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Antibacterial Activity of Zinc Oxide Nanoparticles against Some Multidrug-Resistant Strains of *Escherichia coli* and *Staphylococcus aureus* isolated from milk and milk products

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ABSTRACT

Milk and dairy products contain a variety of nutrients that offer energy, high-quality protein, and essential micronutrients like calcium, magnesium, potassium, zinc, and phosphorus, which are readily absorbed. The important impact on both public health and the economy is due to their decrease caused by harmful bacteria. This study found harmful bacteria in milk from cows with mastitis and dairy products such as cheese and yogurt, investigated the resistance of the isolates to different antibiotics, and evaluated the virulence of *E. coli* isolates using the congo red test. Additionally, experiments were conducted with Zinc oxide (ZnO) nanoparticles to find out the lowest inhibitory and bactericidal concentrations against *Staphylococcus aureus* and *Escherichia coli*.

Out of 100 milk samples (50 milk and 50 milk products) ,76% of milk samples from cattle and 68% from buffaloes tested positive for *S. aureus*, while 36% of yoghurt and 46% of cheese samples contained the bacterium. *E-coli* was detected in milk samples from cows and buffaloes, with proportions of 44% and 24% respectively, as well as in yogurt (24%) and cheese (36%). Milk and dairy products contain valuable nutrients that offer energy and high-quality protein as well as important micronutrients (like calcium, magnesium, potassium, zinc, and phosphorus) that are readily absorbed. The decrease they experienced due to detrimental bacteria has major implications for public health and the economy. This study discovered harmful bacteria in milk from cattle and dairy products like cheese and yogurt, analyzed the resistance of the bacteria to different antibiotics, and evaluated the virulence of *E. coli* isolates using the congo red test. Additionally, Zinc oxide (ZnO) nanoparticles were assessed to find out the lowest inhibitory concentrations (MIC) and lowest bactericidal concentrations (MBC) for *Staphylococcus aureus* and *Escherichia coli.*

The findings from the in vitro sensitivity test revealed that 57.6% of the 59 *S. aureus* isolates demonstrated high resistance to Trimethoprim \ sulphamethoxazole, whereas 71.7% exhibited high sensitivity to Clindamycin. Additionally, out of the 32 *E.coli* isolates, 71.8% demonstrated resistance to Ampicillin/Sulbactam, and 65.6% showed sensitivity to Clindamycin.

The ZnO nanoparticles showed MIC results of 2.5 µg/mL for *E. coli* and 1.25 µg/mL for *S. aureus* when they were separated. The MBC exhibited a concentration of 2.5 µg/mL against both *E. coli* and *S. aureus*. Scientists found that ZnO nanoparticles can be used instead of conventional antibiotics to treat subclinical mastitis in cows caused by *E. coli* and *S. aureus* infections, as well as to address the difficulties presented by bacteria that

are resistant to multiple drugs

1. INTRODUCTION

2. Mastitis is a serious condition characterized by inflammation of the mammary gland caused by physical trauma or bacterial infection [1]. The main cause of bovine mastitis is bacterial infection within the mammary gland, whether it is acquired from the environment or through contagion. Pathogenic bacteria such as *Streptococcus uberis*, coliforms (such as *E. coli*, *Klebsiella spp.*, *Enterobacter spp*.) and *Pseudomonas* spp. can be identified in the bedding and living areas of the herd. However, *Staphylococcus aureus* and *Streptococcus agalactiae* are infectious pathogens that reside on the cow's udder and teat skin, forming colonies and multiplying within the teat canal [2]. The presence of these bacteria that carry milk in dairy products is important for both public health and the economy. Decreased consumer trust could be related to the economic losses faced by the dairy industry, impacting the market for dairy products [3].
3. Mastitis has three types: subclinical, clinical, and chronic mastitis. Subclinical mastitis is the most recognized form and leads to decreased milk output without any apparent clinical signs or irregularities in the milk. The use of antibiotics such as cloxacillin, penicillin, streptomycin, tetracycline, and ampicillin to treat mastitis is frequently expensive and can result in a higher risk of antibiotic resistance transferring from cows to humans [4]. Hence, other treatment approaches must be used to avoid antibiotic resistance from emerging.
4. Metallic nanoparticles such as copper, titanium, magnesium, zinc, gold, and alginate show great potential as a substitute for antibiotics. Because of their large surface-area-to-volume ratio, these nanoparticles show strong bactericidal properties, resulting in increased reactivity. Hence, nanoscale materials provide greater advantages than their larger bulk equivalents [5].
5. Zinc oxide nanoparticles are important in medicine, particularly in fighting bacteria and cancer, due to their ability to produce large amounts of reactive oxygen species (ROS), release zinc ions, and induce apoptosis [6].
6. Zinc oxide nanoparticles demonstrated antibacterial properties in milk samples. More specifically, ZnO nanoparticles were used as antibacterial substances in milk that was tainted with *E. coli* and *S. aureus*, at levels of 5 and 10 mM. Following 8 hours in a controlled setting, the amount of *S. aureus* bacteria in milk treated with ZnO NPs dropped by 2 log CFU/mL in comparison to the untreated sample, whereas the quantity of *E. coli* bacteria was slightly less than that in the control sample. In milk, ZnO NPs demonstrated a stronger antibacterial effect on *S. aureus* compared to *E. coli*. These findings indicate that ZnO NPs exhibit antibacterial properties in milk samples according to Mirhosseini and Firouzabadi [7].
7. Recently, scientists have claimed that the reason ZnO nanoparticles are effective in treating and preventing mastitis in dairy cows is because they can enhance immune response and increase milk production. Hence, ZnO nanoparticles show

potential as dietary additions for animals due to their antibacterial and immune-enhancing properties, as proposed by Beyth et al. [8],Hamilton and Wenlock [9], and Hill and Li [10].

