VRI Bio Med Chem, Volume 2, Issue 1, March 2014

Vedic Research International

BIOLOGICAL MEDICINAL CHEMISTRY

eISSN 2330-7250

JOURNAL HOME PAGE AT WWW.VEDICJOURNALS.COM

RESEARCH ARTICLE

DOI: http://dx.doi.org/10.14259/bmc.v2i1.102

Antimicrobial Activity of Curcumin Against Food-Borne Pathogens

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Article Info: Received: *January 14th, 2014*; **Accepted:** *February 2nd, 2014*

ABSTRACT

Pre and post-harvest practices are very important factors in controlling *Salmonella* and *Escherichia coli* food pathogens in poultry meat. Both these pre-harvest and postharvest practices are controversial and objectionable due to health hazards and allergies associated with them. Ideally, a novel and safer compounds that could be used both in pre- and post-harvest pathogen reduction strategies are needed. Hence our hypothesis tested namely 'Curcumin, a natural product, may replace the controversial substances currently used in pre-harvest and post-harvest practices for controlling food pathogens like *Salmonella* and *Escherichia coli*.' The purpose of this study was to determine the effects of Curcumin on the growth of *Salmonella, E. coli,* macrophage uptake of bacteria, and on bacterial ATPase activity using the BacTiter-Glo^{tm} Cell Viability Assay. This study also evaluated the *In vitro* effect of Curcumin on macrophage phagocytosis. Data from this study showed that growth percentages of *Salmonella* and *Escherichia coli* were inhibited with increasing doses of Curcumin. Our molecular mechanism of action investigations of Curcumin determined that the amount of ATP present in both *Salmonella* and *Escherichia coli* was reduced after treatment with Curcumin. In summary, these data demonstrates promising potential of Curcumin as a universally acceptable pre-harvest and post-harvest alternative.

Keywords: *Curcumin, Antimicrobial, Food borne pathogens, Salmonella, E. coli*

Introduction

Food poisoning is an illness caused by eating foods that contain harmful organisms. These harmful germs can include bacteria, parasites, and viruses. They are mostly found in raw meat, chicken, fish, and eggs, with potential to spread to any type of food. It is normal to find bacteria in the intestines of healthy animals used for food. If bacteria come in contact with meat or poultry during processing, they can contaminate the food. *Campylobacter, Salmonella*, and *E.coli* are important bacteria that cause food poisoning [1].

Salmonellosis is a type of food poisoning caused by the *Salmonella* bacterium. It was found that *Salmonella enteritidis* amongst other bacteria is particullarly found in the commercial chicken industry. High levels of shedding and cecal carriage increase the risk of contamination of poultry meat. Even though *Salmonella enteritidis* can be normally found and isolated from the human gallbladder, it is in an asymptomatic carrier state [2]. Therefore, it is not considered harmful. When individuals with a compromised immune system consume poultry products that are infected with *Salmonella*, they become fatally ill.

Salmonella can infect poultry through contaminated feed, water, dust, feces, or by an infected chicken. The infected poultry and eggs do not significantly exhibit signs of infection or abnormalities. The cecal portion of the gastrointestinal tract is where the infection occurs in the chicken. This infection can last for several months [3]. It becomes a transient passage when it becomes a systematic infection [3]. In 2006, there were 121 cases, 3,300 illnesses reported to the Center for Disease Control (CDC) Foodborne Outbreak Reporting System for

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Salmonella. *Salmonella eneritidis* and *typhimurium* were the two most common serotypes reported [4]. *Escherichia coli (E. coli)* is gram negative, rod shaped, non sporulating, facultative anaerobe, that is able to survive outside the body. Certain serotypes of *E.coli* can cause food poisoning in humans. Just like *Salmonella*, its virulent strains (0157:H7,011:B4) can cause serious illness or death in individuals with a compromised immune system. *Salmonella* and *E. coli* are both major pathogens that play an important role in poultry industries in post harvest processing.

Pre- and post-harvest practices are key risk factors and play an important role in meat contamination. The use of chlorine in post-harvest processing and the use of antibiotics and immunizations during pre-harvest reduce the risk of contamination. Chlorine such as sodium hypochlorite, calcium hypochlorite tablets, and chlorine gas are commonly used in commercial poultry processing plants.

Chlorine has the ability to acidify bacteria cells; hence bacteria can't develop a resistance to it. Thus, chlorine is the most commonly used substance to sanitize equipment and carcasses [5]. The concerns with chlorine are the risk of being incorporated into the food and Trihalomethane (THM) formation. THM is a possible cancer causing agent.

Chlorine has been found to bind readily to unsaturated fatty acids, which enhance its potential for incorporation into the poultry meat [5]. Pre-harvest pathogen reduction strategy includes usage of antibiotics such as penicillin, erythromycin and tetracycline. These antibiotics prevent or reduce sickness and hasten growth of chickens. A novel and safer compounds is needed that could be used both in pre- and post-harvest pathogen reduction strategies to reduce the development of antibiotic resistant strains and to avoid chlorine toxicity. Although disinfectants and sanitation practices can reduce the risk of contamination and infection, there is no single effective and safer preventive that completely eliminates the bacteria.

