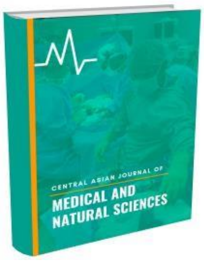

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Article

Improved Histological Effect of *Salmonella Typhimurium* Ghost Vaccine Used in the Treatment of Colon Cancer Induced in Male Albino Rats

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Abstract: Colorectal cancer continues among most serious life-threatening tumors worldwide. Recently, as a result of the progress made in microbial science and its relationship to cancer, and the efforts made to eliminate this dangerous disease without affecting normal cells, the current study aimed to shed light on Bacteria-mediated cancer therapy as a promising therapeutic tool with less side effects and due to its high immune stimulation effect. Colon cancer was induced in male albino rats using Azoxymethane (AOM) at 15mg/Kg bw, the curative effect (post-treatment) of *Salmonella typhimurium* ghosts (STGs), which was prepared using chemical concentrations selected in a special protocol, was studied by inoculated the animal subcutaneously with a 100 µl/rat in single dose with equal volumes (1:1) of STGs +Freund's adjuvant. We vaccinated the animal with (STGs) in 3 cycles. After 42 days of the whole vaccination, the animals were sacrificed, the colorectal tissues were removed and preserved in a diluted formalin solution until performing grossly and histological examinations. The result revealed that histolpathological changes in induced colorectal cancer includes damage and alterations in mucosal crypts architecture, increase spaces between crypts, dysplastic changes in crypts epithelium. In vaccine treated animal showing severe histological alterations, infiltration of inflammatory cells and significant hyperplasia in lymphoid tissue, comparing with the control group. In conclusion, vaccination by STGs vaccine using *S. typhimurium* will enhance the immune response, which is a promising medication for the treatment of colon cancer.

Keywords: bacterial ghost, colorectal cancer, histo-immune stimulation, rats, vaccination

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1. Introduction

Colo-rectum cancer (CRC) is a sever disease characterized as exponential division and uncontrolled growth of colon or rectum cells, and is the second main cause of cancer-related raising mortality throughout the world [1]. Traditional cancer treatments, such as chemotherapy, surgery, and radiotherapy, represent the main treatment option. However, they exhibit remarkable side effects and numerous pharmacological limitations, including the problems of their low selectivity to tumor tissues, reduced penetration to target tissue, the emergence of multiple therapy resistance by cancer cells. In addition, raised rate of tumor returning to patients those have receiving traditional treatments [2].

With the increase in the incidence of various types of cancerous tumors around the world, there has been an urgent need to discover and develop new treatments characterized by their biological effectiveness against cancer, in addition to their ability to reduce the side effects caused by traditional treatments, as Bacteria-mediated cancer therapy (BMCT) has been identified since the first spontaneous regression of tumors at that time of the surgeon Dr. William B. Coley observation to patients exposed to *Streptococci* infection which showed pronounced regression of tumor growth, over the past century, it was the starting and evolution of cancer biological therapy into nowadays immunotherapy. The first reporting to have *Salmonella* an antitumor action was in 1935. Until now, many research discussed its effectiveness as an antitumor agent to be used in the near future [3].

Salmonella are both commensal bacteria, in addition to being pathogenic to human being and animals, such as mammals, fowl, reptiles and insects. It is the main cause for enteritis, systemic infection, and enteric fever diseases if it is transmitted orally when consumption the contaminated food, water, from animals or their products to humans. Salmonellosis is a serious public health disease caused by the serotype *S. typhimurium* in both individuals and animals with the total number of cases increasing yearly because it has the ability to transmitted between healthy and infected animals that carry and shed resistant *Salmonella* to humans or through the food chain [4], [5], [6]. One of the most effective planning of disease prevention is vaccination that has a growing interest in creating cross immunity against it, numerous routs to vaccines production were defined, while inactivated bacteria vaccines represented the potent immunostimulater characterized by safety and effectively preventive route for diseases before take place [7].

