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The Effect of Probiotics on the Structure and Functionality of Enterocytes

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1 Abbreviations

ANOVA: analysis of variance

CLSM: confocal laser scanning microscope

DMEM/F12: 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture

ETEC: enteropathogenic *Escherichia coli*

FBS: fetal bovine serum

EGF: epidermal growth factor

GA: glutaraldehyde

GALT: gut-associated lymphoid tissue

HSP: heat shock protein

LPS: lipopolysaccharide

MRS: DeMan, Rogosa, Sharpe Medium

NRU: Neutral Red Uptake

PB: phosphate buffer

PBS: phosphate-buffered saline

PFA: paraformaldehyde

SCS: spent culture supernatant

TEM: transmission electron microscopy

TJs: tight junctions

TLRs: toll-like receptors

ZO: zona occludens

2 Introduction

Probiotics are of increasing interest due to their proven beneficial effect on the gastrointestinal tract. Most probiotic bacteria are of intestinal origin and belong to a group of lactic acid producing bacteria e.g. *Bifidobacteria*, *Lactobacilli* and *Enterococci* (Dubreuil, 2017). In veterinary clinics all over the world, complementary feeding products containing probiotics are becoming more commonly sought after. These products claim to maintain a healthy digestive system and support the gut after or during a period of digestive upset.

Intestinal disorders are a significant cause of morbidity and mortality amongst populations worldwide; therefore, the knowledge of the molecular and biological epithelial cell functions is of special importance (Nossol *et al.,* 2015). Commercial probiotic products are cheap to purchase, easy to use, have relatively no side effects and are completely natural. Furthermore, prophylactic probiotic treatment has the potential to prevent overuse of antibiotics in veterinary medicine. In turn this could potentially lead to a significant reduction in issues such as antibiotic resistance. Due to the fact that antibiotic resistance is currently a major problem in both human and veterinary medicine, it is surely advisable to research the natural alternatives.

IPEC-J2 cells are porcine enterocytes isolated from the jejunum of a neonatal unsuckled piglet. The IPEC-J2 cell line is unique as it is neither transformed nor tumorigenic in nature; making it an ideal model to study the structure and functionality of enterocytes. Pigs are intensively reared animals, which could greatly benefit from probiotic supplementation, rather than prophylactic antibiotic treatment. Among the challenges that affect the intestinal tract of swine, enteropathogenic *Escherichia coli* (ETEC) represent one of the most common causes of swine diarrhoea (Dubreuil, 2017). ETEC is responsible for neonatal and post weaning diarrhoea, two conditions that lead to growth retardation, require antibiotic therapy and can ultimately result in the death of animals.

The aim of this experimentation is to examine the effect that probiotic bacteria can have on the structure and functionality of enterocytes; and to underline the advantages of their future use in veterinary medicine. Since the use of antibiotics as growth promotors was banned from the European Union in 2006; this has provided probiotics an amble opportunity

to become an integral part of the nutrition program for gut health in animal production. This could potentially lead to healthier guts, healthier animals and more efficient production.

3 Literature review

3.1 Gut: structure and function

The gastrointestinal epithelial layer is composed of several types of cells i.e. enterocytes, enteroendocrine cells, Paneth cells, goblet cells, microfold cells, tuft cells and cup cells (Figure 1). These cells and secreted compounds work together to maintain homeostasis in the gut. The epithelium of the intestine plays a dual role as it absorbs the dietary nutrients and forms a physical barrier against noxious stimuli, secreting cytokines and chemokines as a result of the adverse stimuli (Pitman et al., 2000). The intestinal epithelial barrier is also the first line of defense during an immune response in the gastrointestinal tract.

The enterocyte is one of the most abundant components of the intestinal lining that covers the villi and crypts. They are columnar in shape, with microvilli located on their absorptive surface. These epithelial cells are structurally and functionally polarized, with an apical surface facing the intestinal lumen and a basolateral surface facing the underlying basement membrane and lamina propria (Abreu, 2010). Enterocytes are covered by a thin layer called the glycocalyx, which contains the digestive enzymes. Unique oligosaccharides that form the glycocalyx on the surface of epithelial cells possess high specificity and affinity for lectins, viruses, bacterial toxins, bacteria and immune cells (Cencič and Langerholc, 2010). The absorptive surface of the cell contains transport proteins that facilitate the movement of nutrients into the cell. The lifespan of a typical enterocyte is the time it takes for an undifferentiated stem cell to move from the crypt of Lieberkuhn to the tip of the villus. During this movement, the cell acquires the characterization of a mature cell. The maturation of the digestive tract after birth is crucial because it enables the gradual development of immune cells and prevents occurrence of intolerance and adverse reactions to food allergens (Condette *et al.,* 2014). Cell migration takes around 48 hours in conventional cases but in 'germ free' cases, where antibiotics are used as growth promoters, it can take 96 hours. This means more energy can go towards production and growth (Fekete, 2008).

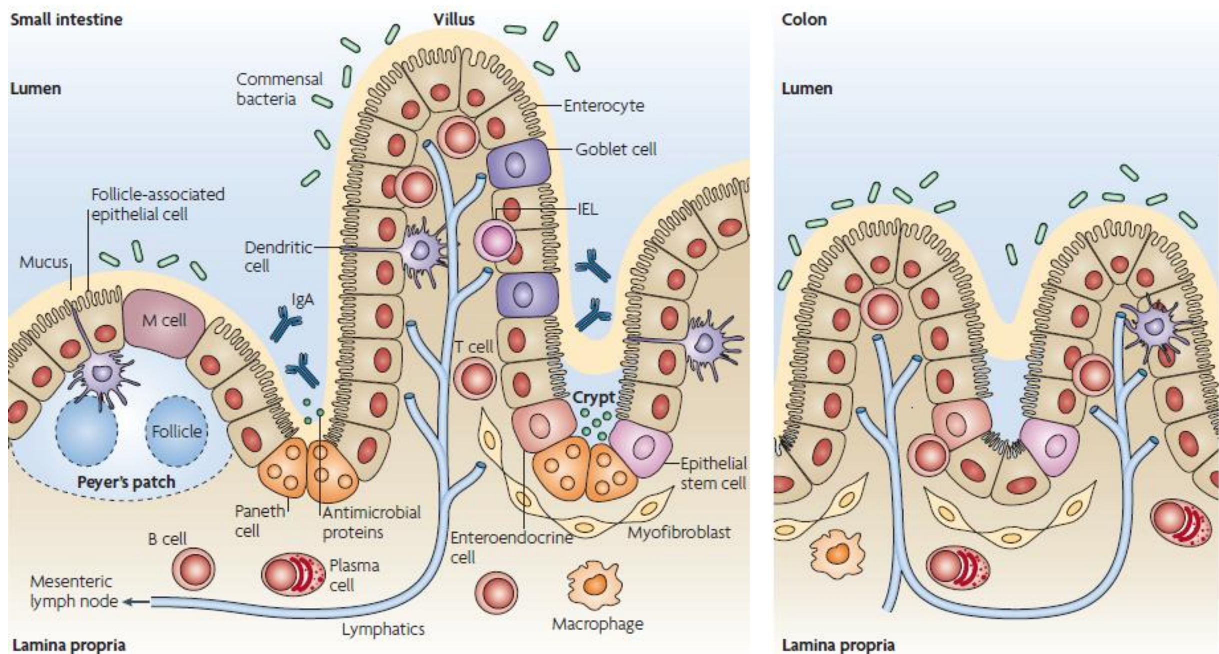

Figure 1. A depiction of the cell types that make up the intestinal lining and how the epithelium responds in an immune reaction (Abreu, 2010).

