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Oral administration of *Bacillus acidophilus* lactic acid bacteria and copper oxide nanoparticles to mice with induced diarrhea- causing serotype of *E.coli* in Samarra-Saladin -Iraq

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Abstract

L. acidophilus bacteria are found in foods and are used in pharmaceuticals to supplement the natural gut flora associated with human digestive health. They also possess excellent therapeutic properties, attributed to their biological activity. These bacteria produce serotypes that inhibit the growth of pathogenic bacteria and adhere to epithelial cells, preventing intestinal pathogens from attaching to them through a process called competitive exclusion. In this study, *L. acidophilus* strains were isolated and identified from local yogurt, and the efficacy of the isolated strains' metabolites against the diarrheal serotype of *Escherichia coli* was investigated. Additionally, some *L. acidophilus* strains were examined for their probiotic properties. The research was conducted in the laboratories of the Department of Biological Sciences and the Central Laboratories at Samarra University from September 1, 2025, to November 1, 2025. In this study, one of the *L. acidophilus* strains was obtained from lactic acid bacteria. The study results showed that the metabolites of the studied strains exhibited good inhibitory activity against the diarrheal serotype of *E. coli*. Regarding the probiotic assay, the results confirmed the ability of all strains to adhere to epithelial cells. The group treated with a combination of pathogenic bacteria and copper oxide nanoparticles (G2) showed a significantly higher level of IL-8 (9.78±3.45 pg/mL), which is considerably higher than G1, suggesting that the nanoparticles may have stimulated a level of inflammatory stress or an immune response. The control group (G3: pathogenic bacteria only) recorded the highest mean IL-8 (12.21 ± 3.09 pg/mL). In comparison, the presence of gut microbiota (G1) appeared to have a modulating effect on the IL-8 response to pathogenic infections, resulting in a marked decrease in its mean concentration (4.54±0.99 pg/mL) compared to the control group (G3) ($p<0.05$). Interestingly, the group treated with pathogenic bacteria and copper oxide nanoparticles (G2) exhibited an exceptionally high mean IL-6 (63.5 ± 22.28 pg/mL), significantly higher than the mean of both the control group (G3: 18.13 ± 9.49 pg/mL) and the gut microbiota group (G1: 9.52 ± 4.11 pg/mL). Copper oxide nanoparticles not only directly and effectively affect bacteria but also influence the host's immune system. The large increase in IL-6 may be due to the recognition of nanoparticles as foreign bodies, causing phagocytic cells to activate and release pro-inflammatory cytokines in an attempt to remove the particles.

Keywords: *Lactobacillus acidophilus*. Probiotic. *Escherichia coli.* Pathogenic bacteria. Interleukins. IL 6. IL 8.

Introduction

Lactic acid bacteria (LAB) have received considerable attention in several fields, as they have occupied a large space in the field of medical, pharmaceutical and agricultural research to solve many health problems, due to their therapeutic properties that have helped in solving many health problems. Lactic acid bacteria are characterized by their lack of toxin production and resistance to acidity, as well as their production of numerous enzymes and vitamins. They have the ability to adhere to intestinal walls and secrete several metabolic substances with an inhibitory effect on pathogenic microorganisms. They are naturally found in the intestines, mouth, and urinary tract, contributing to the healthy balance of these areas **[1]**. These bacteria produce hydrogen peroxide in the presence of oxygen, which exerts an inhibitory effect against many Gram-negative and Gram-positive bacteria, causing mutagenesis of several enzymes .

The bacteriocins produced by lactic acid bacteria are the most important of these bacteria. Studies have indicated that the most important of these bacteria are Bacteriocins, which are antimicrobial substances produced by several strains. They are protein-based and possess a more lethal than inhibitory effect. They are also characterized by their ability to bind to specific sites on the cell wall. Bacteria Lactic acid bacteria include many species, such as *L. acidophilus* **[2]**.