In anti-cancer bacterial immunotherapy methods, bacteria that exist in natural living conditions can be used as whole cells, or in attenuated form, or in a genetically modified form. It is possible to use bacteria as a type of vaccine to stimulate the human immune system to resist cancerous tumors on the same principle in which taking vaccines to combat viral or bacterial diseases [8]. Vaccines are prepared either from modified or attenuated organisms, or from killed or inactivated organisms such as bacteria, viruses, toxoids, or a combination of these organisms to activate the immune system. It causes humoral and/or cellular immunity [9]. Maintaining the effectiveness and safety of vaccines prepared from killed bacteria, in addition to preserving their antigenicity, is due to moderate preparation steps, which can be developed using Ghost preparation techniques, to be a promising alternative to immunotherapy [10].

Bacterial ghosts are bacterial cell membranes that have lost most of their genetic and protein contents and other cytoplasmic components, but have retained their surface properties. They are an excellent tool in producing many kinds of bacterial vaccines, this is due to the fact that they are dead cells, evacuate of contents having a correct three dimensionals structure. These cellular components have the ability to safely stimulate the immune system and producing the appropriate antibody if they enter our bodies or the body of animals, because they are removed from any cytoplasmic component, and then they will lose the ability to replication inside the living body. The current trend is to use Bacterial Ghosts (BGs) in collaboration with drug delivery systems or by interfering with means of activating the immune system, and used in various medical applications [11], [12]. Many researchers have turned to the production of non-living *S. typhimurium* bacterial ghost (STG) using the chemically induced method based on critical chemical concentrations, experimental studies have been conducted using experimental model systems both *in vitro* and *in vivo* in regard to summarised the benefits of STG vaccine candidate as its ability to stimulate the immune system (cellular and humoral) against specific infections in lab animals by stimulating pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), anti-inflammatory cytokines, and stimulating macrophage cells [13]**.**

This study aimed to evaluate the efficiency of STGs (in vivo) in stimulating the immune response within tumor tissue as a therapeutic method for AOM-induced colorectal cancer in male albino rats.

2. Materials and Methods

2.1. Bacterial strain

A sample of *S. typhimurium* was obtained in a ready-made form and activated on selective media for *Salmonella* bacteria from Al-Amin Center for Advanced Research and Biotechnology / Najaf Al-Ashraf. Then the bacterial isolate was transferred to the laboratory and preserved on slanted nutrient agar medium after inoculation and incubation at a temperature of 37°C for 18 hours. The isolate was stored at a temperature of 4°C, and the diagnosis was confirmed using the VITEK2 device.

2.2. Preparation of S. typhimurium ghost

"Sponge Like" (SL) protocol is used in its reduced form "sponge like reduced protocol" (SLRP) for bacterial ghosts (BGs) preparation which depended on critical concentrations for such chemicals that could affect the bacterial cell wall and maintains of correct BGs structure, as mentioned in [14]. According to such protocol, only the chemical compounds and the bacterial cells were used without any genetically modifications or recombinant constituent, the name of (SLRP) was derived from the original protocol "sponge like" after used further steps to producing minor pores in the bacterial cell membrane, mostly the centrifugation steps which leading to release of bacterial cytoplasmic components by squeezing gently on the cells like a sponge in order to release the inner cell content to the external. The sterility and safety of the prepared vaccine were examined and then stored at a temperature of 4°C until used in the subsequent steps of the experiment.

2.3. Animal study and ethical approval

This project includes a study in which rats were used as live experimental animals, and included their experimental injection with the carcinogen, followed by treatment with a bacterial vaccine. The ethical recommendation in using live animals in this study were strictly directed, and this experimental research was approved by the scientific committee, Department of Biology, College of Education for Pure Sciences, University of Kerbala (Reference No. D.A./14/6267 in 12/5/2022). Animal handling and care were in accordance with the National Academy of Sciences Guidelines for the Care and Use of Laboratory Animals [15].

2.4. Housing laboratory animals

In this study, four to 5-week-old of male *Rattus rattus* with body weight between (50-75) g were purchased and housed in the animal house of the College of Pharmacy, University of Karbala. They were housed for a week under monitoring at specific conditions in regarding of sterilized and pathogen free environment under a 12 hours dark/light cycle before being used to ensure their safety, activity, and vitality. The animals were distributed into groups of (10) rat per group in plastic cages with metal covers designated for raising rats, every cage housed with 5 rats. The floors of the cages were spread with sawdust, and care was taken to replace them from time to time. Optimal conditions were provided in terms of temperature, ventilation, appropriate humidity, and clean water. Drinking and lab chow were given freely, consisting of animal feed *ad libitum*, the above instructions were followed with laboratory rats for two weeks before starting the experiment to ensure that the animal adapts to its new environment.

