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Enhancement of Lactic Acid Bacteria by Gamma Radiation to Inhibit Antibiotic Resistance of some *Salmonella* spp.

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Forty nine isolates were isolated from 34 food samples using *Salmonella-Shigella* medium, 10 of them were identified biochemically as *Salmonella* sp., two of them were multidrug-resistant, and they showed a resistance to seven tested antibiotics (ampicillin, streptomycin, gentamicin, nalidixic acid, ciprofloxacin, tetracycline, amoxicillin). Molecular identification of these isolates proved that they were *Citrobacter ferundii* and *Proteus mirabilis*. The antimicrobial activity for *Lactobacillus acidophilus* ATCC 4356 and *Streptococcus thermophiles* ATCC 19987 mixture and their cell-free supernatant mixture were activated by low doses of gamma radiation (5 Gray for lactic acid bacteria & 20 Gray for supernatant). Results proved that on applying the two previously activated mixtures on chicken carcasses, supernatants completely killed the three pathogens (*Citrobacter ferundii*, *Proteus mirabilis* and *Salmonella typhi* ATCC 14028 reference strain) during 4 hours while the lactic acid bacteria mixture killed them after 3 hours.

Keywords: Lactic acid bacteria, Gamma radiation; Salmonella sp., Citrobacter ferundii, Proteus mirabilis

Introduction

Strains of *Salmonella* spp. with resistance to antimicrobial drugs are now widespread in both developed and developing countries. In developed countries, it is now increasingly accepted that such strains are zoonotic in origin and acquire their resistance in the food-animal host before onward transmission to humans through the food chain [1]. High level of antimicrobial resistant *Salmonella* spp. occurrence is probably an indication of their frequent usage both in the animal and public health sectors [2]. Lactic acid bacteria (LAB) are generally recognized as safe (GRAS microorganisms) and play an important role in food and feed fermentation and preservation either as the natural microflora or as starter cultures added under controlled conditions. LAB species with antagonistic activity are used for improving the quality and safety of meat and dairy products [3].

LAB can produce a wide range of antimicrobial metabolites which include organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins. Their antimicrobial activity can contribute in a number of ways towards improving the quality of meat. Microbiological safety and stability, for example, can be improved through controlling the growth of other micro-organisms, including inhibition of pathogenic bacteria such as *Salmonella* spp., *Proteus* spp. and *Citrobacter* spp. and antagonism towards bacterial species such as Enterobacteriaceae which are commonly associated with poultry meat and spoilage of poultry meat products [4]. Furthermore, several LAB strains, including *Lactobacillus acidophillus* and *Lactobacillus spp.*, have been shown to possess in vitro proteolytic and/or antioxidative

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abilities [5], which could have an impact on chemical processes such as proteolysis and lipid oxidation and therefore could influence storage shelf life and quality of poultry meat products.

The aim of the present study is to use LAB in the elimination of antibiotic resistant pathogenic *Citrobacter* sp., *Proteus* sp. and *Salmonella* sp. in food and medical fields

Materials and Methods

Sample collection

A total of 34 different food samples representing different food sources (poultry meat, dairy products and vegetables) were collected from local markets. All the samples were kept in sterile insulated bags iced and transported to the laboratory within three hours.

Sources of bacterial strains

Salmonella enterica serovar Typhi ATCC NO.14028 was obtained from American Type Culture Collection (ATCC).

Isolation of pathogenic strains

Thirty-four different food samples collected from local Egyptian market were tested for the presence of *Salmonella* (*Salmonella* detection) according to the procedures of the World Health Organization [6]. The first step of *Salmonella* detection was carried out by inoculation of 225 ml buffered peptone water with 25 gram of sample followed by incubation at 37^{o}C for 24 hrs. The second step was performed using 10 ml tetrathionate broth medium and 1 ml from the first step was added and then incubated at 37^{o}C for 24 hrs. In the third step 1 ml from the second step was poured in petri dish and directly followed by *Salmonella-Shigella* agar medium (S.S agar medium) (25 ml medium per plate) and incubated at 37^{o}C for 24 hrs. The detected isolates were confirmed morphologically and biochemically to be *Salmonella*.