3.2 Role of junctional complexes and barrier integrity

Junctional complexes are made up of occluding (tight) junctions, anchoring junctions, gap junctions and desmosomes (Figure 2). While the tight junctions (TJs) function as a barrier, anchoring junctions provide structural stability. The gap junctions provide channels for intercellular communication (Condette *et al.,* 2014). Tight junctions are composed of proteins occludin, claudin and junctional adhesion proteins, which allow intracellular signaling. These transmembrane proteins are linked to cytoskeletal components by the zona occludens (ZO) family of proteins, further connecting the TJs to other cell-cell and cell substratum adhesion sites (Condette *et al.,* 2014). In particular, claudin-4 and claudin-7 are associated with barrier function of epithelial cells (Sonoda *et al.,* 1999). Maintaining junctional complexes can be achieved by stabilizing cytoskeletal proteins such as ZO-1. ZO-2 and ZO-3 further make up the ZO family of proteins, sometimes referred to as 'the scaffolding of the cell' (Liu *et al.,* 2015). It has been established that a disruption to TJs brings about an increase in intestinal permeability; in particular ZO-1 and claudin-4 have been shown to be in involved when intestinal barrier dysfunction occurs. Claudins are essential protein regulators responsible for preservation of electrical resistance, paracellular

ionic selectivity and transport mechanisms in both epithelial and endothelial structures (Paszti-Gere *et al.,* 2012).

The gastrointestinal barrier is the largest interface separating the internal and external environment (Dubreuil, 2017). Barrier integrity is essential for proper functioning of the junctional complexes; as this facilitates the cell-cell adhesion of enterocytes, forming a boundary and consequently restricting movement through it. Some diseases of the gut are thought to involve elevated intestinal permeability to macromolecules; which allows leakage from the lumen through the intestinal epithelial barrier. Tight junctions are the most apical structures responsible for controlling permeability of the paracellular pathway (Dubreuil, 2017). The continuity of the adjacent cells can be compromised and intercellular communication weakened if ZO-1 is impaired. Consequently, keeping ZO-1 intact and stable is of vital importance to protect the gut.

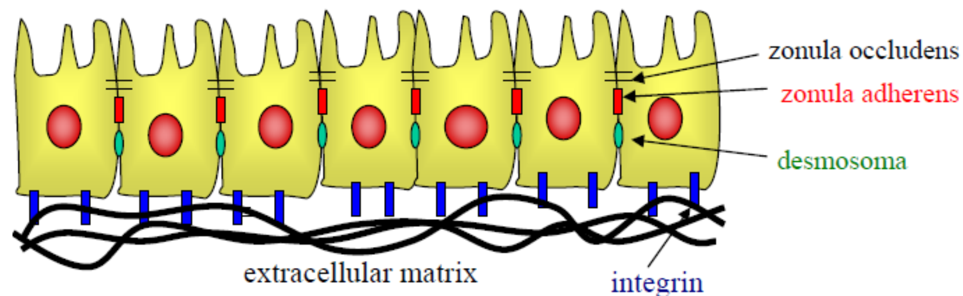

Figure 2. Enterocyte cell monolayer demonstrating junctional complex assembly (Paszti-Gere *et al*., 2013)

Cholinergic signaling has also been demonstrated to have a role in the regulation of the barrier function (Condette *et al.,* 2014). This can be influenced by using cholinergic agonists to alleviate the damage inflicted on TJs as a consequence of penetrating pathogens. Abreu, 2010 stated that toll-like receptors (TLRs) assist the host against exogenous pathogens. TLRs are expressed on many cell types, including epithelial cells and function by detecting molecules recognized as being components of invading pathogens, for example peptidoglycans or LPS. It has been discovered that *Lactobacilli* can communicate by coupling to TLRs, making this bacteria a key promoter for an immune response (Abreu, 2010).

3.3 Importance of a healthy gut

Altered intestinal permeability and structure during neonatal development can have a detrimental impact on the future health status of the animal. It can contribute to adverse clinical outcomes and cause the animal to have an increased susceptibility to infection, inflammation, hypersensitivity and stress (Condette *et al.,* 2014). Mucosal epithelium, lamina propria, glycocalyx and secreted mucus all contribute to the barrier function of the gut (Cencič and Langerholc, 2010).

Consuming the mother's milk postpartum has a profound influence on the functional development of the pig's digestive system. During an infection, such as ETEC, the pathogens can adhere to the brush border of epithelial cells and damage the cell junctions, leading to intestinal inflammation and diarrhoea (Dubreuil, 2017). Further to this, bacterial toxins can disrupt the cytoskeleton, causing reorganization and then leakage of macromolecules through the paracellular route. Probiotics can help keep the gut healthy by antagonizing pathogens and altering their adherence to the epithelium. *Enterococcus faecium* NCIMB 10415 is currently in use on farms and licensed as a feed additive for sows and piglets, as it has shown positive effects in reducing diarrhoea by enhancing barrier function (Dubreuil, 2017). Post-weaning diarrhoea was recorded as 20% after supplementation of the probiotic, compared with 38% incidence in the control group (Dubreuil, 2017).

Considerable advances have been made in swine nutrition over the years, to ensure that the pig's gastrointestinal tract receives the correct nutrients for the stage of development it is at. As the incorrect diet can only lead to the loss of nutrients, subsequently affecting the bioavailability of the diet and causing malnutrition in the animal. Formulating diets to aid the immune status of the pig has been achieved through specific combinations of amino acids, vitamins, minerals and probiotics. The weaning period is one of the most stressful times in a piglet's life, as it involves several major changes. During this time the piglet's immune system is at its most vulnerable stage. The period known as the 'immunity gap' obtains when the passive maternal immunity ends and the piglet's own 'active' immune system is only starting to develop. Commercial pig production has many measures in place to make the transition as smooth as possible and great consideration is given for intestinal function during this time. As a result of the weaning period, a piglet's villi length can significantly decrease,

leaving it with 20-30% less surface area for absorption. To add to this intestinal stress, hydrochloric acid secretion hasn't reached that of an adult yet; therefore, the piglet is less able to control the internal intestinal pH. Enzyme activity is also 40% lower, compared with an adult (Fekete, 2008). These factors combined pose a significant threat to the piglet's intestinal health.

Widespread use of sub-therapeutic levels of feed antibiotics for monogastric animals has been practiced since the 1960's (Fan and Archbold, 2015). However, it is now known that sub-therapeutic levels are only capable of attenuating infections; this is because biologically feed antibiotics do not detoxify the lipopolysaccharide (LPS) endotoxin. It has been established that the elimination of LPS provoked inflammation is of vital importance in pig health management (Farkas *et al.,* 2014).

As the ban on feed antibiotics only occurred within the European Union; in the United States of America, pigs are still exposed to several antibiotics during their lifetime. This is a considerable reason why these pigs would gain from the application of probiotics as an alternative to preventative antibiotic therapy. As a result of intestinal disease, pig farms can suffer significant economic losses which further results in a decrease in yield and lower reproduction rates.

3.4 Role of intestinal microbiota

The intestinal microbiota is hugely important for the efficient functioning of the digestive system, as this has a further knock on effect on the host's overall health. The host's nutrition, environment and genetic background all influence the structure and function of the microbiota (Cencič and Langerholc, 2010). With the use of probiotics, the microbiota colonization of the gut can be modified to have a more beneficial balance of micro-organisms.

Bacterial translocation is the movement of bacteria which normally resides in the gut to extraintestinal sites, such as the lymph nodes and liver. Bacterial translocation is known to contribute to the development of inflammation and several gastrointestinal disorders (Condette *et al.,* 2014). It is the translocation of microorganisms that permits invasion and

spread of pathogens (Dubreuil, 2017). At birth, the animal's epithelial barrier becomes more selective. Bacterial translocation after birth is required to achieve the maturation of gut-associated lymphoid tissue (GALT) (Condette *et al.,* 2014). Bacterial gut colonization, epithelial barrier functioning and the immune system all work together to develop GALT in the neonates.