2.5. Experimental design, induction of colon cancer and vaccination

The experiment was designed according to the steps mentioned in [16], [17] with some modifications. The male rats (30 animals) were grouped randomly into three groups; Group 1 (Normal control), Group 2 (Cancer control), Group 3 (cancer treatment with STGs).

All of the rats were weighed before starting the treatment to calculate their doses. Azoxymethane (AOM) colon carcinogen is used to induce aberrant crypt foci (ACF) formation in rats as a usual method in colorectal cancer examination. The rats (group 2 and 3) were subcutaneous injected with (AOM) at 15 mg/kg body weight once weekly and repeated for two weeks. Group 1 (normal control) injected with N. saline at 15 mg/kg b.w. All animals access feeding and drinking freely, and the rats body weight was measured routinely all over the experiment. After 20 weeks, rats (group 3) were injected with a single subcutaneous dose of equal volumes (1:1) of STGs + Freund's adjuvant at a dose of 100 µl/rat. All animals in this group were vaccinated three times at an interval of two weeks. The STGs vaccine was given as an initial dose on day zero, followed by booster doses on the 14 and 28 days, according to [11]. Rats of groups 1 and 2 were injected with normal saline at a dose of 100 µl/rat. After completing the vaccination process, the experimental rats were dissected and the required tissues were removed for histological examination.

2.6. Preparing Colon for Macroscopic Analysis

2.6.1. Aberrant Crypts Foci estimation

Rats were anesthetized with a high dose of anesthetic (chlorophorm), then they were dissected and the colon specimens were removed from them, washed with cold phosphate buffered saline (PBS). For staining purpose, colon tissues were cut longitudinally starting from the anus and then stained with 0.2% methylene blue dye for 1 minute as stated in for macroscopic inspection [18], Microscopic examination was used to vision and valuation the aberrant crypt foci (ACF) formation [19].

2.6.2. Histological Examination

The histological examination for colorectal tissues samples, which have been collected and preserved previously at the final step, were conducted and processed according to [20]. Beginning with sample fixation in 10% neutral formalin overnight, followed by dehydration passes and then embedded in soluble paraffin until solidify in blocks that undergo sectioning by a rotary microtome into 5 - mm slices and spreading them on glass slides, dried and paraffin removal and then slides staining with hematoxylin & eosin dyes, finally mounting them by using mount media, the slides were observed for histopathological evaluation. The cells infiltration inside colon sections was examined under light microscopy, which were performed by trained pathologists [21].

3. Results

3.1. General observation

During 20 week of CRC induction period, rat's weight in the control group enhanced gradually comparing to the weight of rat in the 2 and 3 group which get lower gradually, and the food intake of rat at that groups decreased significantly. Moreover, over the experiment period, rat number in the control group was (10), in the cancer control group there were four rat perdition (4/10), and one rat died in the STGs vaccine group (1/10).

3.2. Anticancer pathological study

At the end of the study, rat colon tissues were collected and tumor growth was examined:

1) Colon tissue grossly examination from experimental rats revealed the significant anticancer potential of vaccine in the azoxymethane-induced ACF formation in rats. The normal control rats (A) showed the absence of ACF in their colon tissue, the cancer control rats (B) showed multiple polypoid masses, ACFs formation and changes in the size, shape, color, and the characteristic rugated texture in their colon

tissue compared to the STGs treated rats(C) suggesting that STGs vaccine could inhibit the growth of colon tumors (Figure 1).

