Impact of the Ingested Engineered Nanomaterials TiO_2, SiO_2, and Ag on Intestinal Barrier Function in Relation to Inflammatory Gut Diseases

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I dedicate this thesis to my parents

This thesis is dedicated to my parents Ghirmai and Natsnet. This accomplishment would not be possible without their sacrifice and tireless work ethic. Having endured many challenges, they dreamed of a better life and moved us to Canada, forever shifting the course of our lives. I am indeed grateful for their unconditional support and loving prayers throughout all my academic endeavours. Their lives inspire me every day and give me the courage to dream big.

ABSTRACT

Nanoparticles (NPs) are clusters of atoms with at least one dimension below 100 nm. Their high surface area to volume ratio gives them unique properties that are utilized by food and medical industries. Unfortunately, it has been shown that NPs can alter various compartments of human gut intestinal barrier function (IBF), including the epithelial layer, potentially leading to inflammatory bowel disease (IBD). Despite this potential threat to gut homeostasis, humans remain chronically exposed to NPs through ingestion of food additives. Silicon dioxide (SiO_2), titanium dioxide (TiO_2), and silver (Ag) NPs are used in the food additives E551, E171, and E174, respectively. SiO_2 NPs provide anti-caking properties in powdered food products, Ag NPs provide antimicrobial and anti-odorant properties in food and packaging materials, and TiO_2 NPs have optical properties that lighten foods. The rise of human consumption of NPs and increased incidence and prevalence of IBD warrants further investigation on the effects of food NPs on IBF. This study investigates the impact of TiO_2, SiO_2, and Ag NPs on the intestinal epithelial barrier and discusses the implications for gut health and disease. First, NPs are taken through an in vitro simulated digestion process and incubated with the human colon carcinoma HT29 cell line, a common model of the human intestinal epithelial layer. The impact of NPs on cell viability and PLK1 protein expression are determined to elucidate any changes to gut barrier functioning. The current study follows the recommendations of The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) by investigating physiologically relevant doses of NPs and properly characterizing all NPs used. Studies herein demonstrate that NPs cause an increase in PLK1 protein expression in vitro but have little impact on cell viability. This may be used to elucidate mechanisms of NP-induced changes to gut barrier functioning and aid interventions for the treatment of related chronic inflammatory gut diseases.

RESUME

Les nanoparticules (NP) sont des amas d'atomes dont au moins une dimension est inférieure à 100 nm. Leur rapport surface/volume élevé leur confère des propriétés uniques qui sont utilisées par les industries alimentaires et médicales. Malheureusement, il a été démontré que les NP peuvent altérer divers compartiments de la fonction de barrière intestinale (IBF) de l'intestin humain, y compris la couche épithéliale, pouvant conduire à une maladie inflammatoire de l'intestin (MII), notamment le syndrome de l'intestin irritable (IBF). Malgré cette menace potentielle pour l'homéostasie intestinale, les humains restent exposés de manière chronique aux NP par l'ingestion d'additifs alimentaires. Les NP de dioxyde de silicium (SiO2), de dioxyde de titane (TiO2) et d'argent (Ag) sont utilisées dans les additifs alimentaires E551, E171 et E174, respectivement. Les NP de SiO2 offrent des propriétés anti-agglomérantes dans les produits alimentaires en poudre, les NP d'Ag offrent des propriétés antimicrobiennes et anti-odeurs dans les aliments et les matériaux d'emballage, et les NP de TiO2 ont des propriétés optiques qui éclaircissent les aliments. L'augmentation de la consommation humaine de NP et l'augmentation de l'incidence et de la prévalence des MII justifient une enquête plus approfondie sur les effets des NP alimentaires sur l'IBF. Cette étude examine l'impact des NP de TiO2, SiO2 et Ag sur la barrière épithéliale intestinale et discute des implications pour la santé et les maladies intestinales. Tout d'abord, les NP sont soumises à un processus de digestion simulée in vitro et incubées avec la lignée cellulaire HT29 du carcinome du côlon humain, un modèle courant de la couche épithéliale intestinale humaine. L'impact des NP sur la viabilité cellulaire et l'expression de la protéine PLK1 est déterminé pour élucider tout changement dans le fonctionnement de la barrière intestinale. L'étude actuelle suit les recommandations du groupe scientifique de l'EFSA sur les additifs alimentaires et les sources de nutriments ajoutés aux aliments (ANS) en étudiant

les doses physiologiquement pertinentes de NP et en caractérisant correctement toutes les NP utilisées. Les études ici démontrent que les NP provoquent une augmentation de l'expression de la protéine PLK1 in vitro mais ont peu d'impact sur la viabilité cellulaire. Cela peut être utilisé pour élucider les mécanismes des changements induits par les NP dans le fonctionnement de la barrière intestinale et faciliter les interventions pour le traitement des maladies intestinales inflammatoires chroniques associées.

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PREFACE

This manuscript-based thesis has been prepared in accordance with the McGill University thesis preparation and submission guidelines. The experimental section is thus written in the form of original research papers suitable for publication which can be found in chapters 3-6 of this thesis. Each original chapter contains an abstract, introduction, materials and methods, results, discussion, conclusions, and acknowledgement section. The report is also supported by an overall abstract, general introduction, literature review, discussion, conclusions, and recommendations and future applications.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 The use of Nanoparticles in the food industry and potential toxicity

Nanoparticles (NPs) are clusters of atoms with at least one dimension below 100nm in size. NPs have unique properties that are widely utilized by a variety of industries. For example, nanomaterials such as montmorillonite (MMT), zinc oxide (ZnO NPs) coated silicate, kaolinite, silver NPs (Ag NPs) and titanium dioxide (TiO_2 NPs) are used in food packaging [1]. NPs are also highly important in the advancement of drug delivery systems as well as food production [2]. The food industry uses NPs to make additives that generate colours and flavours, improve texture, and even provide antimicrobial properties [3]. Unfortunately, the ingestion of NPs has been linked with the disruption of various components of the human gut intestinal barrier [2]. For instance, the food additive E171 contains titanium dioxide (TiO_2) NPs and was recently deemed as unsafe for use as a food additive due to increasing evidence of its adverse effects systemically [4]. These include concerns regarding genotoxicity, DNA damage, accumulation in tissues, inflammation, dysbiosis, and worsening of a leaky gut in those with pre-existing conditions. Another food additive referred to as E174 contains silver (Ag) NPs known to be toxic to aquatic life but considered safe for use in foods. It's safety is difficult to fully determine due to a lack of the characterization of the food additive in terms of its size distribution [5]. There is data however to suggest potential for cytotoxicity, induction of oxidative stress, inflammatory response, and dysbiosis. Finally, the food additive E551 contains silicon dioxide (SiO_2) NPs which have not been studied sufficiently, in fact the acceptable daily intake (ADI) has yet to be determined [6].

1.2 Intestinal barrier dysfunction, related diseases, and the involvement of nanoparticles

The gastrointestinal tract (GIT) is uniquely positioned between the external and internal environment of the human body. Its ability to maintain controlled interactions between human

tissues and the outside contents sitting in the lumen is essential in protecting the body from harmful substances consumed by the individual [7]. This function of the GIT is defined as the intestinal barrier function (IBF). This IBF consists of four components: the intestinal microbiota, mucus layer, epithelium, and immune system. Dysfunction in the intestinal barrier is linked to many diseases including those which are inflammatory, metabolic, infectious, autoimmune, and even neurologic. While these diseases have complex aetiology involving genetic and psychological factors, these are insufficient to fully explain the increasing occurrence of gut diseases. Environmental factors also play a role, in fact it has recently been postulated that the ingestion of food additives through diet may pose a risk to IBF potentially leading to exacerbation or development of disease. Specifically, NPs have the potential to promote dysbiosis of the gut microbiome and disrupt tight junction protein organization [3]. Such alterations may lead to increased intestinal permeability allowing for the translocation of pathogenic bacteria and other contaminants through the gut barrier, resulting in an inflammatory response. NPs have also been shown to enter epithelial cells, accumulate in endosomes, induce endoplasmic reticulum stress, increase the release of reactive oxygen species, and induce mitochondrial dysfunction. Therefore, NPs are implicated in gut barrier dysfunction potentially leading to inflammatory diseases of the gut.

1.3 Potential use of probiotics for nanoparticle-induced intestinal barrier dysfunction

Probiotics may be used to protect against intestinal barrier dysfunction potentially induced by NP ingestion. NP-induced alterations to the gut microbiota and intestinal barrier have the potential to lead to various inflammatory diseases which are increasingly common [8]. Probiotics such as *Lactobacillus fermentum* have been shown to ameliorate intestinal barrier disruption [9]. Further systematic research must be done to fully determine the impact of human

chronic exposure to NPs and elucidate their exact role in the disruption of IBF in relation to inflammatory gut diseases. Such findings have the potential to facilitate the development of novel treatment strategies for NP induced gut damage. One of which is the development of a novel probiotic formulation targeted to reverse the potentially negative effects of NPs on IBF.

While *in-vivo* experiments provide important information on the toxicity of NPs at the organism level, studies completed in vitro using established cell lines such as the colon carcinogenic HT29 cell line are invaluable for elucidating the specific mechanism of action of NPs and their toxicity [7]. This thesis involves a comprehensive analysis of the effects of the engineered nanomaterials (ENMs) TiO_2, SiO_2, and Ag on an in vitro model of the human intestinal epithelial layer.

1.4 Research Hypothesis:

NPs may disrupt intestinal epithelial barrier functioning. The presence of the probiotic *Lactobacillus fermentum* NCIMB 5221 (Lf5221) may alter the impact of NPs on intestinal epithelial cells. Findings have the potential to inform the design of NP-associated health care measures.

1.5 Research Objectives

This thesis aims to understand the impact of NPs on gut epithelial cells to provide knowledge towards the design of NP associated health care measures. To this end, the human colon carcinogenic HT29 cell line is used to evaluate the impact of NPs on gut IBF. The present work is designed to include characterization of all NPs used in accordance with The European Food Safety Authority (EFSA) panel on Food Additives and Flavourings (FAF). The present research objectives are:

1. To investigate the impact of NPs on gut epithelial cells in relation to human intestinal barrier dysfunction and related diseases.
2. To evaluate intestinal epithelial cell viability and PLK1 protein expression in vitro in response to TiO2 NP treatment at varied doses relevant to the currently reported human exposure levels.
3. To evaluate the dose-dependent effects of SiO_2 NPs on intestinal epithelial cell viability and PLK1 protein expression in relation to gut barrier functioning, in vitro.
4. To measure the impact of Ag NPs at different concentrations using an intestinal in vitro model in relation to cell viability and PLK1 protein expression.
5. To investigate the potentially protective effect of the bacterial probiotic Lf5221 on intestinal epithelial cell viability in the presence of NPs, in vitro.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to nanoparticles and human exposure

2.1.1 Titanium dioxide nanoparticle use in foods

Nanoparticles are clusters of atoms of a single element with sizes below 100nm. TiO_2 NPs have been utilized by the food industry to make food additives which have raised human exposure levels through ingestion. The food additive E171 has been authorized in 48 food categories including 'soups and broths', 'seasonings and condiments', and 'flavoured drinks' [4]. Research has been conducted regarding the occurrence of E171 across 16 of these food categories, and it was found that 32–96% (by weight) of these foods potentially contained E 171. This was used in estimating the human dietary exposure to E171 from its use as a food additive. The maximum exposure estimated for children (3-9 years) was 1.9-11.5 mg/kg body weight (bw)/day. The 95^{th} percentile ranged from 5.9-31.3mg/kg bw/day.

In addition to exposure through consumption of the food additive, humans are also exposed to E171 through medicinal products [4]. However, the level of use of E171 in medicinal products is unknown making it difficult to determine exposure levels. Similarly, exposure to E171 through cosmetics such as toothpaste is also possible, but the estimations for exposure to this have also not been made. Humans are chronically exposed to TiO_2 NPs through means beyond just foods, making exposure levels likely higher than that proposed by current exposure reports.

It is important to note that different kinds of E171 are used in foods which incorporate different amounts of TiO_2 NPs, and therefore it is not possible to precisely determine the exposure to TiO_2 NPs from the use of E171. It can be postulated, however that less than 50% (by number) of the particles in E 171 exist as NPs (minimum external dimension < 100nm) [10].

According to the Regulation EU (No) 231/2012, the content of NPs in E171 is unlimited [10]. Additionally, pristine TiO_2 NPs found in E171 can form agglomerates but can also become deagglomerated depending on the conditions in the food where they are found as well as in the GIT when they are consumed.

2.1.2 Human exposure to silicon dioxide nanoparticles through foods

SiO_2 NPs have been used by the food industry to make food additives like E551 that have been increasingly consumed by humans throughout their lifespan. The food additive E551 can be found in foods like ripened cheese, sugars and syrups, and fine bakery wares. The EFSA Comprehensive European Food Consumption Database (Comprehensive Database) collects nationwide food consumption data. An exposure assessment was done using this database for various population groups (infants, toddlers, children, adolescents, adults, and the elderly) representing 33 dietary surveys completed in 19 European countries. SiO_2 is authorized for use in 22 food categories except for foods made for infants and children for which there are further restrictions on processing and physical properties. 15 of these food categories were considered in the estimation of human exposure to E551 from its use as a food additive. Specifically, the concentrations of SiO_2 (E 551) found in the food category was multiplied by the associated consumption of that food. It was found that children (3-9 years) are exposed to a maximum of 10.2-31.2 mg/kg bw per day, and the 95^{th} percentile ranged from 25 to 79.2 mg/kg bw per day. Beyond the consumption of E 551 through its presence as a food additive, humans are also exposed through its use in food supplements as a source of silicon, and foods for special medical purposes (FSMP). However, exposure estimates were not determined for these exposure scenarios. Finally, E551 can be found in cosmetic products as well as in drugs as aids for the drug manufacturing process.

2.1.3 Silver nanoparticles in the food industry

Ag NPs are used in the food additive E174 which has been authorized for use in three food categories; 'other confectionary' (includes breath-freshening micro-sweets), 'decorations, coatings and fillings', and 'liquor'[5]. Using the EFSA comprehensive database, food consumption data was gathered from 33 different dietary surveys carried out in 19 European countries. The consumption data did not account for E174 found in liqueurs, so this was not included in the E174 exposure estimates. Certain products containing Ag NPs were also not consumed regularly and so the panel estimated a consumption of 10 times per year. Exposure assessments were made based on maximum reported use levels or reported use levels. The former is defined as the maximum level exposure assessment scenario, and this was deemed to be the most conservative estimate considering that humans are exposed to E174 over their lifetime. Given these limitations, the exposure estimates are likely an underestimation of true human exposure. Nevertheless, the estimates were calculated for different population groups including toddlers, children, adolescents, adults, and the elderly. The mean calculated using the maximum reported use levels for the children population was 0.22–2.6 μg/kg body weight (bw)/day and the 95th percentile exposure was determined to be 1.1–12.0 μg/kg body weight (bw)/day.

The total dietary exposure to Ag originating from E174 is only 30% of the total dietary exposure to Ag [5]. Beyond the food additive E174, trace elements of Ag can be found in seafood species, however the majority (82%) has levels below the limit of detection. Molluscs, crustaceans, and offal were found to contain the highest concentrations of Ag. Adults were found to be exposed to 1.29-2.65 μg/kg bw/day, and the 95th percentile of exposure levels was 2.82-4.78 μg/kg bw/day. In children, the mean exposure was found to be higher at 1.60-3.47 μg/kg

bw/day. Molluscs and crustaceans were the main contributors for adults, whereas milk and water were the main contributor for children.

2.2 The impact of nanoparticles on intestinal barrier function in relation to gut diseases

Having established human exposure to NPs through foods, it is now important to consider their effects on IBF. Every component of the intestinal barrier plays a key role in maintaining homeostasis, and it is these components which food NPs have the potential to impact, thereby affecting human health and disease. The mucus layer is important for immobilizing larger unwanted particles including bacteria [11]. The epithelial layer contains villi and microvilli which serve to increase surface area for enhanced absorption [12]. The epithelial layer is made up of specialized cells like goblet cells, responsible for secreting mucus, as well as M-cells, which transport material from the lumen across the epithelial barrier. Any impact NPs may have on the components of the intestinal barrier, such as villus structure or mucus secretion, has the potential to disrupt homeostasis, possibly leading to pathology. Here, various studies on the effects of NPs on the components of the gut intestinal barrier are summarized.

2.2.1 Silver nanoparticle effects on gut barrier function

Ag NPs have the potential to disrupt the intestinal barrier towards inflammatory diseases of the gut. One marker of barrier integrity is the expression patterns of mucin genes as they are often aberrant in various pathologies [13]. MUC genes encode members of the gel-forming mucin protein family which are secreted into the mucus layer [14]. In fact, it has been shown that gel forming mucins play a part in epithelial wound healing and protection, specifically in patients with inflammatory bowel diseases. In one study, Ag NP administration did not affect MUC2 but decreased MUC3 gene expression in the ileum most prominently in female rats [15]. The

microbial recognition toll-like receptor genes TLR2 (in male rats), TLR4 (in female rats), and Nucleotide Binding Oligomerization Domain Containing 2 (NOD2) were downregulated depending on the dose given and sex of the rats [15]. T-cell regulatory genes (FOXP3, GPR43, IL-10, TGF-beta) decreased in expression particularly at low and medium doses [15]. Authors also noted that the observed changes in genetic expression seemed to depend more on Ag NP interactions based on their dose and size rather than their release of ions [15]. Others analyzed fecal microbiota using a smaller size and dose of Ag NPs (12nm, 2.5 mg/kg bw/day) administered to male mice via oral gavage for 7 days [16]. Ag NP treated mice exhibited colitis-like symptoms such as increased disease activity index, histological scores, intestinal epithelial microvilli, tight junction disruption, and increased pro-inflammatory cytokines [16]. Ag NPs can lead to accelerated cell death, increased intracellular reactive oxygen species (ROS) and decreased cellular activity [17]. They have also been shown to cause oxidative DNA damage [18], and increased pro-inflammatory chemokine interleukin-8 (IL-8) secretion [19]. Goblet cell release of mucus granules and abnormal mucus composition has also been shown in response to Ag NP treatment [20].

In contrast, other studies have shown that Ag NPs have little to no impact on IBF. One study using histological analysis did not find any intestinal damage or structural alterations in ileal villi, goblet cells, and the glycocalyx across all groups treated with Ag NPs [21]. Similarly, another study showed that Ag NP (14nm) treated rats experienced no toxicological effects [22]. Furthermore, researchers have shown that Ag NPs can positively impact the GI tract [23]. The researchers first achieved reproducible colitis in mice which showed increased macro- and microscopic damage scores [23]. Administration of Ag NP2 (500 mg/dm3, 100 μl/ animal, once daily) significantly decreased the total macroscopic score effectively attenuating dextran sulfate

sodium (DSS)-induced colitis [23]. In this study, Ag NP1 (500 mg/dm3, 100 µl/animal, i.c., once daily) non-significantly decreased the macroscopic score and significantly reduced the colon damage score [23]. Additionally, microscopic damage (i.e. loss of mucosal architecture, presence of crypt abscesses, and extensive cellular infiltration) that was observed in DSS-treated mice was alleviated after treatment with NPs [23]. The study even showed that NPs alleviated colonic injury in a mouse model mimicking Crohn's disease [23].

2.2.2 Silicon dioxide nanoparticle effects on gut health

SiO_2 NPs have been authorized for use in foods, however they have the potential to damage the intestinal epithelial layer, potentially leading to inflammation. SiO_2 NP-treated mice exhibited significant increases in pro-inflammatory cytokines in the small bowel and the colon [16]. This was confirmed by H and E staining in mice after NP ingestion which revealed severe destruction of the epithelial layer and loss of crypts in colon segments [16]. Moreover, another study orally exposed rats to 100, 1000 or 25000 mg/kg bw/day of synthetic amorphous silica (SAS) or 100, 500 or 1000 mg/kg bw/day of NM-202 (a nanostructured silica) to evaluate the impact on the gut [24]. Elevated tissue silica levels were reported only after 84 days of exposure to SAS accumulating in the spleen [24]. Moreover, after 84 days, liver fibrosis was observed indicating potential long-term effects [24]. SiO_2 NPs have also been shown to cause destruction of the epithelial layer and crypts in colon segments as well as increased pro-inflammatory cytokines [16]. Additionally, they can activate the caspase-1 inflammasome and cause release of IL-1β in macrophages [8, 25]. Additionally, researchers have noted increased major histocompatibility complex II (MHC-II) and cluster of differentiation antigens (CD80 and CD86) on dendritic cells treated with SiO_2 along with increased apoptosis. Such studies indicate there may be negative effects associated with the consumption of SiO_2 NPs.

By contrast, other studies have found silica to have no effect on the GIT. Colloidal silica particles, differing in size (20 nm and 100 nm), were orally administered to Sprague-Dawley rats [26]. A ninety-day repeated dose (2000 mg/kg, 1000 mg/kg or 500 mg/kg) study was conducted [26]. There were no clinical changes, toxic effects, or histopathological findings in any of the rat groups [26]. Similarly, researchers orally administered 2.5 mg/day of amorphous silica NPs to mice, for 28 days, of different diameters and surface properties (70, 300 and 1000 nm) [27]. The three NPs were absorbed in the intestine to different degrees indicating that particle diameter and surface properties are determinants [27]. Moreover, after 28 days, there was no significant difference in hematological, histopathological and biochemical properties in the control mice and mice given silica [27]. These studies thus suggest that silica NPs are safe for food production.

2.2.3 The role of titanium dioxide nanoparticles in gut health and disease

TiO_2 NPs negatively affect IBF, specifically the gut epithelial barrier and immune system. One study showed rutile NPs (a crystalline phase of TiO_2 NPs) increased the length of intestinal villi and caused irregular arrangement of epithelial cells [28]. Of note is that this study used human exposure relevant doses on mice for 28 days [28]. The findings were more pronounced with the use of rutile NPs as compared to anatase NPs [28]. In another study, rats were given 10mg/kg bw/day of food-grade TiO_2, an approved white pigment in Europe, orally for 7 days [29]. Researchers observed intestinal inflammation, preneoplastic lesions and growth of aberrant crypt foci just 100 days after treatment. Researchers noted this indicates an increased risk for IL-17-producing T helper cell (Th17)-driven autoimmune diseases and colorectal cancer [29]. In this way, NPs can negatively impact the health of individuals that are chronically exposed [29]. Studies have also reported epithelial injury, reduced tight junction protein expression, and reduced luminal mucus layer thickness as a result of exposure to TiO_2 NPs [30].