Macrophages act as scavengers that play an important role in adaptive and innate immune immunity. Their main role is to engulf and digest foreign bacteria and debris. Macrophages also recruit other cells in-order to protect the body against attack. When a pathogen or bacteria invades the body, it is engulfed by macrophages and sent to the vacuole of lysozomes for further degradation.

"Unfortunately *Salmonella* and *E.coli* have the capability to survive within a macrophage, it secretes a protein that causes the vacuole composition to change and allows it to escape and replicate in a different compartment" [6]. It uses the macrophage as a vehicle to disseminate from the intestine to deeper tissues within the body [7].

Curcumin derived from Tumeric (curcuma longa plant) is a tropical plant native to southern and southeastern tropical Asia [8]. It is a perennial herb within the ginger family, known for its yellow –orange color and for numerous of therapeutic practices. It is also known for its flavor when used as a spice in Indian curry. Curcumin makes up to 2-5% of the spice turmeric [9]. It is a strong anti-oxidant and anti-inflammatory agent, which also acts as a free radical scavenger [9]. It is insoluble in water, but soluble in Dimethyl Sulfoxide (DMSO), ethanol, and acetone. It was found that in the presence of light and oxygen it is able to kill *Salmonella typhimurium* within 15 minutes. It was found to be more stable in cell culture medium containing 10% fetal calf serum as well as in human blood [8]. "When researchers observed the lining of the intestine after ingestion of curcumin, it was found that CD4+ T-helper and B type immune cells were greater in number, suggesting that it stimulates localize immunity" [10].

Also, under investigation are its proposed abilities to down regulate various proinflammatory cytokines, chemokines, and enhance antibody responses [10]. It can also inhibit the growth of *Salmonella* in a dose-dependent manner. It is thought to assist the macrophages and allow them to engulf the bacteria. Since it is non toxic and antibacterial, it is an ideal model for the use of potential development in medical treatments. The purpose of this study was to investigate the potential of Curcumin as a pre and post poultry harvest and as an alternative disinfectant to current controversial usage of antibiotics and chlorine.

Materials and Methods

Bacteria Culture and Media:

The bacteria used in the study were *Salmonella typhimurium* (ATCC 13311) genomic DNA strain NCTC 74. This strain was isolated from the feces of human food poisoning case. These bacteria were collected from Department of Pathobiology, College of Veterinary Medicine Nursing and Allied Health, Tuskegee University. The *Escherichia coli* PPS P4, which was collected from USDA, Athens, Georgia.

Preparation of Bacterial Culture Medium:

Prepartion of Luria-Bertani (LB) Agar: 20 gms of powered LB Agar was placed in 500 ml of distilled water and mixed by shaking until all contents were dissolved. This was then heated and autoclaved for 15 mins at 120 °C.

Prepartion of Luria-Bertani (LB) Broth: LB broth is a lysogeny broth used specifically for the growth of bacteria. The essential nutrients include peptides, casein, peptones, and vitamin trace elements. For proper sterilization, it is autocalved at 121 °C. It is one of the most common media used for maintaining and cultivating recombinant strains of *E coli*.

Ten grams of powered LB Broth was placed in 500 ml of distilled water and mixed by shaking until all contents were dissolved, followed by heating and autoclaving for 15 mins at 120 °C.

Prepartion of CHROM agar: CHROM agar is a selective and differential medium for isolation and presumptive identification of Salmonella species. It consists of chromogenic substrates that has been added into the medium which facilitates the detection of Salmonella species from other flora. It has been validated by the AOAC research institute under the AOAC *Performance Tested Methods*^{SM} (PTM) program.

Approximately 17.45 grams of powered CHROM agar was placed in 500 ml of boiling water and mixed by shaking until all contents were dissolved and then cooled to 48 °C. It was stored in a dark room wrapped in aluminum foil.

Confirmation of *Salmonella enteritica* and *Escherichia coli*: Bacteria culture was removed from the incubator and streaked on plates containing CHROM agar. The plates were incubated overnight at 37 °C. *Salmonella* confirmation was made by its purple color change of colonies. *E. coli* confirmation was made by its blue color change of colonies [11]. Plates were kept in a sterile refrigerator and used for further inoculations and culturing.

Preparation of Curcumin Stocks: Curcumin C3 compels (R) was a kind gift from Sabinsa Corporation, Hyderabad, India. Approximately 0.74 grams of powdered Curcumin was placed in 20 ml of DMSO to be used as 100mM stock. One ml of the solution was then aliquoted into eppendorf tubes and stored at -30° C to be further used as stock.

HTC Chicken Macrophage Cell Line

HTC cells, also known as transformed cells, are very useful because they have the ability to undergo numerous cell divisions. These cells are able to survive in-vivo or in cell culture due to their ability to naturally mutate in the presence of viruses, chemicals, and genetic manipulation. These cells are also useful in cultivating viruses [12].

The HTC cells were grown at 37 °C in a humidified incubator with 5% CO_2. Cell growth and routine passaging were done in complete RPMI-1640 growth medium supplemented with 5% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 50 µg/ml streptomycin). Viable cell concentration was determined through trypan blue exclusion. Macrophage cells were obtained from USDA Animal Parasitic Diseases Laboratory, Maryland. Cells were split every 48 hours (0.05%) using Trypsin EDTA then transferred into a new flask and fresh media was added.