Identification of isolates:

All the bacterial isolates were identified biochemically according to the Bergey's Manual [7] molecularly in **Sigma laboratory, Cairo, Egypt** [8].

Antimicrobial sensitivity test

The pathogenic isolates were subjected to sensitivity tests. Each isolate was inoculated in nutrient agar separately and incubated for 24 hrs at 37ºC. The culture broth were streaked using sterile cotton swabs on nutrient agar plates. The diffusion discs with antimicrobial drugs were placed on the plates and incubated for 24 hrs at 37ºC. The antibiotics discs (Oxoid, Basingstoke, UK) used were: ampicillin (10 mcg), streptomycin (10 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), ciprofloxacin (5 mcg), tetracycline (30 mcg), amoxicillin (25 mcg), zones of inhibition were measured in millimeter (mm) [9].

Preparation of lactic acid culture suspensions and supernatants

The two lactic acid strains *lactobacillus acidophilus* and the *Streptococcus thermophilus* were rehydrated in MRS broth for 48 hours at 37°C in shaking incubator (200 rpm). The cultures were centrifuged at 4^{0}C and 12752 rpm for 10 min, washed three times and re-suspended in 0.85% NaCl to obtain a cell concentration of at least 10^{8} CFU per ml. The suspensions were freshly prepared when needed [4].

Antimicrobial activity of LAB

The antimicrobial activity of the LAB was determined by the agar well diffusion method. In petri plates with 20 mL of standard count agar medium, previously inoculated with 1.0 × 10^{3}CFU/ml of 24 h bacterial suspensions separately (*Salmonella typhi* 14028, *Proteus* mirabilis and *Citrobacter freundii*), wells were cut into the agar then filled with 100µL of LAB cell free supernatants. After diffusion of supernatants at 4^{0}C for 1 h, the petri plates were incubated at 37^{0}C for 24-48 h. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone (mm) around the well. All experiments were performed in triplicate [10].

Effect of gamma radiation on the antimicrobial activity of LAB

Streptococcus thermophilus and *Lactobacillus acidophilus* were grown in MRS broth medium for 48 hrs at 37°C in shaking incubator (200 rpm). The bacterial cells were centrifugated at 8000 rpm for 10 minutes, washed with sterile saline, and re-suspended in the sterile saline. The re-suspended bacterial cells and the supernatant were distributed into 5ml aliquots in sterile screw capped test tubes. Both cells and cell free supernatant exposed to different doses (0, 2, 5, 7.5, 10, 12.5, 15, 17.5, 20 Gray) of gamma irradiation (Candian cell - ^{137}Cs) separately with dose rate of 0.685 rad/sec., in the

National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. Three replicates were used for each dose. In petri plates with 20 mL of standard count agar medium, previously inoculated with 1.0×10^3 CFU/ml of 24 hrs bacterial suspensions separately (*Salmonella typhi* 14028, *Proteus mirabilis* and *Citrobacter freundii*), wells were cut into the agar and filled with 100µL of either of the irradiated cells or irradiated cell- free supernatant. After diffusion of aliquots at 4^0C for 1 h, the petri plates were incubated at 37^0C for 24-48 hrs. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone around the well. Each experiment was performed in triplicates [4].

Effect of gamma radiation on the antimicrobial activity of LAB and its effect on the viability of the isolated strains in chicken meat:

Streptococcus thermophilus and *Lactobacillus acidophilus* cell free supernatants as well as the bacterial suspensions were prepared as previously described for each strain then were distributed into 5ml aliquots in sterile screw capped test tubes. Both cells and cell free supernatant were exposed to different doses (0 (control), 2.5, 5, 10, 15, 20 Gy) of gamma irradiation (Candian cell - ^{137}Cs) separately with dose rate of 0.685 rad/sec., at NCRRT, Cairo, Egypt. The chicken meat samples were cut into 5 g portions. Overnight cultures of *Salmonella typhi* 14028, *Proteus mirabilis, Citrobacter freundii* were grown in L.B broth at 30^0C. Chicken meat were surface inoculated with 10^3 CFU/ml of *Salmonella typhi* 14028, *Proteus mirabilis* and *Citrobacter freundii* after appropriate 10-fold dilutions of the culture in saline for each. The meat samples were surface inoculated with 10^8 CFU/ml of a combination of *Streptococcus thermophilus* and *Lactobacillus acidophilus* after appropriate 10-fold dilutions of the culture in saline. Bacterial count of the three pathogens was determined on Brilliant green agar plates at 37^0C after 24 for radiation experiments and at 4^0C for timing experiment. Each trial has been performed in triplicate. pH values of the irradiated cell free supernatant were measured.

