EFFECT OF ZINC OXIDE AND SILVER NANOPARTICLES ON INTESTINAL
BACTERIA

A Thesis presented to the Faculty of the Graduate School
University of Missouri

In Partial Fulfillment
of the Requirements for the Degree

Master of Science

by
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DECEMBER 2013

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EFFECT OF ZINC OXIDE AND SILVER NANOPARTICLES ON INTESTINAL
BACTERIA

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ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance and supports of each of following individuals.

I would like to first and foremost thank my advisor, Dr. Azlin Mustapha who was abundantly helpful and offered invaluable assistance, support and encouragement. Her enthusiastic guidance and warm encouragement helped me improve my knowledge and finish this thesis.

I would like to thank Dr. Mengshi Lin and Dr. Zhiqiang Hu for serving on my committee in spite of their busy schedules. I really appreciate their valuable suggestions and comments, without their assistance, this study would not have been successful.

I also would like to express my thanks to Dr. Ingolf U. Gruen, Dr. Andrew Clarke, and Dr. Bongkosh Vardhanabhuti for their enlightening classes; Ms. JoAnn Lewis for her assistance outside the class.

I would like to give a special appreciation to my colleagues in lab, Prashant Prashant, Yuan Yuan, Yarui Liu, Jisu Kang, Jee Hye Lee, Ruoyu Li, Tracy Bish. It was my pleasure to work with them. I also would like to thank Kathleen Kim for her encouragement outside of lab, Wen Wang Ritts and Juliana Pavlovna Arndt, the electron microscopy specialist at EMC for the training on the use of facilities and assistance.

Finally, I would like to express my sincere gratitude to my family for their endless love, encouragement, and support for me.

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THE EFFECTS OF ZINC OXIDE AND SILVER NANOPARTICLES ON INTESTINAL BACTERIA

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ABSTRACT

In this study, the effects of zinc oxide (ZnO) and silver (Ag) nanoparticles (NPs) on the intestinal bacteria, *Escherichia coli*, *Lactobacillus acidophilus*, and *Bifidobacterium animalis* were investigated. All three bacterial strains were inoculated into tryptic soy broth (TSB) or Lactobacilli MRS broth media containing different concentrations of ZnO (0, 12, 16, 20 mM) and Ag (0, 1.8, 2.7, 4.6 mM) NPs and 1% of NP-free solution and incubated at 37 °C for 24 h. The presence and characterization of ZnO and Ag NPs on bacterial cells were investigated by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy-dispersive X-ray spectroscopy (EDS). UV-visible absorbance and the Live/Dead Bacterial Viability assay were performed to assess membrane leakage and viability of bacterial cells before and after treatment with the NPs.

For all bacteria, for up to 12 h of incubation, the numbers of treated cells were within 1 log CFU/mL less than that of the control. Morphological changes of bacterial cells were observed, but many cells remained in normal shapes. Results of UV-visible spectroscopy showed that only a small amount of internal cellular contents were leaked due to the NPs.

Also, more live than dead cells were observed after exposure to the NPs. Results indicate that ZnO and Ag NPs have very mild inhibitory effects on intestinal bacteria.

CHAPTER 1

INTRODUCTIONS

1.1 Need for the research

Nanotechnology is now applied in various industries, including electrical engineering, chemistry, material sciences and cosmetics (Kumar 2006). There is potential power of applications of nanotechnology in the many aspects of food industry such as food safety, disease treatment delivery methods, new tools for molecular and cellular biology, new materials for pathogen detection and protection of the environment (Weiss, Takhistov, and Clements 2006). NPs have been reported for their application in the nanosensor and nanotracer fields in the food industry (Moraru and others 2003; Jin and others 2009). Moreover, the application of nanotechnology to food packaging has been introduced to improve the shelf life of food and to prevent contamination (Duncan 2011; Maillard and Hartemann 2012). There are increasing numbers of foodborne outbreaks over the world, so it is important to control the causes of outbreak. NPs are one of the promising and useful antibacterial agents that could possibly be applied in the food industry. ZnO NPs are unique in that they are not only stable under high temperatures and pressures that are typically needed in food-processing conditions, but they are also generally regarded as safe (GRAS) for human beings and animals relative to organic materials (Sawai 2003; Fu and others 2005). Ag NPs are inorganic antibacterial agents used in the pharmaceutical and medical industries. Ag NPs have a significant potential for a wide range of

biological applications, including as an antifungal and antibacterial agent for antibiotic resistant organisms and for preventing infections.

Recent studies (Sondi and Salopek-Sondi 2004; Brayer and others 2006; Kim and others 2007; Jones and others 2008; Martinez-Castanon 2008; Raffi and others 2009; Liu and others 2009; Jin and others 2009; Tayel 2010; Xie and others 2011; Mirhosseini and Firouzabadi 2013) have demonstrated the antimicrobial activities of ZnO and Ag NPs to pathogenic microorganisms, including *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Salmonella* Enteritidis, *Salmonella* Typhimurium *Bacillus cereus*, *Campylobacter jejuni*, *Botrytis cinerea*, and *Penicillium expansum*. However, not much is known about the behavior of ZnO and Ag NPs upon ingestion and whether they inhibit natural gut microflora.

1.2 Objectives of the study

In this study, we investigated the effect of ZnO and Ag NPs on three important intestinal bacteria, *E. coli*, *L. acidophilus*, and *B. animalis*. The modes of action of ZnO and Ag NPs on the growth of the bacterial cells were also studied by a combination of chemical analytical methods.

CHAPTER 2

LITERATURE REVIEW

2.1 What are nanoparticles?

Nanotechnology is one of the great advancements in various industries that manipulate materials on the atomic or molecular scales. Resulting materials have new characteristics and functions with very small sizes; these smaller materials are referred to as nanomaterials or nanoparticles (NPs) and defined as particles that are less than 100 nm in at least one dimension (Meyer and Kuusi 2002). Nanomaterials have attracted great attention for their unique, superior, and dispensable properties that can be distinguished from conventional macroscopic materials. Their discrete property arises especially from their higher surface to volume ratios and increased percentage of atoms at the grain boundaries. They represent an important class of materials in the development of novel devices that will enable applications in many areas, such as the physical, biological, biomedical and pharmaceutical area (Sigel 1993; Suryanarayana 1995; Gleiter 2000; Lee, Yeo and Jeong 2003).

2.1.1 Types of NPs

There are many types of intentionally produced NPs, and a variety of others are expected to appear in the future. Most current NPs are classified into four types: Carbon-based materials, metal-based materials, dendrimers, and composites (USEPA 2007). The carbon-based materials are composed mostly of carbon, form of spheres, ellipsoid, or

tubes. These types of nanomaterials can be applied to improved films and coatings, stronger and lighter materials. The metal based materials include quantum dots, gold, silver, and metal oxides NPs such as zinc oxide (ZnO), titanium dioxide (TiO₂). The dendrimers are nanosized polymers built from branched units. The surface of dendrimers has numerous chain ends, which can be tailored to perform specific chemical functions which are useful properties for catalysis. Lastly, composites are nanomaterials that combine with two different NPs, or with larger, bulk-type materials. These materials are already applied in the auto parts and packaging industries to improve mechanical, thermal, and barrier properties. Currently, metal-based NPs, especially metal oxide NPs, are among the most highly produced NPs; their available applications include catalysis, sensors, environmental remediation, and personal care products (Kumar 2006). Because of their unique and novel properties, these various types of nanomaterials are highly desirable for applications in diverse area and their possible applications will continue to grow.

2.2 Applications of NPs in the food industry

Nanotechnology is now applied in various industries, including electrical engineering, chemistry, material sciences and cosmetics (Kumar 2006). Medicinal sciences are investigating the use of nanotechnology to improve medical diagnosis and treatments (Bennett and Schuubiers 2005; Howard and Kjems 2007; Andersen and others 2009). Also, there is potential power of application of nanotechnology in the many aspects of food industry such as food safety, disease treatment delivery methods, new tools for molecular and cellular biology, new materials for pathogen detection and protection of

the environment (Weiss, Takhistov, and Clements 2006). NPs have been reported for their application in the nanosensor and nanotracer fields in the food industry (Moraru and others 2003; Jin and others 2009). Moreover, the application of nanotechnology to food packaging has been introduced to improve the shelf life of food and to prevent contamination (Duncan 2011; Maillard and Hartemann 2012). There are increasing numbers of foodborne outbreaks over the world so it is important to control the causes of outbreak. NPs are one of the promising and useful antibacterial agents that could possibly be applied in the food industry.

