

Nanotherapeutics for the mutating multi-drug resistant fecal bacteria

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Abstract

Multi-drug resistant fecal bacteria (MRF) including gram-negative *Escherichia coli* and -positive *Enterococcus faecium* are of prime concern to food safety and public health [1-4]. This study was performed to test efficacy of a natural antimicrobial, polymeric chitosan-based nanoparticles combined with ZnO to *in situ* intervention. Herein, we examined the effects of nanoparticles (NPs) of chitosan, ZnO alone and a combination of chitosan and ZnO, (CZNPs) at 1:1 on co-cultured nosocomial MRFs and a wild type (WT) through the minimal inhibitory concentration (MIC) test conform to National Standards, NCCLS. Toward elucidating visually the mechanistic effects of NPs alone and CZNPs on MRF and WT strains, Transmission Electronic Microscopy (TEM) was performed. While chitosan 1 (C₁) and 2 (C₂) alone with a molecular weight of 3 kDa and 50 kDa, respectively inhibited resistant *E. coli* strain (*E. coli* BAA-2471), they were ineffective at a concentration less than 5 mg/mL on either *E. faecium* strains and the co-cultures. ZnO and chitosan alone did not exhibit optimal effects on MRF strains and cultures alone. However, the MRF co-culture, *E. coli* BAA-2471 and *E. faecium* 1449 was completely inhibited by the C₁ZNPs with an average minimal MIC of 0.781 mg/mL, and a maximal MIC of 1.302 mg/mL. Synergism of C₁ZNPs over C₂ZNPs proved to be predominantly inhibitive of MRF over WT co-cultures. Further TEM analyses demonstrated attachment and lysis of MRFs at 16h past treatment. Conclusively, CZNPs inhibit MRF co-cultures and is a promising *in vivo* intervention agent.

Keywords: Nanotechnology; Multi-drug resistant; Fecal bacteria; Enterococcus faecium; Escherichia coli; Chitosan; Zinc oxide; Synergism; antimicrobial.

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Introduction

Currently, some strains of multidrug resistant *E. faecium* concurrently with *E. coli* pose a serious level of threat to humans [1-4]. While there are several resistant foodborne pathogens originating from animal gut, some strains of MRFs including Vancomycin Resistant *E. faecium* (VRE) remain the leading cause of hospital-acquired infections with 10,000 hospitalized cases and 650 deaths each year in the U.S. [5]. Clonal complex 17 (CC17) is now the prime causative of patient urinary tract infections in clinical settings and could further lead to serious complications primarily in patients with long stay in hospitals [6]. Conjointly in this pathogenic spread, *E. coli* is implicated in millions of extraintestinal infections resulting in more than 100,000 cases of sepsis and 40,000 sepsis-associated deaths [7-9]. Moreover, phenotypic elasticity of these MRF strains mainly *E. faecium* enable them exchange genes with other pathogens such as *Salmonella* and *Campylobacter* genera found in food animals [10-12]. It is also quite possible that resistant MRFs from food animals form a niche in the humans' gastrointestinal tract leading to a reservoir of resistance [14], consequently, jeopardizing the lives of the most immunocompromised populations [15].

Despite the increasing threat of mutating MRFs [5,16], many strains of *E. faecium* are useful lactic

acid bacteria that have been extensively added in food applications for their fermentative ability and health benefits. Despite their probiotic attributes [13], the considerable ability of some *E. faecium* strains to mutate in multiple types of environments has made the use of *E. faecium* as a fermentative strain questionable [15]. Furthermore, the continuous use of the traditional antibiotics has led to the induction of "Super Bugs", that are unresponsive to a wide range of antibiotics [22,23]. Enterococcus exhibited the ability to develop resistance to basically every drug used against them [15]. Thus, developing remediation strategies against these multi-drug resistant bacteria remains a major unmet need.