Assay:

Ten milliliters of cultured bacteria was centrifuged at 1500 rpm for 5 mins. Ten milliliters of LB broth was added to the pellet and vortexed until dissolved. The mixture was serial diluted 1:10 from 10^1-10^{10} in buffered peptone water. The optical density (.371, *Salmonella*; .187, *E. coli*) was recorded and a standard curve of bacterial culture was prepared as 10^3cfu/ml and incubated with 0, 10, 50, 100, 200 µM of Curcumin. The BacTiter-Glo™ was added to the BacTiter-Glo™ substrate and gently mixed to form the BacTiter-Glo™ reagent. One hundred microliters from each curcumin concentration was added in duplicates into a 96 well plate. One hundred microliters of the BacTiter-Glo™ reagent was added to the wells containing the curcumin dosages and placed in luminometer. The ATP was measured by a luminescent signal, and data were recorded.

Statistical analysis: Anova performed Comparisons between groups. A *P* value less than 0.05 was considered significant.

Results and Discussion

Curcumin inhibits growth of *Salmonella enteritica*

To examine Curcumin's ability to inhibit the growth of *Salmonella*, approximately 10^6-10^8 CFU of *Salmonella* were incubated with 0, 10, 50, 100, 200 µM of Curcumin for 4 hrs shaking at 37 °C. After 4 hours the tubes were removed and 100 µl from each tube was plated on LB agar containing 0, 10, 50, 100, 200µM of Curcumin for 18-20 hrs. As a control and also to prove that the inhibition is solely due to Curcumin and not the vehicle DMSO, equal amount of DMSO was incubated with *Salmonella.* Results showed that Curcumin inhibited *Salmonella* growth in a dose-dependent manner (Figure 1A).

With increasing dose of Curcumin, the bacteria colony size and numbers decreased (See the LB agar plates in Figure1A). There was no significant difference between the control and the DMSO treated plate, indicating that the DMSO concentration used did not affect the *Salmonella* growth. When the control plate was compared to the 50µM Curcumin treated plate, the quantity of the colony growth observed was significantly less. When the control plate was compared to the 200µM Curcumin treated plate, there was a significant reduction in the size and number of colonies (Figure 1A.). Colony forming units per millileter (CFU/mL) after treatment with Curcumin were 1.6×10^5, 7.9×10^4, 1.3×10^4, 1.3×10^4, with 0, 10, 50, 100 and 200 µM, respectively (Figure 1B). Percentage of inhibition was found to be 48, 71, 97, and 100% at 10, 50, 100, and 200 µM of Curcumin respectively (Figure 1C). Results shown in Figure 1C are the average of the three replications.

Effect of Curcumin on the growth of *Escherichia coli*

To ascertain whether Curcumin will inhibit the growth of *E.coli* another poultry pathogen, approximately 10^6-10^8 CFU of *E.coli* were incubated with different doses of Curcumin for 4 hours. Approximately 100 µl of the bacteria was cultured in LB agar plate containing different concentration of Curcumin for 18-20 hrs. Results indicated that Curcumin inhibited growth of *E.coli* in a dose dependent manner. The percentages of inhibition were 0, 39, 61, 94, and 97 at 0, 10, 50, 100, 200 µM of Curcumin, respectively (Figure 2). Results shown in Figure 2 are average of the three independent replications. This data clearly demonstrated that Curcumin inhibits growth of both *Salmonella* and *E.coli* in a dose-dependent manner.

Effect of Curcumin on ATP Levels in bacteria

As Curcumin inhibited growth of *Salmonella* and *E.coli* in a dose-dependent manner, the next logical step was to investigate the molecular mechanisms involved in inhibition. Curcumin has been shown as an inhibitor of the ATPase activity of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR). It was hypothesized that Curcumin's ATPase activity could be one of the mechanism for its inhibitory action on bacteria. To further investigate whether the Curcumin depletes ATP in the bacteria, a BacTiter-Glo™ Microbial Cell Viability Assay (Promega) was used. This assay measures the amount of ATP in the bacterial cell. Cultured bacteria were removed from 100 ml of LB broth placed in a incubator that was shaking at 37 °C. Approximately 10^6-10^8 bacteria were incubated with 0, 10, 50, 100, 200 µM of (200 µM data absent) Curcumin for 4 hrs. One hundred microliters from treated and untreated bacterial cultures were added in duplicates into a 96-well plate, and 100 µl of the BacTiter-Glo™ reagent was added to the wells and placed in a luminometer. Results after treatment of *Salmonella* with Curcumin were 100, 66.6, 58.5, and 52.3 luminescence units for 0, 10, 50, and 100µM Curcumin, respectively (figure 3A). (200 µM data absent) Results after treatment of *E. coli* with Curcumin were 100, 55, 46.6 and 41.6 luminescence units at 0, 10, 50, and 100 µM of (200 µM data absent) Curcumin respectively (Figure 3B). Results shown were averages of three replications. These results indicated that Curcumin inhibited the ATP in bacteria.

