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Toxicological Effects of Transition Metal Oxide Nanoparticles on Growth of Lactic Acid Bacteria

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ABSTRACT

Lactic acid bacteria (LAB) are commonly used in food preservation due to their ability to produce lactic acid around which pathogenic bacteria do not thrive. With cost-efficient production of LAB, new research on their agricultural applications is reported at an increasing rate. Transition metal oxide nanoparticles are demonstrated to display antimicrobial activity in this report. However, LAB is able to proliferate in the presence of magnesium oxide nanoparticles at concentrations from 0.25 to 1.0 mg/ml. In comparison, Lactobacillus acidophilus probiotic (LAP) displayed significantly less colony growth when exposed to TMONPs. Furthermore, 4-chlorophenol is a harmful environmental contaminant and priority pollutant and LAB growth decreased slightly with increasing concentration of 4-chlorophenol. Interestingly, the toxicity of 4-chlorophenol to LAB growth was ameliorated in the presence of MgO nanoparticles.

Keywords

Agriculture, 4-chlorophenol, Environment, Food, Lactic acid bacteria, Magnesium oxide, Nanoparticles, Toxicology, Transition metal oxides.

Introduction

Lactic acid bacteria (LAB) comprise a genus of gram-positive, non-sporulating bacteria capable of producing lactic acid as a by-product during the fermentation of carbohydrates [1,2]. LAB is often identified by specific morphological and physiological characteristics such as the bacterial shape, growth, and motility [3]. LAB is traditionally cultured by a common fermentation process using rice and milk [4,5]. A liquid fraction taken from the culture contains a variety of genera including lactobacillus and lactococcus [6]. They are ubiquitous microorganisms in environments that are rich in carbohydrates [7]. LAB is known for their functionality in food preservation [8,9], human health benefits [10,11], and agricultural crops/livestock production [12-15]. LAB assists with the preservation of food as the production of lactic acid lowers the pH to approximately 3-4 at which little to no other microorganisms can thrive. The reduction of pyruvate into lactate and oxidation of nicotinamide adenine dinucleotide go through the Embden-Mayeroff route [16]. One genus of LAB, Lactobacilli, is capable of producing hydrogen peroxide, which has antibacterial properties. Nisin is another bacteriocin produced as a polypeptide by LAB, specifically the *Lactococcus lactis* strains. The antimicrobial protein is effective against gram- positive, pathogenic, spore-producing, and food-spoilage bacteria [17,18]. The LBbb0141 strain contained antimicrobial biopeptides that inhibited the growth of a wide spectrum of Gram-positive and -negative bacteria. The bacteriocin activity attained its maximum value using the MRS agar at pH 7.5 and 30°C incubation temperature [19]. Even the cell-free supernatant of LAB is rich in bioactive compounds that combat *Staphylococcus aureus* infections [20]. Due to its non-harmful properties, the U.S. Food and Drug Administration has given LAB the status of generally regarded as safe [21]. Indeed, LAB has long been approved by the European Food Safety Authority (EFSA) for use as a food additive. Due to all these beneficial properties of LAB, it has been sustainably exploited in agricultural fields as fertilizer for soil organic content amendment [22], plant growth promotion [23], and fungal disease control [23,24]. LAB can bind bacteria physically to affect the peptidoglycan structure of their

cell walls [25]. Due to the production of antifungal metabolites, LAB can inhibit mycotoxin biosynthesis [26]. Recent studies have proposed the efficient bioremediation and detoxification of heavy metals using LAB [27]. All strains demonstrated reactive oxygen species scavenging activity towards superoxide anion, hydroxyl radical and hydrogen peroxide [28,29]. Antioxidant or reducing activity was also found among *Lactobacillus* strains that could be considered a potential for functional food production [30]. Food processors can adopt LAB with natural or synthetic preservatives as a biocontrol solution to proactively to assure the safety of their products [31]. In addition to selecting the right strain, desirable metabolic processes required optimizing and controlling the available nutrients including sugars, peptides, free amino acids, minerals, and vitamins in addition to buffering agents [32]. The antimicrobial resistance in LAB has been reviewed [33], as well as their potential in reducing the negative impacts of climate change during shellfish farming [34].

Transition metal oxide nanoparticles (TMONPs) are increasingly attracting interest in their industrial application as materials for catalysts, cosmetics, pharmacological agents, enzymes purification, and targeted drug delivery [35,36]. With greater interest in the applications of nanotechnology, further advancement in this field of study is gaining momentum. The TMONPs used in the present research work include aluminum oxide, cerium oxide, cobalt oxide, cupric oxide, titanium dioxide, and zinc oxide. Aluminum oxide (Al_2O_3) nanoparticles have been used in personal care products. Unlike other TMONPs, Al_2O_3 nanoparticles exhibit only moderate growth-inhibitory effects at high concentration levels. Its antimicrobial effect comes from the interaction of its surface charge with the bacterial cell wall and cell membrane. Electrostatic interaction between the positive charge of Al_2O_3 nanoparticles and the negative charge of *E. coli* bacteria results in antimicrobial properties, and the production of reactive oxygen species (ROS) can cause cell death [37]. Cerium oxide (CeO_2) nanoparticles are used for mechanical polishing, corrosion protection, and catalysis of fuel oxidation. They have antioxidant properties against gram-positive and gram-negative bacteria mainly due to redox cycling between the 3+ and 4+ states [38]. Cobalt oxide (Co_3O_4) nanoparticles are used in biomedical applications for their anticancer, antimicrobial and enzyme inhibition properties. Under visible light, Co_3O_4 nanoparticles can act as a photocatalyst for organic pollutant degradation [39]. Although Co_3O_4 nanoparticles have fewer research studies compared to other TMONPs, they all have similar antimicrobial properties [40] and gram-positive bacteria such as *Bacillus subtilis* are more susceptible [41]. Copper oxide (CuO) nanoparticles are explored for their semiconducting properties, potential catalysis, and biomedical applications. Their photothermal and photoconductive properties are useful for high-temperature superconductivity and electron correlation applications [42]. CuO nanoparticles are able to kill both gram-positive and gram-negative bacteria in hospitals within 2 hours. However, their capability to produce ROS is a disadvantage in medical treatments due to the potential to induce oxidative stress and damage biological cells [31]. Titanium dioxide (TiO_2) nanoparticles are found as an additive in many consumer products such as food and personal care products [43]. Their photoactivity produces ROS in aqueous media upon exposure to UV light, inducing cell death due to cell membrane damage. TiO_2 nanoparticles can be used in cancer therapy and photodynamic therapy [44]. The buccal mucosa is an absorption route for the systemic passage of food-grade TiO_2 particles [45]. Exposure of TiO_2 nanoparticles increases the concentration of lactic acid bacteria due to the oxidative stress and inflammation [46]. Zinc oxide (ZnO) nanoparticles have been very popular for their use in pharmaceutical formulations, cosmetic products such as sunscreen, photocatalysis, dietary supplements, and antimicrobial agents [47]. ZnO nanoparticles generate ROS that can damage the bacterial cell wall [48]. Lactobacillus spp. have been investigated for their bio-absorption of Zn^{2+} in the intracellular synthesis of ZnO nanoparticles [49]. Through electrostatic interaction, certain species of LAB are comprised of ligand-like functional groups on the cell membrane that attract Zn^{2+}. Interestingly, LAB is tolerant against the toxicity of Zn^{2+} during bio-absorption and bio-accumulation.