Others have reported disruption of microvilli organization [31] as well as reduced colon crypt length and increased macrophages and inflammatory markers [32]. TiO_2 NPs also have also been shown to induce intestinal tumour formation in rats exposed to carcinogens [33]. Moreover, research has also shown that TiO_2 NPs may lead to inflammation [30, 33-36]. TiO_2 NPs can also induce altered tight junction protein expression and increase paracellular permeability by upregulating various efflux pumps and nutrient transporters [37, 38]. These results are concerning since the use of TiO_2 NPs is very popular in candies which is most consumed by children.

Various studies have also investigated the gut microbiome of mice treated with E171 and TiO_2 NPs finding that microbial composition was altered after treatment. One study administered E 171 (0, 2, 10 and 50 mg/kg bw per/day) for 3 weeks finding that their fecal and small intestinal microbiota were unchanged but the release if bacterial metabolites was impacted [39]. By contrast, studies on TiO_2 NPs (25 nm, 50 nm, 80 nm) administered over 7 days at a dose of 1 mg/kg bw per day showed that gut microbial composition was altered [30]. This change in composition was related to physical changes in the distal gut such as epithelial injury, reduced tight junction protein expression, and reduced mucus layer thickness. A more long term 28-day study administered TiO_2 (250 nm) or TiO_2 NPs (25 nm) to mice at doses of 10, 40 and 160 mg/kg bw per day and demonstrated changes in gut microbial composition, particularly those associated with the mucosal barrier [40]. Therefore, it is clear that TiO_2 can alter the composition of the gut microbiome in mice, however this may not always translate into functional differences capable of translating into gut diseases. In fact, the EFSA panel also concluded that there is no agreement whether changes to gut microbial composition should be considered adverse [4].

2.2.4 Iron oxide nanoparticle effects on gut health

Fe_2O_3 NPs have the potential to negatively affect gut epithelial cell morphology towards increased barrier permeability. Researchers investigated iron oxide NPs in food and how their consumption impacts gut morphology in the Bombyx mori silkworm [41]. B. mori were fed 0.3%, 1.5%, and 3% by weight of the iron oxide NPs and fixed with staining for analysis [41]. Results showed morphological changes in the gut including increased amounts of goblet cells for the 1.5% treatment group [41]. In those fed 1.5% NPs, there was pseudostratified epithelium in the gut lining and a loss of goblet cells [41]. Finally in those treated with 3% NPs, the epithelial cells were irregularly distributed and there was apoptosis resulting in increased intracellular space [41].

Fe_2O_3 NPs have also been shown to have no negative effects according to one study. Here, researchers assessed both iron oxide and SiO_2 NPs in Sprague-Dawley rats. One group was orally administered 244.9, 489.8 and 979.5 mg/kg SiO_2 NPs that were 12nm and spherical in shape [42]. Another group received 1030.5 mg/kg Ag NPs and 1000 mg/kg Fe_2O_3 NPs. In this 13-week repeated toxicity study, the SiO_2 and iron oxide were not associated with systemic toxicity or any changes in hematological, serum biochemical, or histopathological lesions [42]. However, the same study showed that Ag NPs increased serum alkaline phosphatase, calcium and lymphocyte infiltration in the liver and kidney [42]. This indicates a potential for Ag NPs to cause systemic toxicity due to its systemic distribution but not SiO_2 and Fe_2O_3 NPs [42]. The toxicity assessments were done according to the Organization for Economic Cooperation and Development (OECD) test guideline 408 [42].

2.2.5 The impact of zinc oxide nanoparticles on gut intestinal lining

It has been shown that ZnO NPs can positively affect IBF. One study compared the effects of 600 mg/kg and 2000 mg/kg ZnO NPs on piglets for 14 days [43]. Antioxidant enzyme

(Cu-Zn superoxide dismutase, glutathione peroxidase) and tight junction protein mRNA expression (zonula occludens protein-1, and occluding) increased in both nano and traditional ZnO treatment groups compared to controls [43]. However, the ZnO NP treatment group had lower expression than the traditional group [43]. Thus, the effect of weaning stress on piglets seems to be better alleviated by traditional ZnO than by the lower dose of Nano-ZnO [43]. The mRNA expression of cyclin-dependent kinase-4 (CDK-4) increased and Caspase3 decreased in both groups compared to controls. However, Nano-ZnO had lower CDK-4 expression compared to the traditionally treated group [43]. CDK4 is a marker for proliferation and Caspases are proteins involved in apoptosis meaning that both nano and traditional ZnO treatments promote proliferation and inhibit apoptosis in enterocytes [43]. Jejunal villus height and the ratio of villus height to crypt depth were unchanged in the nano-ZnO group compared to controls. However, this increased significantly in the traditionally fed group compared to the controls [43]. Crypt depth did not change across all groups [43]. Taken together, this suggested that Nano-ZnO can improve the morphology of the jejunum just as traditional high doses of ZnO can [43]. In another study, researchers green synthesized ZnO NPs from P. tenuifolia root extract and showed them to have antioxidant and anti-inflammatory effects [44]. This shows that there may be natural ways of producing NPs such that they become beneficial and not harmful.

2.2.6 Nanoparticles interact with the immune system to affect intestinal barrier function

Based on the literature, NPs can negatively impact GI health via interaction with the immune system. For instance, a study showed that TiO_2 and SiO_2 NPs caused upregulation of MHC-II, CD80, and CD86 on dendritic cells. The NPs also activated IL-1β-secretion in wild-type (WT) but not Caspase-1-deficient mice [45]. Researchers concluded that silica NPs induced apoptosis and TiO_2 NPs increased ROS production [45]. Interaction with immune cells allows

NPs to alter the secretion of inflammatory cytokines leading to inflammatory diseases of the gut [45]. Another study orally treated rats with food-grade TiO_2 for 7 days, the rats showed decreased levels of T-helper interferon-gamma secretion and increased occurrence of Th1/Th17 inflammatory responses [29]. This was related to induction of autoimmune diseases and colorectal cancer [29].

The literature also shows that NPs can have positive effects on IBF through interaction with the immune system. For instance, mRNA expression of IFN-γ, IL-1β, TNF-α and NF-κB was reduced in piglets treated with ZnO NPs [43]. Thus, ZnO NPs are capable of downregulating proinflammatory cytokines thereby alleviating weaning induced inflammation in piglets [43]. Thus, NPs contribute to disease pathogenesis through interaction with the immune system as well. More research should be done to clarify the relationship between the impact of NP on the immune system in relation to disease pathogenesis.

2.2.7 Overall summary of the impact of nanoparticles on intestinal barrier function

Taken together, the current literature presents conflicting data regarding the impact of inorganic NPs on various components of the intestinal barrier. ZnO was found to have positive effects on the gut while all other inorganic NPs discussed showed the potential to negatively impact IBF. Ag NPs reduced mucus secretion and negatively impacted the intestinal epithelial microvilli and tight junctions leading to colitis like symptoms in mice [15, 16]. In stark contrast, other studies showed that Ag NPs do not damage the intestinal barrier and in fact reduced colon damage scores in mice [22, 23]. SiO_2 NPs led to the destruction of the intestinal epithelial layer and liver fibrosis [16, 24]. However, other studies showed SiO_2 NPs have no impact on the gut deeming them safe for use in foods [26, 27]. TiO2 NP treatment led to irregular arrangement of epithelial cells, increased length of intestinal villi, intestinal inflammation, and preneoplastic

lesions [28] [29]. Fe_2O_3 NPs altered the amounts of goblet cells, caused irregular distribution of epithelial cells, and increased apoptosis resulting in more intracellular space [41]. By contrast, another study declared that Fe_2O_3 NPs did not cause any histopathological lesions [42]. Finally, ZnO NPs increased tight junction protein expression, inhibited apoptosis in enterocytes, and did not alter jejunal villus height and crypt depth [43]. Similarly, in a study of green synthesized ZnO NPs, antioxidant and anti-inflammatory effects were observed [44]. It is important to note however that there is a lack of studies on this, specifically on ZnO NP effects on the gut. Overall, there is a clear disagreement in the literature regarding the harmful nature of inorganic NPs as some studies report damage and others don't. This is likely due to differences in the size and dose of NPs used in each study as well as the physicochemical properties of such NPs at the time of interaction with the model used in each study. Due to these differences, it is clear more studies need to be done to elucidate the specific mechanisms through which NPs elicit their effects on the components of the intestinal barrier towards dysfunction. This must involve consideration of experimental design, NP physicochemical characteristics, size, use of dispersant, and dose as these have been shown to impact toxicity.

2.2.8 The relationship between intestinal barrier dysfunction and inflammatory bowel disease

The negative effects of NPs on IBF cannot be taken lightly due to the strong relationship between intestinal barrier dysfunction and IBD. The dysregulation of Tight junction and pore pathway proteins may play a pathogenic role in IBD. Claudin-2 expression and IL-13 production are increased in patients with Crohn's disease and ulcerative colitis [46-48]. Studies suggest that IL-13 induces claudin-2 expression allowing for the activation of the claudin-2-dependent pore-pathway making the barrier more permeable and leading to apoptosis as well as inhibition of wound healing. This is not however always associated with increased permeability [49].

Additionally, tumor necrosis factor-α (TNF) negatively regulates tight junction function by removing occludin from tight junctions leading to increased epithelial barrier permeability [50]. Patients with active IBD have altered tight junction functioning and protein composition [51]. Accordingly, it has been shown that anti-TNF antibodies can ameliorate Crohn's disease severity and reverse intestinal barrier dysfunction [52]. Furthermore, some studies have linked the loss of tight junction barrier integrity with experimental colitis [53, 54]. Therefore, the observed impact of NPs on barrier function has the potential to lead to diseases like IBD. Table 2.1 summarizes the various ways in which Ag, TiO_2, and SiO_2 NPs have been shown to negatively impact IBF.

Table 1. Impact of Ag, TiO_2, and SiO_2 NPs on various components of the human gut intestinal barrier towards dysfunction

NP	Demonstrated Impact of NPs on IBF
Ag	• ↑ Cell death, ↑ Bax/Bcl-2 ratio and P21 activation [17]. • ↑ Goblet cell discharge of mucus granules and abnormal mucus composition [20]. • ↓ MUC3 gene expression in the ileum [15]. • Induces colitis-like symptoms: ↑ disease activity index, ↑ histological scores, ↑ intestinal epithelial microvilli, ↑ tight junction disruption, and ↑ pro-inflammatory cytokines [16]. • Induces oxidative DNA damage [18]. • ↑ pro-inflammatory IL-8 secretion [19]. • ↑ Intracellular ROS, ↓ cellular activity [17]. • Downregulates TLR2 (in male rats), TLR4 (in female rats), and NOD2 [15]. • Downregulates T-cell regulatory genes (FOXP3, GPR43, IL-10, TGF-beta) [15]. • ↑ GM-CSF [55].

TiO_2	• Epithelial injury, reduced tight junction protein expression, reduced luminal mucus layer thickness [30]. • Increases length of villi, induces irregular arrangement of epithelial cells [28]. • Disrupts microvilli organization [31]. • Reduces colon crypt length [32]. • Potentiates intestinal tumour formation in rats exposed to carcinogens [33]. • Induces preneoplastic lesions, and aberrant crypt foci [29]. • Impairs intestinal immune homeostasis and induces colon microinflammation; ↑ IL-10, IL-1β, IL-8 and TNF-α [29]. • ↑ Colon macrophages, CD8 cells and IL-10, TNF-a and IL-6 mRNA [32]. • ↑ Inflammation [30, 33-36]. • Alters tight junction protein expression and paracellular permeability due to upregulation of various efflux pumps and nutrient transporters [37, 38].
SiO_2	• ↑ Pro-inflammatory cytokines in the small bowel and the colon [16]. • Disrupts epithelial layer and leads to loss of crypts in colon segments [16]. • Activates caspase-1 inflammasome and IL-1β release in macrophages [25] [8]. • Upregulates MHC-II, CD80, and CD86 on dendritic cells and apoptosis [8].

The abbreviations used are: *NP, nanoparticle; IBF, intestinal barrier function; Ag, silver; TiO_2, titanium dioxide; SiO_2, silicon dioxide; ↑, increases; ↓, decreases; IL, Interleukin gene; ROS, reactive oxygen species; TLR2, Toll-like receptor 2; NOD2, nucleotide-binding oligomerization domain 2; MUC3, Mucin 3 gene; TLR2, Toll-like receptor 2 gene; microbial recognition genes; GM-CSF, Granulocyte-macrophage colony-stimulating factor; TNF, Tumor necrosis factors; CD8, differentiation cluster 8 protein; MHC-II, Major histocompatibility complex class II; mRNA, messenger RNA.*

2.3 Gut intestinal barrier function under normal and impaired conditions

This section provides an overview of intestinal barrier anatomy and function under normal and impaired conditions (Fig. 1). The first layer of the intestinal barrier is the mucus layer which is 98% water and contains glycosylated proteins (mucins) and glycolipids [56]. In the colon, the loose outer mucus layer (~100um) is densely colonized by bacteria, fungus, virus, toxins, and allergens. The attached inner mucus layer (~50um) on the other hand is mostly sterile being occupied by immunoglobulins (mostly secretory-IgA), and defensins such as lysozyme. In the small intestine, there is only one single mucus layer which is thinner than that of the colon.

Gut microbes found in the lumen and mucus layers are also involved in protecting against invasion by pathogens through various mechanisms including competition for resources. The next layer is the intestinal epithelial layer which is responsible for nutrient absorption and selective transport of lumen contents across the barrier. Just below the epithelial layer is the lamina propria which includes a diffuse lymphoid tissue consisting of macrophages, dendritic cells, plasma cells, lamina propria lymphocytes, mast cells, eosinophils and occasionally, neutrophils.

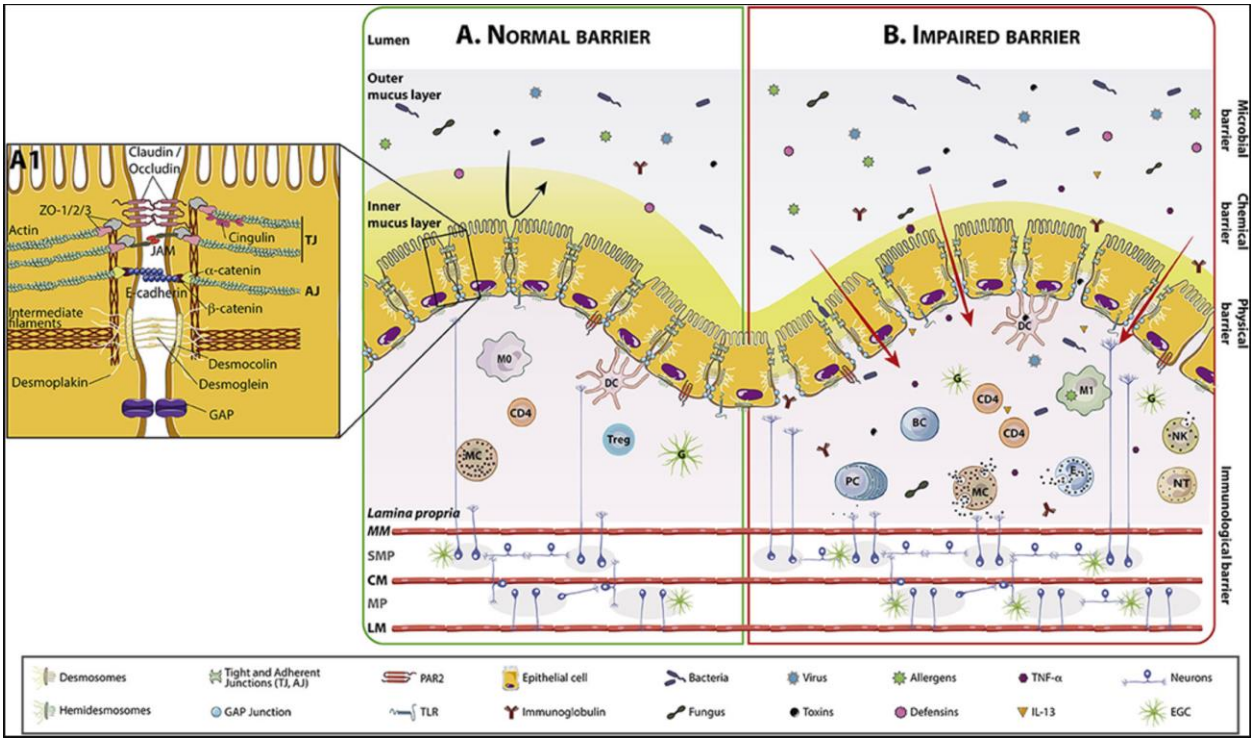

Figure 1. The anatomy and composition of the Intestinal barrier in normal and impaired conditions. (A) The healthy intestinal barrier has cells that are closely attached via intercellular junctions (TJs, adherens junctions, desmosomes, and GAP junctions) as seen in (A1). (B) The Impaired intestinal barrier has increased trans and paracellular passage of lumen contents which triggers the immune system. Legend: AJ, Adherens junctions; BC, B Cell; CD4, Lymphocyte T helper CD4+; CLDN, Claudin; CM, Circular muscle; D, Desmosomes; DC, Dendritic cell; EGC, Enteric glial cell; ENS, Enteric nervous system; IL-13, Interleukin 13; JAM, Junctional adhesion molecule; LM, Longitudinal muscle; M0, Macrophages type 0; M1, Macrophages type 1; MC, Mast cell; MM, Muscularis mucosae; MP, Myenteric plexus; NK, Natural killer; NT, Neutrophil; OCLN, occludin; PC, Plasma cell; SMP, Submucous plexus; TJ, Tight junctions; TNF-α, Tumor

necrosis factor alpha; Treg, T regulatory lymphocyte. Figure adapted from Fortea, M., et al. (2021) [56].

2.3.1 The intestinal barrier and intercellular junctions

The intestinal barrier is a critical junction that separates the external environment from the internal workings of the human body. The barrier must allow for the absorption of essential nutrients from the lumen while preventing the entry of harmful toxins and waste products [57]. A malfunctioning gut barrier fails to do this, and often exhibits increased paracellular and transcellular permeability to damaging compounds [58]. This intricate barrier is supported by the mucosal surface of the epithelial lining and tight junctions that link adjacent cells [59]. Intercellular junctions are responsible for controlling nutrient absorption, water and chloride secretion, and restriction of the passage of specific molecules (4–5 Å at the villus tip, and over 20 Å at the base of the crypt) [56]. There are various kinds of intercellular junctions each composed of unique proteins that serve specific functions. For instance, cells can adhere to the basement membrane through hemidesmosomes. Tight junctions are made up of CLDNs, OCLNs, and JAM proteins which are connected to the cytoskeleton through zonula occludens and cingulin. Adherens junctions are composed of cadherin proteins which connect to the cytoskeleton by binding catenins (α and β). Desmosomes are comprised of desmocollin and desmoglein which connect to intermediate filaments by binding desmoplakin. Epithelial cells are therefore mostly modulated by the cytoskeleton, specifically by actin, myosin, and intermediate filaments. Normal functioning of these intercellular junctions and their components ensures normal barrier integrity. Abnormal functioning on the other hand will lead to increased permeability of the intestinal barrier leading to invasion by pathogens ultimately triggering the immune system and potentially leading to disruption of homeostasis.

2.3.2 Properties of intestinal epithelial cells in relation to intestinal barrier function

Certain mechanical properties of intestinal epithelial cells provide defence mechanisms to maintain healthy gut functioning. The first defence mechanism is the lumen; pancreatic secretions, bile, gastric acids, and intestinal secretions work in conjunction to break and neutralize antigens and bacteria. The next mechanism is the prevention of colonization by pathogenic bacteria due to the commensal bacteria present in the gut [60]. Furthermore, the mucous layer and IgA antibodies respectively allow for the commensal bacteria to grow close to the epithelium thus providing them with a competitive growth advantage [61].

The epithelium physically separates the external luminal environment from the rest of the body. Multiple proteins between the cells form tight junctions that prevent the movement of harmful substances across the layer [62]. Paneth cells exhibit antimicrobial properties that assist to kill pathogenic bacteria [63]. Below the epithelial cells, lies another layer of cells called the lamina propria; this layer consists of neutrophils, T-regulatory cells, macrophages, and mast cells. They consist of components of the innate and adaptive immune system and work together to clear foreign substances from the body and reduce inflammation [64].

The immune cells work together effectively to maintain the delicate balance between the gut microbiota and host body. Often, this balance is disrupted and dysbiosis occurs [65]; this can lead to intestinal inflammation and has been linked to numerous complications such as Alzheimer's disease, diabetes, and depression. A disrupted gut microflora exhibits reduced levels of beneficial bacterial strains such as *F. prauznitzii*. This strain plays an important role in the fermentation of dietary fibers and endogenous intestinal mucous, which are not digested by the human GIT. This is important because such fermentation allows other microbes to grow that produce essential short chain fatty acids such as butyrate [66]. Disruption of such pathways thus interferes with healthy IBF.

2.3.3 Barrier Permeability

A healthy intestinal barrier allows for the passage of water, nutrients, and ions, while restricting the passage of pathogens and toxins. The epithelial lining allows medium sized hydrophilic molecules to move down their respective concentration gradients. In this environment, there is no carrier system involved; thus, increased permeability points to a damaged barrier [67]. Two main methods exist through which solutes can pass the intestinal barrier. As mentioned, the barrier is composed of a layer of epithelial cells, tight junctions, adherens junctions, desmosomes, and gap junctions. Solutes can pass between the cells through the paracellular route, or through the cells themselves, the transcellular route [68]. The paracellular route allows hydrophilic molecules up to 600 Daltons to cross; it is usually impermeable to larger protein sized molecules [69]. The transcellular route allows molecules to passively diffuse through the cells [68]. Due to the amphipathic properties of the cell membrane, only lipid soluble and small hydrophilic molecules can use this pathway. Larger proteins or bacterial products can also be taken up through endocytosis. Once molecules are taken in through endocytosis, they are moved through the cell via transcytosis. Endocytosis and transcytosis are pathways that are exploited by foreign microbes to enter the body. Thus, it is critical that barrier function be maintained to keep harmful pathogens outside the body.