Effect of Curcumin on uptake of bacteria by Macrophages

To determine whether Curcumin aids macrophage phagocytosis of *Salmonella,* HTC chicken macrophages cells were placed in 8 well cell culture slides with 100 µl of growth media and incubated overnight at 37°C in 5% CO_2 atmosphere. Macrophages on the slide were incubated with different concentrations of Curcumin for 4 hrs. Approximately 10^2-10^3 *Salmonella* were added to all slide chamber wells except Slide 2: chamber wells 1, 5 and Slide 3 and were incubated for 2 hrs at 37 °C in 5 % CO_2 atmosphere. Cells were washed twice with PBS, fixed with 70 % and 100 % alcohol, then stained with Giemsa. As shown in Figure 4, macrophages in the absence of Curcumin (Figure 4B) engulfed very few *Salmonella* (Figure 4A represents only untreated macrophages cells only). In the presence of 50 µM Curcumin, there was an increase in the amount of bacteria engulfed by macrophages (Figure 4C). The increased dosages of Curcumin i.e. 200 µM, acted as an aid to the macrophages in the process of phagocytosis (Figure 4D).

To determine whether the macrophages were actually killing the ingested bacteria, the next experiment was designed to measure the amount of live intracellular bacteria in the macrophages and also to observe the amount of bacteria that macrophages engulf and actually kill.

One million chicken macrophage cells in 1 ml of growth medium were added to each well in a 6- well plate. Cells were incubated overnight at 37 °C in 5% CO_2 atmosphere. Specified wells (3,4,5,6,8) were treated with Curcumin and incubated for 2 hrs; then *Salmonella* was added, and plates were

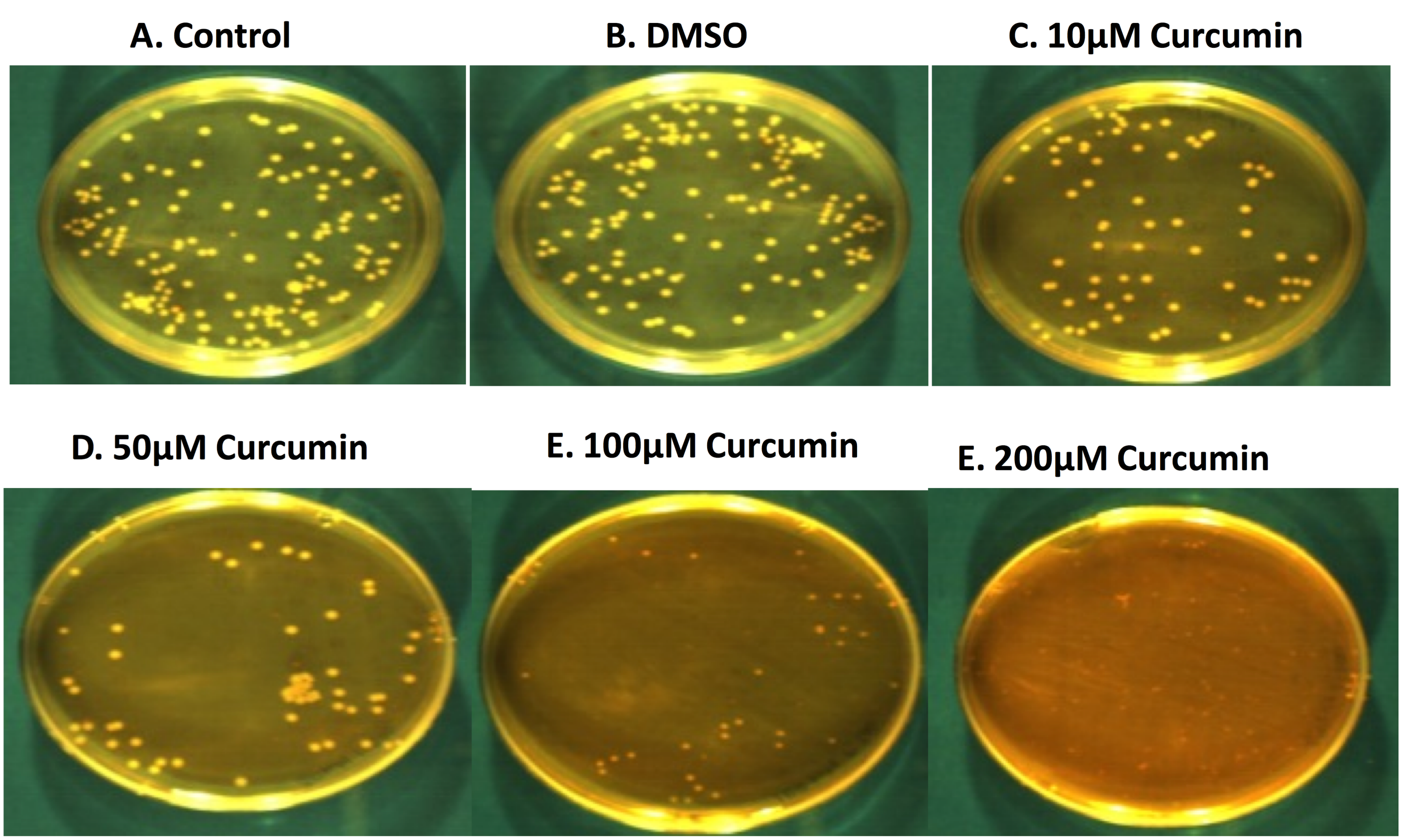

Figure 1A: Effects of Curcumin on the growth of Salmonella .Colony growth inhibition on LB agar plates. A Control; B DMSO; C 10 µM; D 50 µM; E 100 µM; and E 200 µM Curcumin.