Phenol in industrial effluent is a major under-reported pollutant of concern to the aquatic ecosystem. Exogenous bacterial isolates were bio-mined from crude oil polluted soil to reduce phenol residues with nearly 100% efficiency [50]. Chlorophenols contain one or more chlorine atoms at various positions on the aromatic ring. They are industrially used to produce drugs, dyes and pesticides. However, due to waste disposal procedures being far from ideal after its use in anthropogenic activities. These compounds are considered contaminants that are harmful to the environment and extremely toxic to aquatic wildlife. Many chlorophenols are listed as priority pollutants by the United States Environmental Protection Agency. The World Health Organization and the International Agency for Research on Cancers have classified 4-chlorophenol and its derivatives as carcinogenic [51,52]. Early research indicated that biodegradation of chlorophenols is cost-effective with bacteria that utilize them as carbon and energy sources [53]. One study reported mineralization of 4-chlorophenol as a model compound through continuous ozonation followed by aerobic biodegradation [54].

The goal of this research is to investigate the effects of TMONPs on LAB growth and determine the potential of 4-chlorophenol biodegradation by LAB and TMONPs. Magnesium oxide (MgO) nanoparticles have been listed as safe materials according to the US Food and Drug Administration and can herein serve as a standard of comparison with TMONPs. MgO nanoparticles have been investigated for their antibacterial effects on *E. coli* and *Salmonella* [55]. It has been reported that MgO nanoparticles distort the cell membrane and caused the intracellular contents to slowly leak out, leading to bacterial cell death. To increase its bactericidal effects, nisin (a polycyclic antibacterial peptide produced by the bacterium Lactococcus lactis for use as a food preservative) has been added to MgO nanoparticles to observe their synergistic effects as antibacterial agents for enhanced food safety [17]. To achieve lactic acid production at low energy consumption and reactor corrosion rate, four divalent transition metal oxides (Cu, Co, Ni and Zn) were supported on MgO nanoparticles with

hexadecyltrimethylammonium bromide capping. ZnCTAB/MgO exhibited the highest activity with a glucose conversion of 64% at a 100°C and a lactic acid yield of 12% at 140°C after 1 h of reaction time [56]. Magnesium itself is known to be an important non-toxic metal required for bacterial growth; starvation of magnesium can result in the termination of bacterial replication due to environmental stress [57]. However, less attention has been paid to the potential use of MgO for removing microorganisms [58], which is researched more in the present work.

Materials and Methods

Lactic Acid Bacteria Preparation

LAB was grown in rice wash water and 2% milk by following a previously reported procedure [6]. Premium jasmine scented white rice (125 g) was first mixed with deionized water (25 ml) in a glass container. The container was covered with a paper towel to leave the mixture at room temperature (25±2°C) for fermentation. After 72 hours, the rice wash water became turbid and a sweet fermentation smell developed to confirm the growth of LAB. The rice wash water (100 ml) was transferred to another glass container containing 2% skimmed milk (1.0 L). The mixture was allowed to ferment over the course of 10 days, gradually separating into a curd layer (top), liquid layer (middle) and sedimentation layer (bottom). The liquid layer was carefully filtered through a cheesecloth and stored in the fridge at 4±1°C for all subsequent experiments.

Transition Metal Oxide Nanoparticles

All TMONPs were purchased from Sigma Aldrich (Oakville, ON, Canada) with <50 nm in particle size, as listed in Table 1. Standard suspensions of TMONPs (1.0 mg/ml) were prepared using deionized water.

Table 1: Transition metal oxide nanoparticles purchased from Sigma Aldrich.

TMONP	Catalogue number	Lot number
Titanium (IV) oxide	718467-100G	MKBR0084V
Zinc oxide	677450-5G	MKBT7475V
Copper (II) oxide	544868-25G	MKBT8894V
Aluminum oxide	544833-10G	BCBK5287V
Cerium (IV) oxide	700290-25G	MKBV5504V
Magnesium oxide	549649-5G	MKCC6603
Cobalt (II, III) oxide	637025-100G	MKCP7794

Turbidimetric Analysis

The turbidity of TMONP suspensions was analyzed using the TU5 HACH turbidimeter (Loveland, CO, U.S.A.). Suspension samples were prepared by ultrasonic homogenization and allowed to reach room temperature before turbidity measurements. Readings over 700 NTU were above the working range of the turbidimeter and were recorded as "700+NTU".

MRS plate assays

De Man, Rogosa and Sharpe (MRS) agar plates were purchased from Teknova (Hollister, CA, U.S.A.) and stored at 4°C in a fridge until they were used for streak culture plate analysis [59], agar well diffusion analysis, or lawn plate culture analysis. Using sterile equipment, the LAB stock was streaked onto an MRS agar plate in a series of parallel lines to create a bacterial culture. Bacterial growth was allowed over the course of 48 hours before visual examination. Colonies were counted to determine the viability of the bacteria. A suitable counting range was 25-250 colony forming units (CFU) per plate. When plates had more than 250 CFUs, they were considered too numerous to count and might even inhibit the growth of some bacteria. Agar well diffusion analysis was also performed using MRS agar plates. The LAB was stock was inoculated across the surface of the agar plate. Four wells (7 mm diameter) were made in the agar using the larger end of a pasteur pipette. TMONP suspensions (0.25 mg/ml, 0.50 mg/ml, and 1.0 mg/ml) of TMONP suspensions were prepared. Each TMONP suspension (100 µl) was pipetted into one well. A control (100 µl) of deionized water was pipetted into the fourth well. LAB growth across the plates was assessed over the course of 48 hours, based on the distance of the closest bacterial colony from the well that could be measured with a ruler to indicate the strength of antibacterial effect for the TMONP placed in each well. The greater the distance, the greater the inhibition. Lawn plate analysis was last performed on MRS agar plates to determine the bacterial interaction with TMONPs and 4-chlorophenol. Standard (1.0 mg/ml) TMONP suspensions and 4-chlorophenol solutions were mixed together with LAB in various combinations. After incubation at room temperature for 24 hours, each mixture was inoculated on an MRS agar plate to produce a heavy uniform layer over the whole surface of the agar. Bacterial growth, without any individual colonies visible, was assessed by visual examination after 48 hours [60]. All MRS plates after use were autoclaved and safely disposed of.

