International Journal of Medical Toxicology & Legal Medicine **Volume 28, No. 1, 2025**
e-ISSN: 0974-4614
p-ISSN: 0972-0448
https://doi.org/10.47059/ijmtlm/V28I5/032

Salmonella typhimurium ghosts vaccine: The promising enhanced link between immunity modulation and colorectal cancer induced in albino rats

Rana Abdul Hamza Al-Qarraawi[1], Hiyame Abdul Ridha Al –Awade[2]

[1,2]Department of Biology, College of Science, University of Kerbala, Karbala, Iraq
Email: r.alqarawe@uokerbala.edu.iq, heyamkerim@uokerbala.edu.iq

Received: 27.01.2025 Revised: 19.03.2025 Accepted: 21.04.2025

ABSTRACT
Despite the remarkable medical progress in cancer treatment, colorectal cancer remains one of the most diagnosed types and a major cause of death worldwide. This has demand for the development of new, more effective and selective techniques against cancer cells, including immunotherapy using bacteria such as therapeutic bacterial vaccines characterized by their ability to target cancer cells by disrupting immune tolerance within the tumor microenvironment and stimulating a long-term immune response. Therefore, the current study aimed to investigate the therapeutic role of Salmonellatyphimurium ghosts (STGs) vaccine to reduce the effect of Azoxymethane (AOM)-induced colorectal cancer in male albino rats (Rattusrattus), which were used as a model to achieve the required tests, which included two axes: the first axis included evaluating the safety of the locally prepared vaccine (which was prepared using critical chemical concentrations in the sponge-like reduced protocol) and applying the full experimental vaccination program, in addition to studying the effect of the vaccination program on the invasiveness of S. typhimurium by studying the immune response of vaccinated rats and comparing it with the results of the vaccine evaluation for unvaccinated rats, the second axis included investigating the immunomodulatory activity of the vaccine prepared on rats induced with colorectal cancer (before, during and after treatment with the full STGs vaccination program) by measuring the expression level of the immune biomarker Programmed death ligand - 1 (PDL-1), which is a transmembrane protein that acts as a ligand for the immune checkpoint receptor PD-1. It was concluded that the S. typhimurium ghost vaccine (STGs) has an important role as an immune stimulating factor in the body to activate systemic cytokine-secreting T cells, thus recruiting both the innate and acquired immune response for the purpose of improving or modifying the tumor-specific immune response, and as a promising tool for the treatment of colon cancer.

Keywords: Bacterial ghost , Colorectal cancer , PDL-1, Rats, Vaccines, Tumor immunity

INTRODUCTION

Malignant tumors or cancers have their status as one of the leading causes of death worldwide, despite the extensive research that has continued for decades regarding the nature of cancer, its causes and its pathological development. Even if it is not necessarily an inherited disease, it is a genetic disorder characterized by unregulated cell cycle divisions, which leads to un controlling cell growth and proliferation(1). Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive tract resulting from a directed carcinogenic process, with a cumulative mutational effect appearing over an average time period of 10 - 15 years (2).

The stage of cancer initiation and its development until the stage of its metastasis to different areas in the body depends on the components of the tumor microenvironment(TME) of innate and adaptive immune cells that are important in promoting its emergence and inhibition, as the rate of spread and dominance of these components can provide a predictive means for clinical diagnosis (3), and thus these tumor immune infiltrates contribute to being targets for cancer treatment, as the mechanisms related to how to increase anti-tumor immune activity have proven effective in various treatments for different cancers, including lung, breast, and skin cancer (4 -6).

Relying on a single mechanism for cancer immunotherapy is likely to generate resistance in the patient and thus reduce the effectiveness of the treatment, therefore, combined immunostimulating therapy or designed to target immune evasion pathways provides high effectiveness in maintaining the immune cycle of the tumor and acquiring the body's anti-cancer immunity(7). Thus, bacterial therapy emerged based on the use of live

weakened, inactivated or killed bacteria as a stimulant for the immune system through various mechanisms and pathways, and its application has been approved for long periods to fight cancer(8).In general, cancer immunotherapy mediated bybacteria is characterized by its ability to selectively stimulate the body's immunity to track cancer cells, in addition to its high effectiveness and low toxicity compared to other routine treatments(9).

The importance of therapeutic microorganisms for cancer is evident in their ability to selectively reproduce or preferentially accumulate within the tumor microenvironment, in addition to the possibility of employing them as carriers for a variety of antibiotics and therapeutic proteins(10), as well as their unique ability to penetrate and colonize solid tumors, leading to either limiting tumor growth or completely eliminating it(11). Many studies that addressed Salmonella-based treatments have presented the possibility of developing therapeutic or preventive vaccines for various cancers in order to activate anti-tumor immune memory in addition to their primary goal of eliminating solid tumor mass(12).

Salmonella typhimurium is a facultative anaerobic Gram-negative, flagellated, non-spore-forming rod (13). It has gained medical importance for treating various types of cancer by immunomodulation within the tumor environment, as it is a microorganism that can successfully reach its target to trigger a series of immune responses that have an important effect in eliminating cancerous growth and preventing its spread, thus reducing it and increasing the patient's chance of survival for the longest possible period (14,15).

Ghost cells of Salmonellatyphimurium are empty, non-living cell envelopes obtained by chemical induction with a number of simple chemicals at critical concentrations and following a sponge-like reducing protocol(SLRP), causing the formation of microscopic holes in their membranes, emptying the cell from its cytoplasmic and nuclear contents, but retaining its membrane structure and intact surface antigens (16), and thus retaining its ability to stimulate an immune response when used as a vaccine in the body, in addition to being non-toxic and well tolerated by the body(17). The method of chemically lysis of bacterial cells used in preparing bacterial ghosts is characterized by its ease and low cost. It is also useful, fast, and does not involve potential risks (18). The important role of cancer vaccines is limited to inhibiting tumor growth and there metastases through their ability to stimulate the immune system and modify its responses, thus facilitating its recognition and elimination of cancer cells(19).