Results and Discussion

It is widely agreed that *Salmonella* contamination in food products at various stages of production are one of the major factors leading to foodborne illnesses in humans and animals. Isolation was done according to ISO standard methods [11] from thirty-four samples of dairy and dairy products, vegetables, fruits and chicken meat.

Forty-nine isolates were isolated from 34 samples, ten of them showed biochemical characteristics of *Salmonella typhi* like characters. The rest of isolates were suspected to be *S. pullorum* (9 isolates), *S. gallinarum* (5), *S .typhiumrium* (6), *S. chlorasous or S. paraTyphi* (*7)* isolates *Shigella* (12). Chicken carcass was the most contaminated samples by *Salmonella* followed by milk, cheese, tomato, yogurt and orang, lettuce, carrot and herbs respectively as shown in **Figure(1).**

Vegetables, dairy products, spoiled rice, poultry products and bakery products are a direct reflection of the sanitary quality of the cultivation water, harvesting, transportation storage, and processing of the product [12]. Traditionally, most cases of Salmonellosis were thought to originate from meat and poultry products. In addition, environmental factors including contaminated water sources used for irrigation and washing, result in crops that have been implicated in a large number of *Salmonella* species. *Salmonella* is carried by both domesticated and wild animals and can contaminate freshwater by direct or indirect contact, which leads to being attached into vegetables and fruits. These may be factors that make these items more likely to be sources of *Salmonella* [13].

The antibiotic sensitivity test was done for ten target *S. typhi* isolates to select the most antibiotic resistant isolates for further experiments in comparing with *S. typhi* 14028 (reference strain**).** The ten isolates were tested against the common seven antibiotics used in Egypt (ampicillin, amoxicillin, tetracycline, gentamycin, nalidixic acid, ciprofloxacin, streptomycin) using the disc diffusion susceptibility test. It was found that two strains showed a resistance to all seven antibiotics tested, hence highlighting the preponderance of multidrug-resistant isolates. 80% of isolates were resistant to ampicillin. 70% of isolates were resistant to amoxicillin and streptomycin, while 60% of isolates were resistant to nalidixic acid and tetracycline. 50 and 30% of isolates were resistant to gentamycin and ciprofloxacin respectively**(13)**. It was found that 72% of isolates were resistant to one antibiotic at least. The frequency of antibiotic

resistance ranking was in the following order: tetracycline (100%), erythromycin (80%), streptomycin (80%), chloramphenicol (60%) respectively. However, 2 out of 5 isolates were susceptible to ampicillin**.**

The molecular identification proved that the two most antibiotic resistant isolates which were previously isolated from chicken carcass were *Citrobacter freundii* and *Protues mirabilis* except for *Salmonella*. *C. freundii* has recently been reported to express resistance to broad-spectrum antibiotics including piperacillin, piperacillintazobactam, vancomycin and cephalosporins. Isolation of ceftriaxone-resistant *Citrobacter freundii* (CRCF) has been associated with the overprescribed broad spectrum antibiotics [14]. *Citrobacter freundii* is also known to contain in its chromosome a gene coding for cephalosporinase.