2.2.1 Antibacterial effect of metal-based NPs

Antibacterial agents are of great importance to numerous industries, especially in the food industry. The antibacterial agents currently used in the food industry can be classified into two types: organic and inorganic agents (Zhang and others 2007). Inorganic antibacterial agents, such as NPs have received increasing attention in food applications because they are not only stable under high temperatures and pressures that are typically needed in food-processing conditions, but they are also generally regarded as safe (GRAS) for human beings and animals, relative to organic materials (Sawai 2003; Fu and others 2005). Recent studies show that some NPs have selective toxicity to bacteria but minimal effects on human cells (Reddy and others 2007). Because of these properties, NPs, especially metal-based NPs, have attracted much interest for the control of microorganisms, especially pathogens that cause infectious diseases. There are several factors that influence antibacterial effects of NPs on microorganisms.

2.2.1.1 Mechanisms of NPs against bacteria

The exact mechanisms of antibacterial activity of NPs to different types of bacteria are not completely understood. However, several studies have suggested the following mechanisms of antibacterial effects of NPs.

First, NPs are able to attach to the cell membrane of bacteria by electrostatic interaction and break the integrity of the bacterial membrane (Thill and others 2006). The bacterial cell wall is designed to provide strength, rigidity, and shape, and to protect the cell from osmotic explosion and mechanical damage (Singleton 2004). The composition and structure of bacterial cell wall can be categorized into two types: Gram-positive (+) and Gram negative (-). Compared to Gram-positive cell wall, Gram-negative cell wall is more in complex both structurally and chemically. The structure of the cell wall plays an important role in influencing the effect of NPs to bacteria. According to a study by Ashkarran and others (2012), vancomycin (van)-functionalized Ag@TiO₂ NPs have the capacity to target van-sensitive bacteria. The van-sensitive bacterium, *Desulfotomaculum*, was recognized by vancomycin, while the van-resistant bacteria were not recognized by vancomycin. This is because of the van-resistant bacteria cell wall, which has an additional outer membrane that covers the cell surface. There are some components found in Gram-negative bacteria, and not in Gram positives, which can oppose NPs to attachment onto cell walls; the possible mechanism is the extra layer of outer membranes and the pathogen-associated molecular patterns which include lipopolysaccharide, porins, and particular fragments of peptidoglycan. Bacterial cell wall properties can play a crucial role in diffusion of NPs inside biofilm matrixes (Baek and An, 2011).

Second, the mechanisms of NPs against bacteria rely on composition, contact surface, essential properties of NPs and the species of bacteria. Many studies about the antibacterial effects of different types of NPs have reported. According to Baek and An (2011), copper oxide (CuO) NP is more inhibitory to *E. coli*, whereas *S. aureus* and *Bacillus subtilis* are less susceptible. The antibacterial effect of silver (Ag) NP is higher than Cu NP against *E. coli* and *S. aureus* (Lu, Brauer, and Botstein 2009). *S. aureus* and *B. subtilis* are more susceptible than *E. coli* to nickel oxide (NiO) and ZnO NPs. Among the NPs, including CuO, ZnO, NiO, and antimony trioxide (Sb₂O₃) NPs, tested against *E. coli*, *S. aureus*, and *S. aureus*, CuO NP had most antibacterial effect, followed by ZnO (except for *S. aureus*), NiO and Sb₂O₃ NPs (Baek and An, 2011). The concentration of NPs appears to be related to their antibacterial effect. However, some of those studies disagree with one another, which indicate that the mechanisms of NP toxicity to bacteria are very complicated (Bolla and others 2011; Lara and others 2011; Musee and Thwala 2011). Thus, it is difficult to classify the NPs as beneficial NPs and/ or adverse NPs towards bacteria.

Another factor that can influence the growth of bacteria against NPs is the rate of bacterial growth. Bacteria that grow fast are more effectively inhibited by NPs than those that grow slow (Brown, Allison, and Gilbert 1998; Mah and O'Toole 2001). It is possible that the tolerance property of bacteria that grow slow is related to the expression of stress-response genes (Lu and others 2009; Stewart, 2002). Consequently, antibacterial effects highly depend on the particular strain. Another possibility might be the induction

of intracellular reactive oxygen species, hydrogen peroxide which is a strong oxidizing agent that is harmful to bacterial cells (Jones and others, 2008; Sawai, 2003).

2.2.1.2 Antibacterial effect of ZnO NPs

Among the metal-based NPs, ZnO NPs is one of the most studied. In 2006, investigations into the antibacterial effect of ZnO NPs against *E. coli*, done by Brayner and others (2006), showed that as the concentration of ZnO NPs increased, the inhibition of bacterial growth also increased. The results showed that concentrations of 1.3 mM or lower did not significantly affect the growth of *E. coli*. At concentrations of 3.0 to 10 mM, ZnO NPs showed 100-% inhibition of bacterial growth.

Another study by Jones and other (2008) showed that ZnO NPs have inhibitory effect on various bacteria, including, *S. aureus*, *S. epidermidis*, *S. pyogenes*, and *B. subtilis*. Further studies by Jin and others (2009) showed that ZnO quantum dots and NPs of purified powdered ZnO were effective in decreasing numbers of *Listeria monocytogenes*, *S. Enteritidis*, and *E. coli* O157:H7. The reduction of cell number was greater at higher concentrations of ZnO NPs to all three strains. The result showed that 3.2 mg ZnO/mL treatment caused a 5.3 log reduction of *L. monocytogenes* and a 6.0 log reduction of *E. coli* O157:H7 after a 2-day of incubation with ZnO NPs. The results of these studies demonstrated the antibacterial activity of ZnO NPs over a spectrum of bacteria. The growth reduction was greater at higher concentrations of ZnO NPs. According to a study by Liu and others (2009), where ZnO NPs concentrations of 3, 6, and 12 mM were used to examine *E. coli* O157:H7, 3 and 6 mM ZnO NPs resulted in less bacterial growth as

compared to the control while the growth of *E. coli* O157:H7 was completely inhibited by 12 mM ZnO NPs.

Another study by Tayel (2010) showed that Gram-positive strains were more susceptible to ZnO NPs using both qualitative and quantitative assays. ZnO NP was more effective to *S. aureus* which is a Gram-positive bacterium than *S. Typhimurium* which is a Gram-negative bacterium. After exposure to their minimal inhibitory concentration of ZnO NP, *S. Typhimurium* and *S. aureus* cell numbers were reduced to 0 within 8 and 4 h, respectively. Scanning electron micrographs of treated cell showed that the cells completely exploded or lysed. The results of this study also showed that *Bacillus cereus* was the most sensitive strains among all of the examined strains against ZnO NPs, and *Pseudomonas* spp. were the most resistant.

Another study by Xie and others (2011) showed antibacterial activity of ZnO NPs against *Campylobacter jejuni*. The results showed that *C. jejuni* was extremely sensitive to ZnO NPs. The minimum inhibitory concentration (MIC) of ZnO NPs for *C. jejuni* was 0.05 to 0.025 mg/mL, which is much lower than that of *Salmonella enterica* and *E. coli* O157:H7 (0.4 mg/mL). The action of ZnO NPs against *C. jejuni* was determined to be bactericidal, not bacteriostatic. The majority of *C. jejuni* cells, after exposure to ZnO NPs for 16 h had transformed from spiral shapes into coccoid forms.

A study by Mirhosseini and Firouzabadi (2013) demonstrated the antibacterial activity of ZnO NPs in food samples. Specifically, two concentrations, 5 and 10 mM, of ZnO NPs were used as antimicrobial treatments in milk samples inoculated with *E. coli* and *S.*

aureus. As a result, after 8 h of incubation, the growth number of *S. aureus* treated with ZnO NPs in milk was 2 log CFU/mL lower than its control, while the number of *E. coli* was less than 1 log lower than the control. The antibacterial effect of ZnO NPs on *S. aureus* was stronger than *E. coli* in milk. These findings imply that antibacterial efficacy of ZnO NPs in food samples exists.

Also, different sizes of NPs influence their effectiveness against bacterial growth. The study done by Seil and Webster (2012), showed that the smaller size of ZnO NPs, the stronger the antibacterial effect. An increasingly positive surface charge (which results in the NPs being drawn to the negatively charged surface of the bacteria) is another characteristic that generally enhances the antibacterial effects of ZnO NPs.

More studies in recent years have been reported regarding the antimicrobial effects of ZnO NPs and their inhibitory mechanisms towards bacteria and fungi. Although there are no specific conclusions on the mechanisms of action ZnO NPs against bacteria, it is well recognized that the higher the concentrations, the greater the inhibitory effect.