This species belongs to the genus Lactobacillus. The name of this species is derived from two words: Lactobacillus, meaning lactic acid bacteria, and Acidophilus, meaning acid-loving. Therefore, the name of this type of bacteria means acid-loving lactic acid bacteria. This species does not produce ammonia gas (NH_3) **[3]**. *L. acidophilus* does not synthesize the amino acid arginine, it is nitrate test negative and it does not liquefy gelatin. It does not produce the compound indole from the amino acid tryptophan and does not produce H_2S gas (Hydrogen sulfide) **[4]**. The organism can grow in the presence of an acid and it can grow at a wide temperature range of 53-25°C. However, the optimum temperature range for the growth of *L. acidophilus* is 30-40°C.This species does not grow at 15°C.

The importance of this species has emerged in its use as a probiotic, as it is distinguished by its ability to improve immunity and the absorption of substances. It also provides protection against food poisoning and works to enhance the absorption of minerals and vitamins. Furthermore, it works to break down many harmful toxins, some strains of *L. acidophilus* produce low molecular weight bacteriocins, which are called acidophilin. These bacteriocins have a weight of up to 6.5 kDa and are heat-resistant. They are produced in the Log phase and decrease thereafter **[5]**. This study aimed to isolate and identify the bacterium *L. acidophilus*. It also aimed to determine the effect of *L. acidophilus* and copper oxide nanoparticles in treating diarrhea in mice infected with *E. coli*.

Materials and methods

Study Design

This study followed the ARRIVE checklist guidelines for animal studies. Available at: link: https://arriveguidelines.org/arrive-guidelines). Accessed on: September 2025.

Ethical Approval

The research was approved by the animal ethics committee in the laboratories of the Department of Biology, College of Education for Pure Sciences, Samarra University, in 2025. These mice were obtained and reared in the animal house of the College of Education for Pure Sciences, Samarra University.

Isolation of *L. acidophilus*

The research was conducted in the laboratories of the Department of biology, College of Education for Pure Sciences, Samarra University, from September 1, 2025, to November 1, 2025. Milk samples were collected from cattle farms in the Samarra area, south of Salah al-Din Governorate. Male white mice, weighing 100 g and aged one month, were used in this research. These mice were obtained and reared in the animal house of the College of Education for Pure Sciences, Samarra University. *Lactobacillus acidophilus* bacteria were isolated from various sources, primarily fermented dairy products. The samples were placed in sterile containers and transported to the laboratory. To isolate the bacteria, tubes containing 1% Mann-Rogoza-Sharp liquid medium (MRS) were inoculated with the samples and incubated at 37°C for 24 hours. The samples were then cultured on solid MRS containing 1% calcium carbonate. The plates were incubated at 37°C for 48 hours. Colonies with a transparent halo were selected and transferred to solid MRS medium for purification by culture. They were then incubated under the same conditions to obtain pure cultures.

The last sentence regarding *Lactobacillus acidophilus* is likely incorrect and should be deleted. Diagnostic *Lactobacillus acidophilus*: microscopic examination and morphological characteristics of the isolates were based on the initial MRS medium used for identification **[6]**. A range of tests were used to diagnose the isolates. These tests included the Catalase test and the Oxidase test.

For specific types, the following oxidase tests were used: Carbohydrate Fermentation Test Isolates were cultured in test tubes containing Medium Red Phenol containing 1% sugar. The sugar was added after sterilizing the medium with an autoclave and sterilizing the sugars using 0.22 µm Millipore filter paper. The test tubes were incubated under anaerobic conditions for 24-48 hours. A change in the medium's color to yellow indicates a positive test result **[7]**.

CO_2 Production Test

This test was performed by cultured isolates in Medium Basal Sugar containing 2% glucose in a test tube containing a Durham tube. The tubes were incubated for 48 hours. If the medium floated. The upward-pointing tube indicates CO_2 production from glucose fermentation **[8]**.

Ammonia Production Test from Arginine

This test was performed by inoculating tubes containing liquid MRS-arginine medium with 1% of the liquid culture of the bacterial isolate to be tested at 24 hours of age. The tubes were then incubated at 37°C under anaerobic conditions for 7 days. Afterward, 1 ml of the liquid culture was taken and an equal volume of Nissler's reagent was added. The color change from orange to brown indicated the ability of the isolates to hydrolyze arginine and produce ammonia **[9]**.