8. The primary objective of this study was to isolate detrimental bacteria from the milk of cows and buffalos with mastitis, as well as from dairy items such as cheese and yogurt. The research also examined how resistant the bacteria were to antibiotics and evaluated how well ZnO nanoparticles worked against *S. aureus* and *E. coli.*

9. MATERIALS AND METHODS

2 2.1. Sample Collection:

One hundred random samples were collected from El-Menoufia and El-Gharbia governorate. A total of 50 milk samples, consisting of 25 cow milk and 25 buffalo milk, were gathered from different bovine farms with cases of clinical mastitis. Each sample was placed in 10 mL of milk in sterile tubes, then placed in an ice bag and immediately taken to the laboratory for bacteriological testing. Moreover, 25 samples of cheese and 25 samples of yogurt were collected from local grocery stores.

2.2. Isolation and Identification

Around 0.01mL of each milk sample was cultured on different bacteriological media obtained from Oxoid, Ltd., Basingstoke, UK (such as MacConkey agar, Emb agar media, Paired barker agar media, and mannitol salt agar). Following this, they were placed in a controlled environment and maintained at a temperature of 37°C for a duration of 48 hours. The suspected colonies were assessed for physical characteristics such as size, shape, color, pigment production, texture (smooth or rough), and metabolic activity on MacConkey agar (lactose fermenter or non-lactose fermenter). Gram's stain was applied on the suspected colonies in order to prepare them for microscopic examination. The method outlined by Quinn et al. [11]. was used to conduct the biochemical identification evaluation.

2.3. Congo red binding assay:

Congo red stain was dissolved in a powerful water-based solution and then sterilized in an autoclave at 121°C for 15 minutes. Subsequently, the mixture was added to sterilized Brain Heart Infusion agar with sucrose at 55°C. Then, the plates were inoculated with the isolated strains and incubated for 24 to 48 hours at 37°C with oxygen. Dry black colonies with a crystalline texture indicate the presence of biofilm production [12].

2.4. Antibacterial Sensitivity Test :

The susceptibility of the two different strains (*E. coli* and *S. aureus*) to different antibiotics was examined with commercial disks (Hi Media Laboratories Pvt. Ltd., India) following the method outlined by Gloria et al. [13]. Nine antimicrobial discs were used, including cefotrixone (CRD), clindamycin (DA) 2 mcg, tetracycline (TE) 20 mcg, ofloxacin (ofx) 5 mcg, azithromycin (AZm) 15 mg, trimethoprim/sulfamethoxazole (SXT) 125/23.75 mcg, ampicillin/sulbactam (SAM) 20 µg, and penicillin (P) 20 µg.

2.5. Preparation of Zinc Oxide Nanoparticles:

In short, a 1M NaOH solution was slowly added to 500mL Zinc sulfate heptahydrate with constant stirring at a rate of 0.2mL/min until the pH reached a strongly alkaline level of 12 [14,15]. The product was purified through washing with sterile distilled water and centrifuging at 18000 x g for 20 minutes. After that, the end result was once more rinsed with ethanol. Ultimately, ZnO nanoparticles with an average size of 30 nm were obtained through sono-chemical treatment and drying processes [14,16]. The properties of the prepared ZnO nanoparticles were studied through X-ray spectroscopy (XRD), and the surface features were analyzed using scanning electron microscopy (SEM).

The ZnO nanoparticles solution was made by mixing it in sterile normal saline and stirring until a consistent colloidal suspension with a final concentration of 1000µg/mL was achieved, then stored at 4°C until needed.

2.6. Antibacterial activity of ZnO nanoparticles on *S. aureus* and *E. coli* [17]

To assess how effective ZnO nanoparticles are at killing bacteria like *S. aureus* and *E. coli*, the nanoparticles were mixed with sterile normal saline and stirred continuously until a consistent colloidal suspension was achieved. The bacteria samples were introduced into muller hinton broth and incubated at 37°C for a period of 24 hours. The turbidity of collected cultures was standardized at 0.5 Mcfarland (1.5×108 CFU/mL).

2.6.1. Determination of inhibition zone:

The cotton swabs without bacteria were placed in the modified 0.5 Mcfarland suspension containing *S. aureus* and *E. coli*. Next, the swabs were swirled on the surface of nutrient agar. Following removal of

excess moisture, sterile borers were used to cut wells in the selected area of the plates. Next, different amounts of ZnO nanoparticles (20, 10, 5, 2.5, 1.25, 0.6, and 0.3 µg/mL) were injected into the wells in 0.1 ml increments. The ZOI was assessed following 24 hours of incubation. Comparison was made between the antibacterial activity of ZnO nanoparticles.

2.6.2. Determination of the Minimal Inhibitory Concentrations:

Various amounts of ZnO nanoparticles were prepped in sterile eppendorf tubes, including concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.6 µg/mL. Next, 50µL of 0.5 McFarland suspensions of *S. aureus* and *E. coli* were added to individual tubes in two separate groups. After testing, the ZnO nanoparticles were found at concentrations of 20, 10, 5, 2.5, 1.25, 0.6, and 0.3 µg/mL. The tubes were then incubated at 37°C for 24 hours, and turbidity detection was used to visually examine the incubated plate. One well had the culture being tested, while another contained only sterile broth medium as a negative control. The minimum inhibitory concentration (MIC) is the lowest concentration of ZnO nanoparticles which stopped visible bacterial growth following 24 hours of incubation.

The minimum bactericidal concentration was established by inoculating 50 µl from tubes without growth or cloudiness on muller hinton agar and incubating at 37°C for 24 hours. The minimal inhibitory concentration for ZnO nanoparticles, known as the MBC, can halt the growth of bacterial colonies on the plates.