2.2.1.3 Antibacterial effect of Ag NPs

Higher concentrations of Ag NPs have been shown to have the stronger antimicrobial effect. In 2004, a case study using Ag NPs as an antimicrobial agent on *E. coli* as the model for Gram negative bacteria was done by Sondi and Salopek-Sondi (2004). The results showed that a concentration of $10 \mu\text{g cm}^{-3}$ Ag NPs inhibited bacterial growth by 70%, and as the concentration got higher, the number of cells significantly reduced. The concentration of $50\text{-}60 \mu\text{g cm}^{-3}$ caused 100% inhibition of bacterial growth. Also, the

dynamics of bacterial growth was observed in liquid medium with 10^7 *E. coli* cells and 10, 50, and 100 $\mu\text{g cm}^{-3}$ Ag NPs. All of the three different concentrations caused a growth delay of *E. coli* and increasing the concentration of NPs increased the growth delay. According to a study by Kim and others (2007), yeasts and *E. coli* were inhibited at low concentrations of Ag NPs. However, *S. aureus* was less inhibited by Ag NPs compared to yeast and *E. coli* O157:H7. To be more specific, the MIC of Ag NPs against yeast was between 6.6 nM and 13.2 nM and against *E. coli* O157:H7 was between 33 nM and 6.6 nM. As the concentrations of Ag NPs got higher, the stronger inhibitory effects were observed. MIC of Ag NPs against *S. aureus* was estimated to be more than 33 nM. The growth-inhibitory effect was mild against *S. aureus* even in high concentrations of Ag NPs compared to other microorganisms used in this study. A study by Raffi and others (2009) also showed antibacterial effects against *E. coli* 15224 in both liquid and solid growth media. The number of CFU was significantly reduced with increasing concentrations of Ag NPs. The concentrations of Ag NPs, as low as 60 $\mu\text{g/mL}$, showed complete cytotoxicity to the *E. coli* bacterial strain.

A study by Martinez-Castanon (2008) showed that smaller sizes of Ag NPs have larger surface to volume ratio which makes interaction between bacterial cells and NPs and the resulting antibacterial effects stronger. Three different sizes (7, 29, and 89 nm mean value) were synthesized by an aqueous chemical reduction method and the standard microdilution method was used to determine antibacterial activity of Ag NPs. As a result, 7 nm Ag NPs was most effective against both *E. coli* and *S. aureus*. For *E. coli*, there was no significant difference between the MIC of 29 and 89 nm Ag NPs. Also, the MIC of all

samples was lower when testing against *E. coli* than when testing against *S. aureus*. This result is similar to that found by Kim and others (2007). These results can be explained on the basis of the differences in the cell wall composition of each strain explained as section 2.2.1.1 Mechanisms of NPs against bacteria.

Another study showed similar results between *E. coli*, *S. aureus* and Ag NPs (Liu 2010). However, Liu's study also shows that smaller Ag NPs had better antibacterial effects but higher cytotoxicity. In 2011, a study done by Guzman and others showed antibacterial activity of Ag NPs against not only *E. coli* and *S. aureus*, but also, *Pseudomonas aeruginosa*. The average particle sizes used were 9 ± 2 , 14 ± 5 , 24 ± 6 , and 30 ± 7 nm and the antibacterial activity was measured by the Kirby-Bauer method. As a result, the presence of NPs at a certain level inhibited bacterial growth by more than 90%, and clear-zone diameters increased as the concentration of Ag NPs increased in all three bacterial strains. To determine the MIC of Ag NPs against bacterial strains, a standard dilution micromethod was also performed. The smaller Ag NPs, 9 ± 2 and 14 ± 5 nm, showed considerable antibacterial activity. The MIC of Ag NPs on *P. aeruginosa* and *E. coli* were lower than that of *S. aureus*.

More studies have been reported concerning the antimicrobial effects of Ag NPs on a broad spectrum of microorganisms. It is well recognized that the higher the concentration and the smaller the particle size the greater the inhibitory effect on microorganisms. However, the mechanisms underlying inhibitory effects are not yet completely understood.

2.3 Toxicity of NPs

Some believe that nanotechnology will make great developments and enhance the world and quality of life, while others regard nanotechnology as too dangerous and risky for continuing their research due to toxicity issues (Woodhouse 2004). This is because the interaction mechanisms between NPs and living cells are not yet fully understood. In recent years, many studies have been conducted on NP-cell interaction mechanisms. These studies found that cells readily take up NPs via either active or passive mechanisms. However, intracellular mechanisms and pathways are more difficult to understand due to the properties of NPs. Particles of the same material can show completely different characteristics with the slightest differences in surface coating, charge, or size (Li and others 2012). This makes it very difficult to define the behavior and hazard identification of NPs when in contact with biological systems.

Hazard identification of NPs at the in-vivo level is still considered at an early stage. The complexity increases when moving from an in vitro to in vivo model. The lung, gut, and possibly skin are the major entry routes that have been identified (Li and others 2012). However, much more research are needed to better understand mechanisms and pathways of NPs in the body following their ingestion or exposure. Some NPs seem to be able to take a pre-existing transport mechanism through the body using endocytotic mechanisms which is the same method that viruses do (Elsaesser and Howard 2012). Therefore, if the body is exposed to NPs, people have to be aware of the risk of toxicity of NPs.

2.3.1 Toxicity of ZnO NPs

ZnO NP powders are widely used in cosmetics, pigments, coating electronic devices, and catalysts. Despite the widespread use of ZnO NPs, the safety of this compound for humans is still not clear. Studies about the toxicity of ZnO NPs and other metal oxide NPs to mammalian cell and organs have been reported (Wang and others 2008, 2010; Liu and others 2009) ZnO NPs were found to cause more severe damage than other metal oxide NPs in many cases (Lai and others 2008). Skin exposures via products and manufacturers inhalation during processing are predominant exposure ways of ZnO NPs. A study by Jeng and Swanson (2006) showed that mice exposed to ZnO NPs at 100 g/mL or higher concentration became abnormal in size, and resulted in cellular shrinkage. Concentrations of ZnO less than 100 g/ml caused a substantial decrease of mitochondrial function. Also, lactate dehydrogenase leakage and apoptosis were observed in cells that were exposed to ZnO NPs. According to another study done by Wang and others in 2008 through 2010, inhalation of 20 nm ZnO NPs (2.5 mg/kg bw) by rats twice a day for 2 days resulted in an increased Zn content in liver after 12 h and in the kidney after 36 h. Histopathology revealed damage in liver and lung tissues. Also, 20 and 120 nm ZnO NPs were discovered in the bone, kidney, and pancreas in healthy adult mice after exposed to ZnO NPs. Lin and others (2009) found both micro- and nano-sized ZnO particles in human lung epithelial cells, which indicated that showed exposure to both size of ZnO particles could lead to cytotoxicity.

The potential risk of ZnO NPs to human health and environment is an inevitable thing, although scientists continue to look for the mechanisms underlying the toxic effects.

People who are vulnerable to ZnO NPs such as manufacturers, always have to be aware of the risk and handle the materials with extreme caution.

2.3.2 Toxicity of Ag NPs

Ag NPs is now able to access human daily life via a variety of commercial products. The use of Ag NPs in cosmetics and textiles has considerably increased the potential for human skin exposure. According to previous studies, Ag NPs were found to be toxic to keratinocytes and fibroblasts and induced cell death and oxidative stress in human fibrosarcoma and skin carcinoma cells (Lam and others 2004; Poon and Burd 2004; Paddle-Ledinek, Nasa, and Cleland 2006; Arora and others 2008; Arora and others 2009). During its manufacturing, manufacturers are exposed to Ag NPs via inhalation of airborne particles. Currently, the American Conference of Governmental Industrial Hygienist (ACGIH) limits the level of Ag dust in the air at a dose of 100 g/m^3 . Inhalation exposure studies showed that the lung is an the easy target for NPs and that inhaled particles may reach the brain through the nasopharyngeal system (Oberdorster and others 2004). Soto and others (2007) reported that Ag NPs reduced the cell viability of lung epithelial cells. There is a series of inhalation studies focusing on the acute, subacute (28 days) and subchronic (90 days) toxicity of Ag NP in rats (Ji and others 2007; Sung and others. 2009, 2010). Results showed that a high-dose chronic exposure to Ag NP has the potential to cause harm under current guidelines and limits.

Ag NPs have emerged as important nanomaterials for a wide range of industrial and medical applications that have potential risks to human health. To apply not only Ag NPs,

but other NPs safely in various fields, a detailed understanding of the biocompatibility and toxicity of NPs are needed.

