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Ph.D. Thesis

Health-promoting compounds in food and feed: an *in vitro* approach to study dietary bioactives

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“Whenever a theory appears to you as the only possible one, take this as a sign that you have neither understood the theory nor the problem which it was intended to solve.”

— Karl R. Popper

ABSTRACT

The main aim of this thesis was to study the functional properties of health-promoting compounds *in vitro* with an emphasis on milk proteins, vitamin E and micronutrients.

The health-promoting effects of intact whey and casein proteins were evaluated after *in vitro* SGD. Permeate (absorbed fraction) and retentate (intestinal fraction) were obtained and used to study their health-effects *in vitro*. Soya protein was include as non-animal protein in all the experiments performed. Following SGD, the milk proteins exhibited antioxidant activity, ACE-inhibitory activity and tropho-functional properties at the intestinal cell level. The whey protein permeate exhibited a higher ACE-inhibitory activity compared with the casein and soya protein permeate. SGD increased the ACE-inhibitory activity of whey protein and the antioxidant activity of all the protein tested. At specific concentrations, casein, whey and soya proteins were able to modulate intestinal cell viability and the production of intestinal mucus. Moreover, the proliferation of *Lactobacillus casei* was increased by specific concentrations of whey and casein proteins. Modulations of mucus production and probiotic bacteria growth were observed, and casein was the primary protein that was able to stimulate MUC5AC gene expression and promote *Lactobacillus casei* growth. Altogether, the analyses of goblet cell proliferation and prebiotic bacterial growth may represent complementary approaches to study the bioactivities and the functions of food proteins in the gut. Additionally, the comparison of the effect of the two major intact milk proteins performed in this thesis could provide valuable information regarding which is more efficacious in improving health.

α-tocopherol has been demonstrated to play an important role in reducing oxidative stress at cellular level, in different *in vitro* models. Food toxicants, as OTA, have been found to be able of disrupting the cell monolayer and damaging DNA, which leads to cell death. OTA reduced tight junctions protein localization in cell membranes and influenced cell-cell interactions. The immunofluorescence analyses revealed changes in the patterns of occludin and Zo1 proteins in the presence of OTA, compared with control cells. OTA cytotoxic effects were counteracted by the presence of α-tocopherol. The pre-treatment

with α-tocopherol blocked the loss of occludin protein in the tight junctions of kidney cells treated with OTA. α-tocopherol supplementation has demonstrated to counteract short-term OTA toxicity at different cellular levels, supporting the defensive role of this compound in the cell membrane.

Finally, the *in vitro* roles of different micronutrients, specifically choline/methionine and different Zn formulations, were determined. Due to their physiological health-promoting effects, both choline/methionine and Zn are commonly used in animal nutrition. The results of this thesis confirmed that under condition of stress, choline and methionine have important roles in enhancing cell viability and counteracting oxidative stress. Specific concentrations of different Zn sources maintained the viability of human and swine intestinal cells, which underlines the beneficial role of Zn in human and swine intestinal epithelia.

Overall, these results contribute to the identification of the roles of different dietary health-promoting compounds in human and animal target tissues by making *in vitro* models an essential tool. However, further *in vivo* experiments are necessary to extend these *in vitro* results and to clarify the contributions of health-promoting components in animal feed and human formulations. The improved knowledge related to milk protein bioactives, antioxidants and micronutrients represents a crucial subject for future systematic efforts to improve food and feed quality.

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin-Converting Enzyme
BME-UV1	Bovine Mammary Epithelia – University of Vermont – clone 1
CVD	Cardiovascular Disease
EGF	Epidermal Growth Factors
HT29-MTX	HT-29 cells selected by adaptation to methotrexate
IGF-I	Insulin-like Growth Factor-I
INT-407	Intestine 407
MDCK	Madin Darby Canine Kidney
NaACE	Sodium Acetate
NaBt	Sodium Butyrate
NaProp	Sodium Propionate
OTA	Ochratoxin A
SCFA	Short Chain Fatty Acid
SGD	Simulated Gastrointestinal Digestion
TGF-α	Transforming Growth Factor α
TGF-β1	Transforming Growth Factor β1
Zn	Zinc
Zo1	Zonulin 1

PREFACE

Nutritional research commonly employs *in vitro* models to study the roles of health-promoting food compounds. The application of biological *in vitro* assays represents a preliminary step for the development of powerful *in vivo* studies. Among the health-promoting compounds that have received interest in recent years, milk-derived bioactives and antioxidant molecules (e.g., vitamin E) exhibit a wide range of health-promoting applications in humans and animals.

The studies reported focused primarily on the evaluation of the physiological and health-promoting effects of milk bioactives, vitamin E and other micronutrients that were investigated with an *in vitro* approach.

This Ph.D. thesis is composed of three chapters.

Chapter I. In the first chapter, the health-promoting role of *in vitro*-digested milk proteins was investigated with consideration for the antioxidant and ACE-inhibitory effects. Further, the influence of protein digesta was investigated at the gut level in terms of intestinal cell proliferation, the production of the intestinal mucus and the effect on the growth of prebiotic bacteria (*Lactobacillus casei*). The experiments reported in this chapter were conducted in collaboration with the University of Aarhus (DK) and the University of Reading (UK).

Chapter II. In the second chapter, I present three studies related to the health-promoting role of vitamin E as an antioxidant *in vitro*. Specifically, I evaluated the relative bioefficacies of two different forms of α-tocopherol in counteracting the cytotoxicity induced by hydrogen peroxide. To this purpose, *in vitro* models of the mammary gland (BME-UV1) and the kidney (MDCK) were employed. Moreover, the role of α-tocopherol in the counteraction of OTA toxic activity in the kidney was determined. To this purpose, the mechanism of action of OTA at the cellular level (DNA integrity, 8-OHdG adduct formation, DNA methylation pattern) was determined *in vitro*.

Chapter III: In this chapter, I present two studies about cell-micronutrient interactions that I performed during my Ph.D. studies. Specifically, the first study aimed to investigate the roles of choline and methionine in counteracting cytotoxic damage using an *in vitro* mammary cell model. The second study aimed to identify the contribution of Zn in the swine intestine and the human epithelium.

In the **add-on project section**, I report the training activities I performed during may experience abroad at Reading University (UK) as a research scholar. At Reading University, I was involved in the set-up and design of the *in vivo* human study that sought to investigate the acute and chronic effects of milk proteins on heart disease risk markers, such as blood pressure and vascular function. The obtained results will be submitted to a scientific journal after the completion of the data analysis and will be compared with the results of the *in vitro* investigation of the effects of digested proteins that are reported in chapter I of this thesis.

GENERAL INTRODUCTION

Health-promoting compounds

The food industry is one of the most important sectors of the EU economy. Despite the low intensities of research and development (Garcia Martinez, 2000), innovation and novel production development represent important strategies for company growth and the satisfaction of consumer demands (Menrad, 2003 & 2004). Specifically, over the past two decades, consumer 'expectations have become more direct regarding healthier and higher quality foods, and the consumer demand for foods that support functional needs has rapidly expanded. Food components are not only demanded due to their traditional nutritional value but are also demanded for their health-promoting activities. This factor, combined with the increase in unhealthy diet-related disease (e.g., diabetes and cardiovascular disease) (Li Chain, 2015), led to strong development of the concept of health-promoting compounds. Health-promoting compounds may be defined as food ingredients with specific bioactive functions, such as antioxidant, ace-inhibiting, anti-inflammatory, prebiotic and probiotic functions, despite their traditional nutritional roles. Health-promoting compounds represent a sustainable trend in the food market (Bigliardi and Galati, 2013) and an interesting area of research for the scientific community. Numerous bioactive compounds appear to have beneficial health effects *in vivo* and *in vitro*.

In this context, food-derived peptides have high nutritional values and are healthy compounds that are suitable for the development of health-promoting functional foods. Milk proteins are considered one of the most important sources of bioactive peptides and are encrypted in inactive forms in parent protein sequences. Peptides can be released through gastrointestinal digestion. The activities of the gastric and pancreatic enzymes induce the production of bioactive peptides that have been identified as potential ingredients in health-promoting foods. Once released, milk bioactive peptides exert a wide range of physiological effects that differ from those of their parental proteins, including

antihypertensive, antithrombotic, prebiotic and antioxidant effects and the ability to modulate gastro-intestinal cell activity and function (Wada and Lonnerdal, 2014; Petrat-Melin et al., 2015; Plaisancié et al., 2014). The greatest bioavailability and bioactivity occur when the size of a bioactive peptide is approximately 3–20 amino acids, such amino acids are encrypted within the parental proteins and become activated following their release, which is induced by the actions of gastric and pancreatic enzymes (Kekkonen 2009; Korhonen 2009; Korhonen et al., 1998).

Vitamin E, carotenoids and flavonoids have been studied for their potential health benefits particularly those related to antioxidant activity and potential roles in disease prevention (Virtamo et al., 2003 and 2014). Vitamin E is an oil-soluble antioxidant that is associated with several health benefits, such as reducing CVD, diabetes and cancer (Sylvester et al., 2011; Traber et al., 2008). Moreover, vitamin E plays roles in hemoglobin biosynthesis, the modulation of immune responses, and the stabilization of the structures of membranes. Meydani et al. (2000) described the rationale for including food containing high levels of vitamin E in the daily diet in combination with generous amounts of supplemental vitamin E in designed functional foods. Vitamin E, in the context of dairy cow nutrition, has a role as antioxidant that is able to prevent free radical-mediated tissue damage and delay the development of degenerative and inflammatory diseases. Vitamin E seems to be involved in the immune system function of cows. Supra-nutritional levels of the vitamin may result in improved immune responses (Baldi, 2005). Smith and co-workers (1984) established that supplementation with vitamin E, in conditions in which dietary selenium is adequate, significantly reduces the incidences of intramammary infections (IMI) and clinical mastitis. *In vitro*, vitamin E is one of the most important components of cellular antioxidant systems. Vitamin E (tocopherols and tocotrienols) is present in all cell membranes and plasma lipoproteins. Vitamin E is one of the major lipid-soluble chain-breaking antioxidants that protects DNA, low-density lipoproteins (LDLs), and polyunsaturated fatty acids from oxidative damage. Tocopherol has previously been demonstrated to protect the cell from oxidative damage *in vitro* (Baldi et al., 2004) and reduces DNA fragmentation and apoptotic body formation (Fusi et al., 2008). However,

only a few reports in the literature have compared the activities of different tocopherol isomers in terms of *in vitro* oxidative environments.

Scientific food and feed research commonly uses *in vitro* models to study interactions between health-promoting compounds and the intestinal, mammary and renal epithelia. The application of *in vitro* assays represents a useful tool for the development of powerful *in vivo* studies.

In vitro cell culture models

In light of the strong international concern to reduce the use of experimental animals, cell culture technology is reaching growing interest among nutritional and food researchers. *In vitro* models are used to study proliferation, differentiation, toxicology potential of the food and feed compounds, limiting the use of experimental animals. Moreover, considering the high-defined *in vitro* parameters and conditions, cell culture models lead to the production of reproducible results essential for a well design of *in vivo* studies. Cell-based bioassays are representative of the *in vivo* situation and physiology and therefore they are considered a suitable alternative for *in vivo ex*perimentation. The need of reliable *in vitro* cellular models as alternatives to animal studies is considered by the European legislation (http://www.euractiv.com/en/science/ eu-wants-fewer-animals-used-research-news-497607). As the science behind *in vitro* technology advances, and results from new alternatives are shown to be both transferable and reproducible, *in vitro* models continue to gain acceptance among research community and regulatory bodies (Cheli & Baldi, 2011). Cell-based bioassays have many advantages including accessibility, homogeneity of cell population, reproducibility, stable growth rate, and can be designed to reflect cell and tissue responses and address specific experimental conditions and challenges (Langerholc et al., 2011). Nowadays, there are centers throughout the world that focus on the development, validation, and use of alternatives in the biomedical sciences.

The interest of food and pharmaceutical industry to cell-based bioassays is mainly related to toxicological and bioavailability tests of newly developed food ingredients and drugs, inevitable for bringing products to the market. The research regarding the safety and efficacy of additives and new functional food ingredients is an open issue and may take advantage from the use of specific cell-based functional bioassays (Baldi & Cheli, 2011). The use of cell-based models in food research has many advantages; several concentrations of a compound could be assayed at the same time, under strictly control of chemical, physical and physiological conditions, also considering the hormesis concept (Calabrese et al., 1998; Calabrese, 2010). For each food constituent tested, several endpoints can be investigated, such as cell proliferation, viability, cell migration and the regulation of gene-expression, making the *in vitro* cell technology a promising sector of food research. Further, cell-based bioassays represent a low-price and speed response alternative to animal testing, in line with the European Union guidelines (Cheli et al., 2015).

However, the use of cell model is not free of problems. Depending on the model selected, cell cultures may not reflect the *in vivo* situation due to the loss of architecture and three-dimensional tissue-specific interactions between cells. The lack of endocrine and nervous systemic components in the cultural environment represent a limitation with difficulties to maintain cell differentiation. Moreover, *in vitro* studies do not take into consideration the effects of bioavailability, pharmacokinetics, metabolism, distribution and interaction with binding and transport proteins, or other biological processes that occur in the intact organism, all of which may influence the biological effects of food and feed compounds. Cell metabolism is more constant *in vitro* but not fully representative of the *in vivo* tissue. Therefore the results obtained *in vitro* need to be translate in well-design animal and human studies in order to define the physiological effect of bioactive food components (Purup & Nielsen, 2012). *In vivo* experiments provide information on the net effect of a compound in the whole organism, whereas cell-specific answers may be attained with *in vitro* tests. In order to ameliorate the use of *in vitro* model in food research, scientists

should carefully considered the type of cells on the basis of the target organ of the compound to be tested and the most important endpoints to measure.

Type of cell-based cultures

A cell culture is a generic term used to indicate the process by which cells, arising from the breakdown of tissues or isolated from biological fluids such as blood, are grown and maintained strictly controlled conditions.
In vitro systems have been designed to simulate the *in vivo* conditions preserving both the characteristics of the original tissue and the cell-cell interactions (Zucco *et al.,* 2004). A wide range of *cell-based* models is currently available for food researcher including organ cultures, primary cultures, immortalized cells, and three-dimensional models (Zucco et al., 2004).

Organ cultures

Organ culture retains whole or partial (explant) original tissue characteristics and cell–cell interactions *in vitro*. Therefore, organ culture could preserve the differentiated properties of the derived tissue, making this *in vitro* model useful for morphological studies.
However, organ cultures cannot be propagated and do not proliferate rapidly, hence remain in culture for a limited period of time. In addition, organ cultures are characterized by a high intersample variation, with high implication in the reproducibility of the experiments, making difficult to extend the employment of these model in nutritional studies.

Primary cultures

A primary cell culture is the stage of the culture after the isolation of the cells and before the first subculture. The wild-type nature of primary cells make this model useful for mechanicist and morphological studies. However, the reduced lifespan together with the altered cell phenotypes of primary cell cultures, the slow proliferation rates and metabolic capacities limits the applicability of these models in food and nutritional research experiments. Moreover, the cell population of a primary culture is not homogeneous and can be propagated to a limited number of passages (Astashkina et al., 2012).

Immortalized cell cultures

After the first sub-culture, the primary culture become a cell line and may be propagated and subcultured several times. Three-dimensional (3D) models, characterized by a well-defined geometry, maintains cellular interactions and the relationship between structure and function, preserving the cellular interactions. 3D models make use of different substrates and the culture can be homotypic or heterotypic. This system enables the main cellular functions to be maintained for a limited period of time and generally, do not reach a complete stage of differentiation (Mueller-Klieser, 1997).

Barrier systems cultures

Barrier systems are used to study the epithelia (eg. skin, intestine), which are focal points of interaction with xenobiotics and nutrients. The epithelial intestinal barrier is the first gate which actively interact with food compounds, after ingestion. In particular, HT-29 and Caco-2 cell lines have been extensively characterized and widely used for studying the intestinal barrier function, due also to their ability to differentiate in mature enterocytes during long-term culture. HT29-MTX-E12 are also a model of the colonic environment,

with the main property of differentiating in culture into a mucus-producing cell line (Behrens et al., 2001).

Three-dimensional cultures

The immortalized human cells ‘‘jump’’ the senescence barrier, without losing the other physiological properties. These cells undergo cycles of indeterminate replication through the introduction of sequences of DNA tumor viruses, such as SV40, Papilloma virus and Epstain-Barr virus (Zucco et al., 2004). The fast growth rate, the high nucleus/ cytoplasm ratio compared with primary cells and the ability of growth up to high cell density are some of the characteristics of this *in vitro* system. Immortalized cells retain few characteristics of differentiated tissue, and a relative homogeneity; their use facilitates the comparison of results from different laboratories.

Functional *in vitro* models and their application in food and nutritional science

Food science research developed several *in vitro* models to study and reproduce *in vitro* the mammary, kidney and intestinal epithelia. These models represent interesting and efficacious systems to study the interaction between food components and target cells. Despite the primary role of the intestinal epithelium in the food compound uptake and metabolism, the kidney and mammary gland epithelia represent secondary targets of several intestinal metabolites. There is a strict interaction among the gut epithelium and the secondary target tissues, due to chemical and biological signals mediated by food compounds and/or their metabolites. Food compounds modulate primarily the intestinal epithelium after the ingestion, but after the intestinal absorption may influence the function and the physiology of other tissue and organs. Cell-nutrient interaction studies represent a promising system to study and well-describing the role of bioactive at cell level.

In vitro models of the intestinal epithelium

The gastrointestinal tract is the primary target of dietary compounds, and represent the first and most important interface between food and the internal body. therefore, intestinal *in vitro* models are of great interest in food and feed research.

The intestine is an important environment responsible for processing digestive foodstuff, absorbing nutrients and protecting the body against pathogens entering with food. The intestinal mucosa is defined by sheets of epithelial cells connected by tight junctions that form the epithelial barrier of the intestinal wall. The intestinal epithelial monolayer consists of several subsets of epithelial cells that cooperatively constitute a physical and biochemical network for the maintenance of the homeostasis between the body and the luminal environment (Goto & Kiyono, 2012; Cencic & Langerholc, 2010). the predominant cell type in the intestinal epithelium is the enterocytes n the small intestine and colonocyte in the large intestine. Enterocytes are specialized for digesting molecules and transporting small molecules coming from digestion, colonocytes are absorbing cells, mainly implicated in the transport of electrolytes and water.

Among the cell line available, caco-2 cells have been widely used for proliferative and mechanistic studies. They express characteristic and functionality of enterocyte, upon reaching confluence (Langerholc, 2010). The human colon carcinoma HT-29 cells, besides being a model to study the different aspects of the biology of human cancer, they are receiving interest in food digestion and bioavailability studies (Didier et al., 1996; Takahashi et al., 1996).

Along the intestinal tract, goblet cells are present in different number, representing one of the major cell types in the intestine. Goblet cells have a crucial role of secreting mucins, glycoproteins responsible for the viscoelastic properties of the intestinal mucus layer and in maintaining the homeostasis of the intestinal microenvironment and in controlling the localization of commensal bacteria (Goto and Kiyono, 2012). Goblet cells can be mimicked *in vitro* using mucus secreting cell lines, such as HT29-MTX-E12 (**Figure 1**). HT29-MTX-E12 cells were extensively used to study the role of bioactive peptides in gut

function (Martinez-Maqueda et al., 2013) even if they were not fully characterized respect to the major components of the gut.

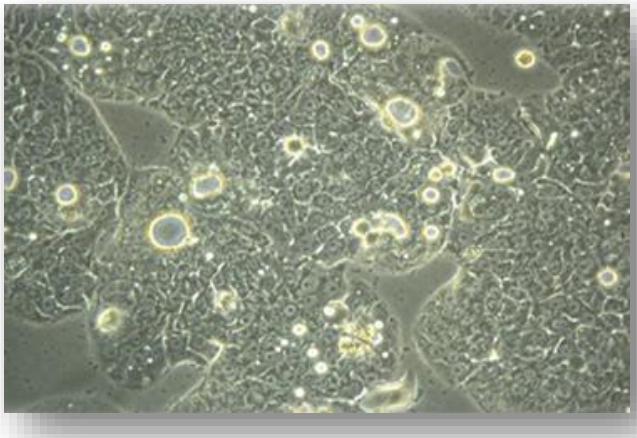


Figure 1*: representative image of HT29-MTX-E12 cell line in culture (40X)*

Since the human intestine is the first target of bioactive molecules entering with food, the identification of the effects of specific bioactive factors at the intestinal cell level may give novel insight into promoting gastro-intestinal development and health, and may have applications for the treatment of a number of diseases.

In vitro model of the mammary gland

Mammary tissue cells or explants have been widely-used over the last years as models to understand the physiological function of mammary gland. The mammary gland is a target organ for several food derived bioactive and therefore a number of mammary cells, in particular of bovine specie, have been established.

Therefore, emphasis has been placed on cell culture methodologies to study growth regulation, hormonal responsiveness or biochemical properties of mammary epithelial cells (MEC). Some of these previous work have led to the development of stable epithelial cell lines of bovine mammary gland (Hu *et al*., 2009).

MAC-T cell lines, BME-UV and BME-UV1 are some of the *in vitro* models widely used to study the biology of the mammary gland of ruminants.

The MAC-T cell line was obtained by transfection of bovine primary epithelial cells with the antigen SV40. This cell line maintains *in vitro* many of the morphological characteristics of breast epithelial cells and can differentiate into a secretory phenotype. This cell model has been widely used to characterize the role of various growth factors in the bovine mammary epithelial cells or to study the interaction of pathogenic bacteria with bovine epithelium. MAC-T cells do not respond to the epidermal growth factor (EGF), a known modulator of mammary function (Zavizion et al., 1996). However, MAC-T is not a homogeneous cell line: the three sub-clones of this cell line differ in size, growth pattern and cytogenetic features (Cheli et al., 1999).

Other *in vitro* models of the bovine mammary epithelium are represented by BME-UV1 (**Figure 2**) and UV2, obtained by microinjection of the large-T antigen of SV40 in epithelial cells of bovine mammary gland in culture (Zavizion et al., 1996). Clones BME BME-UV1 and UV2 assume the "cobblestone" morphology, typical of the mammary epithelial cells. In particular, if they are grown on plastic alone, assume a polygonal morphology, while, on collagen assume a columnar morphology and a domed structure, typical of the organization epithelial cells *in vivo*. Additionally, they are able to form desmosomes and microvilli on the apical surface.

BME-UV cells express functional and biochemical markers of mammary epithelial cells: they are, in fact, competent in the synthesis of α-lactalbumin and αs1casein (Zavizion et al.,1996). The BME-UV1 cells increase their rate of proliferation in the presence EGF and IGF-I (Zavizion et al., 1996) , representing the unique bovine epithelial cell line that responds to EGF. Moreover, BME-UV have been used for studying the effect of a number of bioactive food components, as milk peptides, growth factors, antioxidants as vitamin E and C, considering several endpoints to measure, such as cell metabolic activity, apoptotic body formation and gene expression (Pecorini et al., 2012; Fusi et al., 2008; Baldi et al., 2004). Therefore, these cells represent a valuable *in vitro* system to examine the

interaction between food metabolites and the proliferation, differentiation and cell-to cell communication of the mammary epithelial cell.

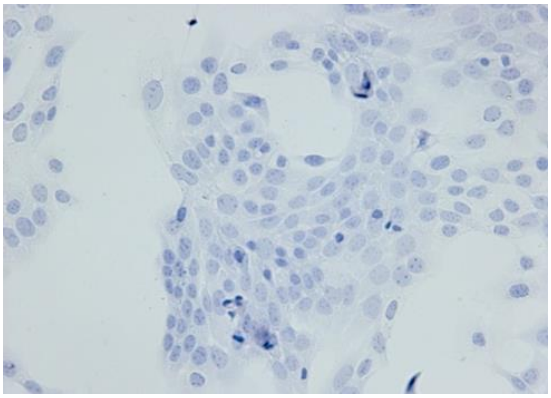


Figure 2*: representative image of BME-UV1 cell line in culture (20X)*

In vitro model of the kidney

Among the models of the kidney epithelium, LLCPK and MDCK cell lines are of the most used and studied in food and nutrition research (Cho et al., 1989)

LLC-PK1 possess the main characteristics of the proximal tubule, such as Na+ dependent transport systems, enzymes located in the apical membrane and maintained *in vitro* the differentiated morphology and had a well developed endocytotic apparatus (Nielsen et al., 1998) These characteristics motivated the wide use of this *in vitro* model for *in vitro* study on the kidney epithelium.

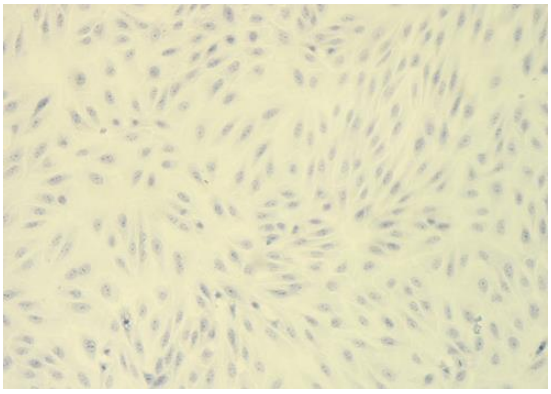


Figure 3*: representative image of MDCK cell line in culture (20X)*

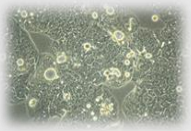
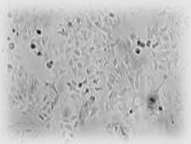
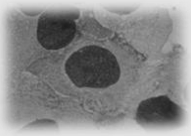
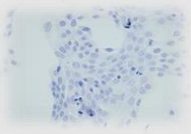
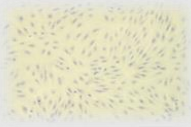
Madin-Darby canine kidney (MDCK) cells (**Figure 3**), derived from the kidney of a normal Cocker Spaniel dog, show monolayer growth in culture and have several functional and anatomical properties like the epithelioid morphology of normal distal tubular epithelial cells (Gaush et al., 1966).

MDCK cells are characterized by apico-basolateral polarity and well defined cell junctions. Due to the clear apico-basolateral polarity, well defined cell junctions and the rapid growth rate, MDCK cell line provides a tractable model for studying cell polarity and junctions (tight, adherens, desmosome and gap) physiology and damage in epithelial cells.

Different strains of MDCK cells are available, as MDCK I, MDCK II, MDCK.1, MDCK.2 and the parental cell line (**Figure 3**). MDCK I cells were isolated from low passage parental MDCK cells while MDCK II cells were obtained from higher passage parental MDCK cells.

MDCK I display high TER values are negative for the tight junction protein claudin-2, displaying an unstable epithelial phenotype. MDCK II display low TER values and are positive for claudin-2. MDCK II are larger and taller than MDCK I and they do not present gap junctions (Dukes et al., 2011).

***Table 1**: Portfolio of cell lines used in this thesis*

Cell line	Cell morphology	Cell origin	Type	Species	References
HT29-MTX-E12		Colon adenocarcinoma	Epithelial	human	Lesuffleur et al., 1990
INT-407		Small intestine	Epithelial	human	Henle & Deinhardt, 1957
IPI-2I		Small intestine	Epithelial	boar	Kaeffer et al. 1993
BME-UV1		Mammary gland	Epithelial	bovine	Zavizion et al., 1996
MDCK		Kidney	Epithelial	canine	Gaush et al., 1966

AIMS OF THE THESIS

The general aim of this thesis was to determine the functional properties of health-promoting compounds *in vitro* with an emphasis on bioactive milk peptides, antioxidants (e.g., vitamin E) and micronutrients.

The specific aims were:

- To study the effect of *in vitro*-digested whey and casein proteins at both the systemic and intestinal levels. The systemic effects were assessed via analyses of the antioxidant and ACE-inhibitory properties of whey and casein digested. The intestinal effects of digested whey and casein were investigated via an *in vitro* intestinal cell model (HT29-MTX-E12) and a prebiotic bacterial strain (*Lactobacillus casei*) that is physiologically present in the gut.
- To study the effect of two different α-tocopherol forms in counteracting the oxidative damage induced by oxidative stressors *in vitro* (e.g., hydrogen peroxide and OTA). To achieve this goal, *in vitro* models of the mammary gland (BME-UV1 cells) and the kidney (MDCK cells) were employed.
- To study the roles of different micronutrients in the proliferation of different cell culture models. To achieve this aim, the first study assessed the roles of choline and methionine in reducing oxidative stress in the mammary gland epithelia, and the second study examined the trophic effects of two different Zn forms in the intestinal epithelia of humans and pigs using INT-407 and IPI-2I cells, respectively.

CHAPTER I: THE HEALTH-PROMOTING ROLE OF *IN VITRO*-DIGESTED MILK PROTEINS

Milk protein digestion

Dairy products, particularly milk, have several and well-known health benefits. Milk represents a rich source of biologically active molecules and possesses a high nutritional value and a wide range of clinical benefits (Hill, 2015). Among the different milk components, the bioactivity of milk is partly derived from intact high-quality proteins and peptides (Fox et al., 2015). Thirty-four grams of protein/liter are present in bovine milk, including 20% whey protein and approximately 80% casein protein. Milk proteins are considered to be the most important sources of bioactive peptides. Beyond the well-known nutritional values of milk proteins, these proteins exhibit an extensive range of bioactivities that promote general health and the functions of specific organs and tissues. The most important health benefits of milk proteins are obtained following gastrointestinal digestion and milk peptides release. Subsequently, the health-promoting functions of peptides are related to their structures, which depend on the parent protein composition and the liberation of peptides following gastrointestinal digestion.

In vivo protein digestion begins when food proteins reach the stomach, and protein digestion is assisted by the highly acidic gastric environment. This acidic environment in the stomach causes the unfolding of proteins, and the gastric enzymes are thus granted easier access to the protein structures to cleave them into smaller pieces. Pepsin is the gastric enzyme that is responsible for the breaking down of dietary proteins into smaller protein portions, i.e., polypeptides. Further, the gastric-protein digesta reaches the small intestine where two pancreatic enzymes, trypsin and chymotrypsin, cleave the polypeptide chain into specific amino acid sequences. Additional enzymes, such as carboxypeptidase and aminopeptidase, which are present in the small intestine, contribute to the intestinal digestion of proteins. Di- and tripeptides can be transported by specific transporters or hydrolyzed into amino acids in the epithelial cells and subsequently absorbed into the

bloodstream and distributed throughout the body where they elicit unique functions (Shimizu, 2004).

In vitro methods for the stimulation of the gastrointestinal protein digestion process are widely used in food research to attempt to simulate *in vivo* physiological conditions in terms of pH, temperature, and digestive enzyme concentration (Minekus *et al*., 2014). These methods are rapid, less expensive and involve fewer ethical restrictions compared with human trials, which are considered to be the gold standard for studying and describing food protein digestion and function. I*n vitro* SGD begins with the mouth phase and then reaches the stomach (gastric phase) where pepsin initiates the degradation of protein in the acidic pH conditions. Furthermore, the digesta are hydrolyzed by pancreatic enzymes (intestinal phase), which results in the production of peptides and amino acid sequences of different sizes. The lengths of the bioactive peptide sequences vary between 3-20 amino acids, and the molecular masses of these peptides are 6000Da (Meisel and Fitzgerald, 2003; Sun et al., 2004). Regulatory peptides may be absorbed via carrier-mediated transport or via paracellular transport to subsequently reach the target tissues where they can elicit their specific health-promoting effects. The absorption is regulated by the intestinal tight junctions and different food substances that may facilitate the paracellular transport of milk peptides (Meisel & Bockelmann, 1999). Milk protein-derived bioactive peptides may act on different intestinal and peripheral target sites of the mammalian organism as exogenous regulatory compounds (Meisel & Bockelmann, 1999).

Milk bioactive peptides obtained by gastrointestinal digestion

It has been widely demonstrated that bioactive peptides derived from milk protein digestion display a wide scope of functions (Hernandez-Ledesma et al., 2014). These properties range from pre-absorptive functions in the gastrointestinal tract (i.e., anti-microbial, prebiotic, and citomodulatory) to post-absorptive functions in the target organs

(ACE-inhibitory, immunostimulating, antioxidant, and hypocholesterolemic functions), and the majority of the peptides display multifunctional properties (Wada and Lonnerdal, 2014).

Antioxidant peptides

Free radicals are physiologically produced in the body and are involved in several positive functions such as the provision of defense against pathogens and infections and signaling roles. The excessive production of reactive oxygen species (ROS) may induce oxidative stress and cell damage at the membrane, protein and DNA levels. Milk protein peptides have been demonstrated to be potent antioxidant molecules with low side effect profiles and high levels of stability and nutritional properties compared with synthetic antioxidants.

Milk-derived peptides exhibit antioxidant properties that counteract radical species or inhibit oxidative reactions (Power *et al*., 2013).

These factors are of particular interest for the development of novel foods and food ingredients with health-promoting functions.