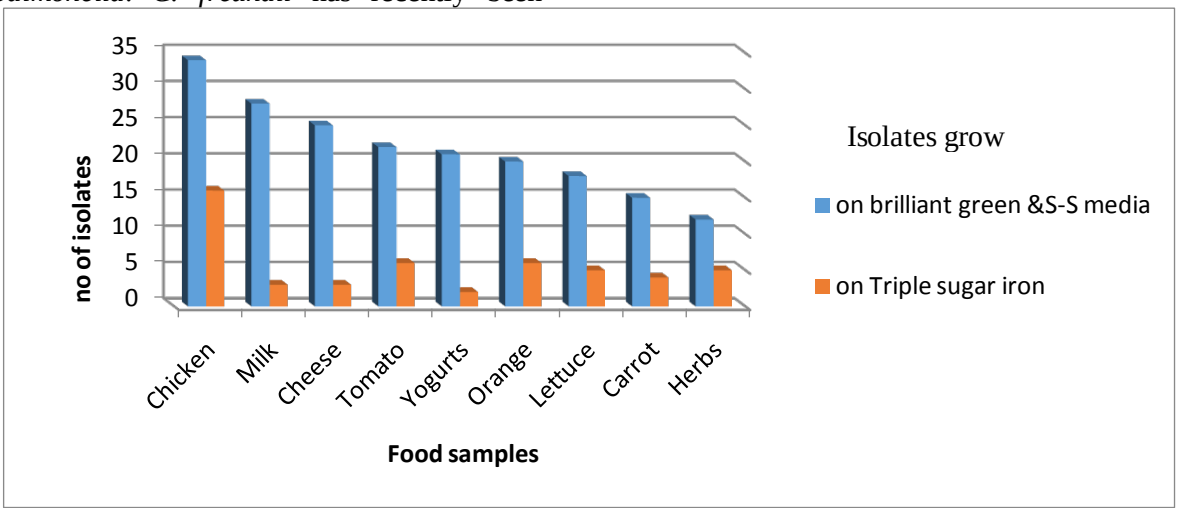


Figure (1): Isolation of foodborne pathogens from different food samples

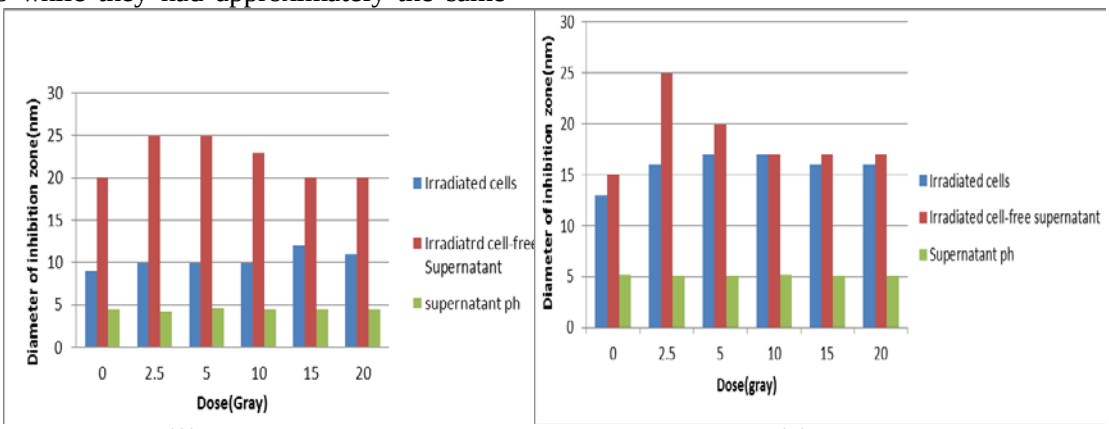
Table (1): Antibiotic susceptibility of salmonella suspected isolates

Isolates sources (No. of isolates)	Diameter of Inhibition zones (mm)							Number of Resistant isolates
	Gn (10 µg)	Am (10µg)	Ax (10 µg)	CIP (5µg)	St (10µg)	NA (30µg)	TE (30µg)	
Tomato (11)	18	30	35	25	R	20	20	1
Tomato(23)	R	15	20	23	19	R	R	3
Chicken(50)	R	R	R	R	R	R	R	7
Herbs(68)	R	R	R	25	R	R	R	6
Lettuce(70)	25	R	R	40	R	R	R	5
Carrot(90)	18	R	R	25	20	20	23	2
Orange(94)	20	R	R	30	R	30	R	4
Carrot(102)	20	R	R	30	R	R	20	4
Milk(159)	R	R	15	R	20	20	40	3
Chicken(194)	R	R	R	R	R	R	R	7
***S. typhi* 14028**	R	18	20	35	R	19	R	3