(A) (B) (C)

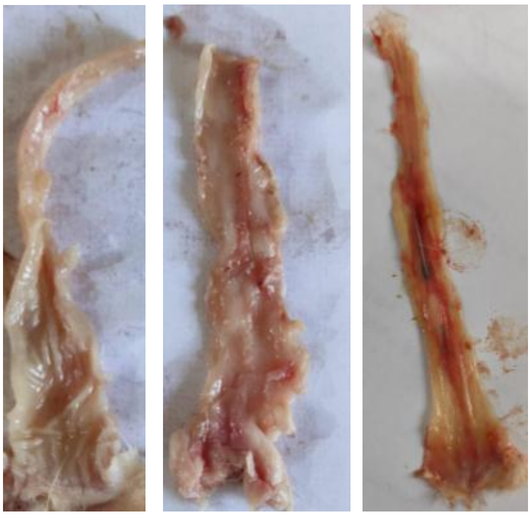

Figure 1. Longitudinally opened rat colorectal tissues illustrating: (A) Normal colon with no change (Control group); (B) The presence of preneoplastic lesions ACF and focal mild epithelial dysplasia (AOM induction CRC group); (C) STGs processing lowered the pre- neoplasms formation in the proximal and distal region of AOM-induced colon cancer in rats (Vaccine treatment group)

2) Colon histopathological changes. The ACF were visualized under light microscope in the paraffin-fixed, sectioned, methylene blue stained colons in which the formation of ACF per colon was recorded under 10x magnifications. The microscopic histological examination of colonic specimens staining with methylene blue of the control group showed normal histological mucosal architecture, normal crypts with lamina propria and normal sub mucosa (Figure 2). While in the group of animals of induced colorectal cancer with 15 mg/kg AOM showing sever inflammatory cells infiltration between crypts, with sever and remarkable hyperplasia in glandular crypts in mucosa, histological crypts changes manifested by Aberrant crypts formation, with large and elongated crypts, darker nuclei of crypts epithelium (hyperchromatism), changes and atrophy in villi with thick epithelial lining (Figure 3-4). While rats that treated with STGs vaccine after colorectal cancer induction which shown severe histological alterations, significant hyperplasia in lymphoid tissue, with infiltration of inflammatory cells (Figure 5).

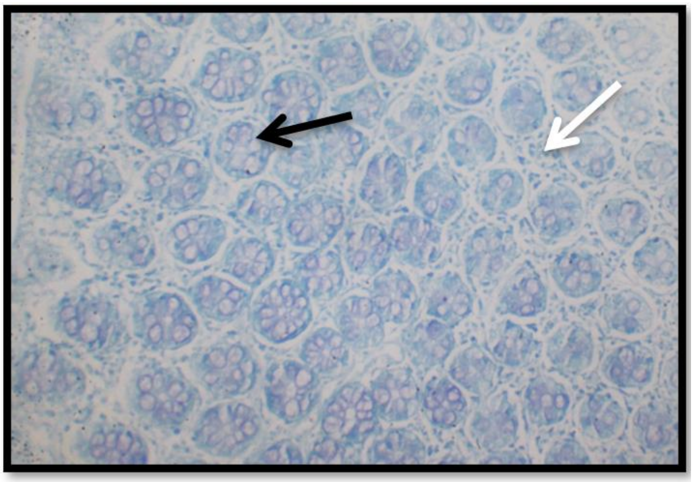

Figure 2. Photomicrograph of colon mucosa for a control animal revealing normal histological mucosal architecture, normal crypts (black arrow) with lamina propria (white arrow) (MB,10 X)

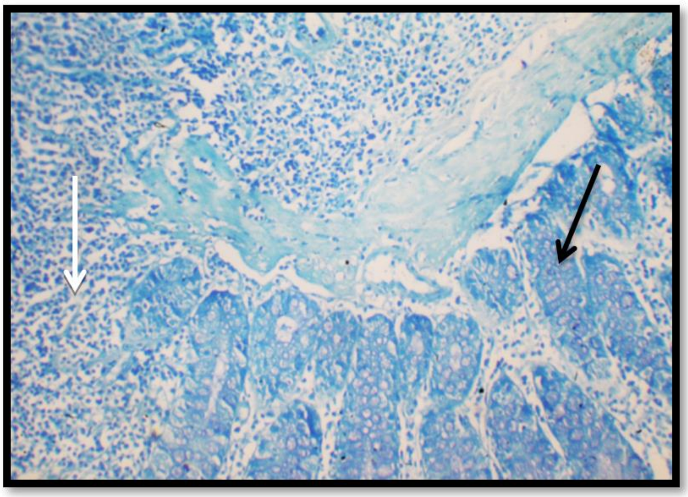

Figure 3. Photomicrograph of colon mucosa of induced colorectal cancer showing sever inflammatory cells infiltration between crypts (white arrow), with sever and remarkable hyperplasia in glandular crypts in mucosa (black arrow) (MB,10 X)