2.6.3. Scanning electron microscope:

The SEM machine from Hitachi in Tokyo, Japan was utilized to study the appearance of bacterial cells pre and post ZnO NPs treatment [18], as conducted at the central lab in the science faculty of Benha University. Initially, cells were mainly treated with a fixative buffer (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M Na-Cacodylate buffer, pH 7.35) for a period of 30 minutes. Afterwards, the samples were washed three times with ultrapure water, then dehydrated using a range of ethanol solutions (10, 30, 50, 70, 90, and 99%). The dried specimens were dehydrated quickly using a critical point dryer (Auto-Samdri-815 Automatic Critical Point Dryer; Tousimis, Rockville, MD), then attached to SEM stubs and coated with gold/palladium using a cool-sputter coater (E5100 II, Polaron Instruments Inc., Hatfield, PA). Segments were subsequently examined using SEM at 8 kV every hour post-treatment. Chosen regions were based on changes in the shape of the cells that had been treated.

3. Results:

3.1. Isolation and Identification of *Staphylococcus aureus* and *E. coli*

Analysis of 50 milk samples from cows and buffaloes and 50 milk products from El- Menoufia and El-Gharbia governorates showed presence of Staphylococcus species and *E. coli* strains (Table 1). In 36 out of 50 milk samples (72%) and 23 out of 50 milk products (46%), *Staphylococcus aureus* was detected, with lower rates of *E. coli* isolation at 34% and 30% in milk and milk products.

3.2. Congo red (CR) Binding test :

The strains that were isolated were examined to determine their growth condition on congo red medium. The response was observed following 18, 24, 48, and 72 hours of being kept at 37˚C and was then left at room temperature for 2 extra days. The isolates that were Congo red positive (CR+) displayed bright or orange red colonies. Various levels of dye absorption were indicated as (+) and (++). Isolates that were Congo red negative (CR-) did not show any dye binding and were seen as white colonies. The majority of isolates showed varying levels of positive results in red color; 10 isolates (31.2%) displayed deep red color, 14 strains (43.7%) showed a light orange hue, and 8 isolates (25%) did not react with the dye.

3.3. Antibacterial Sensitivity test:

The disc diffusion method tested the antimicrobial sensitivity of 59 Staphylococcus aureus isolates to various antibiotics, revealing varying levels of resistance. Penicillins were completely ineffective against all strains (100%), while sulphonamides like trimethoprim sulfamethoxazole showed 57.6% resistance, and tetracyclines and ampicillin/sulbactam both showed 47.4% resistance (table 2, Fig. 1). In contrast, clindamycin showed the highest effectiveness against the isolates at 71.1%, followed by ceftrixone at 57.6% and ofloxacin at 47.4%.

Antimicrobial susceptibility patterns of *E. coli* isolated from milk of cattle and Buffaloes with clinical mastitis and milk product (yoghurt and cheese).

Out of thirty-two *E. coli* isolates, 71.8% showed high resistance to Ampicillin/Sulbactam and 56.25% to Trimethoprim/sulphamethoxazole. On the other hand, they showed a strong response to clindamycin at a rate of 65.6% (Table 3, Fig 2).

3.4. Characterization of Zno nanoparticles:

The XRD pattern of ZnO indicated that the ZnO powder was in hexagonal wurtzite phase with a crystallite size of 19 nm, determined using the Scherrer formula for the (101) diffraction peak. Figure 3 indicated that the lattice parameters a and c were measured at 3.205Å and 5.122Å, respectively.

The scanning electron microscope captured a uniform spherical morphology with some aggregation in the surface image of ZnO nanoparticles powder (fig 4)

3.5. Antibacterial activity of ZnO nanoparticles on *S. aureus* and *E. coli*

3.5.1. Determination of inhibition zone:

The well diffusion method was used to determine inhibition zones against *S. aureus* and *E. coli* by different concentrations of ZnO nanoparticles (20, 10, 5, 2.5, 1.25, 0.6, and 0.3 µg/ml). The inhibitory effect was more pronounced against *S. aureus* compared to *E. coli,* with 10 µg/ml resulting in a 29mm inhibition zone for *S. aureus* and a 19 mm inhibition zone for *E. coli*. In addition, the lowest concentration (0.312 µg/ml) resulted in an inhibition zone of only 15mm against *S. aureus.*

3.5.2. Determination of the Minimal Inhibitory Concentrations and Minimal bactericidal Concentrations:

The minimum inhibitory concentration (MIC) of ZnO nanoparticles was 2.5 µg/ml for isolated *E. coli* and 1.25 µg/ml for *S. aureus*. After incubating *S. aureus* and E. coli with various concentrations of ZnO nanoparticles overnight, both strains exhibited an MBC of 2.5 µg/ml.

3.5.3. Morphological test of bacterial cells Treated with ZnO NPs

Analysis was conducted on SEM images of *S. aureus* and *E. coli* after being incubated in their respective broth medium for 10 hours, both with and without the addition of 20 µg/ml of ZnO NPs. Unprocessed *S. aureus* and *E. coli*, as depicted in Figures (5 and 6), exhibited preserved bacterial cell structure. Following exposure to ZnO NPs for 10 hours, there was a noticeable change in the surface structure and appearance of bacterial cells. Clusters of *staphylococcus* cocci appeared together and lacked clear definition (Figures 7), whereas the cell wall of *E. coli* became twisted and rougher compared to the untreated sample (Figure 8

4.Discussion :

The analysis of milk samples showed that *S. aureus* was found in 76% of cattle and 68% of buffalo . This finding is almost the same as the one reported by Kivaria et al. [19], where they observed an 82% prevalence of *S. aureus* in small-scale dairy farms with bovine clinical mastitis. Chowdhury et al.[20], also found similar results, with a prevalence of 53.85% in milk from cows with mastitis. Yavuz and Esndal. [21], also found *S. aureus* present in 53.3% of cases. Contrary to Kivaria and Noordhuizen . [22], Swai et al. [23], and Gronlund et al. [24], who found lower prevalence rates of *S. aureus* in milk samples at 25.7%, 24%, and 27.2% respectively, Kamal et al. [25] reported a higher prevalence of 94%.