When microbial imbalance occurs, otherwise known as dysbiosis, the immune system is stimulated and cytokines are released. The cytokines produced during the immune reaction can then directly affect cell communication and paracellular permeability in intestinal epithelial cells by altering claudin expression and distribution (Condette *et al.,* 2014). Experimentally induced dysbiosis was shown to be characterized by low numbers of *Lactobacilli* (Condette *et al.,* 2014). This brings about the importance of the balance of *Lactobacilli* and *Escherichia coli* to maintain a healthy gut. Within the large intestine, up to half of its content is made up of micro-organisms. While the activity of *Lactobacilli* decreases the pH and allows optimal functioning of the large intestine, a pH close to 7 will lead to bacterial overgrowth, which provides the ideal conditions for *Escherichia coli.*

3.5 Probiotics

The word 'probiotic' stems from the Greek meaning 'for life' and coincidentally 'antibiotic' has the direct opposite translation. The history of probiotics in the human diet stems back thousands of years to the use of fermented foods, such as milk. Some of the earliest studies on probiotic bacterial strains were accomplished by Élie Metchnikoff on the then called 'Bulgarian bacillus', now referred to as *Lactobacillus delbrueckii subsp. Bulgaricus* (Fuller, 2012). This bacterial strain is currently still used in the food industry for the production of yoghurt. A few years later, it was *Lactobacillus acidophilus* that began to gain attention for its beneficial properties; and in the post war era, the interest in gut flora was revived. It was then the turn of *Bifidobacteria* which became the dominant bacterial strain being examined at the end of the 19th century by Henri Tissier. He demonstrated that these organisms were the dominant species in the gut of breast-fed babies and suggested they would not be found if powdered milk formula was used (Fuller, 2012).

The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Probiotics have grown to become a common supplement in the day to day diet of humans; as the trend of taking charge of one's own health has become progressively popular. Whether preventative or therapeutic, probiotics offer a cheap and risk-free option to help alleviate intestinal upset. Probiotics have already demonstrated their favorable effect on hosts treating enteral inflammations caused by infections such as *Escherichia coli, Clostridium difficile* and Rotavirus. Probiotics can modulate the way the intestine responds to the infection and as a result, decreasing the damage done by the invading pathogen (Dubreuil, 2017). They remain a suitable feed additive to promote both animal health and animal performance.

The methods by which probiotics exert their therapeutic effect could include competitive exclusion, immunomodulation, regulation of cytokine production, production of bacteriocins, maintenance of tight junction complex assembly, antagonism of pathogens, interference with adherence, competition for nutrients, enterotoxin inactivation, modulation of immune response and strengthening of the intestinal barrier. Improved intestinal barrier function is believed to result from varying TJ protein expression and distribution. It has been observed that infection can lead to a loss of cell-cell contact, disruption to structural proteins and even cell death. Pretreatment with *Lactobacillus* species has the potential to eliminate cell death, reduce cell dissociation and retain structural integrity when faced with invading pathogens (Liu *et al.,* 2015). Furthermore, probiotics have shown the ability to stimulate heat shock proteins which have cytoprotective properties that are linked with ZO-1 expression. This method of cytoskeletal reorganization in epithelial host cells has been documented in recent studies; it was demonstrated that *Lactobacilli* species could maintain the correct localization of ZO-1 as well as inhibiting destruction of ZO-1 protein (Dubreuil, 2017).

Heat shock proteins (HSP) are involved in regulatory pathways (mainly at transcription level) and have been found to be influenced by the intestinal microbiota and by probiotics. HSP protect cells, tissues and organs by reducing the stress induced protein denaturation that can occur (Klingspor *et al.,* 2015). In particular, HSP70 is upregulated from the introduction of noxious stimuli, making them stress indicators of cells. HSP27 has the ability to bind to cytoskeleton protein actin and from this stabilize the TJ complex (as a result,

strengthening the barrier). Detecting HSP can be an indicator of an intestinal infection; as it is produced in response to stress. The control of increased proinflammatory cytokines can be due to HSP70 production; in an attempt to regulate barrier properties by influencing TJ proteins and structure/function of the cytoskeleton. The cytoskeleton provides shape and coherence, allowing structural stability, which infections try to alter (Klingspor *et al.,* 2015).

Cytokine production can also be modulated by probiotics. IL-8 is a representative of the proinflammatory response, it has been found to call upon neutrophils to phagocytose antigens and regulate the cell junctions during infection (Dubreuil, 2017). Commensal bacteria can potentially block adhesion of pathogens by competition for common receptors and help maintain the physiological balance (Schierack *et al.,* 2006). Commensal bacteria refer to the bacteria that are part of the natural flora of the gut, also known as "the friendly bacteria"; it is important that the normal gut flora re-establishes itself after infection.

One of the most important ways in which probiotics exert their beneficial effect on its host, is by modifying metabolic processes. According to Fuller, 2012, there are a variety of mechanisms by which they can do so, e.g. suppressing toxic or carcinogenic reactions, stimulating detoxifying reactions, stimulating digestive enzymes and synthesizing vitamins or other essential nutrients. Probiotics are a favorable option in that they act in a prophylactic manner but can also help restore the gut epithelia after damage. It is thought that to be most effective the probiotic bacterial strain should preferably originate from the target host species; as this may help assure the survival and colonization of the bacterial strain in the gut. But to be fully effective they must also resist gastric acid, bile salts and pancreatic enzymes (Dubreuil, 2017).

Oelschlaeger, 2010 stated that probiotics can participate in pathogen elimination due to their anti-invasive, anti-toxin and anti-adhesive properties. These methods can be carried out by inhibitory substances such as organic acids or hydrogen peroxide, which is produced by the probiotics and acts in a bactericidal or bacteriostatic manner. It has also been proven useful to supplement the diet itself with organic acids (lactic acid produced by *Lactobacilli)*, to lower the pH of the digesta which in turn aids protein digestion and stimulates pancreatic enzyme production (De Lange *et al.,* 2010). Butyrate, a short chain fatty acid which is released as an end product from anaerobic microbial fermentation of carbohydrates in the

large intestine of monogastric animals, is a metabolite of probiotics which is already used (Farkas *et al.,* 2014). This short chain fatty acid has an antibacterial effect on the gut, which brings about its ability to strengthen health and performance. Utilizing the metabolites of probiotics such as n-butyrate has demonstrated a promising effect on the gut and can be supplemented in the form of a sodium salt. Anti-inflammatory effect after simultaneous LPS and sodium n-butyrate treatment was noted as it decreased IL-8 mRNA level and protein concentration within the culture (Farkas *et al.,* 2014).

Overall, probiotics have the potential to convey a direct benefit to the host. A variation in the results achieved could be due to the age of the animal, probiotic strain used, length of application or dosage. With continued research and utilization of probiotics in the veterinary field, we can aim to provide a wider choice of preventative options to help avoid particular clinical conditions.