2.3.4 The role of Polo-like kinase 1 protein in relation to intestinal barrier function

Polo-like kinase 1 (PLK1) is a highly conserved serine/threonine-protein kinase that plays a critical role in the cell cycle, specifically in the regulation of cell division and maintenance of genome stability in mitosis [70]. PLK1 has been widely studied as a potential target for cancer therapy, as its inhibition prevents tumor proliferation and induces apoptosis of cancer cells. Increased expression of PLK1 has been associated with diverse human

malignancies, including gastric cancer through mitogen-activated protein kinase (MEK)/ extracellular signal-regulated kinase (ERK) pathway phosphorylation and activation [71]. Data also suggests that PLK1 promotes a pro-inflammatory response through regulation of inflammatory signaling cascades. Inhibition of PLK1 interferes with Toll-like receptor (TLR)-activated MAPK and nuclear factor NF-κB signaling, thereby reducing tumor necrosis factor (TNF)-α expression [72]. Such findings suggest the involvement of PLK1 in innate inflammatory response.

NF-κB is a master regulator of pro-inflammatory responses and can either have a detrimental or protective function, depending on the cell types involved and the specific pathophysiological conditions [73]. Notably, its regulation is required for immune homeostasis at epithelial interfaces such as the intestine [74]. Increased activation of NF-κB in the intestine contributes to intestinal inflammation, as evident in IBD patients. However, it is unclear whether intestinal inflammation is due to NF-κB activity in epithelial or mucosal immune cells. Other research has shown a beneficial role of NF-κB activation in the intestinal epithelium wherein its complete inhibition leads to severe intestinal inflammation. Specifically, ablation of intestinal epithelial cell NF-κB Essential Modulator (NEMO) in mice resulted in severe colitis indicated by TNF-induced apoptosis of colonic epithelial cells, impaired antimicrobial peptide expression and bacterial translocation into the mucosa [75]. This protective function of NF-κB is likely due to its ability to regulate cell apoptosis through induction of antiapoptotic and antioxidative protein expression [74]. Therefore, the activity of NF-κB must be carefully regulated to ensure intestinal homeostasis. Interestingly, the TRAF-associated NF-κB activator (TANK) negatively regulates NF-κB activation and recruits PLK1 to inhibit NF-κB activity induced by TNF-α [76]. PLK1 also prevents NF-κB DNA-binding activity through reduced ubiquitination of NEMO. Thus,

inflammatory response of NF-κB via TNF-α signaling may be regulated by PLK1, however further research is needed to identify mechanisms underlying this interrelationship.

Moreover, recent investigation has highlighted the positive influence of PLK1 on intestinal function and permeability. Gut barrier integrity and regulation of permeability is largely dependent on the balance of cell proliferation and apoptotic activity [77]. Cao et al [78] demonstrated the downregulation of PLK1 in lipopolysaccharide (LPS)-treated HT29 cell line and mice with sepsis-induced intestinal barrier dysfunction. Interestingly, overexpression of PLK1 partly rescued the in vitro apoptosis and proliferation inhibition induced by LPS, suggesting PLK1 to be a potential therapeutic target for resolving intestinal barrier disruption. A later investigation confirmed that targeting PLK1 using the long noncoding RNA known as DANCR improves *in vivo* sepsis-related intestinal barrier dysfunction and alleviates intestinal injury [79]. Findings therefore suggest PLK1 may have a protective function against intestinal barrier dysfunction in the context of sepsis.

2.4 Introduction to probiotics and their potential use in managing nanoparticle-induced barrier dysfunction

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit to the host” [80]. Lactic acid producing bacteria are the most commonly used probiotics and include Lactobacillus, Bacillus, Bifidobacterium, Streptococcus, and Enterococcus species [81]. The clinical implication of probiotics was indicated for the first time in 1954 [82]. Many studies highlighted the role of probiotics in the modulation and reduction of intestinal permeability. Probiotic mediated barrier regulation occurs through various mechanisms [83]. Probiotics actively compete with pathogens for space on the intestinal epithelium through steric hindrance, release of bacteriocins, and competitive exclusion. For

example, Bifidobacteria and Lactobacilli prevent the adhesion of pathogenic bacteria to intestinal mucosa by secreting lectin-like bacteriocins [69]. Probiotics can also prevent epithelial invasion through stabilization of the cytoskeleton and gut barrier [84]. Metabolic products like short chain fatty acids (SCFAs), including butyrate, released by probiotics can also regulate tight junction integrity and expression of tight junction proteins [85]. For example, *Lactobacillus plantarum* (MB452) treatment was reported to increased transcription of cingulin and occludin genes in Caco-2 cells thereby improving tight junction integrity [86, 87].

There are currently no known treatments for NP-induced damage to IBF as it is not an established disorder, however probiotics may be a suitable candidate. The negative effects of NPs on the gut are well documented, especially in the case of TiO_2 NPs, making it important to propose treatments that can protect those who are chronically exposed. The first method for reducing NP induced damage is the limitation of NP exposure through foods. This is done at the level of authorities responsible for limiting NP use in foods. For instance, various EFSA panels have gathered to discuss NP use safety in foods. One such panel concluded that the use of TiO2 NPs in food additives was unsafe. Unfortunately, however, NPs are still used in foods, drugs, fabrics, and many other areas of life making it difficult to eliminate human exposure. Another method relies on the individual consumer to be aware of current literature and avoid certain food products. Unfortunately, however, labelling of products to indicate the presence of NPs has not been enforced making it difficult for individuals to know what foods to avoid. Secondly, many individuals are not aware of the recent literature showing the negative impact of NPs found in food additives. Therefore, current methods for mitigating NP induced barrier dysfunction are not adequate, and greater understanding of NPs and their impact on intestinal barrier functioning will be key in elucidating better methods of NP treatment. One such potential treatment is the use of

probiotic formulations to reverse the known negative effects of NPs. For instance, a probiotic formulation designed to improve IBF can be included in the human diet as a daily method of reversing NP-induced damage. In this way, individuals can reverse chronic assaults on the intestinal barrier as they happen potentially ameliorating the progression of various inflammatory gut diseases.

The intestinal microbiome plays an important role in normal gut functioning [88]. The role of the gut barrier is the most important in this regard, it provides a multifaceted defense system which is capable of separating the intestinal contents from the host tissues and can also modulating the absorption of nutrients and allowing the interaction between the resident microbial flora and the mucosal immune system [89]. When the gut barrier is disrupted, pathogenic microorganisms and food antigens can develop intestinal disorders, which are mainly associated with a local inflammatory response [90]. It has been proposed that probiotics maintain the epithelial barrier function through increased expression of junction mucins and proteins, and promote intestinal epithelial cell activation in response to bacterial infection [91, 92].

2.4.1 Lactobacillus species of probiotics

The gut microbiome plays a critical role in gut barrier functioning, so the use of the probiotic *Lactobacillus fermentum* is discussed here in relation to barrier dysfunction. Epithelial barrier dysfunction is a major factor in IBD onset and progression [93]. Probiotic treatments that include Lactobacillus have been tested on IBD patients and were found to result in remission for ulcerative colitis patients, but not for Crohn's disease patients [94]. One probiotic mixture called De Simone formulation contains Lactobacillus strains and has been shown to induce NFκB nuclear translocation in epithelial cells leading to the release of TNF-α. This stimulates epithelial cell proliferation ultimately reducing epithelial permeability. Therefore, probiotics have the

potential to positively impact regeneration of the epithelial barrier [95]. Another study found that *L. fermentum* was able to prevent and treat GI disorders associated with intestinal epithelial barrier dysfunction [9]. Lactobacillus fermentum CECT 5716 was shown to prevent intestinal barrier dysfunction in newborn rats [9]. The probiotic strain reduced intestinal permeability and increased ZO-1 protein expression suggesting *L. fermentum* is a good candidate for the treatment of intestinal barrier dysfunction and thus prevention of potential NP-induced barrier dysfunction. *Lactobacillus fermentum (CECT 5716),* isolated from human milk, is a potential candidate for strengthening the IBF [96]. Moreover, *L. fermentum* can reduce the prevalence of GI infections in infants [97, 98], as well as protect against GI infections caused by Salmonella species in mice [99]. In another study the oral administration of CECT 5716 prevented psychological stress-induced barrier dysfunction in rat pups [100]. Researchers further suggested that *L. fermentum* could provide a novel tool for the prevention and/or treatment of gastrointestinal disorders associated with altered IBF in newborns. Finally, ferulic acid producing *L. fermentum* NCIMB 5221 (Lf5221) has been shown to reduce markers of metabolic syndrome such as insulin resistance and antioxidant and anti-tumorigenic properties of ferulic acid [101].

2.4.2 Bifidobacterium species of probiotics

Bifidobacterium species can improve various components of the intestinal barrier and have protective effects against invasion. For instance, mouse models of colitis showed reduced colonic permeability and inflammation following treatment with *B. infantis* [83]. Supplementing T84 cell media with *B. infantis* also mitigates TNF-α and IFN-γ–induced increase in intestinal permeability, and prevents dysregulation of intercellular junction proteins through the release of bioactive factors [102]. Moreover, *B. lactis* has been effective in preventing mucosal dysfunction and reducing infection severity [103, 104]. Exogenous Biffidobacteria has also been shown to

reduce translocation of bacteria and endotoxins in rats [105]. Similarly, rat models of necrotizing enterocolitis exhibited improved intestinal integrity following *B. bifidum* treatment [106]. Taken together, *Bifidobacterium species* are capable of eliciting benefits to the intestinal barrier through preservation of tight junction integrity and regulation of immune responses. Such effects of the probiotic Bifidobacterium have the potential to be directly beneficial in preventing NP-induced barrier dysfunction.

2.4.3 Saccharomyces boulardii probiotic yeast

S. boulardii is a strain of *S. cerevisiae* and a probiotic yeast commonly used to treat gut disorders [107]. One study investigated a colitis mouse model to determine the impact of *S. boulardii* on IBF [108]. The probiotic improved mucosal barrier functioning by preserving tight junctions, reducing intercellular space, and protecting the expression of intercellular junction proteins (ZO-1 and occluding). There were also anti-inflammatory effects observed as there were reductions in TNF-α and IL-8. Disease activity index and histological score was also notably reduced. Treating colonic explants with *S. boulardii* enhances E-cadherin expression at the cell surface thereby improving IBF [109]. Another strain called *S. cerevisiae* (UFMG 905), when used in a mouse model of intestinal obstruction, shows ameliorated bacterial translocation, maintenance of gut barrier integrity and induction of immune responses [110]. *S. boulardii* can also maintain tight junction integrity and act as an anti-inflammatory agent [111]. Overall, the Saccharomyces probiotic can promote healthy IBF, thus potentially being useful for the treatment of NP-induced barrier dysfunction.

2.4.4 Escherichia coli Nissle 1917 non-pathogenic probiotic strain

Escherichia coli Nissle 1917, a kind of probiotic, enhances ZO-1 and ZO-2 expression as well as localization thereby protecting barrier function [112-114]. Resistance to pathogens is also conferred by this species [115]. A septic mouse model and a caco-2 cell monolayer has also used to show that this probiotic can alter both expression and localization of tight junction proteins towards protecting IBF [116]. Another study using a murine model of multiple sclerosis showed that Escherichia coli Nissle 1917 could reduce the release of IL-10 in both the central and peripheral nervous systems and repair intestinal barrier integrity [117]. This probiotic can improve loss of IBF related to sepsis and neurological disorders; therefore, it may be useful in the treatment of NP induced damage to IBF.

2.4.5 Probiotic mixtures

Probiotic combinations can also benefit IBF. For example, a mixture called VSL#3 containing Lactobacillus, Bifidobacterium and Streptococcus species stops the reorganization of tight junction proteins (occluding and ZO-1) and prevents apoptosis thereby ameliorating acute colitis in a murine model [118]. It can also increase MUC 2, 3, and 5AC expression and secretion [119] [120]. Table 2 provides a summary of the discussed studies which have demonstrated the benefit of probiotics in regulating IBF. The impact of various probiotic strains and formulations are summarized as well as their impact on the mucus barrier, epithelial barrier, tight junctions, pore forming proteins, immune system cells, and other components of the intestinal barrier.

Table 2. Probiotics with demonstrated benefit for intestinal barrier dysfunction		
Probiotic	**Probiotic effects on components of the intestinal barrier**	**Ref.**
Lactobacillus	• ↑ MUC 2 and 3 expressions. • Linked to remission in colitis patients. • ↓ Intestinal permeability. • ↑ ZO-1 protein expression. • Protects against GI Salmonella infections.	[121] [122] [123] [94] [96] [99]
***L. fermentum* CECT 5716**	• Prevents GI and stress-induced disorders associated with intestinal epithelial barrier dysfunction.	[9] [100]
***L. fermentum* NCIMB 5221**	• Elicits antioxidant and anti-tumorigenic properties via ferulic acid secretion.	[101]
De Simone formulation containining *Lactobacillus* strains	• Induces NFκB nuclear translocation in epithelial cells and releases TNF-α. • Stimulates epithelial cell proliferation. • ↓ Epithelial permeability.	[95]
VSL#3	• ↑ MUC 2, 3, and 5AC expression. • ↑ MUC1, 2, and 3 expression and secretion.	[119] [120]
S. thermophilus* *L. acidophilus	• ↑ TER, ↓ permeability. • Activation of occludins and ZO-1. • Prevents IFN-γ and TNF-α-induced ↓ ion secretion, ↓ TER, and ↑ permeability.	[124] [125]
S. boulardii	• Preserves and protects expression of intercellular junction proteins; ZO-1 and occluding. • Reduces intercellular space. • ↓ TNF-α and IL-8. • ↓ Disease activity index and histological score. • ↑ E-cadherin expression. • Maintains tight junction integrity and acts as an anti-inflammatory agent.	[108] [109] [111]
***S. cerevisiae* (UFMG 905)**	• Ameliorates bacterial translocation. • Maintains gut barrier integrity. • Induces immune responses.	[110]
B. infantis	• ↑ transepithelial resistance, ↓ permeability. • ↑ ZO-1, occludin, ↓ claudin-2 expression. • Prevents IFN-γ and TNF-α induced deleterious effects.	[102]
B. infantis	• Mitigates TNF-α and IFN-γ–induced increase in intestinal permeability. • Reduces translocation of bacteria and endotoxins. • Ameliorates loss of intestinal integrity in necrotizing enterocolitis.	[83] [102] [105] [106]
B. lactis	• Prevents mucosal dysfunction.	[103,

	• Reduces infection severity.	104]
E. coli Nissle	• ↑ TER, ↑ Expression and TJ localization of ZO-2.	[112]
E. coli Nissle	• ↑ ZO-1 expression; prevents DSS-induced decrease in permeability and illness.	[113]
E. coli Nissle	• Resists pathogens. • Improves expression and localization of tight junction proteins. • Reduces release of IL-10 in both the central and peripheral nervous systems. • Repairs intestinal barrier integrity.	[115] [116] [117].
S. boulardii	• Prevents enteropathogenic E. coli-induced apoptosis.	[126]
L. rhamnosus p40, p75	• Inhibits cytokine-induced apoptosis • Inhibits H_2O_2-induced ↓ TER and ↑ permeability.	[127] [128]

Abbreviations used include: ↑, increased; ↓, decreased; GI, gastrointestinal; TER, transepithelial resistance; ZO, zonula occludens; TJ, tight junction; DSS, dextran sodium sulfate; MUC3, Mucin 3 gene; TNF-α, Tumor necrosis factors; NFκB; NF-kappa B; IFN-γ, Interferon gamma; IL, Interleukin gene.

In summary, humans have been exposed to various NPs due to their widespread industrial applications. The most concerning is the use of NPs in additives by the food industry as this allows for direct contact with the gut intestinal lining through direct human ingestion. Such exposures are causing various health concerns as the western diet continues to be correlated with western diseases like inflammatory bowel disease. The interaction of NPs with the gut in general and the intestinal barrier is very important. NPs have been demonstrated to impact many components of the intestinal barrier towards increasing intestinal barrier permeability potentially leading to inflammatory gut diseases. Probiotics have shown some potential in mitigating intestinal barrier dysfunction. However, the role of NPs in barrier dysfunction is yet to be fully revealed, as is the role of probiotics as a mitigating factor. This thesis is about understanding the role of NPs in intestinal barrier function in relation to gut diseases.

PREFACE FOR CHAPTERS 3-5

Presented in the following 3 chapters is the original research that was completed towards achieving the research objectives. To evaluate the impact of food relevant NPs on the human GIT, three inorganic engineered nanomaterials commonly used in three food additives were chosen and are the focus of each chapter. The impact of TiO_2 (E171), SiO_2 (E551), and Ag (E174) NPs were studied in relation to IBF (Chapter 3-5). In each chapter, the effects of NP exposure on HT29 cell viability and PLK1 protein expression is investigated.

Original research articles presented in this thesis:

1. **Ghebretatios**, M., Boyajian, J.L., Islam, P., Abosalha, A., Ahmad, W., Schaly, S., Thareja, R., Prakash, S. (2022). Impact of titanium dioxide nanoparticles on human gut intestinal barrier function in vitro. *Manuscript To be submitted for publication.*
2. **Ghebretatios**, M., Boyajian, J.L., Islam, P., Abosalha, A., Ahmad, W., Schaly, S., Thareja, R., Prakash, S. (2022). Impact of silicon dioxide nanoparticles on human gut intestinal barrier function in vitro. *Manuscript To be submitted for publication.*
3. **Ghebretatios**, M., Boyajian, J.L., Islam, P., Abosalha, A., Ahmad, W., Schaly, S., Thareja, R., Prakash, S. (2022). Impact of silver nanoparticles on human gut intestinal barrier function in vitro. *Manuscript To be submitted for publication.*

Research contributions not included in this thesis

Original articles:

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2. Schaly, S., **Ghebretatios, M**., and Prakash, S. (2021). Baculoviruses in Gene Therapy and Personalized Medicine. Targets and Therapy. https://doi.org/10.2147/BTT.S292692.
3. Boyajian, J.L., **Ghebretatios, M.**, Schaly, S., Islam, P., Prakash, S. (2021). Microbiome and human aging: Probiotic and prebiotic potentials in longevity, skin health and cellular senescence. Nutrients. https://doi.org/10.3390/nu13124550.
4. Abosalha, A., Boyajian, J., Ahmad, W., Islam, P., **Ghebretatios, M.**, Schaly, S., Thareja, R., Arora, K., Prakash, S. (2022). Clinical pharmacology of siRNA therapies: current status and future prospects. Expert Review of Clinical Pharmacology. In press.
5. Abosalha, A., Boyajian, J., Ahmad, W., Islam, P., **Ghebretatios, M.**, Schaly, S., Thareja, R., Arora, K., Prakash, S. (2022). A comprehensive update of siRNA design and delivery strategies for effective targeting and gene silencing. Expert Opinion on Drug Discovery. In press.
6. Islam, P., Schaly, S., **Ghebretatios, M**., Boyajian, J., and Prakash, S. (2022) Nanotechnology in development of next generation of stent and related devices: current and future aspects. *(In Progress).*

Research abstract:

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CONTRIBUTIONS OF AUTHORS

In all the original articles included in the thesis, I am the main author taking full responsibility for the design and conduct of all experiments and data analysis. The reported co-authors have assisted in the revision of manuscripts and provided intermittent laboratory assistance. Dr. Prakash conceived the project idea, assisted in data analysis, and supervised all aspects of the research work. Dr. Prakash is listed as the corresponding author in all articles included in the thesis.

CHAPTER 3

Impact of Titanium Dioxide Nanoparticles on Human Intestinal Barrier Function in Vitro

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Preface

In this first original research paper, a commonly used food nanoparticle is investigated for its potential impact on the human gut intestinal epithelium. Based on a comprehensive literature review of various nanoparticles commonly used in foods, the nanoparticle titanium dioxide (TiO_2) was selected for investigation due to its suspected role in the disruption of the gut barrier function. Cell proliferation is an important part of gut homeostasis that can become dysregulated potentially leading to reduced barrier function and thus inflammatory bowel diseases. The purpose of this research was to determine the impact of TiO_2 NPs on human intestinal cell viability and proliferation. In addition, the role of the probiotic Lactobacillus fermentum NCIMB 5221 (Lf5221) in potential NP toxicity was investigated.

3.1 Abstract

The use of TiO_2 NPs by the food industry has recently been deemed unsafe, however it is still consumed by humans throughout their lifespan. Further studies on TiO_2 NPs and their impact on the gut intestinal barrier are needed to understand the mechanism of their toxic effects and propose novel treatments. This study involves a comprehensive analysis of TiO_2 NP toxicity on the human colon carcinogenic HT29 cell line used as a model of the gut intestinal epithelial layer. The size and zeta potential of TiO_2 NPs found in the E171 food additive was investigated in accordance with EFSA guidelines for NP toxicity studies. TiO_2 NPs were then in vitro digested to simulate human exposure to TiO2 NPs through ingestion before incubation at different concentrations with HT29 cells. Analysis of cell viability using MTT assay showed TiO_2 NPs did not result in any significant loss or gain in cell viability at all tested doses (0.067 $\mu g/cm^2$, 0.34 $\mu g/cm^2$, and 17 $\mu g/cm^2$). Cell viability results remained unchanged following treatment with the probiotic *L. fermentum*. Secondly, PLK1 protein expression analysis using a

Colorimetric Cell-Based ELISA Kit showed that TiO_2 NPs did not significantly affect PLK1 protein expression. There was, however, a non-significant increase in PLK1 protein expression seen in cells exposed to the two highest doses (0.34 μg/cm^2 and 17 μg/cm^2) of TiO_2 NPs. Overall, TiO_2 NPs do not negatively impact HT29 cell viability, but they may lead to increased PLK1 protein expression when exposed at doses relevant to the current human consumption levels. Findings have the potential to inform future therapeutics for TiO_2 NP induced gut damage.