Several assays have been developed to measure the *in vitro* antioxidant potentials of protein/peptide-enriched preparations. However, there is no a validated technique for measuring the total antioxidant activity of a feed or food because of the complex network of antioxidant systems. Additionally, the diversity of methodologies that have been employed makes it difficult to directly compare the data.

Among the available analytical techniques, the trolox equivalent antioxidant capacity (TEAC) assay is among the most frequently used (Zulueta et al., 2009). The mechanism of this assay is based on the scavenging of the 2,2-azinobis- (3-ethylbenzothiazoline-6-sulphonic acid; ABTS) radical cation (ABTS+) by the antioxidant molecules present in the sample.

The ferric-reducing antioxidant power (FRAP) assay measures the ability of a molecule to reduce the ferric ion ferric 2,4,6-tripyridyl-s-triazine complex [Fe(III)-(TPTZ)2]3 to the [Fe(II)-(TPTZ)2]2 ferrous complex at pH 3.6 (Benzie and Strain, 1996).

The FRAP technique exhibits high reproducibility and is simple and rapid to perform (Thaipong et al., 2006).

ACE-inhibitory peptides

CVDs have become an important health issue worldwide. Elevated blood pressure is one of the major risk factors for CVD. In recent decades, there has been a strong interest in the development of functional foods that exhibit beneficial activities that affect the cardiovascular system (Livingstone et al., 2012). The consumptions of milk and dairy products have been demonstrated to be associated with positive effects at the cardiovascular system level, particularly in relation to the control of blood pressure (Fekete et al., 2013). Among the bioactive peptide from milk, ACE-inhibitory peptides have received growing interest in the last two decades due to the high prevalence of hypertensive subjects and CVD in western countries.

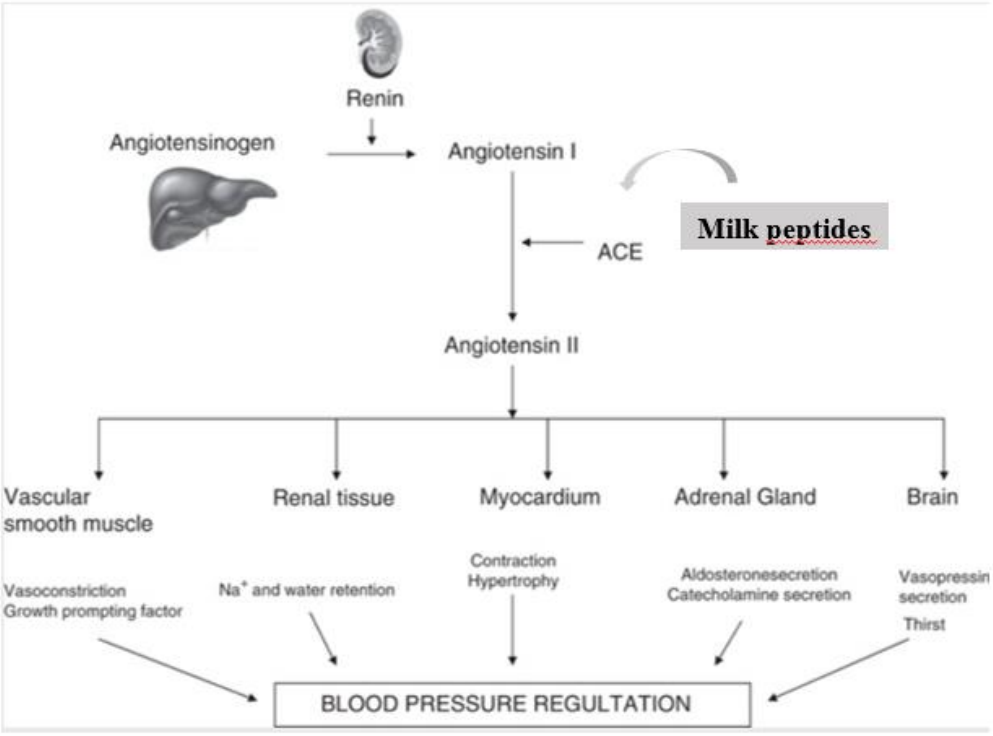


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Figure 1*: Effect of milk bioactive peptides on ACE activity.*

ACE is a multifunctional enzyme that is localized in various tissues and associated with the renin-angiotensin system that controls blood pressure. Specifically, the ACE enzyme converts angiotensin-I to the octapeptide angiotensin-II, which is a potent vasoconstrictor that hydrolyses bradykinin, which is a vasodilating agent (Phelan and Kerins, 2011). The inhibition of ACE can affect various regulatory systems of the body, such as the modulation of blood pressure, the immune system and central nervous system functions (Meisel, 1993). Milk contains many ACE-inhibitory peptides that are able to reduce blood pressure, particularly in hypertensive subjects. Schlimme & Meisel (1995) reported that caseochinine peptides derived from casein fragments exert an antihypertensive effect that reduces blood pressure and increases the local blood flow in the intestinal mucosa. These peptides may originate either from the action of digestive enzymes, such as pepsin, trypsin

and chymotrypsin, or from the actions of proteolytic enzymes at the level of the mammary gland to modulate local blood flow and the functionality of the mammary gland itself (Schanbacher et al., 1997; Madureira et al., 2010). A recent meta-analysis revealed the abilities of lacto-tripeptides (LTPs), obtained from casein digestion, to reduce systolic and diastolic blood pressure (Fekete et al., 2015). Additionally, whey protein enzymatic digestion determines the release of ACE-inhibitor peptides (Moller et al., 2008) as demonstrated by *in vivo* studies conducted in humans (Pal et al., 2010a; Pal et al., 2010b; Pal & Ellis, 2011). It has been shown that the casein proteins and some whey proteins are essential sources of bioactive peptides that elicit positive health effects. However, the EFSA scientific opinion regarding the functional anti-hypertensive activities of some tri-peptides have led to the conclusion that there is still no definitive evidence of a cause-effect relationship between the consumption of these peptides and the control of blood pressure and artery elasticity (EFSA Journal, 2009).

Gut modulating peptides

The GI tract is commonly in contact with food digesta, peptides and growth factors are regularly present in the gut where they are secreted by glands or ingested with foods, such as milk and colostrum (Playford et al, 2000). The gastrointestinal formation of bioactive peptides has been the subject togrowing interest among the food scientists. Several peptides have been demonstrated to modulate different functional properties of various intestinal cells and bacteria. The control of the intestinal epithelial cell growth may be regulated by interactions between growth factors, peptides and the various cell types of the small and large intestinal epithelia. Peptide growth factors and hormones have pivotal roles in the regulation of the functional properties of intestinal cells, including proliferation and differentiation (Dignass & Sturm, 2001). Milk peptides play a role as modulators of intestinal function and exert both prebiotic and citomodulatory roles at the gut level.

Gut modulating peptides: Citomodulatory peptides

Peptides and growth factors are regularly present in the gut where they are secreted by glands or ingested with foods, such as milk and colostrum (Playford et al, 2000). Several regulatory peptides have been found to be present in the intestine and to modulate different functional properties of various intestinal cells. The control of intestinal epithelial cell growth is regulated by interactions between growth factors and the various cell types of the small and large intestinal epithelia.

Peptide growth factors and hormones play pivotal roles in the regulation of the functional properties of epithelial cells, such as proliferation and differentiation (Dignass and Sturm, 2001). In the intestinal mucosa, receptors for IGF, EGF, TGF-α, TGF-β and the lactogenic hormone estradiol-17β have been found, which suggests that these factors may exhibit biological activities in the intestinal environment (Purup et al, 2007; Caiazza et al, 2007; Galluzzo et al, 2007). However, the data regarding the precise roles of these bioactive factors in the regulation of the growth and proliferation of intestinal epithelial cells are limited and conflicting. Further knowledge about the activities of various growth factors and hormones may contribute to the clarification of their roles at the intestinal level.

The human gastrointestinal tract harbors an extensive microbial community, and the colon contains microbe concentrations as high as 10^{11}- 10^{12} microorganisms per gram. The microbial community of the human colon is responsible for the active breakdown of foods, particularly carbohydrates and proteins, which have escaped digestion in the small intestine (Windey et al, 2012). The daily intake of fermentable carbohydrates influences the composition of the colonic microbiota and the synthesis of SCFAs and thus influences colonic health. At the gut level, complex carbohydrates, such as dietary fibers, are metabolized by colonic bacteria and then fermented into SCFAs, including butyrate, propionate and acetate (Tremaroli and Backhed 2012; Brahe et al, 2013). SCFAs are pivotal metabolites of the luminal contents of the large intestine. They provide energy for

colonocytes, which require absorptive processes to take up these bacterial metabolites. It has been demonstrated that butyrate induces an enhancement in epithelial barrier function and the maintenance of colonic health (Peng et al, 2007), whereas, following intestinal absorption, propionate and acetate, are involved in cholesterol metabolism, lipid biosynthesis and lymphocyte proliferation (Hugenholtz et al, 2013; Curi et al, 1993).

Because the human intestine is the first target of bioactive molecules that enter with food, the identification of the effects of specific bioactive factors at the intestinal cell level may provide novel insight into the promotion of gastro-intestinal development and health and may have applications for the treatment of a number of diseases. In this context, cell culture models have been widely used to evaluate the cellular mechanisms related to dietary compounds (Rebucci et al, 2007-2013; Baldi et al, 2004).

Cell growth-modulating peptides derived from milk protein digestion may stimulate the development and maintenance of the health of the gastrointestinal tract. The evaluation of the effects of bioactive peptides on the gastrointestinal health is an arduous task as demonstrated by the lack of clinical studies. *In vitro* studies that use intestinal cells represent a valuable screening tool with moderate predictive power regarding *in vivo* functions. Cell cultures have been used to investigate the potential health effects of bioactive peptides. *In vitro* cell models allow for the evaluation of the citomodulatory effects of milk bioactive compounds in terms of their influences on cell growth, cell differentiation and mucus production by goblet cells. Specifically, goblet cells represent one of the major cell types in the human intestine and have crucial roles in the maintenance of the homeostasis of the intestinal microenvironment and the control of the localization of commensal bacteria (Goto and Kiyono, 2012). Goblet cells can be mimicked *in vitro* using mucus-secreting cell lines, such as HT29-MTX-E12 These cells are a subpopulation of HT29 human colonic adenocarcinoma cells that have been selected for resistance to methotrexate. These epithelial cells can differentiate into mature mucus-producing goblet-like cells (Behrens et al 2001) but can also be used for proliferation and viability activity studies in an undifferentiated state. It is generally accepted that these cells can provide an

important alternative to *in vivo* studies of the gut microenvironment (Behrens et al, 2001). HT29-MTX-E12 cells have been extensively used to study the effects of milk peptide obtained from casein and whey proteins (Martínez-Maqueda et al., 2012; Martínez-Maqueda et al., 2013a; Martinez Maqueda 2013b; Zoghbi et al., 2006).

Given their impressive array of functions, peptides may be incorporated into functional foods and drug preparations. Even if the effects of peptides are less than those of synthetic drugs, they are characterized by less tissue accumulation and reduced side effects compared with synthetic drugs (Li-Chan, 2015).

Gut modulating: Prebiotic peptides

Multiples nutrients that are present in milk have well-recognized prebiotic functions that support the beneficial intestinal microflora. Specifically, milk and colostrum contain a group of bioactive peptides and oligosaccharides that is characterized by a powerful growth promoting activity toward the probiotic microorganisms that are responsible for the maintenance of balanced intestinal microflora (Newburg, 2005; Newburg, 1996). Numerous studies have demonstrated that lactoferrin can exhibit a prebiotic action that stimulates the growth of bacteria of the genera *Lactobacillus* and *Bifidobacterium* (Liepke et al., 2002; Kim et al., 2004). The stimulatory effects on growth are strain-dependent and may be related to the presence of specific receptors for lactoferrin on the surfaces and in the cytosolic fractions of sensitive bacteria (Kim et al. 2004). *In vitro* studies have demonstrated that bovine, human and porcine lactoferrins recombinantly expressed in Pichia pastoris are able to stimulate the growth of four Lactobacillus strains, including the *Lactobacillus casei* ssp *casei* (Pecorini et al.,2005). Oligosaccharides are also effective in modulating the enteric flora, influencing the activities of the digestive tract and modulating inflammatory processes (Kunz et al., 2006; Yu et al., 2012).

For the abovementioned reasons, milk-derived bioactive peptides are considered to be notable candidates for the production of health-promoting functional foods that target the

health of different body levels including the heart, bone and digestive system and also improve immune defense, mood and stress control. The previously discovered positive effects of milk proteins at the systemic and intestinal levels have primarily focused on hydrolyzed peptides. Few studies that have started from intact milk proteins are available. Therefore, study of bioactivities of milk peptides obtained via the *in vitro* digestion of intact milk proteins is the topic of research of the first chapter of this thesis.

The main points addressed in the chapter I are listed below.

- The antioxidant capacities and ACE inhibiting activities of whey, casein and soya protein permeate and retentate, obtained by *in vitro* SGD, were determined.

Further, the effects of digested milk proteins on the human gut epithelium were investigated using the *in vitro* cell model HT29-MTX-E12 and the probiotic bacterial strain *Lactobacillus casei*.

- HT29-MTX-E12 cells were adequately characterized (e.g., medium culture conditions, growth maintenance and effects of growth factors and SCFAs).
- Finally, the citomodulatory and prebiotic roles of whey, casein and soya protein retentate obtained by *in vitro* SGD were determined.

Study I: ***Antioxidant capacity and ACE- inhibitory activity of whey and casein proteins obtained by in vitro SGD***

Aim of study I

The main aim of this study was the further development and the adaption of the *in vitro* SGD protocol described by Mills et al., (2008) for milk protein isolated to study the protein's bioactivities upon digestion. In particular, the antioxidant capacity and the ACE-inhibitory activity of whey, casein protein obtained by *in vitro* SGD were determined.
The soya protein was included as non-animal protein source. Maltodextrin was included as non-protein source.

Materials and methods

Chemicals

Whey protein isolate 90% (Volac International Ltd, 50 Fishers Lane, Orwell, Royston, SG8 5QX, Hertfordshire, UK); calcium caseinate >88% (Cambridge commodities, LTD); soya protein isolate 90 and maltodextrin 100% (My protein, The Hut.com Ltd, UK). All other chemicals were purchased by Sigma-Aldrich (Sigma–Aldrich Company, Dorset, UK), unless otherwise indicated.

In vitro SGD

The SGD of the protein samples was performed according to Mills *et al*. (2008) with slight modifications. Briefly, 20 grams of each protein sample was mixed with 150mL of distilled H_2O in a plastic bag and stomached for 5'. After 12 h of equilibration in the fridge, the samples were maintained at room T for 1 h and a small amount of the undigested proteins were sampled in Eppendorf tubes and snap frozen in liquid nitrogen

before storing at -80 °C for further experiments. The SGD experimental procedure (**Figure 1**) involved three following phases. For the mouth phase 6.66 mg α-amylase in 2.083mL of 1 mM $CaCl_2$, pH 7 were added to the samples and an incubation of 30 min at 37°C on a shaker was performed. The pH was decrease to 2 with HCl 6M. For the gastric phase, 0.9g of pepsin in 8.33 mL of 0.1 M HCl, pH 2. The samples were incubated 120 min at 37°C on a shaker. The pH was increase to 7 with NaOH 6M. For the small intestinal phase 0.18mg pancreatin and 1.16 g bile in 0.5M $NaHCO_3$, pH 7 were added to the samples and a final incubation of 180 min at 37°C on a shaker was performed. Afterwards, the total digesta obtained for each sample was weighed and transferred to 3kDa VIVASPIN 20 Sartorius centrifuge tubes in order to simulate the intestinal absorption. The samples were centrifuged for 16' at 4500 rpm. Permeate (absorbed fraction) and retentate (intestinal fraction) were obtained. Finally, total digesta, permeate and retentate were sampled in Eppendorf tubes and snap frozen in liquid nitrogen before storing at -80 °C for further experiments.

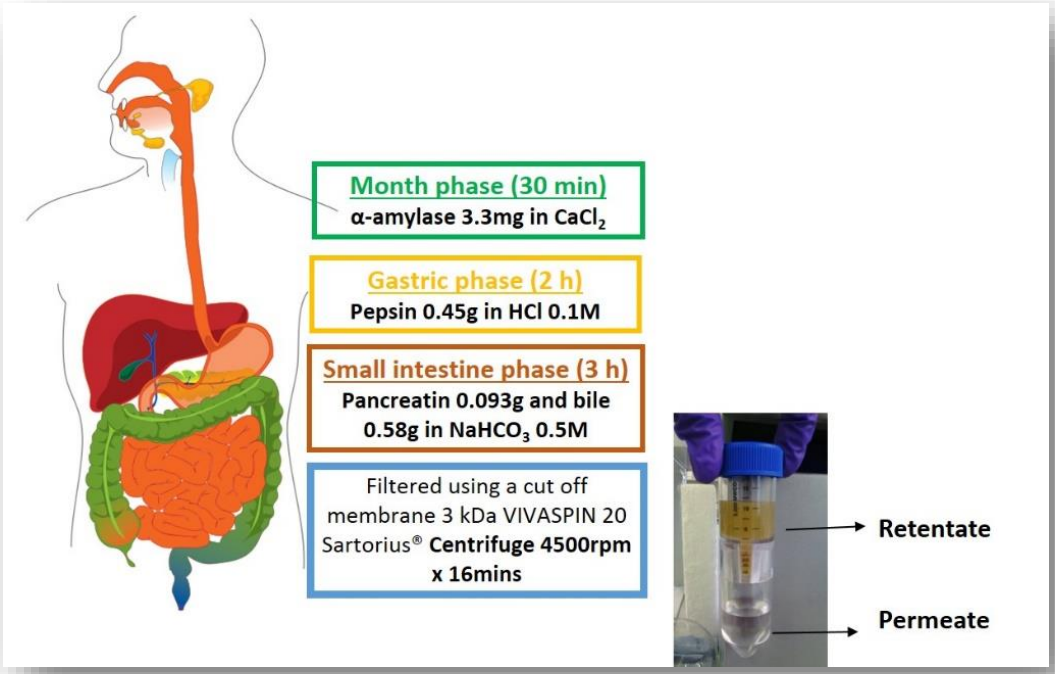


Figure 1*: In vitro SGD procedure performed for whey, casein and soya intact proteins.*

Determination of protein concentration

The quantification of nitrogen content in total digesta, permeate and retentate was performed with Kjeldahl method. The total amount of protein was calculated in each samples and used for the determination of antioxidant and ACE-inhibitory activities.

Antioxidant capacity (AOC): ABTS assay

Measurement of antioxidant activity was performed using the method of Re et al., 1999 with some modifications. 7 mM ABTS stock solution was mixed with 140 mM potassium persulfate and allowed to react in the dark at RT for 12–16 h for the formation of the

ABTS•+ radical . The radical was stable up to 6 days if store in darkness at room T. The ABTS•+ stock solution was diluted in 5 mM phosphate-buffered saline (PBS, pH 7.4) to give an absorbance of 0.70 ± 0.02 at 734 nm in a 1 cm cuvette. Protein samples were diluted 1:10 with 5mM PBS. A volume of 20uL of protein samples or Trolox standard in PBS was mixed with 2mL of ABTS•+ working solution and incubated in dark for 6 min t RT before measuring absorbance at 734 nm on the spectrophotometer (Jasco). Appropriate solvent blanks were run in each assay. Maltodextrin was included as non-protein control. Results are the mean values of three triplicates. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of antioxidants and of Trolox for the standard reference data. The ABTS decolorization properties permitted the calculation of the Trolox equivalent antioxidant capacity (TEAC) of the protein samples. Results are expressed as µmol Trolox equivalent/mg protein.

Antioxidant capacity (AOC): FRAP assay

The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) (Benzie and strain, 1996). The reaction was carried out in a microtiter plate. Protein samples were diluted 1:10 in distilled water. FRAP (300 µl) reagent stock solution (acetate buffer, 2,4,6-tripyridyl-s-triazine and ferric chloride solution) was mixed with 10µL of diluted protein samples and standard in distilled water. The reaction mixture was then incubated at 37°C for 1 min and absorbance was recorded at 595 nm, using a spectrophotometer (UV-VIS Jasco). The concentration of $FeSO_4$was in turn plotted against concentrations of the standard antioxidants (Ascorbic acid). Maltodextrin was included as non-protein control. Results are expressed as µmol ascorbic acid equivalent/mg protein.

ACE inhibitory (ACEi) activity

The measurement of the ACE inhibitory activity of digested proteins was performed with the ACE inhibitory assay using FAPGG as the synthetic substrate for ACE enzyme (Sigma Aldrich). The substrate FAPGG solution (150μL of 0.5mM FAPGG substrate) and samples (10μL of 0.2%w/v protein concentration) were incubated at 37°C for 5' in the Microplate Reader, according to XFluor4 software indications. After the incubation, the ACE enzyme (50 μL of 15mU enzyme) was added to the sample/substrate mixture and the kinetic reaction started. The kinetic reaction was monitored for the following 30' in the Microplate Reader at 340nm. Captopril was used as a positive control. Hydrolysis of FAPGG by ACE enzyme will result in a decrease in absorbance at 340 nm. ACE inhibitory activity was measured by the ability of whey, casein and soya digested proteins to decrease the hydrolysis of FAPGG as previously reported (Theodore & Kristinsson, 2007; Raghavan & Kristinsson, 2009). Maltodextrin was included as non-protein control. A 100% ACE inhibitory activity would indicate complete inhibition of the enzyme and no decrease in absorbance. All experiments were done in duplicates:

$$\%\text{ACE inhibition} = [(Abs_{\text{no sample}} - Abs_{\text{sample}}) / Abs_{\text{no sample}}] \times 100.$$

$Abs_{\text{no sample}}$ is the absorbance of the enzyme-substrate mixture in the absence of digested protein, and Abs_{sample} is the absorbance of the enzyme-substrate mixture in the presence of digested protein.

Statistical analysis

All assays were carried out in three independent experiments and each protein was tested in duplicate. The statistical software SAS (version 9.3) was used to analyze differences between different proteins and stage of digestion by ANOVA Differences were deemed statistically significant if $P<0.0$

Results & Discussion

Antioxidant capacity (AOC): ABTS assay

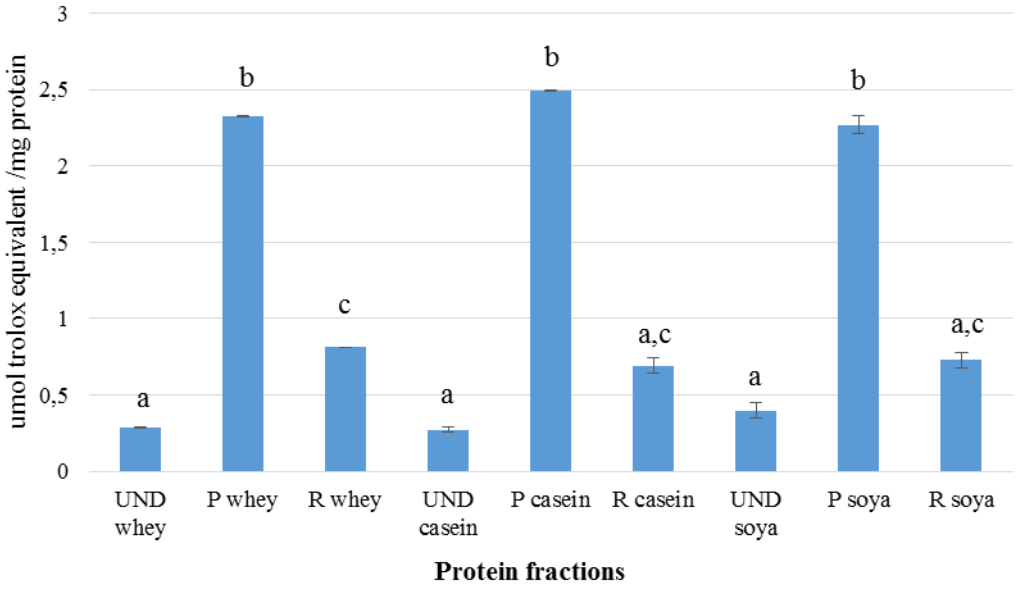


***Figure 2**: Antioxidant activity of protein fractions obtained by SGD of whey, casein and soya proteins. Different superscript letters $^{(a, b, c)}$ indicate statistical difference (P<0.05) (Abbreviations: UND= undigested proteins; P= protein permeate; R=protein retentate).*

The antioxidant activity of undigested proteins, permeate and retentate were first quantified by ABTS°+ decolorization assay and expressed as µmol/mg protein. This method is one of the widely used method for assessing antioxidant properties of extract and food/feed components (Thaipong, et al., 2006).

All undigested proteins had TEAC values below 0.5µmol trolox equivalents/mg proteins. Following SGD, it has been observed a significant increase (P<0.05) in TEAC for whey, casein and soya protein permeate to 2.32, 2.49 and 2.26 µmol trolox equivalents/mg proteins, respectively (**Figure 2**). Whereas, the TEAC values obtained for whey, casein and soya protein retentate were 0.81, 0.69 and 0.71 µmol, respectively. All proteins tested, after the SGD, exhibited higher antioxidant properties. A similar extent was reported for

whey, casein and soya fractions. No antioxidant activity was reported for maltodextrin, as no-protein control (data not shown).

The SGD significantly ($P<0.05$) enhanced antioxidant activity of the proteins. Moreover, it has been observed an overall effect of permeate/retentate separation ($P<0.05$) obtained by ultracentrifugation at the end of the *in vitro* SGD (data not shown). The strongest antioxidant properties have been detected in the permeate fraction of all protein analyzed.

Antioxidant capacity (AOC): FRAP assay

Results obtained with the FRAP assay confirmed the highest antioxidant properties of the permeate fractions compared with undigested proteins. In particular, whey and casein permeates had a similar antioxidant value (4.06 and 4.45 µmol ascorbic acid/mg protein for whey and casein, respectively).

ACE-inhibitory assay

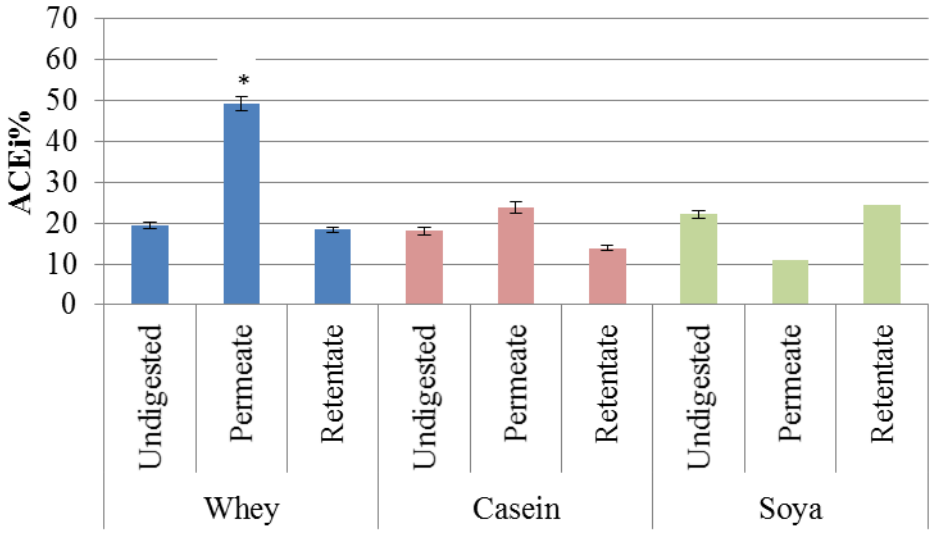


***Figure 3**: %ACEi inhibition of whey, casein and soya protein fractions obtained by SGD.*P<0.05 compared with undigested whey, casein permeate and soya permeate.*

ACE-I inhibitory activity of whey, casein and soya protein fractions was measured for permeate, retentate and undigested proteins. As shown in **Figure 3,** the highest ACE value is reported for the whey protein, compared with casein and soya proteins. The whey protein permeate exhibited a significant ACE-inhibitory activity, compared with soya and casein permeate and compared with undigested whey protein. Casein and Soya protein fractions do not showed significant ACE-inhibitory properties.

TEAC and FRAP results demonstrated the antioxidant activity of whey, casein and soya proteins. ACE inhibition has been detected in the whey protein tested, with the highest value detected in whey protein permeate. The SGD enhanced their antioxidant activity of milk and soya proteins tested and ACE-I inhibitory activity of whey protein. Among the protein considered, the milk permeates, which reflect the systemic/absorbed peptides, possessed the highest antioxidant and ACE-inhibitory properties. The *in vitro* antioxidant and ACE-inhibitory properties of milk proteins were previous demonstrated. Petrat-Melin

et al., 2015 reported values of 0.8µmol of trolox equivalent /mg protein for undigested proteins and an increase in antioxidant potential following digestion. This effect is probably related to the whey protein amino acid composition and it is consistent with the previously described health-benefit of whey proteins on CVD i*n vivo*. The ACE-inhibitory-antioxidant protein fractions may contribute to lower blood pressure and increase the antioxidant defense in humans. Pal et al. (2010a; 2010b) investigated the effects of whey protein isolate and casein protein isolate on cardiovascular health, demonstrating a significant reduction in blood pressure of both whey and casein protein-consumers, compared with the control group. Considering the emerging results related to their properties *in vivo*, we can speculate that milk proteins, after oral administration, gastrointestinal digestion and absorption, may resist to plasma peptidase degradation and can reach their target tissues where they contribute to antioxidant and ACE inhibitory defenses. Beside the traditional nutritional value, the health- promoting properties of milk proteins together with the lack of side effects elect them as potential ingredients of functional foods.

The nutritional strategies that provide a rich source of antioxidant and ACE-inhibitory proteins could help in meeting the nutritional requirements of humans, providing a novel approach to promote healthy aging.

Study II: ***HT29-MTX-E12 cells characterization: set up of the in vitro cell model***

Aim of study II

The aim of the present study was the characterization of HT29-MTX-E12 cell line in order to set up the culture condition for this cell line and use this cell culture as a model of the human intestinal goblet cells. The impact of different growth factors, hormones as well as different combinations of the three major SCFAs on the metabolic activity and proliferation of human undifferentiated HT29-MTX-E12 cells. First, the metabolic and proliferative activity of HT29-MTX-E12 in the presence and absence of IGF-I, EGF, TGF-α, TGF-β1 and the hormone estradiol 17-β were investigated, since an adequate characterization of the response of these cells to several bioactive factors is still lacking. Next, the response to different concentrations of the SCFAs butyrate, propionate and acetate, alone and in combination, was assessed on the metabolic activity of HT29-MTX-E12 cells in culture. The obtained results contribute in the justification for use of a specific cell culture media, rarely provided in published reports.

Materials & methods

Chemicals

AlamarBlue™ and PicoGreen® reagents were purchased from Invitrogen (Taastrup, Denmark). Fetal bovine serum (FBS) was purchased from Bio Whittaker (Lonza Copenhagen Aps, Copenhagen, Denmark). IGF-I and EGF were purchased from Austral Biologicals (AH diagnostics, Aarhus, Denmark). TGF-α and TGF-β1 were purchased from R&D Systems (Abingdon, United Kingdom). Estradiol 17-β, NaBt, NaAce, and NaProp were purchased from Sigma-Aldrich (St. Louis MO, USA). IGF-I was dissolved in culture medium (0.05% FBS) to obtain a stock solution of 50 ng/mL. EGF was dissolved in culture medium (0.05% FBS) to obtain a stock solution of 50 ng/µL. Estradiol 17-β was dissolved in culture medium (0.05% FBS) to obtain a stock solution of 10 mM.

TGF- α was dissolved in culture medium (0.05% FBS) to obtain a stock solution of 10 µg/mL. TGF-β1 was dissolved in culture medium (0.05% FBS) to obtain a stock solution of 1 ng/µL. NaBt, NaProp and NaAce were dissolved in culture medium (0.05% FBS) to obtain stock solutions of 1 M. Stock solutions were stored in the dark at 4°C. All the remaining reagents were purchased from Sigma Aldrich.

Cell line & cell culture

HT29-MTX-E12 cells were kindly donated by Dr. David Brayden and Dr. Sam Maher (UCD Conway Institute, Dublin, Ireland). This human colorectal adenocarcinoma cell line, a clone of HT29 cells, could differentiate into a mucus-producing goblet-like cell line. Cell maintenance was performed in 25 cm^2 flasks using 15 mL of Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L) which was supplemented with 10% of FBS, 1% 1 M HEPES, 1% (v/v) penicillin/streptomycin, 2% Glutamax and 1% MEM NEAA. The cells were cultivated at 37°C in a humidified incubator with 5% CO_2. Cells were subcultured using 0.05% trypsin-EDTA at 60 to 80% confluence. All experiments were carried out using undifferentiated HT29-MTX-E12 cells, within six cell passages (from passage 43 to 49) to ensure reproducibility.