As such, an alternative natural antimicrobial that is nontoxic and sustainable was deemed of prime interest. Chitosan, arguably the most important derivative of chitin, is emerging as a strong, natural antimicrobial that is considered safe for human health [6]. Additionally, chitosan with its exposed -NH₂ groups is involved in specific interactions with metals [6] which not only allows chitosan to behave differently from other polysaccharides due to the positive charge on its surface. In a solid state, chitosan is a semicrystalline polymer that is soluble in acidic solutions, however, its solubility depends on the distribution of acetyl groups along its

polysaccharide chain and the molecular weight [7]. Chitosans have proven to be effective as an antibacterial against a variety of pathogens namely gram-negative strains and biofilms [24]. The effectiveness of such antibacterial activity was found in a previous report to be correlated with chitosan's molecular weight, thus allowing the chitosan biopolymers to be highly effective when compared to chitosan oligomers, which were significantly smaller [32].

Aside from Chitosan, Zinc oxide (ZnO) is emerging as an effective nanocomponent for its effectiveness against some cancer cells [29] in addition to its potential to mitigate some gram-positive strains with regard to its selective toxicity [29]. On the other hand, Zinc oxide nanoparticles seem to inhibit or cause bacterial death more efficiently when they are smaller in size but higher in concentration. A smaller size at higher concentrations provides higher specific surface areas and facilitates the penetration of the antimicrobial agent into the bacterial membrane [5].

A few studies demonstrated antiseptic and chemical attributes of chitosan and ZnO jointly used in cotton fabric in addition to UV protection [30,31], suggesting that a combination of chitosan oligomer and ZnO may provide better anti-septic property compared to either agent alone. However, little is known about the effectiveness of either of these nanoparticles against antibiotic resistant strains and MRFs induced illnesses.

We have been investigating the means of remediation of multi-drug resistant bacteria. Based on the reports that a combination of chitosan and ZnO may lead to better nanoformulations exhibiting anti-bacterial

properties, in this study, the anti-bacterial properties of polymeric chitosan-based nanoparticles and/or ZnO was investigated and the results show that very small oligomers of chitosan effectively mitigate MRFs and VRE. Our results for the first time demonstrates that chitosan oligomeric nanoparticles by themselves or in combination with ZnO provide for the effective remediation of MRFs and VRE.

Materials and Methods

Sample collection and culture conditions

The MRF, gram-negative, *E. coli* BAA-2471 purchased from ATCC and gram-positive *E. faecium* 1449 provided from Moffitt Cancer Center were used as target bacteria for this study. These MRF strains were selected for their broad-spectrum resistance and virulence. Tigecycline was the only available drug effective against these strains. The above isolates were cultured on Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) (Sigma, St. Louis, MO) and incubated at 37°C for 24 hours and maintained at 37°C for 24 hours. Bacterial growth was prepared by adding 100 ul fresh culture having 9.5x10⁸ Colony Forming Units (CFU) on Tryptic Soy Agar (TSA) plates.

The chitosan nanoparticles with three different MWs were provided by Dr. Mohapatra's lab in College of Pharmacy at USF. The ZnO was purchased from Sigma (Sigma, St. Louis, MO).

Serial Dilution and Plate Counts

Single colonies of *E. coli* BAA-2471 and *E. faecium* 1449 were inoculated from TSA plate to 15 mL TSB broth (Sigma, St. Louis, MO), respectively. Each broth culture was incubated at 37°C for 24 hours. A

seven times tenfold series dilution was performed by serially diluting 100 μ L bacteria broth culture into 900 μ L TSB. The 100 μ L of each dilution was plated on TSA and incubated at 37°C for 24 hours.