3.2 Introduction

The GIT plays an important role in human physiology. The four compartments of the intestinal barrier serve to maintain gut homeostasis [7]. Commensal bacteria provide crucial metabolic products by breaking down indigestible nutrients, the mucus layer protects against invasion by microorganisms, the epithelial monolayer serves to facilitate selective absorption of nutrients, and finally the intestinal immune system responds to potential invasion by microorganisms. When these components of the intestinal barrier are impacted, there is a direct impact on gut physiology leading to pathology. For example, Crohn's disease (CD) and ulcerative colitis (UC) are two subtypes of IBD characterised by chronic inflammation of the gut. Such inflammation can eventually increase the risk of colitis associated colorectal cancer (CAC). It has been recently elucidated that mineral particles found in food additives may contribute to the breakdown of IBF, potentially leading to IBD.

Titanium dioxide, also referred to as Titanic anhydride, TiO_2, or Titanium (IV) oxide, can be found in the food additive E171. The European Commission recently requested the Panel on Food Additives and Flavourings (FAF) of the EFSA to assess the safety of the food additive E171. The maximum dose relevant to food related ingestion of E171 by children is 30mg/kg

bw/day. While the absorption of orally consumed TiO_2 is low (0.02–0.1%), the panel on FAF recently concluded that E171 is not safe as a food additive [4]. The small amount that is absorbed can be found in Peyer's patches; a group of cells in the gut-associated lymphoid tissue (GALT). They additionally noted that there have been no studies that have adequately investigated the potential for TiO_2 NPs to cause cancer. Furthermore, mice studies have revealed TiO_2 NPs can be taken up through the paracellular pathway as well as via endocytosis indicating their direct interaction with biological systems at the cellular level. Therefore, it is important for researchers to study the impact of TiO_2 NPs on the gut epithelial barrier to elucidate the mechanisms of TiO_2 NP toxicity and aid future development of treatments.

This study takes into consideration many of the gaps in research identified by the panel on FAF. Firstly, there is a lack of studies on the impact of food NPs on the cells of the GIT as well as the gut microbiome. According to the EFSA panel, 56 studies have assessed micronucleus and chromosomal aberrations, but only 4 of these were completed on intestinal cells [4]. Therefore, this study involves the use of the HT29 colon carcinogenic cell line to determine the effect of TiO_2 NPs found in the E171 food additive. Secondly, several animal studies have been done using TiO_2 NPs smaller than 30nm, however the EFSA determined such sizes represent only 1% (by number) of the particles found in E171 making study findings irrelevant in the safety assessment of E171. Given that less than half of the particles found in E171 have an external dimension below 100nm, this study characterized TiO_2 NPs to ensure their size was relevant for the assessment of the safety of E171.

3.3 Materials and methods

Method for determining the physiological dose relevant to human TiO_2 NP ingestion

The EFSA Panel on Food Additives and Flavourings (EFSA FAF Panel) determined that the maximum dose relevant to food related ingestion of E171 by children is 30mg/kg bw/day. Based on the average weight of a 9-year-old child, it can be assumed that 840mg is consumed per day. Considering the surface area of the small intestine (2.5x10^6 cm^2), an exposure equivalent of 336ng/cm^2 of E171 per day was estimated and applied to HT29 cells cultured in vitro. This calculated dose is referred to as the physiological dose as it represents the true exposure of intestinal epithelial cells to digested NPs. This study exposes HT29 cells to various concentrations relative to the calculated physiological dose (0.02X=6.72ng/cm^2, 1X=336ng/cm^2, and 50X=16 800ng/cm^2).

TiO_2 NP lyophilization method

TiO_2 NPs were purchased from Millipore Sigma (EMPROVE® ESSENTIAL1008050500) and 600mg were diluted in 20mL of 2% sucrose (Bio Basic SB0498) followed by centrifugation for 15 minutes at 10 000rpm. This wash was completed a total of 3 times before the pellet was resuspended in 20mL of 2% sucrose and filtered through a 0.22 µm syringe filter (Celltreat 229747). The sample was then sonicated for 30 minutes at room temperature and left to freeze overnight at -20°C. Samples were then lyophilized to produce a stable powder which was stored at room temperature or diluted in deionized water for immediate use in experiments.

TiO_2 NP Characterization methods

TiO_2 NPs were characterized for size, polydispersity index (PDI), and zeta potential using the Brookhaven Zeta PALS instrument. TiO_2 NPs were dispersed in deionized water to 1 mg/mL for zeta potential measurements and 25 mg/mL for size and PDI measurements. TiO_2 NPs were prepared using sonication for 30 minutes at 50°C and filtration using a 0.22 µm syringe filter

immediately prior to characterization. Error bars represent a mean of standard errors from 10 measurements repeated 3 times.

HT29 cell culture methods

A human colorectal adenocarcinoma cell line with epithelial morphology (HT29) at Passage #18 was generously donated by Prof. Syaram Pandey in the Department of Chemistry and Biochemistry (University of Windsor). Cells were cultured in L-glutamine (0.21 g/L) containing McCoy 5A media (Fisher scientific, MT10050CV) supplemented with 10% FBS (VWR, 76419-584). Cells were passaged using 0.25% Trypsin-EDTA and media was changed every two days. The ATCC 'Protocol for Thawing, Propagating and Cryopreserving of NCI-PBCF-HTB38 (HT-29, ATCC®HTB-38 ™) cells colorectal carcinoma' was followed closely.

Bacterial culture methods

The probiotic strain *L. fermentum NCIMB 5221 (Lf5221)* was purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). Lf5221 was maintained by continuous subculturing in MRS broth at 2% (v/v) in a 37°C static aerobic incubator. Bacterial growth was monitored via colony counting ($2.7x10^8$ CFU/mL), and the stalk was diluted using McCoy 5A Media to a concentration of 263 CFU/cm^2 for cell viability experiments.

Methods for in vitro *digestion of TiO_2 NPs and their use in experiments*

In vitro digestion of TiO_2 NPs was completed following methods previously described by other researchers [129, 130]. TiO_2 NPs were taken through a simulated digestion process. Mouth digestion was simulated using a salt solution (0.1mL of 1M KCl, 0.3mL of 1M CaCl2, and 1.4mL of NaCl). Stomach digestion was simulated using a gastric solution (0.2 g pepsin in 8 mL

of 0.1M HCl). Finally, intestinal digestion was simulated using a pancreatic solution (0.05 g pancreatin and 0.3 g bile extract in 27 mL of 0.1 M $NaHCO_3$). Both 1M $NaHCO_3$ and 1M HCl was used to pH solutions during the digestion process.

Previously lyophilized TiO_2 NPs were first dispersed in deionized water (10mg/mL), and 1mL of this was taken through the digestion process. First, 1.8mL of the salt solution was added for 1-5 minutes, then 1M HCl was used to bring the pH to 2, then 50µL pepsin solution was added followed by incubation horizontally for 1h on a tabletop shaker (150 rpm, 37 degrees, 5% CO2/95% air). The pH was increased to 6 and 250µL of pancreatic solution was added. The pH was adjusted to 7.0 and the volume was brought to 20mL using a 1:1 ratio of NaCl (120mmol/L) and KCl (5mmol/L). The TiO_2 NPs were at this point considered digested and taken though a series of dilutions in McCoy 5A media to generate the different concentrations (0.01X, 1X, 50X) for HT29 exposure.

HT29 cells at P34-36 were seeded in 96 well plates at 20 000 cells/well. TiO2 NP exposure took place 4 days post passage for cell viability experiments and 3 days post passage for PLK1 expression experiments. Cells were exposed to each concentration of digested TiO_2 NPs overnight for 23 hrs (0.067 µg/cm^2, 0.34 µg/cm^2, and 17 µg/cm^2). Following this, media containing NPs was removed. One group was treated with the probiotic *L. fermentum* diluted in McCoy 5A medium at 263cfu/cm^2 for 4 hrs, and another group received only the complete McCoy 5A medium.

Methods to measure HT29 cell viability using MTT assay

MTT reagent was dissolved in PBS (0.5mg/mL, CAT. #: T0793) and filtered using a 0.22 µm syringe filter. 50µL of this was added to each well of the 96-well plate followed by incubation

for 1 hour at 37°C under 5% CO2/95% air. 100µL of isopropanol-HCL solution (35µL of 6M HCl in 10 ml isopropanol) was used to dissolve the crystals. Absorbance was read at 560nm using a Perkin Elmer HTS 7000 Bioassay Reader. Absorbance values of experimental groups were normalized to the control group treated with McCoy 5A media alone and results represented as % viability.

Methods to measure PLK1 protein expression

The PLK1 Colorimetric Cell-Based ELISA Kit (Assay Biotechnology, CB5577) was used to measure relative PLK1 protein expression in HT29 cells. In short, cells were fixed with 4% paraformaldehyde and quenched using Quenching Buffer. Binding sites were blocked using Blocking Buffer. PLK1 protein was targeted using Anti-PLK1 Primary Antibody (rabbit monoclonal) and HRP-Conjugated Anti Rabbit IgG secondary Antibody. Positive control wells were incorporated targeting the GAPDH protein using the Anti-GAPDH Primary Antibody (mouse, monoclonal) and HRP-Conjugated Anti-Mouse IgG secondary Antibody. Negative controls were incorporated containing the secondary antibodies alone. Substrate was added and the colorimetric reaction read at 450nm using a Perkin Elmer HTS 7000 Bioassay Reader. Following this, crystal violet cell staining was utilized on the same plate to stain nuclei and absorbance was measured at 595nm. Absorbance representing PLK1 protein expression and cell amounts (450nm/595nm) were calculated to normalize for differences in cell amount. Results are presented relative to the control treated group.

Statistical analysis

Data is expressed as mean ± standard deviation. Analysis of cell viability and PLK1 expression was completed using the data analysis and graphing software Origin 2021b. Results were

analysed using pairwise comparison using the paired comparison plot app (v3.60) for pairwise comparison of experimental groups. Statistical significance is indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Error bars represent standard deviation. The mean comparison method used was one-way analysis of variance (ANOVA) with Turkey's post test.

3.4 Results

TiO_2 NP characterization

Zeta potential, hydrodynamic size, and polydispersity index (PDI) of undigested TiO_2 NPs were characterized using the Brookhaven Zeta PALS instrument. TiO_2 NPs had an effective diameter of 141 nm and a polydispersity index (PDI) of 0.226 ± 0.017 (Fig. 2). The zeta potential was determined to be -26.7mV.

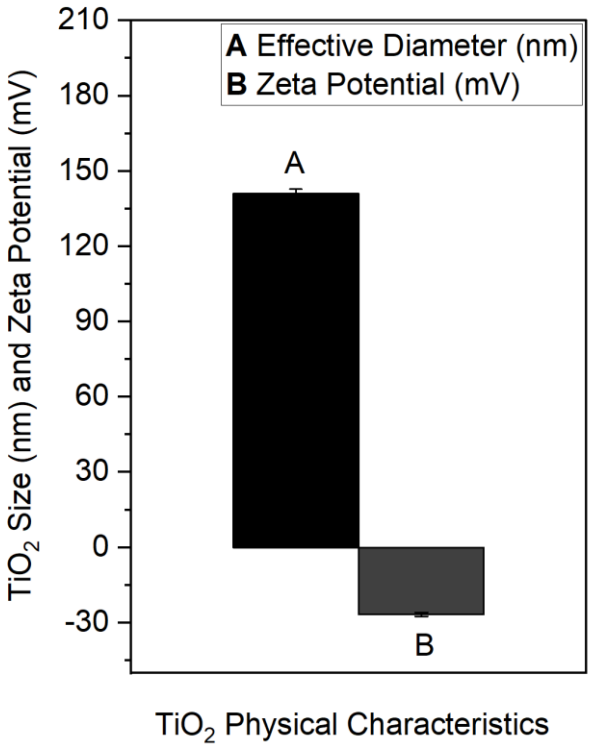

Figure 2. TiO_2 nanoparticle (NP) size and zeta potential characterization. TiO_2 NPs were characterized using a Brookhaven Zeta PALS instrument following dissolution in deionized water; 1 mg/mL for zeta potential and 25 mg/mL for sizing. NPs were sonicated for 30 minutes at 50°C and filtered (0.22 µm) immediately prior to characterization. Results showed TiO_2 NPs had a zeta potential of -27 mV and average hydrodynamic diameter of 141 nm. Error bars represent a mean of standard errors from 10 measurements repeated 3 times.

The effect of TiO_2 NP exposure on HT29 cell viability

TiO_2 NPs, at physiologically relevant doses, have no significant impact on HT29 cell viability. Data is presented relative to a control group treated with McCoy 5A media. Exposing HT29 cells to TiO_2 NPs at 0.067 µg/cm^2, 0.34 µg/cm^2, and 17 µg/cm^2 did not cause any significant gain or loss in HT29 cell viability when compared to the digest control (Fig. 3). The digest treated control group exhibited reduced cell viability by 19% compared to media treated control groups.

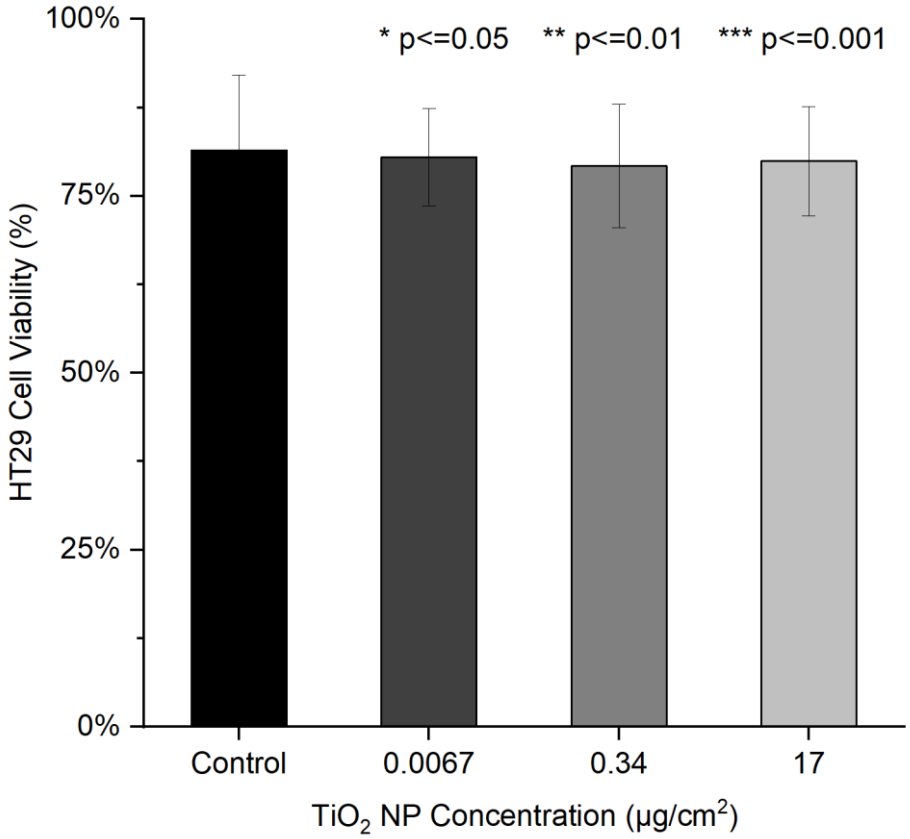

Figure 3. Impact of different concentrations of TiO_2 nanoparticles (NPs) on HT29 cell viability. HT29 cells were incubated with digested TiO_2 NPs diluted in McCoy 5A media at various concentrations (0.067 µg/cm^2, 0.34 µg/cm^2, and 17 µg/cm^2). Results show no significant changes in cell viability. Control groups received digest components without TiO_2 NPs. Results are normalized relative to a control group treated with McCoy 5A complete media. Error bars indicate standard deviation (n=6).

The effect of probiotic and TiO_2 NP treatment on HT29 cell viability

Treatment with *L. fermentum NCIMB 5221 (Lf5221)* and TiO_2 NPs does not affect HT29 cell viability. HT29 cell viability (%) is normalized to cells treated with McCoy 5A media alone. Treating HT29 cells with 0.067 µg/cm^2, 0.34 µg/cm^2, and 17 µg/cm^2 TiO_2 NPs for 23 hrs followed by the probiotic Lf5221 does not result in any statistically significant difference in cell viability when compared to both probiotic and digest controls (Fig. 4). Cell viability of TiO_2 and

probiotic treated groups (Fig. 4) were not significantly different from TiO_2 NP treated groups (Fig. 3). The probiotic control showed the highest cell viability (97%) while the digest control exhibited reduced cell viability (81%) comparable to that seen in NP exposed groups. HT29 cell viability seems to decrease with increasing TiO_2 NP concentration, however these differences are not statistically significant (Fig. 3).

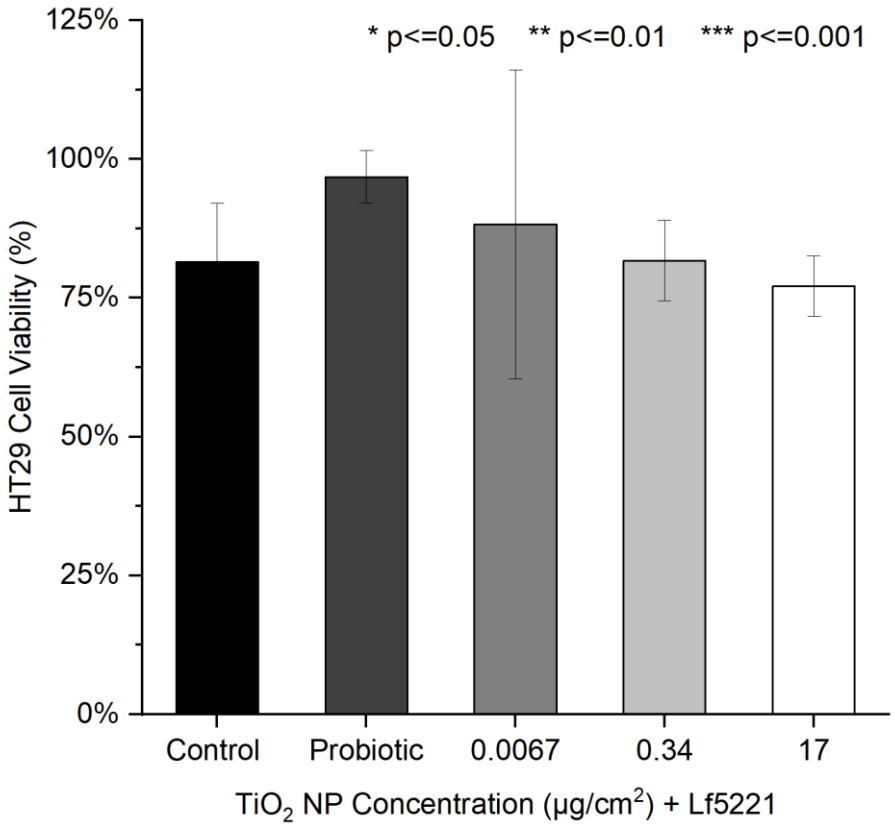

Figure 4. Impact of *L. fermentum NCIMB 5221 (Lf5221)* on HT29 cell viability following incubation with TiO_2 nanoparticles (NPs). HT29 cells were incubated for 23 hrs with TiO_2 NPs at various doses (0.067 µg/cm^2, 0.34 µg/cm^2, and 17 µg/cm^2) followed by incubation with Lf5221 at 2.63 x 10^2 CFU/cm^2 for 4 hrs. Cell viability was then measured using the colorimetric MTT assay. Results show no significant differences in HT29 cell viability among treatment groups. Control group represents cells exposed to digest components without TiO_2 NPs, the probiotic group represents cells exposed to Lf5221 alone with no prior exposure to NPs. Results are normalized to a control group treated with McCoy 5A complete media. Error bars indicate standard deviation (n=6).

The effect of TiO_2 NP exposure on PLK 1 protein expression

TiO_2 NPs non-significantly increase the expression of PLK1 protein in HT29 cells in a dose dependant manner. PLK1 protein expression was measured using an ELISA based colorimetric immunoassay and normalized to cell amounts. Data is presented relative to the PLK1 protein expression of the control treated group. Exposure to TiO_2 NPs at 0.067 μg/cm^2, 0.34 μg/cm^2, and 17 μg/cm^2 did not affect PLK1 protein expression in a statistically significant manner. However, cells exposed 0.34 μg/cm^2 and 17 μg/cm^2 TiO_2 NPs expressed higher levels of PLK1 protein in a dose dependent manner compared to the control (Fig. 5). This contrasted with the lowest dose of TiO_2 NP (0.067 μg/cm^2) treated group, which showed lower PLK1 protein expression compared to the control.

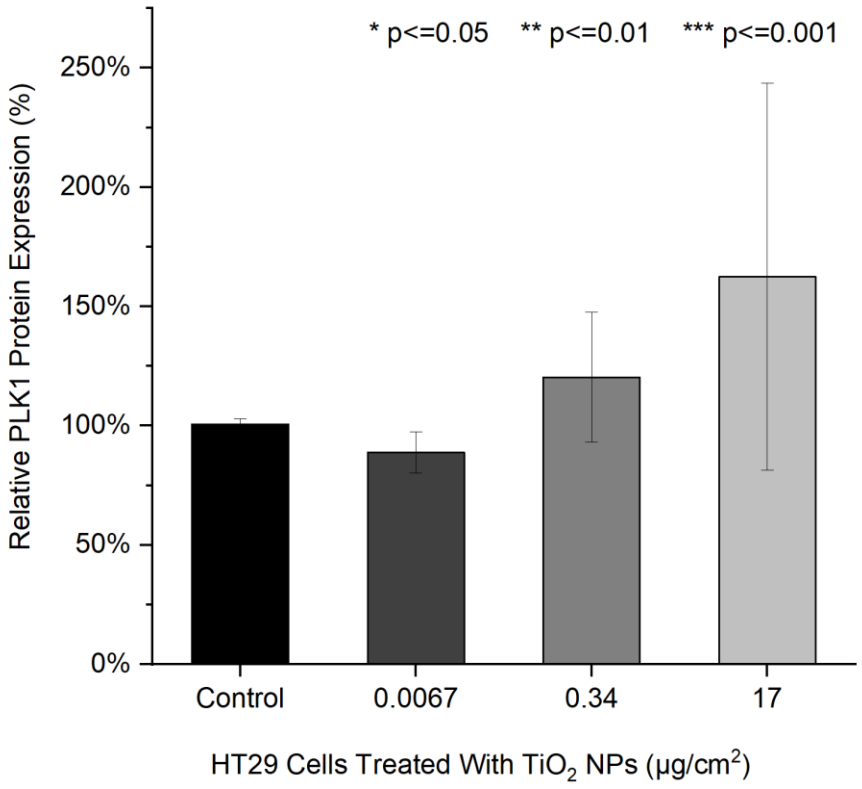

Figure 5. Relative expression of PLK1 protein in HT29 cells treated with different doses of TiO_2 nanoparticles (NPs). HT29 cells were incubated with different concentrations (0.067 µg/cm^2, 0.34 µg/cm^2, and 17 µg/cm^2) of TiO_2 NPs for 26 hrs. An ELISA-Based Colorimetric Immunoassays was used to measure PLK1 protein expression (OD 450nm). Results showed no significant differences in PLK1 protein expression across all groups. Data was normalized to cell amounts (OD 450nm/OD595nm) and analysed relative to a control group receiving no treatment (McCoy 5A Media alone). The control bar represents cells exposed to digest components alone without NPs. Error bars indicate standard deviation.