Results

Turbidity Analysis and Sedimentation

Turbidity analysis measures the lack of clarity of an aqueous suspension due to the presence of particles that obstruct the transmission of light. The size, shape and colour of the suspended particles will affect the light transmittance via scattering. Sedimentation evaluation allows for observational determination of the particle settling rate. Large sediment formation can be indicative of suspended solids. For bacteria the increased opacity, measured in nephelometric turbidity units (NTU), is an assessment of bacterial growth [61]. In the present work, wash water of the rice contained colonies of LAB along with other microorganisms. Allowing 2-3 days for casein to aggregate and settle to the bottom [62] created a supernatant wash water where LAB was the predominant species. Milk was added on day 3 as a food source for LAB proliferation. Over the next 10-11 days, coagulation of the milk protein (mostly casein and partly whey) by lactic acid occurred, forming a soft white substance (or curd) that settled down to cover the rice at the bottom. Production of LAB from milk sugar (or lactose) fermentation was indicated by the turbidity, colour, and smell of the supernatant. As shown in Figure 1, the supernatant displayed an opaque green colour and exuded a sweet fermentation smell. After filtration through a cheesecloth, the LAB culture in a pale green filtrate was ready for use in all subsequent assays.

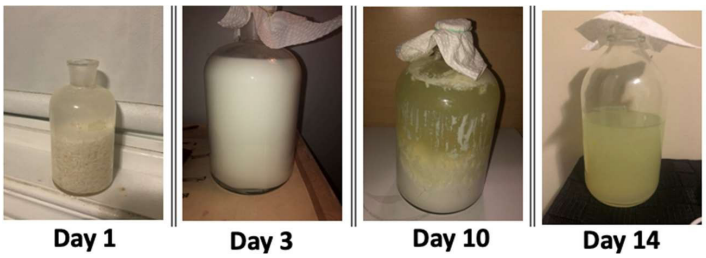

Figure 1: Lactic acid bacterial growth from fermentation at 25°C. Day 1: white rice in wash water. Day 3: addition of 2% milk. Day 10: sedimentation of aggregated casein and whey protein. Day 14: LAB culture in pale green filtrate of supernatant through a cheesecloth.

Interaction of LAB filtrate (10 ml) with TMONPs (100 mg) at 25°C was carefully monitored during the course of 24 hours. TMONPs that were initially dark in coloration, such as CuO and Co_3O_4 (Figure 2), displayed little to no sediment formation (Table 2). Most of the changes in TMONP interaction with LAB occurred within the first hour. ZnO and MgO produced the thickest layers of sediments after 24 hours. Turbidity measurements were higher than 700 NTU for TiO_2, CuO and Co_3O_4 in LAB (Table 3). MgO in LAB displayed the lowest turbidity value comparable to the control. Interestingly, CeO displayed a turbidity value lower than the blank control, hence, this is considered an outlier. CaO was included as a positive control for validation of the turbidity measurement even though it was not considered a TMONP.

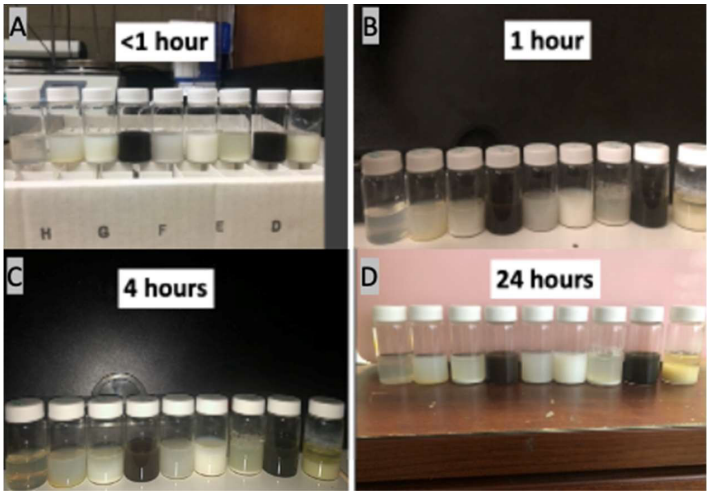

Figure 2: Interaction of suspended TMONPs (100 mg) with LAB filtrate (10 ml) at 25°C over (a) less than an hour, (b) one hour, (c) 4 hours, and (d) 24 hours. From left to right: blank control, TiO_2, ZnO, CuO, Al_2O_3, CeO_2, MgO, Co_3O_4 and CaO.

Antimicrobial Susceptibility

Antimicrobial susceptibility is determined by the effects of antibiotic agents on a bacteria culture. The most common method for testing susceptibility is to evaluate the microbial growth on agar plates. The media chosen for the agar is dependent on the

Table 2: Observations of various TMONP (100 mg) suspensions in LAB filtrate (10 ml) at 25°C.

TMONP	<1 hour	1 hour	4 hours	24 hours
Control	· Translucent · Dull yellow/clear · No sedimentation	NOC*	NOC	NOC
TiO_2	· Less translucent than control · Dusty yellow · Thin layer of sedimentation already formed, white powdery like layer (~1mm)	NOC	NOC	NOC
ZnO	· White, opaque, milky · Thicker layer of sedimentation than titanium (~2.5mm)	· Thin residual layer formed on top · Gradient layer of translucence formed in supernatant	NOC	· Residual layer at top disappeared · Very small change in sedimentation (~2.6mm)
CuO	· Dark purple-black, opaque · Sedimentation cannot be seen	NOC	NOC	NOC
Al_2O_3	· Similar translucence to titanium, slightly grey/ yellow colour · Sedimentation layer formed (~1mm)	NOC	NOC	NOC
CeO_2	· White, opaque, milky · Sedimentation not seen/formed	NOC	NOC	NOC
MgO	· Similar characteristics to titanium and aluminum · Yellow/green colour · Sedimentation layer formed (~1.5mm)	· Bubbles in photo are caused by sample slightly shaken	NOC	· Lighter yellow/green colour
Co_3O_4	· Dark purple-black, opaque · Sedimentation cannot be seen	NOC	NOC	NOC
CaO	· Opaque yellow/green-white · No sedimentation seen	· Separation of supernatant and sediment can be seen to be formed. Sediment remains white (4mm), supernatant has oily features to it (similar colour, transparent, shiny	· Sedimentation layer thickened (~0.5mm)	NOC

* NOC = no obvious changes

microbe being grown, as ingredients for optimal growth conditions can vary. Specifically, De Man, Rogosa and Sharpe (MRS) agar is ideal because it favors the growth of *Lactobacilli* while suppressing many other bacteria. In the present study, susceptibility testing included streak plating, agar well diffusion and lawn plate analysis. Streak plating involves inoculating the plate with the microbe in a series of parallel lines so as to isolate single colonies from a bacterial culture. Spot plating has a similar working principle to streak plating, except it is done with a single drop or more of the microbe being studied. This will give a clear view of the dense colonies growing. Spot plating of the LAB filtrate on an MRS plate is shown in Figure 3. Several colonies displayed characteristics such as concave, shiny, milky white or orange. After growing for 48 hours, the plate exuded a sweet smell of fermentation as well.