Minimal Inhibitory Concentration (MIC) assays

These overnight bacteria cultures were diluted 1 in 1,000 in fresh TSB or LB. Sterile 96-well plates were loaded with a co-cultured *E. coli* BAA-2471 and *E. faecium* 1449 (5×10^5 cfu/mL) and screened against chitosan, ZnO alone and CZNPs at decreasing concentrations by 1:2 dilution to equal a total volume of 100 μ L in each of the wells. All assays were performed in triplicate with identical results. Care was taken to not add more than 1.0% (CZNPs) to any well. The plates were incubated at 37°C overnight. The MICs were determined after 24 hours by visual determination of the minimum concentration of compound to inhibit growth. Inhibition of growth was determined by lack of turbidity in the wells.

TEM assay

Routine preparation of bacteria by negative staining would require pelleting the bacteria to rinse them in order to remove growth media protein through rinsing. Exposing unfixed bacteria to high-speed centrifugation could alter damaged surface membrane structure. Aldehydes, typically employed to fix and stabilize bacteria prior to observation in the electron microscope, could not be used to stabilize bacteria, as the aldehydes would crosslink proteins in the growth media to the bacterial surface, obscuring surface damage to the bacterial membranes, if present. A new method of fixing the bacteria in osmium tetroxide

prior to pelleting was employed to stabilize the bacterial membranes. Osmium would not crosslink any protein in the culture medium to the bacteria, but would preserve the membrane structure of the bacteria throughout the centrifugation process, allowing rinsing to remove the culture media proteins necessary to prepare the bacteria for TEM, and impart electron density similar to that of uranyl acetate or other negative stains used to observe bacteria in the electron microscope.

Aliquots of bacteria in growth media were initially fixed in equal volume of 2% osmium tetroxide in distilled water for 10 minutes at 4°C. Following fixation, the bacteria were rinsed in distilled water and pelleted at 5000RPM for 10 minutes. This rinse step was repeated three times. A proper dilution of bacteria was obtained to yield approximately 2000-3000 bacteria per drop, and one drop of a sample was applied to a carbon-formvar coated copper grid. The grid was allowed to air dry. This procedure was repeated for each sample. Once dry, the grids were observed and photographed in the electron microscope.

Results

The antimicrobial efficacies of chitosan tested after 24 hours incubation at 37°C are shown in Table 1. The chitosan 1 and 2 was not effective against both *E. faecium* culture and the co-culture as well as the WT cultures. However, chitosan 1 and 2 showed a significantly inhibitory antimicrobial ability (0.0488 mg/mL) against multidrug resistant *E. coli* BAA-2471 culture compare to non-effective against WT *E. coli* MCC 13 culture (Figure 1).

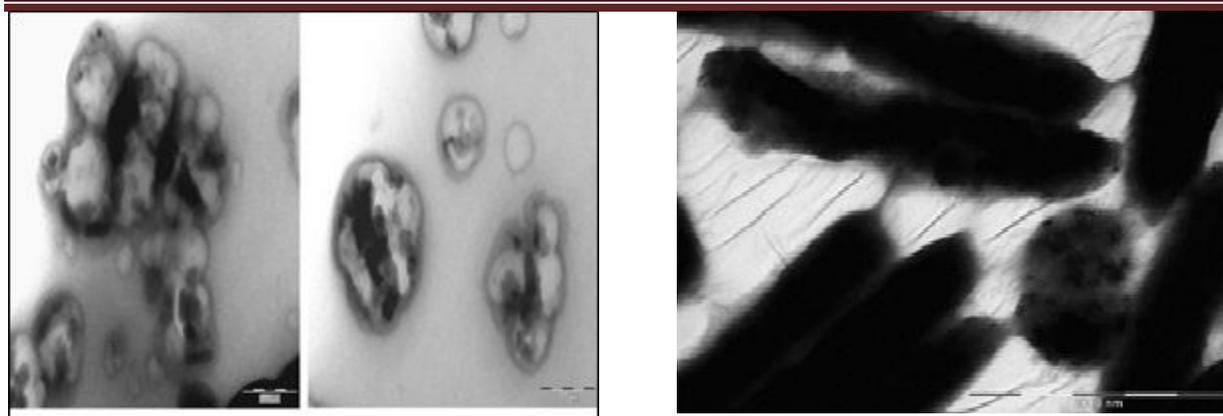
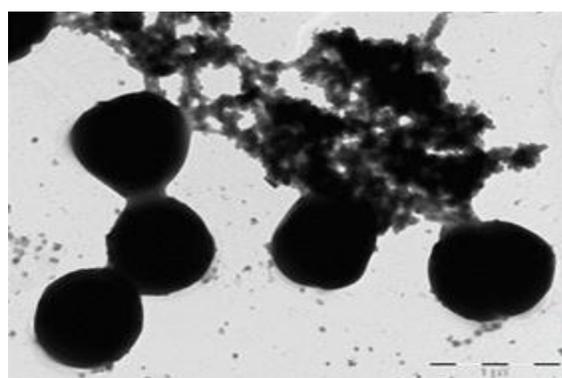