3.5 Discussion

Exposing HT29 cells to TiO_2 NPs alone or following up with probiotic treatment did not result in any significant loss or gain in cell viability. This was maintained regardless of the concentration of TiO_2 NPs used (0.067 μg/cm^2, 0.34 μg/cm^2, and 17 μg/cm^2). It was also noted that the probiotic control showed no loss in cell viability while the digest control exhibited reduced cell viability comparable to that seen in TiO_2 NP exposed groups (Fig. 4). This means that the digest solution caused a loss in cell viability by 19%. It is unclear what component of the digest caused this loss in viability, but it could be the pancreatin, bile, pepsin, HCl and/or $NaHCO_3$. These components must negatively interact with HT29 cells leading to reduced cell viability. Consistent with this, the probiotic control group (which was not in vitro digested) showed no reduction in cell viability (97%). It would be interesting to determine whether digested probiotics would show reduced viability, however this was not assessed in this study. TiO_2 NPs, at any dose, did not affect PLK1 protein expression in HT29 cells. However, groups receiving the highest two doses of TiO_2 NPs (0.34 μg/cm^2, and 17 μg/cm^2) showed slightly higher PLK1 protein expression, but this was not significant. Therefore, it may be possible that TiO_2 NPs lead to increased PLK1 protein expression.

Consistent with the findings of this study, some findings suggest TiO_2 NPs have no negative impact on the GIT. One such study showed that TiO_2 NPs can have no impact on intestinal barrier functioning. Specifically, researchers have reported no adverse effects of E171 at doses as high as 100mg/kg per day and even 267mg/kg bw/day [131] [132]. Furthermore, researchers have reported no increase in ACF due to E171, although there were limitations to this study with regard to sampling [132].

By contrast, an overwhelming number of studies have also reported many negative effects of NPs. The food additive E 171 can impact immune-related and inflammatory markers, potentially due to the presence of a small fraction of TiO_2 NPs. Thus, studies have been completed on both E171 and TiO_2 NPs. Researchers have studied the short- and long-term effects of TiO_2 NPs. After 5 days [35] and 7 days [30] of exposure, researchers have found that NPs (47nm) could elicit an inflammatory response in the stomach at various doses (5, 50 or 500 mg/kg bw). The longer-term studies lasting 90 days showed that TiO_2 NPs at 20 or 40 mg/kg bw per day caused changes in immunological parameters [36]. In another study, exposure of Caco-2 cells to 50 μg/mL of TiO_2 NPs did affect paracellular permeability of the epithelia [133]. By contrast, mice experienced changes to tight junction protein expression and altered paracellular permeability of intestinal epithelia in response to a one-time ingestion of 12nm TiO_2 NPs (12.5 mg/kg b.w). Caco-2 cells acutely exposed to 50 μg/mL of anatase (12 nm) or rutile (20 nm) TiO2-NPs displayed upregulation of various efflux pumps and nutrient transporters [38]. This indicated that NPs were able to affect the role of the intestinal barrier in absorbing nutrients and removing harmful substances. Consistent with those findings, another study found that exposure of the Caco-2/HT29-MTX coculture to 30 nm TiO_2 NPs resulted in altered tight junctions and reduced microvilli which are involved in nutrient absorption [37]. Here, researchers used doses of 10^6, 10^8 and 10^{10} particles/cm^2 for acute exposure experiments, and three times these concentrations for chronic exposure experiments. E171 at 100 ng/cm^2 (i.e. 350 ng/mL) disrupts microvilli organization in Caco-2 cells making them appear limp and fewer in number [31]. Authors considered this may be due to particle sedimentation on the microvilli, however disruption of microvilli persisted even when sedimentation was prevented. This ultimately shows that E171 can alter epithelial morphology biologically. Furthermore, studies have demonstrated

reductions in colonic crypt length and increased colon macrophages and CD8 cells in IL-10, TNF-a and IL-6 mRNA at even lower doses of 10 and 50 mg/kg bw per day of TiO_2 [32]. Researchers have demonstrated the ability of E171 to increase parameters related to inflammation at doses as low as 10 mg/kg bw/day [29], 5 mg/kg bw per day [33] and 2 mg/kg bw per day [34]. One study reported that a dose of 10 mg/kg bw per day TiO2 led to the induction of preneoplastic lesions and aberrant crypt foci (ACF) in the male rat colon [29]. Similarly, the food additive E 171 at the same dose caused an increase in the number of carcinogen induced ACF. Taken together, most of the literature suggests that the protective and absorptive roles of intestinal epithelial cells are impaired by TiO_2 NP exposure along with other components of IBF.

The TiO_2 NPs (141 nm) used in this study had a low PDI of 0.226 ± 0.017 which shows that the sample was uniform with respect to particle size. NP size was considered relevant for the evaluation of the potential effects of ingesting the E171 food additive according to the EFSA guidelines. Zeta potential provides valuable information about the physical property of NPs which can be used to determine colloidal stability [134]. The zeta potential for TiO_2 NPs dispersed in deionized water was -26.7mV. The high magnitude of the charge allows for electrostatic repulsion that facilitates TiO_2 NP dispersion resulting in a more stable solution. While the TiO_2 NPs are stable under these conditions, it is important to recognize that this property changes with other dispersion mediums. In this study, TiO_2 NPs were taken through a series of pH changes and exposed to various digestive enzymes during the in vitro digestion process which likely resulted in agglomeration and aggregation of NPs which impacts their biological effects as well.

3.6 Conclusion

In conclusion, it has been shown that TiO_2 NPs do not negatively impact intestinal epithelial cell viability at the calculated physiologically relevant dose as well as 50X above and below the physiological dose. This response is the same when cells are exposed to the probiotic strain Lf5221 following TiO_2 NP exposure. Accordingly, PLK1 protein expression is unaffected by exposure to TiO_2 NPs at all doses tested. Of note is the statistically insignificant increase in PLK1 protein expression seen with increasing dose of TiO_2 NP exposure.

3.7 Acknowledgements

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CHAPTER 4

Impact of Silicon Dioxide Nanoparticles on Human Intestinal Barrier Function in Vitro

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Preface

In Chapter 3, the impact of TiO_2 NPs on human intestinal epithelial cell proliferation and viability was investigated. The findings revealed that TiO_2 NPs did not alter intestinal epithelial cell viability at physiologically relevant doses. Additionally, the exposure of cells to the probiotic Lf5221 did not make a difference. Similarly, PLK1 protein expression was unaffected by exposure to TiO_2 NPs at all doses tested. Of note was the statistically insignificant increase in PLK1 protein expression seen with increasing dose of TiO_2 NPs. Given this finding, another nanoparticle commonly used in foods was selected for further investigation. Based on the previous findings, it was postulated that different food NPs could alter PLK1 protein expression, an indicator of cell proliferation. In this original research paper, SiO_2 NPs at various doses were investigated with regards to their effects on human intestinal epithelial cells, specifically on their ability to alter proliferation related signaling protein expression.

4.1 Abstract

SiO_2 nanoparticles (NPs) can be found in the additive E551 used by the food industry for its anticaking properties. Despite continuing human consumption, SiO_2 NPs have been shown to alter various components of the human intestinal barrier function. SiO_2 NPs can disrupt the gut microbiome, alter intestinal permeability, and interact with biological systems at the cellular level leading to cytotoxicity and the release of reactive oxygen species. While their use as a food additive is deemed safe based on current data, there is a call for more studies to assess their toxicity. In this work, the impact of SiO_2 NPs on intestinal barrier function is investigated using the colon adenocarcinoma HT29 cell line. SiO_2 NPs were in vitro digested to simulate human consumption through ingestion and prepared at different concentrations relative to currently reported human exposure levels (79 mg/kg bw per day). The effect of SiO_2 NPs was investigated

on HT29 cells via analysis of cell viability and PLK1 protein expression. The effect of the presence of the probiotic *L. fermentum NCIMB 5221* (Lf5221) following incubation with SiO_2 NPs was also assessed. Finally, SiO_2 NPs were characterized to determine their size and zeta potential following two methods of preparation to confirm NP relevance to the food additive. It was found that in vitro digested SiO_2 NPs (128 nm) do not impact HT29 cell viability at all concentrations tested relative to the physiological dose (0.02X, 1X, 50X). The administration of the probiotic Lf5221 following HT29 treatment with NPs did not result in any changes in cell viability. Most notably, relative PLK1 protein expression significantly increased in cells treated with 1X and 50X SiO_2 NPs compared to control treated and 0.02X SiO_2 NP treated groups. Relative PLK1 protein expression increased with higher SiO_2 NP exposure, but not significantly. Findings can be used to elucidate the mechanism of SiO_2 NP induced changes to the intestinal barrier function and aid the design of future therapeutics.

4.2 Introduction

NPs are clusters of atoms with at least one dimension in the nano scale (< 100 nm). Their high surface area to volume ratio provides them with unique and useful properties which various industries utilize. The food industry specifically has incorporated SiO_2 NPs into food additives for their unique anticaking properties. The food additive E551 contains SiO_2 NPs and is used in powdered food products. The main food categories contributing to human exposure to SiO_2 NPs includes chewing gum, sauces, flavored drinks, processed nuts, soups/broths, desserts, fine bakery wares, ripened cheese and more. The widespread incorporation of SiO_2 NPs into food products has allowed NPs direct access to the human GIT through ingestion.

Researchers have found that some inorganic NPs have the potential to increase colonisation by pathogens and reduce beneficial strains, something that is commonly seen in

patients with IBD [135]. SiO_2 NPs however have been shown to enrich the diversity of the microbiome as indicated by higher proportions of Firmicutes and Proteobacteria, and reduced Bacteroidetes and Lactobacillus [136]. In addition, various biological and toxicological studies have shown that SAS and intentionally engineered nano-SAS have little to no toxic effects [6]. Despite such positive results, there is a severe lack of toxicity studies for SiO_2, information regarding current use levels, and the current ADI is labelled as 'not specified' [6]. Furthermore, the EFSA panel on FAF concluded there are uncertainties regarding how representative the NPs thus far studied are to the food additive E551. Consequently, the panel on FAF did not confirm the current ADI.

This study follows the guidelines of the EFSA panel on FAF regarding NP toxicity studies. Firstly, it was noted that a lack of characterization of the particles studied makes it difficult to fully translate toxicity data to the food additive E551. Thus, this study will include the characterization of all NPs used. Secondly, food additives are not made up of intentionally engineered nano-SAS prepared using amorphous SiO_2, however the current specifications would allow their use as a food additive. Additionally, only the amorphous form of SiO_2 (synthetic amorphous silica (SAS)), not the crystalline silica, has been authorized as a food additive. Therefore, this study will focus on the toxicity of intentionally engineered fumed amorphous silica to remain representative of the food additive E551. Finally, the EFSA panel expressed that the relevance of current studies to the risk assessment of SAS as a food additive was lacking due to the use of unlikely routes of administration and unusually high doses up to 200X the estimated exposure for an adult population. This study therefore involves careful calculation of a physiologically relevant dose and in vitro simulated digestion of SiO_2 NPs to simulate human ingestion at the currently reported levels of exposure to SiO_2 NPs.

This in vitro study evaluates the effect of SiO_2 NPs on intestinal epithelial barrier functioning. Cell viability and PLK1 protein expression are measured to investigate the potential effects of SiO_2 NPs at different doses on gut barrier functioning in relation to inflammatory diseases of the gut.

4.3 Materials and methods

Methods for determining SiO_2 NP physiological doses

The EFSA Panel on Food Additives and Flavourings (EFSA FAF Panel) determined that the maximum dose relevant to food related ingestion of E551 by children is 79 mg/kg bw per day. Based on the average weight of a 9-year-old child, it can be assumed that $2.2x10^3$ mg is consumed per day. Considering the surface area of the small intestine (2.5x10^6 cm²), an exposure equivalent of 0.89 µg/cm^2 of E551 per day was estimated and applied to HT29 cells cultured in vitro. This calculated dose is referred to as the physiological dose as it represents the true exposure of small intestinal cells to digested NPs. This study exposes HT29 cells to various concentrations relative to the above calculated physiological dose (0.02X= 0.17 µg/cm^2, 1X=0.89 µg/cm^2, and 50X=44 µg/cm^2).

Methods for SiO_2 NP lyophilization

Fumed Silica was purchased from Millipore Sigma (SiO_2 NPs, S5130) and 600mg were diluted in 20mL of 2% sucrose (Bio Basic SB0498) followed by centrifugation for 15 minutes at 10 000 rpm. This wash was completed a total of 3 times before the pellet was resuspended in 20mL of 2% sucrose and filtered through a 0.22 µm syringe filter (Celltreat 229747). The sample was then sonicated for 30 minutes at room temperature and left to freeze overnight at -20°C. Samples were

then lyophilized to produce a stable powder which was stored at room temperature or diluted in deionized water for immediate use in SiO_2 NP exposure experiments.

Methods for SiO_2 NP Characterization

SiO_2 NPs purchased from Millipore Sigma were characterized for size, polydispersity index (PDI), and zeta potential using the Brookhaven Zeta PALS instrument. SiO_2 NPs were dispersed in deionized water to 1 mg/mL for zeta potential measurements and 25 mg/mL for size and PDI measurements. SiO_2 NPs were prepared using sonication for 30 minutes at 50°C and filtration using a 0.22 µm syringe filter immediately prior to characterization. In another preparation method, the filtration step was removed. Error bars represent a mean of standard errors from 10 measurements repeated 3 times.

Methods for HT29 cell culture

A human colorectal adenocarcinoma cell line with epithelial morphology (HT29) at Passage #18 was generously donated by Prof. Syaram Pandey in the Department of Chemistry and Biochemistry (University of Windsor). Cells were cultured in L-glutamine (0.21 g/L) containing McCoy 5A media (Fisher scientific, MT10050CV) supplemented with 10% FBS (VWR, 76419-584). Cells were passaged using 0.25% Trypsin-EDTA and media was changed every two days. The ATCC 'Protocol for Thawing, Propagating and Cryopreserving of NCI-PBCF-HTB38 (HT-29, ATCC®HTB-38 ™) cells colorectal carcinoma' was followed closely.

Methods for Bacterial cell culture

The probiotic strain *L. fermentum NCIMB 5221* (Lf5221) was purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). Lf5221 was maintained by continuous subculturing in MRS broth at 2% (v/v) in a 37°C static aerobic

incubator. Bacterial growth was monitored via colony counting ($2.7x10^8$ CFU/mL), and the stalk diluted using McCoy 5A Media to a concentration of $2.63x10^2$ CFU/cm^2 for cell viability experiments.

Methods for In vitro *digestion of SiO_2 NPs, preparation of doses, and incubation with cells*

In vitro digestion of SiO_2 NPs was completed following methods previously described by other researchers [129, 130]. SiO_2 NPs were taken through a simulated digestion process. Mouth digestion was simulated using a salt solution (0.1mL of 1M KCl, 0.3mL of 1M CaCl2, and 1.4mL of NaCl). Stomach digestion was simulated using a gastric solution (0.2 g pepsin in 8 mL of 0.1M HCl). Finally, intestinal digestion was simulated using a pancreatic solution (0.05 g pancreatin and 0.3 g bile extract in 27 mL of 0.1 M NaHCO3). 1M $NaHCO_3$ and 1M HCl was used to pH solutions.

Previously lyophilized SiO_2 NPs were first dispersed in deionized water (10mg/mL), and 1mL of this was taken through the digestion process. First, 1.8mL of the salt solution was added for 1-5 minutes, then 1M HCl was used to bring the pH to 2, then 50µL pepsin solution was added followed by incubation horizontally for 1h on a tabletop shaker (150 rpm, 37 degrees, 5% CO2/95% air). The pH was increased to 6 and 250µL of pancreatic solution was added. The pH was adjusted to 7.0 and the volume was brought to 20mL using a 1:1 ratio of NaCl (120mmol/L) and KCl (5mmol/L). The SiO_2 NPs were at this point considered digested and taken though a series of dilutions in McCoy 5A media to generate the different concentrations relative to the previously calculated physiological dose (0.01X, 1X, 50X) for HT29 exposure. Specifically, this was 0.17 µg/cm^2, 0.89 µg/cm^2, and 44 µg/cm^2.

HT29 cells at P34-36 were seeded in 96-well plates at 20 000 cells/well. SiO_2 NP exposure took place 4 days post passage for cell viability experiments and 3 days post passage for PLK1 protein expression experiments. Cells were exposed to each concentration of digested SiO_2 NPs for 23 hrs. Following this, media containing NPs was removed. One group was treated with the probiotic Lf5221 diluted in McCoy 5A media at $2.63x10^2$ CFU/cm^2 for 4 hrs, and another group received only the complete McCoy 5A medium.

Methods for measuring HT29 cell viability using MTT Assay

MTT reagent was dissolved in PBS (0.5mg/mL, CAT. #: T0793) and filtered using a 0.22 µm syringe filter. 50µL of this was added to each well of the 96-well plate followed by incubation for 1 hour at 37°C under 5% CO2/95% air. 100µL of isopropanol-HCL solution (35µL of 6M HCl in 10 ml isopropanol) was used to dissolve the crystals. Absorbance was read at 560nm using a Perkin Elmer HTS 7000 Bioassay Reader.

Materials and methods for PLK1 protein expression

The PLK1 Colorimetric Cell-Based ELISA Kit (Assay Biotechnology, CB5577) was used to measure PLK1 expression in HT29 cells. In short, cells were fixed with 4% paraformaldehyde and quenched using Quenching Buffer. Binding sites were blocked using Blocking Buffer. PLK1 protein was targeted using Anti-PLK1 Primary Antibody (rabbit monoclonal) and HRP-Conjugated Anti Rabbit IgG secondary Antibody. Positive control wells were incorporated targeting the GAPDH protein using the Anti-GAPDH Primary Antibody (mouse, monoclonal) and HRP-Conjugated Anti-Mouse IgG secondary Antibody. Negative controls were incorporated containing the secondary antibodies alone. Substrate was added and the colorimetric reaction read at 450nm using a Perkin Elmer HTS 7000 Bio Assay Reader. Following this, the crystal

violet cell staining was utilized on the same plate to stain nuclei and measure absorbance at 595nm. Absorbance values representing PLK1 protein expression were normalized to absorbance values representing cell amounts (450nm/595nm). Results were then presented relative to the control treated group which received media alone.

Statistical analysis

Data is expressed as mean ± standard deviation. Analysis of cell viability and PLK1 expression was completed using the data analysis and graphing software Origin 2021b. Results were analysed using pairwise comparison using the paired comparison plot app (v3.60) for pairwise comparison of experimental groups. Statistical significance is indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Error bars represent standard deviation. The mean comparison method used was one-way analysis of variance (ANOVA) with Turkey's post test.

4.4 Results

Physicochemical characterization of SiO_2 NPs

Zeta potential, hydrodynamic size, and polydispersity index (PDI) of undigested SiO_2 NPs were characterized using the Brookhaven Zeta PALS instrument following two methods of NP preparation. SiO_2 NPs that had been sonicated and filtered using a 0.22 µm syringe filter had a hydrodynamic size of 128 nm and a zeta potential of -23.6 mV (Fig. 6). Omitting the filtration step resulted in a higher average size of NPs (164 nm) as well as a slight reduction in the magnitude of the zeta potential (-21.4 mV). TEM image can bee seen which confirms the presence of SiO_2 nanoparticles (Fig. 7).

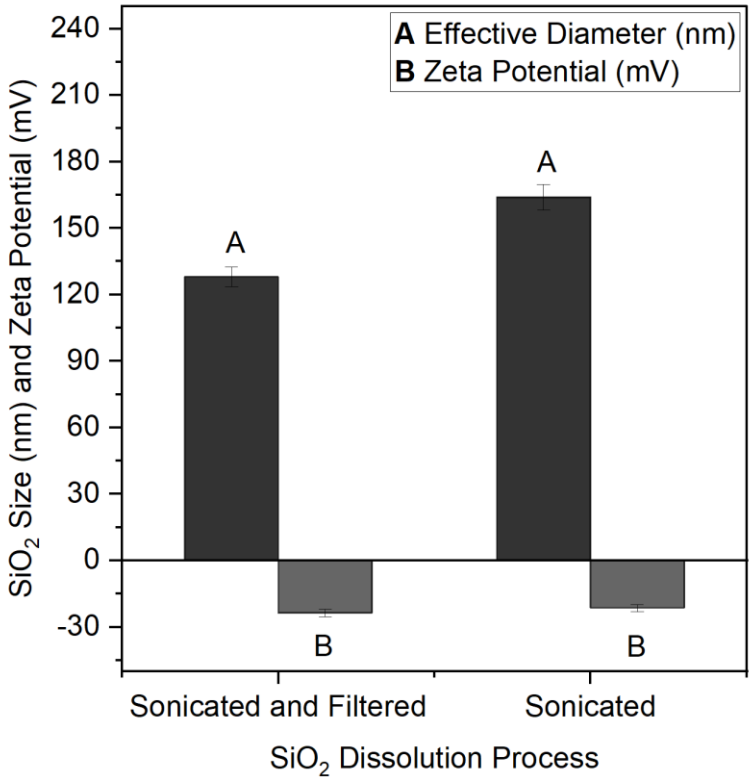

Figure 6. SiO_2 NP size and zeta potential characterization following two methods of preparation. In the first method, SiO_2 NPs were dispersed in deionized water (1 mg/mL for zeta potential and 25 mg/mL for size), sonicated for 30 minutes at 50°C, and filtered (0.22 μm). Analysis using the Brookhaven Zeta PALS showed a hydrodynamic size of 128 nm and zeta potential of -23.6 mV. In the second method, the filtration step was omitted, and results showed reduced size (164 nm) and zeta potential (-21.4 mV). Error bars represent a mean of standard errors from 10 measurements repeated 3 times.