2.4 Intestinal bacteria

The human gut is the natural habitat for a large and dynamic bacterial community. Many species of bacteria have evolved and are adapted to live and grow in the human intestine. An individual's intestine contains 300-500 different species of bacteria (Simon and Gorbach 1984; Borriello 1986). The stomach and small intestine contain only a few species of bacteria adhering to the epithelia and some other bacteria in transit. In contrast, the large intestine contains a complex and dynamic microbial ecosystem with high densities of living bacteria, with concentrations of up to 10^{11} or 10^{12} cells/g. These concentrations are similar to those found in colonies growing under optimum conditions over the surface of a laboratory plate (Levison 1990). The constant interaction between the host and its microbial system can infer important health benefits to the human host (Salminen and others, 1998).

2.4.1 Functions of intestinal bacteria

Major functions of the gut microflora include metabolic activities, trophic effects, and protective functions (Guarner and Malagelada, 2003).

Metabolic activities: A major metabolic function of colonic microflora is fermentation of non-digestible dietary residue and endogenous mucus produce by the epithelia (Roberfroid and others 1995). Various kinds of microbes provide different kinds of enzymes and biochemical pathways that are distinct from the host's own constitutive

resources. Another role of colonic microbes is synthesizing vitamins and helping in the absorption of calcium, magnesium, and iron (Roberfroid and others 1995; Miyazawa and others 1996; Younes and others 2001)

Trophic activities: An important trophic effect is controlling epithelial cell proliferation and differentiation. All three major short-chain fatty acids stimulate epithelial cell proliferation and differentiation in the large and small intestine (Franke and others 1994).

Interactions between gut bacteria and host immunity are another trophic activity. The intestinal mucosa is the main interface between the immune system and the external environment. The interaction between host and bacteria at the mucosal interface plays an important role in development of a competent immune system (Guarner and Malagelada 2003).

Protective activities: Intestinal bacteria are important in that they prevent species that are harmful to the host from colonizing the host and they protect against invasion by external microbes which is called barrier effect (Guarner and Malagelada 2003).

2.4.2 Types of intestinal bacteria

Not all microbial species in the gut have been identified because of the difficulty of identification. The genera of intestinal bacteria include, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus*, which are the predominant ones in humans. Also, there are sub-dominant genera, such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus*, and others. (Simon and Gorbach 1984; Salminen and others 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of intestinal bacterial strains

Escherichia coli K-12, *Lactobacillus acidophilus* ADH, and *Bifidobacterium animalis* Bif-6 were provided by the Food Microbiology Laboratory at University of Missouri, Columbia, MO. *E. coli* K-12 was grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY; Difco Labs., BD Dignostics Systems, Sparks, MD, USA). *L. acidophilus* ADH and *B. animalis* Bif-6 were grown in Lactobacilli MRS broth (Difco Labs.) supplemented with 0.05% cysteine. All three strains were freshly prepared by transferring 100 μ L of each culture into separate tubes of 10 mL respective broth media. Tubes were incubated –aerobically for *E. coli* and anaerobically for the other two strains for 18 to 20 h at 37 °C ($\sim 10^9$ CFU/mL).

3.2 Preparation of zinc oxide and silver nanoparticles

ZnO NP suspensions with an average particle sized of 77 nm were purchased from Alfa Aesar (Ward Hill, MA, USA). The concentration of the original suspensions was 12 M.

Ag NPs were synthesized by using a chemical reduction method (Ratyakshi and Chauhan 2009). Silver nitrite (AgNO_3) and sodium citrate ($\text{C}_6\text{H}_5\text{O}_7\text{Na}_3$) were prepared in deionized water. Then, 300 mL of 1 mM AgNO_3 and 300 mL of 20 mM of sodium citrate were separately heated to boiling. When both solutions reached 100 °C, sodium citrate was added slowly to AgNO_3 . The mixture was boiled with continuous stirring. The

solution was heated for 8 min until it turned a pale yellow color. The solution was cooled to room temperature and centrifuged at $3,290 \times g$ for 20 min. Then, 90% of the supernatant was removed from the centrifuged solution. The final concentration of Ag NP solution was 9.2 mM. The size of ZnO NPs and Ag NPs were determined by analyzing transmission electron microscopy (TEM) images using ImageJ software available at <http://rsb.info.nih.gov/ij/>.

3.3 Effect of ZnO NPs on the growth of *E. coli* K-12, *L. acidophilus* ADH, and *B. animalis* Bif-6

All three strains, incubated overnight in respective broth media, TSBY and MRS 0, were inoculated into broth media containing different concentrations (0, 12, 16, 20 mM) of ZnO NP suspensions, and 1% of NP-free solution. The NP-free solution was prepared by filtering ZnO NP suspensions through an anodisc inorganic membrane with a 20 nm pore size (Whatman Inc., Clifton, NJ, USA). Both ZnO NP suspensions and the NP-free solution were added to the broth media before autoclaving.

After inoculating the cultures to the broth media, TSB or MRS, containing ZnO NP suspensions and NP-free solution, were incubated at 37 °C in a shaking incubator. The tubes inoculated with *L. acidophilus* ADH and *B. animalis* Bif-6 were placed in an anaerobic jar with GasPak (BD, GasPak EZ Anaerobe Container System) and incubated in a shaking incubator (Lab-Line 3528 Shaking incubator). The reason for using a shaking incubator was to avoid the aggregation of ZnO NPs in the broth and to allow a consistent contact between the bacterial cells and ZnO NPs. The samples were diluted

with peptone water and plated on respective agar media, Tryptic Soy Agar supplemented with yeast extract for *E. coli* K-12 and Lactobacilli MRS agar supplemented with cysteine for *L. acidophilus* ADH and *B. animalis* Bif-6, at 0, 6, 7, 8, 9, 10, 11, 12, 15, and 24 h.

3.4 Effect of Ag NPs on the growth of *E. coli* K-12, *L. acidophilus* ADH, and *B. animalis* Bif-6

All three strains, incubated overnight in respective broth media, TSBY and MRS, were inoculated into broth media containing different concentrations of Ag NP solution (0, 1.8, 2.7, 4.6 mM), and 1% of NP-free solution. For concentrations of Ag NPs, mg/mL unit was converted into mM unit. The NP-free solution was prepared by filtering the Ag NP solutions using an anodisc inorganic membrane (Whatman Inc.) with a pore size of 20 nm. Both Ag NP suspensions and the NP-free solution were added to the broth media before autoclaving.

After inoculating the cultures into the TSB or MRS broth media containing Ag NP suspensions and NP-free solution, samples were incubated at 37 °C in a shaking incubator. The tubes inoculated with *L. acidophilus* ADH and *B. animalis* Bif-6 were placed in an anaerobic jar with GasPak (BD) and incubated in a shaking incubator. The samples were diluted with peptone water and plated on respective agar media, Tryptic Soy Agar supplemented with yeast extract for *E. coli* K-12 and Lactobacilli MRS agar supplemented with cysteine for *L. acidophilus* ADH and *B. animalis* Bif-6, at 0, 1, 2, 3, 4, 5, 6, 9, 12, and 24 h.

3.5 Morphological test of the bacterial cells

Scanning electron microscopy (SEM) was used to examine morphological changes of the bacterial cells before and after treating with ZnO NPs and Ag NPs. The three bacterial strains treated or untreated with NPs were fixed with a primary fixative (2.5 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M Na-Cacodylate buffer, pH 7.4). The samples were then rinsed three times with ultrapure water, followed by dehydration with a series of ethanol solutions (10, 30, 50, 70, 90, and 100%). The dehydrated samples were immediately dried by a critical point dryer (Auto-Samdri 815 Automatic Critical Point Dryer; Tousimis, Rockville, MD, USA), mounted on SEM stubs and coated with a thin layer of carbon using a sputter coater (K575X Turbo Sputter Coater; Emitech, Ltd, Kent, UK). The coated samples were observed under SEM (FEI Quanta 600, FEI Company, Hillsboro, OR, USA).