Cancer cells use multiple ways to escape the immune system and thus grow, colonize and develop cancer, among these methods is their ability to activate various immune checkpoint pathways that have immunosuppressive functions, the most important of which are programmed death protein -1 (PD-1) and programmed cell death ligand-1 (PDL-1), which are the main immune checkpoint molecules(20).It is known that activated $CD8^+$ T cells express the PD-1 receptor protein, and once this receptor specifically binds to PDL-1 on cancer cells, this binding will lead to inhibition of $CD8^+$ T cell function and proliferation, consequently, blocking the interaction of PD-1 with its ligands can increase the activity of $CD8^+$ T cells against tumors, thus, PD-1 shows a high immunogenic expression level in activated and killer T cells when compared to other immune checkpoints(21).

Research has shown that Salmonella has the ability to reduce the gene expression of a transmembrane protein that acts as a specialized mediator of tumor immune tolerance (PD-L1) (22) , which binds to the immune checkpoint receptor PD-1 (one of the main gatekeeper of the immune response) (23) . This binding in the tumor state is likely to suppress the activation of effector T cells and TCR-mediated proliferation and reduce the production of cytokines coupled with increased expression of the surface protein PD-L1 associated with PD-1 T cells (24) . All this process is reflected in the case of resorting to Salmonella as a treatment (22).

The current study aimed to evaluate the effectiveness of STGs (in vivo) in stimulating or modulating the immune response within tumor microenvironment as a therapeutic toolin male albino rats which induced colorectal cancer with AOM.

MATERIALS AND METHODS

Bacterial strain and cultivation condition

Salmonellaenterica serovar Typhimuriumstrain was used in this study for BGs productionwhich provided byAl-Amen Center for Advanced Research and Biotechnology / Najaf /Iraq, the strain purity was confirmed by morphology of its colony onXLD selective media for Salmonella. The bacterial isolate was transferred to the laboratory and inoculation on slanted nutrient agartube followed by incubated at 37°C for 18 hrs. Thenthe isolate was stored at 4°C, the identity was confirmed by the automated VITEK2system.

Determination of the minimum inhibitory concentration (MIC) and minimum growth concentration (MGC) for NaOH, SDS, H_2O_2 and $CaCO_3$

The concentration for each materials was determined by using standard criteria as stated in (25). (10%) of each of NaOH and SDS was prepared as the stock solution, as well as H_2O_2 (30%). The standard serial dilutions method was applied to determine the values of (MIC) and (MGC) representing the diluted concentration sequence immediately after the MIC value (in which the first growth of the studied bacterial strain appears and

is distinguished).In case of $CaCO_3$, MIC and MGC were not determined and the used quantities equal to +1 which was 1.05 μg /mL and -1 was 0.35 μg /mL.

Preparation of STGs

The Salmonellatyphimuriumwas cultivated under static conditions in one-liter flask contains 500 ml Nutrient Broth for 72 hrs at 37°C,after the incubation period, the biomass was collected by centrifuging the medium at 4000 rpm/10min, then washed with (0.5%)physiological salt solution, then the cells biomass was collected , re-suspended in distilled water in order to give the final image of the prepared biomass for each of the NaOH, SDS and H_2O_2compounds used.

For S. typhimurium Ghosts (STGs)preparing, the Sponge Like Reduced Protocol(SLRP) was used by following two experiments with different parameters (Table 1), and the two experiments were chosen from the results gained by (25) with the aim of obtaining the most favorablestatus for preparing bacterial ghosts (BGs), and they are essentially experiments No. (I and II) in the original SLP and as shown in the SLRP according to what was stated in (26).

Table 1 : Preparation of STGs ghosts using the reduced sponge-like protocol.

Experiment variables				
Experiment.No.	NaOH	$H_2O_2CaCO_3$	SDS	Shaking rate /temperature
I -1	1	1	1	
II -1	-1	-1	1	1

Evaluationand Characterization of STGs

The high-quality of STG preparations was proved by using standard criteria depending on staining with crystal violet for 2 min and then visualized by the aid of light microscope by using amplification power of 100 x to investigate the cellular external surface 3D shape integrity as either intact or deformed,and the morphology of STGs in comparison with live S. typhimurium cells was examined by scanning electron microscope (SEM) in order to visualization of the formed trans-membrane tunnels. Additionally, the quality of produced ghosts was determined by the quantification of released DNA and protein concentrations in the supernatant before washing process using spectrophotometrat 260 and 280 nm, respectively(27).

To determinetheSTGs viability and to tested for the chancefor the presence of any viable cells ,samples of the prepared STGs were cultured on MacConkey agar, SS agar and/or XLD selective agar medium, the cultured plates were incubated at 37°C for 2 days to ensure the purity of the vaccine and its absence of live bacteria and contamination(26).After the examination to the sterility and safety of the prepared STG vaccine ,it stored at a 4°C temperature until used in the following steps of the study.

Agglutination test for STGs surface antigen-antibodies reaction (the Rapid Slide test) was performed using standard antisera prepared by Mediaclone Company to determine the reaction of the surface antigen of STGs and its specific antibodies, and to determine the specific antibodies against Salmonella, according to the method of (28).

Experimental animals and Housing requirement

In this study , experimental male Rattus rattus (4 to 5 week-old) and with body weight between (50-75) gram were used for colorectal cancer induction by injection with carcinogenic material AOM and followed by treatment with bacterial ghosts as vaccine. All rat experiments were conducted according to the ethical recommendation for work with live animals and it was strictly directed following the scientific committee - Kerbala University Number D.A./14/6267 in 5/12/2022. And accordance to the National Academy of Sciences Guidelines for the Care and Use of Laboratory Animals(29). Male albino rats were housed in the animal house - College of Pharmacy - Kerbala University. Initially , they were housed for a week under monitoring at certain conditions in regard to environmental sterilization and microbial safety under a 12 hrs dark/light cycle before being used to ensure their activity and vitality. The experimental rats including 35 rat were distributed into groups in plastic cages ,every cage housed with 5 rat and designated for ranching them in which there floors were spread with sawdust with replace them from time to time. Essential conditions in regard of temperature, ventilation, appropriate humidity, and clean water were conducted. Animal feed ad libitum and drinking were given freely , the above instructions were followed with laboratory rats before starting the experiment to ensure that the animal adapts to its new environment(along 2-weeks).