Table 3: Turbidity analysis of supernatant from LAB filtrate (10 ml) and various TMONPs (100 mg) after 24 hours at 25°C.

TMONP	Turbidity Readings (NTU)			Average (NTU)
LAB control	173	173	173	173
TiO_2	700+	700+	700+	700+
ZnO	463	464	464	464
CuO	700+	700+	700+	700+
Al_2O_3	528	528	527	528
CeO_2	92.7	92.6	92.6	93
MgO	333	333	331	332
Co_3O_4	700+	700+	700+	700+
CaO	543	545	546	545

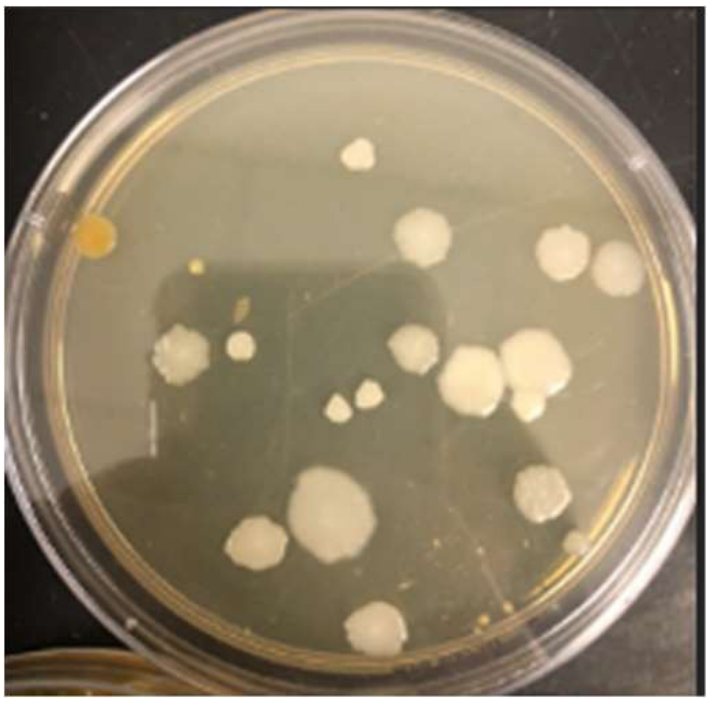

Figure 3: Spot plating of LAB filtrate on MRS plate after 48 hours.

Agar well diffusion examines the antimicrobial activity of microorganisms. After inoculating the agar surface with the microbe, a small volume of the antibiotic agent will be introduced into several wells at different concentrations. The microbe is then allowed to incubate at the desired temperature, and diffusion of the antibiotic agent occurs across the agar plate. The distance of microbial growth around each well compared to the control will provide a clear indication of how susceptible the inoculated microbe is to the antibiotic agent [59]. Figures 4.1-4.3 show the agar well diffusion growth on MRS plates of LAB that were exposed from zero to 48 hours to three concentrations of various TMONPs at 25°C. In Figure 4.3, the control plate displayed a lack of bacterial growth around the four wells of deionized water, whereas MgO displayed the largest LAB growth compared to all other TMONPs over 48 hours. Interestingly, ZnO displayed a characteristic growth of minuscule LAB colonies around the wells. The other TMONPs displayed similar bacterial growth and similar colony counts among themselves. The colony counts in Figure 5 indicate that lower concentrations of TMONP sometimes resulted in higher colony counts. Table 4 compares the colony counts of LAB growth versus the zone of inhibition distances for the three concentrations of each TMONP tested. A common trend can be seen where the higher the TMONP concentration, the longer the zone of inhibition distance.

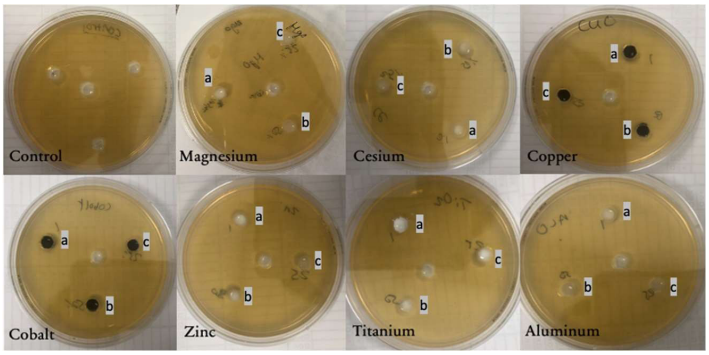

Figure 4.1: Agar well diffusion plates of LAB exposed to various TMONPs at zero hour and 25°C in three concentrations: (a) 1.0 mg/ml (b) 0.5 mg/ml (c) 0.25 mg/ml. Middle well was a control with 0 mg/ml of TMONP present.

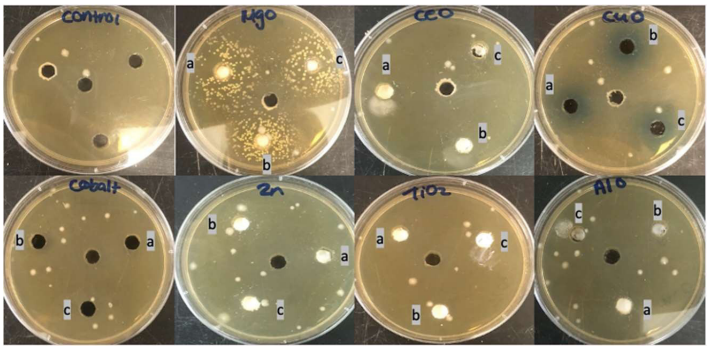

Figure 4.2: Agar well diffusion plates of LAB exposed to various TMONPs at 24 hours and 25°C in three concentrations: (a) 1.0 mg/ml (b) 0.5 mg/ml (c) 0.25 mg/ml. Middle well was a control with 0 mg/ml of TMONP present.

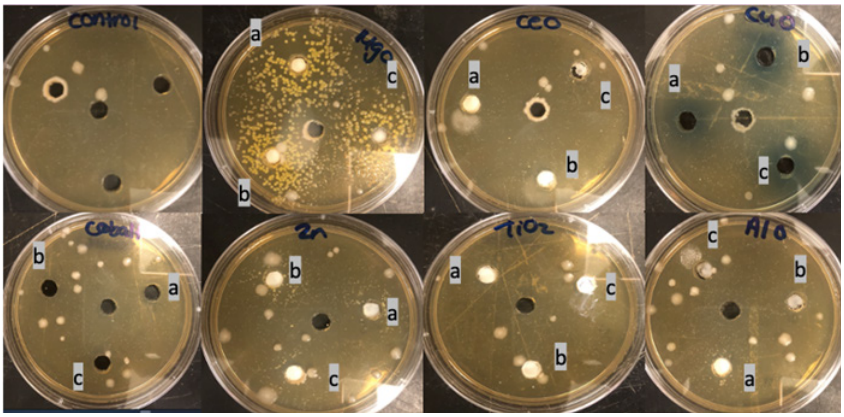

Figure 4.3: Agar well diffusion plates of LAB exposed to various TMONPs at 48 hours and 25°C in three concentrations: (a) 1.0 mg/ml (b) 0.5 mg/ml (c) 0.25 mg/ml. Middle well was a control with 0 mg/ml of TMONP present.

