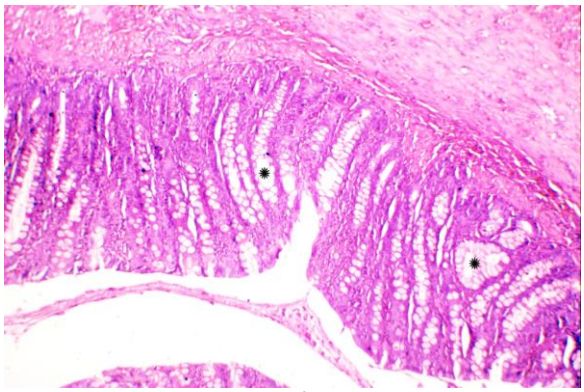

Fig. 5 Represent (Group V): Colon tissue of late UC+ probiotic treated rats, showing mucosal crypts are elevated, hyperplastic with increase in numbers of goblet cells. H&E stain x 100.

4. DISCUSSION

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease (IBD) that affects the large intestine (Ungaro *et al*., 2016). Increased development of reactive oxygen species (ROS) causes oxidative stress, which, in combination with cytokines, causes the destruction of tight junctions in the intestinal barrier, resulting in increased permeability, tissue damage, and gastrointestinal mucosal wall dysfunction (Dvořák et al., 2020).

Acetic acid colitis is characterized by increased neutrophil penetration, crypt abscesses, granulomatous inflammation with fibrosis, and massive submucosal thickening, both of which are associated with local inflammation (Wang et al., 2013). Probiotics have been shown to reduce the side effects of the intestinal mucosa by decreasing the levels of

anti-inflammatory cytokines and neutrophil infiltration in many studies. (Depommier et al., 2019). Oxidants play a key role in the inflammatory response in experimental colitis, raising the number of neutrophils and macrophages. Endogenous antioxidants are effective targets for Ulcerative colitis treatment since they degrade highly reactive oxygen species (Guan and Lan, 2018).

Myeloperoxidase (MPO), a haemenzyme found primarily in neutrophil granulocytes, produces hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2), and chloride anion (Cl) causes colon damage, resulting in neutrophil and macrophage activation (Chamanara *et al.,* 2019). The obtained results revealed that, MPO activity was increased in the acetic acid-induced UC compared to control as reported by Matuszyk *et al*., (2018). Interestingly, after Probiotic treatment to ulcerative colitis induced rats, the results revealed a substantial decrease in MPO activity. Similarly, Liu *et al*. (2020) showed that VSL#3 (L plantarum, L Bulgaricus, L casei and L. acidophilus) (a mixture of 8 probiotic bacteria) had adjunctive therapeutic effect by down regulating MPO activity. A significant increase in MDA level, indicator of lipid peroxidation in the colon tissue, has been reported as a result of the findings in experimental UC models. UC is induced and triggered by oxidative stress in two ways: oxidative damage to intestinal mucosal cells and upregulation of inflammatory cytokines. (Al-Rejaie et al., 2013).

In the current study a significant decrease in MDA concentration was observed in probiotics treated rats either before or after ulcerative colitis induction. Probiotics did not lick steroids so probiotics can be used as supportive therapy to classical therapy with their antioxidant effects (Utku *et al*., 2020). In the current study, a significant decrease in glutathione concentration and Catalase activity was observed in acetic acid-induced ulcerative colitis. This result nearly matched to that described by Wang *et al*. (2013), who reported that intra-rectal administration of acetic acid decrease antioxidant defensive system, as acetic acid causes severe oxidative stress in colonic tissue, which is manifested as an enhancement in lipid peroxidation that occurs via an increase in the L-MDA as well as decreasing CAT activity in tissue, simply GSH and CAT acts to scavenge the free radicals from cells to ameliorate the happening oxidative stress that can destruct the cells and to return Redox balance to our cells. This evidence was reported by Soliman *et al.* (2019). Meanwhile, GSH and CAT transcription levels substantially increased in the B. Bifidum ATCC 29521 treated group compared to the colitis group, implying that B. bifidum ATCC 29521 as a UC treatment (Din *et al.,* 2020).

The obtained results demonstrated a significant up-regulation in relative expression of IL-1β gene was observed ulcerative colitis –induced animal groups (Owusu *et al*., 2020). Increased production of pro-inflammatory mediators like TNF- and interleukins (e.g., IL-1 and IL-17) induced inflammation by stimulating monocytes, macrophages, and intestinal immune cells, which then elicited immune responses by inducing the secretion of other cytokines, resulting in tissue damage in the pathologic progression of colitis, according to published studies (Impellizzeri et al. 2018). Lactobacillus casei OLL2768 was found to reduce the expression of IL-1 by inhibiting NF-B signaling pathways in bovine intestinal epithelial (BIE) cells, which attenuated the ETEC-induced pro-inflammatory response (Takanashi *et al.,* 2013). The present study agrees with previous study on effect of probiotics on UC (*Bifidobacterium animalis*) MB5L. rhamnosus GG Probiotics Secreted factors inhibition of nitric oxide transmigration by suppression of chemokine regulators, IL-1β and TNFα. (Llewellyn and Foey, 2017). In the present study, a significant up-regulation of Caspase-3 downregulation of Bcl-2 gene relative expression were observed ulcerative colitis induced rats groups. These results were agreed with (Masoumi et *al*., 2020) Caspase-3, which is known to be expressed in enterocytes, has also been shown to be increased in animal models of induced colitis, according to the researchers. Caspase 3 is activated by the EPS of A. halaphytica, which induces apoptosis. The capacity of Lactobacillus spp. EPSs to induce apoptosis was associated with an upregulation of Caspase 3, and 9 and a downregulation of Bcl-2, according to gene and protein expression findings (Jiang *et al*., 2017). Secretion metabolites of probiotic yeast, the effects of Pichiakudriavzevii AS-12 secretion metabolites on the expression level of 6 essential genes involved in extrinsic and intrinsic apoptosis (BAD, Bcl-2, Caspase-3, Caspase-8, Caspase-9, and Fas-R) induce apoptosis pathways in human colorectal cancer cell lines (Saber *et al*., 2017).

5. CONCLUSION

It could be concluded that, treatment with probiotics alleviates the colon oxidative stress and inflammation associated with UC, mediated by adjustment of inflammatory mediators, and increasing antioxidants as well as attenuating oxidant/antioxidant imbalance. In fact, probiotics may be a novel treatment of UC, exerting its anti-inflammatory, anti-apoptotic effect through suppression of caspase3 and IL-1B, and the enhancement of IL-1β signaling pathways**.**

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