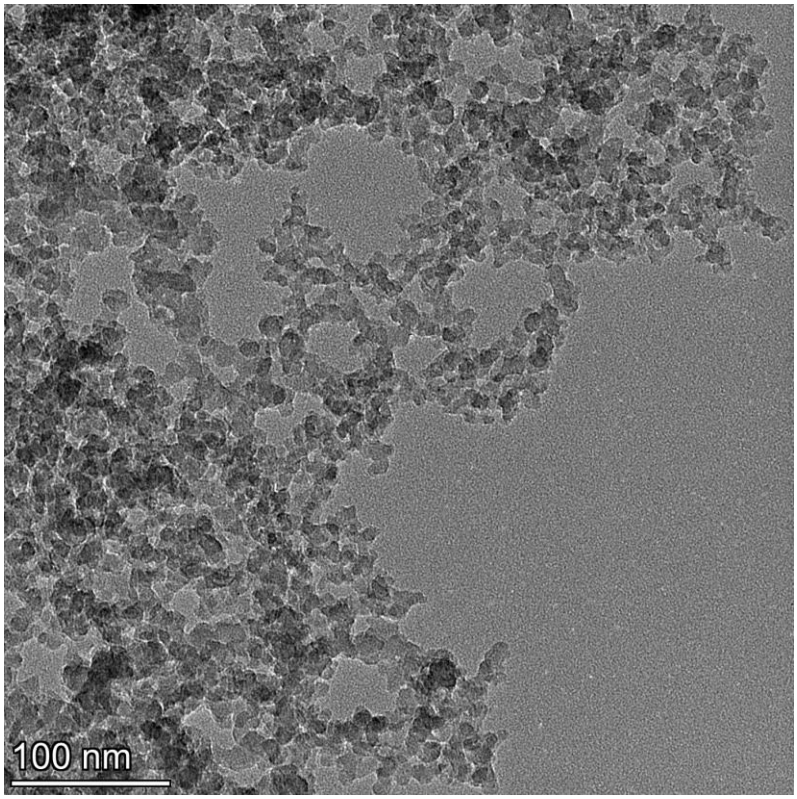

Figure 7. TEM image of SiO_2 Nanoparticles taken using the Thermo Scientific Talos F200X G2 (S) instrument.

The effect of SiO_2 NP exposure on HT29 cell viability

SiO_2 NPs, at all physiological doses, had no effect on HT29 cell viability. After exposing HT29 cells to in vitro digested SiO_2 NPs at various concentrations (0.17 µg/cm^2, 0.89 µg/cm^2, and 44 µg/cm^2), it was found that cell viability was not impacted negatively by each concentration tested (83%, 92%, and 81%, respectively) when compared to the digest control (81%) (Fig. 8). Control cells receiving digest components showed reduced cell viability compared to cells receiving the media control by 19%.

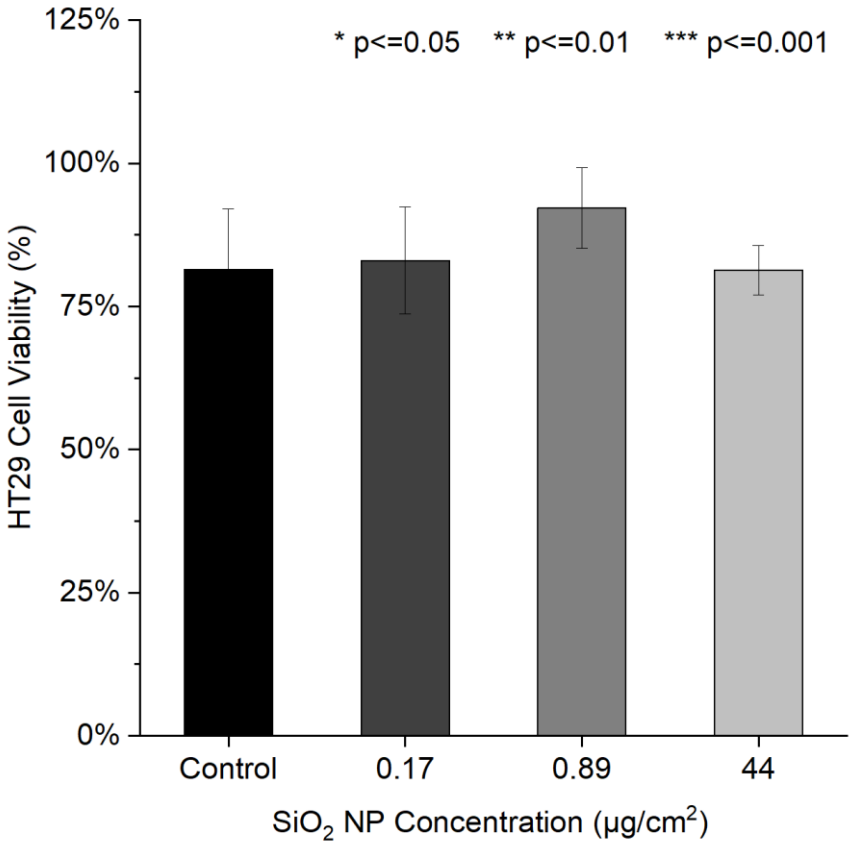

Figure 8. Effect of SiO_2 NPs at different concentrations on HT29 cell viability. Following 23-hour incubation of HT29 cells with SiO_2 NPs at 0.17 µg/cm^2, 0.89 µg/cm^2, and 44 µg/cm^2, cell viability was measured using an MTT assay. Results showed no significant change in cell viability. Control group represents cells incubated with digest components without NPs. Results are normalized to a control group treated with McCoy 5A media alone. Error bars indicate standard deviation (n=6).

The effect of SiO_2 NP exposure on HT29 cell viability following Probiotic treatment

HT29 cell viability is not impacted by treatment with both *Lf5221* and SiO_2 NPs. The probiotic was introduced to HT29 cells after HT29 incubation with different concentrations of SiO_2 NPs (0.17 µg/cm^2, 0.89 µg/cm^2, and 44 µg/cm^2). MTT assay was used to measure cell viability. There was no significant difference in cell viability of groups exposed to SiO_2 NPs at the various concentrations (84%, 85%, and 81%, respectively) followed by Lf5221 compared to those

receiving the digest control (81%) (Fig. 9). Additionally, the cell viability exhibited by groups receiving NPs and probiotics was comparable to that seen in groups exposed to NPs alone (Fig. 8). Of interest was the statistically significant increase in cell viability of cells exposed to probiotics alone (97%) compared to all other experimental and control groups (<85%).

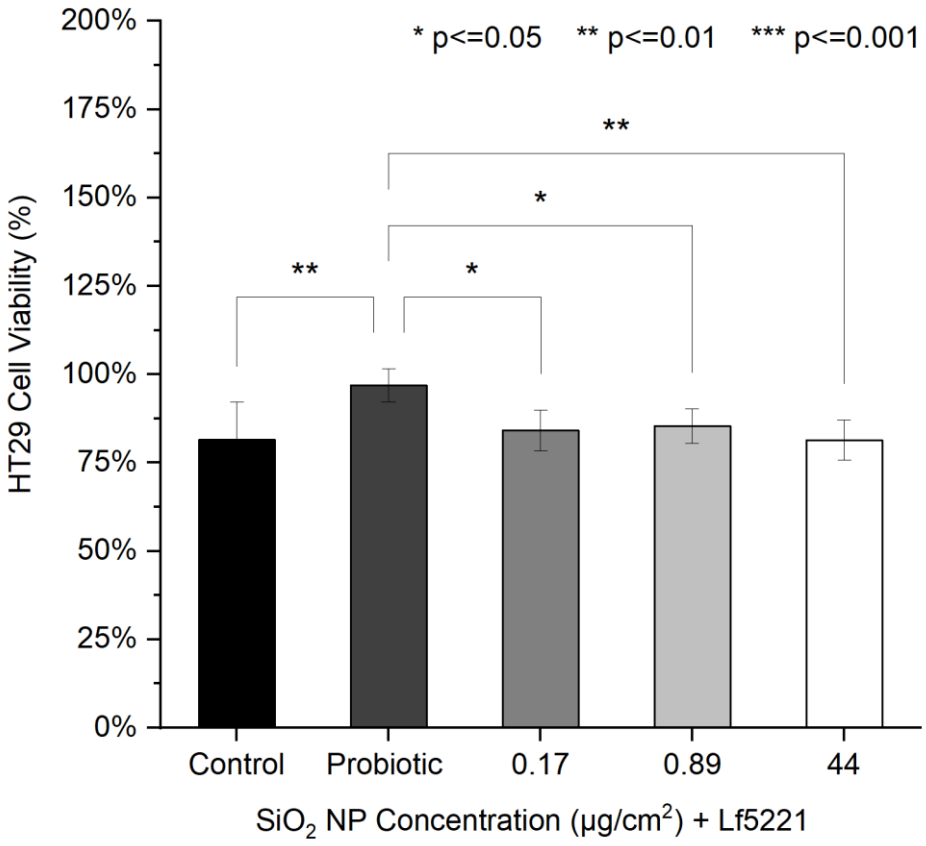

Figure 9. Impact of *L. fermentum NCIMB 5221 (Lf5221)* on HT29 cells previously treated with SiO_2 NPs at different doses. HT29 cell viability was measured using MTT assay after 23 hrs of incubation with SiO_2 NPs (0.17 µg/cm^2, 0.89 µg/cm^2, and 44 µg/cm0^2) followed by 4 hrs of incubation with Lf5221 at 263CFU/cm^2. Results show no significant change in cell viability because of SiO_2 NPs. Control represents cells exposed to digest components without NPs and probiotic control represents cells exposed to the probiotic Lf5221 with no prior exposure to NPs. Data is normalized to a control group treated with McCoy 5A complete media. Error bars indicate standard deviation (n=6).

The effect of SiO_2 NP exposure on PLK 1 protein expression

SiO_2 NPs cause significant increase in PLK1 protein expression of intestinal epithelial cells in vitro. PLK1 protein expression was investigated via immunostaining after incubating HT29 cells with SiO_2 NPs at different concentrations for 26 hrs. It was found that SiO_2 NP exposure at 44 $\mu g/cm^2$ caused a significant increase in relative PLK1 protein expression compared to the digest control and 0.17 $\mu g/cm^2$ SiO_2 NP treated groups (Fig. 10). There seems to be higher relative PLK1 protein expression with increasing dose of SiO_2 NP treatment, however this was not statistically significant. The difference in PLK1 protein expression between 0.17 $\mu g/cm^2$ and 44 $\mu g/cm^2$ SiO_2 NP treated groups was significant suggesting a potential dose dependent response.

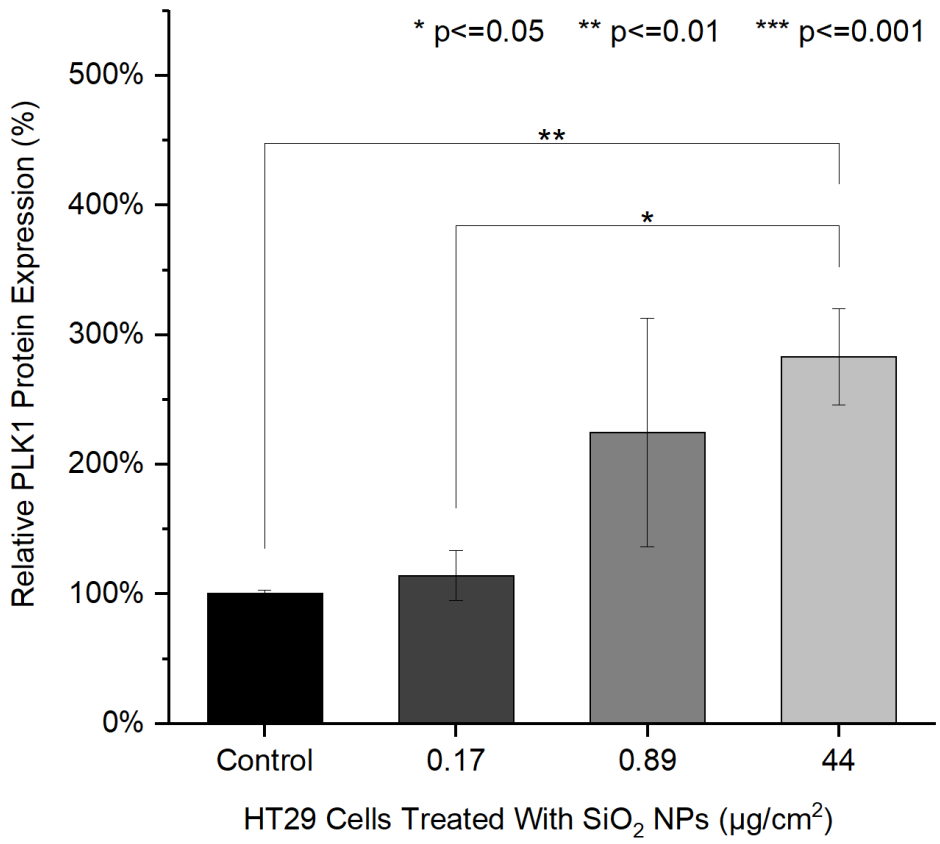

Figure 10. Relative PLK1 protein expression of intestinal epithelial cells following treatment with SiO_2 NPs at different doses relevant to current human exposure levels. HT29 cells were incubated for 26 hrs with SiO_2 NPs at different concentrations (0.17 µg/cm^2, 0.89 µg/cm^2, and 44 µg/cm^2). PLK1 protein expression was then measured using a colorimetric ELISA immunoassay (OD 450nm) normalized to account for cell amounts (OD 450nm/595nm). Data was analysed relative to a control group receiving no treatment (McCoy 5A Media). Results show significant increase in PLK1 protein expression for cells exposed to 44 µg/cm^2 SiO_2 NPs, as well as non-significant SiO_2 NP dose dependent increase in PLK1 expression. Control groups represents cells incubated with digest components alone without NPs. Error bars indicate standard deviation.

4.5 Discussion

Various studies have assessed the impact of SiO_2 NPs on in vitro models. One study showed that SiO_2 NPs can upregulate MHC-II, CD80, and CD86 on dendritic cells [8]. This was shown to induce apoptosis and activate the inflammasome, a protein complex involved in innate immunity, resulting in the secretion of IL-1β. Another study incubated Caco-2 derived cells with 100 mg/cm² silica NPs and found NPs internalized in the cytoplasm of cells but not in the nucleus [137]. Others combined exposure to TiO_2 and SiO_2 NPs and found unexpected activation of the caspase-1 inflammasome and IL-1β release in macrophages [25]. Interestingly, only SiO_2 NPs were found localized in lysosomes, not TiO_2 NPs. Furthermore, SiO_2 and TiO_2 NPs aggregated more easily as individuals and become stable at ~250 nm when combined in the presence of cations. In conclusion, the study found that SiO_2 and TiO_2 NPs work together to induce an inflammatory response.

In contrast to the literature findings which show increased apoptosis, the current study reports there was no impact of SiO_2 NPs on HT29 cell viability. This may be due to many differences in experimentation. The first is the use of one NP alone rather than in combination with other inorganic NPs. This may also be due to differences in NP size and dose used in other studies. It is important to note that the doses used in other studies is not physiologically relevant, often well above normal consumption levels. Additionally, studies using 70nm and 300nm silica particles have shown that intestinal absorption was dependent on particle surface properties as well as particle diameter [27]. Similarly, researchers using human intestinal follicle-associated epithelium (FAE) have found that silica absorption is higher when using silica NPs (3.94%) compared to bulk silica materials (2.95 %) [138]. This could translate to differing biological effect of silica based on size considering that nano-sized particles can be actively transported

through M cells; cells located in the epithelium which serve to transport luminal antigens thereby initiating an immune response. Given all these factors, it is possible that SiO_2 NPs may impact cells differently depending on the environmental surroundings, surface properties, size, and dose used.

Serine/threonine-protein kinase, also known as polo-like kinase 1 (PLK-1), is an enzyme encoded by the PLK1 gene. The Nuclear Factor (NF)-κB pathway is a major player in mucosal barrier functioning and a regulator of inflammatory gene expression (IL-6) and tumor necrosis factor (TNF)-α. The exact effect of NF-κB activation on cell apoptosis is debated. On one hand, it may induce expression of proteins that inhibit apoptosis, but it may also promote inflammatory factors such as TNF-α and IL-6 which promote apoptosis. The balance of epithelial cell proliferation and apoptosis is important for proper IBF [139]. Cell death by necroptosis can trigger inflammation via the release of danger associated molecular patterns (DAMPs). In the case of intestinal epithelial cells, even cell death by apoptosis can lead to inflammation as the loss of cells disrupts barrier functioning leading to bacterial invasion. Indeed, epithelial cell death is an important factor of human inflammatory diseases including Crohn's disease and Ulcerative colitis. For example, it is thought that intestinal inflammation in IBD results from TNF-induced death of IEC's which is why anti-TNF treatment has been shown to inhibit cell death in Crohn's disease and Ulcerative colitis. One study revealed that down regulation of PLK1 activates the NF-κB pathway leading to apoptosis in HT29 cells [140]. Specifically, in HT29 cells with lipopolysaccharide (LPS)-induced intestinal sepsis, PLK1 was downregulated leading to apoptosis. This was supported by the increase in markers of apoptosis (pro-caspase-3 and pro-caspase-9). Accordingly, inhibition of the NF-κB pathway reduced LPS-induced apoptosis. Therefore, researchers established PLK1 as an upstream regulator of NF-κB during

sepsis making its downregulation critical for NF-κB induced intestinal barrier dysfunction. In this study, it was shown that SiO_2 NPs at 50X the physiological dose caused significant increase in PLK1 protein expression in HT29 cells. Based on the discussed ways in which PLK1 can exert its effects on the cell, it is difficult to determine whether a NP induced increase in PLK1 protein expression is beneficial or harmful to the cell. Further research must be done on the other signaling molecules involved in both diseased and healthy models to elucidate the role of the SiO_2 NPs in barrier functioning.

As mentioned, it is important to consider the environment surrounding SiO_2 NPs in the assessment of their toxicity as their physicochemical characteristics can impact their ability to interact with biological systems. These environmental factors include the parameters of the food matrix in which NPs are incorporated, or the GIT once they have been ingested such as the pH and presence of solutes in each compartment of the GIT. Researchers have created in vitro models of the GIT to simulate the process of human digestion starting from the mouth to the stomach and intestines. Using this kind of model, one study found that nano-sized silica remained present during the mouth phase of digestion, then became agglomerated in the gastric phase, only to return during the intestinal digestion stage [141]. Authors explain that the agglomeration of silica NPs during the gastric phase may be due to the low pH and high electrolyte concentrations accompanied by the gastric phase of digestion. Researchers have shown that these larger amorphous silica particles that have agglomerated due to being dispersed in food matrix containing simulated intestinal fluids (size > 1,000 nm) are less likely to be absorbed or transport through human Caco-2 cells [142]. By contrast, the fasted-state simulated intestinal fluids (lacking the food matrix) did not result in agglomeration of the silica particles (50, 100 or 200 nm), allowing for greater absorption and transport by Caco-2 cells. In this study,

the SiO_2 NPs were not incorporated into a food matrix, which means that there may have been greater absorption by HT29 cells than would be the case under normal human exposure conditions where the NPs are incorporated into foods and thus are more likely to be agglomerated. Therefore, one limitation to this study is the use of SiO_2 ENMs alone rather than in combination with a food matrix as this clearly affects its behaviour and interaction with biological systems [6]. Additionally, due to the in vitro digestion process utilized in this study, involving the addition of various solutes and several pH changes, it is likely that SiO_2 NPs were subject to many physicochemical changes which may have affected their ability to negatively impact the intestinal model at the cellular level. This digestion process is a close representation of the environments encountered by food NPs following human ingestion, thus making the present study relevant to the study of food NP toxicity.

4.6 Conclusion

In conclusion, it has been shown that SiO_2 NPs do not negatively impact intestinal epithelial cell viability at all exposure relevant doses tested. This response is the same when cells are exposed to the probiotic strain Lf5221 following SiO_2 NP exposure. Finally, relative PLK1 protein expression of intestinal cells increased significantly following treatment with 50X the currently reported SiO_2 NP exposure level (50X=44 $\mu g/cm^2$). There was also a statistically insignificant increase in PLK1 protein expression with increasing dose of SiO_2 NPs.

4.7 Acknowledgements

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assistance provided by Michael Yitayew (Ph.D. Candidate) which made NP characterization possible. We thank the Facility for Electron Microscopy Research of McGill University for help in microscope operation and data collection. We thankfully acknowledge financial support provided by the Natural Sciences and Engineering Research Council (NSERC) of Canada.

Chapter 5

Impact of Silver Nanoparticles on Human Intestinal Barrier Function in Vitro

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Preface

In chapter 4, SiO_2 NPs (44 μg/cm^2) were shown to significantly increase relative PLK1 protein expression in human intestinal epithelial cells. However, in chapter 3, TiO_2 NPs were shown to have no impact on PLK1 protein expression at all doses tested. In all cases, NPs had no impact on cell viability. In the following chapter, another common food NP is selected for similar investigation to determine weather it has an impact on signaling proteins implicated in proliferation of intestinal epithelial cells. Additionally, the role of a probiotic in this process is investigated. This study involved the use of various doses of NPs relevant to the currently reported human consumption levels.