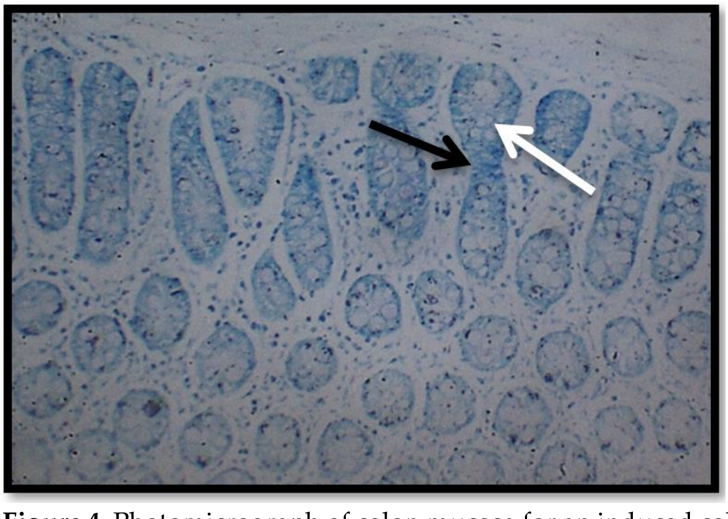

Figure 4. Photomicrograph of colon mucosa for an induced colorectal cancer animal showing histological crypts changes, manifested by aberrant crypts formation (black arrow) with thick epithelial lining (white arrow) (MB,10 X)

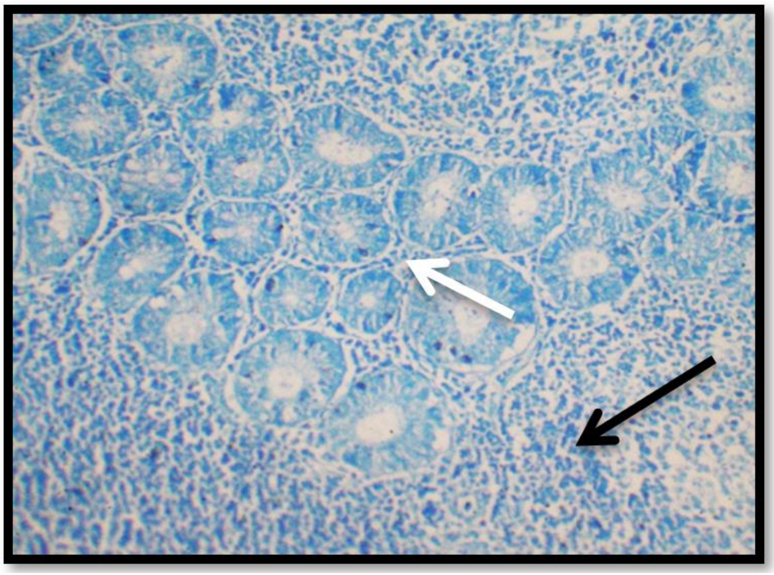

Figure 5. Photomicrograph of colon mucosa for a vaccine treated animal showing severe histological alterations, significant hyperplasia in lymphoid tissue (black arrow), with infiltration of inflammatory cells (white arrow) (MB,10 X)

The curative potentials of STGs vaccine were also examined via the current histological results accomplished by staining colon tissue with (H&E), revealed that the histological sections for colorectum of control group showing the regular histological architecture, significant normal mucosa, circular and rounded crypts with marked lumen, normal laminae propria, remarkable muscular layers and normal goblet cells (Figure 6).

The results showed that the AOM treated rat showing loss normal histological architecture, remarkable hyperplastic changes(ACF) and significant serration in ducts lumen and sever damage in mucosa, significant erosion in epithelial lining of the crypts with marked lumen dilation, sever infiltration of inflammatory cells in laminae propria, sever elongation in the crypts with marked hyperplasia in epithelial lining, remarkable change in crypts mucosa, represented by increase in goblet cells and present in different direction inside lumen with significant crowding in crypt lumen, sever hyperplasia in colonic lymphoid tissue and sever thickening in muscular layer with degeneration, other histological sections showed alterations on mucosal architecture, remarkable shortening and broadening of villi, adjacent villi are atrophied (Figure 7-9).