then incubated for 1 hr, at 37 °C in 5% CO_2 atmosphere. The cells were then treated with gentamicin to eliminate the extracellular bacteria. Since gentamicin penetrates the cell wall poorly, it does not harm the intracellular bacteria. The cells were washed twice with PBS and then lysed using distilled water. The cell lysates (100 µl) were plated onto the LB agar plate, incubated overnight, and the colonies were counted. *Salmonella* did not grow when macrophages were not lysed with water compared to the presence of water except for the control wells (7 and 8). The control wells that contained no gentamicin (7 and 8) had a significantly increased amount of growth colonies on the plates, indicating the presence of extracellular bacteria. Within the Curcumin treated plates, the *Salmonella* growth increased with dosage from 10µM- 100 µM, but with 200 µM the colonies decreased in number. This indicated that 200 µM of Curcumin eliminated the bacteria by the macrophages similar to the results shown above. The CFU/mL results after treatment with Curcumin were 31.6, 123.3, 155.6, 305.6, and 205 at the concentrations of 0, 10, 50, 100 and 200 µM, respectively (Figure 5). The results shown in the figure 5 are the averages of three replications. To observe and compare the amount of bacteria being killed by macrophages, the

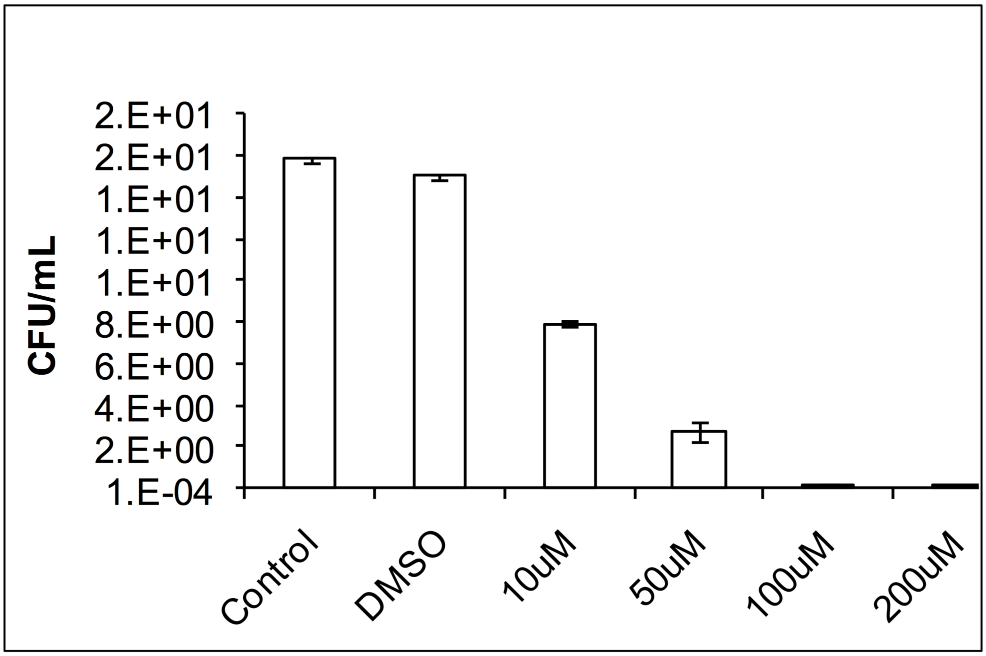

Figure 1B: Effect of different concentrations of Curcumin were 1.6 x 10^5, 7.9 x 10^4, 1.3 x 10^4, 1.3 x 10^4, with 0, 10, 50, 100 and 200 µM respectively on the growth of Salmonella.