Growth at 45°C

Growth at 45°C was tested by inoculating tubes containing liquid MRS medium with 1% of the bacterial isolate to be tested. The tubes were incubated at 45°C for 24 hours. The result is positive when turbidity is present. Growth at 4% sodium chloride concentration: Liquid MRS medium containing 4% sodium chloride was inoculated with the isolate to be tested, then incubated at 37°C for 24 hours. Growth was observed with turbidity and compared to the control **[10]**.

Growth at pH 3

The effect of pH on the growth and activity of the studied bacterial isolates was investigated using solid MRS medium at pH 3. The medium was poured into plates in duplicate, inoculated with bacterial suspension, and incubated at 37°C for 48 hours. The results were then interpreted **[11]**.

Life environment Tests

Tolerance to acidic conditions: Each isolate of *L. acidophilus* was cultured in a tube containing liquid MRS medium under anaerobic conditions at a temperature of the tubes were incubated at 37°C for 24 hours, then centrifuged at 6000 rpm for 10 minutes to obtain the bacterial precipitate. This precipitate was then resuspended with 0.9% physiological saline (NaCl) until a bacterial suspension with a density equal to that of a standard McFarland solution (0.5). Four test tubes were prepared, each containing 2 ml of physiological saline. Three of the tubes were incubated with 0.1 N HCl in varying amounts to achieve different pH ranges (pH 2, pH 3, and pH 4). The fourth tube was left untreated and served as the control. 0.5 mL of the bacterial suspension was then added to each of the four test tubes. The tubes were incubated at 37°C for 1 hour, with three replicates per tube, and then repeated for 1.5 and 3 hours. The number of viable cells in all tubes was then determined. The number of live cells in all studied samples was obtained using the decimal dilution and culture method. The percentage of viable cells was determined according to the following equation **[12]**.

Bile salt tolerance

The *L. acidophilus* isolates were grown in a tube with liquid MRS media under anaerobic conditions at 37°C for 24 hours. Each tube was then centrifuged at 6000 rpm for 10 minutes and the bacterial precipitate from each isolate was collected. The precipitate was then resuspended with 0.9% physiological saline (NaCl) until a bacterial suspension with a density equal to that of a standard McFarland solution was obtained. Six test tubes were prepared, each containing 5 mL of sterile liquid MRS medium. Bile salts were then added to five of the tubes in different amounts to obtain five different **[13]**.

Concentrations of bile salts.

These are 0.1%, 0.3%, 0.5%, 0.7%, and 0.9%. The sixth tube received no added salts and served as the control sample. The pH of all six tubes was then adjusted to pH 6.5, and 100 µL of bacterial suspension was added to each tube. The tubes were incubated at 37°C for 24 hours. The optical density (OD) of the samples was measured at a wavelength of 600 nm. The experiment was repeated for each isolate. The percentage of bile salt resistance was calculated according to the following equation, as described in **[14]**.

Nano-cuprate copper oxide (CuO)

CuO can be classified as a transition metal oxide, as the copper ion in copper oxide has an oxidation number of +2. It is a semiconductor with a monoclinic crystal structure and possesses many interesting properties, including high thermal conductivity, high stability, excellent photoelectric properties, and antimicrobial activity The addition of trace elements is

of great importance to animal health, especially when added in carefully calculated amounts. It helps stimulate high bioavailability and improve animal immunity. Numerous previous studies have demonstrated various correlations between the physical and chemical properties of metallic nanoparticles and their health effects **[15,16]**.

These properties can influence the dosage of nanoparticles that reaches target organs. Furthermore, it is believed that the surface properties of copper nanoparticles can change upon entering the body or during transport **[17]**. Copper and its oxides are important for animals and play a crucial role in growth and fertility by reducing free radical activity in the body. Copper acts as a cofactor in the synthesis of important antioxidants, which mitigate the damaging effects of free radicals. These antioxidants consist of both enzymatic and non-enzymatic systems **[18]**.