Figure 1. Chitosan particles carrying zinc oxide on the collapsing outer membranes of resistant strains.

The MIC values of ZnO tested after 24 hours incubation at 37°C are shown in Table 1. ZnO exhibited varying MICs against different cultures. According to the results, ZnO was more effective against WT *E. coli* MCC 13 (7.292 mg/mL) and WT *E. faecium* ATCC 35667 (0.391 mg/mL) than resistant *E. coli* BAA-2471 (13.54167 mg/mL) and *E. faecium* 1449 (3.125 mg/mL), respectively. However, ZnO showed a slightly lower MIC of 5.208 mg/mL against resistant co-culture than the WT co-culture with a MIC of 6.25 mg/mL (Figure 2).



A. Wild type bacteria in contact with zinc oxide particles, showing no adverse effects from exposure to the zinc oxide at 4 hours

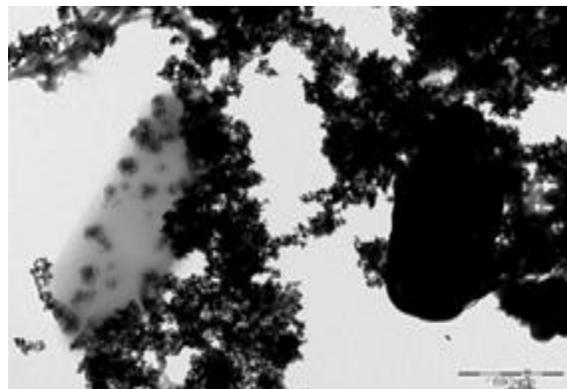
B. MRFs including *E. coli* BAA-2471 and *E. faecium* 1449 showing adsorbed chitosan on their cell membranes. The resistant bacteria have begun to lyse and collapse.

Figure 2. Effects of CZNPS on MRFs compared to wild type control exposure (A, B)

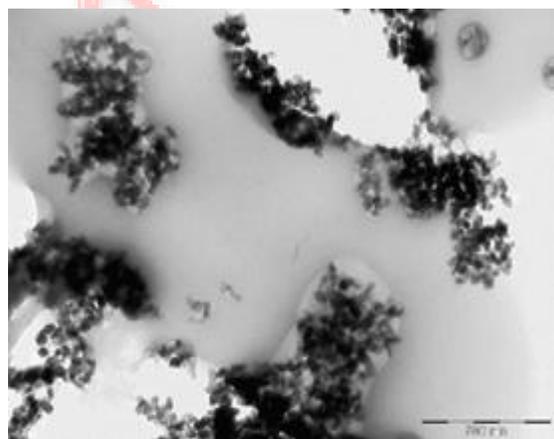
The antimicrobial efficacies of synergism of chitosan and ZnO tested after 24 and 48 hours incubation at 37°C are shown in Table 1. Both C₁ZNPs and C₂ZNPs presented markedly higher antimicrobial efficacy against resistant co-cultures than WT co-cultures with a minimal average MIC of 0.781 compared to 2.083 mg/mL, respectively. Furthermore, the effectiveness of the CZNPs complex was closely related to the molecular weight of chitosan. Indeed, the MIC values of C₁ZNPs against resistant *E. coli* BAA-2471, *E. faecium* 1449 and co-culture were 0.0488, 1.563 and 0.781 mg/mL, respectively. However, the MIC values of C₂ZNPs against resistant *E. coli* BAA-2471, *E. faecium* 1449 and co-culture were 0.0488, 1.042 and 1.302 mg/mL, respectively. Interesting, C₁ZNPs with low MW (3 KDa) was much more effective than C₂ZNPs with high MW (50 KDa) against resistant co-culture. The MW was one of key factors of NPs to impact the