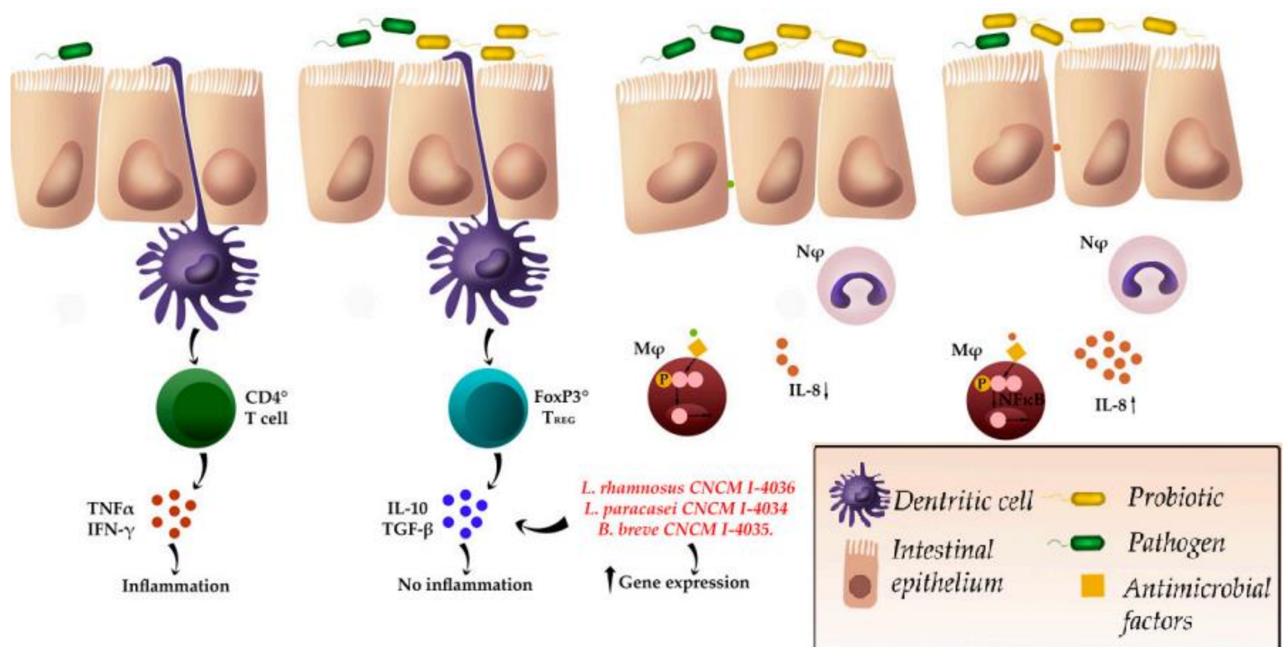

Figure 3. A depiction of the action of probiotics during a gastrointestinal infection. Probiotics help inhibit the adhesion of pathogens to the epithelial surface and enhance the ability of the epithelium to prevent penetration of invading pathogens. (Plaza-Díaz et al., 2018).

3.6 In vitro model

It is important to limit live animal experimentation for ethical reasons; therefore *in vitro* models make a great alternative. Intestinal cell models are particularly useful for the food and pharmaceutical industry, as they allow products to be risk assessed. Cencič and Langerholc, 2010, stated that cell models offer an inexpensive and convenient method to study toxicity and bioavailability of new substances; along with the interactions between the host, pathogens and intestinal microflora. The cell model can also be tailored to the specific experimentation taking place; whereas live animal research isn't always as flexible. Previous studies concerning pathogenesis in swine that have been conducted on animal infection models have failed to investigate the exclusive response of the cell line to the infection (Schierack *et al.,* 2006). As the epithelial cell has an essential role in innate immunity during enteric infections, an *in vitro* model could assist in further understanding the mechanisms behind this. Epithelial cells produce cytokines which act as growth and differentiation factors, alarm signals for the inflammatory response, chemotactic factors and modulators of immune cell function. The Caco-2 and HT-29 human colon cancer cell lines are most commonly used to model the human small intestinal mucosa in research studies and in the pharmaceutical industry, respectively.

The cell line featured in this study was IPEC-J2; a suitable model for determining the functional and structural changes as a result of probiotic application. Capable of forming mostly single cell monolayers comprised mostly of cuboidal cells, interspersed with flat cells and no goblet cells; the IPEC-J2 can mimic the physical barrier which restricts movement between the lumen of the intestines and the underlying mucosa (Schierack *et al.,* 2006). This epithelial origin of IPEC-J2 was confirmed by the detection of cytokeratins (distinctive cytoskeletal proteins) in studies carried out by Schierack *et al.,* 2006. Being non-carcinogenic in nature, the use of IPEC-J2 eliminated the major differences that could occur between interlaboratory data from tumor derived cell lines. The advantage of using IPEC-J2 over other tumorigenic cell lines is due to their glycosylation pattern, proliferation rate and colonization ability; as these attributes, characterize better the *in vivo* conditions of the gut (Cencič and Langerholc, 2010). Due to the broad adhesion spectrum of the *in vitro* model, it can easily recreate a physiological model when relevant bacterial strains are applied (Schierack *et al.,* 2006).

4 Goals

The aim of this work was to provide scientific data about the positive effect of probiotics on the structure and function of porcine enterocytes. Our research focused on the followings:

- improve our *in vitro* intestinal model consisting of IPEC-J2 porcine jejunal cells in order to test the effect of metabolites from different probiotic bacterial strains on the structure and function of enterocytes

  - culture the cells on Aclar film for transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM)
  - detection of tight junction proteins (claudin-4, occludin) and cytoskeletal proteins (p34, β-actin) which stabilize TJs

- select the parameters which are worthy to follow up when we would like to characterize the effect of different probiotic bacteria on the enterocytes

- test the effect of metabolites of three different bacterial strains isolated from swine on IPEC-J2 cells

  - *Enterococcus faecium* (NCIMB 10415)
  - *Lactobacillus rhamnosus* (DSM7133)
  - *Lactobacillus plantarum*

- set the proper treatment periods and concentrations of spent culture supernatants derived from these bacteria.

5 Materials and methods

5.1 Cell line and culture conditions

The IPEC-J2 cell line was a kind gift from Dr. Jody Gookin, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA. IPEC-J2 cells were stored in liquid nitrogen. For our experiments we put 1 ml of frozen IPEC-J2 cells into a cell culture flask of 25 cm^3 containing 10 ml of DMEM/F12, which is a complete medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 5µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor (EGF) and 1% penicillin-streptomycin (Biocenter Kft., Szeged, Hungary). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and the cell culture medium was changed every second day.

For cell viability determination with the Neutral Red Uptake (NRU) method, cells were cultured onto a 96-well plate. Each well was inoculated by 200 µl cell culture suspension. Cells were cultured until confluency was reached.

For transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) cells were seeded onto a 24-well cell culture plate containing Aclar film (Electron Microscopy Sciences, PA, USA). Each well contained 500 µl cell culture suspension and cells were cultured until confluency was reached.

5.2 Culture conditions of bacteria and preparation of spent culture supernatants

Applied bacterial strains included: *Enterococcus faecium* (NCIMB 10415), *Lactobacillus rhamnosus* (DSM7133) and *Lactobacillus plantarum*. The bacteria were submitted from our research partner Dr. Zoltán Kerényi (Magyar Tejgazdasági Kísérleti Intézet, Mosonmagyaróvár, Hungary). These bacteria were grown in DeMan, Rogosa, Sharpe (MRS) broth. Inoculation was accomplished with a stationary culture of a probiotic strain (1% inoculum). The bacteria were placed into the incubator and were grown for 24 hours at 37°C and sub-cultured twice prior to experiments. Spent culture supernatants (SCSs)

were prepared by centrifugation of the bacterial suspension at 3000g at 5°C for 10 minutes. The pH values of SCSs were set to 7 using NaOH (1M). SCSs were then passed through a sterile 0.22 µm pore size filter unit.

Different concentrations of SCSs (3%, 6%, 12%, 24%) were prepared. For solution DMEM/F12 medium without supplementation was used (plain DMEM/F12). 3% treatment solution was prepared using 300 µl supernatant with 9.7 ml plain DMEM/F12, 6%: 600 µl supernatant with 9.4 ml plain DMEM/F12, 12 %: 1200 µl supernatant with 8.8 ml plain DMEM/F12 and finally 24%: 2400 µl supernatant with 7.6 ml plain DMEM/F12.

5.3 Treatment of IPEC-J2 cells with spent culture supernatants

Before treatment, cells were washed with phosphate buffered saline (PBS). Cells were treated with SCSs (derived from *Lactobacillus plantarum*, *Enterococcus faecium* or *Lactobacillus rhamnosus*) of different concentrations (3%, 6%, 12%, 24%). SCSs were removed according to the applied treatment time (1 hour, 2 hours, 4 hours, and 24 hours). Number of samples was six in each group. As a control, plain DMEM/F12 medium was used. Effect of MRS in different concentrations was also tested. After having removed the SCSs supplemented DMEM/F12 culture medium was added to the samples.

5.4 Viability measurement with Neutral Red Uptake method

Twenty-four hours after the beginning of treatment, the viability of IPEC-J2 cells was measured with the NRU method. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes (Repetto *et al.,* 2008). The dye cannot bind to dead cells. It is extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye can be measured using a spectrophotometer at 540 nm. There is a linear relationship between the number of cells and the absorbance.