Consumption of yoghurt and cheese is widespread globally, with potential health risks if contaminated with pathogens like *S. aureus* [26]. Bacterial analysis of cheese found *S. aureus* present in 56% of samples, similar to the 24% reported by Elmaghraby et al. [27]. In contrast, Kamal et al. [25] discovered a higher prevalence of 93%. The reason for *Staph. aureus* being in kareish cheese is due to the fact that this cheese is produced from unprocessed skimmed milk and is not exposed to high temperatures. Additionally, using dirty utensils and improper storage can also lead to contamination of this cheese with Staph. aureus and other harmful pathogens. This outcome aligned with the findings of Sadek et al. [28].

Examination of yoghurt showed *S. aureus* presence at a rate of 36%. Previous studies by Zakary et al. [29] reported a prevalence of 14%, whereas Meshref et al. [30] found a much higher incidence of 88%.

In this study, *E.coli* was found in 34% of milk samples that were isolated. Ahmed [31] studied 203 milk samples and found a high incidence of *E. coli* (27.09%) among the isolated bacteria, which was similar to the findings. Additionally, it aligned with Zaki et al. [32] in their examination of *E. coli* isolation from 100 clinical mastitic cases, revealing a prevalence rate of (25.3%). A research conducted in Northern Ireland on 264 cases of both acute and per acute toxic mastitis showed that *E. coli* was found in 50% of milk samples from the cases [33]. Kerro-Dego and Tareke [34], found a lower rate of 22% of *E.coli* in clinical mastitis cases, while Ibrahim et al. [35], reported a higher prevalence of 52% in raw market milk.

In this study, *E.coli* was found in 24% of yoghurt samples and in 36% of cheese samples. Abushaala et al. [36], found that locally produced dairy products tested for *E. coli* using Sorbitol MacConkey agar showed 24% (6/25) positive samples in cheese and 12% (3/25) in Supermarket Plain yogurt and Small dairies Plain yogurt. Ibrahim and colleagues [36], found the highest prevalence in cheese (48%), with the lowest prevalence found in large-scale yoghurt and large-scale Laban rayeb samples (8%).

***S. aureus* found in milk samples showed resistance to seven various antibiotics, as observed in this study. Narmeen et al. [37] also reported a comparable discovery. It was discovered that no isolate was sensitive to all antibiotics, as *S. aureus* demonstrated resistance to multiple antibiotics. The examined strains showed resistance to Tetracycline and Trimethoprim-Sulfamethoxazole at rates of 47.4% and 57.6%, respectively (Table 4). Wang et al. [38] reported resistance rates of 38.2% and 6.1% for the antibiotics used previously. Narmeen et al. [37], also reported a comparable discovery. It was discovered**

that there were no isolates sensitive to all antibiotics as *S. aureus* showed resistance to multiple antibiotics. The examined isolates showed resistance to Tetracycline and Trimethoprim-Sulfamethoxazole at rates of 47.4% and 57.6%, respectively (Table 4). Wang et al. [38], reported resistance rates of 38.2% and 6.1% for the antibiotics used previously.

The sensitivity test conducted in vitro on 32 *E.coli* isolates showed high resistance (71.8%) to Ampicillin/Sulbactam and high sensitivity (65.6%) to Clindamycin. The existence of bacteria resistant to multiple drugs is concerning as they pose a significant threat to public health, according to Zouhairi et al. [39]. This was in line with the study by Ibrahim et al. [35] which identified that 58.8% of E. coli strains met the criteria for multi-antibiotic resistance (MAR), being resistant to at least one antimicrobial in three distinct antibiotic classes. Erythromycin had the highest resistance rate at 100%, followed by oxacillin at 94%, cefepime at 82%, penicillin G at 76.5%, and ampicillin at 58.5%. Nalidixic acid had a resistance rate of 52.9% and cefazolin at 47.1%. These findings highlight the significant threat posed to public health, emphasizing the crucial need for implementing hygiene practices across all stages of the dairy industry from production to consumption to safeguard human health.

New developments in nanotechnology, specifically in creating metal oxide nanoparticles with high ionic properties in any size and shape, could result in the creation of novel antibacterial treatments. Nanomaterials made of metal oxides caused more cell death at higher concentrations, impacting mitochondrial function, leading to leakage of lactate dehydrogenase, and causing unusual cell shapes even at low concentrations of 50–100 mg/L [40].

The ZnO NP exhibited a significantly higher level of antibacterial activity compared to ZnO powder. This can be clarified as smaller particles typically have a higher surface area to volume ratio, allowing for a more effective way of carrying out antibacterial functions [41]. The antibacterial activity of Zno nanoparticles is further explained by the production of hydrogen peroxide (H2O2) once they enter the bacteria[42]. It is possible to suggest that as the particle size decreases, the concentration of H2O2 produced on the surface likely increases due to the greater number of ZnO powder particles in a given volume of powder slurry. Initial research as detailed in this report has revealed that ZnO metal oxide nanoparticles exhibited a notable decrease in growth under standard laboratory conditions [43]. In this study, two bacterial strains, one Gram positive and one Gram negative, were utilized to examine the antimicrobial potential of ZnO.

Additionally, the findings showed that the effectiveness of ZnO nanoparticles in fighting bacteria varied greatly based on their concentration. The MIC and MBC values of ZnO nanoparticles were measured to assess their antimicrobial effects on drug resistant strains of *E. coli* and *S. aureus* cows. The MIC values for ZnO nanoparticles against isolated *E. coli* and *S. aureus* were shown in Table 4 to be 2.5 µg/mL and 1.25 µg/mL, respectively. These results support Palanikumar et al. [44] findings which highlighted the significance of ZnO nanoparticles concentration and size in influencing the antimicrobial properties of the particles.