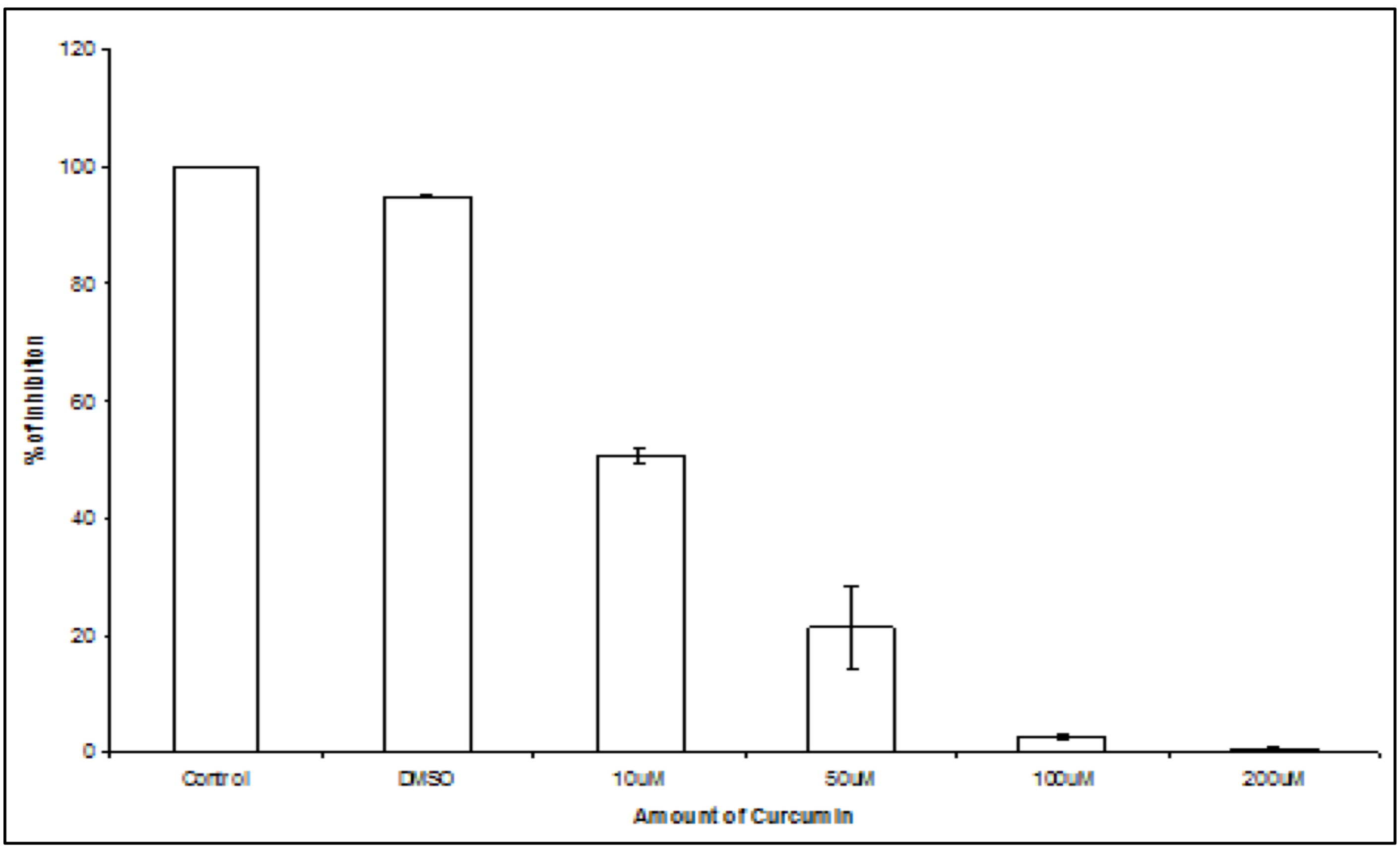

Figure 1C: Percentage of inhibition was found to be 48, 71, 97, and 100% at 10, 50, 100, and 200 µM of Curcumin respectively.

plates were incubated for 5-6 hrs and also overnight. There was no growth of *Salmonella* when the macrophages were incubated with *Salmonella* 5-6 hours overnight and treated with Curcumin at the lower doses (data not shown). These data indicate that in the presence of Curcumin, *Salmonella* were not only engulfed by macrophages but were killed.

Data provided further evidence for the promising potential of Curcumin as a pre-harvest and post-harvest alternative agent in controlling *Salmonella* and *E. coli*. As a safe and non toxic substance, it may have the potential for replacing controversial antibiotic and chlorine usage in pre-harvest and post-harvest disinfection procedures in the poultry industry.

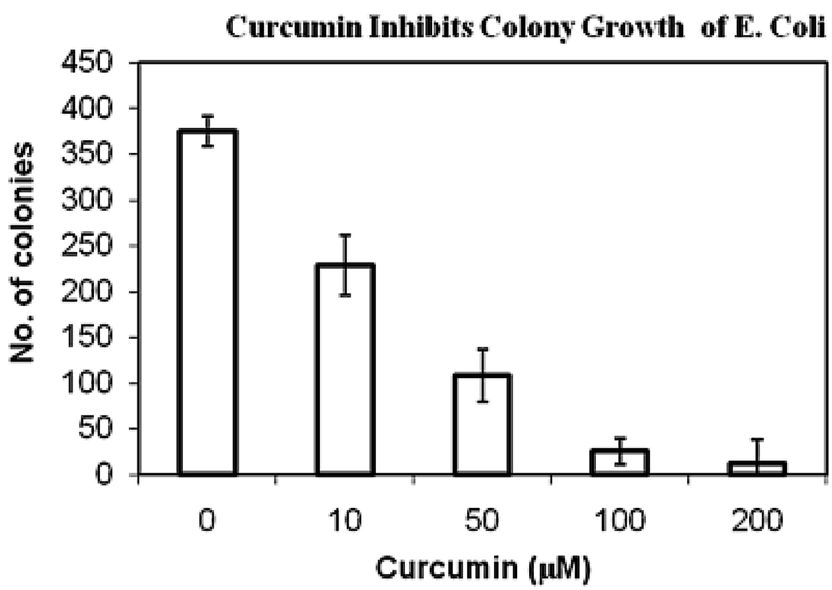

Figure 2: The percentages of inhibition results were 0, 39, 61, 94, and 97 at 0, 10, 50, 100, 200 µM of Curcumin respectively.