The culture medium was aspirated and the cells were washed with PBS. Neutral Red medium (100 µl) was added to each well on the 96-well tissue culture plate. The concentration of the Neutral Red dye in the medium was 40 µg/ml. The incubation period was 2 hours at 37°C in a humidified atmosphere of 5% CO_2. After 2 hours the Neutral Red

medium was removed and cells were washed with PBS solution. After having removed the PBS solution 75µl of Neutral Red destain solution was added to each well. The destain solution used contained 50% ethanol (96%), 49 % deionized water and 1% glacial acetic acid. The 96-well tissue culture plate was shaken for 10 minutes in a microtiter plate shaker. The absorbance of Neutral Red extract was measured in a spectrophotometer (EZ Read 400, Biochrom) at 540 nm.

5.5 Fixation and immunohistochemistry

Full DMEM/F12 culture medium was aspirated off and 1000 µl of buffered fixative was added. For TEM assays the fixative contained 0.1% glutaraldehyde (GA) and 4% paraformaldehyde (PFA) in 0.1M Phosphate Buffer (PB). For light microscopy we used the same fixative without GA. The fixation was performed at 4°C for 20 minutes followed by two washing steps with PBS (pH 7.4).

For examination with TEM sections were postfixed with 1% OsO_4 in 0.1M PB, washed with 0.1 M PB, dehydrated in an increasing series of ethanol (50-100%) and embedded in epoxy resin. Section were cut on a Reichert ultramicrotome, mounted on 300 mesh copper grids, contrasted with lead citrate (Ultrostain II, Leica) and examined with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany). From the images produced by the TEM, Image J 1.52a software was used to further analyze the features of the epithelial cells.

For immunohistochemistry cells were permeabilized with PBS containing 0.5% triton and blocked with 10% normal donkey serum (NDS) - PBS 0.1% triton. Primary antibodies were mouse anti-claudin 4, mouse anti-actin, rabbit anti-occludin and rabbit anti-p34 (all from Sigma, Germany). Applied dilution rate was 1:250 for β-aktin, p-34 and 1:100 for occludin, claudin-4. Secondary antibodies were donkey anti rabbit Alexa488, donkey anti mouse Cy3, donkey anti mouse Alexa488, donkey anti rabbit Cy3 (all from Izinta Kft, Budapest, Hungary). Applied dilution was 1:100. Immunofluorescence microscopy was performed with a Leica SP2 CLSM.

5.6 Statistical analysis

Data were tested for normality of distribution and statistical analysis was performed by R 2.11.1 software package (2010). The data are given as mean values ± S.E.M (n) where *n* refers to the number of parallel measurements. Differences between means were evaluated by one-way analysis of variance (ANOVA) with post-hoc Turkey test when data were of normal distribution and homogeneity of variances was confirmed or Kruskal-Wallis nonparametric test A *p* value of <0.05 was accepted to indicate statistical significance.

6 Results

6.1 Effect of spent culture supernatants on the viability of IPEC-J2 cells

After treatment with different concentrations of SCSs, viability of IPEC-J2 cells was detected. MRS did not influence the viability of cells (data not shown). As it is summarized in figure 4, there was a significant difference between the control and the cells that received *Enterococcus faecium* NCIMB 10415 supernatant.

Treatment of 1 hour showed significant increase in cell viability in case of each SCS concentration ($p \leq 0.05$). Moreover, groups treated with 3%, 6% and 12% SCS were different from the control at 99.9% significance level. Four hours treatment resulted in significant elevation in the viability of IPEC-J2 cells. The absorbance values of samples treated with 3%, 6% and 12% SCS were more than double of control samples. Contrarily, treatment with 24% SCS for 4 hours caused no significant alteration in the number of living cells compared to the control. While 3% and 6% treatment for 24 hours caused a significant elevation in the number of living enterocytes, the effect of 12% SCS was not significant.

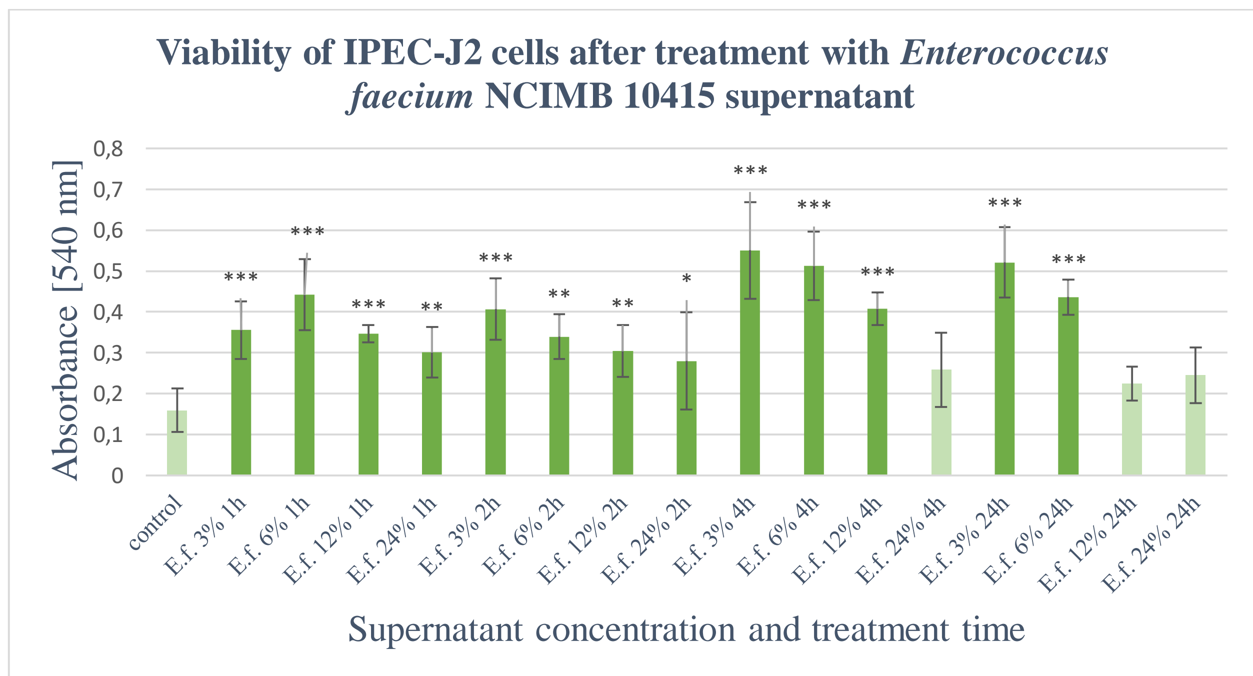

Figure 4. Results obtained from viability measurements of IPEC-J2 after treatment with *Enterococcus faecium* NCIMB 10415 supernatant. *: $P \leq 0.05$ **: $P \leq 0.01$ *** $P \leq 0.0001$ n=6 E.f: *Enterococcus faecium*

Figure 5 shows the results of *Lactobacillus rhamnosus* DSM7133 supernatant treatment. It seems that less than 50% of the applied treatment concentration and incubation times resulted in a significant improvement in the number of viable IPEC-J2 cells compared to the control. It is conspicuous, that 12% and 24% treatment with metabolites of *Lactobacillus rhamnosus* did not change the number of living enterocytes compared to the control. Administration of 3% and 6% SCS produced significant increase in the absorbance values, the only exception was the effect of 6% and 24 hours treatment time. In the case of 3% treatments, 2 hour, 4 hour and 24 hour treatment time groups differed significantly from the control at the level of 99.9.