HT29-MTX-E12 cells were plated at low density (1000 cells/well) in 96-well plates and cultured for 48 hours. Preliminary experiments were carried out and growth curves were obtained with different concentrations of FBS for 6 to 8 days of culture to ensure that cells were in the sigmoid phase of the growth curve when components to be tested were added to the culture medium.

Several concentrations of FBS (from 0 up to 10%) were tested on the cells and the optimal concentration to support cell attachment was found to be 0.05%. Therefore, this FBS concentration was added to the treatment medium in all experiments.

Treatment medium was composed of basal medium DMEM and 0.05% FBS containing IGF-I, TGF-α of EGF at different concentrations (0.1, 1, 2.5, 5, 10, 50 and 100 ng/mL); TGF-β1 at different concentrations (10, 50, 100, 500, 1000, 5000, 10000 pg/mL); estradiol 17-β (0.1 pM, 1 pM, 0.01 nM, 0.1 nM, 1 nM, 0.01 µM, 1 µM, 0.1 mM); or NaBt, NaProp or NaAce at different concentrations (0.5, 1, 5, 10, 25, 50 and 100 mM). In this study, NaBt, NaProp or NaAce were also added to the treatment medium in combination.

Two combinations of SCFAs defined the low SCFA combination (LowSCFAs) and the high SCFA combination (HighSCFAs) were chosen in accordance with Le Gall et al, (2009) in order to reproduce the physiological ratio of SCFAs in the human digestive tract. These combinations are shown in **Table 1**.

Table 1*: Estimation of physiological and sub-physiological concentrations of SCFAs produced by intestinal fermentation in vivo (Le Gall et al., 2009).*

	Physiological [ ] mM	Sub-physiological [ ] mM
ACETATE	70	35
PROPIONATE	18	9
BUTYRATE	8	4

Treatment medium (200 µL per well in 96-well plates) was added to individual wells.

Preliminary time-course experiments showed that all treatments affected cell metabolic activity and proliferation at 72 hours but not at 24 and 48 hours. Therefore, incubation for 72 hours was used in all experiments of this study.

Cell viability: metabolic activity and proliferation

The first part of the study investigated the effect of IGF-I, EGF, TGF-α, TGF-β and 17-β estradiol on the metabolic activity and proliferation rate of HT29-MTX-E12 cells whereas the second part of this study examined the activity of butyrate, propionate and acetate on cell metabolic activity. Two methodologies were used in these studies. The first method considered was the AlamarBlue™ (AB) assay, used to assess cell metabolic activity. AB is a tetrazolium-based dye, incorporating resazurin and resorufin as oxidation–reduction indicators that yield colorimetric changes and a fluorescent signal in response to metabolic activity of the cell; the blue non-fluorescent oxidized form (resazurin) becomes pink and fluorescent (resorufin) upon mitochondrial reduction in metabolically active cells. Briefly, after the removal of treatment medium, 110 µL aliquots of AB solution (dilution 1:10 in PBS) were added to each well and incubated with the cells for 2 hours. The color change was monitored, and a fluorimetric evaluation of cell vitality was performed using an EnVision fluorometer (Perkin Elmer). The fluorescence values were detected at 544 nm (excitation) and 596 nm (emission). As the negative control, AB was added to medium without cells.

The second methodology used was the PicoGreen® (PG) assay in order to assess cell proliferation. The Quant-iT™ PicoGreen® dsDNA reagent is fluorescent nucleic acid stain for highly selective quantitating double-stranded DNA (dsDNA) over single-stranded DNA and RNA in solution. Upon binding to dsDNA the dye exhibits fluorescence enhancement.

72 hours after the addition of treatment medium to the cells, the PicoGreen® assay was performed according to the manufacturer's instruction. Medium was removed and 100 µL of TE buffer was added to each well. To lyse cells, plates were stored in -80°C freezer for 45 minutes and then thawed. After three times of freezing and thawing, 100 µL of PG working solution (Quant-iT™ in TE) were added to each well and the cells were incubated in the dark at room temperature for 5 minutes. The fluorescence signal was detected using

a fluorescent plate reader (EnVision fluorometer) at 485 nm (excitation) and 535 nm (emission).

Statistical analysis

Results were depicted as the mean value ± standard error of three independent experiments. A one-way ANOVA, using the GLM procedure (PROC GLM) of SAS 9.3 (SAS Institute, Cary, NC, USA), was used to evaluate the effects of various treatments. Differences between means were considered statistically significant at $P<0.05$ (*) and $P<0.0001$(**).

Results & discussion

First of all, the effect of increasing concentrations (from 0 up to 10%) of FBS in the culture medium was examined to test how cell growth was affected by the presence of serum in the culture medium. Cell proliferation increased in undifferentiated HT29-MTX-E12 cells in a dose dependent manner (data not shown). In light of these observations, a concentration of 0.05% FBS was chosen as the concentration of FBS in treatment medium when growth factors, hormones and SCFAs were examined in order to support and simulate physiological cell growth.

Effects of IGF-I, EGF, TGF-α, TGF-β, and estradiol 17-β on metabolic activity and proliferation of HT29-MTX-E12 cells

At 72 hours of incubation, IGF-I stimulated the metabolic activity of HT29-MTX-E12 cells in a significant manner ($P<0.0001$; $P<0.05$) compared with control cells (0 ng/mL IGF-I) (**Figure 1a**). The maximal stimulation was detected in the range from 10 up to 100 ng/mL of IGF-I. IGF-I dose dependently increased the proliferation rate of HT29-MTX-

E12 cells (**Figure 1b).** Concentrations of 5 and 10 ng/mL of IGF-I significantly (P<0.05) increased cell proliferation by 20%, whereas the highest concentrations tested (50 and 100 ng/mL) significantly (P<0.0001) increased proliferation by 30%.

EGF in concentrations ranging from 0 to 100ng/mL did not affect the metabolic activity or the proliferation rate of HT29-MTX-E12 cells (**Figure 1c and 1d**).

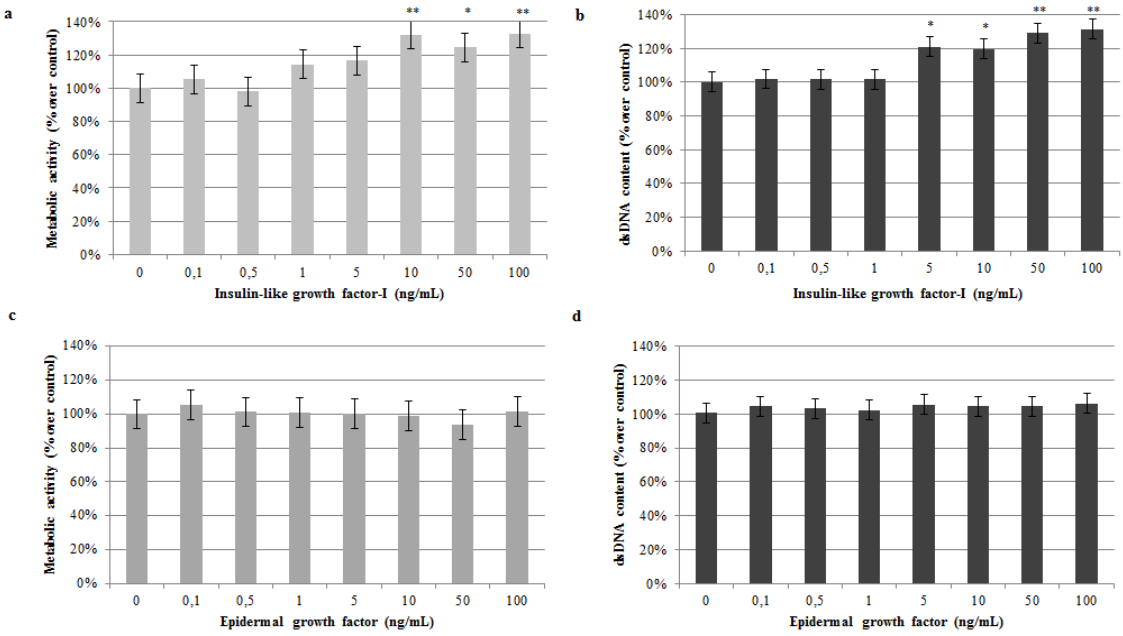


Figure 1*: Effect of increasing concentrations of IGF-I and EGF added to the culture medium for 72 hours on metabolic activity (**a, c**) and proliferation (expressed as dsDNA content) (**b, d**) in* HT29-MTX-E12 *cells. Results are from three independent experiments with quadruplicate wells and presented as least square means ± SEM relative to metabolic activity/proliferation obtained in basal medium (0 ng/mL). Values significantly different from the metabolic activity/dsDNA content obtained in basal medium are indicated by * (P<0.05) or ** (P<0.0001).*

Both TGF-α and TGF-β1 affected the metabolic activity of the intestinal cells. In particular, this inhibition was about 24% in the presence of 1 ng/mL of TGF-α (*P*<0.05) (**Figure 2a**). TGF-α treatment at 10 ng/mL showed a significant (*P*<0.05) impact on cell

proliferation (**Figure 2b**). However, no significant effects were obtained with TGF-β1 on cell proliferation (**Figure 2d**).

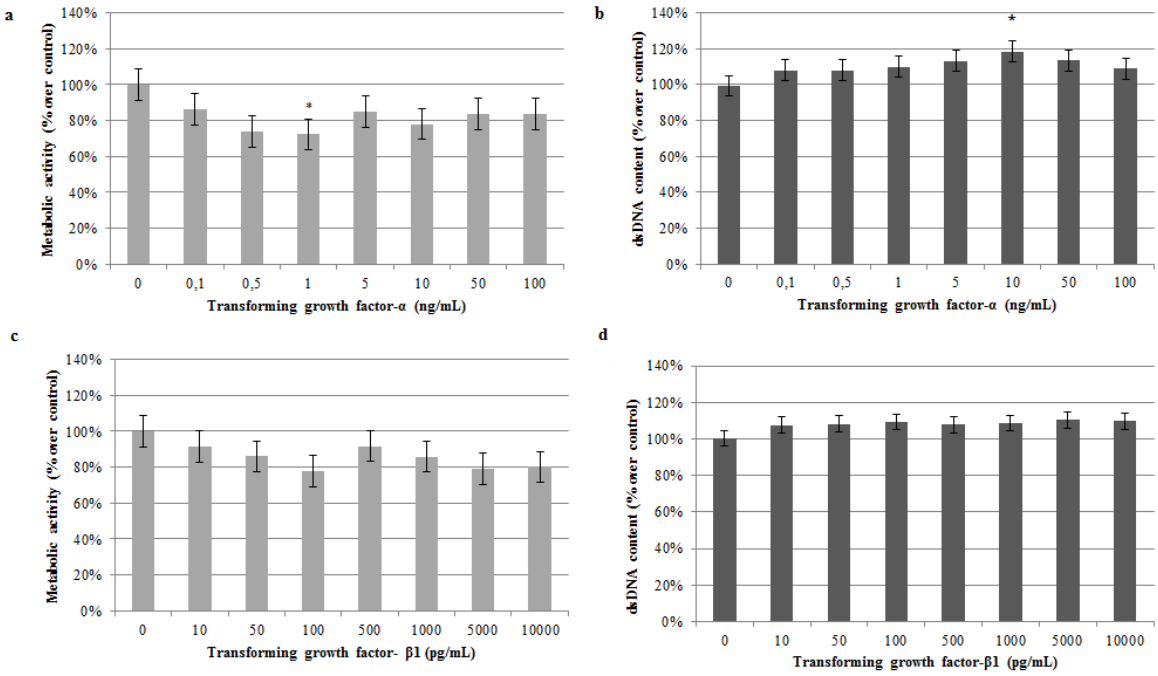


***Figure 2**: Effect of increasing concentrations of TGF-α and TGF-β1 added to the culture medium for 72 hours on metabolic activity **(a, c)** and proliferation (expressed as dsDNA content) **(b, d)** in* HT29-MTX-E12 *cells. Results are from three independent experiments with quadruplicate wells and presented as least square means ± SEM relative to metabolic activity/proliferation obtained in basal medium (0 ng/mL) (0 pg/mL). Values significantly different from metabolic activity/dsDNA content obtained in basal medium are indicated by * ($P<0.05$).*

The metabolic activity of HT29-MTX-E12 cells was significantly ($P<0.05$) stimulated by specific concentrations of estradiol 17-β (from 0.1nM up to 1µM), while the highest estradiol 17-β concentration tested (0.1 mM) strongly inhibited the metabolic activity ($P<0.0001$; **Figure 3a**). However, the lowest concentration of estradiol 17-β increased the proliferation of HT29-MTX-E12 cells to 129%, while the highest concentration of estradiol 17-β significantly ($P<0.0001$) reduced the proliferation rate to 66% (**Figure 3b**).

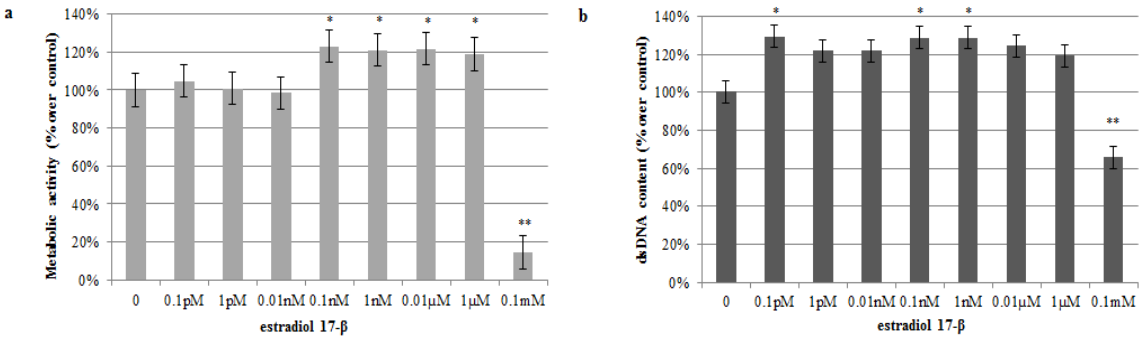


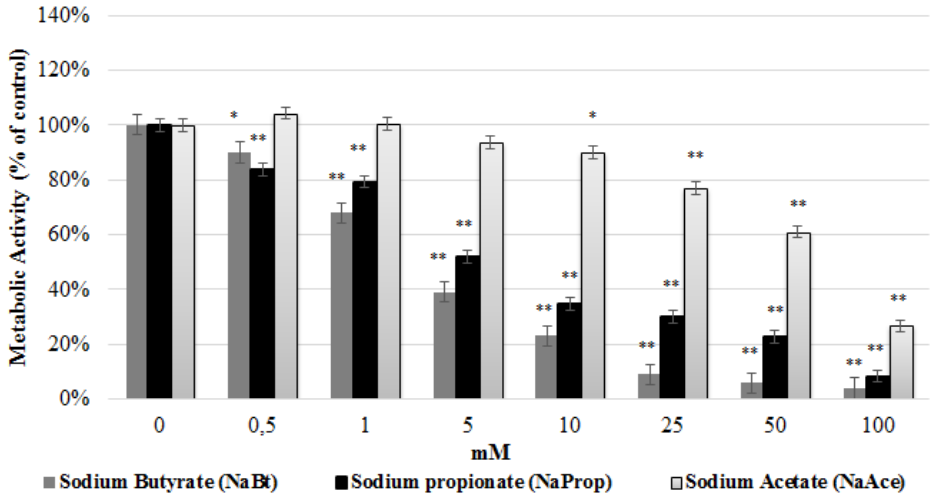
***Figure 3**: Effect of increasing concentrations of estradiol 17-β added to the culture medium for 72 hours on metabolic activity (**a**) and proliferation (expressed as dsDNA content) (**b**) in* HT29-MTX-E12 *cells. Results are from three independent experiments with quadruplicate wells and presented as least square means ± SEM relative to metabolic activity/proliferation obtained in basal medium (0). Values significantly different from metabolic activity/dsDNA content obtained in basal medium are indicated by * (P<0.05) or ** (P<0.0001).*

Effect of butyrate, propionate and acetate and combinations on the metabolic activity of HT29-MTX-E12 cells

In preliminary experiments the metabolic activity assay (AlamarBlue™) showed to fit for the purpose of describing the effect of SCFAs on intestinal HT29-MTX-E12 cells. Therefore, undifferentiated HT29-MTX-E12 cells were grown alone or in the presence of several concentrations of either NaBt, NaProp or NaAce and cell metabolic activity was evaluated.

The addition of NaBt alone in the culture medium caused a dose dependent decrease in the metabolic activity of the cells. **Figure 4** clearly shows that NaBt exerted the strongest effect in terms of inhibition, after 72 hours of incubation, compared to both NaProp and NaAce.

Concentrations of NaBt above 1 mM significantly (P<0.0001) inhibited the metabolic activity of HT29-MTX-E12 compared with control (0 mM). Indeed, the addition of NaProp at concentrations ≥1 mM led to weaker inhibition compared with the same concentrations of NaBt. Also, increasing the NaAce concentration caused a reduction in the metabolic activity of HT29-MTX-E12 cells in a dose dependent manner. However, NaAce, among the SCFAs considered in this study, was devoid of any significant effect at the lower concentrations. Only concentrations higher than 25 mM significantly (P<0.0001) inhibited metabolic activity; in particular, the presence of 50mM of NaAce reduced the metabolic activity by 39% compared with control cells (0mM).



b

Figure 4*: Effect of increasing concentrations of NaBt, NaProp and NaAce added to the culture medium for 72 hours on metabolic activity in* HT29-MTX-E12 *cells. Results are from three independent experiments with quadruplicate wells and presented as least square means ± SEMs, relative to metabolic activity obtained in basal medium (0 mM). Values significantly different from metabolic activity obtained in basal medium are indicated by * (P<0.05) or ** (P<0.0001).*

The effect of the addition of the combinations of the SCFAs on HT29-MTX-E12 cell metabolic activity is shown in **Figure 5a and 5b.** These results indicate an additive interaction effect of the three SCFAs at both high (HighSCFAs) and low (LowSCFAs)

concentrations. In particular, the dose dependent inhibitory effect of increasing concentrations of NaBt was significantly (P<0.0001) enhanced by the addition of specific concentrations of NaAce and NaProp.

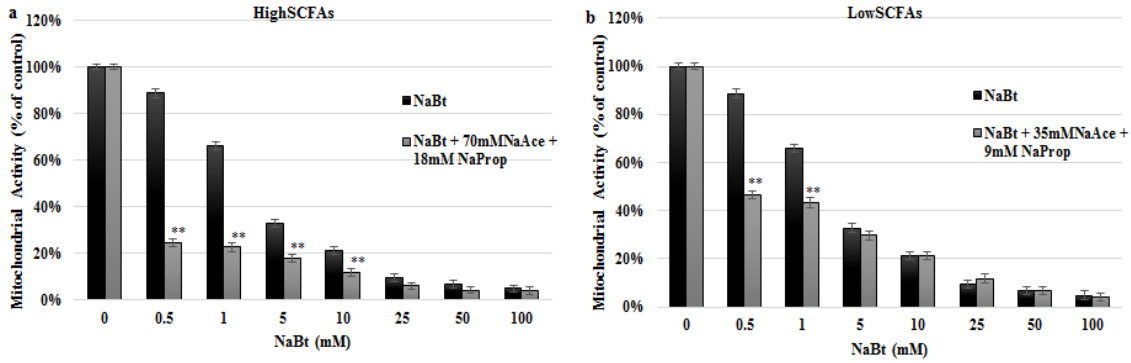


Figure 5*: Effect of the HighSCFAs combination (**a**) and the LowSCFAs combination (**b**) added to the culture medium for 72 hours on metabolic activity of* HT29-MTX-E12 *cells. Results are from three independent experiments with quadruplicate wells and presented as least square means ± SEMs, relative to metabolic activity obtained in NaBt alone. Values obtained with NaBt combined with other SCFAs significantly different from values of obtained with NaBt alone are indicated by ** (P<0.0001).*

In this study, it is reported the impact of growth factors, estradiol 17-β and SCFAs on the metabolic activity and proliferation (dsDNA content) of the undifferentiated HT29-MTX-E12 intestinal cell line.

HT29-MTX-E12 can be a useful model for food and nutritional *in vitro* studies, even though this cell line has not yet been adequately characterized with respect to bioactive components. The first finding of this study is that HT29-MTX-E12 cells respond to IGF-I. IGF-I stimulates the growth and proliferation of several cell types, as demonstrated in previous studies (Theodorou et al, 2011; Meng et al, 2007; Purup et al, 2007). This growth factor is responsible for several cellular physiological processes through binding with IGF-R1 located on the cell surface that stimulates a signal transduction cascade, which is

involved in cell viability and proliferation (Rodon et al, 2008). Furthermore, at the intestinal level, IGF-I is involved in several functions such as the regulation of carbohydrate and protein metabolism, and it has a key role in gastrointestinal growth and development by its effects on the proliferation of intestinal cells (Luo et al, 2011). However, it has previously been demonstrated that IGF-I does not affect intestinal cell differentiation (Sanderson and Walker, 1999). The results obtained in this study confirm that IGF-I stimulates both the metabolic activity and proliferation of intestinal HT29-MTX-E12 in a dose dependent manner. In addition, estradiol 17-β had a significant effect on HT29-MTX-E12 intestinal cells, increasing both metabolic activity and proliferation in a concentration dependent manner, whereas the highest amount of estradiol 17-β significantly reduced cell viability and proliferation. However, this inhibitory effect was detected at a concentration above the physiological range that could reach the colon. Other studies have previously demonstrated the gut to be an estrogen-responsive tissue (Caiazza et al, 2007; Galluzzo et al, 2007). Thomas et al. (1993) demonstrated the presence of estrogen receptors (ERα and ERβ) in the intestine, even though the abundance of estrogen receptors in the intestinal epithelium is relatively low compared to the reproductive tissue. Estradiol levels may affect intestinal tissues at times other than during reproduction (Caiazza et al, 2007), such as during the consumption of dietary compounds such as milk and colostrum.

These findings suggest that both IGF-I and estradiol 17-β may have a crucial role in up-regulating the proliferation and metabolic activity of intestinal epithelial cells.

TGF-α inhibited cell metabolic activity at specific concentrations. This inhibition could not be attributed to direct toxicity since the proliferation rate of the cells treated with TGF-α was maintained throughout the 72 hours of culture at all concentrations tested. TGF-α expression in the small and colonic intestinal epithelium occurs mainly in non-proliferative zones. This finding suggests that its role may be to regulate different processes rather than cell proliferation. TGF-β1 is structurally different from TGF-α, and its major site of expression is the superficial proliferative zone of the intestine. Therefore,

it may be involved in cell proliferation processes and in maintaining epithelial homeostasis (Playford et al, 2000). Koyoama et al. (1989) revealed the complementary role of TGF-α and β1 in balancing proliferation and differentiation processes in the intestinal epithelium. Other studies have shown that TGF-β1 inhibits the proliferation of intestinal epithelial cells, whereas TGF-α promotes the growth and development of the gastrointestinal tract (Booth et al, 1995; Kurokowa et al, 1987).

In the present study, EGF did not produce any significant effect, suggesting that EGF is not a mitogen in this cell line. These data are consistent with the results of Kurokowa et al. (1987), which demonstrated that the role of EGF in the enhancement of intestinal cell proliferation is minor compared to other growth factors, such as IGF-I.

Such variations in the effects of growth factors support the hypothesis of a narrow range at which each growth factor and hormone present in milk, colostrum and in other nutrients may stimulate gut epithelial cells.

The major types of complex carbohydrates in the human colon are cellulose, pectin and hemicellulose, commonly referred to as dietary fibers (Cummings, 1981). SCFAs are produced by bacterial fermentation of carbohydrates, which occurs in the colon. Colonic-derived SCFAs provide from 5% up to 15% of the total energy requirement by humans, depending on nutrient intake and the composition of the microbiota (Bergman, 1990). The major products of the saccharolytic fermentation are butyrate, propionate and acetate. They are involved in several physiological processes such as blood pressure regulation (Pluznick et al, 2013), inflammation (Tedelin et al, 2007), lipid metabolism, mineral and ammonia absorption (Fava et al, 2008; Hamer et al, 2008). They can prevent the overgrowth of pathogenic bacteria, lower the pH in the colon (Roy et al, 2006) and they have a protective role against tumors by modulating cell proliferation and apoptosis (Scharlau et al, 2009). Butyrate is the main energy source for colonocytes, as demonstrated by Donohoe et al. (2011) who discovered that the lack of butyrate in germ-free mice induces autophagy in colonocytes in order to extract amino acids for energy metabolism. Besides being an essential energy source for large intestine epithelial cells, butyrate is

involved in a wide array of cellular functions such as the promotion of apoptosis and gene expression modulation (Meijer et al, 2010; Purup and Larsen, 2007; Brahe et al, 2013).

The metabolic activity assay AlamarBlue^{TM} (AB) measures the mitochondrial activity at a given time point which is related to the number of viable cells. The PicoGreen® assay gives the amount of DNA at the end of the experiment, which, if the amount of DNA in a cell is constant, measures the total number of cells (viable or not). In preliminary experiments conducted with SCFAs, a detrimental effect of either NaBt, NaProp or NaAce on metabolic activity of HT29-MTX-E12 cells was demonstrated. AB showed to fit for the purpose of describing the effect of SCFAs on intestinal HT29-MTX-E12 cells, and, therefore, it has been used in the second part of this study.

The present study showed the anti-proliferative role of butyrate, which was the most effective SCFA able to inhibit HT29-MTX-E12 metabolic activity, exerting a strong dose dependent effect on these cells. Our findings show that propionate could also modulate cell growth and proliferation. Previous studies reported the role of propionate at the cellular level. Vecchia et al. (1992) demonstrated the inhibitory role of propionate in cancer cells, whereas Curi et al. (1993) reported that specific concentrations of propionate cause the inhibition of lymphocyte proliferation. Acetate is the most abundant SCFA in the human intestine. It is a precursor for the biosynthesis of lipids and increases phospholipid and cholesterol synthesis. Vecchia et al. (1997) reported that acetate exerts an antineoplastic effect, while Hague et al. (1995) found an apoptotic effect induced by 80 mM acetate on a colorectal cell line, similar to 4 mM butyrate. This observation is in line with our findings in which only high concentrations of acetate strongly compromised HT29-MTX-E12 metabolic activity.

The concentrations of SCFAs employed in the present study are in accordance with amounts encountered in the gastro-intestinal tract of humans (Le Gall et al., 2009). Specifically, Cummings et al (1987) reported butyrate levels around 24 mmol/kg digesta and acetate levels around 60 mmol/kg digesta in the proximal colon of humans. From a recent study conducted in swine, levels of 9-16 mmol/kg wet digesta of butyrate in the

colon have been reported (Nielsen et al, 2014). In this study, when SCFAs were tested in combination, the concentrations chosen were comparable (HighSCFAs) and below (LowSCFAs) those amounts found in the intestinal lumen. The effect of butyrate, propionate and acetate in combination at concentrations comparable to those found in the human bowel was also observed in another cell line (Vecchia et al, 1997). With regard to SCFA combinations, propionate and acetate enhanced the inhibitory activity of butyrate on HT29-MTX-E12 cells, suggesting an additive effect of the three major SCFAs on intestinal epithelial cells.

Taken together, the inhibitory effect of SCFAs on the HT29-MTX-E12 cell line observed in this study is also interesting from the point of view that HT-29 cells are of cancer origin. The data presented herein suggest a protective effect of SCFAs against the proliferation of colon cancer cells, underlining the beneficial role of a fiber-rich diet. Of the SCFAs tested, butyrate was the most effective and its effect was enhanced by the addition of propionate and acetate. These data support the proposition that nutritional manipulation with a fiber-rich diet could have a crucial role in controlling the occurrence of colorectal cancer and other gastrointestinal disorders.

Another interpretation of the results reported here is the fact that the HT29-MTX-E12 cells were kept in a medium containing glucose as the main energy source. Treatment with alternative energy sources, such as butyrate, propionate or acetate, might be affected by the presence of glucose in the culture medium. Singh et al. (1997) reported that butyrate, in the presence of glucose, inhibits the growth of HT29 cells, whereas it stimulates cell proliferation in glucose-depleted medium. Whether the effects of the three major SCFAs occurred due to the presence of glucose in the culture medium remains to be examined in glucose-depleted HT29-MTX-E12 cells.

Overall, this study showed that several bioactive compounds and fermentation metabolites have an essential role in the growth and development of the human intestinal epithelium. In addition, these results contribute to the characterization of the response of HT29-MTX-

E12 cells with respect to dietary compounds, which can pass to the intestine in intact form, and to fermentation metabolites produced by colonic bacteria.

Undifferentiated HT29-MTX-E12 cells provide an opportunity to examine the potential role of growth factors, hormones and SCFAs in the regulation of metabolic activity and proliferation of the intestinal cells. HT29-MTX-E12 cells have been shown to be a suitable *in vitro* model for cell-nutrient interaction studies.

Study III: ***Citomodulatory and prebiotic roles of whey, casein and soya protein retentate obtained by in vitro simulated gastro-intestinal digestion***

Aim of study III

The aim of the study was to investigate the *in vitro* effects of milk protein retentate on human gut health, after *in vitro* SGD.

Specifically, citomodulatory roles of whey and casein protein retentate were determined via the use of human intestinal HT29-MTX-E12 cells, and the prebiotic activities of the protein retentate were determined according to their abilities to modulate *Lactobacillus casei* proliferation. Soya protein was included in all experiments as a non-animal protein source.

Material & methods

All chemicals were purchased from Sigma Aldrich (St. Louis MO, USA) unless otherwise indicated.

Cells & culture conditions

The HT29-MTX-E12 cells were kindly donated by Dr. David Brayden and Dr. Sam Maher (UCD Conway Institute, Dublin, Ireland). This human colorectal adenocarcinoma cell line, which is a clone of HT29 cells, is able to differentiate into a mucus-producing goblet-like cell line. Cell maintenance was performed in 25-cm^2 flasks using 15 mL Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L) and supplemented with 10% of FBS, 1% 1 M HEPES, 1% (v/v) penicillin/streptomycin, 2% Glutamax and 1% MEM NEAA. The cells were cultivated at 37°C in a humidified incubator with 5% CO_2. The cells were subcultured using 0.05% trypsin-EDTA to at 60 to 80% confluence. All experiments were performed using HT29-MTX-E12 cells within six cell passages (passages 40 to 45) to ensure reproducibility.

Undifferentiated HT29-MTX-E12 cells were plated at a density of $1.5x10^4$ cells/well in 96-well plates and cultured for 24 hours. For the viability assay, the cells were in the sigmoid phase of growth when the protein retentate were added to the culture medium. The treatment medium was composed of basal DMEM and 0.05% FBS containing whey, casein and soya protein retentate at different concentrations (0.39 up to 50 µg/µl) that were chosen based on preliminary tests on this cell line to reproduce the physiological ratios of protein retentate that may be reached the human colon. Retentate stocks were obtained by SGD as indicated in the study I of this chapter.

MTT assay: Cell viability

The viability of the HT29-MTX-E12 cells after 3 and 24 h of treatment was calculated with the MTT test. MTT assay measures the production of the chromophore formazan from 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide (MTT). Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase. Three replicates per treatment were employed, and the experiments were repeated at least twice.

Specifically, the cell viability percentages induced by the protein retentate treatments were calculated as follows:

% cell viability = (mean optical density of treated cells/ mean optical density of control cells) x 100.

Effect of whey, casein and soya protein retentate on HT29-MTX-E12 mucin production: PCR of the MUC5AC gene and cyclophilin reference gene

HT29-MTX-E12 cells were maintained for 21 days in culture. It has been reported that, after 21 days of culture, HT29-MTX cells express a stable amount of mucus (Lesuffluer 1993; Martized-Maqueda et al., 2012).

To control the mucus production, HT29-MTX-E12 cells were seeded into 2-well chamber slides at 2×10^4 cells/cm^2 and maintained in complete medium without passaging for up to 21 days. At selected time points, mucin production was detected using Alcian blue stain. The cell monolayers were first fixed with chilled 99% methanol for 10 minutes and washed 3 times with PBS. Next, incubation with 1% Alcian blue/3% acetic acid for 3 h at room temperature was performed. The cells were subsequently washed three times with PBS to remove any residual stain, and mucin production (assessed based on the level of blue staining) was examined by light microscopy. Paint Shop Pro 7.04 software was used to capture the images.

The HT29-MTX-E12 cells were also plated at a density of $5x10^5$ cells/well in 12-well plates and maintained for up to 21 days in complete medium. At day 21, the cells were washed with PBS, and the medium was replaced with 0.05% FCS medium for 24 h (cell starvation). The treatment medium (0.05%FCS) with or without whey, casein or soya protein retentate at different concentrations (0.78 up to 3.12 µg/µl) was added to the cells, which were then incubated in a controlled atmosphere (37°C / 5% CO_2). The concentration range was chosen based on the MTT test results.