(GN): Gentamicin resistance (10 µg) (MIC ≤ 16 µg/ml), **(AM):** Ampicillin resistance (10 µg) (MIC ≤ 14 µg/ml), **(AX)**: Amoxicillin resistance (10 µg) (MIC ≤ 16 µg/ml), **(CIP):** Ciprofloxacin resistance (5 µg) (MIC ≤ 17 µg/ml), **(ST):** Streptomycin resistance (10 µg) (MIC ≤ 19 µg/ml), **(NA)**: Nalidixic acid resistance (30 µg) (MIC ≥ 16 µg/ml), **(TE):** Tetracycline resistance (30µg) (MIC ≤ 14 µg/ml)

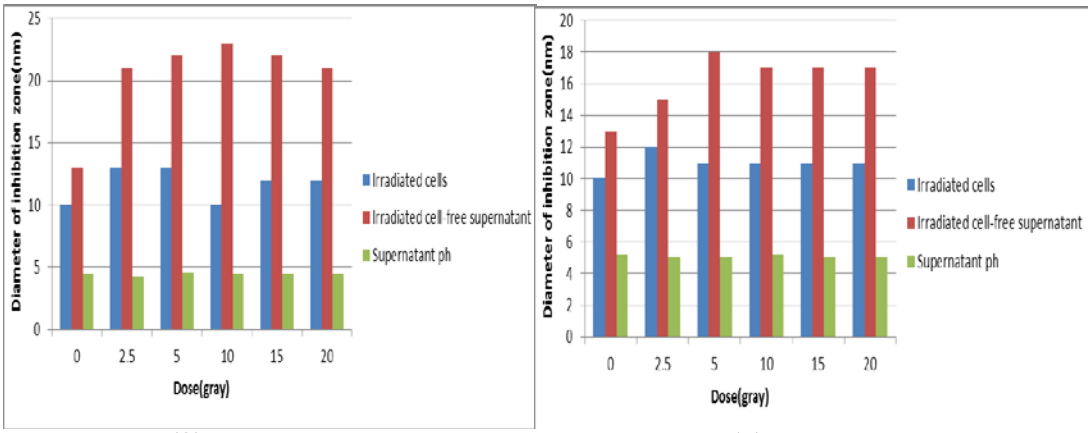
This enzyme hydrolyses the −CO−NH− bond in the lactam ring of cephalosporins and cephamycis thus, rendering the bacteria resistant to this type of antibiotics [15,16] didn't find a single antibiotic to be effective bactericidal agent against uropathogenic *P. mirabilis* strains collected from patients in Polish.

Gamma radiation was used as a tool to increase the antimicrobial activity of *L. acidophilus* and *S. thermophilus* towards the three pathogens. From **Figures. (2), (3) & (4)** show that radiation increased the antimicrobial efficiency of the free cells supernatants of the two LAB strains more than the irradiated suspended LAB cells. The activated *L. acidophilus* supernatant and its suspended cells were more effective than the corresponding *S. thermophilus* in killing *Proteus mirabilis* while they had approximately the same antimicrobial activities on *Citrobacter freundii* and *Salmonella typhi* 14028. The best activated doses for *L. acidophilus supernatants* cells free were 2.5, 10 and 20 Gray and 5,15,20 Gray for its cells against C. *freundii*, *P. mirabilis* and *S. typhi* 14028 respectively. While the best activated doses for *S. thermophiles* supernatants cells free were 2.5, 5, and 20 Gray and 2.5, 5 and 20 Gray for the *S. thermophiles* cells against C. *freundii*, *P. mirabilis* and *S. typhi* 14028 respectively. No remarkable change was noticed in the irradiated supernatants pH for the two LAB strains, but the *L. acidophilus* supernatants were more acidic than *S. thermophiles.* These results indicate that the antimicrobial activity of *Lactobacillus acidophilus* may be due to production of other antimicrobial compounds beside the organic acids.