Transmission electron microscopy (TEM) was used to characterize the size of the NPs and to observe the morphology of bacterial cells after treatment with ZnO NPs and Ag NPs. Three samples of bacterial cells treated with and without NPs were fixed with a primary fixative and microwaved under vacuum conditions in a Pelco Biowave (Ted Pella, Inc., Redding, CA, USA) at 120 W. The samples were rinsed with 0.1 M cacodylate buffer and embedded in histogel, followed by a secondary microwave fixation with a buffered (0.1 M cacodylate, 0.01 M of 2-mercaptoethanol, and 0.13 M of sucrose) 1% osmium tetroxide. The samples were then quickly rinsed three times with 0.1M 2-mercaptoethanol, 0.13M of sucrose and then rinsed three times with ultrapure water. Then, samples were dehydrated with ethanol solutions (20, 50, 70, 90, and 100%) and

100% acetone solution. The samples were infiltrated with Spurr's resin and polymerized at 60 °C for 24 h. The sample blocks were processed in 85 nm thin sections with Leica Ultracut UCT ultramicrotomes (Leica Microsystems GmbH, Wetzlar, Germany). The sections were placed onto 200 mesh thin bar grids and post-stained for 20 min with 5% uranyl acetate and 10 min with Sato's triple lead stain. Stained samples were then observed in JEOL 1400 (JEOL, Ltd, Tokyo, Japan).

3.6 Determination of membrane leakage

Overnight cultures of the three strains were inoculated into the respective broth medium containing different concentrations of ZnO NPs (0, 12, 16, 20 mM) and allowed to sit for 10 h at 37 °C. Similarly, strains were exposed to Ag NPs (0, 1.8, 2.7, 4.6 mM) for 6 h at 37 °C. After incubation, 1 mL of the treated bacterial suspension was centrifuged at $18,200 \times g$ for 5 min and resuspended in peptone water. The light absorbance of the suspensions was examined using a UV-visible spectrophotometer (UV-1650 PC, Suzhou Instruments Manufacturing Co. Ltd, Suzhou, China) at a wavelength of 260 nm (for DNA absorbance) and 280 nm (for protein absorbance). All experiments were replicated twice.

3.7 Viability of bacterial cells

To determine the viability of the treated cells, 1 mL of the samples was centrifuged at $18,200 \times g$ for 5 min. Cell pellets were washed with 1 mL of 0.85% NaCl and stained using the *BacLight*[™] Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal volumes of Component A (SYTO 9 dye) and component B (propidium iodide) were mixed thoroughly and 3 µL of the dye mixture

was added to each mL of the bacterial suspension. The samples were incubated at room temperature in the dark for 15 min and 8 μ L of the stained bacterial suspension were placed between a glass slide and 170 μ m thick coverslip. Then samples were observed under a Zeiss LSM 510 META (Zeiss LSM 510 META NLO, Carl Zeiss Ltd, Jena, Germany).

3.8 Statistical data analysis

The SAS GLM procedure (SAS 9.2, Copyright 2002-2007; SAS Institute Inc., Cary, NC, USA) was used to evaluate the effects of ZnO and Ag NPs on growth of bacterial strains. Tukey's test was applied to determine differences between different concentrations of NPs with a significance level of 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of ZnO NPs on the growth of *E. coli* K-12, *L. acidophilus* ADH, and *B. animalis* Bif-6

The same concentrations (3, 6, 12 mM) of NPs were used to assess the effect of ZnO NPs on *E. coli*, *L. acidophilus*, and *B. animalis*. Increasing antimicrobial effects against *E. coli* O157:H7 were found as the concentration of ZnO NPs increased, and a treatment of 12 mM showed complete inhibition of the organism. No significant differences ($P \leq .05$) between the control and treatments of 3 and 6 mM ZnO NPs on all three bacteria were found (data not shown). Hence, the higher concentrations (12, 16, 20 mM) of ZnO NPs were used for further experiments. Figure 4.1 shows the growth curves of *E. coli*, *L. acidophilus*, and *B. animalis* with different concentrations of ZnO NPs and NP-free solution. As shown in Figure 4.1A, the *E. coli* growth curve of the control and all treated samples including the NP-free sample, exhibited very similar patterns. Even at the highest concentration of ZnO NPs (20 mM), no significant effects on *E. coli* ($P \leq .05$) were observed. For *L. acidophilus*, for up to 12 h of incubation, the numbers of treated cells were within 1 log CFU/mL less than that of the control. All three treatments of ZnO NPs showed similar growth patterns. After 12 h, the cell numbers of treated samples picked up slowly and by the end of 24 h, their numbers showed no differences as compared ($P \leq .05$) to the control. This result can be explained by previous study (Martinez-Castanon 2008) that showed that not only particle size and concentration of NPs, but also time can

Figure 4.1 Effects of ZnO NPs on the growth of *E. coli* (A), *L. acidophilus* (B) and *B. animals* (C).

affect the antibacterial activity of NPs. For *B. animalis*, until 7 h of incubation, cell numbers of treated samples were less than 1 log CFU/mL as compared to the control. After 8 h, the number of treated cells followed similar patterns of the control and NP-free sample. By the end of 24 h, the numbers of treated cells were within 1 log CFU/mL that of the control. As shown in Table 4.1, *L. acidophilus* after 10 h of exposure to ZnO NPs showed 11.4% reduction in the number of cells which was the highest percentage compared to the other exposure times. For *B. animalis*, between 6 and 7 h of exposure to ZnO NPs about 10% of reduction was observed. After that, less than 5% of reductions were observed. However, at the end of 24 h of exposure, more than 10% reduction in the number of cells was observed.

The results (Figure 4.1) indicated that concentrations of ZnO NPs higher than 12 mM showed mild inhibition effects on growth of *L. acidophilus* and *B. animalis*, which are gram-positive bacteria, and no inhibition effects on growth of *E. coli*, which is a gram-negative bacterium. Similar results were observed in recent a study by Baek and An (2011). *S. aureus* and *B. subtilis* (gram-positive) were more susceptible than *E. coli* (gram-negative) to nickel oxide (NiO) and ZnO NPs. It is currently impossible to explain the species sensitivity in terms of bacterial classification (Gram + and -). The biosorption of metal NPs to bacterial cells depends on not only the types of NPs, but also the microbial species (Hassen and others 1998). One possible mechanism of antimicrobial effects of ZnO NPs is suggested in several studies (Sawai 2003; Ghule and others 2006; Jones and others 2008; Li and others 2008). Antimicrobial effects of NPs are generally triggered by the induction of oxidative stress by free radical formation, ROS, and result in

cell death. According to Pan and others (2010), ZnO NPs have weak mutagenic properties that induce frameshift mutations in *S. Typhimurium*. The presence of the S9 fraction is necessary to cause frameshift mutations using ZnO NPs. S9 fraction is the product of an organ tissue homogenate that used to assess the mutagenic potential of chemical compound (Sakura and others 2004). It is possible that the S9 fraction increased the internalization of NPs and increases the generation of reactive oxygen species (ROS) that induce frameshift mutations in the bacteria. However, there are many reports about the antibacterial effect of NPs that contradict one another which indicate that the mechanisms of antimicrobial are very complicated and depend on many other factors. Also, there are several bacteria that naturally adapt to toxins or NPs that are present in the environment and become tolerant to the compounds (Wu and others 2010). There is a possibility that this might be the reason for our observations of no inhibitory effects of ZnO NPs on *E. coli* in this study.

Table 4.1 Number of *L. acidophilus* before and after treatment with 20 mM of ZnO NPs.

Time of incubation (h)	Number of colonies (CFU/mL)		% of Reduction
	Control	Treatment with ZnO NPs	
0	8.9×10^6	1.1×10^7	-1.3
6	3.0×10^8 _a	3.4×10^7 _b	11.1
7	4.1×10^8 _a	4.5×10^7 _b	11.1
8	4.3×10^8	1.4×10^8	5.5
9	1.6×10^9	7.1×10^8	3.8
10	4.1×10^9 _a	3.3×10^8 _b	11.4
11	8.7×10^9 _a	6.6×10^8 _b	11.2
12	6.3×10^9 _a	4.9×10^8 _b	11.3
24	3.5×10^9	5.4×10^9	-1.99

_{ab} Different letters between columns indicate significant differences at a level of 0.05.

Table 4.2 Number of *B. animalis* before and after treatment with 20 mM of ZnO NPs.

Time of incubation (h)	Number of colonies (CFU/mL)		% of Reduction
	Control	Treatment with ZnO NPs	
0	4.5×10^7	2.1×10^7	3.2
6	3.8×10^8 _a	9.2×10^7 _b	9.7
7	4.4×10^8 _a	8.2×10^7 _b	10.6
8	4.5×10^8	1.9×10^8	4.7
9	5.8×10^8	2.1×10^8	2.9
10	6.1×10^8	1.8×10^8	3.5
11	6.9×10^8	2.8×10^8	3.8
12	5.7×10^8	3.0×10^8	3.1
24	6.0×10^9 _a	6.9×10^8 _b	12.4

_{ab} Different letters between columns indicate significant differences at a level of 0.05.