At the end of the incubation, total RNA was extracted from the treated and untreated HT29-MTX-E12 cells using a nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocols. The RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad), and the resulting cDNA was used as a template for the qualitative and quantitative PCRs. MUC5AC was amplified by PCR with primer sequences that have previously been published by Martínez-Maqueda et al., 2012 (**Table 1**), and cyclophilin was included as a reference gene. Qualitative PCRs were performed on all samples under the following thermocycling conditions: 10' initial denaturation at 95°C; followed by 40 cycles of amplification at 95°C for 15-s, 1' of annealing at 60°C and 1' extension at 72°C; and a final 3' elongation step at 72°C. The PCR products were analyzed by gel electrophoresis in 2% agarose to detect the cyclophilin, β-actin and MUC5AC rRNA bands (Image Lab™, BioRad, software analysis).

***Table 1**: Primers for qualitative and real time PCRs*

Gene (&bp)	primers	References
MUC5AC (240bp)	5'- CGACCTGTGCTGTGTACCAT-3′ 5′-CCACCTCGGTGTAGCTGAA-3	Martínez-Maqueda et al., 2012
β-actin (197bp)	5′-CTTCCTGGGCATGGAGTC-3′ 5′GCAATGATCTTGATCTTCATTGTG-3′	Martínez-Maqueda et al., 2012
Cyclophillin (160bp)	5′-CTTCCTGGGCATGGAGTC-3′ 5′-GCAATGATCTTGATCTTCATTGTG-3′	Martínez-Maqueda et al., 2012

Effect of whey, casein and soya protein retentate on HT29-MTX-E12 mucin production: Real-time PCR

Real-time PCR quantification analyses were performed with SYBR green methodology using a real-time PCR system (Strategene Mx3000p).

Each reaction tube contained 2X SYBR Green real-time PCR Master Mix, gene-specific forward and reverse primers and the cDNA. The master mix included Maxima® Hot Start *Taq* DNA polymerase, dNTPs in an optimized PCR buffer, and SYBR® Green I dye supplemented with ROX passive reference dye. The primers and lengths of the amplified fragments are listed in table 1. One microliter of cDNA was used for each sample. The samples were tested in duplicate and non-reverse transcribed controls and no-template controls were included in the assays. The thermal profile began with 2' at 50°C and 10' at 95°C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1'. The comparative CT method was used (Livak and Schmittgen, 2001) to determine the fold changes in gene expression, which were calculated with the threshold method (2-ΔΔCT). Relative quantification was performed, and the values were normalized to the internal reference gene. The cyclophilin gene and β-actin gene were tested as reference genes. Cyclophilin was amplified to calculate the relative expression because it has previously been demonstrated to be stable under these conditions.

Prebiotic activity

Lactobacillus casei ssp casei (Orla-Jensen) Hansen and Lessel (ATCC® 393™) was maintained at the Health, Animal Science and Food Safety department of the University of Milan (Italy). Stock cultures were maintained in Man Rogosa Sharpe (MRS) with acetate and manganese, which act as specific growth factors for lactobacilli. The MRS composition is indicated in the **Table 2**.

***Table 2**: Man Rogosa Sharpe (MRS) medium composition*

Ingredients	**Grams/Litre**
Peptone	10.0
Meat extract	8.0
Yeast extract	4.0
D(+)-Glucose	20.0
Dipotassium hydrogen phosphate	2.0
Sodium acetate trihydrate	5.0
Triammonium citrate	2.0
Magnesium sulfate heptahydrate	0.2
Manganous sulfate tetrahydrate	0.05
Final pH 6.2 +/- 0.2 at 25°C	

A series of *in vitro* experiments were conducted to evaluate the influences of whey, casein and soya protein retentates on the growth of *Lactobacillus Casei*. The bacteria were grown to the stationary phase in MRS broth and then serially diluted in peptone water to 10^4-10^5 CFU. Protein retentate stock solution (100 µg/µL) was diluted in peptone water until the treatment concentrations were achieved. In 96 well-plates, 25 µL of the different concentrations (from 0.39 up to 3.12 µg/µL) of each whey, casein and soya retentate were mixed with 225 µL of 10^4-10^5 CFU lactobacilli culture, and the plate was then incubated

at 37°C for 48 h in anaerobic conditions. At the end of the incubation period, the treated bacteria were serially diluted 10-fold in peptone water, and 100-µL aliquots of each dilution were spread in duplicate onto the surfaces of the MRS agar plates. The MRS agar plates were incubated anaerobically at 35°C. After 48 h, the number of viable bacterial cells/colonies was counted. The entire experiment was repeated twice, and each treatment was performed in triplicate to confirm the results.

Statistical analysis

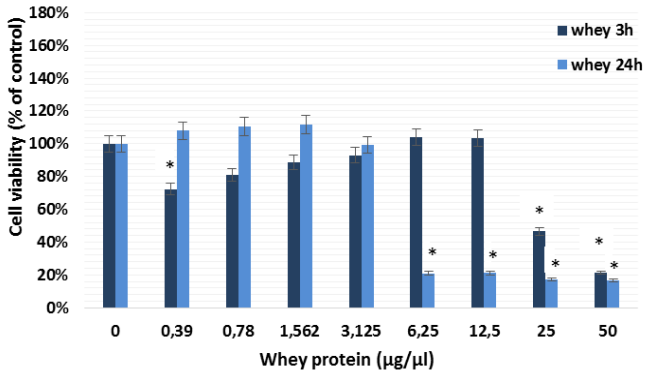
The data were analyzed as the mean values ± the standard errors of three independent experiments using one-way ANOVA. SAS 9.3 software (SAS Institute, Cary, NC, USA) was used to identify the significant differences between treatment and control data. The differences between means were considered statistically significant at $P<0.05$ (*).

Results & discussion

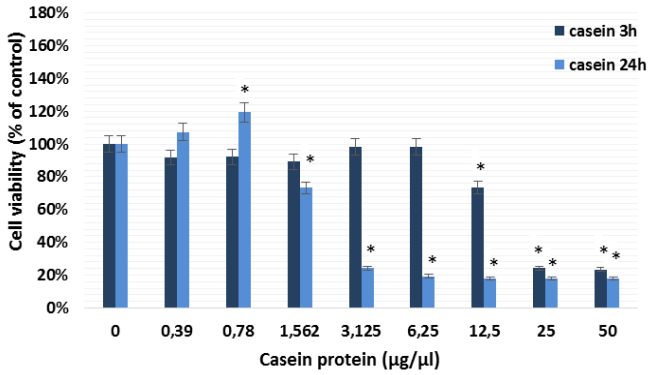
The HT29-MTX-E12 cells were first used in this study in the undifferentiated state to test the proliferative roles of *in vitro*-digested milk and soya proteins and after 21 days of culture to study the modulation of mucus-production by protein retentate treatments.

MTT assay: Cell viability

A



B



C

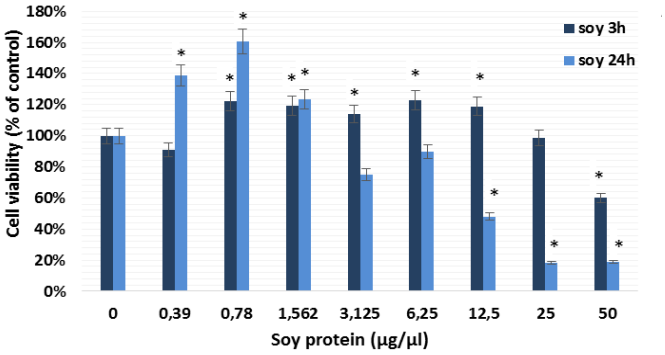
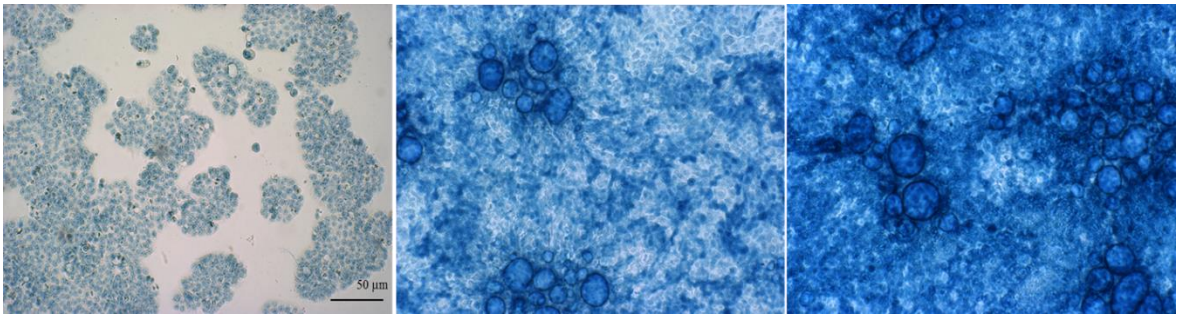


Figure 1: *Effects of whey protein retentate (A), casein protein retentate (B) and soya protein retentate (C) on cell viability after 3 and 24 h. The values that were significantly different from the cell viability obtained in the control cells (0 µg/µl) are indicated by * (P<0.05)*

When the lower range of concentrations tested (0.39-1.56 µg/µl) was considered, the soya protein retentate appeared to be the most effective (**Figure 1**) in enhancing HT29-MTX-E12 cell viability compared with the untreated cells. The whey and casein protein retentates in the lower range of the tested concentrations maintained intestinal cell viability. In contrast, at the higher concentration range (3.12-50 µg/µl), all of the protein retentates reduced the viability of the HT29-MTX-E12 cells in dose-dependent manners. Ganjam et al., 1997 demonstrated the ability of β-casomorphin-7, which is a casein peptide, to modulate the proliferation and induce apoptosis in rat and human intestinal cells. The inhibitory roles that were detected at the specific concentrations of the protein retentates may be interesting from the perspective that the HT29-MTX-E12 cells have a cancerous origin. Thus, the milk and soya peptides may exhibit anti-proliferative effects in colon cancer cells as has been previously reported (Meisel & FitzGerald, 2003).

The results presented here indicated that all of the protein retentates, at specific concentrations, were able to modulate the viability of the HT29-MTX-E12 cells and to exert trophic effects on the intestinal epithelia. The citomodulatory effects of the food-derived peptides require further investigation before the full influence of their physiological effects at the intestinal cell level can be determined. Based on the viability assay, a range of concentrations (0.78-3.12 µg/µl) for the mucus-production evaluation was selected.

Assessment of HT29-MTX-E12 morphology and mucus production



7 days 14 days 21 days

Figure 2: *Representative Images of mucin production by HT29-MTX-E12 cell lines at days 7, 14, and 21, 20X objective. Mucin production was visualized by Alcian blue staining.*

Effects of whey, casein and soya protein retentates on HT29-MTX-E12 mucin production: PCR for the MUC5AC gene and cyclophilin

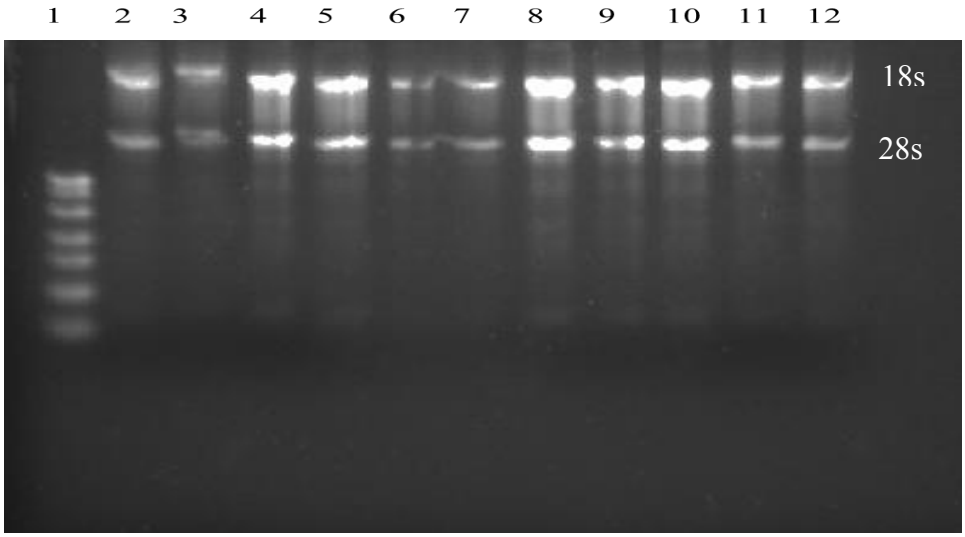


Figure 3*: Total RNA was isolated with Nucleospin RNA II (Macherey-Nagel, Dü ren, Germany) from HT29-MTX-E12 cells treated (24 h) with DMEM alone (CTR, LINE 2-3); 0.78, 1.56, 3.12* µg/µL *casein protein retentate (LINEs 4-6), 0.78, 1.56, and 3.12* µg/µL *whey protein retentate (LINEs 7-9), or 0.78, 1.56, and 3.12 µg/µL soya protein retentate (LINEs 10-12). RiboRuler low Range RNA Ladder Thermo Fisher Scientific (LINE1).*

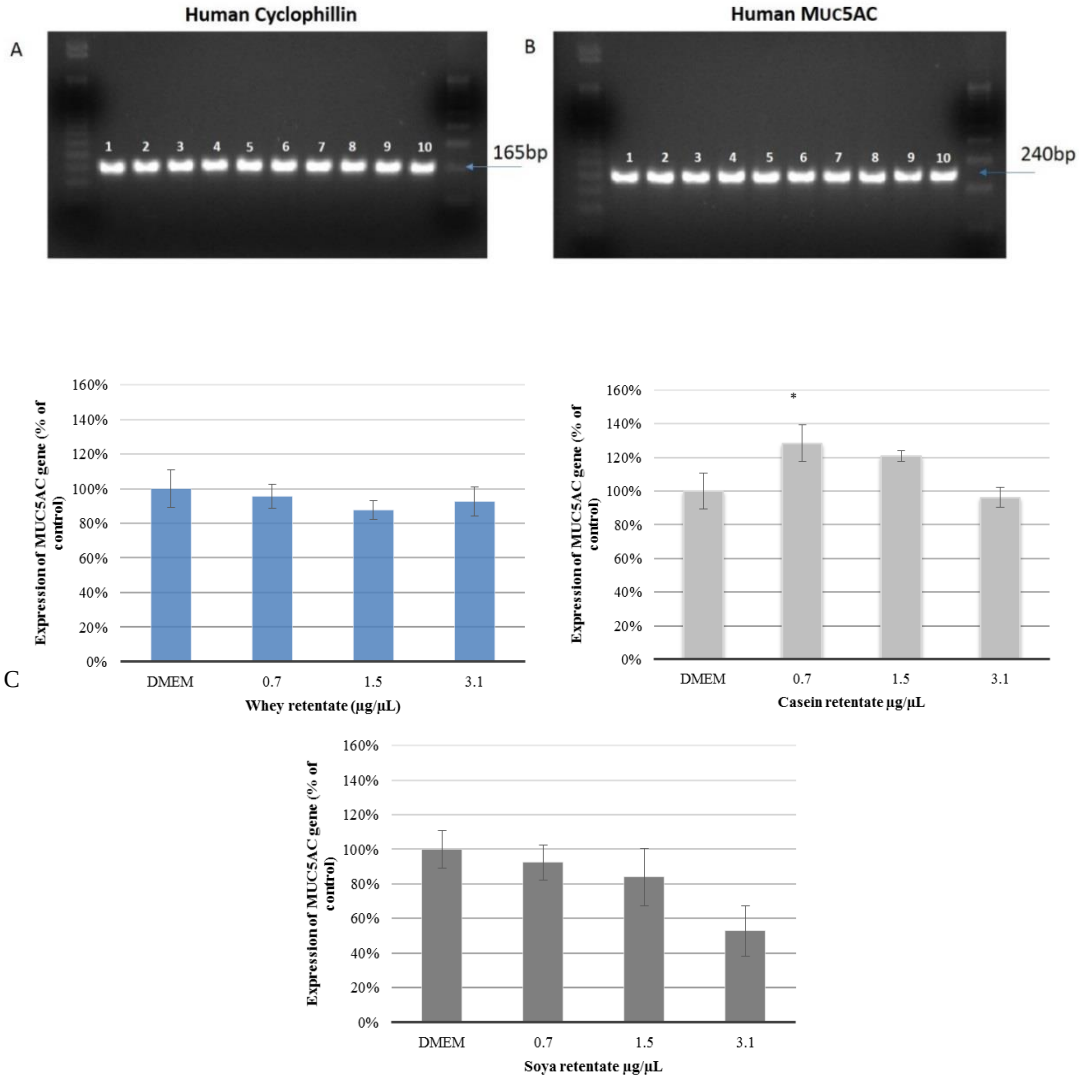


Figure 4*: The presence of the control and MUC5AC genes were tested by qualitative PCR. (A) Representative gel of cyclophilin cDNAs obtained from the HT29-MTX-E12 cells exposed to DMEM alone (CTR, LINE 1), 0.78, 1.5, and 3.12 µg/µL casein protein retentate (LINEs 2-4), 0.78, 1.56, and 3.12 µg/µL whey protein retentate (LINEs 5-7), or 0.78, 1.56, and 3.12 µg/µL soya protein retentate (LINEs 8-10. (B) Representative gel of the MUC5AC cDNAs obtained from the HT29-MTX-E12 cells exposed to DMEM alone (CTR, LINE 1), 0.78, 1.56, and 3.12 µg/µL casein protein retentate (LINEs 2-4), 0.78, 1.56, and 3.12 µg/µL whey protein retentate (LINEs 5-7), or 0.78, 1.56, and 3.12 µg/µL*

soya protein retentate (LINEs 8-10). (C) MUC5AC gene expressions in the HT29-MTX-E12 cells treated with whey, casein and soya retentate determined by quantitative RT-PCR. The data are expressed as the relative MUC5AC expression levels relative to the controls (untreated cells, DMEM). Each point represents the mean of three independent experiments that were performed in duplicate.

The mucus gel layer is part of the intestinal mucosa and acts as a protective barrier against pathogenic microorganisms, food toxins and hard dietary constituents; it protects the intestinal epithelium from the corrosive activities of gastric juice and bacterial and endogenous proteases. The intestinal mucus is composed of 95% water and electrolytes ($Ca^{2+,}$ Mg2+, Na+), carbohydrates glycoproteins, peptides and lipids. The viscosity of the mucus gel derives from glycoproteins called mucins that are primarily secreted by goblet cells in the colon.

To determine whether the whey, casein and soya retentate affected mucin expression and secretion, HT29-MTX-E12 cells were treated for 24 h with each of the compounds. As demonstrated in **Figure 4,** casein protein retentate modulated the expression of MUC5AC mRNA at the concentration of 0.78 µg/µl, but the higher concentrations tested were not able to significantly affect MUC5AC mRNA after 24 h of treatment. Soya protein retentate reduced the expression of MUC5AC mRNA (**Figure 4**). These results accord with those of previous studies that have demonstrated the roles of milk peptides in modulation of gastrointestinal mucus production. Martinez-Maqueda et al. (2013) reported that casein hydrolysate stimulates HT29-MTX-E12 cells and promotes the expression of MUC5AC. The same group demonstrated the mucin-secreting role of whey protein hydrolysate in HT29-MTX-E12 cells. However, the whey protein peptide β-lactorphin increases the synthesis of mucin proteins without eliciting differences in MUC5AC gene expression (Martinez-Maqueda 2012). Plaisancie et al. (2013) demonstrated increases in mucin secretion and MUC2 and MUC4 gene expression in HT29-MTX-E12. β-casomorphin-7 increases MUC5AC mRNA expression and the secretion of this mucin, as demonstrated by Zoghbi et al. (2006).

Prebiotic activity

The results revealed that, at specific concentrations, whey and casein protein retentate were effective in promoting the growth of *Lactobacillus casei in vitro,* compared with soya protein retentate. Casein retentate was the most effective in promoting *Lactobacillus casei* growth compared with whey and soya. Specifically, the 1.56 µg/µL of the casein retentate significantly ($P<0.05$) enhanced bacterial growth, compared with the control (0 µg/µL) (**Figure 5, 6**). From a health-promotion perspective, this finding is very important due to the growing interest in the promotion of the probiotic bacteria along the gastrointestinal tract. Probiotic bacteria play a role in the enhancement of gut health by competitively excluding pathogens and by producing positive substances for the gut microenvironment. However, the specific mechanism by which casein retentate increased *Lactobacillus casei* proliferation needs to be further identified. This proliferative property may be related to the antioxidant peptides contained in the digesta, which can modulate oxidative stress in the medium via the creating of a more beneficial environment for the growth and proliferation of the bacteria.

This preliminary finding suggests that milk proteins may have a role in the maintenance and/or increase in the numbers of prebiotic bacteria in the human gut, leading to a more protective environment.

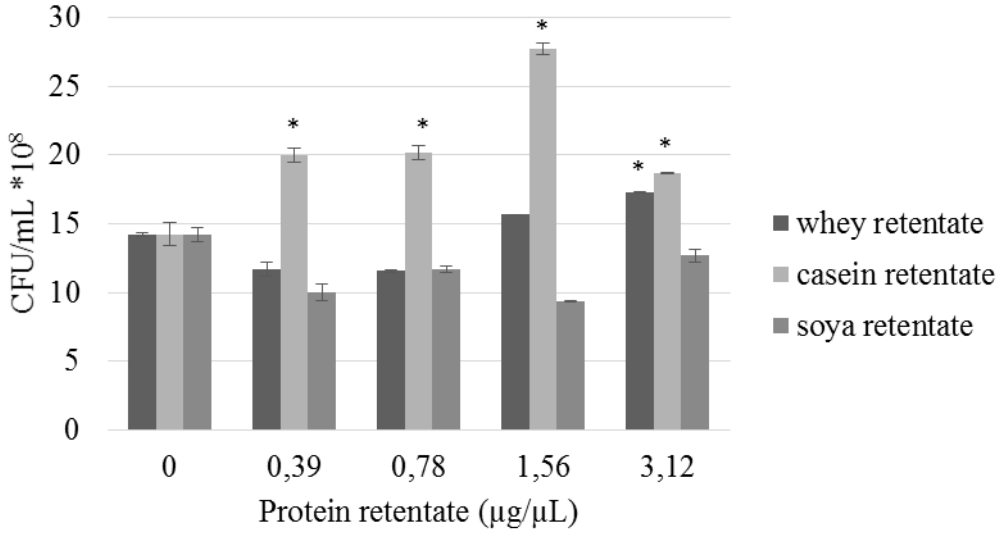


Figure 5: *Effects of increasing concentrations (µg/µl) of whey protein retentate, casein protein retentate and soya protein retentate on Lactobacillus casei proliferation (prebiotic activity) after 48 h of incubation in an anaerobic environment. The results are expressed as colony-forming units (CFU/mL), and the values that significantly differed from those obtained in the control (0 µg/µl) are indicated by * (P<0.05).*

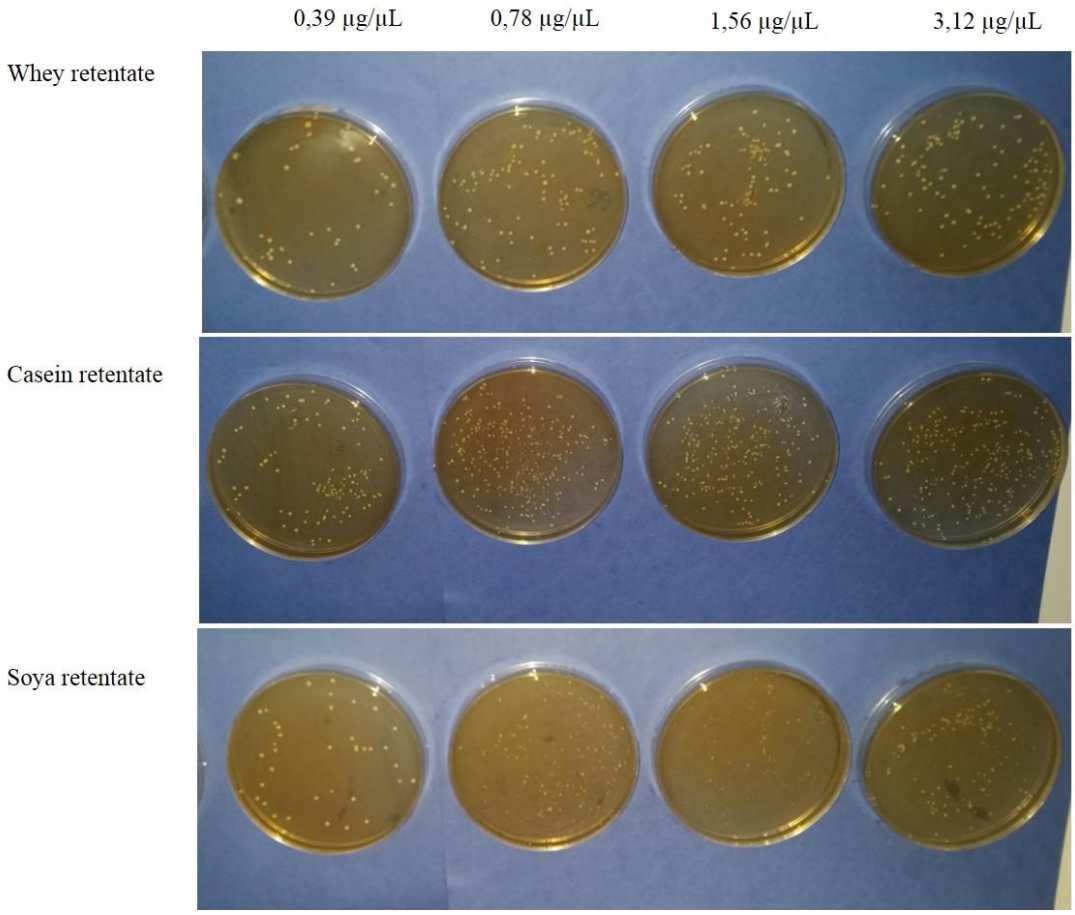


Figure 6: *Effects of increasing the concentrations of whey protein retentate, casein protein retentate and soya protein retentate on Lactobacillus casei proliferation (colony formation) after 48 h of incubation in MRS agar in an anaerobic environment.*

Altogether, the analyses of prebiotic bacteria and goblet intestinal cell viability may represent a complementary and valuable tool, in addition to the available *ex vivo* and *in vivo* strategies, for the study of the bioactivities of food components in the gut. Although the *in vitro* results may be questionable compared with those obtained *in vivo*, the results obtained with the prebiotic bacteria and goblet cells *in vitro* may contribute to clarify and better describe the effects observed *in vivo*, when variance in responses between species or sexes are observed. However, the results from *in vitro* cell-based model studies should be considered as such and further studies in animal and human models are needed (Purup

& Nielsen, 2012). However, further studies on mRNA and mucin proteins expression will be performed to confirm the modulation of mucus production that is induced by milk protein digested.

CHAPTER II: VITAMIN E ROLE AS HEALTH PROMOTING FOOD INGREDIENT AND ITS ABILITY IN CONTROLLING OTA INDUCED TOXIC EFFECTS *IN VITRO*

In the past decades, important advances have been made in understanding the adequate vitamin E status in animal and human diet, by an appropriate nutrition. The interest in vitamin E originates from its role as antioxidant able to counteract oxidative stress. However, only partial results are available regarding the role of vitamin E at cellular and molecular level (Abid-Essefi et al., 2003 Baldi et al., 2004; Fusi et al., 2008). The regulation of cell proliferation, gene expression, cell death and DNA damage are emerging functions of vitamin E, stimulating the current research on vitamin E in the target tissues, as the kidney and the mammary gland (Baldi, 2005).

Vitamin E structure and functions

The increased interest and research in micronutrient supplements derives from the potential roles of the antioxidant micronutrients (i.e., vitamin C, vitamin E, and the carotenoids) in balancing oxidative stress (Rock et al., 1996). Vitamin E is a fat-soluble vitamin that is naturally synthesized by plants (seeds and oils) and is widely used as functional ingredient in foods, pharmaceuticals, and cosmetic preparations (Chiu & Yang, 1992).

The term vitamin E is used to describe a family of eight molecules that have a chromanol ring and a phytol side chain as common structural features (Gonnet et al., 2010).

Vitamin E is present in nature in eight different forms that include four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ). All of these compounds occur in a variety of isomers (e.g., eight stereoisomers of α-tocopherol result from the synthesis of this compound). Among all forms of vitamin E, α-tocopherol is the most active in biological systems. The bioavailabilities of the eight stereoisomers have been investigated in several animal species, and it has been concluded that the bioactivity is related to ages, species

and assessment criteria (Blatt et al., 2004). The differences in bioavailability are also related to the stereoisomer composition (Dersjant-Li and Peisker, 2010).

Although the other naturally occurring forms of vitamin E (β-, γ-, and δ-tocopherols and the tocotrienols) are absorbed, they are not converted to α-tocopherol in humans, and they are recognized poorly by the α-tocopherol transfer protein (α-TTP) in the liver.

The natural form of α–tocopherol is composed of 100% RRR-α-tocopherol and is produced by plants, whereas the synthetic form (all-*rac*-α-tocopherol) consists of a mixture of eight stereoisomers in equal amounts. Indeed, when α-tocopherol is chemically synthesized, eight possible stereoisomers are produced due to the three stereocenters in the molecular structure. Four stereoisomers exhibit 2R configurations (RRR, RRS, RSR, and RSS), and four exhibit 2S configurations (SRR, SSR, SRS, and SSS) (**Figure 1**). All of the stereoisomers exhibit antioxidant activities, but only the 2R stereoisomers exhibit high biological activities (Traber & Steven, 2011; Vagni et al., 2011). In food and feed, vitamin E activity is expressed in International Units (IU). The relative activities of RRR and all-*rac*-tocopherol were been calculated in 1979 by the United States Pharmacopenia (USP) as 1 mg for all-*rac*-α-tocopherol acetate (all-*rac*-α-tocopheryl acetate, equal to 1 IU), and all-*rac*-α-tocopherol has a bioactivity of 1.1 IU. *RRR*-α-tocopherol has a bioactivity of 1.49 IU, and *RRR*-α-tocopherol acetate has a bioactivity of 1.36 IU (Vagni et al., 2011). The institute of Medicine (IOM) redefined the USP conversion factors for human due to the instability of the 2S stereoisomers of all-*rac*-α-tocopherol in human plasma and tissues (National Academy of Science, IOM, 2000). However, Jensen et al., (2007) and Dersjant-Li & Peisker (2009) suggested that studies aimed at reconsidering the bioavailabilities of α-tocopherol isomers should be required to consider livestock animals and that appropriate revisions of the conversion factors should be performed.

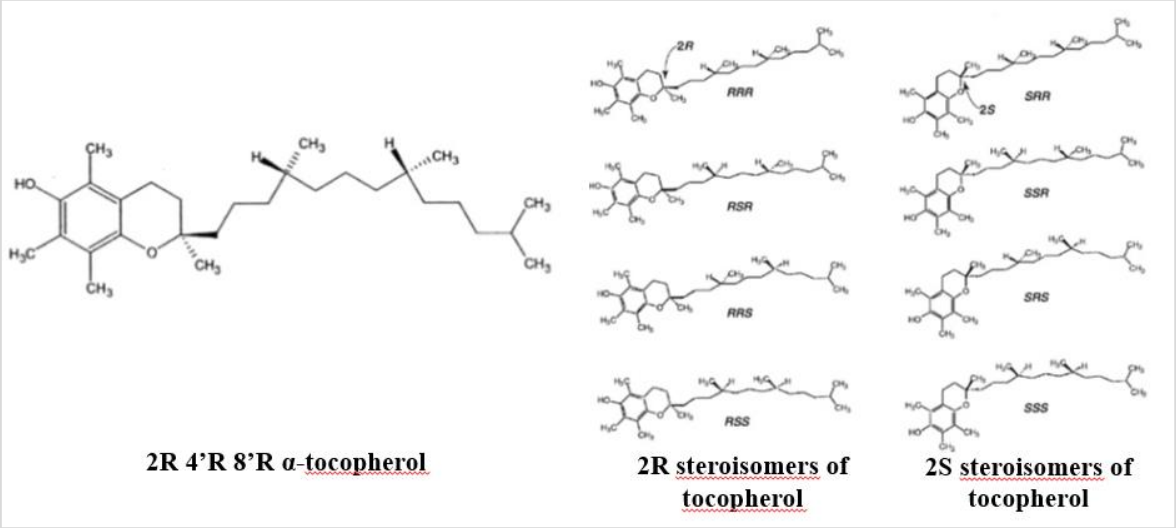


Figure 1*: Natural and synthetic tocopherol stereoisomers*

Antioxidant activity of α-tocopherol: *in vitro* evidences

Vitamin E is a potent radical scavenger that prevents the propagation of free radicals in cell systems and, particularly, protects polyunsaturated fatty acids (PUFAs) within membrane phospholipids and plasma lipoproteins (EFSA, 2015). Polyunsaturated fatty acids (PUFAs) of the cell membranes are vulnerable to attack by ROS, and such attacks can initiate a chain reaction of lipid peroxidation that damages the membrane of the cell. In the cellular environment, ROS-mediated free radicals damage can be reversible or irreversible and targets the molecules such as DNA, lipids and proteins. High levels of ROS generation are due to their abilities to propagate the oxidative reactions initiated by single radical species. A balance of antioxidants is needed to control high levels of ROS generation and the consequent oxidative damage and related implications before they become irreversible. The physiological antioxidant defense systems of the cell are normally able to detoxify reactive intermediates or repair damage caused by ROS generation. However, imbalances between ROS production and the physiological antioxidant system lead to oxidative stress.