The MIC obtained for *S. aureus* and *E. coli* was 1.25 µg /ml and 2.5 µg/ml, respectively, matching the MIC reported by Reddy et al. [45] for ZnO nanoparticles against *S. aureus* at 1 mm. Confirmation of bactericidal effect of MBC ZnO nanoparticles against *S. aureus* and *E. coli* (2.5µg/ml) was established.

On the other hand, the earlier data on bacterial susceptibility to ZnO revealed that Gram-positive bacteria were more susceptible to ZnO powder and NPs compared to Gram-negative bacteria. It was suggested that ZnO's antibacterial effect occurs by interacting with certain cell compounds that are more abundant in or enhanced in Gram-positive bacteria compared to Gram-negative bacteria. Possible candidates for these compounds include the external thick peptidoglycan layer, its amino acid components, surface proteins like adhesins, and teichoic acids combined with lipids to create lipoteichoic acids. These substances function as chelating agents and play a role in various adhesion processes. (Ibrahim and colleagues [46]; Alekish and team [47]. Moreover, Karvani and Chehrazi . [48], Alekish et al. [47], and Walaa et al. [49], all demonstrated in their research that gram positive bacteria exhibit greater sensitivity to ZnO nanoparticles compared to gram negative bacteria. Nonetheless, the results obtained contradict the previous research by Slavin et al. [50], which demonstrated that ZnO nanoparticles exhibit stronger antibacterial effects against gram-negative bacteria (*K. pneumoniae* and *E. coli*) compared to gram-positive bacteria (*S. aureus*). The difference in the cell wall composition between gram-negative and gram-positive bacteria could be the reason for this occurrence [50].

Examining SEM pictures of untreated *S. aureus* and *E. coli* (Figures 8 and 9) versus those treated with ZnO NPs for 10 hours (Figure 10 and 11) showed a noticeable variation in the surface structure and form of the bacterial cells. The breakdown of the membrane structure in *S. aureus* and *E. coli* leads to metal loss, leading to the creation of irregularly-shaped holes in the bacteria's outer membrane. Additionally, zinc is thought to attach to proteins' functional groups, causing them to unfold.

3. Results:

3.1. Isolation and Identification of *Staphylococcus aureus* and *E. coli*

Analysis of 50 milk samples from cows and buffaloes and 50 milk products from El- Menoufia and El- Gharbia governorates showed presence of Staphylococcus species and *E. coli* strains (Table 1). In 36 out of 50 milk samples (72%) and 23 out of 50 milk products (46%), *Staphylococcus aureus* was detected, with lower rates of *E. coli* isolation at 34% and 30% in milk and milk products.

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3.5. Antibacterial activity of ZnO nanoparticles on *S. aureus* and *E. coli*

3.5.1. **Determination of inhibition zone:**

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3.5.2. Determination of the Minimal Inhibitory Concentrations and Minimal bactericidal Concentrations:

The minimum inhibitory concentration (MIC) of ZnO nanoparticles was 2.5 µg/ml for isolated *E. coli* and 1.25 µg/ml for *S. aureus*. After incubating *S. aureus* and E. coli with various concentrations of ZnO nanoparticles overnight, both strains exhibited an MBC of 2.5 µg/ml.

3.5.3. Morphological test of bacterial cells Treated with ZnO NPs

Analysis was conducted on SEM images of *S. aureus* and *E. coli* after being incubated in their respective broth medium for 10 hours, both with and without the addition of 20 µg/ml of ZnO NPs. Unprocessed *S. aureus* and *E. coli*, as depicted in Figures (5 and 6), exhibited preserved bacterial cell structure. Following exposure to ZnO NPs for 10 hours, there was a noticeable change in the surface structure and appearance of bacterial cells. Clusters of *staphylococcus* cocci appeared together and lacked clear definition (Figures 7), whereas the cell wall of *E. coli* became twisted and rougher compared to the untreated sample (Figure 8)

Table 1: Incidence of *Staphylococcus aureus* and *E. coli* strains in milk samples from cows and buffaloes with clinical mastitis and dairy products (yogurt and cheese):

Samples	Origin of Milk	No of samples	*Staphylococcus aureus*		*E. coli*	
			No.	%	No.	%
Milk (Clinical Mastitis)	Cattle	25	19	76%	11	44%
	Buffalo	25	17	68%	6	24%
Total		**50**	**36**	**72%**	**17**	**34%**
Milk products	Yoghurt	25	9	36%	6	24%
	Cheese	25	14	56%	9	36%
Total		**50**	**23**	**46%**	**15**	**30%**

Table 2: Antibiotic resistance profiles of *Staphylococcus aureus* found in the milk of cows and buffaloes with mastitis, as well as in dairy products like yogurt and cheese.

Antimicrobial class	Antimicrobial agents	Disc	Conc.	Resistant (%)	Inter
Quinolones	Ofloxacin	Ofx	5	12 (20.3)	19
Tetracycline	tetracycline	TE	20	28 (47.4)	9
Cephalosporins	Ceftrixone	CRD	-	8 (13.5)	18
Lincomycin	Clindamycin	DA	2	12 (20.3)	5
Macrolides	Azithromycin	AZm	15	14(23.7)	18
Sulfonamides	Trimethoprim\ sulphamethoxazole	SXT	14	34 (57.6)	19
B –lactams	Ampicillin/Sulbactam	SAM	20	28 (47.4)	11
	Penicillin	p	20	59(100%)	

Table 3: Antibiotic resistance patterns of *E. coli* found in milk from cows and buffalo suffering from clinical mastitis, as well as in dairy products like yogurt and cheese.