4.2 Effect of Ag NPs on the growth of *E. coli*, *L. acidophilus*, and *B. animalis*

Different concentrations (0.1, 0.4, 0.9, 1.3 mM) of Ag NPs were used to assess their effects of on *E. coli*, *L. acidophilus*, and *B. animalis*. No significant differences ($P \leq .05$) between the control and treated samples were observed up to 1.3 mM of Ag NPs for the three bacteria (data not shown).

There are several possible mechanisms of antibacterial activity by Ag NPs. One of them is because of Ag^+ ions from Ag NPs (Sondi and others 2004; Morones and others 2005; Choi and others 2008). These reported studies showed that Ag NPs released Ag^+ ions in the presence of oxygen and water (Equation 1).

After Ag^+ ions are released from the Ag NPs, there is a possibility that they affect membrane-bound enzyme functions in bacterial cells and facilitate the generation of ROS (McDonnell and others 1999; Pal and other 2007). Also, the electrostatic forces between Ag^+ ions and the negatively charged cell membrane or wall may result in the inhibition of respiratory chain enzymes, a change in membrane permeability and ultimately cell lysis and death (Ratte 1999; Sambhy and others 2006).

In the case of *E. coli*, the number of cells grew rapidly within 6 h of incubation using a shaker incubator. Therefore, one of the reasons for the ineffectiveness of Ag NPs on *E. coli* might be that *E. coli* used up all the oxygen in the tube so quickly that there was no chance for Ag NPs to be in contact with oxygen and to allow for release of Ag^+ ions.

Therefore, another set of experiment was performed using the same concentrations of Ag

NPs but with larger flasks which could contain more oxygen than the test tubes. The results were very similar to that of the previous experiment in that there was no significant effect of Ag NPs on *E. coli* (data not shown). Therefore, the higher concentrations (1.8, 2.7, 4.6 mM) of Ag NPs were used for further experiments. Figure 4.2 shows the growth curves of *E. coli*, *L. acidophilus*, and *B. animalis* with different concentrations of Ag NPs and NP-free solution. Ag NPs at 1.8, 2.7 and 4.6 mM have a significant effect ($P \leq .05$) on *E. coli* for up to 9 h. As the concentrations of Ag NPs increased to 1.8, 2.7, and 4.6 mM, the growth of *E. coli* was more greatly inhibited for up to 10 h of incubation. After 10 h, their numbers showed no differences when compared to the controls. Compared to *E. coli*, *L. acidophilus* and *B. animalis* showed less inhibitory effects by Ag NPs. The growth curve of treated *L. acidophilus* and *B. animalis* showed very similar patterns as those of their controls and NP-free controls. Concentrations of Ag NPs up to 1.8 mM had no significant effects on *L. acidophilus* and *B. animalis*. Ag NPs at 2.7 and 4.6 mM showed a significant effect ($P \leq .05$) on *L. acidophilus* and *B. animalis* for up to 9 h. No significant effects of Ag NPs after 12 h of exposure were observed for *L. acidophilus* and *B. animalis*. As shown in Tables 4.3, 4.4, and 4.5, all the three strains showed higher reduction numbers of cells between 3 to 6 h of exposure to Ag NPs. After 9 h of exposure to Ag NPs, less than 5% of cell reductions were observed. The mechanisms of the inhibitory effects of Ag NPs on microorganisms are partially known. Some studies (Dragieva and others 1999; Hamouda and others 2000; Dibrov, Dzioba, and Hass 2002) have reported that the positive charge on the Ag^+ ion is crucial for its antimicrobial activity through the electrostatic attractions between the negatively

Figure 4.2 Effects of Ag NPs on the growth of *E. coli* (A), *L. acidophilus* (B) and *B. animals* (C).

charged cell membrane of microorganisms and positively charged Ag NPs. In this study, *L. acidophilus* and *B. animalis* showed less inhibitory effect by Ag NPs compared to *E. coli*. As explained earlier, Ag NPs release Ag^+ ions when in the presence of oxygen and water. However, because they are both facultative anaerobes, *L. acidophilus* and *B. animalis* were incubated in anaerobic conditions. Hence, there was not enough oxygen available for the Ag NPs to release Ag^+ ions. This could explain why these two bacteria were less inhibited by Ag NPs as compared to *E. coli*. Without oxygen, Ag NPs cannot release Ag^+ ions which would have affected the growth of the bacterial cells. The slight inhibitory effect on the growth of *L. acidophilus* and *B. animalis* may be due to the Ag NPs exposure to oxygen during the preparations and experiments release of Ag^+ ions. Additionally, compared to Ag NPs, ZnO NPs showed higher inhibitory effect on *L. acidophilus* and *B. animalis*. This is because ZnO NPs can release Zn^{2+} ions regardless of the presence of oxygen. However, a study by Sondi and Salopek-Sondi (2004) used negatively charged Ag NPs which cannot support the antimicrobial mechanisms of positively charged Ag NPs. Therefore, another possible mechanism as described by Amro and others (2000) can explain the antimicrobial effect of Ag NPs. They suggested that metal depletion may cause the formation of irregularly shaped pits on the outer membrane of cells that can change the membrane permeability and cause leakage of internal cell contents. However, the exact mechanisms of antibacterial property of NPs to different types of bacteria are still not completely understood.

Unlike the results of ZnO NPs on *E. coli* (Gram negative), which showed higher antimicrobial effects than *L. acidophilus* and *B. animalis* (Gram positive) to Ag NPs,

other studies (Kim and other, 2007; Martinez-Castanon, 2008) showed similar results as those observed in this study. *E. coli* O157:H7 cells were inhibited by Ag NPs at lower concentrations as compared to *S. aureus* (Gram positive). Also, the MIC of Ag NPs was lower when testing against *E. coli* than when testing against *S. aureus*. As mentioned above, there is no supporting evidence to explain the species sensitivity in terms of bacterial classification (Gram+ and-) yet.

Table 4.3 Number of *E. coli* before and after treatment with 4.6 mM of Ag NPs.

Time of incubation (h)	Number of colonies (CFU/ mL)		% of Reduction
	Control	Treatment with Ag NPs	
0	1.7×10^7 _a	2.7×10^6 _b	10.8
1	3.8×10^7 _a	7.3×10^6 _b	9.4
2	4.0×10^7 _a	7.0×10^6 _b	9.9
3	4.5×10^8 _a	3.7×10^7 _b	12.6
4	4.2×10^8 _a	3.4×10^7 _b	12.6
5	6.1×10^8 _a	8.2×10^7 _b	9.9
6	1.6×10^9 _a	9.4×10^8 _b	13.3
9	3.1×10^9	2.2×10^9	1.6
12	3.1×10^9	2.3×10^9	1.4
24	3.3×10^9	3.6×10^9	-0.4

_{ab} Different letters between columns indicate significant differences with a significant level of 0.05.

Table 4.4 Number of *L. acidophilus* before and after treatment with 4.6 mM of Ag NPs.

Time of incubation (h)	Number of colonies (CFU/ mL)		% of Reduction
	Control	Treatment with Ag NPs	
0	1.2×10^7	4.4×10^6	6.3
1	1.3×10^7	4.5×10^6	6.3
2	1.7×10^7	6.8×10^6	5.6
3	3.4×10^7_a	7.6×10^7_b	9.3
4	5.1×10^7	2.5×10^7	4.1
5	7.1×10^7_a	1.3×10^7_b	10.3
6	2.4×10^8_a	3.7×10^7_b	10.6
9	7.1×10^8	4.0×10^8	2.8
12	1.1×10^9	4.8×10^8	3.9
24	2.5×10^9	1.2×10^9	3.7

^{ab} Different letters between columns indicate significant differences at a level of 0.05.

Table 4.5 Number of *B. animalis* before and after treatment with 4.6 mM of Ag NPs.

Time of incubation (h)	Number of colonies (CFU/ mL)		% of Reduction
	Control	Treatment with ZnO NPs	
0	1.2×10^7	1.4×10^7	-0.9
1	1.4×10^7 _a	3.8×10^6 _b	7.9
2	1.9×10^7 _a	4.5×10^6 _b	8.7
3	3.0×10^7 _a	6.7×10^6 _b	8.6
4	8.0×10^7	5.0×10^7	2.6
5	7.3×10^7	2.4×10^7	6.1
6	2.8×10^8 _a	5.1×10^7 _b	8.8
9	5.8×10^8	3.0×10^7	3.3
12	6.4×10^8	4.2×10^8	2.0
24	4.8×10^8	5.0×10^9	-0.2

_{ab} Different letters between columns indicate significant differences at a level of 0.05.