α-tocopherol is localized in the cell membrane and is associated with lipoproteins due to its lipophilic nature. α-tocopherol performs its antioxidant role by scavenging the peroxyl radical and blocking free radical propagation. Reactions between lipid peroxyl radicals and α-tocopherol result in α-tocopheroxyl radicals and lipid peroxides. Subsequently, ascorbic acid can reduce α-tocopheroxyl radicals back to α-tocopherol by donating electrons (Azzi et al., 2000; Nakamura & Omaye, 2009). The ability of α-tocopherol to scavenge ROS is greater than those of other tocopherols (Zingg, 2007).

In contrast to other cellular antioxidants, α-tocopherol activity is nonenzymatic, rapid, and appears to depend on the microenvironmental conditions, including its concentration and the presence of oxidants compounds from foods and the environment.

Among the environmental- and food-mediated oxidative stressors, OTA represents a cause of ROS generation that may compromise antioxidant defense mechanisms.

OTA is produced by filamentous mold species belonging to the genera Aspergillus and Penicillium. This toxin has aroused public concern due to its wide range of toxicological effects and its widespread distribution in feed and food (EFSA, 2006). OTA is considered to be a potent nephrotoxic, hepatotoxic and teratogenic substance in different animal species and exhibits a long half-life in human blood (Stojković et al., 1984). After small intestinal absorption, OTA enters in bloodstream where it strongly binds serum proteins and is subsequently distributed to different tissues and organs, including the kidney, which is the primary target (Hagelberg et al., 1989; Palli et al., 1999; Pfohl-Leszkowicz and Manderville, 2011). However, lower OTA concentrations can be detected in different tissues, such as the liver, adipose tissue and muscles. Additionally, detectable amounts of OTA have been found in the milk of several species, including humans, rabbits, rats and ruminants, which suggests that the mammary glands could be among the potential targets of this mycotoxin (Fink-Gremmels, 2008; Sorrenti et al., 2013). At the cellular level, several OTA mechanisms of action have been proposed, and metabolic activity, cell membrane damage, oxidative stress and DNA damage have been studied. However, the mechanisms by which OTA elicits its cytotoxic action vary from tissue to tissue. OTA can express its toxicity through the covalent binding of OTA metabolites to DNA or via an

indirect mechanism of action (Pfohl-Leszkowicz and Manderville, 2011; Ali et al., 2011). Accumulating evidence has demonstrated that, *in vitro*, OTA expresses its toxicity through an indirect mechanism that primarily consists of the overproduction of reactive oxidative species (ROS) that cause oxidative stress (Palma et al., 2007; Zheng et al., 2013). ROS overproduction leads to a wide array of oxidative cell lesions at membrane, mitochondria and DNA levels that can induce cell death. Indeed, according to the cell type examined and the time of exposure, the oxidative effects of OTA may cause apoptosis, necrosis or cancer (Costa et al., 2007).

ROS production has been correlated with DNA oxidation and with the subsequent formation of DNA adducts. One of the major OTA-induced oxidative DNA damage products is 8-hydroxy-2′-deoxyguanosine (8-OHdG). 8-OHdG is an abundant base modification in mammalian DNA that is associated with oxidative stress (Cheng et al., 1992) and is commonly used as an *in vitro* biomarker for assessments of OTA-induced oxidative damage (Zheng et al., 2013).

Increasing evidence suggests that OTA is responsible of the perturbation of cell-cell interactions and cell-cell signaling (Mally et al., 2006; González-Mariscal et al., 2005; Mc Laughlin et al., 2004; Schramek et al., 1997)

Tight junctions represent the most apical components of the intercellular junctional complex, which also includes adherens junctions, desmosomes and gap junctions. Tight junctions are composed of multiple transmembrane, scaffolding and signaling proteins between which occludin and Zo1 interact to connect with the actin cytoskeleton (Harhaj et al., 2004).

Several strategies, including some nutrient supplementations, have been proposed to reduce OTA toxicity (Denli et al., 2010; Sorrenti et al., 2013).

Among these strategies, antioxidant molecules, such as vitamin E, vitamin A, lycopene, and phenolic compounds, have demonstrated different beneficial effects in combating OTA toxicity *in vivo* and *in vitro* and thus exhibited key roles in promoting health (Sorrenti et al., 2013). The preferential localization of α-tocopherol to the cell membrane enhances its functional role as a lipid antioxidant and membrane stabilizer (Wang et al., 1999).

Baldi et al. (2004) evaluated the protective effects of α-tocopherol in OTA-induced oxidative damage. A panel of five well-characterized human and animal cell lines stressed with OTA, SK-N-MC (human neuroblastoma), MDCK (Madin Darby canine kidney), AML-12 (mouse liver hepatocytes), LLC-PK1 (pig kidney), and BME-UV1 (bovine mammary epithelium) cells have been successfully used to investigate the effects of α-tocopherol in counteracting ROS production. The counteracting effect of α-tocopherol has been demonstrated based on significant differences in cell sensitivities to OTA, and BME-UV1 and MDCK cells are the most sensitive of the tested cell lines.

However, much remains to be investigated regarding the cellular bioactivities and membrane stabilizing roles of vitamin E and specifically α-tocopherol. In light of the EFSA recommendations for research (EFSA, 2015), which suggest the performance of studies to develop various biomarkers and endpoints as indicators of the α-tocopherol health-promoting requirements *in vivo*, a comprehensive knowledge of the *in vitro* antioxidant mechanisms of α-tocopherol represents an unavoidable focus of research. Additionally, few reports in the literature have compared the activities of different tocopherol isomers with respect to the *in vitro* oxidative environment.

Therefore, the second chapter of this thesis is composed of three studies in which the main aim was to evaluate the roles of two different α-tocopherol forms in counteracting the oxidative damage induced by oxidative stressors (e.g., hydrogen peroxide and OTA) *in vitro*.

- First, the *in vitro* relative bioefficacies of RRR-α-tocopherol (RRR- α-T) and all-*rac*-α-tocopherol (all-*rac*-α-T) in counteracting the cytotoxic effects induced by oxidative challenges, specifically hydrogen peroxide (H_2O_2), were investigated in BME-UV1 and MDCK cells.
- Further, the oxidative damage induced by OTA *in vitro* was characterized in the selected *cell* models while considering different endpoints.
- The obtained results, together with the well-established nephrotoxic action of OTA, led to the study of the role of *all-rac*-α-tocopherol in MDCK cells subjected to oxidative stress induced by OTA.

Study I: ***Relative bioefficacy of RRR-α-tocopherol (RRR- α-T) versus all-rac-α-tocopherol (all-rac-α-T) in in vitro cell culture models***

Aim of study I

In light of the conversion factors recently revised in human, the main aim of this study was to investigate the *in vitro* relative bioefficacy of RRR-*α*-tocopherol (RRR- *α*-T) versus all-*rac*-*α*-tocopherol (all-*rac*-*α*-T) in counteracting the cytotoxic effect induced by hydrogen peroxide (H_2O_2) in Bovine Mammary Epithelial cell line (BME-UV1) and Madine Darby Canine Kidney cell line (MDCK).

In this study two commercial forms of *α*-tocopherol were considered:

1) (±) *α*-tocopherol prepared by organic synthesis (all-*rac*-*α*-T);

2) naturally occurring (±) *α*-tocopherol (RRR- *α*-T) delivered entirely from soybeans

Material & methods

Cell lines & culture conditions

BME-UV1 cells were routinely cultivated in 75-cm^2 plastic culture flasks in complete medium, which is a mixture of 50% DMEM-F12, 30% RPMI-1640 and 20% NCTC-135 supplemented with 10% Foetal Bovine Serum (FBS), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1.0 µg/ml insulin, 5.0 µg/ml transferrin, 1.0 µg/ml hydrocortisone, 0.5 µg/ml progesterone, 10.0 µg/ml L-ascorbic acid and 5ml/L pen/strep.

MDCK cells were routinely cultivated in 75-cm^2 plastic culture flasks in complete medium, which is a mixture of MEM (EBSS), 10% Foetal Bovine Serum (FBS), 2mM Glutamine, 1% Non Essential Amino Acids (NEAA) and 2 ml/L of pen/strep.

Cells were maintained at 37°C into an incubator in a humidified atmosphere containing 5% CO_2. The cells were incubated until the monolayer was sub-confluent (3 to 5 days). Medium were changed every 2 days.

After reaching the sub-confluence, cells were trypsinized and seeded (seeding density: BME-UV1 2 x 10^5cells/mL; MDCK 1 x10^5 cells/mL) into 96-wells tissue culture plates. After 24h of incubation at 37°C in an atmosphere of 5% of CO_2 a sub-confluent layer of cells was reached.

RRR-α-tocopherol (RRR- α-T) and all-rac-α-tocopherol (all-rac-α-T) dose-response curve

In the first set of experiments a dose-response curve, with several tocopherol concentrations was performed in order to determine the appropriate concentrations for further oxidative challenge experiments. In subdued lighting, the two forms of tocopherol were dissolved in ethanol to give 10 mM solutions. Further dilution in serum-free culture medium produced concentrations, ranging from 100 µM to 1nM. The concentrations of RRR- *α*-T and all-*rac*- *α*-T selected for oxidative challenge experiments were 100µM and 1nM for BME-UV1 and for MDCK cell line. The selection has been made also on the base of previous results and on the data obtained in studies conducted by Baldi and collaborators (2004). Work solutions were stored in the dark before addition to cultures. Cells incubated with only basal medium, specific for each cell model, were used as control. The effect of RRR- *α*-T and all-*rac*-*α*-T on both BME-UV1 and MDCK cell lines was determined by MTT test.

MTT assay measures the production of the chromophore formazan from 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide. Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase. Three replicates per treatment were employed and the experiment was repeated at least twice.

In particular, the percentage of cells viability induced by protein retentate treatments was calculated as follows

% cell viability = (mean optical density of treated cells/ mean optical density of control cells) x 100.

Evaluation of RRR-α-tocopherol (RRR- α-T) and all-rac-α-tocopherol (all-rac-α-T) activity in counteracting the cytotoxic effect induced by oxidative challenges

Preliminary experiments were performed to determine H_2O_2 cytotoxicity, the LC_{50} was calculated in both cell lines (MTT test).

In the second set of experiments a putative difference in the protective effect of the two forms of tocopherol against oxidative challenges was evaluated. Cells were trypsinized and inoculated into wells of 96-cell tissue culture plates (seeding density: BME-UV1 2 x 10^5 cells/ml; MDCK 1 x10^5 cells/ml). Cells were pre-incubated for 3 h with the selected RRR- *α*-T and all-*rac*- *α*-T concentrations and then exposed to increasing H_2O_2 concentrations ranging from 250 to 750 µM for BME-UV1 cell line and from 125 to 175 µM for MDCK cell line for the following 24h.

The effect of RRR- *α*-T and all-*rac*- *α*-T on both BME-UV1 and MDCK cell lines in counteracting H_2O_2 toxicity was determine with the MTT and LDH tests in order to evaluate cell viability and membrane stability, respectively.

The LDH assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. In order to examine cell membrane damage induced by the different treatments Lactate dehydrogenase enzyme release (LDH) assay was performed using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) as instructed by the manufacturer. Briefly, after the treatments, supernatants were removed and centrifuged for 5 minutes at 1500 × g (4°C). 50 µL of each supernatant was transferred to a new 96 well plate. Cellular monolayers were lysed by adding 15 µL of 9% Triton X-100 solution in water per 100 µL of basal medium (EMEM), followed by an incubation for 1 h at 37 °C. Cells debris were removed by centrifugation for 5 min at 1500 × g (4 °C) and 50 µL of each cell lysate was transferred to 96 well plate. Then, 50 µL of LDH substrate was added to the supernatants and cell lysates. After the incubation for 30 minutes at room temperature in the dark, the enzymatic assay was stopped by adding 50 µL of 1 M acetic acid and the plate was read at 490 nm using a Biorad 680 microplate reader.

The percentage of LDH release was calculated as follows:

%LDH release= LDH medium/ (LDH medium + LDH cell lysate).

Statistical analysis

At least three replicates at each incubation time were performed and all experiments were performed three times. Results are expressed as mean ±SEM. The effect of various treatments were evaluated by one-way analysis of variance using the GLM procedure of SAS (SAS institute Inc, NC, USA). Values significantly different from controls are indicated as $P<0.05$.

Results & discussion

RRR-α-tocopherol (RRR- α-T) and all-rac-α-tocopherol (all-rac-α-T) dose-response curve

After 24 h of incubation a significant ($P<0.05$) stimulatory effect of all-*rac*- α-T and RRR- α-T (**Figure 1**) on BME-UV1 cells viability was observed. A higher percentage of cell viability in BME-UV1 cells treated with all-*rac*- α-T and RRR- α-T compared with control cells (DMEM), was observed. The proliferative effect induced by RRR- α-T form was higher at the lowest concentrations (1 nM and 0.01 µM) than all-*rac*-α-T.

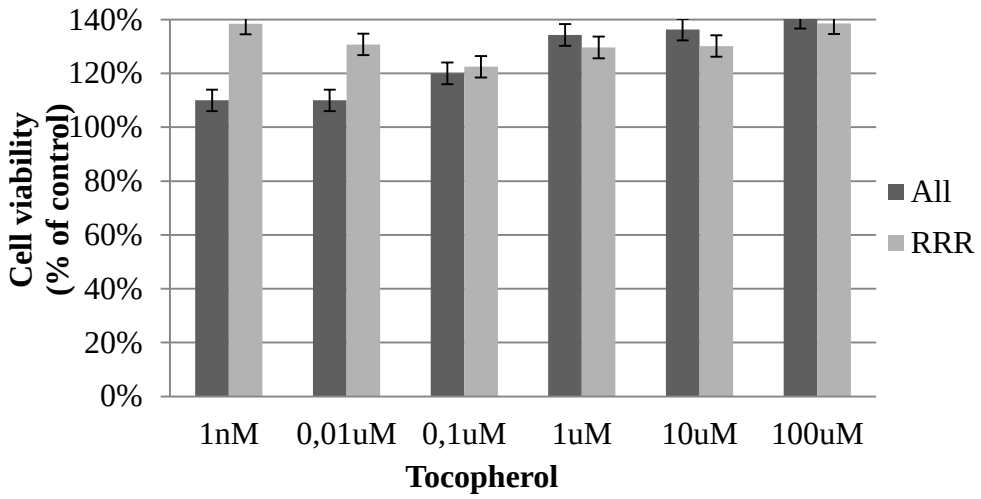


Figure 1*: Effect of all-rac- α-T (All) and RRR-α-T (RRR) (concentrations ranging from 1nM to 100 µM) on BME-UV1 cell viability. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to cell viability obtained in basal medium (DMEM).*

In MDCK cell line, after 24h of treatment with increasing concentrations of all-*rac*- *α*-T and RRR- *α*-T (**Figure 2**) a maintenance of the cell viability was demonstrated.

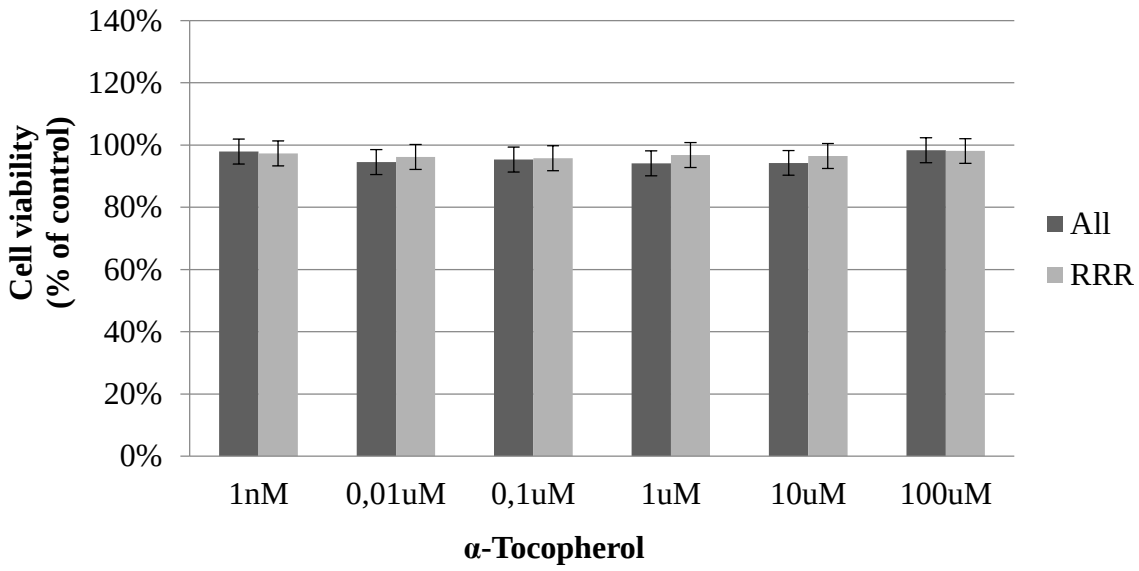


Figure 2*: Effect of all-rac- α-T (All) and RRR-α-T (RRR) (concentrations ranging from 0.01nM to 100 µM) on MDCK cell viability. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to cell viability obtained in basal medium (MEM).*

Evaluation of RRR-α-tocopherol (RRR- α-T) and all-rac-α-tocopherol (all-rac-α-T) activity in counteracting the cytotoxic effect induced by H_2O_2

In order to set up the challenge we have first investigated the half-lethal concentration (LC_{50}) of H_2O_2 in both cell lines. In BME-UV1 cells LC_{50}, after 24h of H_2O_2 treatments, was 375.87 µM, while in MDCK cells, LC_{50} was 140 µM after 24h of H_2O_2 exposure.

Effect on cell viability: MTT test

In the second set of experiments, the ability of RRR- *α*-T and all-*rac*- *α*-T forms to counteract the damage induced by the oxidative stressors as H_2O_2 was assessed, considering the cell viability and the stability of the cell membrane.

In BME-UV1cells (**Figure 3**) pre-treatments with 100 µM of RRR- *α*-T and 100 µM all-*rac*- *α*-T were able to significantly (*P*<0.05) counteract the oxidative effect induced by the highest H_2O_2 concentration tested (750 µM).

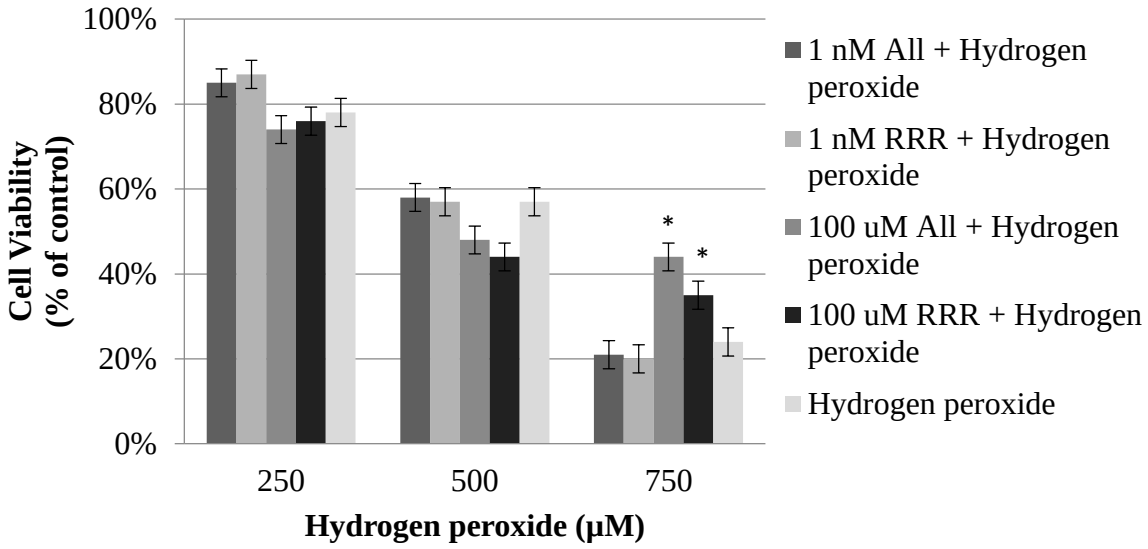


Figure 3*: Effect of pre-treatment with all-rac- α-T (All) and RRR- α-T (RRR) against H_2O_2 cytotoxicity in BME-UV1. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to cell viability obtained in basal medium (DMEM). Values significantly different from cell viability obtained in H_2O_2 alone are indicated by * (P<0.05).*

In MDCK cells (**Figure 4**) pre-treatments with 1nM of all-*rac*-*α*-T were able to significantly (P<0.05) reduce the oxidative effect of H_2O_2 when it was supplemented at 125 µM and 150 µM.

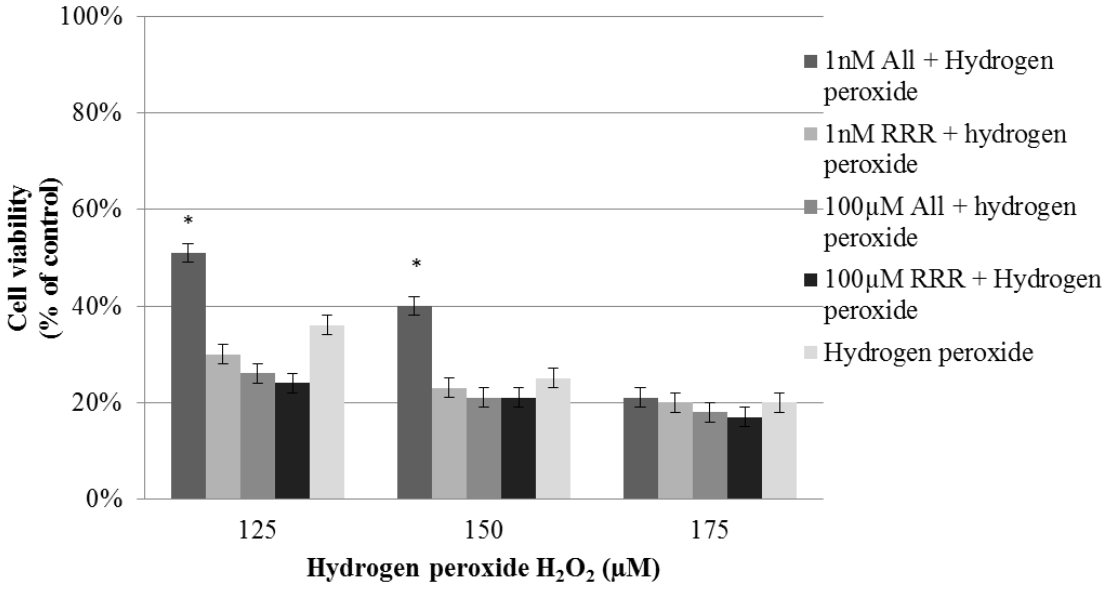


***Figure 4**: Effect of pre-treatment with all-rac- α-T (All) and RRR- α-T (RRR) against H_2O_2 cytotoxicity in MDCK. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to cell viability obtained in basal medium (MEM). Values significantly different from cell viability obtained in H_2O_2 alone are indicated by * ($P<0.05$).*

Effect on membrane stability: LDH test

The preferential localization of α-tocopherol in the cell membrane enhances its functional role as lipid antioxidant. Therefore, the membrane stabilizer ability of both all-*rac*-*α*-T and RRR-*α*-T was evaluated by LDH release in BME-UV1 and MDCK cells. After 24 h of co-incubation with all-*rac*-*α*-T or RRR-*α*-T and H_2O_2 solutions, in BME-UV1 cell line no differences in LDH release were detected. In MDCK cells, the pre-incubation with all-*rac*-*α*-T determined a significant ($P<0.05$) reduction of the membrane damage, induced by 175 µM of H_2O_2.

In conclusion, RRR-*α*-T and all-*rac*-*α*-T demonstrated to have antioxidant activity *in vitro*. The dose-response curve experiments showed that RRR-*α*-T and all-*rac*-*α*-T tocopherols were able to maintain (MDCK cells) and increase (BME-UV1 cells) the cell viability. In this study the oxidative cellular stress was simulated by H_2O_2 treatment. It has been observed that adequate concentrations of RRR-*α*-T and all-*rac*-*α*-T could reduce the oxidative damages induced by H_2O_2 in both BME-UV1 and MDCK cells as demonstrated by cell viability and membrane stability results. However, further investigations will help in describing their specific mechanism of action *in vitro*.

RRR-α-T form has been demonstrated to have higher biological activity compared with all-rac-α-T form *in vivo* (Weiss et al., 2009) and it could be due to the different tissue uptake of the tocopherol forms and to the different stereoisomers composition. It has been established that after consumption of all-rac-α-T, *2R*-α-tocopherols are well retained in circulation; however, *2S*-α-tocopherols are actively metabolized and eliminated in humans (Traber et al., 1998). Further work is required toward translation of the *in vitro* findings to improve the *in vivo* impact of RRR-α-T and all-rac-α-T intake, and their health-promoting effect in animals and humans.

Study II: ***Cytotoxicity, apoptosis, DNA damage and methylation in mammary and kidney epithelial cell lines exposed to OTA***

Aim of study II

In the present study the cytotoxicity of OTA was first investigated by the evaluation of metabolic activity, membrane stability and apoptotic cell rate in BME-UV1 and MDCK cell lines. Further, the effect of the addition of OTA has been evaluated at DNA level by the determination of the DNA integrity, by the quantification of DNA adduct formation (8-OHdG) and by the assessment of the global DNA methylation status (5-mC) in order to improve the available data regarding OTA mode of action *in vitro*.

Material & methods

Chemicals

OTA and all other chemicals were purchased by Sigma-Aldrich (St. Louis MO, USA), unless otherwise indicated.

Cell culture & treatments

BME-UV1 and MDCK cell were maintained in culture as previously describe in the study I. Cell lines were maintained at 37°C in a humidified 5% CO_2 incubator until sub-confluence. Medium was replenished every second day for both cell lines and cells were passaged for exposure to treatment media while in the logarithmic growth phase. OTA was dissolved in methanol (100%) to obtain a stock solution of 10,000 µg/mL. The treatment concentrations of OTA were composed of serum free basal medium (DMEM for BME-UV1 and EMEM for MDCK cells) contained OTA at several concentrations (from 0.15 µg/mL up to 10 µg/mL). Preliminary experiments showed that the

concentration of methanol employed in the serum free OTA dilutions had no effect on cell metabolic activity. Portion (150 µl) of cell suspension (2.5 x 10^5 cell/mL for BME-UV1 and 1 x 10^5 cell/mL for MDCK) were dispensed into 96 well-plates (NuncClon Surface, Nunc, Denmark). Under these conditions, a sub-confluent monolayer was observed after 24 hours of incubation at 37°C in an atmosphere of 5% CO_2. At sub-confluence, complete medium was removed from each well and BME-UV1 and MDCK cells were exposed to treatment concentrations of OTA (150 µL/well) for an incubation time of 24 hours.

Cell viability: MTT test

Cell viability was determined after incubation with OTA by measuring the production of the chromophore formazan from 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide. Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase.

After 24 hours of OTA treatment, medium was removed and 150 µL MTT stock solution (5 mg/ mL) in Phosphate Buffer Saline (PBS, Euroclone) were added to each well and the plate was incubated for 3 h at 37°C in a humidified chamber. After incubation time MTT solution was removed to stop the reaction and 150 µl of dimethyl sulfoxide was added to dissolve the formazan. The optical density of the dimethyl sulfoxide solution at 570 nm was determined on a Biorad 680 microplate reader (Biorad, Veenendaal, The Netherlands). In particular, the percentage of cells viability after OTA treatments was calculated as follows

% cell viability = (mean optical density of treated cells/ mean optical density of control cells) x 100.

From MTT assay results, the Half Lethal Concentration 50 (LC_{50}) of OTA was calculated for each cell line.

Membrane stability: LDH test

BME-UV1 and MDCK cell lines were treated with selected concentrations of OTA (0.3, 0.6 and 1.25 µg/mL) chosen on the basis of LC_{50}. In order to examine cell membrane damage induced by OTA treatments Lactate dehydrogenase enzyme release (LDH) assay was performed using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) as instructed by the manufacturer. Briefly, after 24 hours of OTA treatment, supernatants were removed and centrifuged for 5 minutes at 1500 × g (4°C). 50 µL of each supernatant was transferred to a new 96 well plate.

Cellular monolayers were lysed by adding 15 µL of 9% Triton X-100 solution in water per 100 µL of basal medium (EMEM), followed by an incubation for 1 h at 37 °C. Cells debris were removed by centrifugation for 5 min at 1500 × g (4 °C) and 50 µL of each cell lysate was transferred to 96 well plate. Then, 50 µL of LDH substrate was added to the supernatants and cell lysates. After the incubation for 30 minutes at room temperature in the dark, the enzymatic assay was stopped by adding 50 µL of 1 M acetic acid and the plate was read at 490 nm using a Biorad 680 microplate reader. The percentage of LDH release was calculated as the amount of LDH in the supernatant over total LDH from both supernatant and cell lysate.

Apoptosis: Tunel (TdT-mediated dUTP nick end labeling) test

BME-UV1 and MDCK cells were seeded at a density of 0.3×10^6 and 0.2×10^6 cells/mL, respectively, in a two-well chamber slides (Nunc Lab-Tek, Nunc, Denmark) and maintained for 24 hours. Afterward, the medium was removed and the cell monolayers washed twice with PBS. Based on results obtained from previous assays, the concentrations of 0.8 µg/mL and 1 µg/mL of OTA were selected and added to BME-UV1 and MDCK cells respectively for the following 24 hours.

At the end of the incubation, the medium was removed and the cells fixed with refrigerated methanol for 10 min at -20°C. The Tunel assay was performed using the DeadEnd Colorimetric Apoptosis Detection kit (Promega, Madison, WI, USA). The monolayers were washed twice with PBS and permeabilized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 min at room temperature. After washing with PBS, cells were incubated with biotinylated nucleotide mixture together with terminal deoxynucleotidyl transferase enzyme. Horseradish peroxidase–labeled streptavidin (streptavidin HRP) was then added to bind to these biotinylated nucleotides, which are detected using the peroxidase substrate hydrogen peroxide and the stable chromogen diaminobenzidine (DAB). Afterward, to distinguish and calculate the apoptotic and non apoptotic cells, haematoxylin staining was performed. Images (20X) were captured under an Olympus BX51 microscope. For each experiment, ~500 cells were counted in randomly selected fields, and the percent of Tunel-positive cells were calculated.

DNA damage: agarose gel electrophoresis and 8OH-dG adduct formation

BME-UV1 and MDCK cells were cultured in 25 cm^2 flasks (Sarstedt, Germany) at the seeding density of $1x10^6$cells/mL and left untouched for 24 hours at 37°C and 5% of CO_2. Cells were treated with the treatment medium containing the selected concentrations of OTA (0.3-1.25µg/mL) and the subsequent day cells were collected for purification and quantification of the DNA.

DNA from BME-UV1 and MDCK cells was extracted using the Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. The concentration and the quality (λ260/280) of the total DNA was determined by UV spectrophotometry at 260nm and by agarose gel electrophoresis followed by Image Lab™ software analysis (BioRad).

Specifically, loading buffer was added to 5µg DNA for each treatment and to 5µg of DNA ladder (Molecular Weight Marker II, Roche) and the samples were analysed by

electrophoresis on a 1% agarose gel (1.30 hour at 65V/200mA) with 1X Tris/Boric acid/EDTA (TBE) buffer (BioRad). DNA was subsequently used for the further analysis investigating DNA damage induced by OTA treatments.

To assess DNA damage induced by OTA treatment, HT 8-oxo-dG ELISA kit II (Trevigen®) was employed. After DNA collection and quantification, a concentration of 500 µg/mL of BME-UV1 and MDCK cell DNA was fragmented in single nucleotides and processed according to the manufacturer's protocol. Results are expressed as [8-OHdG] (nM) / DNA (µg/mL).

Global DNA methylation: 5-methyl-Cytosine (5-mC)

The same DNA samples were employed for the analysis of 5-mC in BME-UV1 and MDCK cell lines.