These trace elements help stimulate high bioavailability and improve animal immunity. Nano-copper is an important element, as copper contributes to strengthening the immune system to resist infections and repair damaged tissues **[19]**. Furthermore, it supports the neutralization of free radicals that cause severe cell damage. Copper appears to be partially absorbed by the stomach, and the vast majority is absorbed in the small intestine **[20]**.

Immune mediators

IL 6 & IL 8 test A - Basic principle. This ELISA kit uses the Sandwich-ELISA method.

Immunological Indicators

Immune Mediators

Dilution of standard solutions for IL-6. All of the reagents were brought to room temperature before use. To create a stock solution of 180 pg/mL from the first 270 pg/mL preparation, the lyophilized TNF-α standard was first reconstituted with 150 µL of standard diluent. To guarantee full dissolution, the mixture was gently mixed for 15 minutes. Concentrations of 120 pg/mL, 60 pg/mL, 30 pg/mL, and 15 pg/mL were then prepared by serial two-fold dilutions in small tubes using the standard diluent. The zero concentration (0 pg/mL) was achieved by using the standard diluent alone. 50 µL of each created dilution was added in duplicate to the microplate wells. Any leftover stock solution was utilized within a month and kept at -20 °C. Figure 1: Dilution of standard solutions for IL-6 180 pg/mL Standard No.1 300 µL Original Standard + 150 µL Standard Diluent 120pg/mL Standard No.2 300µL Standard No.1 + 150µL Standard Diluent 60pg/mL Standard No.3 150µL Standard No.2 + 150µL Standard Diluent 30pg/mL Standard No.4 150µL Standard No.3 + 150µL Standard Diluent 15pg/mL Standard No.5 150µL Standard No.4 + 150µL Standard Diluent. 180pg/mL Standard No.1 300µL Original Standard + 150µL Standard Diluent 120pg/mL Standard No.2 300µL Standard No.1 + 150µL Standard Diluent 60pg/mL. Standard No.3 150µL Standard No.2 + 150µL Standard Diluent 30pg/mL Standard No.4 150µL Standard No.3 + 150µL Standard Diluent 15pg/mL.

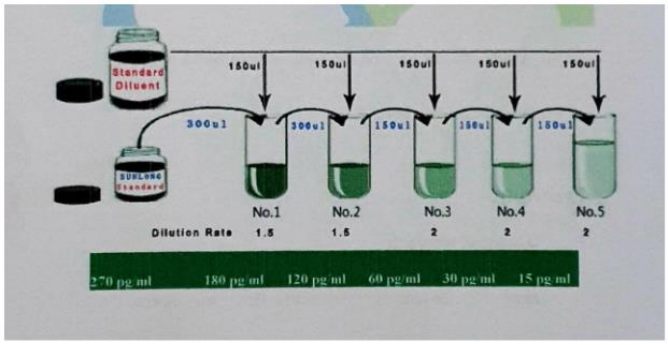

Figure 1. Dilution of standard solutions for IL-6.
Source: Own authorship.

Interleukin-8 (IL-8):

Many cells produce the cytokine IL-8, including: mononuclear cells, macrophages, T lymphocytes, neutrophils, hepatocytes, and endothelial and epithelial cells. In general, IL-8 plays an important role in host defense mechanisms. Intestinal epithelial cells secrete IL-8 in response to intestinal infection with *E. coli* **[21]**.

Experimental Design

This experiment includes 12 animals divided into four groups as follows:

Mice were given 0.5 mL of bacterial suspension containing 4 x 10^6 using a gastric tube to three groups expect number 4:

1. Positive Control Group: 3 mice treated with *E. coli* bacteria for 7 days and with water for 21 days only, administered orally during the experiment .
2. Second Group: 3 mice treated orally with *E. coli* bacteria for 7 days and with *L. acidophilus* at a dose of 100 mg/kg for 21 days .
3. Third Group: 3 mice treated with *E. coli* bacteria for 7 days and with copper oxide nanoparticles orally for 21 days .
4. Negative Control Group: 3 mice treated with a buffer solution orally for 21 days. Blood was withdrawn from the slaughtered mouse after the dosing period was completed and placed in special tubes and the required tests for this experiment were performed, namely, IL 6, IL 8.