5.1 Abstract

Silver nanoparticles (Ag NPs) can be found in the food additive E174 which is used in liquor, confectionary, and other foods. Unfortunately, some studies have shown that Ag NPs can induce oxidative stress, an inflammatory response, and lead to dysbiosis of the gut microbiome. Further investigation of Ag NPs relevant to foods is needed to contribute to the currently conflicting literature. In this study, Ag NPs were investigated to determine their effects on intestinal barrier functioning. Ag NPs were digested in vitro at different doses relative to current human exposure levels (0.02X, 1X, 50X) and incubated with HT29 cells, a human colon adenocarcinoma cell line. Cell viability was examined as well as PLK1 protein expression. Ag NPs at 0.02X and 50X did not alter cell viability when compared to the digest, however 1X Ag NP treated cells had significantly higher viability compared to the digest control and 0.02X Ag NP group. Treatment with the probiotic *L. fermentum NCIMB 5221* (Lf55221) following 1X NP exposure reduced cell viability back to that seen in the digest control groups. Additionally, the probiotic control groups exhibited a significant increase in cell viability compared to digest

control groups. Most notably, exposure to Ag NPs at 1X and 50X currently reported exposure levels caused a significant increase in relative expression of PLK1 protein when compared to those treated with 0.02X Ag NPs or controls. There was no significant difference between the 1X and 50X Ag NP exposed groups. Ag NPs were also characterized for size and zeta potential to ensure relevance to NP sizes found in the food additive E174. Ag NPs that were only sonicated showed a significantly higher size (249 nm) compared to Ag NPs that had been filtered using a 0.22 μm filter (90 nm). Ag NPs were also found to be stable with a zeta potential of -23mV before filtration and -17 mV after filtration. Study findings have the potential to aid the formulation of future therapeutics for Ag NP related damage to the intestinal barrier.

5.2 Introduction

Ag NPs can be found in the food additive E174 commonly utilized as a preservative due to its antimicrobial activity. Based on an acute oral toxicity study, spherical Ag NPs (10-20 nm, 5,000 mg/kg bw) did not lead to mortality or acute toxic signs in ICR mice (a strain of albino mice) [143]. By contrast, repeated long term oral administration of Ag NPs (22, 42, 71 nm) led to organ toxicity [144]. Thus, Ag NPs may cause organ toxicity in mice despite not showing short term and subchronic toxicity. Studies on rats has also shown that Ag NPs (300 mg Ag NPs /kg bw/day) can lead to liver damage. Moreover, a histochemical study of intestinal mucins of the rats showed that they induce discharge of mucus granules and abnormal mucus composition in goblet cells. The small and large intestinal lamina propria showed a simultaneous dose dependent accumulation of Ag NPs with abnormal mucus composition in goblet cells [20, 145]. Furthermore, a 90-day study displayed the treatment-related effects of bile duct hyperplasia in the liver due to Ag NPs, although the EFSA panel considered this to have inadequate research backup [145, 146]. Finally, Ag NPs have been shown to damage the intestinal epithelia of mice,

including the loss of microvilli, thereby reducing their absorptive capacity [147]. It is therefore clear that Ag NPs have the capacity to impact IBF.

As is usually accompanied by nickel with a known sensitizing property, the reports of allergy associated with them were considered to be irrelevant for food additives [148]. Mice orally treated with Ag NPs repeatedly showed an increase in cytokine levels causing organ toxicity and inflammatory responses, however this has not been reported in rats [144, 149]. Ag nano colloids were observed to reduce monocyte counts in mice blood leading to reduced proliferation of lymphocytes and phagocytosis [150]. Hamilton et al showed that 20 nm NPs produce more toxicity to macrophages and epithelial cells compared to 110 nm NPs as smaller particles experience rapid dissolution in acidic phagolysosomes following the principles of Ag ion mediated toxicity [151]. Ag NPs increase intracellular levels of ROS, initiate the release of neutrophil extracellular traps, and inhibit nitric monoxide formation and protein phosphatase activity [152]. Though these studies were inconsistent, the EFSA panel concluded that Ag NPs may have an effect on the immune system [148].

Given such findings on the impact of Ag NPs on the GIT, this study aims to add to the current literature by investigating the effects of Ag NPs on HT29 cell viability as well as PLK1 protein expression, a protein involved in the homeostasis between cell proliferation and apoptosis. Given established benefits of the *Lactobacillus fermentum* probiotic on the amelioration of intestinal epithelial barrier dysfunction [9], this study also investigates the use of *Lactobacillus fermentum NCIMB 5221* (Lf5221) in the treatment of potential Ag NP induced gut damage.

5.3 Materials and Methods

Methods to determine a physiological Ag NP dose relevant to human consumption

The EFSA Panel on Food Additives and Flavourings (EFSA FAF Panel) determined that the maximum dose relevant to food related ingestion of E174 by children is 12 µg/kg bw/day. Based on the average weight of a 9-year-old child, it can be assumed that 336mg is consumed per day. Considering the surface area of the small intestine (2.5x10^6 cm^2), an exposure equivalent of 0.13 ng/cm^2 of E174 per day was estimated and applied to HT29 cells cultured in vitro. This calculated dose is referred to as the physiological dose as it represents the true exposure of small intestinal cells to digested NPs. This study exposes HT29 cells to various concentrations relative to the calculated physiological dose (0.02X= 0.0027 ng/cm^2, 1X= 0.13 ng/cm^2, and 50X= 6.7 ng/cm^2).

Method of Ag NP preparation and lyophilization

Ag nano-powder was purchased from Millipore Sigma (Ag NPs containing PVP as dispersant; 576832) and 600mg were diluted in 20mL of 2% sucrose (Bio Basic SB0498) followed by centrifugation for 15 minutes at 10 000rpm. This wash was completed a total of 3 times before the pellet was resuspended in 20mL of 2% sucrose and filtered through a 0.22 µm syringe filter (Cell treat 229747). The sample was then sonicated for 30 minutes at room temperature and left to freeze overnight at -20°C. Samples were then lyophilized to produce a stable powder which was stored at room temperature or diluted in deionized water for immediate use in Ag NP exposure experiments.

Method of characterizing Ag NP size and zeta potential

Ag NPs purchased from Millipore Sigma were characterized for size, polydispersity index (PDI), and zeta potential using the Brookhaven Zeta PALS instrument. Ag NPs were dispersed in

deionized water to 1 mg/mL for zeta potential measurements and 25 mg/mL for size and PDI measurements. Ag NPs were prepared using sonication for 30 minutes at 50°C and filtration using a 0.22 µm syringe filter immediately prior to characterization. In another preparation method, the filtration step was removed. Error bars represent a mean of standard errors from 10 measurements repeated 3 times.

Methods for culturing HT29 cells

A human colorectal adenocarcinoma cell line with epithelial morphology (HT29) at Passage #18 was generously donated by Prof. Syaram Pandey in the Department of Chemistry and Biochemistry (University of Windsor). Cells were cultured in L-glutamine (0.21 g/L) containing McCoy 5A media (Fisher scientific, MT10050CV) supplemented with 10% FBS (VWR, 76419-584). Cells were passaged using 0.25% Trypsin-EDTA and media was changed every two days. The ATCC 'Protocol for Thawing, Propagating and Cryopreserving of NCI-PBCF-HTB38 (HT-29, ATCC®HTB-38 ™) cells colorectal carcinoma' was followed closely.

Methods for culturing bacteria

The probiotic strain *L. fermentum NCIMB 5221* (Lf5221) was purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). Lf5221 was maintained by continuous subculturing in MRS broth at 2% (v/v) in a 37°C static aerobic incubator. Bacterial growth was monitored via colony counting ($2.7x10^8$ CFU/mL), and the stalk diluted using McCoy 5A Media to a concentration of $2.63x10^2$ CFU/cm^2 for cell viability experiments.

Method for simulated in vitro *digestion of Ag NPs and incubation with HT29 cells*

In vitro digestion of Ag NPs was completed following methods previously described by other researchers [129, 130]. Ag NPs were taken through a simulated digestion process. Mouth digestion was simulated using a salt solution (0.1mL of 1M KCl, 0.3mL of 1M CaCl2, and 1.4mL of NaCl). Stomach digestion was simulated using a gastric solution (0.2 g pepsin in 8 mL of 0.1M HCl). Finally, intestinal digestion was simulated using a pancreatic solution (0.05 g pancreatin and 0.3 g bile extract in 27 mL of 0.1 M $NaHCO_3$). 1M $NaHCO_3$ and 1M HCl was used to pH solutions.

Previously lyophilized Ag NPs were first dispersed in deionized water (10mg/mL), and 1mL of this was taken through the digestion process. First, 1.8mL of the salt solution was added for 1-5 minutes, then 1M HCl was used to bring the pH to 2, then 50µL pepsin solution was added followed by incubation horizontally for 1h on a tabletop shaker (150 rpm, 37 degrees, 5% CO2/95% air). The pH was increased to 6 and 250µL of pancreatic solution was added. The pH was adjusted to 7.0 and the volume was brought to 20mL using a 1:1 ratio of NaCl (120mmol/L) and KCl (5mmol/L). The Ag NPs were at this point considered digested and taken though a series of dilutions in McCoy 5A media to generate the different dosages (0.0027 ng/cm^2, 0.13 ng/cm^2, 6.7 ng/cm^2) for incubation with HT29 cells.

HT29 cells at P34-36 were seeded in 96 well plates at 20 000 cells/well. Ag NP exposure took place 4 days post passage for cell viability experiments and 3 days post passage for PLK1 expression experiments. Cells were exposed to each concentration of digested Ag NPs overnight for 26 hrs at 0.02X, 1X, and 50X the calculated physiological exposure. Following this, media containing NPs was removed. One group was treated with the probiotic *L. fermentum* diluted in McCoy 5A medium at 2.63x10^2 CFU/cm^2 for 4 hrs, and another group received only the complete McCoy 5A medium.

Methods to determine HT29 cell viability using MTT Assay

MTT reagent was dissolved in PBS (0.5mg/mL, CAT. #: T0793) and filtered using a 0.22 µm syringe filter. 50µL of this was added to each well of the 96-well plate followed by incubation for 1 hour at 37°C under 5% CO2/95% air. 100µL of isopropanol-HCL solution (35µL of 6M HCl in 10 ml isopropanol) was used to dissolve the crystals. Absorbance was read at 560nm using a Perkin Elmer HTS 7000 Bioassay Reader.

Methods to measure relative PLK1 protein expression

The PLK1 Colorimetric Cell-Based ELISA Kit (Assay Biotechnology, CB5577) was used to measure PLK1 expression in HT29 cells. In short, cells were fixed with 4% paraformaldehyde and quenched using Quenching Buffer. Binding sites were blocked using Blocking Buffer. PLK1 protein was targeted using Anti-PLK1 Primary Antibody (rabbit monoclonal) and HRP-Conjugated Anti Rabbit IgG secondary Antibody. Positive control wells were incorporated targeting the GAPDH protein using the Anti-GAPDH Primary Antibody (mouse, monoclonal) and HRP-Conjugated Anti-Mouse IgG secondary Antibody. Negative controls were incorporated containing the secondary antibodies alone. Substrate was added and the colorimetric reaction read at 450nm using a Perkin Elmer HTS 7000 Bio Assay Reader. Following this, the crystal violet cell staining was utilized on the same plate to stain nuclei and measure absorbance at 595nm. Results were then calculated as a ratio of absorbance values representing PLK1 protein expression and absorbance values representing cell amounts (450nm/595nm). These results were then normalized to the media treated control groups.

Statistical analysis

Data is expressed as mean ± standard deviation. Analysis of cell viability and PLK1 expression was completed using the data analysis and graphing software Origin 2021b. Results were analysed using pairwise comparison using the paired comparison plot app (v3.60) for pairwise comparison of experimental groups. Statistical significance is indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Error bars represent standard deviation. The mean comparison method used was one-way analysis of variance (ANOVA) with Turkey's post test.

5.4 Results

Ag NP characterization

Ag NP size and zeta potential was found to be dependent on the method of NP preparation. Ag NPs were dispersed in deionized water followed by sonication and/or filtration and the effects on size and zeta potential measured. Ag NPs had a significantly smaller average size when filtered after sonication (90.1nm) compared to when NPs were only sonicated and not filtered (249 nm) (Fig. 11). Similarly, the zeta potential was also smaller in magnitude after filtration (-16.5mV) compared to when filtration was omitted from sample prep (-21.7 mV). The presence of Ag nanoparticles is also confirmed by a TEM image (Fig. 12).

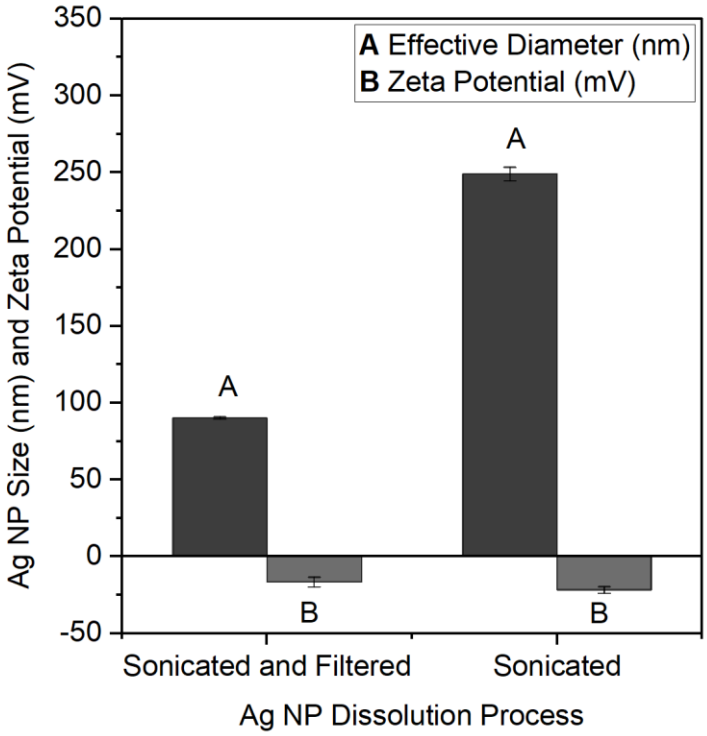

Figure 11. Ag NP characterization of size and zeta potential following two methods of preparation. In the first method, Ag NPs were dispersed in deionized water (1 mg/mL), sonicated for 30 minutes at 50°C, and filtered using a syringe filter (0.22 µm). In the second method, the filtration step was omitted. The Brookhaven Zeta PALS instrument was used to measure size and zeta potential. Results showed Ag NPs have a hydrodynamic size of 249 nm and zeta potential of -21.7 mV when sonicated, but they decrease to 90 nm and -17 mV following filtration. Error bars represent a mean of standard errors from 10 measurements repeated 3 times.

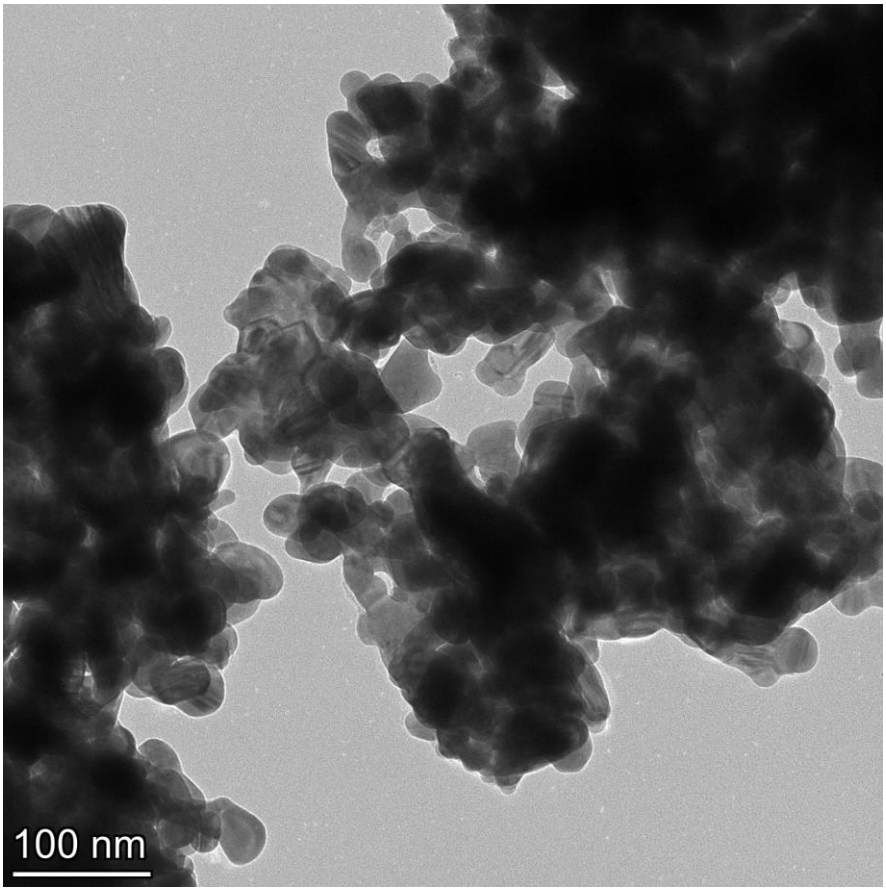

Figure 12. TEM image of silver nanoparticles taken using the Thermo Scientific Talos F200X G2 (S) instrument.

The effect of Ag NP exposure on HT29 cell viability

Ag NPs can cause significant increase in HT29 cell viability. After exposing HT29 cells to in vitro digested Ag NPs, it was found that cell viability was reduced after treatment with the digest control alone (Fig. 13). Additionally, HT29 cells treated with 0.13 ng/cm^2 Ag NPs showed significantly increased cell viability compared to the control. All other concentrations of Ag NPs tested (0.0027 ng/cm^2 and 6.7 ng/cm^2) did not alter cell viability when compared to the control.

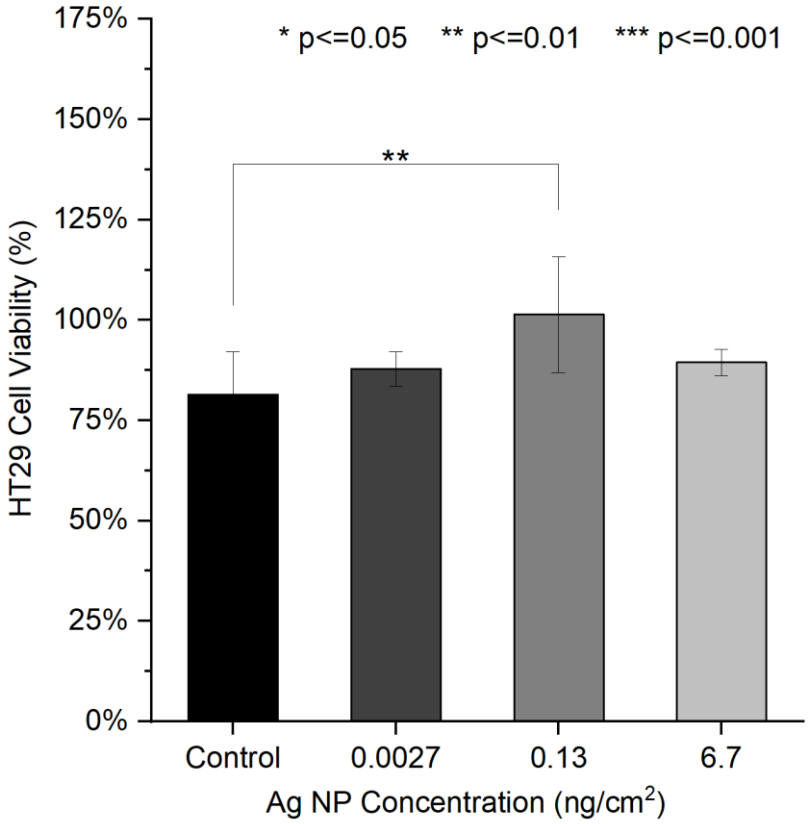

Figure 13. Impact of Ag NPs at various doses on HT29 cell viability. MTT assay was used to measure HT29 cell viability following 23 hrs of incubation with digested Ag NPs at different doses (0.0027 ng/cm^2, 0.13 ng/cm^2, 6.7 ng/cm^2). Results show significant increase in HT29 cell viability in response to 13ng/cm^2 Ag NPs, but not the other two doses tested. Control group represents treatment with digest components without NPs. Results are normalized to a control group treated with McCoy 5A media alone. Error bars indicate standard deviation (n=6).

The effect of Ag NP exposure on HT29 cell viability following Probiotic treatment

Intestinal epithelial cell viability is unaffected by treatment with *Lf5221* and Ag NPs. The probiotic Lf5221was introduced to HT29 cells that were incubated with different concentrations of Ag NPs. There was no significant difference in cell viability following treatment with the probiotic Lf5221 in cells treated with Ag NPs at various doses (0.0027 ng/cm^2, 0.13 ng/cm^2, and 6.7 ng/cm^2) compared to the digest control group (Fig. 14). however, the probiotic control treated group exhibited a significant increase in cell viability compared to all other groups tested.

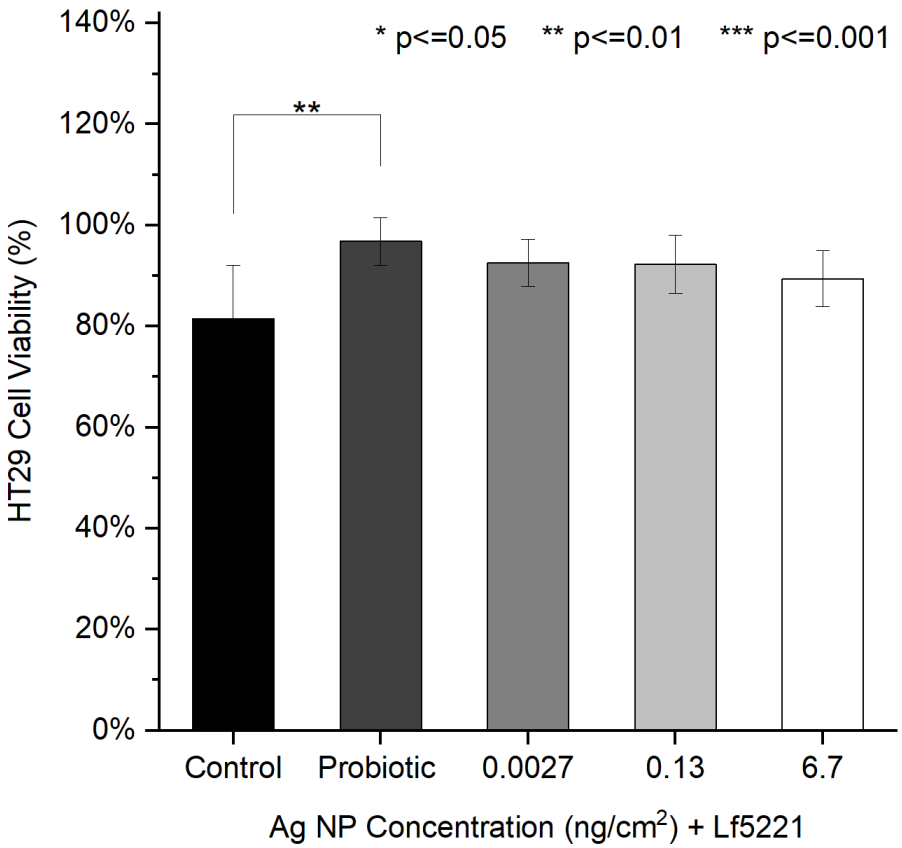

Figure 14. HT29 cell viability in response to treatment with Ag NPs at different doses followed by the probiotic *L. fermentum* NCIMB 5221 (Lf5221). HT29 cells were incubated for 23 hrs with Ag NPs at various concentrations (0.0027 ng/cm^2, 0.13 ng/cm^2, 6.7 ng/cm^2) followed by incubation for 4 hrs with Lf5221 at 263CFU/cm^2. Results show that co-exposure to Ag NPs and a probiotic has no impact on cell viability. Control group represents cells exposed to digest components without NPs and the probiotic group represents cells exposed to the probiotic Lf5221 alone with no prior exposure to Ag NPs. Results are normalized to a control group treated with McCoy 5A complete media. Error bars indicate standard deviation (n=6).