In particular, in order to investigate if OTA treatment and the DNA damage could be linked to change in the global DNA methylation, MethylFlash Methylated DNA 5-mC quantification (Epigentek) was performed as indicated by the manufacturer. The percentage of 5-mC was calculated in 100ng DNA extracted BME-UV1 and MDCK cells treated with OTA using the second-order regression equation of the standard curve that was constructed with negative control and positive controls in the same experiment. Results are expressed as 5-mC percentage over the total DNA.

Statistical analysis

Results are expressed as mean value ± standard error of three independent experiments in triplicate wells.

Statistical differences between control and treated cells for all experiments were evaluated by one way analysis of variance (ANOVA) using the General Linear Model procedure of SAS 9.4. The level of significance at P <0.05 was considered statistically significant.

Results & Discussion

Graphical representation of results

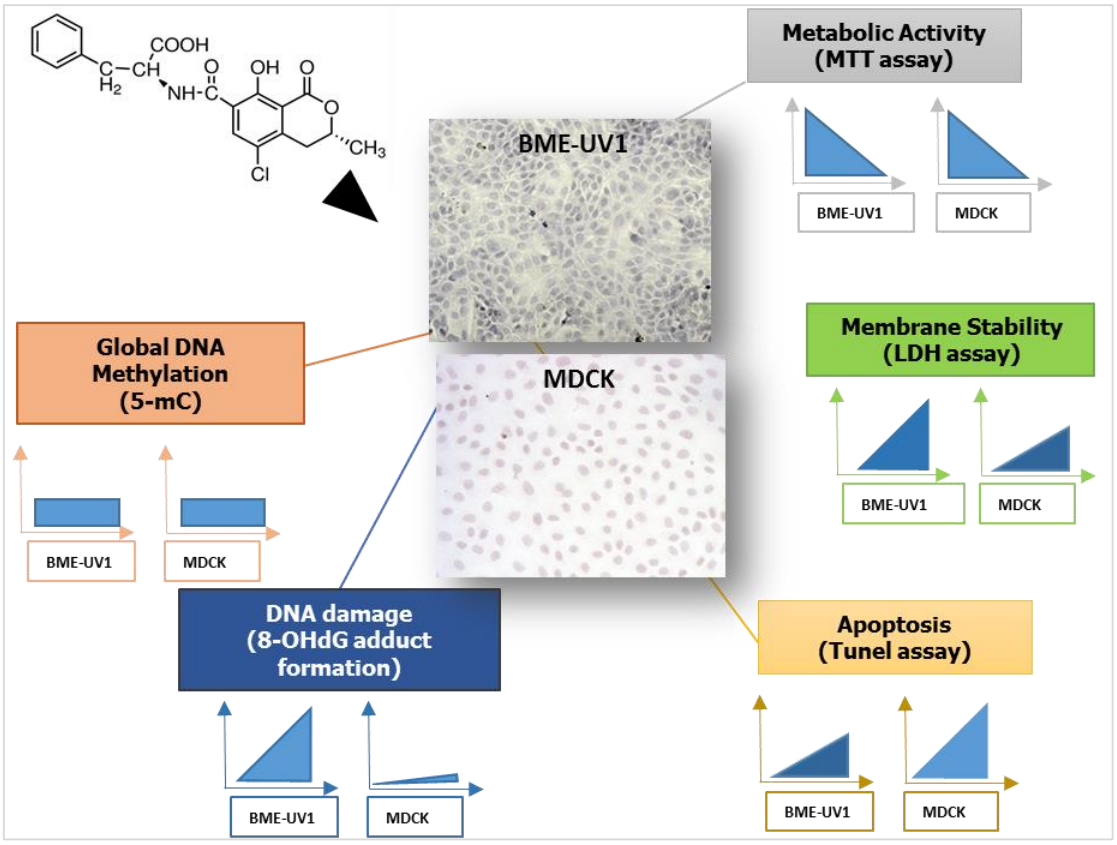


***Figure 1**: Graphical summary of the study II results*

Cell Viability: MTT test

Cytotoxicity of OTA was first assessed by the determination of cell viability in BME-UV1 and MDCK cells.

In particular, the incubation with increasing concentrations (ranging from 0 up to 10 µg/mL) of OTA for 24 hours determined a dose dependent inhibition of cell viability1 in both cell lines (**Figure 2A** and **B**). LC_{50} was calculated in both BME-UV1 and MDCK cells after 24 hours of OTA incubation and reported in Table 1.

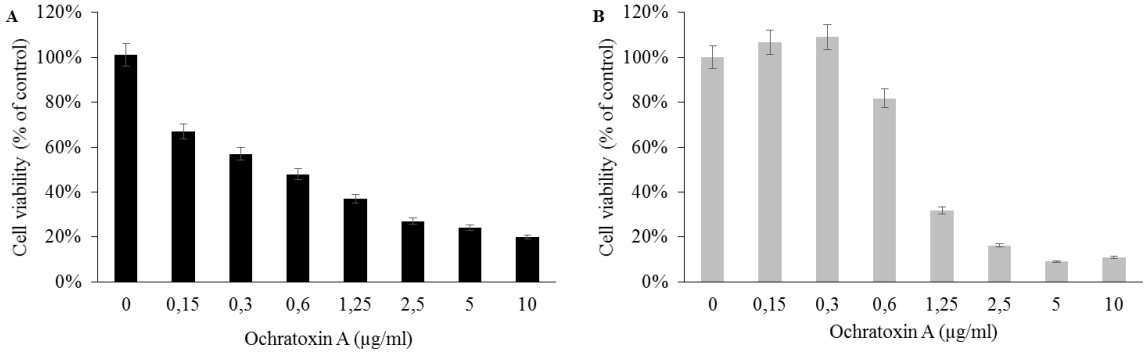


Figure 2*: Effect of increasing concentrations of OTA added to the culture medium for 24 hours on cell viability (MTT test) in BME-UV1 cells (A) and MDCK cells (B). Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to cell viability value obtained in basal medium (0 µg/mL OTA).*

Table 1*. LC_{50} values of OTA in BME-UV1 and MDCK cell lines as determined by MTT test.*

	LC_{50} (µg/mL)
BME-UV1	0.8
MDCK	1

Considering the LC_{50} values, the range of OTA concentrations for the further experiments was determined in 0.3 -1.25µg/mL for both BME-UV1 and MDCK cells.

Membrane stability: LDH release assay

The cell membrane damage induced by OTA on BME-UV1 and MDCK cells was investigated by the release of LDH, a biomarker of membrane integrity. As showed in **Figure 3A** and **3B**, 24 hours incubation with OTA induced a dose dependent LDH release in both cell lines, indicating that OTA caused cell membrane damage. OTA significantly (P<0.05) increased LDH release in BME-UV1 cells at all the concentrations tested, compared with control cells (0 µg/mL OTA). In particular, a percentage of 46% of LDH release has been observed in BME-UV1 cells treated with 1.25 µg/mL of OTA. In MDCK cells, 1.25 µg/mL of OTA induced 35% (P<0.05) LDH release, compared with control cells (0 µg/mL of OTA).

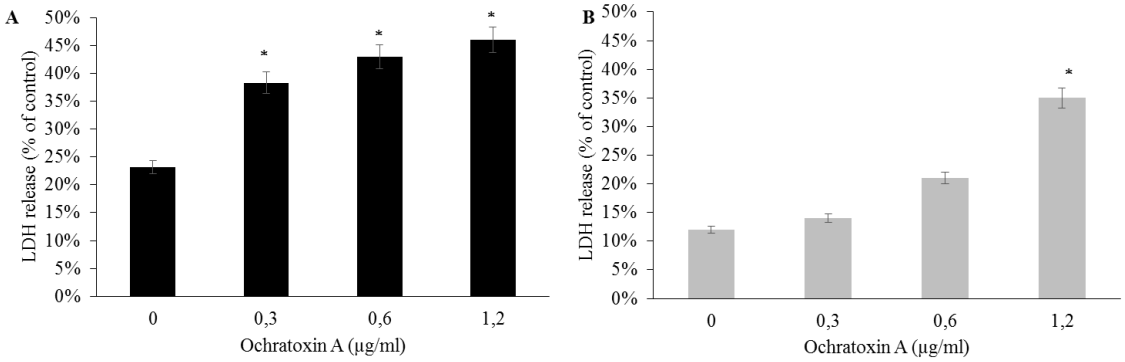


Figure 3: *Effect of increasing concentrations of OTA added to the culture medium for 24 hours on membrane stability (LDH release) in BME-UV1 cells (A) and MDCK cells (B). Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to membrane stability in basal medium (0 µg/mL OTA). Values significantly different from membrane stability obtained in basal medium are indicated by * (P<0.05).*

The MTT assay was performed to measure the cell viability after OTA treatment, the quantification of the LDH release was used as a biomarker of the integrity of the cell membrane. The above-mentioned assays together may provide a picture of the cytotoxicity effect of OTA in BME-UV1 and MDCK cells.

The first finding of this study confirmed the cytotoxicity of OTA in BME-UV1 and MDCK cells, as previously observed (Baldi et al., 2004). According to LC_{50}, both cell lines cells showed a higher sensitivity to OTA compared with other cell models. Hepatocellular (Hep3B) cells exhibited a LC_{50} of 104µM after 24 hours of OTA treatment (Anninou et al., 2014). In OTA treated-PK15 cells the LC_{50} obtained by MTT and Trypan Blue assays were 14 And 20 µM, respectively (Klarić et al., 2012). The sensitivity of BME-UV1 cells is significant considering that OTA and its metabolites were detected in milk of several species (Sorrenti et al., 2013), even if their carry over in ruminant milk is limited, due to the rumen microflora hydrolysis. However, rumen detoxification capacity is saturable and can vary with changes in the diet, the duration of OTA exposure, and the health and production status of the animal (Cheli et al., 2015; Fink-Gremmels, 2008), leading to a more consistent OTA transfer to milk in ruminants.

Apoptotic cell rate: Tunel (TdT-mediated dUTP nick end labeling) assay

The number of apoptotic cell nuclei induced by OTA treatment on BME-UV1 and MDCK cells was investigated by Tunel assay. Representative photos of BME-UV1 and MDCK cells morphology incubated with complete medium alone (control cells, 0 µg/mL of OTA) for 24 hours are showed in **Figure 4A** and **C**, respectively.

In the absence of OTA, the uniformity of monolayers, the typical cell-cell interactions in cultures were evident in both cell lines tested, with only a small amount of apoptotic nuclei detected. The average percentage of apoptotic nuclei calculated in the selected fields for BME-UV1 control cells was of 1.37± 0.88 (**Figure 4A**), while for MDCK control cells was 2.2±0.88 (**Figure 4C**).

In BME-UV1 cells co-incubated with 0.8 µg/mL of OTA for 24 hours, the average percentage of apoptotic nuclei was 10± 0.86 (P<0.05). In addition the uniformity of the monolayers was greatly compromised and floated cellular debris have been detected in all the observed fields (**Figure 4B**).

In MDCK cells co-incubated with 1 µg/mL of OTA for 24 hours, the average percentage of apoptotic nuclei was 24.86 ± 0.88 (P<0.05), as demonstrated by the high presence of dark brown nuclei in the **Figure 4D**.

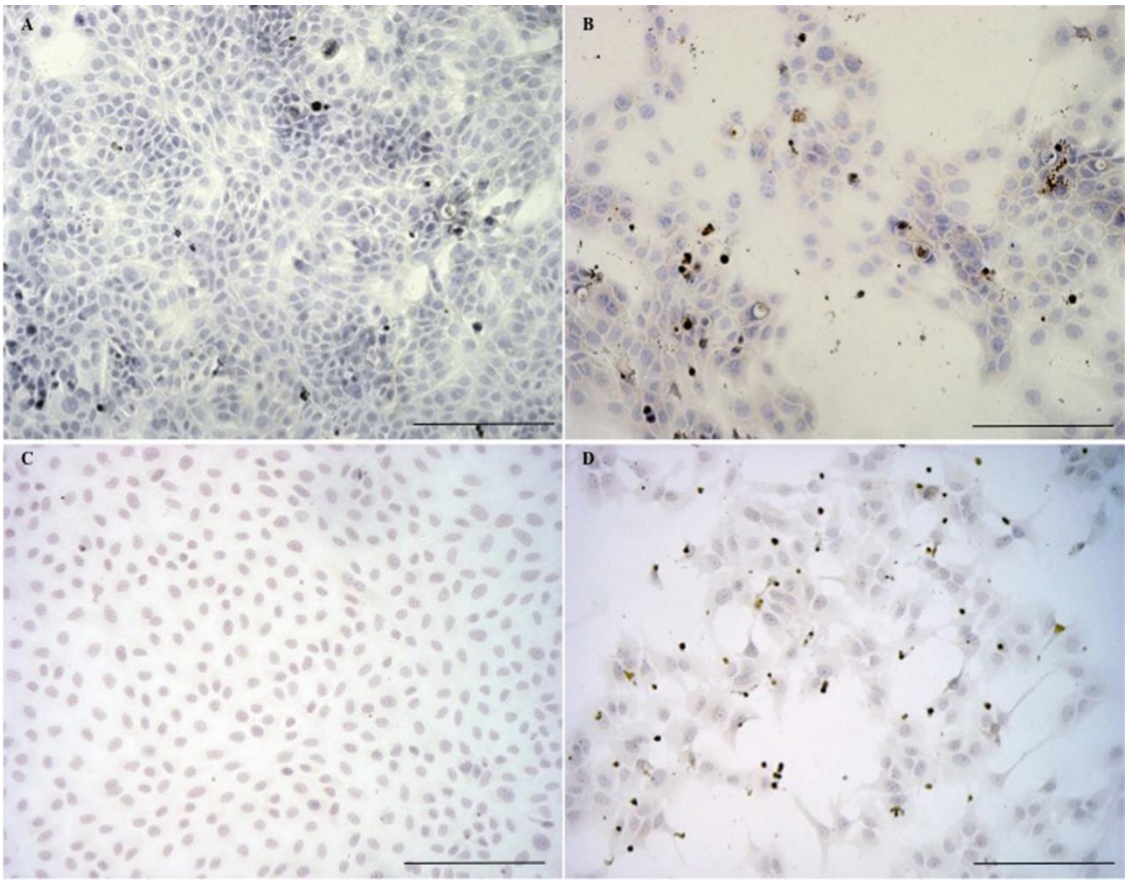


Figure 4*: Representative photos of TUNEL-stained BME-UV1 and MDCK cells. The TUNEL-positive nuclei, indicating apoptotic cells, are stained in dark brown, while the vital nuclei are stained violet (haematoxylin). (A) BME-UV1 cells in culture for 24 h (0 µg/mL OTA); (B) BME-UV1 cells incubated with 0.8 µg/mL OTA for 24 h; (C) MDCK*

cells in culture for 24 h (0 µg/mL OTA); (D) MDCK cells incubated with 1 µg/mL OTA for 24 h. (Bars 100 µm)

In the present study, the Tunel assay, widely used for detecting apoptotic cells at earlier stages, demonstrated that the cellular monolayers observed were compromised and the cellular debris were present, underlining the OTA-induced cytotoxicity in both BME-UV1 and MDCK cells. Tunel positive apoptotic nuclei were observed in MDCK cells and this finding is consistent with the kidney as the main target of OTA and in line with previous findings (Gekle et al., 2000). After OTA incubation, an increased number of apoptotic cells has been described in different kidney *in vitro* models (Schwerdt et al., 1999; Kamp et al., 2005), and in cells from different sources (Cui et al., 2010). In this study, a lower but consistent number of apoptotic cells was also detected in BME-UV1 cells incubated with 0.8µg/mL OTA. However, given the amount of detached cells and cellular debris floated in the observed fields we can speculate that a different mechanism of death intervened in OTA-treated BME-UV1 cells, also considering the high level of membrane damage previously described by LDH release assay in this cell line.

Moreover, since the cell death is a multi-stage procedure, quantifying and characterize the apoptotic process need several techniques and remains to be elucidated in BME-UV1 cell lines.

DNA damage: agarose gel electrophoresis and 8OH-dG adduct formation

The DNA was extracted from BME-UV1 and MDCK cells incubated with selected concentrations of OTA and a qualitative analysis was performed by agarose gel electrophoresis. OTA incubation caused a dose-dependent DNA degradation in BME-UV1 cells, as illustrated by the appearance of DNA ladders in **Figure 5A**. No specific DNA degradation was detected in MDCK cells treated with OTA.

Further, the effect of OTA on the formation of DNA adduct was investigated in the DNA of BME-UV1 and MDCK cells. The formation of 8-OHdG was significantly increased (P<0.05) in BME-UV1 cells treated with specific OTA concentrations, compared with control cells (**Figure 5B**). In particular, a 24 hours exposition of 1.25µg/mL OTA led to a concentration of 9.03nM of 8-OHdG adduct formation in BME-UV1, whereas in the BME-UV1 control cells (0 µg/mL OTA) the concentration of 8-OHdG was 2.23 nM. No difference was detected in MDCK cells treated with OTA compare with control, suggesting a different pathway of OTA toxicity in this cell line.

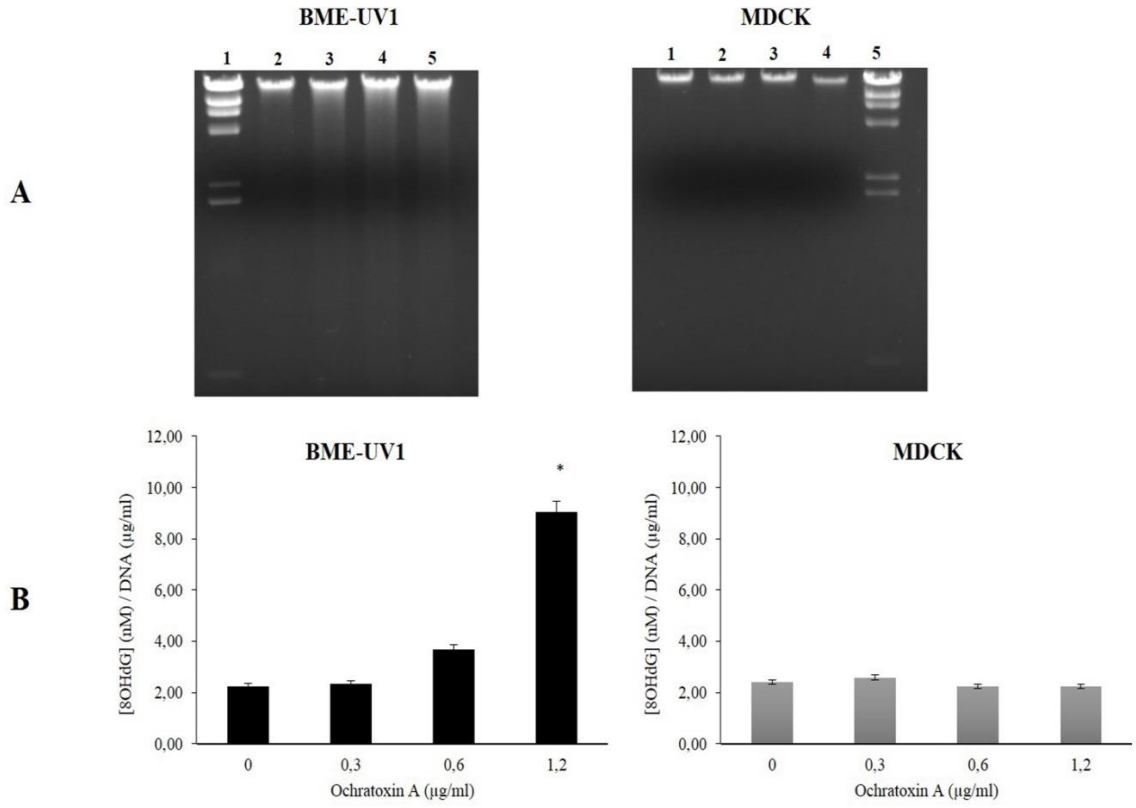


***Figure 5**: (A) Effect of increasing concentrations of OTA evaluated on the integrity of DNA extracted from BME-UV1 and MDCK cells and loaded on agarose gel electrophoresis. Line 1A:DNA Ladder; Line 2A DMEM (0 µg/mL OTA); Line 3: 0.3 µg/mL OTA; Line 4: 0.6 µg/mL OTA; Line 5: 1.2 µg/mL OTA; Line 1B: MEM (0 µg/mL OTA); Line 2B: 0.3 µg/mL OTA; Line 3B: 0.6 µg/mL OTA; Line 4B 1.25 µg/mL OTA; Line*

*5B:DNA Ladder. (B)Effect of increasing concentrations of OTA on 8-OHdG adduct formation in BME-UV1 cells and MDCK cells. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to 8-OHdG adduct formation in basal medium (0 µg/mL OTA). Values significantly different from 8-OHdG adduct formation obtained in basal medium are indicated by * (P<0.05).*

The capability of OTA and its metabolites to bind DNA is still conflicting and OTA-DNA damaging potential was mainly related to free radical overproduction and oxidative stress. Previous studies demonstrated the ability of OTA to induce DNA damage *in vitro*, such as by the chromosomal aberration and the formation of DNA strand breaks, DNA adducts and DNA-DNA cross links (Mally & Dekant, 2005; Yang et al., 2015; Rutigliano et al., 2015). OTA-induced adduct formation has been investigated in different cell lines. Zheng et al. demonstrated the toxic effect of 25µM of OTA in HepG2 cells with a significant increase in the formation of 8-OHdG and an induction of global DNA hypo-methylation. Further, DNA degradation and 8.8ng/mL 8-OHdG adduct formation was found in kidney LLC-PK1 cells after 60µM of OTA treatment (Costa et al., 2008). OTA caused the formation of 8-OHdG also in HPBMCs cells, as demonstrated by Liu et al. (2012).

However, biological data associated to the activity of OTA on the mammary gland tissue are rather limited, especially those regarding the induction of DNA damage. The results here obtained demonstrated the genotoxic effect induced by OTA *in vitro* with a significant DNA degradation and 8-OHdG adduct formation in the DNA of BME-UV1 cells after 24 hours of treatment. Considering the *in vitro* condition, we can speculate that the 8-OHdG formation occurred independently of metabolic activation. The investigation of the mechanism of adduct formation, by the covalent binding of OTA metabolites to DNA or by an indirect mechanism (ROS-mediated DNA damage) in BME-UV1 cells is complex and requires further qualifiers and different endpoint to measure as HPLC techniques and ^{32}P-postlabelling assays (Obrecht-Pflumio & Dirheimer, 2000).

However, a different scenario turned up when MDCK cells were treated with OTA, with no DNA degradation detected by the agarose gel-electrophoresis and no significant 8-OHdG adduct formation occurred. This may be owed to a different cell response to the mycotoxin activity, as also suggested by the different LDH release results and number of apoptotic cell detected with Tunel assay. In addition, although 8-OHdG is one of the most studied oxidative biomarker and widely considered a simple and a valid method to estimate the cellular oxidative damage related to the genotoxic potential of OTA (Zheng et al., 2013; Kasai et al., 1997; Faucet et al., 2004), different forms of DNA damage are known to be produced *in vitro*. Therefore, it becomes important to make effort in the investigation of other cell biomarkers in BME-UV1 and MDCK cells to describe and characterize OTA-induced damages at DNA level.

Global DNA methylation: 5-methyl-Cytosine (5-mC)

Methylation of cytosine in the DNA of BME-UV1 and MDCK cells was investigated in order to determine whether the cytotoxic action of OTA and the subsequent DNA damage induction could also affect the pattern of global DNA methylation in these cell lines. In both BME-UV1 and MDCK control cells (0 μg/ml OTA) the average percentage of 5-mC was below 6%. No detectable changes in the global DNA methylation pattern have been observed after OTA treatment in BME-UV1 and MDCK cells (**Figure 6**).

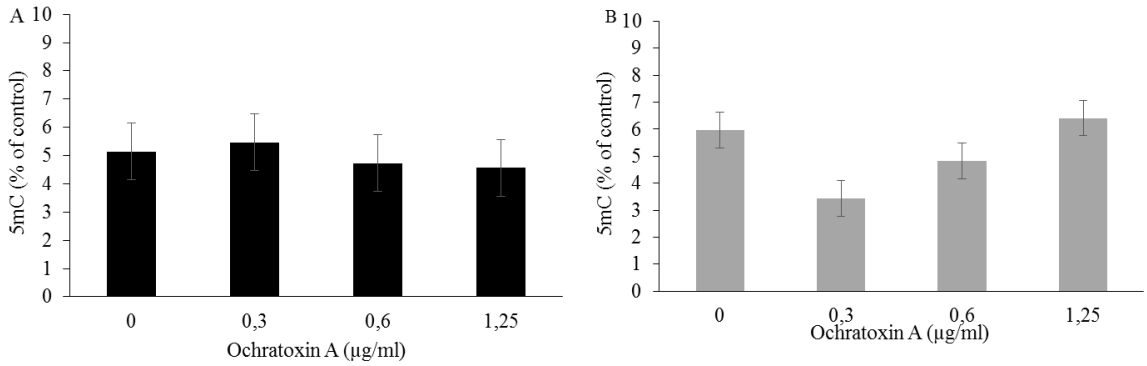


Figure 6*: Effect of increasing concentrations of OTA on DNA global methylation in BME-UV1 cells (A) and MDCK cells (B). The level of global DNA methylation is indicated by the percentage of 5-mC in the total cytosine. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to membrane stability in basal medium (0).*

The methylation of the 5th position of cytosine to form 5-mehtyl Cytosine is one of the most important epigenetic biomarker in mammalian cell genome, and it is commonly associated with DNA integrity. Specifically, if the DNA adduct occurs in a methylated portion of the DNA it could alter the cytosine methylation profile at this site. Adduct formation by OTA may impair the global methylation of the DNA as suggested for other mycotoxins (Kouadio et al., 2007). Zheng *et al*. (2013) demonstrated a link between OTA treatment and the decrease of 5-mC percentage, leading to a global DNA hypo-methylation in hepatic cells.

However, in the present study the global DNA methylation appears not to be affected in BME-UV1 and MDCK cells treated with OTA. The 8-OHdG adduct formation observed in BME-UV1 incubated with OTA was not associated with global DNA methylation alteration. This finding suggests that the *in vitro* relationship between OTA incubation, adduct formation and DNA methylation is not resolved yet. Other epigenetic markers,

higher OTA concentrations and different *in vitro* models need to be used in order to have a complete understand of OTA mode of action at the DNA level.

Overall, the cytotoxicity potential of OTA impaired BME-UV1 and MDCK cell viability, cell membrane integrity, DNA integrity and increased the number of apoptotic cells. The major difference in the OTA-induced toxicity was a more consistent formation of DNA adduct in BME-UV1 cells, compared with MDCK cell line. However, the high apoptotic cell number detected in MDCK cells is consistent with the kidney as the primary target of OTA *in vivo*. These findings could help in ameliorate the characterization of OTA mode of action *in vitro*, suggesting that a different mechanism of toxicity may occur in these cell lines and merits a deeper characterization. Given the high OTA cytotoxicity in MDCK cells together with the high number of apoptotic cells detected, we can speculate that a predominant cytotoxic mechanism of OTA rather than a genotoxic mechanism of action occurs, with marked effects on cell metabolic activity, membrane and tight junction's integrity.

Study III: ***α-tocopherol role in counteracting OTA toxic activity in MDCK cells: effects on cell viability and tight junctions***

Aim of study III

Kidney represents one of the main target organs of OTA toxicity. Increasing evidence suggested that OTA is responsible of the perturbation of cell-cell interactions as well as cell-cell signaling. At kidney level, tight junctions varies along the nephron. The level of complexity of the tight junction increases moving forward the distal tubule. MDCK cells represent an estimable model of the distal tubule/collecting duct (Feldman et al., 2005) and for the OTA toxicity studies. Therefore, the aim of this study was to evaluate the OTA effects on cell viability, apoptotic rate and occludin and Zo1 localization and the role of α-tocopherol in counteracting its effects in MDCK cells.

Material & methods

Chemicals

OTA, all-racemic α-tocopherol and other chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA), unless otherwise specified. OTA was dissolved in methanol (100%) to obtain a stock solution of 10,000 μg/mL. The treatment concentrations of OTA were composed of basal medium (EMEM, serum free) contained OTA at several concentrations (from 0.15 μg/mL up to 5 μg/mL). Preliminary experiments showed that the concentration of methanol employed in the serum free OTA dilutions had no effect on cell metabolic activity

In subdued lighting, α-tocopherol was dissolved in ethanol to give 10 mM solutions. Further dilutions with basal EMEM medium produced micromolar (10 μM) and nanomolar (1 nM) concentrations. These diluted stock solutions were stored in the dark before addition to cultures accommodated in a cabinet without lighting.

Cell culture

Madin Darby canine kidney (MDCK) cell line was purchased and maintained as previously indicated in the study II and III. At confluence, cells were detached from the flasks by trypsinisation and re-suspended in culture medium to a concentration of 1 x 10^5 cell/mL. Portions of cell suspension were dispensed in the most suitable supports for the following experiments. Cells used in this study were between passage number 22 and 25.

Determination of cell viability and LDH release in the presence of OTA and α-tocopherol

In dose response experiments, MDCK cells were exposed to increasing concentrations of OTA (0-5 µg/mL). Cell culture medium, in fact, was replaced with red phenol and serum free media in which serial concentration of OTA was diluted. After 24 h of incubation, cell viability was detected by measuring the production of the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) (Promega, Madison, WI, USA). CellTiter 96® AQ_{ueous} solution were added to each well and the plates were incubated for 3 h at 37°C in a humidified chamber. To measure the amount of soluble formazan produced by viable cells the absorbance at 490 nm on a Biorad 680 microplate reader (Biorad, Veenendaal, The Netherlands) was recorded. Cells incubated with EMEM alone, representing 100% viability, were included as negative controls in all experiments.

The half Lethal Concentration (LC_{50}) was calculated considering the percentage of cytotoxicity (1- mean optical density in the presence of OTA/mean optical density of EMEM alone).

In another set of experiments, MDCK were cultured in the presence of OTA doses around LC_{50} determined as previously described, in the presence or absence of α-tocopherol (1 nM or 10 µM). Cells were first pre-incubated for 3 h with α-tocopherol and then exposed

to increasing concentrations of OTA (0-1.2 µg/mL) for the following 24 h. Cells were also exposed to antioxidant alone or ethanol (the α-tocopherol solvent) alone to evaluate any non-specific effects. Cell viability was evaluated by the CellTiter 96® AQ_{ueous} assay, as previously described.

To determine the LDH release, the MDCK cells were exposed to OTA solutions (concentration range: 0-1.2 µg/mL) in the presence or absence of α-tocopherol (1 nM or 10 µM). Cells were treated as mentioned above. At the end of the incubation period the media were removed and cells washed with PBS twice. LDH release was detected by CytoTox 96® Non Radio-active Cytotoxicity Assay (Promega, Madison, WI, USA), as described by manufacturer. Briefly, after treatments supernatants were removed and centrifuged for 5 min at 1500 × g at 4 °C. 50 µL of each supernatant was transferred to a 96 well plate. Cells were lysed by adding 15 µL of 9% Triton X-100 solution in water per 100 µL of culture medium, followed by incubation for 1 h at 37 °C. Cells debris were removed by centrifugation for 5 min at 1500 × g at 4 °C and 50 µL of each sample was transferred to 96 well plate. Then, 50 µL of LDH substrate was added to the supernatants and cell lysates. After incubation for 30 min at room temperature in the dark, the enzymatic assay was stopped by adding 50 µL of 1 M acetic acid. The optical density at 490 nm was determined on a microplate reader. The percentage of LDH release was calculated as the amount of LDH in the supernatant over total LDH from both supernatant and cell lysate.

Detection of apoptotic cells induced by OTA alone and after the treatment with α-tocopherol by TUNEL (TdT-mediated dUTP nick end labeling) assay

MDCK cells were seeded at a density of 2×10^5 cells/mL in chamber slides (Nunc Lab-Tek, Nunc, Denmark). Cells were cultured in complete medium for 24 h. Afterward, the media were removed and the MDCK monolayers washed twice with PBS. Based on results obtained from previous assays, the cells were exposed to OTA solutions (0.6 µg/mL) in the presence or absence of α-tocopherol (1 nM or 10 µM, 3 h pre-treatment)

for the following 24 h. At the end of the incubation, the media were removed and the cells fixed with ice cold methanol for 10 min at -20°C. The monolayers were washed twice with PBS and permeabilized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 min at room temperature. The TUNEL assay was performed using the DeadEnd Colorimetric Apoptosis Detection kit (Promega, Madison, WI, USA). After washing with PBS, cells were incubated with biotinylated nucleotide mixture together with terminal deoxynucleotidyl transferase enzyme. Horseradish peroxidase–labeled streptavidin (streptavidin HRP) was then added to bind to these biotinylated nucleotides, which are detected using the peroxidase substrate hydrogen peroxide and the stable chromogen diaminobenzidine (DAB). Afterward, to visualize and estimate the apoptotic and normal cells, haematoxylin staining was performed. Images (20X) were captured under an Olympus BX51 microscope. For each experiment, ~500 cells were counted in randomly selected fields, and the percent of TUNEL-positive cells was calculated.