While the colon mucosa of rats treated with vaccine was showing severe histological alterations, significant hyperplasia in lymphoid tissue, with infiltration of inflammatory cells, epithelial damage and desquamation, injury in submucosal crypts and muscular layer thickening (Figure 10).

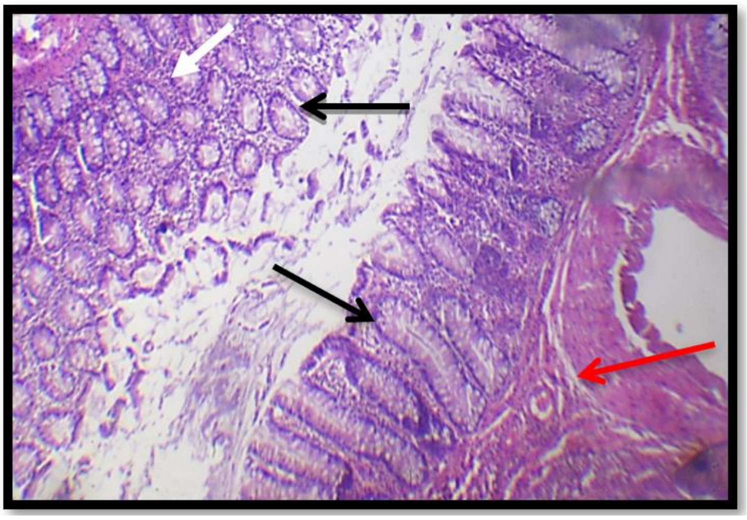

Figure 6. Photomicrograph of histological section for colorectum of normal rat showing the regular histological architecture, significant normal mucosa, circular and rounded crypts with marked lumen (black arrow), normal laminae propria (white arrow) and remarkable muscular layers (red arrow) (H & E, 10X)

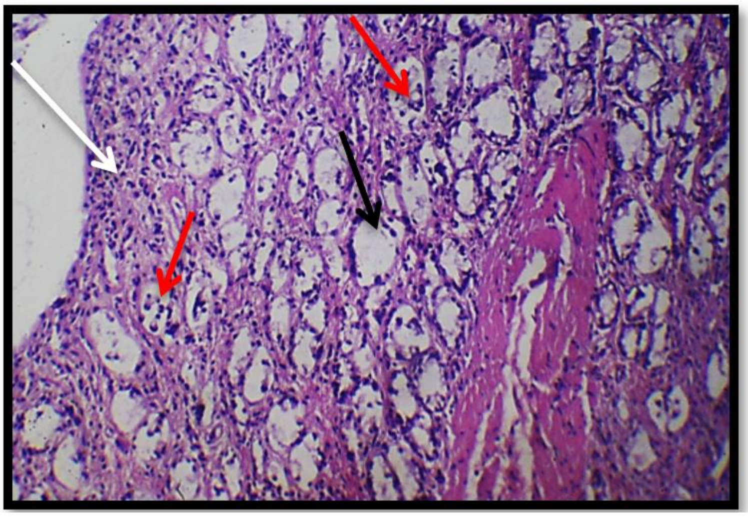

Figure 7. Photomicrograph of colorectal histological section for a AOM treated rat showing loss normal histological architecture, sever damage in mucosa, manifested by significant erosion in epithelial lining of the crypts (red arrow) with marked lumen dilation (black arrow), sever infiltration of inflammatory cells in laminae propria (white arrow) (H & E, 10X)