Antimicrobial class	Antimicrobial agents	Disc	Conc.	Resistant (%)	Intermediate (%)	Sensitive (%)
Quinolones	Ofloxacin	Ofx	5	3 (9.37)	18 (56.25)	11 (34.3)
Tetracycline	tetracycline	TE	20	17 (53.1)	10 (31.2)	5 (15.6)
Cephalosporins	Ceftrixone	CRD	-	5 (15.6)	18 (56.25)	9 (28.1)
Lincomycin	Clindamycin	DA	2	9 (28.1)	2 (6.25)	21 (65.6)
Macrolides	Azithromycin	AZm	15	3(9.37)	13 (40.6)	16 (50)
Sulfonamides	Trimethoprim\ sulphamethoxazole	SXT	14	18 (56.25)	9 (28.1)	5 (15.6)
B –lactams	Ampicillin/Sulbactam	SAM	20	23 (71.8)	3(9.3)	6 (18.75)
	Penicillin	P	20	32(100%)	-	-

Table 4: showing the growth behavior of *E. coli* and *S. aureus* when treated with ZnO nanoparticles to find their Minimum Inhibitory Concentration (MIC).

Mode of effect **Concentration (mg/ml)**	***S. aureus*** **Concentration (mg/ml)**	***E.coli***
0.3	Turbid	Turbid
0.625	Turbid	Turbid
1.25	Non Turbid (MIC)	Turbid
2.5	Non Turbid (MBC)	Non Turbid(MIC) (MBC)
5	Non Turbid	Non Turbid
10	Non Turbid	Non Turbid
20	Non Turbid	Non Turbid

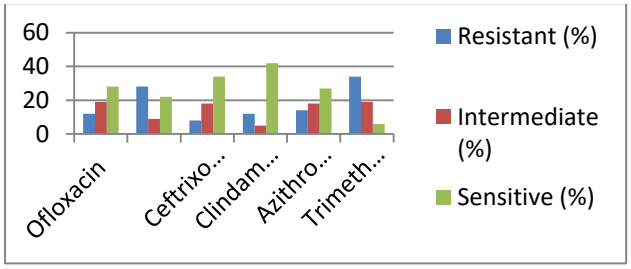

Fig 1: Antibiotic resistance profiles of *Staphylococcus aureus* from cows and buffaloes with mastitis and dairy products (yogurt and cheese

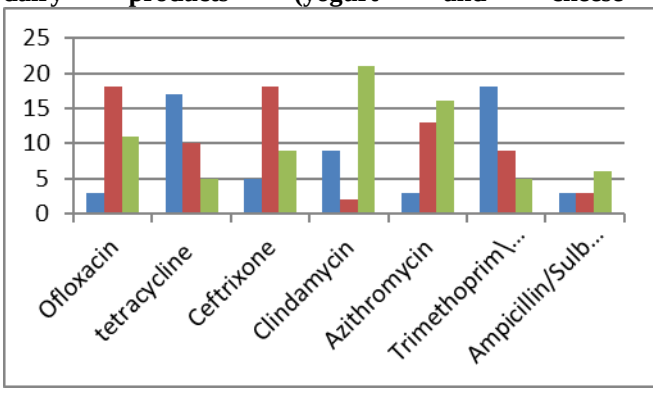

Fig (2): Antibiotic resistance of *E.coli* found in milk from cows and buffaloes with mastitis and dairy products (yogurt and cheese

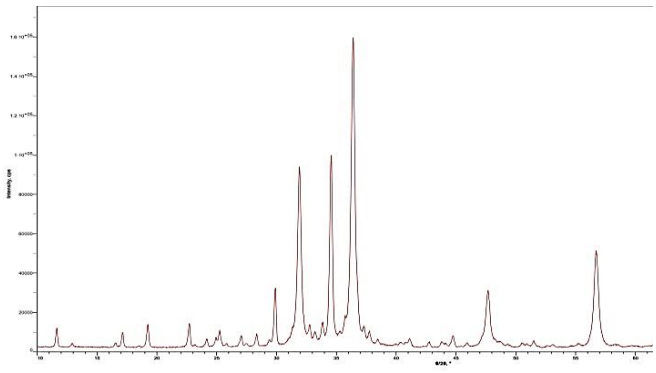

Fig (3): XRD patterns for ZnO powder charts

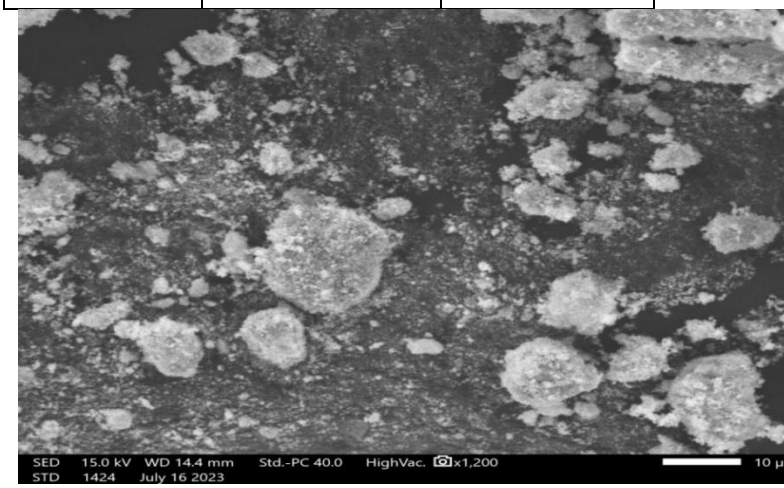

Figure (4): SEM surface image of nanoparticles ZnO powder.