Experimental Design , Induction of coloncancer and vaccination

1- **Experimental protocol for vaccination with STGs**

The animals of this experiment were randomly divided into three groups, with five animals for each group, as follows (17):

A. The first group: The control group was injected subcutaneously with normal saline solution only according to the experimental design.
B. The second group: A single dose was given orally in equal volumes (1:1) of STGs + Freund's adjuvant at a dose of 100 µl/rat.
C. The third group: A single dose was injected subcutaneously in equal volumes (1:1) of STGs + Freund's adjuvant at a dose of 100 µl/rat.

All experimental animals were vaccinated three times at two-week intervals. The STGs vaccine was given with a primer dose on day 0, followed by booster doses on days 14 and 28 (30). Serum was collected from each animal before each vaccine dose and after completion of the vaccination program(42 days) and serum antibodies were tested using agglutination test.

2- Studying the effect of the vaccine on the invasiveness of a bacterium S. typhimurium (Challengetest)

After completing the entire vaccination program as mentioned in the above paragraph (1-C), each rat was infected by subcutaneous injection with 1 ml of live S. typhimurium bacterial suspension at a concentration of (10^8 cells/ml) once, then 1g of stool samples were collected from each group within one week of infection from the first day of the challenge experiment with live bacteria and cultured on (SS-agar) medium, and after 7 days all rats were sacrificed and dissected and a part of the liver and spleen was taken separately, then mashed with 1ml of phosphate buffer saline (PBS) and the resulting suspension was homogenized by a rotary mixer, and cultured on (SS-agar) medium, all plates were incubated at 37°C for 24 hrs and the culture results were read by calculating the bacterial count for the purpose of further confirmatory tests (17,18).

3- Study of the interaction between vaccination with STGs and live cells of S. typhimurium and the carcinogen (AOM) in laboratory rats

The study was conducted from February 2023 to August 2023, where 20rats were allocated for this experiment, divided into four groups, with 5 rats for each group, according to (31), as follows:

A. First group: negative control that non injected or dosed with any substance and has the standard feed and drank tap water. Blood samples were collected and animal was dissected when needed during the experimental conditions.
B. Second group: positive control that was injected with 15 mg/kg of animal weight with AOM , animals were injected once a week for two consecutive weeks. Blood samples were collected and animals was dissected according to the experimental conditions.
C. Third group: animals that were injected with a single dose subcutaneously with equal volumes (1:1) of STGs + Freund's adjuvant at a dose of 100 µl/rat after 20 weeks from the second injection of the carcinogenic AOM. The entire vaccination program continued as stated in paragraph (1-C). Then blood samples were collected and dissected after that.
D. Fourth group: animals treated with the carcinogenic AOM according to the method mentioned in paragraph (B) above, which were subjected to interference with live bacteria by subcutaneous injection with 1mlof live S. typhimurium bacterial suspension at a concentration of 10^8 cells/ml once after the completion of the total vaccination program, after which stool and blood samples were collected and dissected as stated in paragraph 2.

Measurement of PDL-1 Levels

The level of PDL-1 in the serum samples collected on day0, 14,28,42 of the experimentalrats under study (as in paragraph 3 above) was estimated using direct enzyme-linked immunosorbent assay (ELISA Sandwich) according to the method of the diagnostic kit prepared by (ELK Biotechnology /China) which is the immunological standard Rat PDL-1 (Programmed Cell Death Protein Ligand -1) ELISA Kit with serial number (ELK0661).The concentration of PDL-1 was determined in each sample by a standard curve and multiplied by the initial dilution factor, levels of PDL-1 were expressed as pg/mL. A standard curve was constructed by plotting the mean absorbance for each standard against its concentration on semilogarithmic graph paper with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

Statistical analysis

Statistical analysis of the data for estimating the expression level of immune parameter was performed using the Statistical Package for Social Sciences (SPSS) version 26 program, and the values of the standard deviation ± Standard Deviation (SD) were calculated for measuring four replicates, as the association between the studied parameter was evaluated using the Anova (Tukey's pairwise) test at the probability level ($p \leq 0.05$).

RESULTS

Determination of MIC and MGC

The main step for the purpose of producing chemically induced Salmonella ghosts (STGs) depends on determining the (MIC) and (MGC) of the different chemicals used. The MIC and MGC of NaOH and SDS were determined using the two-fold broth dilution method on(NB)media. The current study showed that the MIC and MGC results were 0.1 mg/ml and 0.01 mg/ml for NaOH and SDS, respectively. The MIC and MGC of hydrogen peroxide were 0.03% and 0.003%, while the MIC and MGC of calcium bicarbonate for the amount used at +1 were 1.05 µg/ milliliters while the value of -1 was 0.35 µg / milliliter.

STGs quality evaluation andIdentification tests

The high quality of Salmonella ghosts was determined by measuring the concentration of both DNA and protein released from the cells during the preparation steps using the NanoDrop spectrophotometer at 260 and 280 nm, respectively. The results showed that the cytoplasmic content for both DNA and protein was properly and correctly discharged during the preparation steps, as shown in Table (2).

Table 2: DNA and protein concentration at experiment one and twoforSTGs preparation

Basic Experiment			**H_2O_2 Step**		**Ethanol step**		
Experiment	**Protein %**	**DNA**	**Protein**	**DNA**	**Protein**	**DNA**	**(BGQ)**
No.	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	
I	2961.94	211.21	3535.47	278.62	3286	176.7	100
II	3452.8	387.51	3765.43	495.76	3221	288.6	95

The quality of the ghost cells was determined using light microscopy, as STG cells showed high bacterial ghost quality (BGQ) after staining with crystal violet and examining them under a microscope. The scanning electron microscope was also used to verify the integrity of STGs cells, which is an additional step to study the bacterial ghost quality (BGQ). As Figure (1) shows that the ghost cells of S. typhimurium contain intact outer shells and at least one membrane tunnel in the form of very small pores responsible for evacuating the cells from their contents of large molecules, as the cells were of good quality and maintained their three-dimensional structure.