The effect of Ag NP exposure on PLK 1 protein expression of intestinal cells

Ag NP treatment of HT29 cells results in a significant dose-dependent increase in PLK1 protein expression. HT29 cells were treated with Ag NPs at 0.0027 ng/cm^2, 0.13 ng/cm^2, and 6.7 ng/cm^2. It was found that 0.13 ng/cm^2, and 6.7 ng/cm^2 Ag NP treated groups experienced significantly increased expression of PLK1 when compared to the control and the 0.0027 ng/cm^2 Ag NP

treated group (Fig. 15). There was no significant increase in PLK1 protein expression following HT29 treatment with the lowest dose of Ag NPs (0.0027 ng/cm^2).

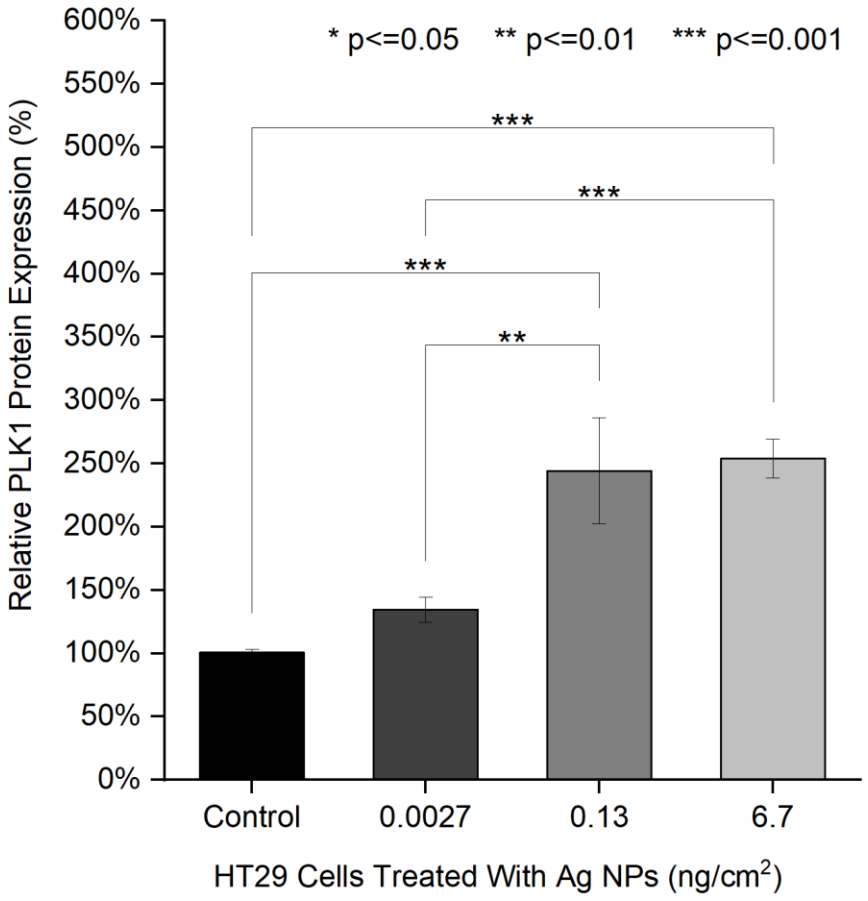

Figure 15. Relative PLK1 protein expression of intestinal epithelial cells in response to treatment with Ag NPs at different doses. HT29 cells were treated with Ag NPs for 26 hrs at different concentrations (0.0027 ng/cm^2, 0.13 ng/cm^2, 6.7 ng/cm^2). PLK1 protein expression was determined using a colorimetric ELISA immunoassay and a Perkin Elmer plate reader. Results are normalized to cell amounts (OD 450nm/595nm). Data was analysed relative to a control group receiving no treatment (McCoy 5A Media). Control group represents cells incubated with digest components alone without NPs. Error bars indicate standard deviation.

5.5 Discussion

The impact of physiologically relevant doses of Ag NPs on human intestinal epithelial cells was investigated in relation to cell viability and signaling protein expression. Cell viability

was reduced after treatment with the digest control alone due to the toxic effects of the digest components on HT29 cells. Interestingly, this reduction in cell viability was not apparent for cells treated with the 1X physiological dose of Ag NPs which exhibited normal viability near that of the media control group. It is unclear why this is the case; however, it may be that the 1X exposure scenario is more diluted compared to the 50X exposure scenario, making digest components diluted as well. This does not explain, however, why the 0.02X exposure scenario also had reduced viability. All other concentrations tested (0.02X and 50X) did not have altered cell viability when compared to the digest showing that Ag NPs likely do not impact HT29 cell viability. There was also no significant difference in cell viability of cells exposed to NPs and probiotics compared to those receiving the digest control. The probiotic control group exhibited significantly increased viability compared to the digest control which is likely because probiotics did not undergo simulated digestion and thus did not contain digest components.

There are various studies on the impact of Ag NPs on IBF. One such study assessed the impact of 7.74nm Ag NPs on caco-2 cells. It was found that the NPs were taken up by the cells, however NPs did not translocate to the basolateral chamber. There was significant increase in oxidative DNA damage, but no significant effects on monolayer integrity or permeability [18]. Another study used an ex-vivo model of ileal tissue to assess the effects of Ag NPs on intestinal inflammation and barrier functioning [55]. Researchers reported gender specific cytokine expression in response to 20nm Ag NP treatment such that male samples showed increased Granulocyte-macrophage colony-stimulating factor (GM-CSF), a substance involved in host defences against pathogens, while female samples showed no changes in GM-CSF secretion. Another study found that 110nm Ag NPs were less toxic than 20nm Ag NPs potentially indicating that smaller particles may be able to dissolve more rapidly in phagolysosomes [151].

Finally, in a histochemical study, researchers found that Ag NPs dose dependently accumulated in the lamina propria of the small and large intestine. Ag NP exposure caused goblet cells to release mucus granules more than did control cells. Researchers also noted abnormal mucus composition in Ag NP treated goblet cells [20].

Studies have also shown that pro-inflammatory chemokine IL-8 (IL-8) secretion towards the apical compartment increases in Caco-2 cells incubated with Ag NPs [19]. This secretion was found to be a response to oxidative stress. Researchers noted the involvement of Ag^+ ions present in the Ag NP suspension, therefore the impact observed may not be specific to NPs alone. Another study used normal and cancerous human colon cells (NCM460 and HCT116 respectively) to test the toxicity of different concentrations of Ag NPs [17]. Intracellular ROS increased and cellular activity decreased with increasing concentration of Ag NPs. Researchers also observed accelerated cell death via an increase in the Bax/Bcl-2 ratio and activated P21. By contrast, a low concentration of Ag NPs (<15 μg/mL) did not have toxic effects. In the current study, it was found that treatment with 1X and 50X the physiological dose of Ag NPs resulted in significantly increased expression of PLK1 proteins when compared to intestinal cells treated with 0.02X the physiological dose of Ag NPs or controls. There was no significant difference between the 1X and 50X Ag NP exposed groups. This is an interesting finding; however, it is difficult to determine the mechanism underlying this change in protein expression due to the role of PLK1 in both proliferation and apoptosis. Further investigation must be done to elucidate this mechanism as the innerworkings of cell signaling are very complicated involving various upstream and downstream components.

Methods have been developed to screen foods for nanomaterials, such a method has been validated for Ag NPs to facilitate food safety assessments [153]. Others have characterized Ag

NPs found in different E174 containing products [154]. Despite these efforts, there is still a lack of characterization of the particles found in the E174 food additive. In this study, Ag NPs had a significantly smaller average size when filtered after sonication (90.1nm) compared to sonication alone (249 nm). This large difference demonstrates the effectiveness of a 0.22 μm filter in narrowing the size distribution of the Ag NP solution by removing aggregated particles. The zeta potential was also reduced when the filtration was incorporated (-16.5mV) compared to when filtration was omitted from sample preparation (-21.7 mV).

5.6 Conclusion

In conclusion, a physiologically relevant dose of Ag NPs (0.13 ng/cm^2) can prevent digest induced loss in HT29 intestinal cell viability, but not when NP treatment is followed by treatment with the probiotic Lf5221. At all other Ag NP doses tested (0.0027 ng/cm^2, 6.7 ng/cm^2), there was no impact on HT29 cell viability. Most notably, Ag NP exposure at 1X (0.0027 ng/cm^2) and 50X (6.7 ng/cm^2) the physiologically relevant dose leads to significantly increased expression of the PLK1 signaling protein. Higher PLK1 expression was observed with increasing Ag NP exposure, however differences among concentrations were not significant except between the 0.02X and 50X Ag NP exposed groups. Overall, Ag NPs have the potential to alter intestinal epithelial cell signaling relevant to gut homeostasis and barrier functioning.

5.7 Acknowledgments

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CHAPTER 6: SUMMARY OF OBSERVATIONS

6.1 Summary of Observations

This thesis investigated the potential effect of three inorganic NPs (TiO_2, SiO_2, and Ag NPs) found in food additives (E171, E551, and E174 respectively) at various concentrations relative to the reported human exposure scenarios for each NP (0.02X, 1X, and 50X). It comprised of a series of studies each focused on one NP and its effects on HT29 cell viability and PLK1 protein expression. All NPs were characterized for size, polydispersity index, and zeta potential to ensure relevance of study findings to the assessment of the toxicity of the relevant food additive.

1. Results clearly showed that treatment with Ag NPs at 1X the calculated physiological dose led to increased HT29 cell viability compared to the digest control. All other concentrations tested (0.02X and 50X) did not alter cell viability.
2. Epithelial cells treated with TiO_2 NPs did not exhibit any significant loss in cell viability at all concentrations tested (0.02X;80%, 1X;79%, 50X;80%). This was clear as the digest control treated cells showed similar viability (81%).
3. HT29 cells treated with SiO_2 NPs did not experience any loss in cell viability at all doses (0.02X;83%, 1X;92%, 50X;81%) when compared to the digest control (81%).
4. Treatment with the probiotic Lf5221 following epithelial cell's exposure to TiO_2 NPs did not result in any increase in cell viability when compared to both probiotic and digest control groups. This is clear as results show that digest control groups exhibited reduced cell viability (81%) comparable to that seen in NP exposed groups (0.02X;88%, 1X;82%, 50X;77%).

5. For SiO_2 NP exposed groups followed by Lf5221 treatment, there was no significant differences in cell viability of cells exposed to NPs and probiotics (0.02X;84%, 1X;85%, 50X;81%) compared to those receiving the digest control (81%).
6. For Ag NP exposed groups followed by treatment with Lf5221, there was no significant difference in cell viability compared to those receiving the digest control. All concentrations of Ag NPs and probiotic exposed groups showed the same viability as the probiotic control group.
7. The epithelial cell viability exhibited by groups receiving TiO_2 and SiO_2 NPs and probiotics was comparable to that seen in groups exposed to the NPs alone.
8. The increase in epithelial cell viability seen in groups treated with 1X Ag NPs was reversed following treatment with the probiotic Lf5221.
9. Treatment of epithelial cells with SiO_2 and Ag NPs at 1X and 50X the physiological dose resulted in a significant increase in PLK1 protein expression compared to control groups.
10. The observed increase in PLK1 protein expression following NP exposure appears to be concentration dependent as the groups exposed to 50X SiO_2 NPs exhibit higher PLK1 protein expression than the groups treated with 1X SiO_2 NPs, although this was not statistically significant.
11. Exposure to TiO_2 NPs at all concentrations tested did not affect PLK1 protein expression of epithelial cells in a statistically significant manner. However, cells exposed to 1X and 50X the physiological dose of TiO_2 NPs expressed higher levels of PLK1 protein compared to groups exposed to 0.02X TiO_2 NPs, media control, and digest control treated groups.

12. Control groups of cells treated with digest components caused reduced cell viability compared to media treated control groups by 19%. Probiotic control treated groups showed the highest cell viability (97%) because probiotics did not undergo simulated digestion and were thus free from digest components. Thus, digest components cause reduced epithelial cell viability.
13. The method of NP dissolution affects size and zeta potential measurements. SiO_2 NPs that were sonicated and filtered had a hydrodynamic size of 128 nm and a zeta potential of -23.6 mV. By contrast, SiO_2 NPs that were only sonicated were 164 nm in size and had a zeta potential of 21.4mV.
14. Including a filtration step in the preparation of Ag NP solutions results in a drastically reduced size distribution profile. Ag NPs that were sonicated and filtered were 90 nm in size and their zeta potential was -16.5mV. Ag NPs that were only sonicated were 249 nm in size and their zeta potential was -21.7 mV.

CHAPTER 7: GENERAL DISCUSSION

The use of nanoparticles (NPs) has been popularized in recent years owing to their unique properties useful across many industries [4-6]. The most concerning for human health is the use of NPs by the food industry to create various food additives with attractive properties for the consumer such as coloring and flavouring. Three inorganic NPs (TiO_2, SiO_2, and Ag) were the focus of this investigation as they are widely incorporated in the food additives E171, E551, and E174, respectively. Due to the use of NPs in foods, NPs can directly access the human GIT via ingestion. Furthermore, NPs have become a vital component in the design of drug delivery systems. Such widespread use and the chronic exposure to humans across their lifespan makes it necessary to investigate the potential toxicity of these food NPs, specifically their impact on the human GIT.

The human GIT has become increasingly recognized for its role in human health and disease [155]. Intestinal barrier function is a highly important property of the human gut as it protects the body from foreign pathogens that reside in the gut lumen. Additionally, the intestinal barrier promotes gut homeostasis with regards to microbial composition, mucus secretion, and immune response. When this barrier is dysfunctional, the gut becomes leaky which destroys gut homeostasis resulting in various inflammatory diseases including Crohn's disease and ulcerative colitis [156-159]. Barrier dysfunction is correlated with the destruction of the intestinal epithelial layer which is held together by intercellular junctions. This occurs through reduced expression of tight junctions holding together adjacent epithelial cells, as well as through apoptosis of epithelial cells creating gaps in the gut lining. Additionally, loss of mucus can also compromise barrier function. Interestingly, NPs have been shown to impact these various components of gut barrier function. However, there are conflicting findings in the current literature with regards to NP toxicity and calls have been made for further studies. This work therefore involved an

investigation of the impact of various food relevant NPs on intestinal epithelial cell viability and cell signaling. The gut microbiome, consisting of trillions of microbes including bacteria, has been recognized for its ability to influence gut health. Because of this, various probiotics have been shown to benefit intestinal barrier function [30, 83, 108, 118, 127]. Given that NPs have the potential to cause intestinal barrier dysfunction, and probiotics have been shown to reverse gut barrier damage, this study also investigated the potential use of a well-known probiotic *L. fermentum* in ameliorating NP induced barrier dysfunction.

This work has shown that intestinal epithelial cell viability is not impacted by treatment with SiO_2 and TiO_2 NPs, however it was shown that cell viability increases following treatment with Ag NPs at a concentration relevant to the currently reported human exposure levels [4-6]. NPs have been demonstrated by many studies to have pro-inflammatory effects [29-31, 33-36]. Additionally, paracellular permeability of the gut epithelia has been shown to be negatively affected by NPs through tight junction regulation [37, 38, 133]. By contrast, other studies on the effect of NPs on the gut report little to no negative effects [131, 132]. This shows that there are variabilities in the current literature regarding NP toxicity, likely due to differences in experimentation, NP doses, and gut models used. This work will contribute to the current literature as it is a comprehensive analysis of the effect of food relevant NPs on gut barrier functioning with consideration for the relevance of NP size and dose to currently reported human exposure levels. This work also recognizes the importance of NP physiochemistry as it involves in vitro digestion of NPs to simulate the state of NPs following human exposure through ingestion.

This work has shown that epithelial cells treated with NPs followed by treatment with *Lf5221* do not experience any changes in cell viability. Probiotics have been shown to positively regulate

IBF [9, 30, 56, 83, 107, 160]. However, in this study, NPs did not induce any loss in intestinal epithelial cell viability, thus it could not be determined whether probiotics would have ameliorated such NP-induced damage. This work has also shown that intestinal epithelial cells, particularly HT29 cells, are negatively impacted by digest components such as pepsin and pancreatin as evidenced by their loss in cell viability.

The most striking observation in this thesis is that SiO_2 and Ag NPs, but not TiO_2 NPs, significantly increase PLK 1 protein expression. PLK1 is involved in cell proliferation and apoptosis among other signaling pathways making it relevant to the study of the intestinal epithelial barrier as the gut relies on a balance of cell proliferation and cell death to maintain homeostasis [70-72, 79, 161]. The results of this work are in line with other studies which have shown NPs can affect the expression of genes involved in gut barrier functioning including cellular proliferation and signaling [162].

The various components of the intestinal barrier serve to maintain gut homeostasis, and recent studies have clearly shown that food relevant NPs have the potential to impact some components of barrier function. Such findings indicate that NPs have the power to affect gut barrier functioning potentially leading to inflammatory gut diseases such as IBD. This investigation focused on one component of gut intestinal barrier function, the intestinal epithelial layer, and assessed the impact of three NPs frequently used in foods. Findings may be used to further elucidate the mechanisms through which NPs impact gut health. Additionally, this work can inform the future design of NP associated health care measures.

CHAPTER 8: CONCLUSION

There has been increasing incorporation of NPs into foods due to their unique and favourable properties for consumers. Unfortunately, recent studies have found some NPs to be toxic thus making it necessary to understand the mechanisms through which NPs impact human health and develop ways to ameliorate such damage. In this study, the effect of three NPs was investigated on intestinal barrier function in relation to inflammatory gut diseases. NPs at all doses tested did not impact HT29 intestinal cell viability, except for Ag NPs which caused increased cell viability at a physiologically relevant dose. All NP exposure doses tested still had no impact on HT29 cell viability following treatment with the probiotic Lf5221. The only exception was that the increase in cell viability seen in Ag NP treated groups became decreased again after probiotic treatment. Most remarkably, SiO_2 and Ag NPs, but not TiO_2 NPs, caused significantly increased PLK1 protein expression at 1X and 50X the calculated physiological exposures. Study findings imply that NPs do not impact HT29 cell viability, but they can alter cell signaling proteins related to proliferation and apoptosis which can have more prominent effects if exposure persists over a longer period. Such findings have the potential to aid the future development of treatments for NP related intestinal barrier dysfunction.

CHAPTER 9: RECOMMENDATIONS AND FUTURE STUDIES

9.1 Investigate NP effects on the gut microbiome using a gut model **in vitro**

While the focus of this work has been on the impact of NPs on intestinal epithelial barrier functioning, there is immense data on the role of the gut microbiome in maintaining gut barrier function [15, 135, 163, 164]. Therefore, future studies should utilize the Simulated Human Intestinal Microbial Ecosystem (SHIME), a full model of the GIT, to determine changes in microbial composition in response to food NPs. Given that changes to microbial composition may not translate to meaningful changes in physiology, it will be important to also measure the microbial metabolites relevant to healthy gut functioning such as short chain fatty acids and key metabolic enzymes. This model can simulate the GIT microbiome of specific population groups including individuals with IBD, celiac disease, and colorectal cancer [165, 166]. Therefore, SHIME can be used to elucidate any mechanisms of NP toxicity in vulnerable populations. This would inform dietary health care measures for individuals already suffering from gut barrier dysfunction.

9.2 Study of NP toxicity in populations with impaired gut intestinal barrier function

The effects of NPs may depend on the health status of the host, therefore susceptible models of gut barrier dysfunction should be used in measuring NP toxicity. For example, one study found that Ag NPs caused more damage to the epithelial barrier when it was inflamed compared to when it was healthy [167]. This demonstrates the need to assess NP safety in the context of specific diseased intestinal conditions. Results suggest an increased vulnerability of the inflamed epithelial barrier towards Ag NPs underlining the importance of considering the intestinal health status in the safety assessment of nanomaterials. Additionally, researchers have noted synergistic effects of gliadin peptides and Ag NPs on Caco-2 monolayer integrity and tight junction protein expression [168]. The combination induced cytokine production in celiac

disease biopsies but not healthy biopsies. NPs therefore may be promoting the passage of immunogenic substances into the lamina propria thereby increasing the availability of antigens leading to activation of the immune system. This would suggest that celiac patients would be at greater risk of NP induced barrier damage than the healthy population. The toxicity of NPs in the context of inflammatory gut diseases must therefore be further studied.

9.3 Study of other components of barrier dysfunction in relation to a combination of food NPs

While this study focused on the impact of NPs on cell viability and PLK1 related cell signaling, the literature clearly shows that NPs can impact IBF in a multitude of ways. Therefore, it would be beneficial to do a comprehensive study involving measurement of tight junction proteins, inflammatory markers, pore forming proteins, mucus composition, and microbial and metabolic composition. This would provide a broader and more complete understanding of the mechanism for NP induced intestinal barrier dysfunction. As mentioned previously, some studies have shown that NPs can work together to produce toxic effects, therefore it is also important to study all food NPs in concert to determine the true risk for toxicity currently posed by the incorporation of a variety NPs in the food and medical industry.

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