antimicrobial efficacy against bacteria cultures

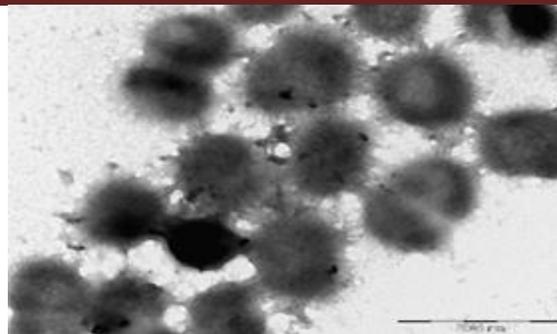
(Figure 3).



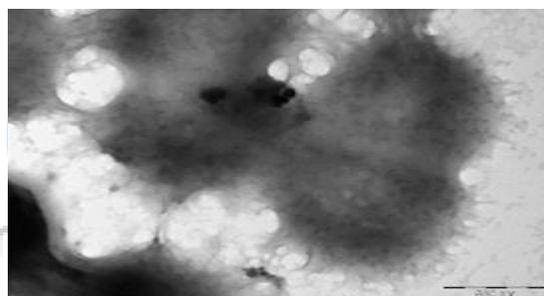
A. *E. coli* BAA-2471 exposed to zinc oxide nanoparticles and chitosan for 16 hours. The bacterium on the left shows chitosan adsorbed to the cell membrane and zinc oxide nanoparticles attached to the membrane. Low electron density indicates that the bacterium is lysing. An intact *E. coli* BAA-2471 is on the right.



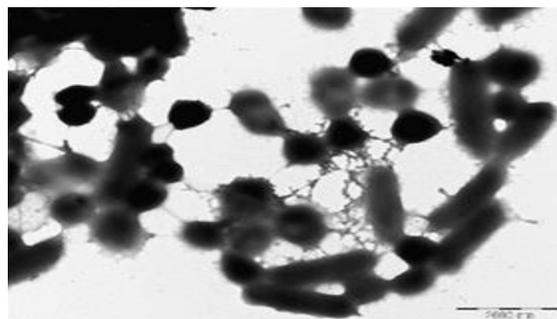
B. *E. coli* BAA-2471 exposed to chitosan and zinc oxide nanoparticles, showing adsorption of chitosan to cell membrane of bacteria and adherence of zinc oxide to bacteria. ZnO is causing pitting of the bacterial membrane.



C. *E. faecium* 1449 exposed to chitosan and zinc oxide. An intact *E. faecium* 1449 is in the lower left. Small high-density regions inside the cell are indicative of the nanoparticles. Presence of asterisk-like fragments detail lyses and disintegration of the cell wall.