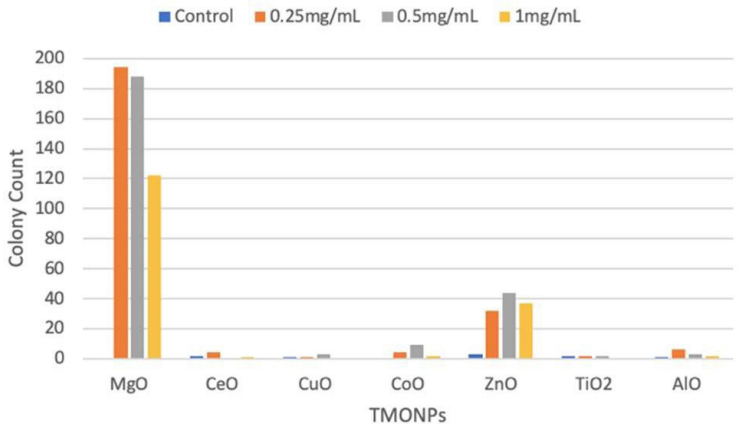

Figure 5: Colony counts of LAB growth after exposure to three concentrations (0.25 mg/ml, 0.50 mg/ml, 1.0 mg/ml) of various TMONPs for 48 hours at 25°C.

Table 4: Colony counts and zone of inhibition distances (mm) of LAB growth on MRS plates after exposure to three concentrations (0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml) of various TMONPs for 48 hours in the agar well diffusion test.

TMONP	Control		0.25 mg/L		0.50 mg/L		1.0 mg/L	
	Colony Count	Inhibition Distance (mm)	Colony Count	Inhibition Distance (mm)	Colony Count	Inhibition Distance (mm)	Colony Count	Inhibition Distance (mm)
MgO	0	0	194	0	188	0	122	0
CeO_2	2	5	4	4	0	N/A	1	20
CuO	1	4	1	2.8	3	15	0	N/A
Co_3O_4	0	N/A*	4	N/A	9	7	2	11
ZnO	3	3	32	0	44	0	47	0
TiO_2	2	7	2	1	2	0	0	N/A
Al_2O_3	1	N/A	6	1	3	6	2	14

*Not applicable indicates no distance measurement was made due to the lack of sufficient bacterial growth.

Table 5: Colony counts and zone of inhibition distances (mm) of LAP growth on MRS plates after exposure to three concentrations (0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml) of various TMONPs for 48 hours in the agar well diffusion plate test.

TMONP	Control		0.25 mg/L		0.50 mg/L		1.0 mg/L	
	Colony Count	Inhibition Distance (mm)	Colony Count	Inhibition Distance (mm)	Colony Count	Inhibition Distance (mm)	Colony Count	Inhibition Distance (mm)
MgO	0	N/A*	0	N/A	4	6	1	0
CeO_2	0	N/A	0	N/A	0	N/A	0	N/A
CuO	0	N/A	0	N/A	0	N/A	0	N/A
Co_3O_4	0	0	1	9	1	12	1	12
ZnO	0	N/A	0	N/A	0	N/A	0	N/A
TiO_2	0	N/A	7	9	0	7	0	N/A
Al_2O_3	0	N/A	0	N/A	4	5	1	1.5

*Not applicable indicates no distance measurement was made due to the lack of sufficient bacterial growth.

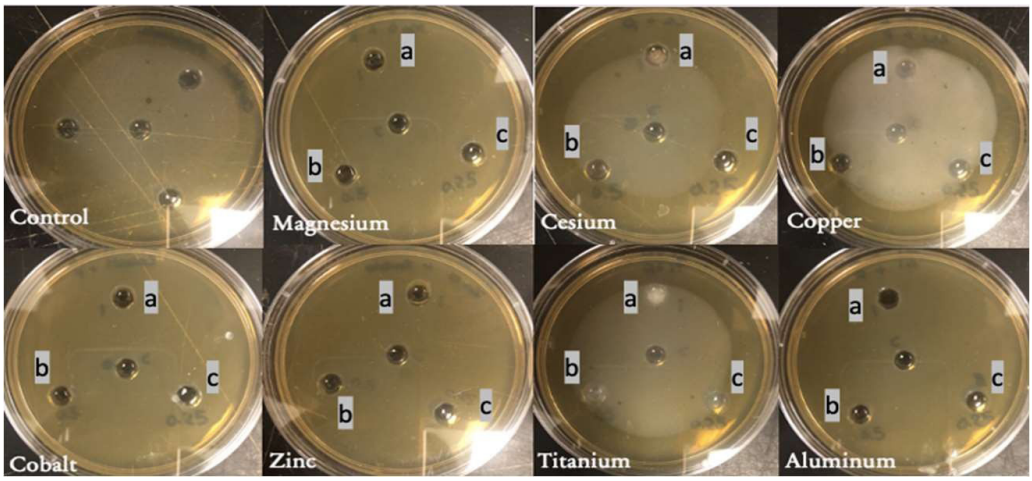

Figure 6.1: Agar well diffusion plates of LAP exposed to various TMONPs at zero hour and 25°C in three concentrations: (a) 1.0 mg/ml (b) 0.5 mg/ml (c) 0.25 mg/ml. Middle well was a control with 0 mg/ml of TMONP present.

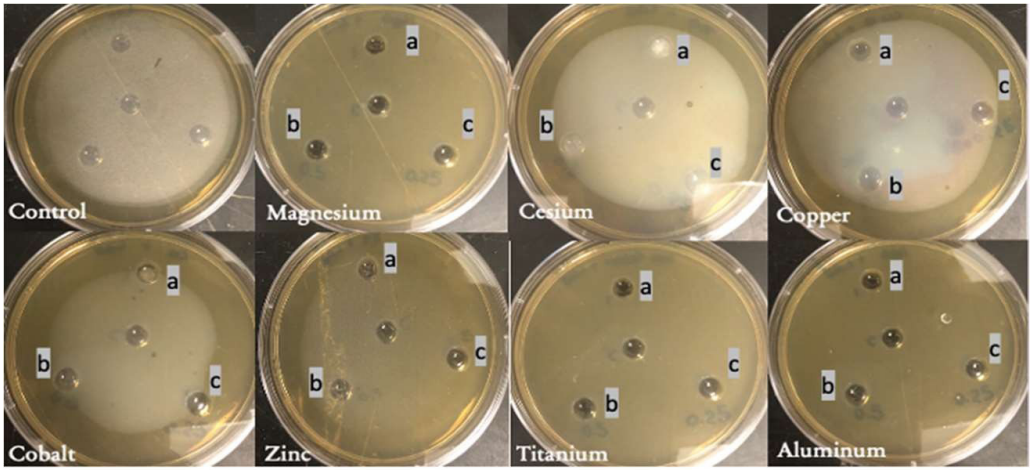

Figure 6.2: Agar well diffusion plates of LAP exposed to various TMONPs at 24 hours and 25°C in three concentrations: (a) 1.0 mg/ml (b) 0.5 mg/ml (c) 0.25 mg/ml. Middle well was a control with 0 mg/ml of TMONP present.