4.3 Characterization of ZnO and Ag NPs

The size and morphology of ZnO and Ag NPs were determined by TEM. The original ZnO NPs suspensions were diluted with ultra purified water and observed under TEM (Figure 4.3). Most of ZnO NPs were in either round or oval shape with an average size of 77.9 nm. The software ImageJ was used to analyze the sized of NPs. Figure 4.4 showed the histogram of the size distribution of ZnO NPs, and the majority of ZnO NPs measured fell in the range of 60 to 80 nm in diameter. ZnO NPs may be present in the form of agglomerates due to synthetic processing (Zhang and others, 2007). Therefore, ultrasonication and dispersants, such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) are often used to disintegrate NPs agglomerates (Brayner and others, 2006). However, as shown in Figure 4.3, ZnO NPs were relatively well dispersed with slight agglomeration in ultra-purified water without sonification or dispersant. Also, a shaking incubator was used to avoid the aggregation of ZnO NPs in the broth.

Fig. 4.3 TEM image of ZnO NPs diluted with ultra-purified water.

Fig. 4.4 Size distribution of ZnO NPs with an average size of 70.9 nm in diameter.

The Ag NPs synthesized by using a chemical reduction method were observed under TEM (Figure 4.4). The shapes of Ag NPs observed under TEM were uniformly spherical and well dispersed. The majority of the Ag NPs measured fell in the range of 30 to 50 nm in diameter with an average size of 40.2 nm, and a histogram of the size distribution of Ag NPs is shown in Figure 4.5.

Fig. 4.5 TEM image of Ag NPs.

Fig. 4.6 Size distribution of Ag NPs with an average size of 40.2 nm in diameter.

4.4 Morphological test of bacteria cells

4.4.1 Treated with ZnO NPs

The SEM and TEM images (Figures 4.7 and 4.8) of *E. coli*, *L. acidophilus*, and *B. animalis* incubated in respective broth medium for 10 h, with and without the presence of 20 mM of ZnO were analyzed.

As shown in Figures 4.7A and 4.7B, no significant changes in bacterial morphology (e.g. size, shape, appearance, etc) were observed after ZnO NPs treatment for 10 h. Also, ZnO NPs were observed to adhere to the *E. coli* cells (Figure 4.7B). The sizes of ZnO NPs attached to bacterial cells appeared larger and clustered as compared to those shown in the corresponding TEM image (Figure 4.3). This may be due to agglomeration of ZnO NPs during the incubation time. Figures 4.8A and 4.8B are TEM images that allow direct visualization of intracellular morphological changes of bacterial cells before and after treatment with ZnO NPs. Figure 4.8A shows the intracellular structures of *E. coli* control sample. The bacterial cells were normal in size with intact intracellular structures and well-maintained intracellular contents. Deformations of intracellular structures of bacterial cells were observed in Figure 4.8B. However, not all cells were damaged which could explain the result of the previous experiment where no effect of ZnO NPs on *E. coli* was demonstrated.

Figure 4.7 SEM images of *E. coli* without the treatment of ZnO NPs (A) and with 20 mM ZnO NPs (B).

Figure 4.8 TEM images of *E. coli* without the treatment of ZnO NPs (A) and with 20 mM ZnO NPs (B).

Figures 4.9 and 4.10 are SEM and TEM images of *L. acidophilus* before and after treatment of ZnO NPs. As shown in Figure 4.9A (control) and 4.9B (treated), obvious changes in bacterial morphology were observed after treatment with ZnO NPs. ZnO NPs adhered to the cells and deformed the cell morphology into a spiral shape. Many spiral or twisted cells were found in the sample treated with ZnO NPs. TEM images (Figure 4.10A and 4.10B) show morphological changes of intracellular of *L. acidophilus*. No damaged cells and very similar shape (rod-shape) of cells as shown in the SEM image were observed in the control sample (Figure 4.10A). On the contrary, treated samples (Figure 4.10B) were clearly damaged by ZnO NPs. The membranes of bacterial cells and intracellular structures were deformed. *B. animalis* showed very similar morphological changes as *L. acidophilus* (Figure 4.11, 4.12). Both SEM and TEM images showed significant differences between the external appearance and intracellular structures of bacterial cells between the control and treated samples. Many deformed cells and spiral shaped cells, were observed in the treated samples. Damaged cells in the treated samples indicated leakage of cell contents. The results of the effect of ZnO NPs on *L. acidophilus* and *B. animalis* can be explained by the damages of intracellular structures observed in Figure 4.10B and 4.12B.

Figure 4.9 SEM images of *L. acidophilus* without the treatment of ZnO NPs (A) and with 20 mM ZnO NPs (B).

Figure 4.10 TEM images of *L. acidophilus* without the treatment of ZnO NPs (A) and with 20 mM ZnO NPs (B).

Figure 4.11 SEM images of *B. animalis* without the treatment of ZnO NPs (A) and with 20 mM ZnO NPs (B).

Figure 4.12 TEM images of *B. animalis* without the treatment of ZnO NPs (A) and with 20 mM ZnO NPs (B).

Energy dispersive spectroscopy (EDS) is a common technique for analysis of the elemental composition of a specimen. It is also capable of generating a map of multiple chemical elements of interest at specifically pointed spots. EDS coupled with SEM were used for elemental analysis and it was effective in locating and identifying NPs attached to the bacterial cells.

Figure 4.13 shows SEM-EDS elemental analysis of ZnO NPs attached to *E. coli* cells. Control cells and ZnO NPs attached to cells were spotted to analyze the elements. Three spots were selected in each sample to analyze the elements. Results showed that no Zn elements were identified in the control cells, Figure 4.13A, which was as expected. Zn elements were observed in samples treated with ZnO NPs (Figure 4.13B and 4.13C) which indicated the presence of ZnO NPs. Also, relatively large amounts of platinum elements were observed in all samples. This is due to the thin layer of platinum coating to reduce the charges when observing under SEM.

Figure 4.13 Energy-dispersive X-ray spectroscopy spectrum of *E. coli* without the treatment of ZnO NPs (A), with 20 mM ZnO NPs (B) and *B. animalis* with 20 mM ZnO NPs (C).

4.4.2 Treated with Ag NPs

As shown in Tables 4.3, 4.4, and 4.5, bacterial strains exposed to Ag NPs for 6 h showed the highest antimicrobial effect. Therefore, SEM and TEM images of *E. coli*, *L. acidophilus*, and *B. animalis* incubated in the respective broth medium for 6 h, with and without the presence of 4.6 mM of Ag NPs, were observed.

Figures 4.14A and 4.14B showed SEM images of *E. coli* before and after treatment with Ag NPs. As shown in Figure 4.14A and 4.14B, no significant changes in the external appearance of the cells were observed after treatment with Ag NPs for 6 h. Ag NPs were attached to bacterial cells (Figure 4.14B) in clusters instead of a single particle, as shown in Figure 4.5. Similar agglomeration was observed in ZnO NPs too. Figure 4.15A showed intracellular structures of the *E. coli* control sample. The bacterial cells were normal in size with intact intracellular structures and well-organized intracellular contents. However, deformation of cell membrane and intracellular structure were observed in Figure 4.15B. Bacterial cells were surrounded by Ag NPs and null bacterial cells were found in bacterial sample treated with Ag NPs, indicating that intracellular contents had leaked from cells due to damage to the cell membrane.

Figure 4.14 SEM images of *E. coli* without the treatment of Ag NPs (A) and with 4.6 mM Ag NPs (B).

Figure 4.15 TEM images of *E. coli* without the treatment of Ag NPs (A) and with 4.6 mM Ag NPs (B).

The SEM images of *L. acidophilus* before and after treatment with Ag NPs are shown in Figure 4.16. Very similar morphology of cells was observed for *B. animalis* samples (Figure 4.18). As shown in Figure 4.16A (control) and 4.16B (treated), no significant changes in bacterial morphology were observed before and after treatment with Ag NPs. However, TEM images of *L. acidophilus* shown in Figure 4.17 showed differences between treated and untreated cells. Unlike the control sample, treated samples showed distortion of cell membranes and leakage of internal contents of cells. However, not all cells were damaged, there were still many cells in normal size with intact intracellular structures and well-organized intracellular contents. The results in Figure 4.2B, compared with the plate count number of about 10^7 CFU/mL, which is only 1 log CFU.mL less than the control shown previously are supportive of one another, which show. This indicates not all cells were affected by Ag NPs and resulting leakage of internal contents did not occur with all the cells.

Figure 4.16 SEM images of *L. acidophilus* without the treatment of Ag NPs (A) and with 4.6 mM Ag NPs (B).

Figure 4.17 TEM images of *L. acidophilus* without the treatment of Ag NPs (A) and with 4.6 mM Ag NPs (B).