Conclusion

Pre- and post-harvest practices are very important factors in controlling *Salmonella* and *E. coli* food pathogens in poultry meat. Poultry pre-harvest pathogen reduction strategy includes usage of antibiotics such as penicillin, erythromycin and tetracyclines. They also prevent or reduce sickness and hasten growth of chickens. Poultry post-harvest practices use chlorine forms, such as sodium hypochlorite, calcium hypochlorite tablets and chlorine gas. However, all these agents are controversial and objectionable. Novel and safer compounds that could be used both in pre and post-harvest meat processing for pathogens with minimal side effects are needed. These data suggest that the molecular mechanism of action investigations of Curcumin's anti-microbial activity demonstrated promising potential for Curcumin as a universally acceptable alternative for antibiotics used as feed additives and chlorine in poultry processing.

The yellow color of Curcumin may be esthetically controversial for the public; hence field testing with "white" Curcumin will probably enhance the chances of public acceptability. Ideally, consumers do not want to consume potentially harmful/hazardous compounds like bleach (a chlorine compound) even at low parts per million. If consumers were fully aware of the potential hazard of trace amounts of chlorine compounds on the poultry, the poultry industry is likely to suffer a financial loss.

The findings in this study are encouraged to be fully tested as a potential alternative to

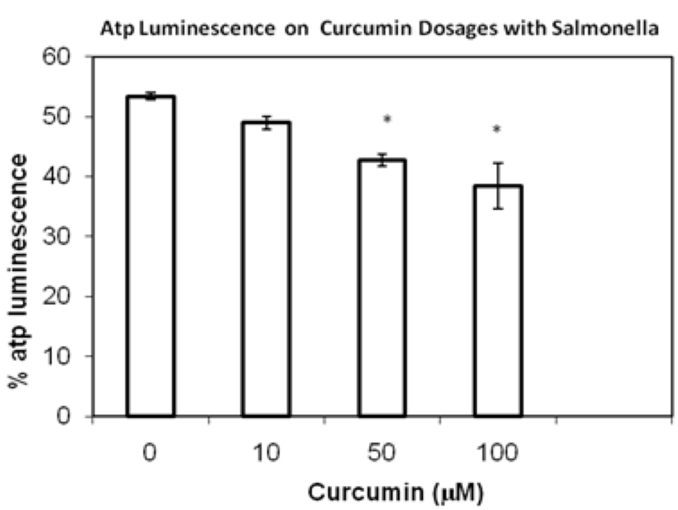

*Figure 3A. Results after treatment of Salmonella with Curcumin were 100, 66.6, 58.5, and 52.3 Luminescence units for 0, 10, 50, and 100µM Curcumin respectively (Figure 3A) (200 µM data absent) *Indicates P value is statistically significant at P < 0.05*

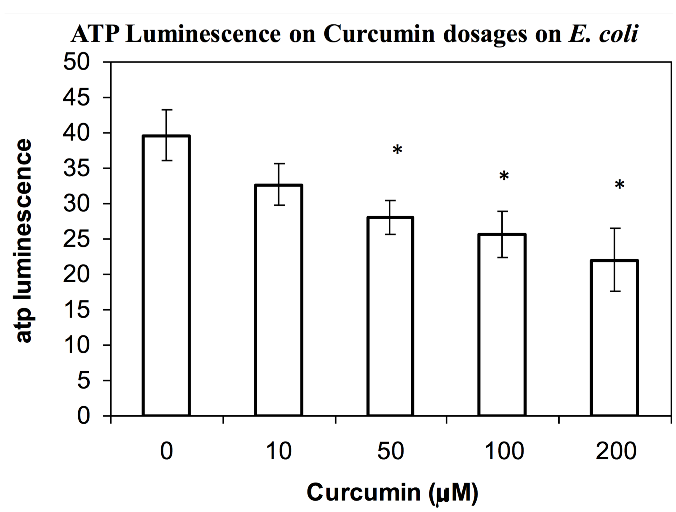

*Figure 3B: Results after treatment of E. coli with Curcumin were 100, 55, 46.6 and 41.6 luminescence units at 0, 10, 50, and 100 µM of Curcumin respectively *Indicates P value is statistically significant at P < 0.05.*

currently used chlorine compounds.