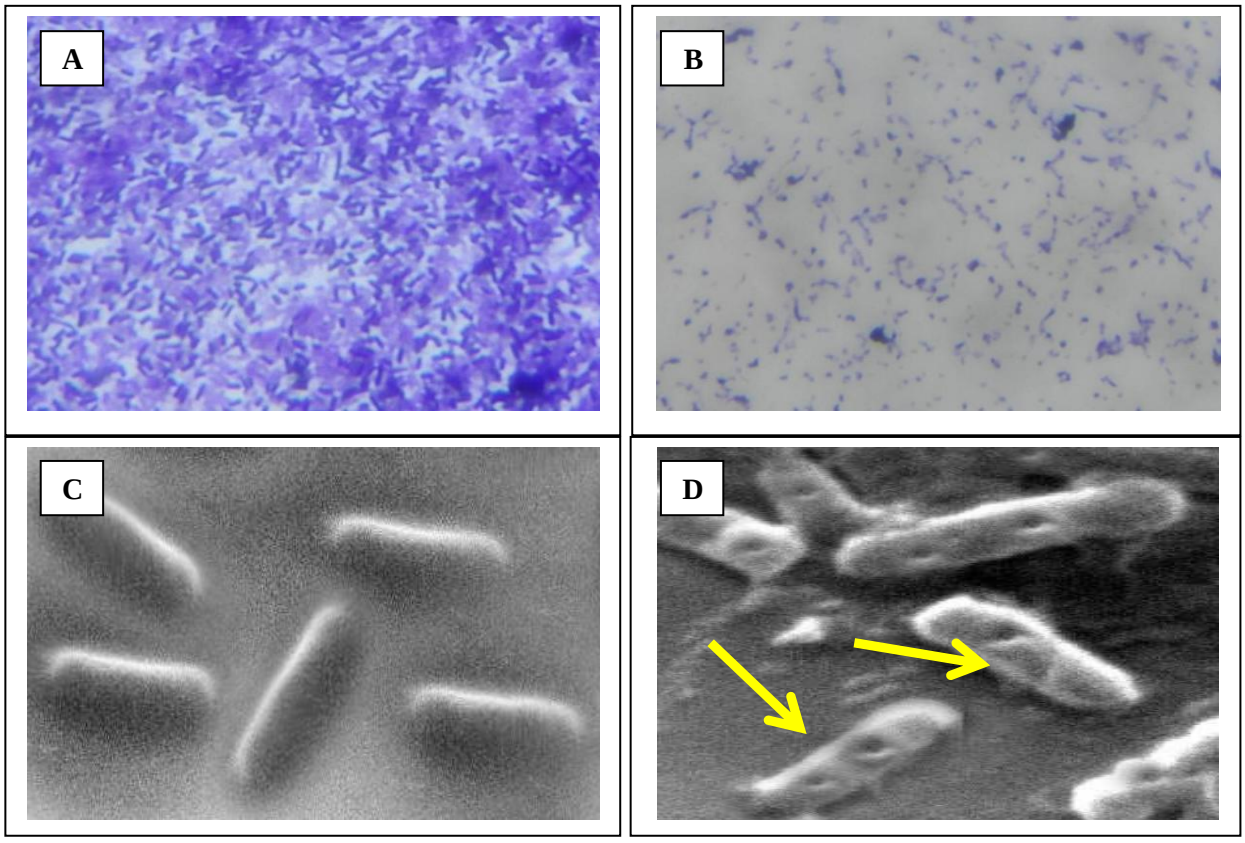

Figure 1: Live cells of S. typhimurium (A) and STGs (B) under light microscopy showing their intact three-dimensional structure (crystal violet stain, 100x magnification). Scanning electron microscope of live cells of S.

typhimurium (C) and STG ghosts (D) Yellow arrows show trans-membrane tunnels in the STGs (50,000x magnification).

To verify the viability of the produced STGs vaccine, and to ensure that the bacterial cells lose their ability to multiply and thus lose their ability to cause disease (pathogenicity) while still retaining their immunogenicity due to the presence of their surface antigens, this test was performed, and no bacterial growth was observed on culture media plates such as nutrient agar, MacConkey agar, and SS agar on which STGs swabs were cultured after incubation for two days at 37°C, which indicates that the prepared vaccine is not contaminated with the bacteria.

In order to determine the integrity of STGs surface antigen through interaction with its specific antibodies, an agglutination test was performed on S. typhimurium cells and STGs using the Rapid Slide test and using standard antisera. The results of the current study showed that agglutination was clearly evident when using standard antibodies with S. typhimurium cells and STGs prepared through the presence of granular aggregation, while the agglutination ability was absent in the negative control group, as shown in Figure (2). The appearance of agglutination is evidence of the presence of intact surface antigens on the surface of STGs prepared using the relevant protocol.