Figure 4.18 SEM images of *B. animalis* without the treatment of Ag NPs (A) and with 4.6 mM Ag NPs (B).

Figure 4.19 TEM images of *B. animalis* without the treatment of Ag NPs (A) and with 4.6 mM Ag NPs (B).

The results of the SEM-EDS elemental analysis of Ag NPs are shown in Figure 4.20. Like ZnO NPs samples, three spots were selected in each sample to analyze the elements. No Ag elements were observed in all control samples (Figure 4.20A), while Ag elements were identified in all treated samples (Figure 4.20B, 4.20C). The presence of other element such as silicon (Si) was unexpected, which was most likely contributed by the pre-treatment of the SEM specimens, the sample holder or contamination during the preparation.

Figure 4.20 Energy-dispersive X-ray spectroscopy spectrum of *L. acidophilus* without the treatment of Ag NPs (A), with 4.6 mM Ag NPs (B), *B. animalis* with 4.6 mM Ag NPs (C).

4.5 Determination of membrane leakage

To determine membrane leakage, a UV-visible spectrophotometer was used to monitor the absorbance at wavelength of 260 nm (DNA absorbance). The absorbance is related to the leakage amount of intracellular DNAs. Figure 4.21 shows the absorbance of bacterial strains after treatment of ZnO NPs for 10 h. For all three bacteria, the absorbance at 260 nm shows a slight increase after 10 h of exposure to ZnO NPs. Higher concentrations of ZnO NPs resulted in a higher absorbance. Figure 4.22 showed the absorbance of bacterial strains after treatment of Ag NPs for 6 h. The results were similar to the ZnO NPs results. Absorbance at 260 nm of all three bacteria increased slightly as compared to the control. The absorbance increased due to the leakage of nucleic acids from bacterial cells when their cell membranes are damaged. However, there were no significant differences ($P \leq .05$) between the control and treated samples.

Figure 4.21 Absorbance of intracellular DNA leakage (260 nm) from *E. coli* (A), *L. acidophilus* (B), and *B. animalis* (C) after treatment with ZnO NPs

Figure 4.22 Absorbance of intracellular DNA leakage (260 nm) from *E. coli* (A), *L. acidophilus* (B), and *B. animalis* (C) after treatment with Ag NPs

4.6 Viability of bacterial cells

The BacLight™ Bacterial Viability Kit and fluorescence microscopy demonstrated to observe viability of bacterial cells after treated with ZnO and Ag NPs. The viability of bacterial cells of *E. coli*, *L. acidophilus*, and *B. animalis* incubated in respective broth medium for 10 h, with and without the presence of 20 mM of ZnO were analyzed. For treatment with Ag NPs, as shown in Tables 4.3, 4.4, and 4.5, bacterial strains exposed to Ag NPs for 6 h, showed the most antimicrobial effect. Therefore, *E. coli*, *L. acidophilus*, and *B. animalis* incubated in their respective broth medium for 6 h, with and without the presence of 4.6 mM of Ag NPs were used to perform the cell viability assay using fluorescence microscopy. Figure 4.23 shows the fluorescence microscopic images of the cells untreated (Figure 4.23 A, C, E) and treated (Figure 4.23 B, D, F) with ZnO NPs. *E. coli* control (A) and treated (B) samples as shown in Figure 4.23, demonstrated green fluorescence in both images which indicates live cells. There were very few red fluorescent cells, indicating the presence of dead cells in the treated sample (4.23B). This result supports with previous results (Figure 4.1A) which showed no significant effects ($P \leq .05$) of ZnO NPs on *E. coli* growth.

L. acidophilus and *B. animalis* showed similar morphological changes as those observed in SEM images (Figure 4.23D and F) after treatment of ZnO NPs. Green fluorescent cells were observed in the control image (4.23C and E) with straight rod shape formed in a chain while, treated cells were formed in clusters or twisted around one another. However, not all twisted cells showed red fluorescence, which indicates that not all deformed cells were dead. The results support the plate count numbers in Figure 4.2B and 4.2C, that

showed about 10^7 CFU/mL of bacterial cells. This indicates that not all cells were affected by ZnO NPs and resulted in leakage of internal contents and death of cells. Fluorescence microscopic images of cells treated with Ag NPs and the controls were shown in Figure 4.24. The control samples of *E. coli* shown in Figure 4.24A, mostly fluoresced green, indicating live cells. Many red fluorescence cells were observed in Ag NPs treated samples (Figure 4.24B) which indicate dead cells. Not only were there red cells observed, there were also green fluorescent cells. However, as compared to ZnO NPs treatment which showed very few dead cells, many dead cells were observed following Ag NP treatment.

Figure 4.24 showed *L. acidophilus* and *B. animalis* untreated (Figure 4.24C, E) and treated (Figure 4.24D, F) with Ag NPs. Mostly, green fluorescent cells with straight rod shapes were observed in the untreated sample image (Figure 4.24C, E). Both green and red fluorescent cells, in large clusters, were observed in the treated samples (Figure 4.24D, F). However, more green cells than red were observed in the treated samples, which indicated more live cells were present.

Thus, the results of viability of cells showed that not all cells were affected by ZnO or Ag NPs and resulted in cell death.

Compared to cells without NP treatment which showed well-organized single cells and non-clustered cells, cells treated with ZnO or Ag NPs agglomerated together and formed large clusters. Possible explanation may be electrostatic attractions between the negatively charged cell membrane of microorganisms and positively charged Zn^+ or Ag^+ ions from NPs. In the case of Ag NPs, these cannot release Ag^+ ions without oxygen and *L. acidophilus* and *B. animalis* were incubated in anaerobic condition. In spite of the

anaerobic conditions, *L. acidophilus* and *B. animalis* similarly showed agglomerations.

This may be due to the exposure of Ag NPs to oxygen and release of Ag⁺ ions, during the preparations of bacterial viability assay and fluorescence microscopy, that subsequently reacted with the negatively charged bacterial surfaces.

Figure 4.23 BacLight™ fluorescence microscopic images of cells untreated and treated with 20 mM ZnO NPs (A) *E. coli* untreated, (B) *E. coli* treated, (C) *L. acidophilus* untreated, (D) *L. acidophilus* treated, (E) *B. animalis* untreated, (F) *B. animalis* treated.

Figure 4.24 BacLight™ fluorescence microscopic images of cells untreated and treated with 4.6 mM Ag NPs (A) *E. coli* untreated, (B) *E. coli* treated, (C) *L. acidophilus* untreated, (D) *L. acidophilus* treated, (E) *B. animalis* untreated, (F) *B. animalis* treated.

CONCLUSIONS

In summary, ZnO and Ag NPs exhibited mild antibacterial effects against intestinal bacteria, *E. coli* K-12, *L. acidophilus* ADH, and *B. animalis* Bif-6. Inhibitory effects slightly increased as the concentrations of NPs increased.

Results showed that bacterial strains exposed to ZnO NPs for 10 h and Ag NPs for 6 h suffered the most antimicrobial effect. However, the number of treated cells were within 1 log CFU/mL less than that of the control and the reduction percentage in the number of cells were about 10% or less. Therefore, SEM and TEM were performed after strains were exposed to ZnO NPs for 10 h and Ag NPs for 6 h. The results of SEM and TEM images and EDS demonstrated the morphological changes of the cells and the adherence of NPs to bacterial cells. Some externally and internally damaged cells were observed. However, not all cells were damaged, and there were still many cells in normal size with intact intracellular structures and well-organized intracellular contents which correlated with the results of numbers of cells reduction. Also, results of the UV absorbance and cell viability assay correlated with the previous experiment results. The results of the UV absorbance indicated that no significant amounts of internal cellular contents were leaked due to NPs. Finally, the viability assay of bacterial cells confirmed that more live than dead cells were present after treatment with NPs. Overall results indicate that not all cells were affected by NPs.

According to all results obtained in this study, ZnO and Ag NPs have very mild inhibitory effects on intestinal bacteria as compared to those of pathogenic

microorganisms that have been done in previous studies (Sondi and Salopek-Sondi 2004; Brayer and others 2006; Kim and others 2007; Jones and others 2008; Martinez-Castanon 2008; Raffi and others 2009; Liu and others 2009; Jin and others 2009; Tayel 2010; Xie and others 2011; Mirhosseini and Firouzabadi 2013). The contamination level of ZnO and Ag NPs in food sample, if present, is very low, and in this study, a much higher concentration of NPs were tested. The data obtained from this study indicate that the food contaminated with ZnO and Ag NPs offers a negligible threat to the beneficial gut microflora.

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