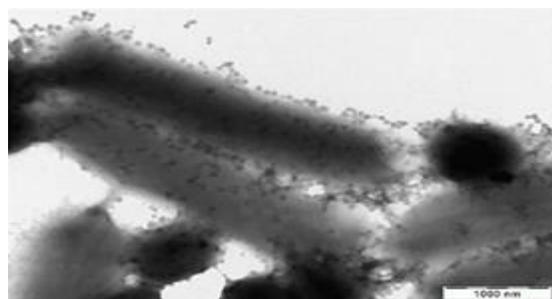


D. Higher magnification photograph of the disintegration of the cell wall of *E. faecium*.



E. Resistant co-culture, MRF of *E. faecium* 1449 and *E. coli* BAA-2471 subjected to the combinatory nanoparticles. Nanoparticles surrounding the outside

of the cells demonstrate clearly the formation of a synergistically formed meshing. Lower electron density indicates lyses of the bacteria, which is noticeable in both bacterial strains though notably more soon *E. coli* BAA-2471 in this panel.



F. Higher magnification photo of the co-cultured MRFs detailing the presence of nanoparticles surrounding and inside both bacterial species.

Figure 3. Effects of C_1ZNP_S on resistant *E. coli* BAA-2471 and *E. faecium* 1449 alone and co-cultured after 16 hours exposure (Figures B, D, and F present the mechanism effects of C_1ZNP_S under higher magnification photograph. (A-F)

The antimicrobial efficacies of chitosan tested after 24 hours incubation at 37°C are shown in Table 1. The chitosan 1 and 2 was not effective against both *E. faecium* culture and the co-culture as well as their WT cultures. However, chitosan 1 and 2 showed a significantly inhibitory antimicrobial ability (0.0488 mg/mL) against multidrug resistant *E. coli* BAA-2471 culture compare to non-effective against WT *E. coli* MCC 13 culture.

The minimal inhibitory concentration (MIC) values of the antimicrobials tested after 24 hours incubation at 37°C are shown in Table 1. Zinc oxide and chitosan were tested alone and in equal combined level (1:1) against separate cultures and co-cultures of *E. coli* strain ATCC BAA-2471 and *E. faecium* strain 1449. Chitosan 1, and 2, which vary in molecular weights (3 and 50 kDa, respectively) were ineffective at low concentrations (<5%) against either strains of bacteria as well as against the co-culture.

| Antimicrobials | Strains of MRFs and Wild Type Counterparts | | | | | |
|---------------------|--|-------------------------------------|----------------------------------|--|---------------------------------|---|
| | <i>Escherichia coli</i> BAA-2471 | <i>Escherichia coli</i> MCC 13 (WT) | <i>Enterococcus faecium</i> 1449 | <i>Enterococcus faecium</i> ATCC 35667(WT) | Co-culture of BAA-2471 and 1449 | Co-culture of MCC 13 and ATCC 35667(WT) |
| MIC: mg/mL | | | | | | |
| Chitosan 1 (3 kDA) | <0.0488 | Not Detected (>50) | Not Detected (>50) | Not Detected (>50) | Not Detected (>50) | Not Detected (>50) |
| Chitosan 2 (50 kDA) | 0.04883 | Not Detected (>50) | Not Detected (>50) | Not Detected (>50) | Not Detected (>50) | Not Detected (>50) |

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| | | | | | | |
|---------------------|----------|-------|-------|-------|-------|-------|
| ZnO | 13.54167 | 7.292 | 3.125 | 0.391 | 5.208 | 6.25 |
| C ₁ ZNPS | <0.0488 | 2.604 | 1.563 | 3.125 | 0.781 | 2.083 |
| C ₂ ZNPS | <0.0488 | 2.083 | 1.042 | 2.604 | 1.302 | 3.125 |

Table 1. Minimum Inhibitory Concentration (MIC) values of Chitosan and ZnO against

E. faecium and *E. coli*.

Notes:

^aValues are mean of three replicates.

^bCZNPS: Chitosan combined with Zinc monoxide Nanoparticles.

^cChitosan: C1, C2 with different Molecular Weights of 3kDa and 50 kDa, respectively.

^dND: not detected.

^eCo-cultured MRFs: multidrug resistant *E. coli* BAA-2471 combined with *E. faecium* 1449.

^fWT: wild type.