Determination of the LD50 in mice and bacterial counts:

The LD50 was determined according to the method of Reed & Muench (1938) [22]. *E. coli* bacteria were grown in trypton soya broth at 37°C for 24 hours, then concentrated using centrifugation at 3000 rpm for 15 minutes. The bacteria were washed three times with phosphate buffer for 5 minutes each time, and the clear liquid was discarded. The bacteria were diluted to a tenth dilution using phosphate-salt buffer. Seven tenth dilutions were tested: 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1}. Twelve mice were used, divided into four equal groups of three mice each, with the last group serving as the control group. Food was withheld for 24 hours, and each group was given 1 mL of one of the dilutions, while the last group received 1 mL of phosphate-salt buffer. The number of deaths and survivals for each dilution was counted over 14 days.

The number of live bacteria per dilution was determined using the spreading method with MacConkey agar plates. Three plates per dilution were used for this purpose, and the plates were incubated for 24 hours at 37°C. Based on the number of colonies per plate and their average (number of germs = average number of colonies for the three plates multiplied by 10 and multiplied by the reciprocal of the dilution), the efficient dose was determined to be 4 x 10^6.

Statistical Analysis

The ANOVA (2005) statistical software was used for data analysis, and the Complete Randomize Design (CRD) was used for data analysis.

Results

A one-way ANOVA was used to confirm that the difference between groups was statistically significant for IL-8 levels (F (2,14) = 7.013, p -value = 0.008). The post hoc Tukey HSD test showed that the mean of IL8 in G3 was significantly higher than G1 (p-value = 0.006).The difference between Group 1 (G1) and Group 2 (G2) approached but did not reach statistical significance (p-value = 0.068), and no significant difference was found between Group 2 (G2) and Group 3 (G3) (p-value = 0.327), according to Tables 1 and 2.

For IL6, the results were showed a highly significant difference between groups, F (2, 14) = 20.0, p-value < 0.05. Tukey HSD post hoc tests identified that Group 2 (G2) had significantly higher IL-6 levels than both Group 1 (G1) (p-value < 0.05) and Group 3 (G3) (p-value<0.05). There was no significant difference in IL6 levels between Group 1 (G1) and Group 3 (G3) (p-value = 0.680), according to Tables 1 and 2.

Table 1. Effects of Microbiota and Copper Oxide Nanoparticles on Pathogen-Induced Inflammatory Cytokine Production (IL-8 and IL-6).

Parameters	Pathogenic bacteria + Microbiota (G1)	Pathogenic bacteria +Nanoparticle copper oxide (G2)	Pathogenic bacteria (G3)	F	p-value
	M±SD	M±SD	M±SD		
IL-8	4.54±0.99	9.78±3.45	12.21±3.09	7.01	0.05
IL-6	9.52±4.11	63.5±22.28	18.13±9.49	20	0.05
M, mean SD, Standard Deviation F, Variance					

Source: Own authorship

Table 2. Tukey HSD post hoc tests among study groups.

Dependent Variable	(I) GROUP	(J) GROUP	Mean Difference (I-J)	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
IL-8	G1	G2	-5.24	0.05	-10.8551	0.3684
		G3	-7.67*	0.05	-13.0479	-2.3021
	G2	G1	5.24	0.05	-0.3684	10.8551
		G3	-2.43	0.05	-6.7177	1.8544
	G3	G1	7.65*	0.05	2.3021	13.0479
		G2	2.43	0.05	-1.8544	6.7177
IL-6	G1	G2	-53.99*	0.05	-81.7458	-26.2375
		G3	-8.61	0.05	-35.1838	17.9613
	G2	G1	53.99*	0.05	26.2375	81.7458
		G3	45.38*	0.05	24.1828	66.5780
	G3	G1	8.61	0.05	-17.9613	35.1838
		G2	-45.38*	0.05	-66.5780	-24.1828