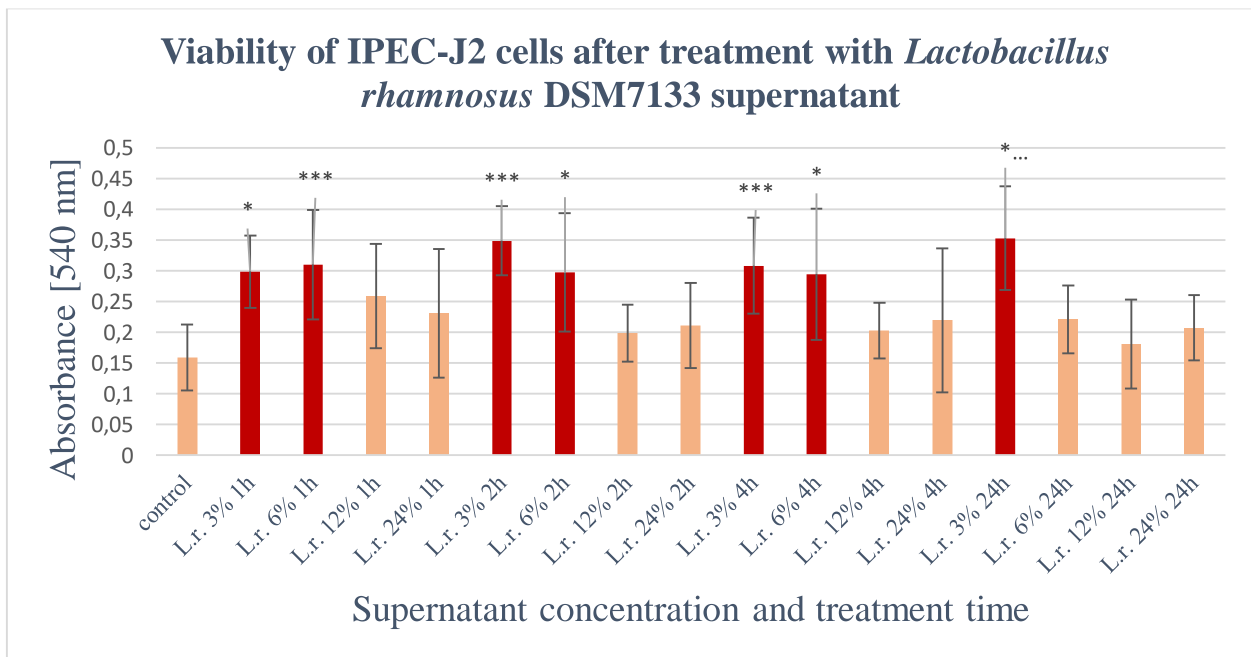

Figure 5. Results obtained from viability measurements of IPEC-J2 after treatment with *Lactobacillus rhamnosus* supernatant. * = P ≤ 0.05. **: P ≤ 0.01 *** P ≤ 0.0001 n=6 L.r: *Lactobacillus rhamnosus* DSM7133

Effect of *Lactobacillus plantarum* administration (Figure 6) on the viability of IPEC-J2 cells is similar to *Lactobacillus rhamnosus* DSM7133 treatment. Incubation with 12% and 24% SCS did not influence significantly the viability of IPEC-J2 cells, only in case of 12% and 4 hours treatment time. Treatment with 6% supernatant increased the number the viable cells most of all. Incubation with 3% of SCS for 1 hour, 2 hours and 24 hours caused a significant increase in enterocyte viability as well.

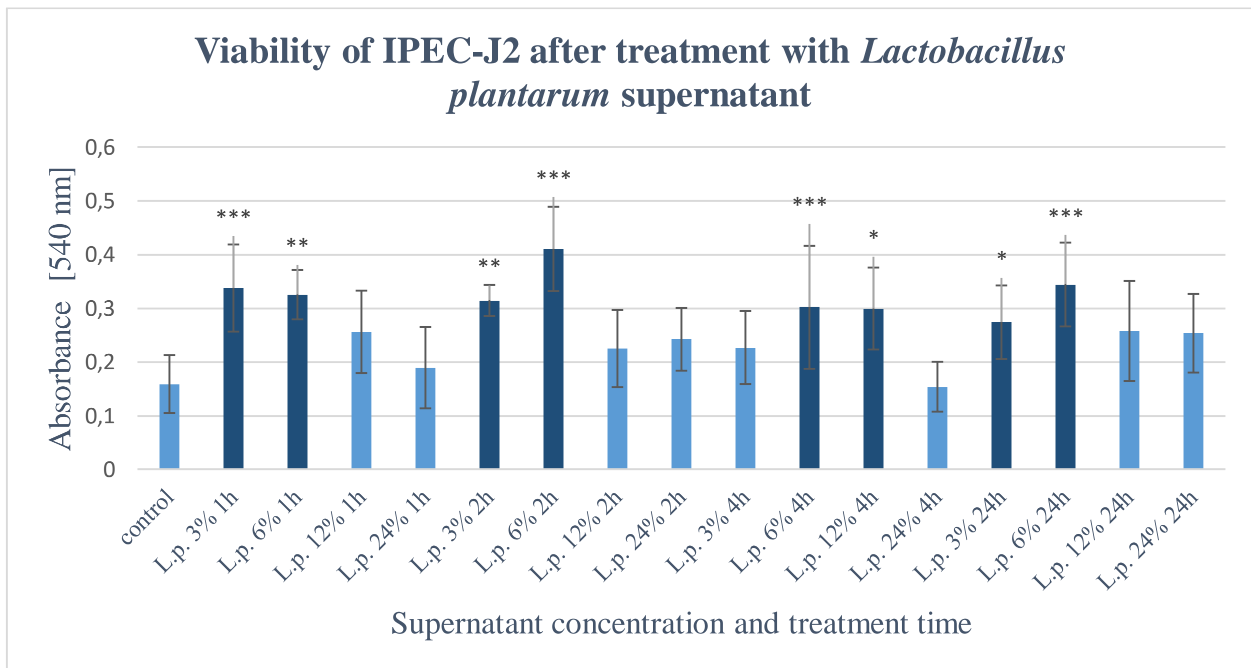

Figure 6. Results obtained from viability measurements of IPEC-J2 after treatment with *Lactobacillus plantarum*. * = $P \leq 0.05$. **: $P \leq 0.01$ *** $P \leq 0.0001$ n=6 L.p.: *Lactobacillus plantarum*

6.2 Structural studies on IPEC-J2 cells

At first, growing of IPEC-J2 cells on Aclar film was examined. The TEM images revealed enterocyte-like cells. Despite not using Transwell collagen-coated membrane inserts, the cells produced a confluent monolayer and formed microvilli of differing lengths and widths (Figure 7A). The formation of cell adhesion structures between neighbouring cells can be observed (Figure 7B).

On figure 7, untreated IPEC-J2 cells are shown. Vacuoles, ribosomes and lipid drops can be also detected. It could be seen in all control samples, that vacuoles were formed in IPEC-J2 cells. Nuclei and nucleoli could also be observed (pictures not shown).

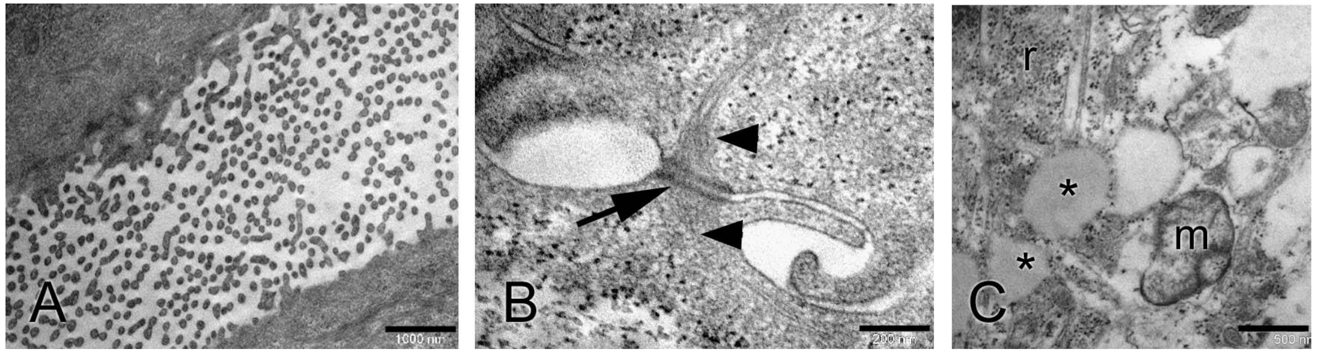

Figure 7. Electron micrographs showing subcellular details and components of IPEC-J2 cells with high magnification: (A) the microvilli of the cells can be seen in cross section. IPEC-J2 cells also develop intercellular junctions (B, arrow) with associated tonofilaments (arrowheads). Their cytoplasm also contain several mitochondria (m), free ribosomes (r) and moderately electron dense granules (asterisks) Scale bar: 1 µm (A), 0.2 µm (B), 0.5 µm (C)