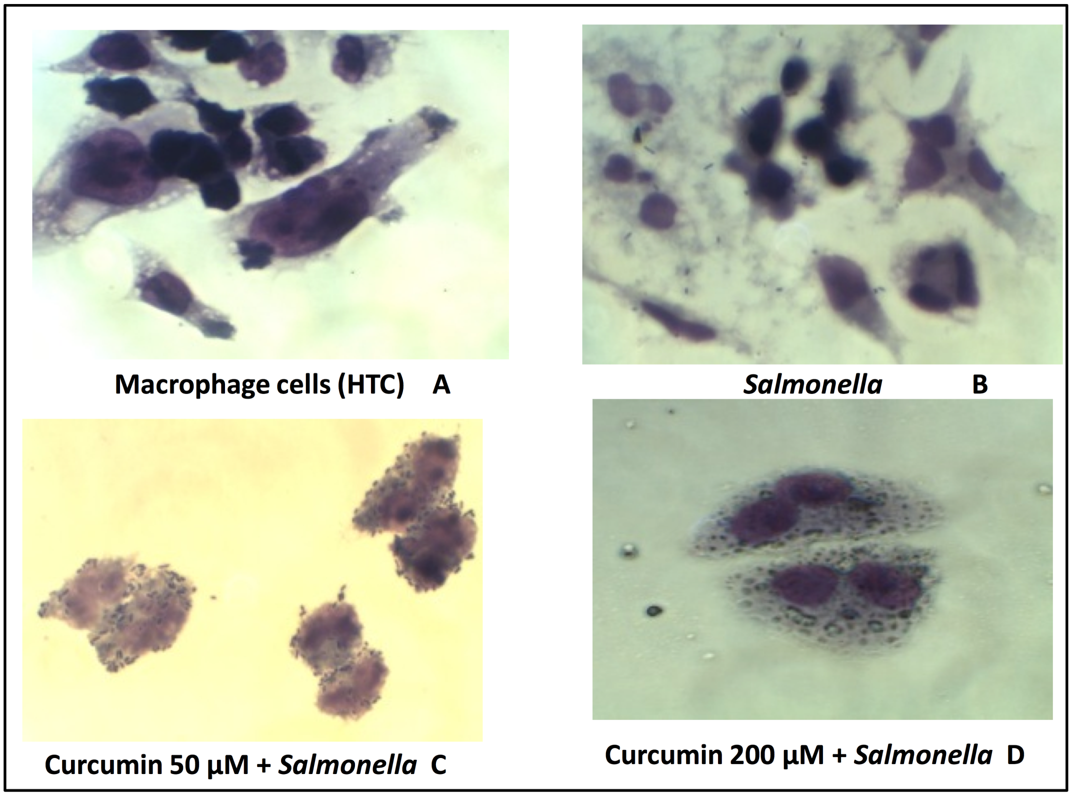

Figure 4: Curcumin enhanced uptake of Salmonella by macrophages

A. Control: Macrophage cells (HTC) only. B. Treatment: Macrophage cells (HTC) with Salmonella. C. Treatment: Macrophage cells (HTC) with 50µM of curcumin. D. Treatment: Macrophage cells (HTC) with 200µM of curcumin.

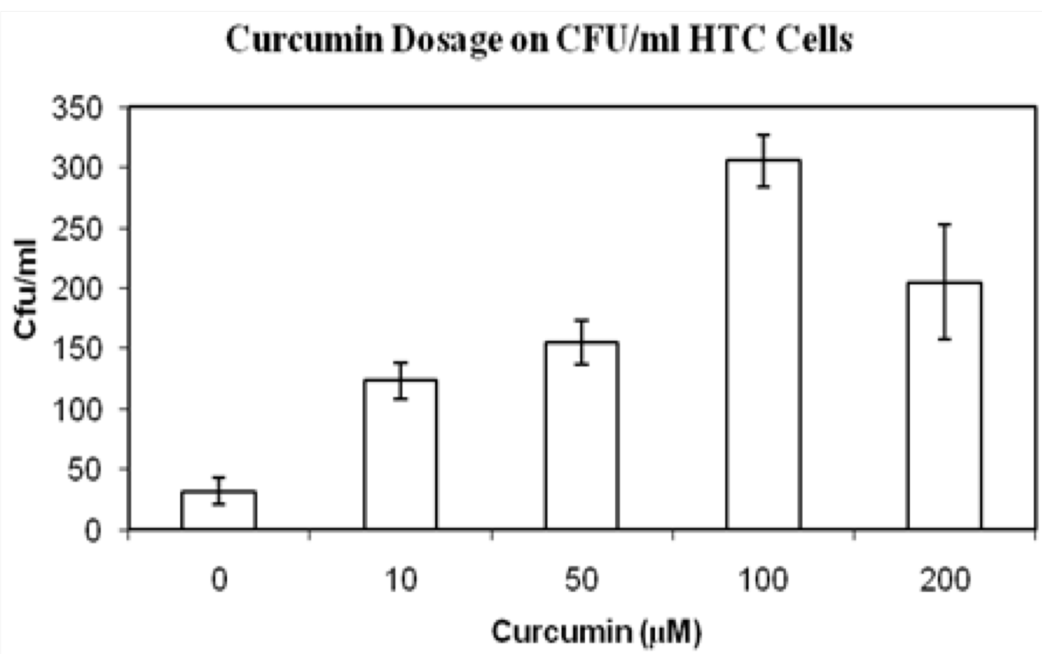

Figure 5: The CFU/mL results after treatment with Curcumin were 31.6, 123.3, 155.6, 305.6, and 205 at 0, 10, 50, 100 and 200 µM concentrations respectively. Treatment of curcumin at 0hr, 5-6hrs, and overnight; 5-6hrs, overnight not graphed above due to absence of colony formation on LB agar plates.

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Research Interests:
Molecular mechanisms of immunosuppression induced by stress and viruses such caprine arthritis-encepahalitis virus, immunomodulation by cytokines, effect of single nutrients such as copper and zinc on immune responses of domestic animals, rapid detection of foodborne pathogens by immunomagnetic separation and RT-PCR.

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