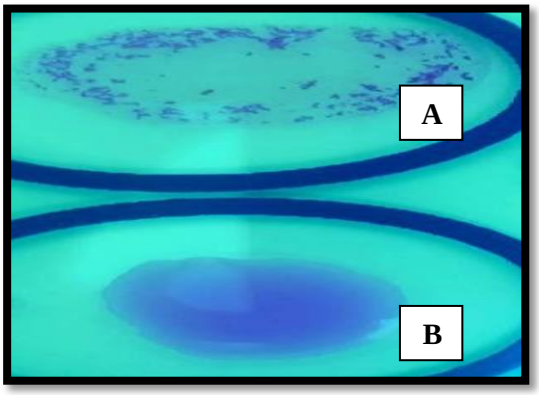

Figure 2:(A) Agglutination reaction of BGs prepared from S. typhimurium ,(B)negative control

Experimental protocol for vaccination with STGs

The results of the current study showed that the positive agglutination result on the glass slide was through the presence of milky-colored granular fog, as the ability of the antibodies present in the serum to interact with the antigen of the live bacteria was measured using the agglutinating ability test, which is shown in Table (3), as all tests of the subcutaneous vaccination group gave clear agglutination of the antigen-antibody reaction after treatment with the STGs vaccine (within one minute), compared to the oral vaccination group, which showed a weak agglutinating ability with the serum after treatment with the STGs vaccine and in the periods before the third treatment and after the end of the vaccination program (28 and 42) days, respectively, while the agglutinating ability with the serum was absent in the control group, which was treated with normal saline solution.

Table 3: Agglutination test for groups of sera treated with live S. typhimurium before and during the STGs vaccination program.

Groups	Agglutination test time	Results
Control	Before the first treatment (zero) day	-
	Before the second treatment (14) day	-
	Before the third treatment (28) day	-
	After the end of vaccination (42) day	-
Orally Vaccination	Before the first treatment (zero) day	-
	Before the second treatment (14) day	+
	Before the third treatment (28) day	+*
	After the end of vaccination (42) day	+*
SC Vaccination	Before the first treatment (zero) day	-
	Before the second treatment (14) day	+
	Before the third treatment (28) day	+
	After the end of vaccination (42) day	+

* Weak agglutination.

Bacterial Challenge test

In order to determine the effectiveness and immunological effect of the prepared vaccine against the pathogenic S. typhimurium, all rats in the experimental groups were injected subcutaneously with liveS. typhimurium at a concentration of (10^8 cells/ml) in two weeks after the last vaccination (the seventh week). The results as in Figure (3) showed that the experimental rats injected with the ghost immunogenic preparation remained alive and in good health after the injection, while most of the animals in the control group showed signs of the disease such as loss of desire to eat, weight loss, poor movement, and some of them died at different times after the injection.

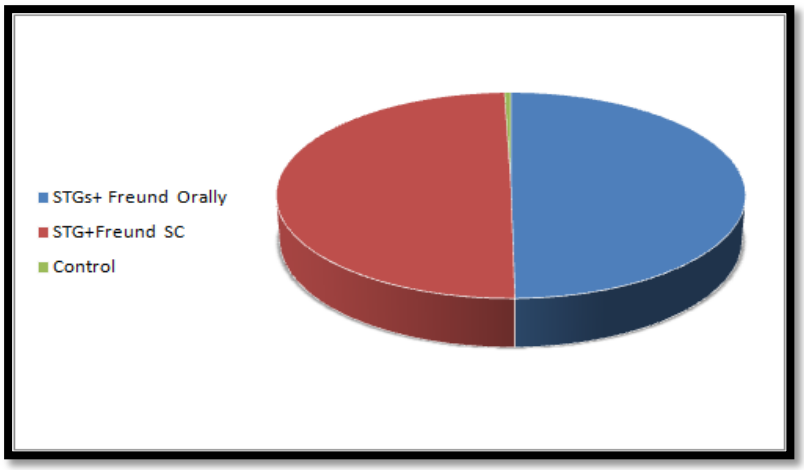

Figure 3: Percentage of live rats between vaccinated and non vaccinated groups according to the challenge experiment.

the interaction between vaccination with STGs and live cells of S. typhimurium and the carcinogen (AOM) in laboratory rats (Immunological study)

When looking at the results of the current study through Table (4), we find that the negative control group showed average levels of PDL-1 before treatment, and over time a gradual decrease in PDL-1 levels was observed, and at the end of the monitoring period, i.e. after 42 days, there was a rate and standard deviation indicating a relative stability in PDL-1 levels, but with a noticeable decrease. In contrast, the positive control group in which colon cancer was induced started with a low PDL-1 level before treatment, and after 14 days this group witnessed a significant increase in PDL-1 levels, indicating a strong response to the development of the disease after 42 days. Despite this decrease, the values remain high, indicating a clear effect of tumors on the PDL-1 level. As for the induced - vaccinated group, it showed high levels of PDL-1 before the start of treatment, after 14 days these levels continued to be relatively stable, then increased slightly after 28 days, to show a decline after 42 days compared to the other groups. When the results were statistically analyzed for multiple comparisons test, the results showed that the probability value for the negative control group was 0.8105, indicating no significant differences ($P>0.05$), while the probability value for the positive control group was 0.0102, indicating strong significant differences ($P<0.05$). As for the induced-vaccinated group, it showed a p-value of 0.00091, which also indicates a clear significant difference ($P<0.05$).

Table 4: Measurement the level of PDL-1 in the serum of rats induced with colon cancer and vaccinated with the STGs vaccine compared with positive control rats and healthy rats according to the serial time of vaccination

Groups / Experiment		Control Negative Mean ±SE	Control Positive Mean ±SE	Induced-vaccination Mean ±SE
Pre-treatment	**PDL-1 (Pg/ml)**	722.29 ± 10.85 A	362.83 ±30.134 B	1146.71±87.404 A
After 14 day of vacc.		647.15 ±21.599 A	952.505 ±9.04 A	1148.28 ±4.651 A
After 28 day of vacc.		523.04 ±22.48 A	1036.43 ±42.44 A	1151.51 ±35.88 B

After 42 day of vacc.		590.60 ±21.122 A	978.88 ±9.304 A	974.815 ±17.017 C
p-value		0.8105	0.0102 *	0.00091*

***Indicates significant differences at a significance level (P<0.05)**
SE = Standard Error
Different capital letters in the vertical direction indicate significant differences

DISCUSSION

Despite the progress in the CRC therapeutic strategies , patients suffering from the long-term and poor prediction of with the disease remains terrible ; therefore, it is still urgent to identify useful drugs with less side effects for the curative and prognosis of CRC. This study based on the AOM-derived CRC rat model indicated that STGs suppresses the growth of CRC, helping in better survival rate of CRC patients.