Based on the Neutral Red Uptake viability test, 6% SCS concentration was chosen to treat IPEC-J2 cells. Incubation periods were 1 hour, 2 hours, 4 hours and 24 hours, respectively. In the first experiment, only the effect of *Enterococcus faecium* and *Lactobacillus plantarum* metabolites was followed up. Evaluation of some samples has already been occurred. In order to produce enough data for statistical calculations and find statistically relevant differences in certain structural attributes of enterocytes treated by different SCSs, more samples are needed; analysis of further samples is now in progress. Some examples of cells with different incubation times can be seen in figure 8. Microvillus formation and differences in vacuole formation can be observed. We noticed, that the size and number of vacuoles increased with longer exposure to the SCS (examples are shown).

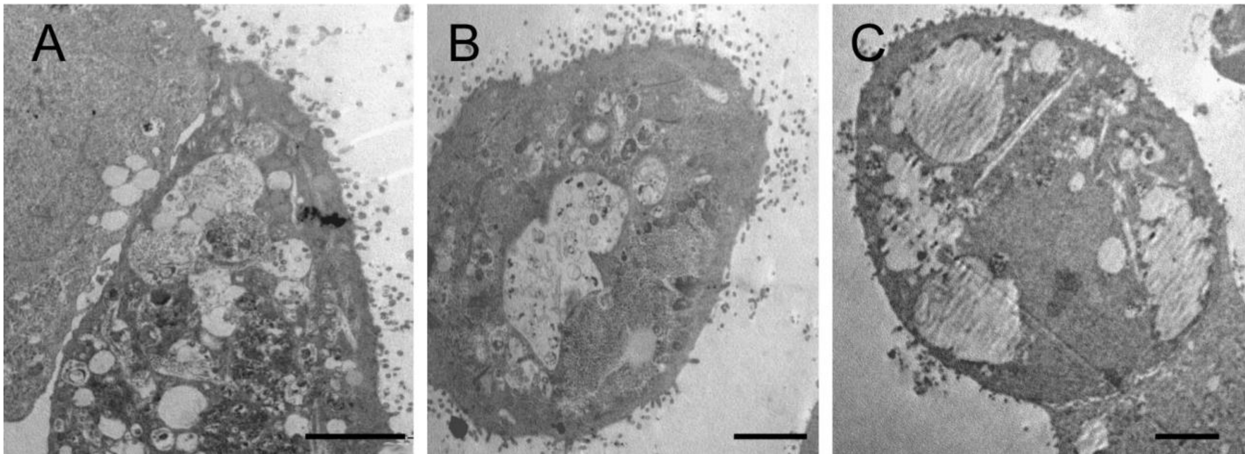

Figure 8: Morphological changes of IPEC-J2 cells upon incubation with SCS of *Enterococcus faecium* for 1 (A), 2 (B) and 4 (C) hour. Scale bar 0.2 µm

Immunofluorescence showed that tight junction proteins claudin-4 and occludin were located in the cell membranes and cytoskeletal proteins β-actin and p34 were present in the cytoplasm (Figure 9).

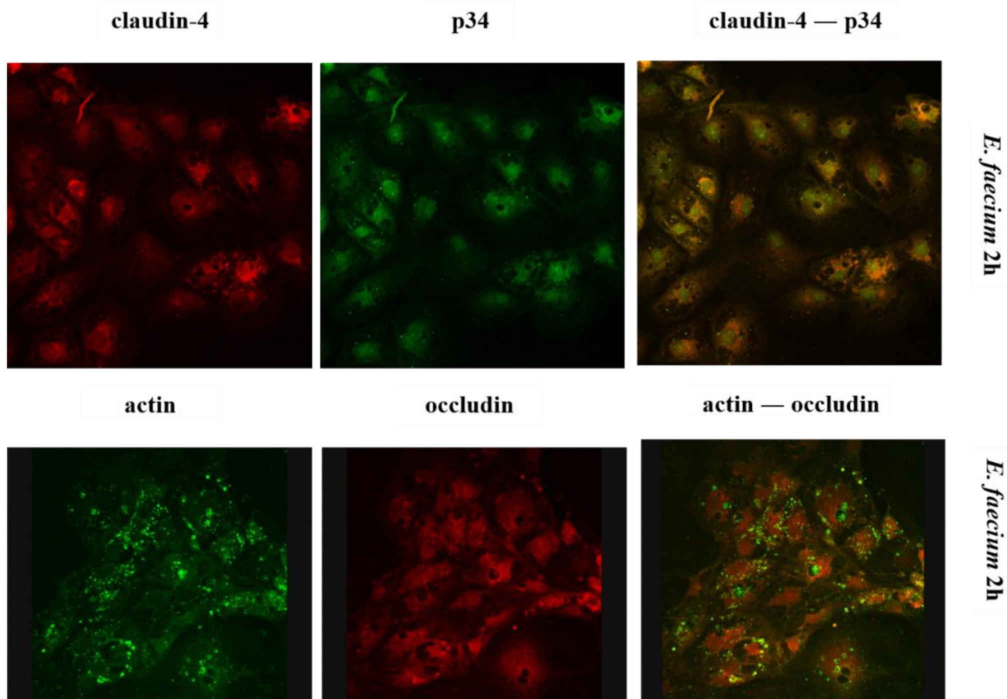

Figure 9: Immunofluorescence localization of claudin-4, occludin, actin and p34 in IPEC-J2 cells. Cells were treated with *Enterococcus faecium* supernatant for 2 hours.

7 Discussion

Probiotics have grown to become a common supplement in the day to day diet of humans; as the trend of taking charge of one's own health has become progressively popular. With continued research and utilization of probiotics in the veterinary field, we can aim to provide a wider choice of therapeutic options to treat a variety of clinical conditions. Furthermore, with current issues such as antibiotic resistance, it is an important research issue to find alternative natural ingredients that can enhance gastrointestinal health. As an impaired intestinal barrier caused by an infection can allow pathogens and toxins to enter the body. Tight junctions, which are a component of junctional complexes, are responsible for controlling permeability of the paracellular pathway, preventing leakage from the lumen and facilitating the cell-cell adhesion of enterocytes.

In this work the following three probiotic bacterial strains were examined: *Enterococcus faecium* NCIMB 10415, *Lactobacillus rhamnosus* (DSM7133) and *Lactobacillus plantarum.* Firstly, the application of different incubation times and concentrations of the spent culture supernatants on IPEC-J2 porcine epithelial cell culture were examined. Viability measurement was carried out using the Neutral Red Uptake method. The primary goal was to determine the safe combinations for TEM and CLSM studies, but we received some unexpected results. It was discovered that all three bacterial strains exerted a significant beneficial effect on the cell viability of IPEC-J2 cells. Liu et al., 2015 found that 12 hours of incubation with different probiotic bacteria partly affected the survival of IPEC-J2. The number of live cells were not different after *Lactobacillus johnsonii* and *Lactobacillus reuteri* treatment compared with the untreated control; whereas *Lactobacillus rhamnosus* GG significantly decreased the cell viability. This result differs from our observations, but in the above mentioned study, living bacteria were used to treat cell cultures. It was shown in a previous study by the department that metabolites of *Lactobacillus plantarum* 2142 were effective in decreasing the LPS-induced inflammatory response in an IPEC-J2 monoculture (Farkas *et al.,* 2014). Using the IPEC-J2 *in vitro* model, there are two ways of completing the experimentation: either treating the monolayer directly with a probiotic bacterium species or incubating with SCS. According to Klingspor *et al.,* 2015 the advantage of using the SCS allows to avoid the pH decrease in the medium due to

the rapidly growing bacteria. The disadvantage would be that SCS cannot fully mimic the effect that live bacteria would have; therefore this excludes potential direct interactions of living bacteria with epithelial cells. Future examinations could include comparing effect of live bacteria and their metabolites on enterocytes. It was demonstrated (Lodeman et al., 2015) that if the application time of the live bacteria was reduced, then the pH of the cell culture media had less time to decrease. They also found that *Enterococcus faecium* alone increased transepithelial electric resistance of Caco-2 colon adenocarcinoma cells. Preincubation with *Enterococcus faecium* had no effect on the transepithelial resistance decrease induced by ETEC. Using a slightly different protocol, *Enterococcus faecium* ameliorated the transepithelial electric resistance decrease induced by ETEC at 4 hours in IPEC-J2 and at 2 hours, 4 hours, and 6 hours in Caco-2 cells. Reproducibility of the results is, however, limited when experiments are performed with living bacteria over longer periods.