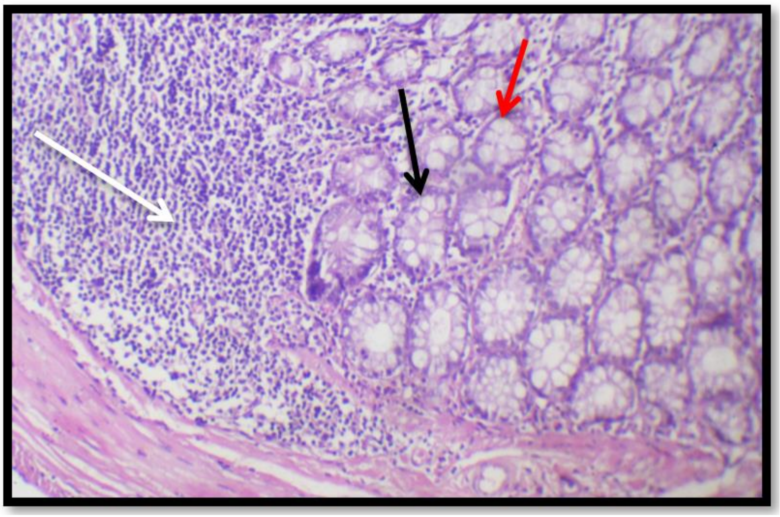

Figure 8. Photomicrograph of colorectal histological section for a AOM treated rat revealing significant histological alterations, remarkable change in crypts mucosa, represented by increase in goblet cells and present in different direction inside lumen (red arrow) with significant crowding in crypt lumen (black arrow) and sever hyperplasia in colonic lymphoid tissue (white arrow) (H & E, 10X).

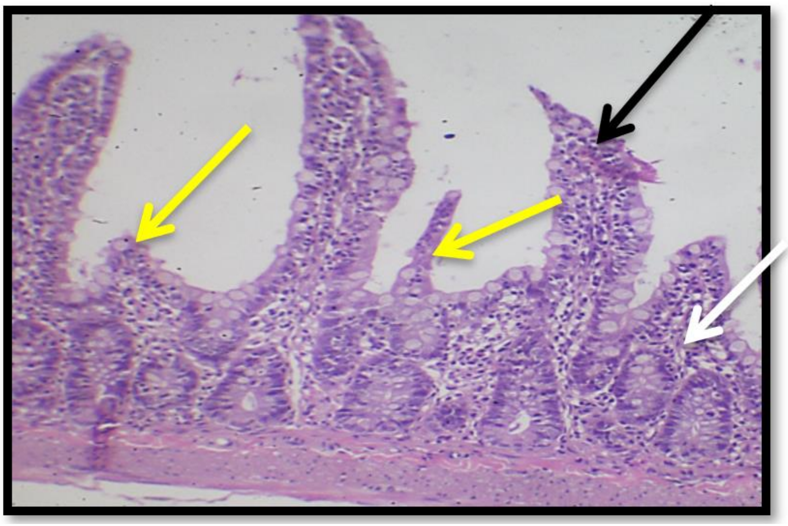

Figure 9. Photomicrograph of colon mucosa for a AOM treated rat showing histopathological alterations on mucosal architecture, remarkable shortening and broadening of villi (black arrow), adjacent villi are atrophied (yellow arrow), with sever inflammation in lamina propria (white arrow) (H & E, 4X).

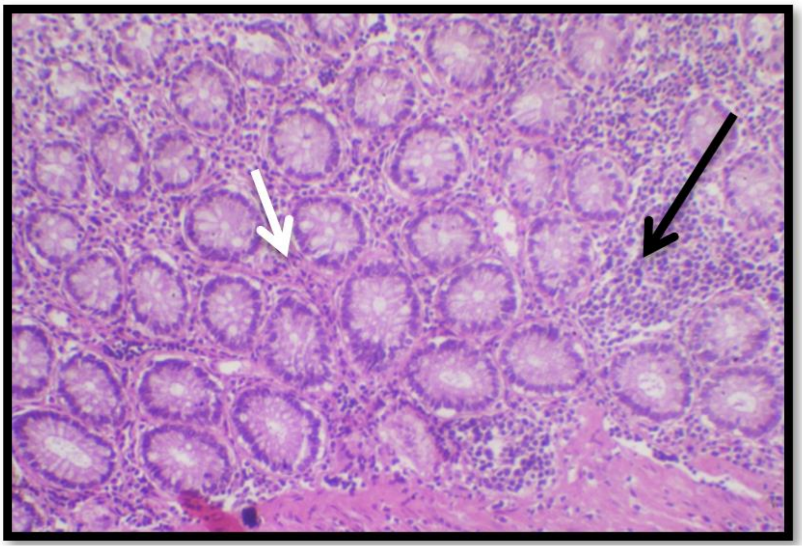

Figure 10. Photomicrograph of colon mucosa for a vaccine treated animal showing severe histological alterations, significant hyperplasia in lymphoid tissue (black arrow), with infiltration of inflammatory cells (white arrow) (H & E,10 X).