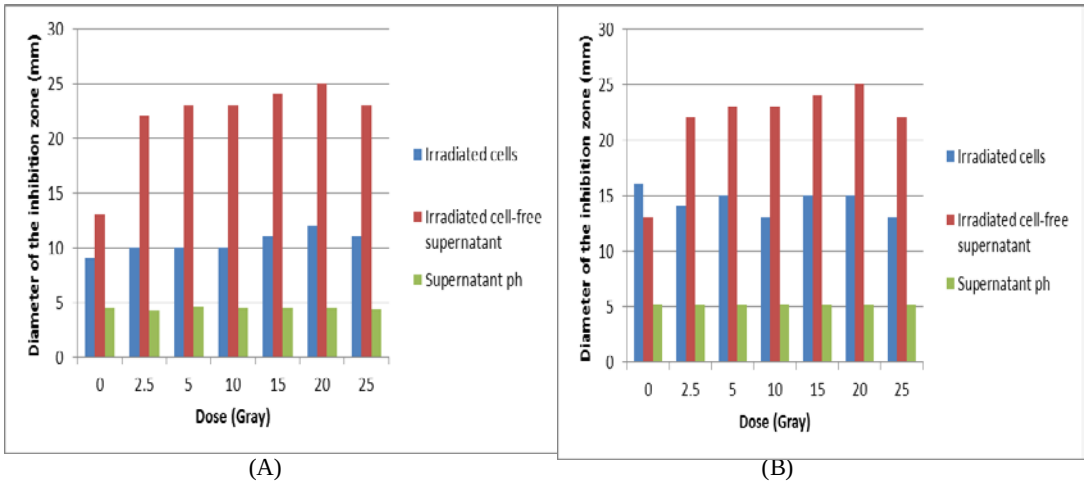
(A) *Lactobacillus acidophilus* (B) *Streptococcus thermophiles*

Figure(2): Antimicrobial activity of either of the irradiated cells or the irradiated cell- free supernatant of *L. acidophilus* (A) and *S. thermophilus* (B) on *C. freundii* after exposure of different doses of gamma radiation



(A) *Lactobacillus acidophilus* (B) *Streptococcus thermophiles*

Figure(3): Antimicrobial activity of either the irradiated cells or the irradiated cell- free supernatant of *L. acidophilus* (A) and *S. thermophiles* (B) on *Proteus mirabilis* after exposure of different doses of gamma radiation

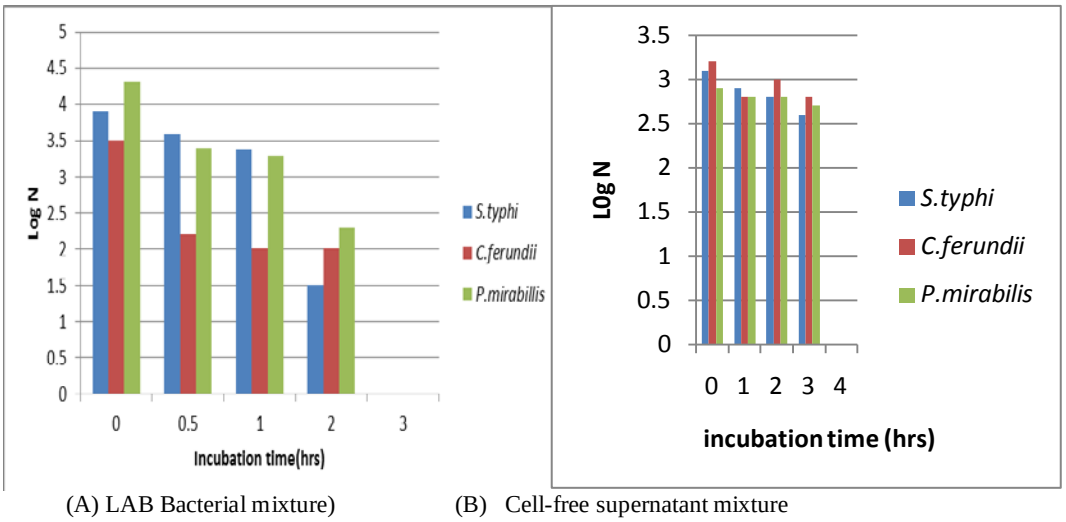


(A) *Lactobacillus acidophilus* (B) *Streptococcus thermophiles*

Figure(4): Antimicrobial activity of either of the irradiated cells or the irradiated cell- free supernatant of *L. acidophilus* (A) and *S. thermophilus* (B) on *Salmonella typhi* after exposure to different doses of gamma radiation