Further structural and functional studies on enterocytes were carried out using TEM and CLSM with immunohistochemistry. IPEC-J2 cells were grown in a confluent monolayer and formed microvilli of differing lengths and widths. Cell adhesion structures between neighbouring cells could also be observed.

Our investigations with TEM and immunohistochemistry foreshadow that the treatment with probiotics had an effect on the ultrastructure of IPEC-J2 cells that may also have functional relevance. Vacuole formation could be observed on all the images taken by TEM. Cytoplasmic vacuolization is a frequently observed phenomenon during exposure to pharmaceutical agents and other chemicals. The reason for vacuolization is very complex, both physicochemical and biochemical properties are considered to play a role in the process. Compartments on endosome to lysosome pathway, compartments on autophagy to lysosome pathway and the endoplasmic reticulum can be vacuolated. In order to elucidate the origin of vacuoles further experiments including electron microscopic observations, immunocytochemical analyses with appropriate organelle markers are needed (Aki *et al*., 2012).

As it was mentioned in the results section, evaluation of microscopy samples are still in progress. The dimensions of the enterocytes, presence and size of vacuoles, number of microvilli should be analyzed; control and treated samples should be compared. Changes in

quantity and structure of tight junction and cytoskeletal proteins are planned also to be investigated quantitatively. Other junctional proteins such as ZO-1 is planned also to be studied. Culturing IPEC-J2 cells on membrane inserts might also be implemented; differences in cell structure can be compared with samples derived from enterocytes growing on Aclar film. Further studies will focus on the effect of SCSs when IPEC-J2 cells are triggered by bacterial lipopolysaccharides or pathogenic bacteria. Cooperation with research partners (Magyar Kísérleti Tejkutató Intézet, Mosonmagyaróvár) will result in data about other properties such as stress tolerance, production exo-polysaccharides and other anti-microbial compounds, which can also be used for selecting the most effective strains in order to maintain the gut's structure and function in pigs.

8 Summary

Since the 1960's, wide spread use of sub-therapeutic levels of feed antibiotics for monogastric animals has been practiced. This has led to serious issues with antibiotic resistance and subsequently in 2006 the use of antibiotics as growth promotors were banned in the European Union. In the case of food producing animals, for example pigs, intestinal disease results in significant economic loss. Therefore, it is an important research issue to find alternative natural ingredients that can maintain the health of the gut. The use of probiotics offers an alternative to enhance gastrointestinal health.

One of the most important ways in which probiotics exert their beneficial effect on their host, is by modifying metabolic processes. Probiotics have also been found to strengthen the intestinal barrier against noxious stimuli. However, some of the underlying mechanisms are still undiscovered.

In this work the *in vitro* effect of three probiotic bacterial strains (*Enterococcus faecium, Lactobacillus rhamnosus, Lactobacillus plantarum*) will be investigated. The cell line featured in this study is IPEC-J2, isolated from the jejunum of a neonatal unsuckled piglet. Our main research question is on the one hand to determine the appropriate treatment conditions (probiotic bacterial supernatant concentration and applied treatment time) and on the other hand to examine the effect of probiotic treatment on the structure and function of enterocytes.

IPEC-J2 cells were treated with spent culture supernatants (SCS) derived from the probiotic strains using different incubation times and concentrations. Afterwards, viability measurement was carried out using the Neutral Red method. Structural and functional studies on enterocytes were carried out with electron microscopy and immunohistochemistry. Size of enterocytes, vacuoles, number of microvilli and presence of tight junction proteins were studied as well.

We found that all three bacterial strains exerted a significant beneficial effect on the cell viability of IPEC-J2 cells, although alterations in the extent could be observed. Our investigations with electron microscopy and immunohistochemistry confirmed that the treatment with probiotics had an effect on the structure and function of IPEC-J2 cells.

9 Összefoglalás

Az antibiotikumok alacsony dózisban történő hozamfokozás célú megjelenése a takarmányozásban az antibiotikum rezisztencia növekedésének problémaköréhez vezetett, ezért az EU döntéshozói 2006-ban betiltották az alacsony dózisú antibiotikumok hozamfokozóként való alkalmazását. Haszonállatok, pl. sertések esetében a bélrendszeri megbetegedések súlyos gazdasági károkat is jelentenek. Éppen ezért fontos kutatási cél olyan természetes eredetű kiegészítők keresése, amelyek képesek fenntartani a bélrendszer egészséges állapotát. A probiotikumok egy alternatív megoldást jelenthetnek azáltal, hogy elősegítik a bélrendszer egészséges működését.

A probiotikumok sokféle módon kifejthetik jótékony hatásukat, pl. módosíthatják a metabolikus útvonalakat, erősítik a bél barrier funkcióját is az ártalmas hatásokkal szemben. Hatásmechanizmusuk azonban részben még mindig ismeretlen.

A kutatás során három probiotikus baktériumtörzs (*Enterococcus faecium* NCIMB 10415*, Lactobacillus rhamnosus* DSM7133*, Lactobacillus plantarum*) hatását vizsgáltuk *in vitro* körülmények között. A kísérleteket IPEC-J2 egészséges sertés jejunum sejttenyészeten végeztük. Kutatásunk során célkitűzésünk volt egyrészről a megfelelő kezelési feltételek (úgymint a probiotikus felülúszó koncentráció és a kezelési idő) meghatározása, másrészt tanulmányozni kívántuk a probiotikumokkal történő kezelés hatását az enterociták szerkezetére és funkciójára.

Az IPEC-J2 sejteket különböző koncentrációjú probiotikus felülúszóval kezeltük különböző időtartamokon keresztül. A kezelést követően a sejtek életképességét a Neutral Red módszer segítségével határoztuk meg. A bélhámsejtek szerkezetében és funkciójában bekövetkező változást elektronmikroszkóppal, illetve immunhisztokémiai vizsgálattal követtük nyomon. Tanulmányoztuk az enterociták méretét, a vakuólumokat, a mikrovillusok számát, továbbá a tight junction fehérjék jelenlétét.

Kísérleteink során kimutattuk, hogy mindhárom probiotikum kedvezően befolyásolja az IPEC-J2 sejtek életképességét. Elektronmikroszkópos és immunhisztokémiai vizsgálataink alátámasztották, hogy a probiotikumokkal történő kezelés változást idéz elő az IPEC-J2 sejtek szerkezetében és funkciójában.

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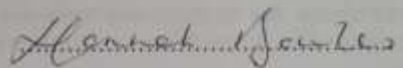

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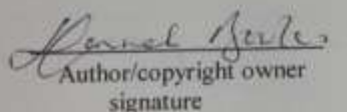

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Name and title of thesis reviewer: ______

Written assessment:

1.) Considerations regarding formatting, length and style

______

2.) Professional assessment

______

3.) Further comments

____

____

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Questions proposed to the candidate:

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Grade proposed by members of the Assessment Committee: ________

Final grade: ________

Comments: ______________________________

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