Evaluation of the effect of OTA and α-tocopherol pretreatment on occludin and Zo1 localization

To determine localization of occludin and Zo1, MDCK cells were seeded, cultured and treated as described above. At the end of the treatment with α-tocopherol solutions (1 nM and 10 µM) combined with OTA (0.6 µg/mL) or OTA solution alone, cells were washed in PBS, fixed and permeabilised with Triton. Cells were subsequently, incubated with polyclonal rabbit anti occludin (1: 400 dilution in PBS, Invitrogen, US) or anti Zo1 (1: 400 dilution in PBS, Invitrogen, US) antibodies overnight at 4°C. The cellular monolayers were washed in PBS and then secondary antibody DyLight®594 Anti-rabbit IgG (9 µg/ml of 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.08% sodium azide, Vector Laboratories Inc., UK) and DyLight®488 Anti-rabbit IgG (9 µg/ml of 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.08% sodium azide, Vector Laboratories Inc., UK) was applied for 90 minutes at room temperature, respectively. Finally, the monolayers were embedded in Vecta- shield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser

Scanning Microscope (FluoView FV300, Olympus, Italy). The immune-reactive structures were excited using Argon/Helio-Neon-Green lasers with the excitation and barrier filters set for the used fluorochromes. Images showing the superimposition of the fluorescence were obtained by sequentially acquiring an image slice from each laser excitation or channel. To quantify occludin and Zo1 - immunoreactivities in cell monolayers, pixel intensities were determined using the histogram/area functions of the FluoView software, which assigned gray levels (GL) within a 0–256 gray scale. Data were presented as mean fluorescence intensities.

Double immunofluorescence tests were conducted to evidence possible co-localizations. Briefly, after permeabilitation, the cellular monolayers were incubated with polyclonal rabbit anti-occludin (1: 400 dilution in PBS, Invitrogen, US) antibodies overnight at 4°C. The cells were washed in PBS and then incubated for 90 minutes at room temperature with secondary antibody DyLight®594 Anti-rabbit IgG (Vector Laboratories Inc., UK). The cells were then washed in TBS and incubated with rabbit IgG for 2 h (Vector Laboratories Inc.) to inhibit the binding of the anti-Zo1 antibody to the goat anti-rabbit IgG used in the first sequence. For the second step, the cells were treated with anti-Zo1 antibody, washed in PBS and incubated for 90 minutes at room temperature with DyLight®488 Anti-rabbit IgG (Vector Laboratories Inc., UK). Finally, the monolayers were embedded in Vecta - shield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300, Olympus, Italy). The specificity of the immunofluorence reactions has been verified by incubating cellular monolayers with PBS instead of the second step antibody procedure. The results of these controls were negative (i.e. staining was abolished).

For quantification of occludin and Zo1 peaks of immunofluorescence, the laser power and photomultiplier tube voltage were constant so that fluorescence intensities of various cellalar monolayers could be compared. Images were digitized under constant gain and laser offset, with no postcapture modifications. Before quantification, the images were digitally zoomed three times. Pixel intensities were determined using the histogram/area

function of the FLUOVIEW software, which assigned the gray levels (GL) within a 0–256 Gy scale. Data were presented as mean fluorescence intensity.

Statistical analysis

The data are expressed as means and standard errors (SE). At least three replicates at each incubation time were performed, and all the experiments were performed twice. The data obtained were analyzed by one way ANOVA (GLM) (SAS); Duncan's post hoc multiple range test was used with $P \leq 0.05$ considered statistically significant.

Results & discussion

Determination of cell viability and LDH release in the presence of OTA and α-tocopherol

After 24 h of incubation in the presence of OTA, MDCK showed a LC_{50} of 0.94 µg/ml. The effect of 3 h pre-incubation with µ-tocopherol on OTA cytotoxicity is shown in **Figure 1**. α-tocopherol at 1 nM significantly ($P < 0.05$) reduced OTA cytotoxicity by 20%, only when the mycotoxin was present at 0.3 µg/mL. In presence of higher OTA concentrations used, no reduction of OTA cytotoxicity was detected.

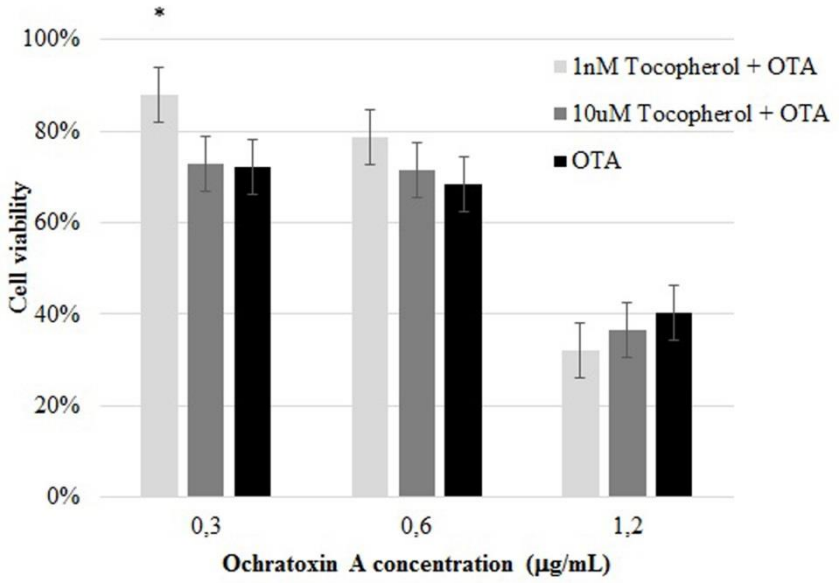


Figure 1*: Effect of pre-treatment with α-tocopherol 3 h against OTA induced cytotoxicity in MDCK cells at 24 h. Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to cell viability obtained in basal medium (DMEM). Values significantly different from cell viability obtained in OTA alone are indicated by * (P<0.05).*

Moreover, these data have been confirmed by the results obtained in the LDH assay. In fact, the presence of 1 nM α-tocopherol was able to significantly ($P < 0.05$) reduce the LDH release in MDCK cells, when OTA was present at 0.3 and 0.6 µg/mL (**Figure 2**).

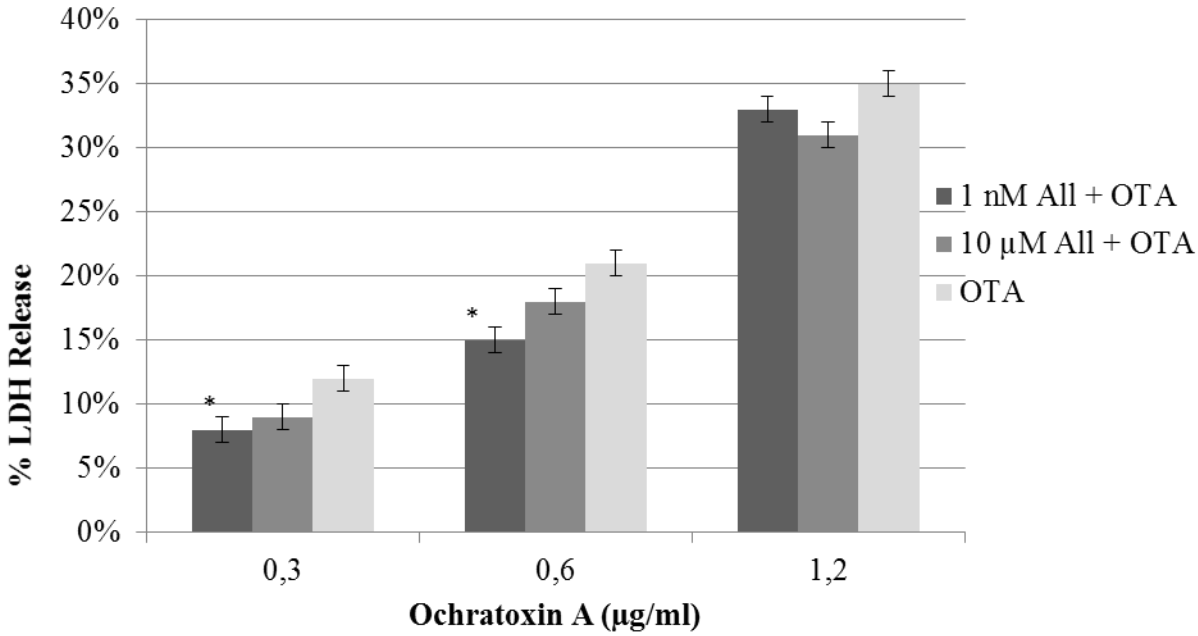


Figure 2: *Effect of pre-treatment with α-tocopherol 3 h against OTA induced* LDH release in MDCK cells at 24 h. *Results are from three independent experiments with triplicate wells and presented as least square means ± SEM relative to LDH release obtained in basal medium (MEM). Values significantly different from LDH release obtained in OTA alone are indicated by * (P<0.05).*

Detection of DNA damage induced by OTA alone and after the treatment with α-tocopherol by TUNEL (TdT-mediated dUTP nick end labeling) assay

Figure 3 shows representative photos of the morphology and the nuclear stain of MDCK cells maintained in culture media and incubated with OTA with and without a 3 h pre-incubation with α-tocopherol (1 nM and 10 µM) for 24 h, respectively. In particular, MDCK cells maintained in culture media for 24 h form a uniform cellular monolayer, characterized by the typical cell-cell interaction, in which the percentage of apoptotic cells was 1.37 ± 0.89 (**Figure 3a**). In the presence of OTA (0.6 µg/mL), the monolayers were disrupted and the percentage of TUNEL-positive cells was 11.01 ± 0.89, as demonstrated

by the presence of dark brown nuclei in the **Figure 3b**. The pre-incubation with α-tocopherol significantly ($P < 0.05$) reduced the percentage of apoptotic nuclei in MDCK cells induced by the presence of OTA. In fact, the 1 nM α-tocopherol pre-incubation was able to decrease the number of apoptotic cells, as showed by the reduction of the percentage of TUNEL-positive cells to 2.67 ± 0.89 (**Figure 3c**). MDCK cell pre-incubation with 10 μM α-tocopherol induced a slight decrease of the percentage of apoptotic nuclei to 7.43 ± 0.89 (**Figure 3d**).

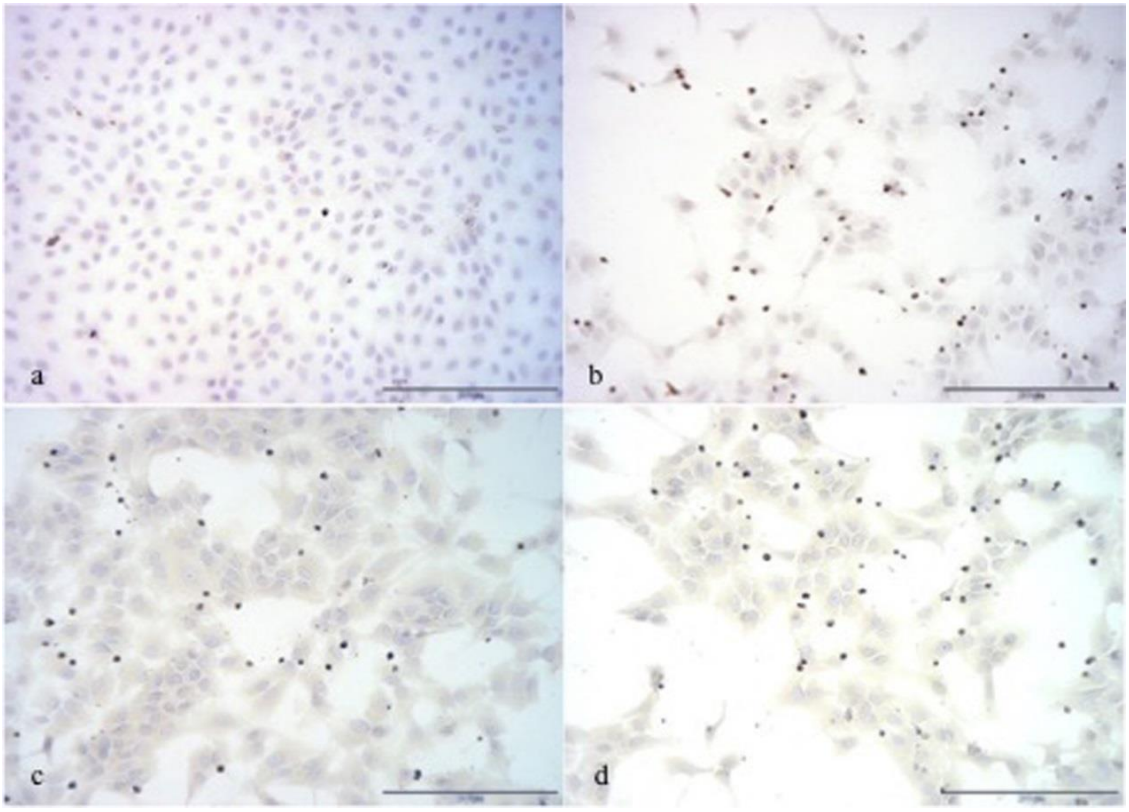


Figure 3*: Apoptosis induced by OTA in MDCK cells. TUNEL-positive cells showed dark brown nuclei staining, while the vital cell nuclei presented violet (haematoxylin) staining. Representative photographs of selected fields are shown. (a) MDCK cells maintained in culture medium for 24 h. (b) MDCK cells incubated for 24h with 0.6 μg/mL OTA. (c) MDCK cells pre-incubated with 1 nM α-tocopherol and treated with 0.6 μg/mL OTA for the following 24 h. (d) MDCK cells pre-incubated with 10 μM α-tocopherol and treated with 0.6 μg/mL OTA for the following 24 h. Bars = 100 μm.*

Evaluation of the effect of OTA and α-tocopherol pretreatment on occludin and Zo1

In the observed cellular monolayers, immunofluorescence staining revealed that both occludin and Zo1 were present in the thin peripheral cytoplasmic rim of the MDCK cells and partially co-localised (**Figure 4a**). When OTA treated cells have been observed at confocal microscopy, reduction in the intensity of occludin and Zo1 have been detected (**Figure 4b**). Immunofluorescence peaks confirmed that occludin and Zo1 presence in MDCK monolayers maintained with culture media (control) were significantly ($P < 0.05$) higher than those detected in the monolayer treated with OTA. The 3 h pre-incubated cells with 1 nM α-tocopherol showed peaks of occludin and Zo1 significantly ($P < 0.05$) higher than those revealed in OTA (0.6 µg/mL) treated cells. The pre-incubation with 10 µM α-tocopherol determined only a numerical increase of occludin and Zo1 peaks respect to those present in OTA treated cells (Table 1).

Table 1. *Comparison between mean fluorescence intensity peaks of the cellar monolayers exposed to control medium, OTA (0.6 µg/mL), α-tocopherol (1 nM and 10 µM) and OTA (0.6 µg/mL) for 24 h.*

	Control	OTA (0.6 µg/mL)	10 µM α-tocopherol and OTA	1 nM α-tocopherol and OTA
Occludin	151.42 ± 12.72^{a}	76.94 ± 12.72^{b}	$104.18 \pm 12.72^{b, c}$	$139.19 \pm 12.72^{a, c}$
Zo1	218.22 ± 15.34^{a}	117.94 ± 15.34^{b}	120.85 ± 15.34^{b}	171.54 ± 15.34^{c}

^{a,b,c} Means within a row with different superscripts differ ($P < 0.05$)

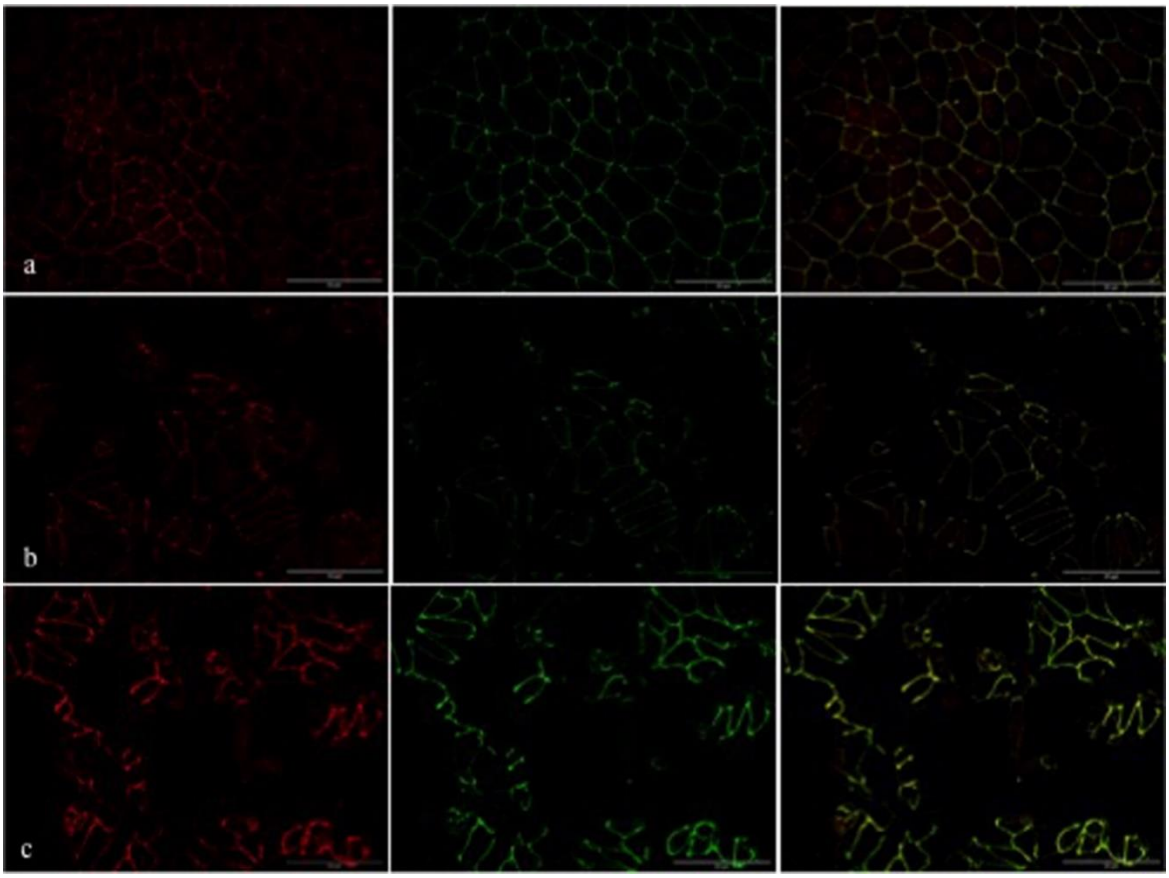


Figure 4. *Immunofluorescence analysis of occludin and Zo1 in MDCK cells. In cells maintained in culture medium (a), the presence of occluding (red dots) and Zo1 (green dots) and their co-localition (yellow dots) are evident. In the cells incubated with OTA (0.6 µg/mL) for 24 h (b), the signals for occludin, Zo1 and their co-localization are reduced compared with the cell pre-incubated with 1 nM α-tocopherol (c). Bars = 20 µm.*

At kidney level, it is well know that OTA at µM concentrations could affect several cellular processes, such as direct and unspecific inhibition of macromolecules (DNA, protein) synthesis, reduced cell growth and decreased cell viability (Gekle et al., 2005). Oxidative stress represents one of the mechanism by which this compound is able to exert its toxicity. As described in the review of Sorrenti *et al.*2013, growing evidence of the role of several antioxidants in the modulation of OTA toxicity support their protective function *in vitro* models. In particular, the α-tocopherol at nM concentrations has been able to

counteract the OTA toxicity in primary cells (Fusi et al.,2010) and established cell lines (Baldi et al.2004).

The release of cytosolic LDH represents a valuable marker of cellular membrane integrity and could be used to evaluate the mechanism of action by which OTA could exert its action and the role of α-tocopherol in counteracting its toxicity. The amount of LDH release increased in association with increasing concentration of OTA (**Figure 2**), in accordance with previous studies conducted using MDCK cells (Gekle et al., 2000) and other cell models (Petrik et al., 2005).

Overall, our results indicate that the pre-incubation with α-tocopherol could preserve the cell viability, due to its position and movements in the cellular membrane, and its activity in cytosolic reduction reactions that recycles tocopheroxyl radicals, thus preventing lipid peroxidation (Traber & Atkinson, 2007).

As demonstrated by several studies (Gekle et al., 2005; Petrik et al., 2005; Gekle et al., 2000), OTA was able to induce apoptosis in MDCK cells, confirming that nM concentration led to renal damage, but the mechanisms by which it has been manifested are multiple and complex.

In this framework, α-tocopherol could find a key role. In fact, it could suppress lipid peroxidation and cell death induced by OTA and other oxidative stressors in various cell types, stabilizing the cellular membrane and breaking the chain propagation, taking place at this level. One possible explanation of the more protective action exerted by the lower concentration of α-tocopherol (1 nM) could be due to its intracellular concentration and not to its concentration added to medium, as reported by Niki (2014). Moreover, the function of α-tocopherol could be not explained by only its chain breaking activity (Zingg, 2007; Azzi, 2007), but it could monitor the oxidative environment and could act as sensor, transferring the information to the cell, as affirmed by Azzi (2007).

As reported by Blasig *et al.*(2011) occludin is sensitive to oxidative stress, appearing as an early and specific target for reactive species. Due to the close structural and functional

interactions between occludin and the other proteins of the tight junctions, in particular Zo1, this sensitivity influenced the process involved in cell-cell interaction. Because lipid peroxidation represents one of the mechanisms of action of OTA, the integrity of cellular monolayers may be affected resulting in the disruption of connection, as evidenced by the reduction in the intensity of immunofluorescence staining.

In this study, the immunofluorescence analyses (**Figure 4**) revealed changes in the pattern of occludin and Zo1 in the presence of OTA. The oxidative stress could influence the interaction between occludin and Zo1 (Rao, 2008). This relationship is very important for the maintenance of epithelial tight junctions. The disruption of the occludin-Zo1 complex in the MDCK monolayers treated with OTA has been confirmed by the lower intensity of the fluorescence in the co-localization (**Figure 4b**) of these proteins. Moreover, the presence of OTA was able to alter cell adhesion and gap junction in MDCK cells. In fact, as demonstrated by Mally *et al.* (2006) the presence of OTA at µM concentration (> 25µM) has been able to influence the intercellular communication and change the pattern in b-cadherin and e-cadherin labelling, supporting the role of OTA in altering cell adhesion and cell-cell signalling, that may contribute to explain its toxicity.

Oxidants, indeed, reduce occludin specific membrane localization and influence the regulatory activity in cell-cell interaction, which could be counteract by the presence of antioxidants. Considering the data obtained with the immunofluorescence staining, we could speculate that the presence of α-tocopherol has been able to counteract OTA effects on occludin and Zo1 location. These results are in accordance with those obtained by Lee *et al.*(2006). In fact, primary brain micro-vessel endothelial cells, isolated from rats, exposed to oxidative stimuli (hydrogen peroxide) after the 24 h pre-incubation with α-tocopherol have been able to maintain the occludin continuous pattern at cell-cell boundaries. The pre-treatment with α-tocopherol blocked the loss of occludin presence in the tight junctions.

Overall, these results confirmed that OTA is able to induce dose-related damages in MDCK cells and the protective role of α-tocopherol in counteracting its toxicity. In

particular, LDH release, cell viability as well as apoptotic events induced by OTA have been counteracted by the presence of nM concentration of α-tocopherol. Moreover, α-tocopherol may play an important role to reduce the negative impact of the oxidative stress at cell-cell interaction level. In fact, OTA demonstrated to be able to disrupt cell-cell interaction in the monolayers, involving occludin and Zo1. α-tocopherol was able to influence the functional interactions between these proteins of the tight junctions, supporting its basic properties in the cellular membranes and in cellular metabolism.

CHAPTER III: CELL-MICRONUTRIENT INTERACTION STUDIES

Study I: ***Role of choline and methionine in Bovine Mammary Epithelial cell line exposed to hydrogen peroxide***

Choline and methionine are considered methyl group sources and are nutritionally interrelated. Choline is considered to be a vitamin-like compound that is essential for mammals when the supply in methionine and folates is not adequate (Zeisel, 2000). This consideration led to the concept of choline as vitamin-like compound despite it does not satisfy the standard definition of vitamin (McDowell, 1989). Functions of choline in the organism can be divided into two areas: choline per se, and choline as a source of methyl groups (Pinotti et al., 2008). Choline per se is a constituent of all cell membranes in the form of phosphatidylcholine, lysophospatidylcholine, choline plasmalogen or sphingomyelin and it plays a crucial role in lipid metabolism. Choline is also an important source of labile methyl groups for the biosynthesis of other methylated compounds. Based on this second function choline and methionine are interchangeable, as sources of methyl groups. However, as choline and methyl-group metabolism are closely interrelated, it has been difficult to separate the effects of choline deficiency from methyl-groups deficiency.

In this context, ruminants have represented an interesting model due to some specific features. In adult ruminants, choline (Baldi and Pinotti, 2006; Pinotti et al., 2008; baldi et al., 2011) and methionine (National research Council, 2001; Baldi et al., 2008; Jenkins & McGuire, 2006) are degraded in the rumen, and can be limiting nutrients for milk production. This is particularly true at the onset of lactation, when the mammary gland represents an important site of utilisation of both choline and methionine. Moreover, at this stage, mammary gland is exposed to significant stressors, such as free radicals, metabolic stress, oxidative and toxic compounds (Baldi, 2005), that associated with differentiation of secretory parenchyma and intense mammary gland growth (Sharma et al., 2011), make the availability of limiting nutrient even more crucial.

Aim of study I

Starting with these assumptions, we determined the contribution of choline and methionine on BME-UV1 cells viability upon hydrogen peroxide-induced stress. We determine the contribution of choline and methionine on BME-UV1 cells viability upon hydrogen peroxide-induced stress.

Material & methods

Cell line and cell culture

The BME-UV1 cell line is maintained in the Laboratory of Cell Culture-Department VESPA (University of Milan, Italy). The origin of the BME-UV1 established bovine mammary epithelial cell line has been described previously (Zavizion et al., 1996). Cells are routinely cultivated in plastic flasks (Corning Glass, Corning, NY, USA) in complete medium, which is a mixture of 50% DMEM-F12, 30% RPMI-1640 and 20% NCTC-135 supplemented with 10% foetal bovine serum (FBS) (BioWhittaker, Cambrex, Belgium), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1 µg/ml insulin, 5 µg/ml transferrin, 1 µg/ml hydrocortisone, 0.5 µg/ml progesterone, 10 µg/ml L-ascorbic acid and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). The cells are maintained at 37°C in a humidified 5% CO2 incubator until sub-confluence. All medium supplements are from Sigma-Aldrich. The cells used in the present work were at the passage numbers between 34-44.

Determination of the half-lethal concentration (MTT assay) induced by hydrogen peroxide

In order to calculate the half-lethal concentration (LC50), that correspond to the concentration of H2O2 which is lethal to 50% of the cells after 24, 48 and 72 h of

exposure, a dose-response curve was set up. BME-UV1 cells were dispensed into wells of 96-well tissue culture plates ($2.5x10^5$ cells/ml, in 150 µl of complete medium). After 24 h of incubation at 37°C in an atmosphere of 5% CO_2, medium was removed and cell monolayer was exposed to increasing concentrations of H_2O_2 (0-500 µM) for the following 24, 48 and 72 h.

The effects of H_2O_2 treatments on cells viability were determined using MTT. MTT test is a colorimetric assay based on the production of the chromophore formazan from 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide. Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase. The standard protocol with minor modifications, as described by Baldi et al.(2004), was used. Briefly, at the end of the incubation, the media were removed and 150 µL MTT stock solution (5 mg/ml) in PBS was added to each well, and the plates were incubated for 3 h at 37 °C in a humidified chamber. Afterward, 150 µL dimethyl sulfoxide was added to each well to dissolve formazan. Absorbance at 540 nm was determined on a Biorad 680 microplate reader (Bio-rad, Veenendaal, The Netherlands). Cells incubated with culture medium alone, representing 100% viability, were included as negative controls in all experiments. The percentage cytotoxicity was calculated as follows: Percentage cytotoxicity = (1 – mean optical density in presence of hydrogen peroxide /mean optical density of negative control) × 100.

Determination of the effect of choline and methionine against hydrogen peroxide-induced toxicity

The identification of the most suitable combinations of choline and methionine for further experiments with H_2O_2, was established by a dose-response curve (MTT test).

BME-UV1 cells were dispensed into wells of 96-well tissue culture plates as described above. The cell monolayers were exposed for 24 h to serial concentrations of choline,

ranging from 125 to 1000µM according to Chao et al.(1988), and methionine, ranging from 178 to 1430µM.

Two combination of choline and methionine, Low Choline/Methionine dosage (LCM) and High Choline/Methionine dosage (HCM), were chosen in order to keep the ratio between them constant and equilibrate; the proportion between choline and methionine has been established on molar basis (Sharma & Erdman, 1988). Insulin (1 µg/ml) has been included in all experiments in order to support their uptake (Rilemma, 2004).

To the purpose to evaluate the ability of choline and methionine combined in counteracting H_2O_2 cytotoxicity, two ranges of H_2O_2 were identified according to LC50 previously calculated. The first range, defined low H2O2 concentrations (from 15.62 µM to 62.5 µM) and the second range, defined high H_2O_2 concentrations (from 83.2 µM to 333 µM).

BME-UV1 cells have been first pre-incubated with LCM and HCM. Subsequently, cells have been exposed to low and high ranges of H_2O_2 for 24 h, 48 h and 72 h.

Cells exposed to H_2O_2 alone have represented the control. Effect of choline and methionine combined in counteracting H_2O_2 cytotoxicity on BME-UV1 viability was evaluated by MTT test, whose procedure has previously described.

Statistical analysis

At least three replicates at each incubation time were performed and all experiments were performed at least twice. Results are expressed as mean and SD. The effect of various treatments was evaluated by one-way analysis of variance using the GLM procedure of SAS (SAS institute Inc, NC, USA). Values significantly different from controls are indicated as $P<0.05$.

Results & discussion

Determination of the half-lethal concentration (LC_{50}) induced by hydrogen peroxide

The effect of H_2O_2 on BME-UV1 cell viability was evaluated by a dose-response curve (MTT test). As shown in **Figure 1** up to 125 µM of H_2O_2, at all incubation time, cell viability was maintained over 50%, while higher H_2O_2 concentrations have produced a strong reduction of BME-UV1 viability, indicating a dose-dependent sensitivity of the cell line tested. Based on these data, we have calculated the LC_{50} at all incubation time, corresponding to 376.5 µM, 249.9 µM and 244.9 µM after 24h, 48h and 72h, respectively (**Figure 1**). BME-UV1 cell line has shown a dose and time-dependent sensitivity.

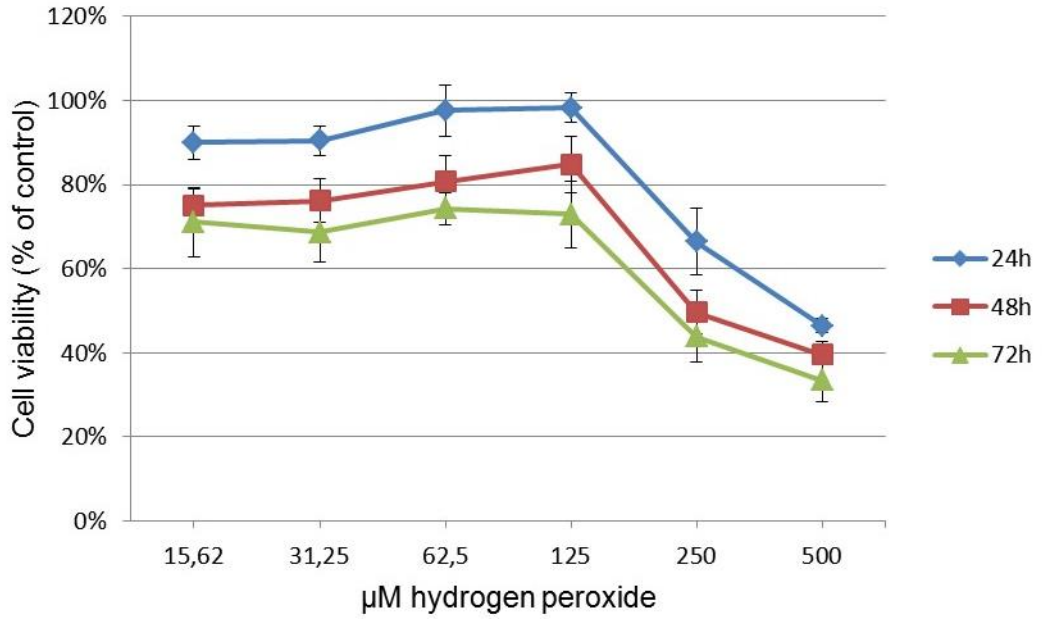


Figure 1*: evaluation of cell viability (% of control) over time of BME-UV1 cells exposed to increasing concentrations of hydrogen peroxide. Cell viability has been evaluated by MTT test*

Determination of the effect of choline and methionine against hydrogen peroxide -induced toxicity

The most effective combinations of choline and methionine in term of maintenance of cell viability after 24 hours were: 500 µM and 715 µM for choline and methionine and 1000 µM and 1430 µM for choline and methionine (**Figure 2**), even if no significative effects have been observed. These two selected amounts of choline and methionine were named Low Choline/Methionine dosage (LCM) (500/715 µM) and High Choline/Methionine dosage (HCM) (1000/1430 µM).