protection of mice urinary tract in laboratory from *Proteus mirabilis* infections by *Lactobacillus acidophilus* [17, 18] *Salmonella* enterica serovar *Enteritidis* (*Salmonella Enteritidis*) in broiler chicks by 3 ATCC lactobacilli [19] and [20] recorded the ability of live bacterial cultures and probiotic organisms to also reduce colonization of opportunistic microorganisms in the gastrointestinal tract by production of some active antimicrobial substances.

In this study, mixtures of *L. acidophilus* and *S. thermpohilus* as well as their supernatants were applied on cubs of chicken carcass which was artificially contaminated by the three pathogens individually and stored in refrigerator at 4°C for 4 hrs to determine the killing time for each mixture. According to the previous results, the LAB mixture was activated by 5 Gy of gamma radiation while the supernatant mixture was activated by exposure to 20 Gy. Results shown in **Figs. (5A & 5B)** indicate that the irradiated viable cells mixture was stronger than the supernatant mixture. The irradiated bacterial mixture killed all the three pathogens during three hours while it takes four hours to kill the supernatant mixture.



(A) LAB Bacterial mixture) (B) Cell-free supernatant mixture

Figure(5): Effect of mixture of *L. Acidophilus* and *S. thermpohilus* (A) exposed to 5 Gy and their cell-free supernatant mixture activated by 20 Gy (*B*) against pathogenic strains supplemented to chicken carcasses

Ionizing radiation can affect DNA either directly, by energy deposition in this critical target, or indirectly, by the interaction of radiation with other atoms or molecules in the cell or surrounding the cell like water. In particular, radiation interacts with water, leading to the formation of free radicals that can diffuse far enough to reach and damage DNA [21]. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants.

In the present study, the activated free radicals formed by radiation of water in the supernatant mixture may have two targets, the pathogens cells and the carcass protein. So, the irradiated supernatant mixture takes more time to kill the pathogens (four hours) than the LAB mixture. The antibacterial activity of the cell free supernatant was due to the production of acetic, lactic acids that lowered the pH of the medium and antimicrobial substances including bacteriocins that have ability to inhibit pathogenic and food spoilage bacteria [22, 23].

While in case of the bacterial mixture fermentation process, the only target is the pathogens cells. LAB utilize chicken carcas proteins as their prime source of essential and growth-stimulating amino acids[24]. LAB proteolytic system is very efficient in releasing encrypted molecules. These encrypted peptides are able to control infection of pathogenic microbial inhibition. Peroxide radical is usually considered to be free radical species for the oxidation of protein[25].

Lactic acid bacteria isolated from poultry carcasses showed statistically significant reduction in *Salmonella* population on the 5th day in brain heart broth medium and after 6th day on the chicken skin[26], while the reduction on the chicken meat was slightly lower. Also, identifying a single or a mixture of probiotic bacteria that inhibit the growth of spoilage and pathogenic bacteria is of growing interest for research to improve the shelf-life and safety of the meat products [27].

The LAB increased shelf-life and sustainability, palatability, and nutritional value. Application of LAB in the food/ feed biotechnology industry seems promising and requires further research [26].

Conclusion

Streptococcus thermophilus and *Lactobacillus acidophilus* mixture or their cell free supernatant successfully eliminated the antibiotic resistant *Citrobacter freundii, Salmonella typhi* 14028 and *Proteus mirabilis* from chicken carcass within 3 to 4 hours. Very low dose (5 Gy) could initiate the efficiency of *Streptococcus thermophilus* and *Lactobacillus acidophilus* and their supernatants for eliminating the three pathogens.

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