Zinc oxide only had relative effectiveness against *E. faecium* at a concentration of 3.125 mg/mL. Zinc oxide effectiveness at low concentrations against *E. coli* or against the co-culture was not detectible. Further tests can confirm if the co-cultures treated with zinc oxide inhibited only the growth of *E. faecium* or if both microbes grew relatively unhindered. The values denoted for combinatory antimicrobials are of total antimicrobial and therefore the concentration of each antimicrobial in these trials are half of the total concentration of antimicrobials. Detectable MICs were observed for the combination of antimicrobials against both separate cultures and the co-cultures. The C₂ZNPs was effective against *E. coli* and *E. faecium* alone with an MIC of 0.024mg/mL and 12.5 mg/mL, respectively.

These trials give evidence for a synergistic property between the two antimicrobials as trials showed effective inhibition of *E.coli*, *E. faecium*, and the co-culture as compared to the trials using just one antimicrobial, which were not detected (>5%). The MIC of C₁ZNP against *E. faecium* was a fourth of the MIC of zinc oxide against *E. faecium*. The MIC of the combined antimicrobial C₁ZNPs and C₂ZNPs in the co-culture was twice that of the MIC needed against the separate cultures. These values give evidence that these MRF strains increase their resistance to combined chitosan and Zinc oxide when in the same culture. This gives evidence that the molecular weight of chitosan can greatly alter the effectiveness of the antimicrobial. Further tests can confirm the optimal molecular weight for this antimicrobial against a particular strain.

Discussion

This report assessed the potential for remediation of multi-drug resistant bacteria of two different nanoparticles, such as the chitosan and the ZnO. Although both of these have been evaluated previously either individually or in combination for their anti-bacterial properties, hitherto neither of these have been examined for potential for remediation of the multi-drug resistant bacteria.

Since the association between the molecular weight of chitosan and its anti-bacterial property has been controversial [32] and this has not been studied for MRF and VRE strains, we initiated our investigations using two different chitosan nanoparticles, one oligomeric chitosan (3kDA) nanoparticles and a high molecular weight (50kDA) chitosan. Chitosan presents distinct mechanisms according to whether a bacteria is gram-positive or gram-negative; in previous studies, electron micrographs for *S. aureus* and *E. coli* interacting with chitosan show how the cell membrane of *S. aureus* was “weakened or even broken, while the cytoplasm of *E. coli* was concentrated and the interstice of the cell were clearly enlarged” [7], which provides ample evidence to suggest two main antibacterial mechanisms performed by chitosan. For gram-positive bacteria, chitosan forms a polymer membrane around the cell’s surface preventing any nutrients from entering while for gram-negative bacteria, chitosan with lower molecular weight entered the cell through pervasion [6]. Chitosan seems to work more efficiently against gram-negative bacteria due to how its positively-charged structure adsorbs the electronegative substance in the cell and flocculate it to disturb the physiological activities of the bacteria and inhibit it.

However, chitosan is naturally a large molecule, therefore the use of chitosan oligomers present a stronger choice if the molecules would be combined with another compound for a synergistic effect. Although chitosan oligomers do not have the same level of antibacterial activity as chitosan, their smaller size allows for a lower molecular weight and the facilitation of penetrating the bacterial surface. The effect of this property is seen as chitosan oligomers seemed to have an increased antibacterial activity against gram-negative bacteria at lower molecular weight such as 1 kDa, where Kyoon No et al., 2002 found that the growth of *E. coli* was reduced by 1 to 3 log cycles at a 1.0% concentration of chitosan oligomer with various degrees of polymerization [8]. Moreover, the ZnO nanoparticle effect is more pronounced “against gram-positive bacterial than gram-negative bacterial strains” [9]. Gram-positive bacteria have a thick layer of peptidoglycan polymer that encircles the cell and a much thicker cell wall, while gram-negative have two thin cell membranes divided into an outer membrane and a plasma membrane [2], therefore they both require different agents against their different structures. Chitosan oligomers’ polysaccharide structure not only contain transcellular properties to cross cellular membranes, they also contain mucoadhesive and bioadhesive properties that contribute to their absorption improving effects [10]. The positively-charged chitosan does not only bind to negatively-charged oxides, it also can bind to different drugs or peptide hormones like calcitonin to deliver a specific dose effectively, partly due to its bioadhesive properties with gram-negative bacterial membranes or negative mucus in tissue membrane in the case of calcitonin delivery [10]. Since chitosan