Source: Own authorship

Discussion

The results statistically compared the mean amount of inflammation, as measured by interleukin levels (IL-8 and IL-6), in three separate trials of treatment with pathogenic bacteria. The results indicate that the treatment conditions had a statistically significant effect on the amount of these pro-inflammatory cytokines. The most interesting finding was the strong pro-inflammatory effect of the control group (G3: pathogenic bacteria only), which showed the highest mean IL-8 level (12.21 ± 3.09 pg/mL). This is consistent with the established literature, as the pathogenic bacteria used in this study are well documented as potent inducers of the immune response in phagocytic cells, usually by activating

pattern recognition receptors such as TLRs, thereby triggering the release of IL-8, which is a strong inflammatory attractant for phagocytic cells and neutrophils to the site of infection **[1,2]**.

The combined treatment group using pathogenic bacteria and copper oxide nanoparticles (G2) also showed a similarly high IL-8 response (9.78 ± 3.45 pg/mL), although not as strong, and still significantly higher than G1. This may suggest that the nanoparticles also induce some degree of inflammatory stress or enhance the immune response to the pathogens themselves, as has been previously shown for other metal oxide nanoparticles **[23,24]**.

In contrast, the addition of microbiota (G1) appeared to modulate the IL-8 challenge response with a much lower mean concentration of 4.54±0.99 pg/mL compared to the control group (G3) ($p<0.05$), indicating an inhibitory effect. This is consistent with the concept of "anti-colonization" and immune modulation by commensal bacteria, which have been shown to suppress excessive inflammatory responses to pathogens in various ways, including through the production of anti-inflammatory molecules and competitive exclusion **[25]**.

Another, more pronounced factor is the pattern observed in IL-6, an important cytokine in the acute phase response and fever (Figure 4C and 4D). Statistically analyzed results showed a stronger therapeutic effect on IL-6 than on TNFα ($p = 0.05$). The group infected with pathogenic bacteria + copper oxide nanoparticles (G2) exhibited a very high mean value of IL-6 (63.5±22.28 pg/mL), a level significantly higher than both the control group (G3: 18.13±9.49 pg/mL) and the microbiota group (G1: 9.52±4.11 pg/mL). It appears that copper oxide nanoparticles not only act against bacteria but also primarily and directly activate the host immune system in response to infection **[26]**.

The dramatic increase in IL-6 may result from the host cells engulfing the nanoparticles as foreign bodies, leading to phagocytic cell activation and the production of pro-inflammatory cytokines in an attempt to clear the particles **[27]**. Some metallic nanoparticles have been shown to induce oxidative stress and activate inflammation (a key pathway for the maturation and secretion of IL-1β and IL-6) via the NLRP3 inflammasome **[28]**. The microbiota group (G1) again exhibited the lowest proi-nflammatory IL-6 levels, and the microbiota appears to be crucial in protecting the host from pathogenic infections. This protection can occur by primed immune responses to reduce the pathogen load or by activating protective responses within the host cells themselves **[29]**.

Conclusion

It was concluded that copper oxide nanoparticles not only directly and effectively affect bacteria but also influence the host's immune system. The large increase in IL-6 may be due to the recognition of nanoparticles as foreign bodies, causing phagocytic cells to activate and release pro-inflammatory cytokines in an attempt to remove the particles.

CRediT

Author contributions: Conceptualization; Data curation; Formal Analysis; Investigation; Methodology; Project administration; Supervision; Writing - original draft; Writing - review & editing - Rawya Fadhil Mohammed and Harith Ahmed Mustafa.

Acknowledgment

Not applicable.

Ethical Approval

The research was approved by the animal ethics committee in the laboratories of the Department of Biology, College of Education for Pure Sciences, Samarra University, in 2025. These mice were obtained and reared in the animal house of the College of Education for Pure Sciences, Samarra University.

Informed Consent

Not applicable.

Funding

Not applicable.

Data Sharing Statement

All referenced sources are accessible through the respective journals or public repositories.

Conflict of Interest

The authors declare no conflict of interest.

Similarity Check

It was applied by Ithenticate®.

Application of Artificial Intelligence (AI)

Not applicable.

Peer Review Process

It was performed.

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