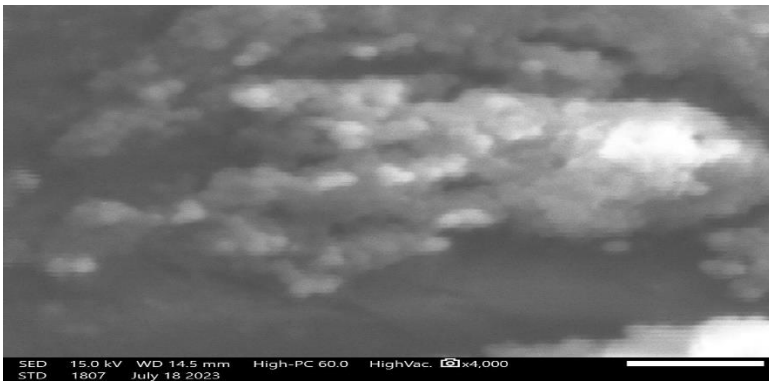

Figure (5) : *S.aureus* (control sample) under scan electron microscope at a magnification power of 500×.

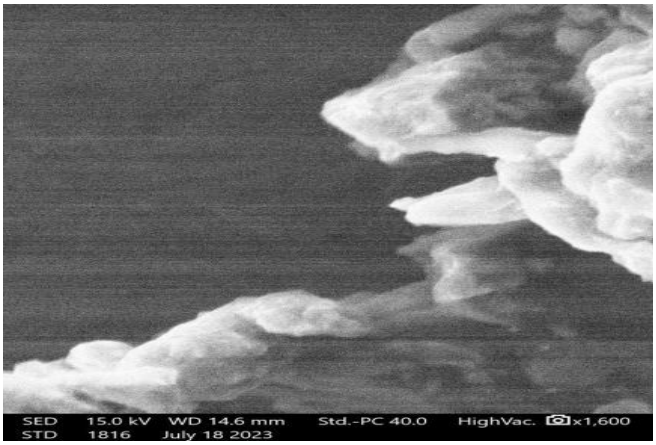

Figure (6) : *E-coli* (control sample) under scan electron microscope at a magnification power of 500

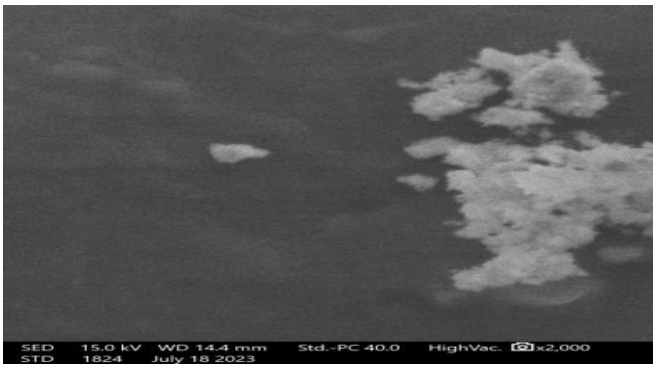

Figure (7): *S. aureus* treated with ZnO nanoparticles under scan electron microscope).

Figure (8) : *E.coli* treated with ZnO nanoparticles under scan electron microscope

4.Discussion :

The analysis of milk samples showed that *S. aureus* was found in 76% of cattle and 68% of buffalo . This finding is almost the same as the one reported by **Kivaria et al. [19],** where they observed an 82% prevalence of *S. aureus* in small-scale dairy farms with bovine clinical mastitis. **Chowdhury et al**.[20], also found similar results, with a prevalence of 53.85% in milk from cows with mastitis. **Yavuz and Esndal**. [21], also found *S. aureus* present in 53.3% of cases. Contrary to **Kivaria and Noordhuizen . [22], Swai et al. [23],** and **Gronlund et al. [24],** who found lower prevalence rates of *S. aureus* in milk samples at 25.7%, 24%, and 27.2% respectively, **Kamal et al. [25]** reported a higher prevalence of 94%.

Consumption of yoghurt and cheese is widespread globally, with potential health risks if contaminated with pathogens like *S. aureus* [26]. Bacterial analysis of cheese found *S. aureus* present in 56% of samples, similar to the 24% reported by Elmaghraby et al. [27]. In contrast, Kamal et al. [25] discovered a higher prevalence of 93%. The reason for *Staph. aureus* being in kareish cheese is due to the fact that this cheese is produced from unprocessed skimmed milk and is not exposed to high temperatures. Additionally, using dirty utensils and improper storage can also lead to contamination of this cheese with Staph. aureus and other harmful pathogens. This outcome aligned with the findings of Sadek et al. [28].

Examination of yoghurt showed *S. aureus* presence at a rate of 36%. Previous studies by Zakary et al. [29] reported a prevalence of 14%, whereas Meshref et al. [30] found a much higher incidence of 88%.

In this study, *E.coli* was found in 34% of milk samples that were isolated. Ahmed [31] studied 203 milk samples and found a high incidence of *E. coli* (27.09%) among the isolated bacteria, which was similar to the findings. Additionally, it aligned with Zaki et al. [32] in their examination of *E. coli* isolation from 100 clinical mastitic cases, revealing a prevalence rate of (25.3%). A research conducted in Northern Ireland on 264 cases of both acute and per acute toxic mastitis showed that *E. coli*

was found in 50% of milk samples from the cases [33]. Kerro-Dego and Tareke [34], found a lower rate of 22% of *E.coli* in clinical mastitis cases, while Ibrahim et al. [35], reported a higher prevalence of 52% in raw market milk.

In this study, *E.coli* was found in 24% of yoghurt samples and in 36% of cheese samples. Abushaala et al. [36], found that locally produced dairy products tested for *E. coli* using Sorbitol MacConkey agar showed 24% (6/25) positive samples in cheese and 12% (3/25) in Supermarket Plain yogurt and Small dairies Plain yogurt. Ibrahim and colleagues [36], found the highest prevalence in cheese (48%), with the lowest prevalence found in large-scale yoghurt and large-scale Laban rayeb samples (8%).