MIC and MGC determination is one of the important analytical step in STGs preparation in order to determine the main effect of variables that positively or negatively affected the quality of bacterial ghosts BGs preparation and to obtain a clearer picture to the effect of the chemical compounds on the Salmonella cell wall components in the points where the bacteria will bekilling or survive (26,32).

The results of the current study showed that a correct discharge process was achieved for both DNA and protein during the preparation steps, and high concentrations of the released protein were recorded in a higher quantity than the amount of DNA, especially in the treatment step with H_2O_2. This was proven by the study (17) when evaluating the quality of the produced STGs ghosts, as a high amount of proteins and DNA were released, amounting to 2975 µg/ml and 786 µg /ml, respectively, compared to untreated cells, which dose not recorded of any proteins or nucleic acidsrelease. The results of the current study also showed that the amount of DNA released in the second experiment when using H_2O_2 with a value of (-1) was greater than that in the first experiment (+1), as it amounted to 495.76 and 278.62 µg /ml, respectively, as shown in Table (2).

Salmonella cell walls are highly sensitive to SDS, which is responsible for destabilizing the cell wall and plasma membrane and forming pores, while treatment with hydrogen peroxide (H_2O_2) ensures the analysis and destruction of any remaining DNA (33). Accordingly, the protocol in question was called Sponge-like, as the chemical compounds contributed to creating microscopic pores in the bacterial cells during the steps of preparing Salmonella ghosts (STGs). The cells were gently pressed like sponges to release their contents without distorting their three-dimensional structure, which is the most important event,the basic idea behind this technique is to apply a simple method to prepare BGs by using critical concentrations of selected active chemical compounds and using them either individually or in combination in a precise combination (to ensure preventing cross-reactions), the method also included several steps of washing and low-speed centrifugation (enabling precise pressure on the cells) to ensure complete removal of the cell's cytoplasmic contents, an important step determines the viability of the cells or not based on determining the concentrations of MIC and MGC, one of which is responsible for cell death, while the other is responsible for determining and limiting the lethal effect of the chemicals used at the lowest concentration to allow the cells to remain viable (17, 18).

(34) reported that Chemically derived BGs undergo morphological and structural changes compared to their parent bacterial cells, and morphological analysis of these ghosts is usually performed using electron microscopy such as scanning electron microscopy (SEM), while maintaining morphological similarity and they retain the surface properties and structure of the parent bacterial cells, including outer membrane proteins, adhesion factors, and other surface components.

The chemically prepared S. typhimurium ghosts vaccine has the ability to induce humoral and cellular immune responses when given subcutaneously, the reason for the simple or low immune response to the BG vaccine given orally was explained by the mechanisms of gastrointestinal digestion in addition to the possible deliberate reflux by the animal itself (17). The results of the agglutination test for antibodies in the immune sera treated with live S. typhimurium before and during the vaccination program with the STGs vaccine for the current study showed that the level of antibodies was high, which indicates a high immune response from the animals injected with the antigens and then their production of antibodies at this high level, which indicates that the antigens of the bacteria under study are good immune stimulants in terms of activating the immune system and modifying the immune response at multiple levels.

Vaccines prepared from bacterial ghosts (BGs) of several pathogenic bacterial genera provide acceptable and safe protection for experimental animals, many scientific studies have aimed to determine the immunological efficacy of vaccines against the same pathogenic bacteria from which the vaccine was prepared, both studies (35 , 36) recorded 100% survival rates for experimental animals after the bacterial challenge experiment, and the vaccinated animals maintained their full vitality compared to the unvaccinated ones.

Many scientific studies have indicated the effect of bacteria as an immunotherapy on some immune parameters in cases of cancer, whether as a treatment with genetically weakened or killed bacteria used as a vaccine. In a study (37) on the role of weakened Salmonella in enhancing immunotherapy for PD-L1 trapped in a model of colorectal cancer, the effect of a reduced dose of Salmonella on the expressive capacity of inhibitory checkpoint

molecules represented by PD-1 and PD-L1 on tumor T cells was analyzed using flow cytometry, the results showed that giving a dose of Salmonella led to a significant decrease in the percentages of TILs $CD4^+$ and $CD8^+$ cells expressing PD-1 checkpoint molecules to reach an average of approximately 23% and 7% among TILs $CD4^+$ and to approximately 51% and 33% among TILs $CD8^+$ cells,considering these percentages together, the low percentage of T cells expressing ligands Inhibitory checkpoint ligands within tumor tissues may contribute to the reversal of inhibitory factors imposed on T cells and thus activate anti-cancer immunity. The association of Salmonella-induced downregulation of PDL-1 in tumor tissues with inhibition of the AKT/mTOR/p70S6K signaling pathway after treatment of tumor-induced mice with attenuated Salmonella was explained.

Study (5) provided additional mechanisms to explain the increase in the number of tumor-specific reactivated T cells, and in particular showed that the negative regulation of tumor PDL-1 by Salmonella contributed to the reactivation of tumor-specific T cells, the role of this negative regulation of PDL-1 in increasing T cell activity was evaluated by conducting a co-culture experiment using tumor-bearing mouse models,the results showed that the number of activated $CD8^+$ T cells increased significantly while the number of T cells undergoing apoptosis decreased.