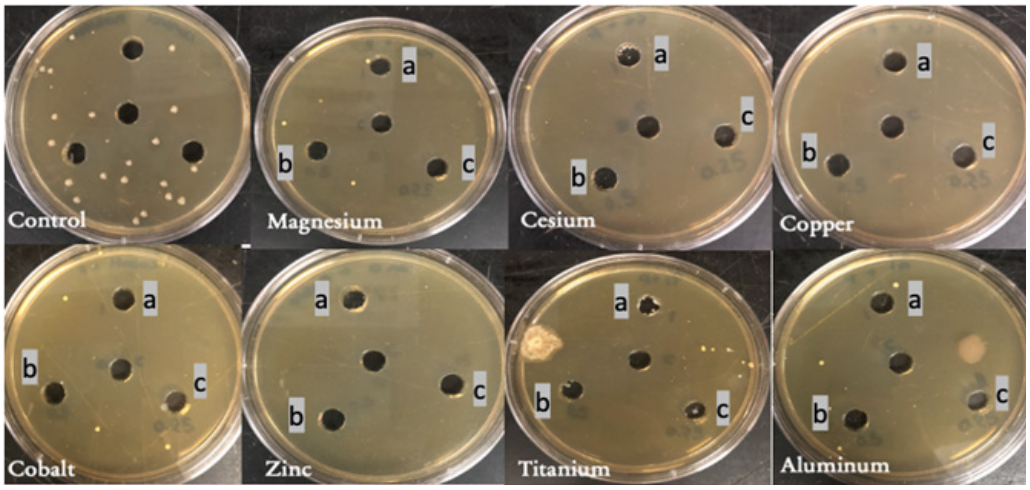

Figure 6.3: Agar well diffusion plates of LAP exposed to various TMONPs at 48 hours and 25°C in three concentrations: (a) 1.0 mg/ml (b) 0.5 mg/ml (c) 0.25 mg/ml. Middle well was a control with 0 mg/ml of TMONP present.

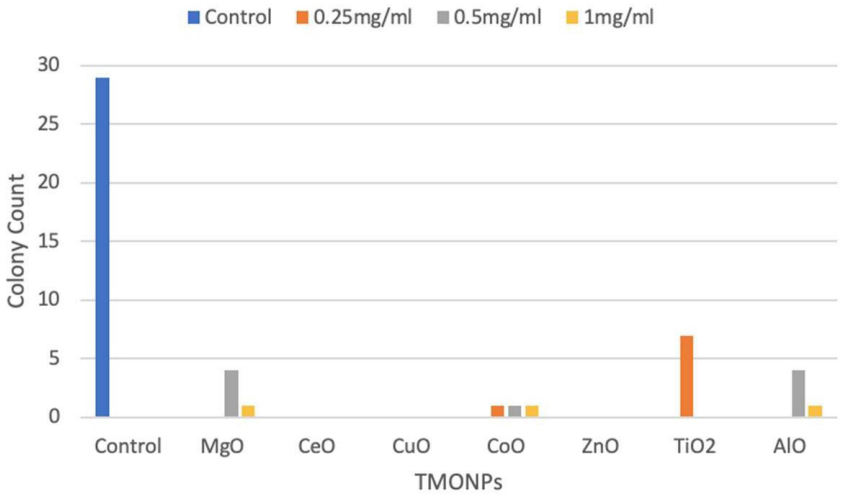

Figure 7: Colony counts of LAP growth after exposure to three concentrations (0.25 mg/ml, 0.50 mg/ml, 1.0 mg/ml) of various TMONPs for 48 hours at 25°C.

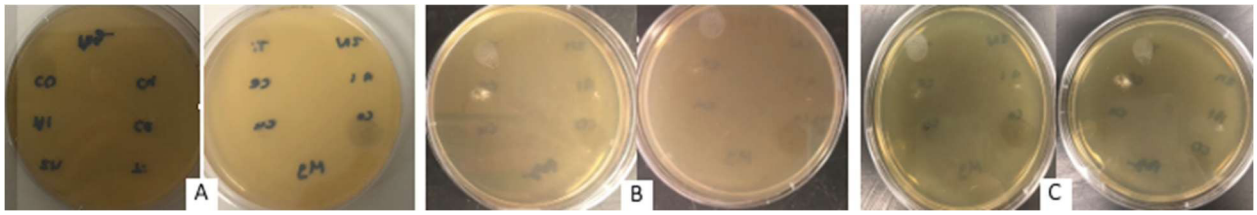

Figure 8: Spot treatment test of various TMONPs (1.0 mg/ml) on MRS plates for (A) 24 hours, (B) 48 hours, and (C) 72 hours.

Comparison with Lactobacillus Acidophilus Probiotic

By comparison, Figures 6.1-6.3 show the agar well diffusion growth on MRS plates of *Lactobacillus* acidophilus probiotic (LAP) that were exposed from zero to 48 hours to three concentrations of various TMONPs at 25°C. After 48 hours, the control displayed a good amount of bacterial growth. However, exposures of LAP to CeO_2, CuO and ZnO displayed a lack of bacterial growth. Al_2O_3, Co_3O_4 and MgO displayed a bacterial growth of several colonies only. Blatantly, TiO_2 displayed a large contamination. Figure 7 compares the colony counts of LAP growth versus the three concentrations of each TMONP tested. Essentially not much bacterial growth was developed, with all TMONP counts substantially below the control count Table 5. Compares the colony counts of LAP growth versus the zone of inhibition distances for the three concentrations of each TMONP tested. Like MgO, only Al_2O_3 and Co_3O_4 displayed some potential bacterial growth that had no contamination.

To determine if the TMONPs used were contaminated with bacteria, a spot treatment test was performed. As shown in Figure 8, the lack of bacterial growth after 72 hours of exposure on the MRS plates verified that no significant contamination of the TMONPs by alternative bacteria species was found.