S. aureus found in milk samples showed resistance to seven various antibiotics, as observed in this study. Narmeen et al. [37] also reported a comparable discovery. It was discovered that no isolate was sensitive to all antibiotics, as *S. aureus* demonstrated resistance to multiple antibiotics. The examined strains showed resistance to Tetracycline and Trimethoprim-Sulfamethoxazole at rates of 47.4% and 57.6%, respectively (Table 4). Wang et al. [38] reported resistance rates of 38.2% and 6.1% for the antibiotics used previously. Narmeen et al. [37], also reported a comparable discovery. It was discovered that there were no isolates sensitive to all antibiotics as *S. aureus* showed resistance to multiple antibiotics. The examined isolates showed resistance to Tetracycline and Trimethoprim-Sulfamethoxazole at rates of 47.4% and 57.6%, respectively (Table 4). Wang et al. [38], reported resistance rates of 38.2% and 6.1% for the antibiotics used previously.

The sensitivity test conducted in vitro on 32 *E.coli* isolates showed high resistance (71.8%) to Ampicillin/Sulbactam and high sensitivity (65.6%) to Clindamycin. The existence of bacteria resistant to multiple drugs is concerning as they pose a significant threat to public health, according to Zouhairi et al. [39]. This was in line with the study by Ibrahim et al. [35] which identified that 58.8% of E. coli strains met the criteria for multi-antibiotic resistance (MAR), being resistant to at least one antimicrobial in three distinct antibiotic classes. Erythromycin had the highest resistance rate at 100%, followed by oxacillin at 94%, cefepime at 82%, penicillin G at 76.5%, and ampicillin at 58.5%. Nalidixic acid had a resistance rate of 52.9% and cefazolin at 47.1%. These findings highlight the significant threat posed to public health, emphasizing the crucial need for implementing hygiene practices across all stages of the dairy industry from production to consumption to safeguard human health.

New developments in nanotechnology, specifically in creating metal oxide nanoparticles with high ionic properties in any size and shape, could result in the creation of novel antibacterial treatments. Nanomaterials made of metal oxides caused more cell death at higher concentrations, impacting mitochondrial function, leading to leakage of lactate dehydrogenase, and causing unusual cell shapes even at low concentrations of 50–100 mg/L [40].

The ZnO NP exhibited a significantly higher level of antibacterial activity compared to ZnO powder. This can be clarified as smaller particles typically have a higher surface area to volume ratio, allowing for a more effective way of carrying out antibacterial functions [41]. The antibacterial activity of Zno nanoparticles is further explained by the production of hydrogen peroxide (H2O2) once they enter the bacteria[42]. It is possible to suggest that as the particle size decreases, the concentration of H2O2 produced on the surface likely increases due to the greater number of ZnO powder particles in a given volume of powder slurry. Initial research as detailed in this report has revealed that ZnO metal oxide nanoparticles exhibited a notable decrease in growth under standard laboratory conditions [43]. In this study, two bacterial strains, one Gram positive and one

Gram negative, were utilized to examine the antimicrobial potential of ZnO.

Additionally, the findings showed that the effectiveness of ZnO nanoparticles in fighting bacteria varied greatly based on their concentration. The MIC and MBC values of ZnO nanoparticles were measured to assess their antimicrobial effects on drug resistant strains of *E. coli* and *S. aureus* cows. The MIC values for ZnO nanoparticles against isolated *E. coli* and *S. aureus* were shown in Table 4 to be 2.5 µg/mL and 1.25 µg/mL, respectively. These results support Palanikumar et al. [44] findings which highlighted the significance of ZnO nanoparticles concentration and size in influencing the antimicrobial properties of the particles.

The MIC obtained for *S. aureus* and *E. coli* was 1.25 µg /ml and 2.5 µg/ml, respectively, matching the MIC reported by Reddy et al. [45] for ZnO nanoparticles against *S. aureus* at 1 mm. Confirmation of bactericidal effect of MBC ZnO nanoparticles against *S. aureus* and *E. coli (*2.5µg/ml) was established.

On the other hand, the earlier data on bacterial susceptibility to ZnO revealed that Gram-positive bacteria were more susceptible to ZnO powder and NPs compared to Gram-negative bacteria. It was suggested that ZnO's antibacterial effect occurs by interacting with certain cell compounds that are more abundant in or enhanced in Gram-positive bacteria compared to Gram-negative bacteria. Possible candidates for these compounds include the external thick peptidoglycan layer, its amino acid components, surface proteins like adhesins, and teichoic acids combined with lipids to create lipoteichoic acids. These substances function as chelating agents and play a role in various adhesion processes. (Ibrahim and colleagues [46]; Alekish and team [47]. Moreover, Karvani and Chehrazi . [48], Alekish et al. [47], and Walaa et al. [49], all demonstrated in their research that gram positive bacteria exhibit greater sensitivity to ZnO nanoparticles compared to gram negative bacteria. Nonetheless, the results obtained contradict the previous research by Slavin et al. [50], which demonstrated that ZnO nanoparticles exhibit stronger antibacterial effects against gram-negative bacteria (*K. pneumoniae* and *E. coli*) compared to gram-positive bacteria (*S. aureus*). The difference in the cell wall composition between gram-negative and gram-positive bacteria could be the reason for this occurrence [50].

Examining SEM pictures of untreated *S. aureus* and *E. coli* (Figures 8 and 9) versus those treated with ZnO NPs for 10 hours (Figure 10 and 11) showed a noticeable variation in the surface structure and form of the bacterial cells. The breakdown of the membrane structure in *S. aureus* and *E. coli* leads to metal loss, leading to the creation of irregularly-shaped holes in the bacteria's outer membrane. Additionally, zinc is thought to attach to proteins' functional groups, causing them to unfold.

5. Conclusion

Recent developments in nanotechnology, specifically the capability to create metal oxide nanoparticles with high ionic properties in various sizes and forms, could result in the creation of novel antibacterial substances. Initial research as described in this document has shown that ZnO metal oxide nanoparticles exhibited a notable decrease in growth under typical lab conditions.

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