CONCLUSION

The results of the study revealed a new vision about the possibility of modifying the microenvironment of colorectal cancer immunologically using cancer vaccines, including STGs vaccine were produced by chemical induction with simple materials using the SLRP, which retained their surface antigens and intact morphological characteristics, through the ability of serum to agglutinate with live Salmonella surface antigens after following the complete vaccination program. Also it is regarded as a promising platform for many application areas, including as vectors for chemotherapy and antibiotics to treat tumors and a wide range of infectious and non-infectious diseases. In addition, it reflects an advanced image of the possibility of using the vaccine in a safe and intact manner, as it maintains its antigenic immunogenicity and its distinguished efficiency in presenting the antigen and targeting specific in immunotherapy. Salmonellatyphimuriumghost vaccines play their role in immunotherapy or their preventive role in cancer by activating T cells specific to the tumor antigen, which are the main killer monitor of cancer cells. The cancer vaccine is also important in changing the properties of immune cells in terms of function and appearance within the tumor environment, thus enhancing the effectiveness and response rate of the immune parameter, the programmed cell death ligand 1(PDL-1), so Vaccination with STGs caused a decrease in the expression level of the immune protein PDL-1 in vaccinated rats induced with colorectal cancer compared to the positive control group which showed a significant response to the effect of the disease on PDL-1 levels. Finally , Chemically induced S. typhymurium ghosts could represent a promising vaccine that contributed to enhancing the immune response even in the presence of colon cancer. The results suggest the possibility of using the vaccine as an adjuvant therapy to improve the immune status of rats with cancer.

ACKNOWLEDGMENT

The author is grateful to the University of Kerbala /College of Sciences , and to the training center of the Iraqi Biotechnology Company in Al-Qadisiyah Governorate , for all the facilities to achieve this study.

CONFLICT OF INTEREST

The authors declare that there is no collision of interest.

REFERENCES

1. Pangilinan, C.R and Lee, C.H.(2021). Highlights of Immunomodulation in Salmonella-Based Cancer Therapy. Biomedicines. 29;9(11):1566. doi: 10.3390/biomedicines9111566. PMID: 34829795; PMCID: PMC8615479.
2. Yu W, Zhang J, Chen Z, Wang S, Ruan C, Zhou W.&et al.(2020) .Inhibitory Effect of a Microecological Preparation on Azoxymethane/Dextran Sodium Sulfate-Induced Inflammatory Colorectal Cancer in Mice. Front. Oncol. 10:562189. doi: 10.3389/fonc.2020.562189.
3. Fridman, W.H.; Zitvogel, L.; Sautès–Fridman, C.; Kroemer, G.(2017**).**The Immune Contexture in Cancer Prognosis and Treatment. Nat. Rev. Clin. Oncol., 14, 717–734. [CrossRef]
4. Kuan, Y.-D. and Lee, C.-H. **(**2016**).**Salmonella Overcomes Tumor Immune Tolerance by Inhibition of Tumor Indoleamine 2, 3-Dioxygenase 1 Expression. Oncotarget, 7, 374–385. [CrossRef] [PubMed]
5. Chen, M.-C.; Pangilinan, C.R.; Lee, C.-H. **(**2019**).**Salmonella Breaks Tumor Immune Tolerance by Downregulating Tumor Programmed Death-Ligand 1 Expression. Cancers, 12, 57. [CrossRef] [PubMed]
6. Lee, C.-H.; Hsieh, J.-L.; Wu, C.-L.; Hsu, P.-Y.; Shiau, A.-L. **(**2011).T Cell Augments the Antitumor Activity of Tumor-Targeting Salmonella. Appl. Microbiol. Biotechnol., 90, 1381–1388. [CrossRef]