4. Discussion

The danger of colorectal cancer lies in the fact that it is a silent disease that is difficult to diagnose, and develops over the long term within the patient's body. Despite the progress of strategies for treating CRC, there is still need to identify and detect useful treatment without any or little side effects. The basis of this study depends on the colorectal cancer induction by AOM in rat model which indicated that STGs vaccine help in limiting the growth of CRC, leading to better survival rate of CRC patients.

The results of Microscopic examination of the current study mention that rats' colon treated with AOM were showing significant alterations in mucosal crypts architecture and significant damage with increase spaces between crypts ,sever inflammatory cells infiltration between crypts, with sever and remarkable hyperplasia in glandular crypts in mucosa, Aberrant crypts formation, with large and elongated crypts, this take place due to Azoxymethane administration which metabolize indirectly producing a toxic metabolites to the cells [22], [23].

Azoxymethane (AOM) used in this study is a potent, organ-specific colon carcinogen and ACF initiation and progression, that widely used to study cancer research, it is an indirect carcinogenic compound, and a gene mutation agent causing sever alteration in the nucleic acids, it is activated in the liver, producing metabolites which induce inflammation, its cytotoxicity is by mediate oxidative stress [24], [25].

The ACF induced by AOM in rodents persist and grow as clusters of abnormal tube like glands in the lining of the colon and rectum, so the growth features of ACF have been used to identify initiators and modulators of colon cancer progression, and may represent one of the earliest changes that can be seen in the development of colon cancer. These foci do not appear immediately after the carcinogenic chemical is administered, but rather take time as their number increases over time. The researcher [26] found that when he injected laboratory rats with AOM at a concentration of 15 mg/kg, the abnormal foci appeared 6 weeks after the second injection with AOM. The researchers [27] found that the appearance of the abnormal foci occurred 8 weeks after the second injection with AOM at a concentration of 15 mg/kg for the laboratory rats.

The rat model used in this experiment is better valued by interested in the study of colon tumor development in the setting of genetically predisposed to a high incidence of tumors and cancers and inflammation, the extent of disease noticed in rodents can vary considerably depending on animal strain and housing conditions (feeding, crowded cage, pollutions) [28], [29].

Many studies have shown the role played by bacteria as therapeutic organisms in dealing with colon cancer induced in laboratory rats. The researcher [30] reported that the cells and components of *Lactobacillus acidophilus* have a role in reducing the number of ACF, which are among the biomarkers studied in the colon of laboratory rats during the initiation stage of colon carcinogenesis.

Colon mucosa sections in rat treated with STGs vaccine showing severe histological alterations, significant hyperplasia in lymphoid tissue with infiltration of inflammatory cells. The intact structure of *S. typhimurium* ghost which represented by bacterial cell membrane with its critical components such as lipopolysaccharide (LPS) that could be definitive in the activation and mobilization of immune cells and production of TNF-α, the TLR4 signaling pathways involved in the cytokine expression that induced by *Salmonella*. Moreover, preventing the proliferation of cancer cells by encouraging the apoptosis pathway, and preventing the formation and development of tumors by enhancing and strengthening the immune system [31, 32], enhancing and support the infiltration of immune cells to the tumor microenvironment like T and B lymphocytes, neutrophils, natural killer cells and macrophages [33-37].

In a study [38] in which vaccine of dead *S. typhimurium* cells mixed with an adjuvant then applied to mice, results of mice immunization with the mixture revealed elevated cellular immunity which induces both humoral and cell-mediated immunity, which could be monitoring by mononuclear cells infiltration and histopathological lesions**.**

5. Conclusion

The main goal of this research is to highlight the successful role from introduction bacteria in its reduced or dead form as safe and promising strategies for immunotherapy of colorectal cancer and thus identify new treatment strategies. In conclusions, our results provide experimental evidence about the toxic and carcinogenic effects of Azoxymethane (AOM) on colon tissue of laboratory rats, diagnosed through the appearance of inflammation and hyperplasia, as well as revealing the role of STGs in inhibiting tumor development through regulation the tumor microenvironment with regard immunity and induction of inflammation.

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Conflict of interest

The authors announce that there is no discord of interest.

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