Effects of 4-chlorophenol and MgO nanoparticles

For the overall growth of LAB, a lawn plate has the filtrate spread on top of the agar with a sterile spreader so colonies will grow evenly on the surface. This would display the potential of bacterial growth in normal room conditions. Figure 9 shows the control lawn plate assessment of LAB growth before and after the addition of 4-chlorophenol at three different concentrations. Bacterial growth can be seen on all plates containing LAB, decreasing slightly with increasing concentration of 4-chlorophenol.

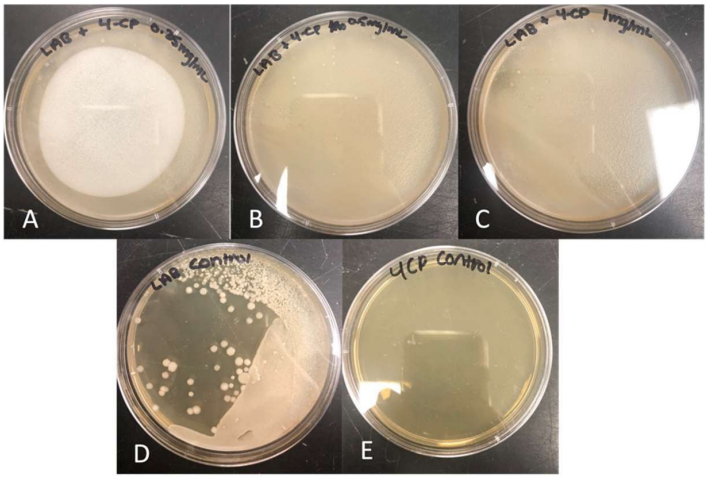

Figure 9: Lawn plate assessment on MRS plates incubated at 25°C for 48 hours: (A) LAB with 0.25 mg/ml 4-chlorophenol, (B) LAB with 0.50 mg/ml 4-chlorophenol, (C) LAB with 1.0 mg/ml 4- chlorophenol, (D) LAB control, and (E) 4-chlorophenol control.

Figure 10 shows the control lawn plate assessment of LAB growth before and after the addition of MgO nanoparticles at three different concentrations. Bacterial growth can be seen on all plates containing LAB, increasing rapidly with higher MgO concentration.

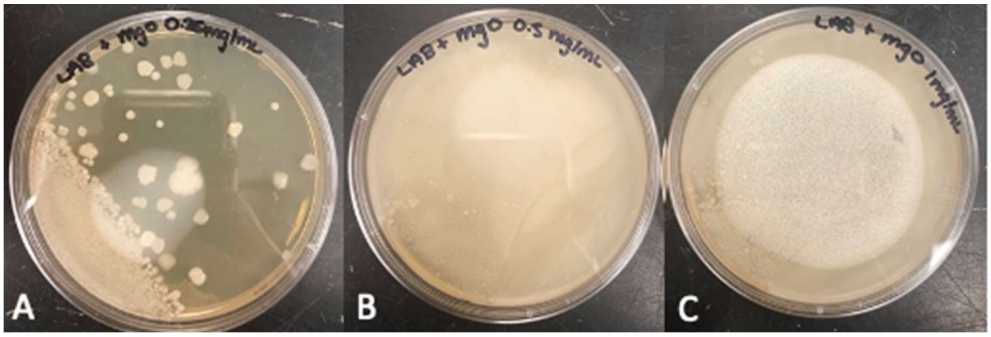

Figure 10: Lawn plate assessment on MRS plates incubated at 25°C for 48 hours: (A) LAB with 0.25 mg/ml MgO nanoparticles, (B) LAB with 0.50 mg/ml MgO nanoparticles, and (C) LAB with 1.0 mg/ml MgO nanoparticles.

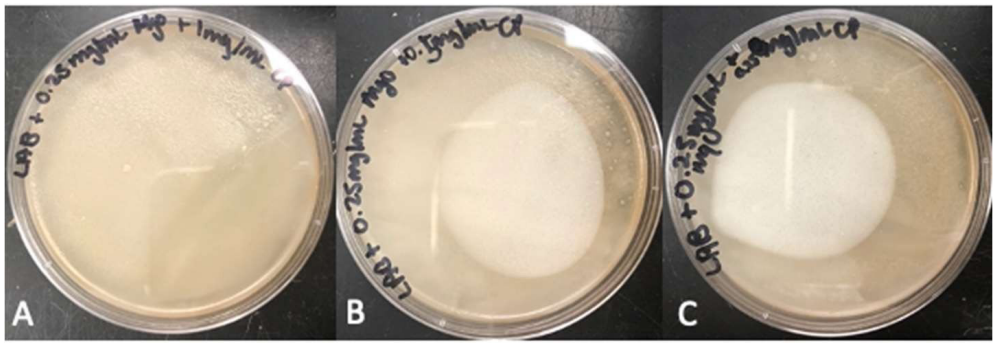

Figure 11.1: Lawn plate assessment of LAB and 0.25 mg/ml of MgO nanoparticles with different concentrations of 4-chlorophenol: (A) 1.0 mg/ml, (B) 0.50 mg/ml, and (C) 0.25 mg/ml on MRS plates incubated at 25°C for 48 hours.

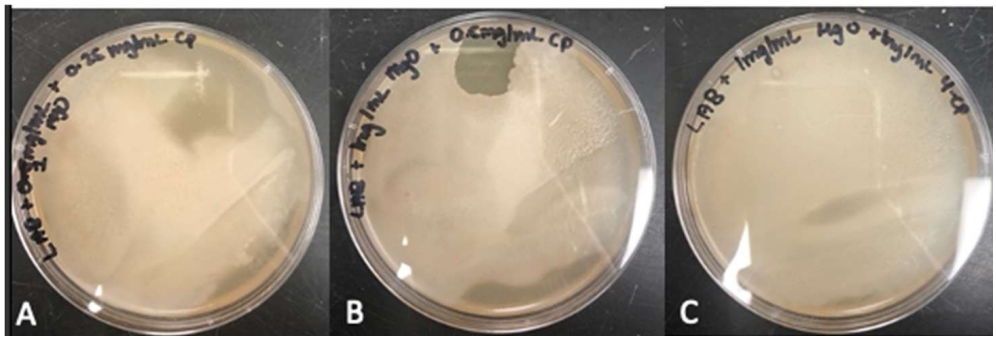

Figure 11.2: Lawn plate assessment of LAB and 0.5 mg/ml of MgO nanoparticles with different concentrations of 4-chlorophenol: (A) 0.25 mg/ml, (B) 0.50 mg/ml, and (C) 1.0 mg/ml on MRS plates incubated at 25°C for 48 hours.