7. Wu Z, Li S, Zhu X. (2021).The mechanism of stimulating and mobilizing the immune system enhancing the anti-tumor immunity. Front Immunol.; 12: 682435.
8. Zhou X, Ni Y, Liang X, Lin Y, An B, He X, et al. (2022).Mechanisms of tumor resistance to immune checkpoint blockade and combination strategies to overcome resistance. Front Immunol.; 13: 915094.
9. Wang Y. (2023). Bacterial therapy: a promising strategy for cancer immunotherapy. Cancer Biol Med.;20(11):795–8. doi: 10.20892/j.issn.2095-3941.2023.0292. PMID: 37964508; PMCID: PMC10690880.
10. Kikuchi T, Shimizu H, Akiyama Y, Taniguchi S. In situ delivery and production system of trastuzumab scFv with Bifidobacterium. Biochem Biophys Res Commun. 2017 Nov 4;493(1):306-312. doi: 10.1016/j.bbrc.2017.09.026. Epub 2017 Sep 7. PMID: 28890351.
11. Kiaheyrati N, Babaei A, Ranji R, Bahadoran E, Taheri S, Farokhpour Z. Cancer therapy with the viral and bacterial pathogens: The past enemies can be considered the present allies. Life Sci. 2024 Jul 15;349:122734. doi: 10.1016/j.lfs.2024.122734. Epub 2024 May 22. PMID: 38788973.
12. Saxena, M.; van der Burg, S.H.; Melief, C.J.M.; Bhardwaj, N. Therapeutic Cancer Vaccines. Nat. Rev. Cancer **2021**, 21, 360–378. [CrossRef] [PubMed].
13. Esfahani, K.; Roudaia, L.; Buhlaiga, N.; del Rincon, S.V.; Papneja, N.; Miller, W.H. (2020**).**A Review of Cancer Immunotherapy: From the Past, to the Present, to the Future. Curr. Oncol., 27, 87–97.
14. Popoff, M.Y.; le Minor, L.E.(2015).Salmonella. In Bergey's Manual of Systematics of Archaea and Bacteria; Wiley: Hoboken, NJ, USA,.
15. Pangilinan, C.R. and Lee, C.-H. (2019**).**Salmonella-Based Targeted Cancer Therapy: Updates on A Promising and Innovative Tumor Immunotherapeutic Strategy. Biomedicines, 7, 36.
16. Kudela P, Koller VJ, Lubitz W. (2010).Bacterial ghosts (BGs)-Advanced antigen and drug delivery system. Vaccine.;28:5760–7. doi:10.1016/j. vaccine.2010.06.087. PMID:20619379.
17. Rabea ,S.; Yassin, A. S. ; Mohammed ,A. F. ; Salem- Bekhit, M. M. ; Alanazi, F. K. &et al. (2022). Immunological characterization of the chemically prepared ghosts of Salmonella Typhimurium as a vaccine candidate. BMC Veterinary Research;18:72.https://doi.org/10.1186/s12917-021-03112-4 .
18. Vinod, N.; Noh, H. B.; Oh, S.; Ji, S.; Park, H. J.; Lee, K.-S.; Kim, S. C.; Park, H.-O.; Yang, J.-S.; Choi, C. W. (2017).A Salmonella Typhimurium Ghost Vaccine Induces Cytokine Expression in Vitro and Immune Responses in Vivo and Protects Rats against Homologous and Heterologous Challenges. PLOS ONE, 12 (9), e0185488. https://doi.org/10.1371/journal.pone.0185488.
19. Zhao J, Xu Y, Ma S, Wang Y, Huang Z, Qu H, &et al. (2022). A Minimalist Binary Vaccine Carrier for Personalized Postoperative Cancer Vaccine Therapy. Adv Mater.;34(10):e2109254. doi: 10.1002/adma.202109254. Epub 2022 Jan 31. PMID: 34984753.
20. Lin X, Kang K, Chen P, Zeng Z, Li G, Xiong W, &et al. (2024). Regulatory mechanisms of PD-1/PD-L1 in cancers. Mol Cancer ;23(1):108. doi: 10.1186/s12943-024-02023-w. PMID: 38762484; PMCID: PMC11102195.
21. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, &et al. (2015).PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. ;372:2509–2520. doi: 10.1056/NEJMoa1500596.
22. Chen, M.-C.; Pangilinan, C.R.; Lee, C.-H. (2019).Salmonella Breaks Tumor Immune Tolerance by Downregulating Tumor Programmed Death-Ligand 1 Expression. Cancers, 12, 57. [CrossRef] [PubMed]
23. He, X. and Xu, C. (2020).Immune Checkpoint Signaling and Cancer Immunotherapy. Cell Res., 30, 660–669. [CrossRef] [PubMed]
24. Blank, C.; Gajewski, T.F.; Mackensen, A. (2005).Interaction of PD-L1 on Tumor Cells with PD-1 on Tumor-Specific T Cells as a Mechanism of Immune Evasion: Implications for Tumor Immunotherapy. Cancer Immunol. Immunother., 54, 307–314. [CrossRef] [PubMed]
25. Amara, A. A.; Neama, A. J.; Hussein, A.; Hashish, E. A. & Sheweita, S. A.(2014). Evaluation the surface antigen of the Salmonella typhimurium ATCC 14028 ghosts prepared by ―SLRP. The Scientific World Journal ; ID 840863 :1-6.
26. Amara, A.A.; Salem-Bekhit, M.M. & Alanazi, F.K. (2013). Sponge-like: a new protocol for preparing bacterial ghosts. Sci World J; 1-10. ID 545741.
27. Brown, A.E. &Auburn, U. (2012).Benson's Microbiological Applications Laboratory Manual in general Microbiology Complete Version. 12th Ed. McGraw- Hill.
28. Cruickshank, R. (1965).Medical Microbiology, E. and S. Livingstone, London, UK, 11th edition.
29. Garber JC; Barbee RW; Bielitzki JT; Clayton LA; Donovan JC; Hendriksen CF; Kohn DF; Lipman NS; Locke PA; Melcher J; et al. Guide for the Care and Use of Laboratory Animals; The National Academic Press: Washington, DC, USA, 2011; ISBN978-0-309-15400-0.
30. Vinod , N.; Oh , S.; Kim , S.; Choi, CW; Kim, SC; Jung, CH. (2014).Chemically induced Salmonella enteritidis ghosts as a novel vaccine candidate against virulent challenge in a rat model. Vaccine.; 32: 3249±55. https://doi.org/10.1016/j.vaccine.2014.03.090 .

31. Jabbar, A.A.; Ibrahim, I.A.A.; Abdullah, F.O.; Aziz, K.F.; Alzahrani, A.R.; Abdulla, M.A. (2023). Chemopreventive Effects of Onosma mutabilis against Azoxymethane- Induced Colon Cancer in Rats via Amendment of Bax/Bcl-2 and NF-_B Signaling Pathways. Curr. Issues Mol. Biol.; 45: 885–902. https:// doi.org/10.3390/cimb45020057 .
32. Park, SY. (2023). Chemically induced bacterial ghosts: a novel approach for advancing biomedical applications. Mol. Cell. Toxicol. ;19: 657–665 https://doi.org/10.1007/s13273-023-00389-4.
33. Amara, A. A. A. F. & El-Baky, N. A. (2022).Evacuation of Yeast and Fungal Cells Using the Sponge-like Protocol: A New Approach for Fungal Control. Journal of Applied Pharmaceutical Science; 12(4): 199–200.https://doi.org/10.7324/japs.2022.120421.
34. Ma, Y.; Cui, L.; Wang, M.; Sun, Q.; Liu, K.&Wang, J. (2021). A Novel and Efficient High-Yield Method for Preparing Bacterial Ghosts. Toxins (Basel) ;13(6):420. doi: 10.3390/toxins13060420.
35. Bahy, R.; Gaber, A.; Zedan, H. & Mabrook, M. (2023). New typhoid vaccine using sponge-like reduced protocol: development and evaluation. Clinical and Experimental Vaccine Research; 12(1):70-76.
36. Sheweita, S.A.; Amara, A.A.; Gamal, H.; Ghazy, A.A.; Hussein, A. & Bahey-El-Din, M. (2022).Bacterial Ghosts of Pseudomonasaeruginosa as a Promising Candidate Vaccine and Its Application in Diabetic Rats. Vaccines; 10: 910. https://doi.org/10.3390/ vaccines10060910.
37. Al-Saafeen, B.H.; Al-Sbiei, A.; Bashir, G.; Mohamed, Y.A.; Masad, R.J.; Fernandez-Cabezudo, M.J.& Al-Ramadi, B.K. (2022). Attenuated Salmonella potentiate PD-L1 blockade immunotherapy in a preclinical model of colorectal cancer. Front Immunol.;13:1017780.