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has the structural ability to bond to metal oxide compounds such as the ionic ZnO, a possibility exists to develop an antimicrobial agent that can efficiently work against both types of bacteria by bonding these two compounds in order to create a synergistic combination that targets a broader range of bacteria found in food products without risking human health. The applicability of ZnO as an antimicrobial agent is due to its morphology that broadens the uses of it against various bacteria. The compound's structure allows for an easier biocompatibility over other metal oxides, solubility in alkaline medium, and the Zn–O terminated polar surfaces [3]. Zinc oxide nanoparticles seem to inhibit or cause bacterial death more efficiently when they are smaller in size but higher in concentration. A smaller size at higher concentrations provides higher specific surface areas and facilitates the penetration of the antimicrobial agent into the bacterial membrane [5]. Virtually every unique property of ZnO proves to be beneficial in regards to antibacterial activity. Although ZnO is usually insoluble in water due to its high polarity, it can be managed efficiently in an aqueous cell culture media such as tryptic soy broth, also known as TSB. Zinc oxide possesses photo-oxidizing and photocatalysis impacts on chemical and biological species [2], which combined with its bio-safe composition, provides a safe interaction on food products where they come in contact with bacteria to inhibit and/or kill it to prevent food-related diseases.

Additionally, this study adds a novel perspective on the increased effectiveness of chitosan by molecular weight when in the presence of ZnO. While the oligomer of chitosan (3kDA) in literature shows the least potential as a possible effective antimicrobial,

the effectiveness of chitosan oligomers against MRFs was substantially amplified by the addition of ZnO. The discrepancy between our data and the current literature may be due to the presence of ZnO along with the structural modifications of the chitosan oligomers used to generate optimal conditions.

Combination of chitosan oligomers with ZnO demonstrated synergistic effects in remediation of important food-borne bacteria including the resistant strains. Current literature has shown the synergistic antimicrobial properties of chitosan and ZnO [30], as well as detailed the synergistic properties of chitosan and ZnO individually against wild type bacteria [31]. However, there has been no quantitative data thus far on the precise concerted effect of these nanoparticles against multi-drug resistant microbes. This experiment has demonstrated the effect against both multi-drug resistant gram-positive and gram-negative fecal bacteria while comparing the combinatory antimicrobial properties to the individual nanoparticles. The overall objective of this experiment was to ascertain the efficacy of CZNPs synergism on co-cultured MRF strains through validated MIC tests and TEM assays. Specific goal was to validate a nanotherapeutic agent to further *in situ* intervention.

This research study has novelty demonstrated this success of CZNPs against both multi-drug resistant gram-positive and gram-negative fecal bacteria while comparing the combinatory antimicrobial properties to the individual nanoparticles. Synergism of CZNPs primarily C₁ZNPs proved to be successfully suppressive to MRFs over WT strains. It is concluded that C₁ZNPs has therapeutic potential to *in situ* intervention.

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Acknowledgements

The Research, Innovation & Economic Development of USF is thankfully acknowledged for its grant support to this research study. The authors also thank the Department of Integrative Biology at USF for providing their TEM core facility access and service. The College of Pharmacy is also genuinely recognized for its assistance in nanotechnology.

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Citation: Limayem A, Micciche A, Haller E, Zhang C and Mohapatra (2015) Nanotherapeutics for multating multi-drug resistant fecal bacteria. J Nanotec Nanosci 1:100106

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