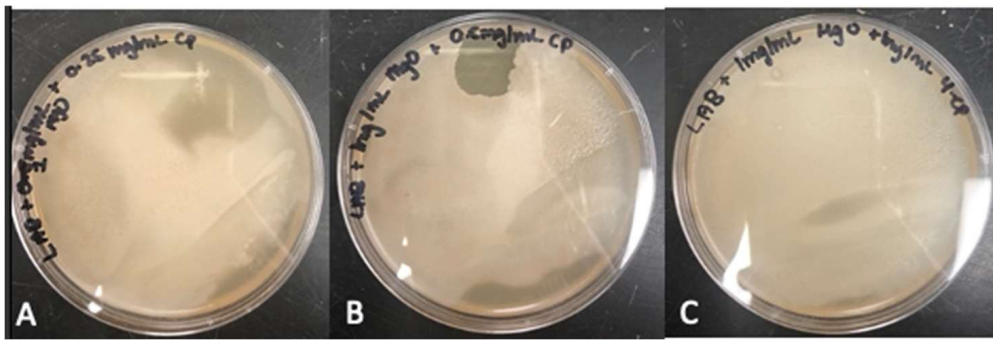

Figure 11.3: Lawn plate assessment of LAB and 1.0 mg/ml of MgO nanoparticles with different concentrations of 4-chlorophenol: (A) 0.25 mg/ml, (B) 0.50 mg/ml, and (C) 1.0 mg/ml on MRS plates incubated at 25°C for 48 hours.

Lawn plate assessments of LAB growth at 25°C for 48 hours was performed using three different concentrations of MgO nanoparticles in combination with three different concentrations of 4- chlorophenol. As shown in Figures 11.1-11.3, healthy growth of LAB could develop in all the plates.

Discussion

Turbidity Analysis and Sedimentation

Turbidity analysis provides a reading that measures the lack of clarity of a colloidal sample and indicates the presence of any suspended nanoparticles that can obstruct the incoming light via scattering. In microbiology, an increase in turbidimetric value over time is definitely an assessment of bacteria growth. Sedimentation evaluation allows for observational determination of the particle settling rate. Large sediment formation can result from the agglomeration of suspended solids; in the case of bacteria, bacterial aggregation occurs before settling to the bottom [62]. In the present study, by measuring the turbidity changes, the effects of TMONP on LAB growth could be monitored. Surely, the LAB control displayed an average reading of 173 NTU after 24 hours of incubation (see Table 3). Compared to the LAB control, all TMONP mixtures with LAB resulted in higher turbidity readings except for the CeO_2 mixture with LAB as a low outlier. Since the suspensions were allowed to settle for 24 hours, the supernatant contained both the LAB colonies and any unsettled nanoparticles remaining in suspension [63]. On the other hand, Co_3O_4, CuO and TiO_2 mixtures with LAB resulted in readings beyond the turbidimeter's upper limit of 700 NTU. For CuO and Co_3O_4 mixtures with LAB, the dark nanoparticles in suspension caused very high turbidity readings due to absorption of the light being transmitted through the sample [64]. These high outlier readings could not be used for monitoring the bacterial growth.

Antimicrobial Susceptibility

Spot plating of the LAB filtrate (see Figure 1) on an MRS plate (see Figure 3) was used to assess if there was any potential growth of the bacteria prior to its use in all subsequent experiments [60]. Bacterial growth did occur with the convex colony morphology expected for LAB, milky-white colour, and smooth texture [65]. The spot treatment test (see Figure 8) was later performed to determine if there were any bacterial contaminations from the TMONPs used. After 72 hours of incubation on the MRS plates, no bacterial growth had occurred. This indicates that bacterial contamination of the nanoparticles was highly unlikely. Next, agar well diffusion plates were created to determine the potential antibiotic effects of each TMONP applied at different concentrations against LAB over a period of 48 hours at 25°C (see Figure 4.1- 4.3). Colony counts of LAB were similar between all TMONPs with limited bacterial growth, except for ZnO and MgO nanoparticles (Table 4). This was expected, as TMONPs had been considered bactericidal since they demonstrated the potential to kill various types of bacteria [66]. In contrast, all concentrations of ZnO nanoparticles with LAB displayed relatively high colony counts due to the number of single colonies formed around the wells (see Figure 4.3). Due to their tiny size and transparency, these single colonies could not be specified as LAB only. Interestingly, all concentrations of MgO nanoparticles with LAB exhibited very rapid bacterial growth with no inhibition zone. Lawn plate assessment was performed as well to verify that MgO nanoparticles did not inhibit the growth of LAB at all (see Figure 10). This finding can be explained by the significant importance of magnesium as a nutritional element that specific cell membrane proteins transport across the cell membrane for use in enzymatic reactions. Without its presence, cell growth would cease and apoptosis could occur [67]. Actually, LAB produces a bacteriocin known as nisin and is often used in agriculture for better soil maintenance. MgO also exhibits inhibitory effects and prevent pathogenic bacterial growth. Synergistic effects of nisin and MgO have been reported to yield great results for causing pathogenic bacterial cell death [68]. This finding is indeed very encouraging, consistent with the previous report that significant and positive influence was achieved by MgO nanoparticles on macronutrients like nitrogen, phosphorus and potassium in the growth and development of cotton plants [69]. Reportedly, MgO nanoparticles have a huge potential to increase the productivity of crops if applied in optimal concentrations [70].

The Agar well diffusion plate experiment was conducted on LAP (see Figures 6.1-6.3) to assess the antibacterial effects of TMONPs. There was no bacterial proliferation as excessive as that observed above for LAB with MgO nanoparticles (see Table 5). This could either be due to the low concentration of LAP in the initial stock, or the high antibacterial effects of TMONPs on this specific strain of bacteria. It had been indicated in previous studies that only 4% of the active 10% LAB were considered to have strong colony growth activity.

Last, LAB colony growth was assessed on lawn plates in the presence of MgO nanoparticles (see Figures 11.1-11.3). The bacterial growth was not affected by the three concentrations of 4-chlorophenol added. Apparently, the toxicity of 4-chlorophenol to LAB growth was ameliorated in the presence of MgO nanoparticles. This may be explained by 4- chlorophenol being used as a potential energy source for the growth of LAB. Control experiments for LAB and 4-chlorophenol were performed separately, and no deviation results were seen (see Figure 9). Further studies would be required to determine the potential biodegradation of 4- chlorophenol by LAB.

Conclusion

The goal of this research was to determine the antimicrobial effect of TMONPs and the toxic effect of 4-chlorophenol on LAB growth. It is evidenced that the low TMONP concentration (1 mg/ml) caused a measurable growth inhibition. In contrast, MgO nanoparticles promoted a rapid growth of LAB. The chemical toxicity of 4-chlorophenol proved to have little effect on the bacterial growth. Combination of 4-chlorophenol with MgO nanoparticles did not display any inhibition of the rapid LAB growth.

Potential directions of our future study include performing 16SrRNA gene analysis to determine the specific lactic acid

bacterial strains prepared in the LAB filtrate and proliferating on the MRS plates. In terms of chemical toxicology, environmental contaminants such as quinolone antibiotics can be assessed at relevant concentrations for their impact on LAB growth in the presence of TMONPs. Better utilization of MgO as a nano-fertilizer can go a long way towards agronomic efficiency despite all organic contaminants in soil.

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