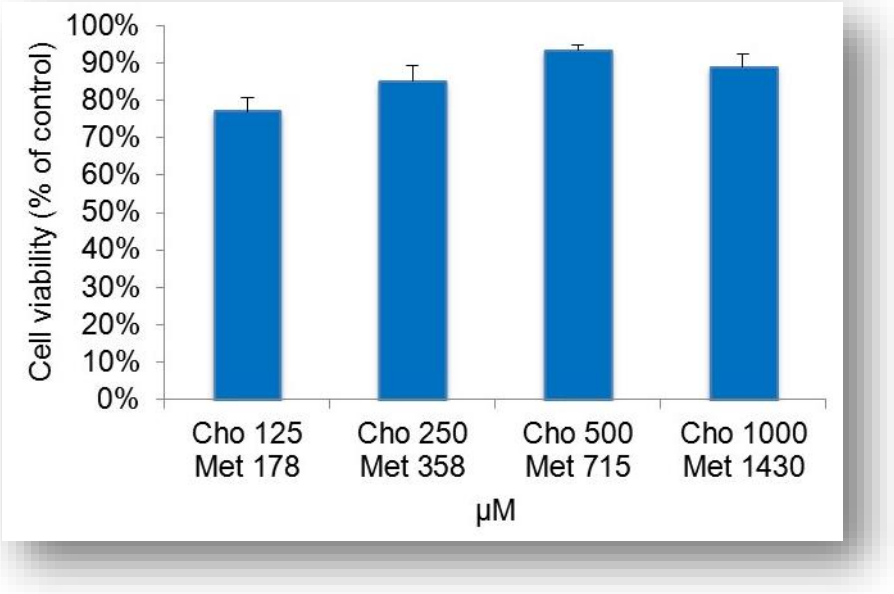


***Figure 2**: Evaluation of cell viability (% of control) of BME-UV1 cells exposed to increasing concentrations of choline and methionine for 24 hours. Cell viability has been evaluated by MTT test*

BME-UV1 cell line was treated with combinations of LCM and HCM and two H_2O_2 levels for the following 24, 48 and 72 h. The experiment was set up in two different steps.

In the first set of experiments, we have tested LCM and HCM effects in a weak condition of stress represented by low H_2O_2 concentrations (ranging from 15.62 to 62.5μM). We found that choline and methionine significantly ($P< 0.05$) enhanced cell viability on average by 21% and 25.8% after 48 and 72h, respectively, whereas their effect at 24h was limited (**Figure 3**). The effects were independent of the choline/methionine concentrations used.

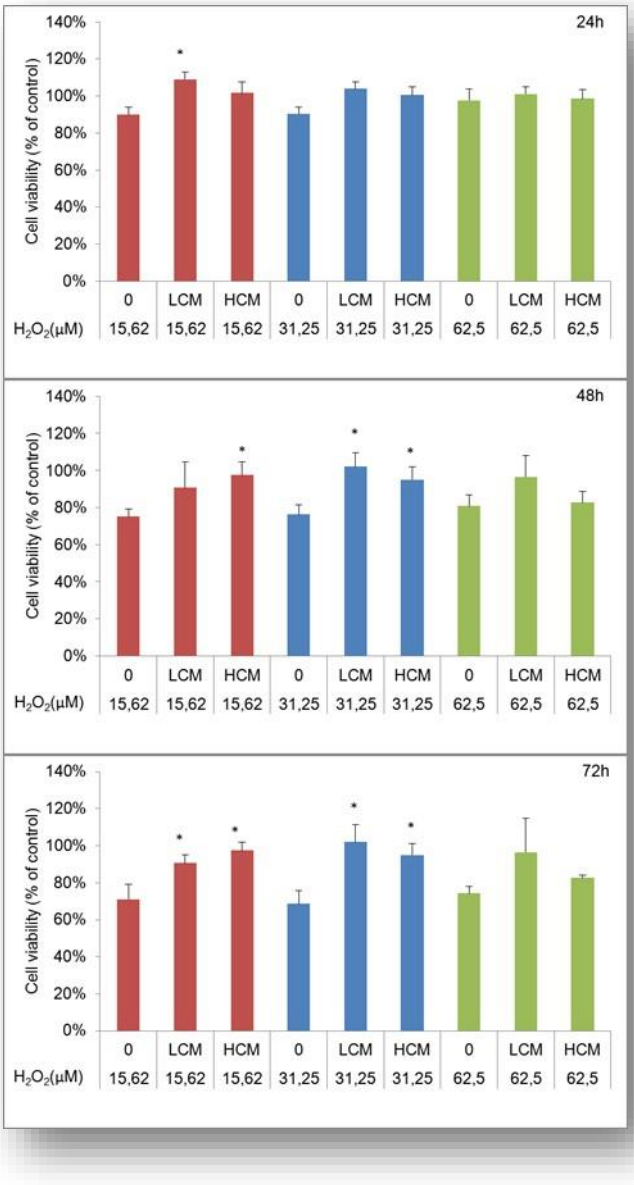


Figure 3*: Evaluation of the effect of Low Choline/Methionine dosage and High Choline/Methionine dosage against low concentrations of hydrogen peroxide (ranging from 15.62 to 62.5 µM). The role of Low Choline/Methionine dosage and High Choline/Methionine dosage has been evaluated by MTT test. (*P<0.05)*

In the second set of experiments, we have tested LCM and HCM effects in a condition of stress around LC50, represented by high H_2O_2 concentrations (ranging from 83.2 to 333

μM). In this range of H_2O_2 tested, choline and methionine significantly ($P< 0.05$) enhanced cell viability on average by 15% and 17% after 48 and 72h, respectively. No effects were observed at 24h (**Figure 4**), indicating a time dependent activity of LCM and HCM. The effects, again as in the previous experiments, were independent of the choline/methionine concentrations used.

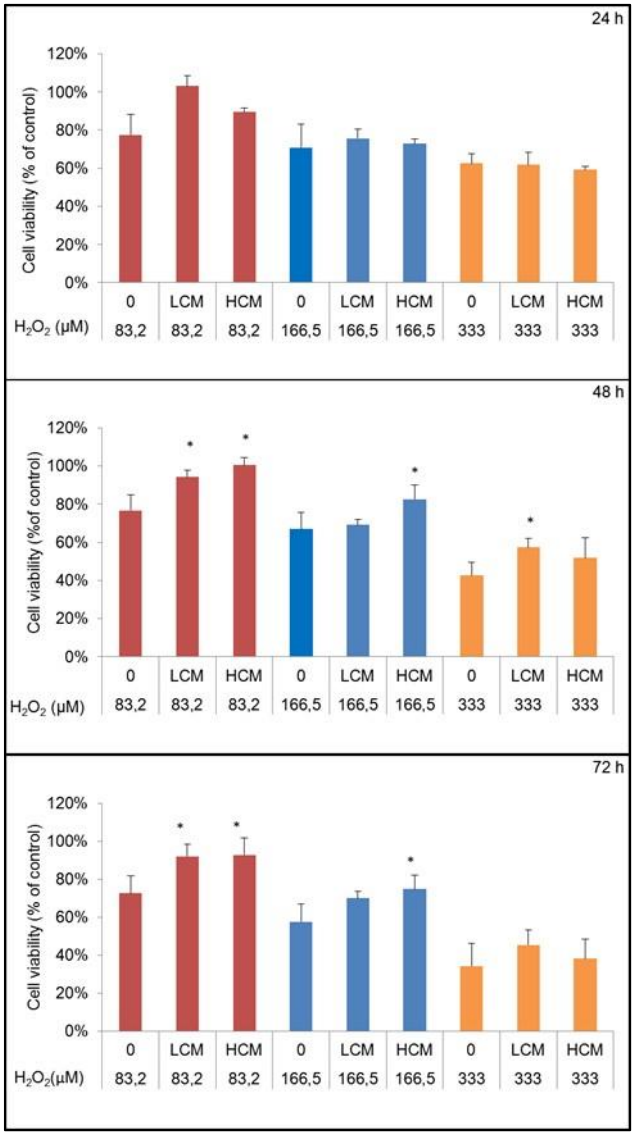


Figure 4*: Evaluation of the effect of Low Choline/Methionine dosage and High Choline/Methionine dosage against low concentrations of hydrogen peroxide (ranging from 83.5 to 333 µM). The role of Low Choline/Methionine dosage and High Choline/Methionine dosage has been evaluated by MTT test. (*P<0.05)*

Irrespective of the methionine and choline concentrations used, after 48 and 72h of incubation, cell proliferation was enhanced by choline and methionine presence in the medium, indicating a possible modulating effect exert by the two nutrients against H_2O_2. On the contrary, after 24h of incubation, choline and methionine did not influence the

viability of cells exposed to H_2O_2. The observed positive effects on cell viability were higher at the lowest range of H_2O_2 concentration tested, compared to the highest concentrations. Therefore, in the present study it has been observed that choline and methionine could play a crucial role in counteracting oxidative damage induced by H_2O_2 in bovine mammary epithelial cells, even though the real mechanism is not clear.

Oxidative stress occurs when the presence of oxidizing agents, free radicals and reactive oxygen species (ROS), exceeds the antioxidant capabilities of the organism. This lack of balance between oxidants and antioxidants leads to tissue injuries and to a wide range of degenerative diseases. Therefore, it is important to assess the damaging effects of oxidative stress on *in vitro* models and to understand protective mechanisms by which the cells respond to stress. Miranda et al. (2011) reported that the effect of H_2O_2 is different in relation to the cell type tested. In fact, some cell types respond to H_2O_2 with an increase in cell proliferation (Sigaud et al., 2005), while other cell types, including bovine mammary cells (Miranda et al., 2011), respond to H_2O_2 treatment by decreasing viability rate as supported by data herein presented.

In mammalian cells, oxidative degradation of membrane lipids can result in loss of membrane integrity, defective membrane transport mechanisms, and increased permeability. It has demonstrated that high concentrations of H_2O_2, cause cell membrane damage to Caco-2 cells with an increase in lipid peroxidation and a decrease of cell viability (Wijeratne et al., 2005).

Choline and methionine have been identified as required nutrients and play various biological functions on cells, including regulation of growth and proliferation (Guo et al., 2004; Pinotti, 2012). Our results showed that, under a condition of stress, choline and methionine have an important role in enhancing viability of BME-UV1 and, if supplemented in the culture medium in adequate concentrations, they have a possible role in counteracting oxidative stress induced by H_2O_2. In this respect, previous *in vivo* studies have indicated a potential relation between methyl-group and oxidative status. In particular, Sachan et al. (2005) have reported that choline and other methyl group sources

supplementation have reduced oxidative stress, and have promoted conservation of retinol and α-tocopherol in humans. In the same way Pinotti et al. (2003) and Baldi and Pinotti (2006) have reported a smaller depletion in vitamin E concentrations in choline supplemented dairy cows than in controls, suggesting a possible effect of choline on antioxidant status. However in these studies the real mechanisms by which choline has produced these interactions with antioxidant status have been not fully elucidated, and in most of the cases methionine has been directly involved. Thus based on present results it can be speculate that, in a condition of adequate bioavailability of methionine, the use of choline per se can be enhanced, which may affect both cell membrane stability and in turn cell response to stressor, such as H_2O_2.

The results presented here suggest that choline and methionine could play a role in counteracting oxidative damage induced by H_2O_2 in bovine mammary epithelial cells. The cytotoxicity, induced by H_2O_2 addition, could be reduced by supplementation of these two limiting and essential nutrients, i.e. choline and methionine. This effect seems to be time and dose-dependent. In particular choline/methionine treatment is effective in a weak condition of oxidative stress, and at 48 and 72 h of exposure. However, present results are referred to viability only, and therefore the real mechanism of choline and methionine on oxidative stress in a bovine mammary epithelial cell deserves further investigations.

Study II: ***Effect of Zn Oxide and Zn Chloride on Human and Swine Intestinal Epithelial Cell Lines.***

Zn is an abundant trace element in the body recognized as essential micronutrient. At intestinal level, Zn is involved in the modulation of immune system, affecting both non-specific and acquired immunity (Fraker et al., 2000; Frieke, 2000), in mucosal resistance to infection, in the restoration of mucosal barrier integrity and the promotion of antibody production against intestinal pathogens (Sargeant et al., 2011). The absorption of Zn occurs primarily in the small intestine and is influenced by different dietary factors such as the presence of Zn antagonists (e.g. calcium, phytate, fibers). Low molecular weight binding ligands such as citrate, picolinate and amino acids enhance its absorption (Lönnerdal, 2000). Zn is supplemented in human multivitamin formulations and in animal feedstuff in either inorganic or organic forms. In pig livestock, Zn has been included as nutritional additive at concentration lower than 150ppm as indicated in the Reg. CE 1831/2003. Whereas, pharmacological concentrations of Zn >1000ppm are commonly used as alternative to in-feed antibiotics (Poulsen, 1995). Generally, inorganic Zn salts (e.g. Zn oxide) are the most commonly forms supplemented in animal diets. It has been previously demonstrated that in swine livestock the supplementation of 150ppm of Zn Oxide (ZnO) improves gut health (Rosselli et al 2005). Also, ZnO at pharmacological concentrations reduces the incidence of diarrohea in the weaned piglets (Owusu-Asiedu et al., 2003). New approaches are necessary to optimize Zn supplementation in feedstuffs in order to improve animal health and to control Zn release in the environment. Moreover, further investigations into the mechanism by which Zn supplementation improves intestinal swine condition may shed light on the role of Zn in human gut level. Studies conducted *in vitro* reported that ZnO inhibits bacterial growth, plays a pivotal role in maintaining epithelial barrier integrity and function, may improve mucosal repair and paracellular permeability (Roselli et al, 2003). Nevertheless, despite the wide use of Zn oxide in *in vivo* trials, few studies were focused on its effect at cellular level (Sargeant et al., 2010; Sargeant et al., 2011). The importance of intestinal cell models of human and

pig origins in *in vitro* platforms for preclinical research is well recognised (Langerholc et al., 2011). In this context, *in vitro* studies can provide evidences regarding the mechanism of action of specific dietary compounds at cellular level, as well as discover any cytotoxic effects of these molecules. Cell culture models have been often used to evaluate the cellular mechanisms of several compounds (Baldi et al, 2004; Rebucci et al, 2007). In addition, *in vitro* animal and human cell models represent a suitable alternative to *in vivo* animal experiments (Cencič & Langerholc, 2010) for the determination of Zn supplements effects.

Aim of the study II

The aim of the present study was to assess the effect of two widely used additives in feedstuff, on the intestinal epithelium. In particular, the effect of ZnO and $ZnCl_2$ was investigated in human (INT-407) and porcine (IPI-2I) cell line models.

Materials and Methods

Cell line and cell culture conditions

The Human embryonic intestinal cell line (INT-407), a non-transformed epithelial cell line, originally derived from ileum of a 2-month-old human embryo, was obtained from the American Type Culture Collection. INT-407 were routinely cultivated into 75 cm2 tissue culture flasks in RPMI-1640 medium supplemented with 200mM glutamine, 1% non-essential amino acids (NEAA) and 10% Fetal Bovine Serum (FBS). Cells used in this work were between passages 36-40.

The IPI-2I cell line, derived from the ileum of an adult boar and immortalized by transfection with an SV40 plasmid (pSV3) (Kaeffer et al., 1993), was obtained from American Type Culture Collection. IPI-2I were routinely cultivated into 75cm2 tissue

culture in DMEM-F12 supplemented with 4mM glutamine, 0.024UI/ml insulin and 10% Fetal Bovine Serum (FBS). Cells used in this work were between passages 5-12.

Cells were cultured in an atmosphere of 5% CO2 at 37 °C until sub-confluence. Cell monolayers were washed with phosphate buffered saline (PBS) and trypsinized with 0.25% trypsin-EDTA. After 48 hours, cells were detached and re-suspended in culture medium to a concentration of 2.5 x 105 cells/ml. Portions (200 µl) of cell suspension were dispensed into sixty wells of a ninety-six-well tissue culture plates.

Zn oxide and Zn chloride solutions

A stock solution of 400mM ZnO (Zn content 80.34%) was prepared in 5% of acetic acid. Starting from this stock, ZnO treatment solutions (50, 200, 1000 and 4000 µM) were dissolved in serum-free medium.

A stock solution of $ZnCl_2$ (Zn content 47.97%) was prepared as described in Merck Index instructions (1989). In detail, 1 g of $ZnCl_2$ was dissolved in 0.25 ml of 2% HCl. Starting from this stock, $ZnCl_2$ treatment solutions (50, 200, 1000 and 4000 µM) were dissolved in serum-free medium.

IPI-2I and INT-407 were exposed to treatment solutions in concentration mentioned above (200µl) of ZnO and $ZnCl_2$ for the following 3 and 24h. In particular, for each cell lines we tested four concentrations of Zn sources in triplicate (3 wells per treatment) at two incubation times (3 and 24h). Moreover, at least two independent experiments were performed. The concentrations of ZnO and $ZnCl_2$ were selected on the basis of preliminary assays (data not shown) whereas the times of exposure were selected on the basis of the intestinal transit time in according to Roselli et al. (2003).

Evaluation of the effect of different Zn sources on cell viability: MTT test

The effect of Zn sources on IPI-21 and INT-407 cell lines was evaluated by MTT test using an incubation period of 3 and 24 hours under serum-free conditions. In particular, after removing the treatment solutions, 150µl MTT stock solution (5mg/ml) in PBS was added to each well and the plates were incubated for 1.5 h in a humidified chamber. The reaction was accomplished by removing the incubation solution and adding 150µl dimethyl sulfoxide to dissolve the formazan. The optical density of dimethyl sulfoxide (540 nm) was determined on a Biorad 680 microplate reader. Cells incubated with culture medium alone representing 100% viability, were included as negative controls in all experiments. This assay measured the production of the chromophore formazan from (4,5 – dimethylthiazol – 2 - yl) - 2,5 -diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich). Formazan was produced in viable cells by the mitochondrial enzyme succinate dehydrogenase.

Statistical analysis

At least three replicates (3 wells per treatments) at each incubation time were performed and two independent experiment were conducted. The data are presented as means and standard error (SE) and analyzed by one-way ANOVA (SAS, 2010). Duncan's post-hoc multiple range test was used, with $P < 0.05$ considered statistically significant. Different concentrations of the two Zn sources were tested using a model including the systematic effects of the source of Zn (n = 2), concentrations of Zn (four levels). The effect of the assay (n = 2) was included as a blocking factor.

Results & discussion

INT-407 cells have been exposed to a wide range of ZnO and $ZnCl_2$ concentrations in order to establish the cell viability in response to Zn treatments (**figure 1**). In particular, INT-407 cell line treated for 3h with 50μM of ZnO increased cell viability up to 120% of the control. The same tendency in term of enhancement of cell viability was observed after 3 and 24h of exposure to 50 μM of $ZnCl_2$. Higher concentrations of ZnO and $ZnCl_2$ have affected cell viability in a dose- dependent way. A significative ($P < 0.05$) reduction of cell viability was observed.

As shown in **Figure 2**, IPI-2I have shown a different susceptibility to ZnO and $ZnCl_2$ treatment. In particular after 3h of incubation, ZnO did not significantly inhibited IPI-2I cell viability, which remained about 80% at 50, 200 and 1000μM. Whereas, a significative ($P < 0.05$) reduction in IPI-21 cell viability was observed at the highest Zn oxide concentration tested (4000 μM). A significative reduction ($P < 0.05$) was observed at all concentrations of ZnO tested after 24h of incubation. A similar dose and time dependent effect was also observed when IPI-21 cells were treated with increasing concentrations of $ZnCl_2$ for 3 and 24h.

Overall, a dose-response effect was observed after ZnO and $ZnCl_2$ treatments in both the intestinal cell lines considered suggesting that *in vitro* cell models are suitable for studying the effect of Zn additives on cell viability. In particular, it was observed that after 3 hours of incubation, which correspond to the transit time for several nutrients, cell viability was maintained by the lowest ZnO and $ZnCl_2$ presence in the medium.

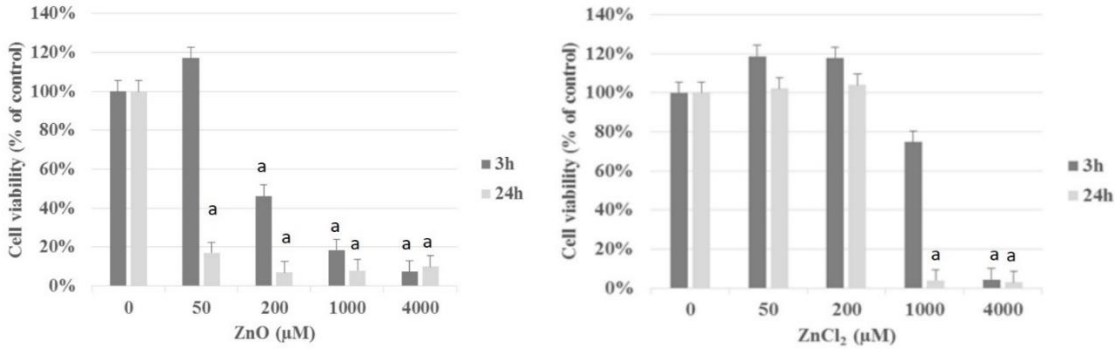


***Figure 1**. Effect of increasing concentrations of ZnO and $ZnCl_2$ on cell viability of INT-407 after 3h and 24h of treatment measured by MTT test. The graph shows the percentages of cell viability over control. Values significantly different from proliferation obtained with 0 µM Zn sources (control medium 100% viability) are indicated: a, $P < 0.05$*

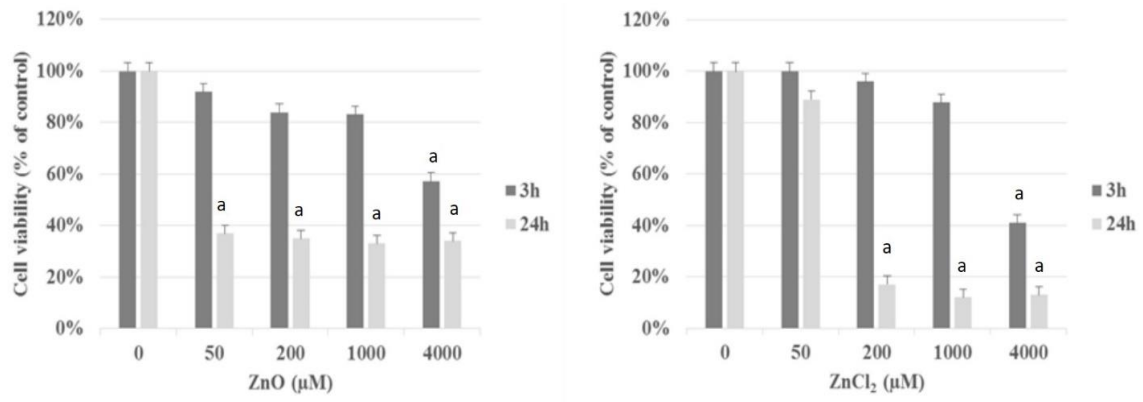


***Figure 2**. Effect of increasing concentrations of ZnO and ZnCl2 on cell viability of IPI-2I after 3h and 24h of treatment measured by MTT test. Values significantly different from proliferation obtained with 0µM Zn sources (control medium 100% viability) are indicated: a, $P < 0.05$*

MTT assay is a viability assay that measures the metabolic activity the cells. MTT assay used in this study represent a simple and useful method to preliminary qualify the effect of a large number of compounds (Zn, tocopherol, probiotics) at cellular level (Baldi et al., 2004; Rebucci et al., 2007). Faller et al. (2002) reported that this assay showed a better correlation with *in vivo* assay than other bioassays. Cell-based model represents simplify

biological system and may be used in the evaluation of nutritional additives effects. In this study, two intestinal epithelial cell lines from different species have been considered. This is essential in order to better characterize the response of dietary compounds on a specific epithelium. Despite the wide use of Zn as animal feed supplement, little studies have focused on its effect *in vitro*. The intestinal cell lines INT-407 and IPI-2I showed to be suitable models of the intestine and represent a simple tool to investigate the role of Zn supplements.

INT-407 showed to be the most sensible model to Zn supplements considered in this study, whereas IPI-2I showed to be the most resistant. Viability of IPI-2I cells is reduced at ZnO and $ZnCl_2$ concentrations >1000uM, whereas Sargeant et al. (2010) found a reduction of IPEC-J2 cell viability at concentrations >100μM of ZnO. However, the lowest concentrations of both ZnO and $ZnCl_2$ considered in this study have maintained the viability of INT-407 and IPI-2I underlining the beneficial role of Zn on human and swine intestine. In particular, it was observed that after 3 hours of incubation, which correspond to the transit time for several nutrients, cell viability was enhanced by the lowest ZnO and $ZnCl_2$ concentrations tested, indicating that these compounds may have a beneficial role for human and swine intestinal epithelium. Overall, these results contribute to determine the role of Zn in human and swine intestinal epithelium. In general, this study confirm that the use of *in vitro* cell-based models for screening the biological activity of single compounds at specific concentrations and in a strictly controlled environment could offer novel insight in the field of human and animal nutrition, making *in vitro* models an essential tool in biological studies. However, cell-based models may not reflect the *in vivo* condition of the intestinal cells in their natural state in the intact organism, where bioavailability, metabolism, binding and transport proteins may influence the biological effect of Zn sources. Therefore, further *in vivo* experiments are necessary in order to extend the results obtained *in vitro*, to clarify the contribution of Zn supplements in gut health, and to improve Zn supplementation in animal feed and in human formulations.

ADD-ON PROJECT -***Health-promoting role of intact milk proteins: in vivo human intervention study***

Introduction

Diet, obesity and ageing population increase the risk of chronic disease. Animal products are a staple source of nutrients in the human modern western diet. High blood pressure – a modifiable risk factor of CVD – is responsible for 13% of all deaths in the world annually and hence hypertension is one of the five leading global risks for mortality (Givens, 2010; Givens et al., 2014).-In this context, milk and dairy products are reported to possess a wide range of biological properties (Huth et al., 2006; Wada & Lönnerdal, 2014) and are therefore potential neutraceutical (health-promoting food) ingredients. Several target of such health-promoting food components are proposed as cardiovascular health, bone health, weight management, immune defense, digestive health. Further, milk peptides have previously demonstrated to contribute in the reduction of the CVDs and colorectal cancer (Cho et al., 2004; Aune et al., 2012; Fekete et al., 2013). Evidence from human intervention studies suggests that both whey and casein may be effective in lowering blood pressure. However limited data are available on the impact of intact milk proteins on vascular function. Pal & Ellis (2011) and Pal et al. (2010a; 2010b) performed one of the few studies investigating the differential effect of whey and casein on vascular function and reported that mainly whey protein appeared to improve vascular reactivity. However, to date, no adequately powered randomised control intervention studies have been performed (Givens et al., 2010; Fekete et al., 2013). Therefore, the study aims to investigate the acute and chronic effect of milk proteins on heart disease risk markers as blood pressure and vascular function. In particular, this study has been designed to test the hypothesis that the incorporation of whey protein in the habitual diet for 8 weeks will result in an increase in vascular reactivity and a reduction in blood pressure, plasma lipids and markers of insulin resistance compared with casein and maltodextrin (as non-protein control).

Material & methods

Study protocol

People with primary, prehypertension and stage 1 hypertension (i.e. blood pressure within range 120/80 to 159/99) were recruited (9). Twenty-one males and twenty-one females were recruited from the Reading area by emails to University staff and from local companies such as Prudential, local council etc, posters, use of Hugh Sinclair Unit of Human Nutrition and Sensory Dimensions participant databases, leaflets in local papers, and internet advertisements. Subjects were selected if they meet the study criteria. All participants gave their informed consent before taking part in the study.

Inclusion criteria:

- ✓ A signed consent form
- ✓ Blood pressure: 120/90-159/99
- ✓ Age: 30-65 years
- ✓ BMI 20-35 kg/m2
- ✓ Glucose <7 mmol/l (Not diagnosed with diabetes)
- ✓ Chol <8 mmol/l
- ✓ TAG <4 mmol/l
- ✓ Normal liver and kidney function
- ✓ Haemoglobin (>130 g/l)

Exclusion criteria:

- ✓ Milk allergy, lactose allergy, collagen allergy
- ✓ Cardiovascular, renal, gastrointestinal, respiratory, endocrine, liver disease or cancer
- ✓ Surgery in the previous 6 months
- ✓ Secondary hypertension
- ✓ Excess alcohol consumption (drinking >28 unit/wk man; >21 unit/wk women)
- ✓ Smoker
- ✓ Vegan

- ✓ Taking nutritional supplementation (e.g. fish oil, proteins)
- ✓ Anaemia
- ✓ Coeliac disease

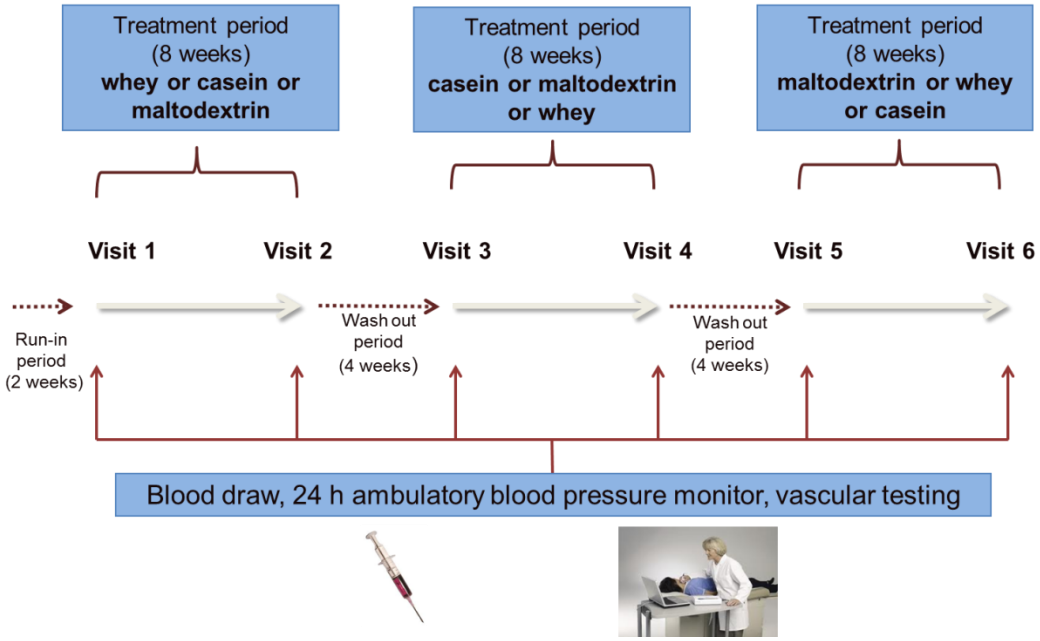
Potentially suitable participants were identified and asked to attend a screening session during which a fasting blood sample was taken and their height, weight (Tanita Europe BV, Middlesex, UK) and waist circumference were measured. One blood sample has been sent to the Royal Berkshire Hospital's Department of Clinical Biochemistry (Reading, UK) for full blood count analysis and the second was used to analyse fasting blood lipids (total cholesterol, HDL-cholesterol and TAG) and glucose, as well as liver and kidney function enzymes (glomerular filtration rate, alkaline phosphatase, alkaline transferase, □-glutamyl transferease, serum creatinine, total bilirubin and uric acid) using the Ilab 600 (Instrumentation Laboratories Ltd., Warrington, UK). After 20-30 mins rest, blood pressure (BP) was measured 4-6 times for up to an hour (10-15 mins intervals) by an electronic sphygmomanometer in order to gain typical values and minimise 'white coat effect'.

Chronic study days

In the chronic study subjects was randomised to one of three feeding groups (G1: whey/maltodextrin/casein, G2: casein/whey/maltodextrin, G3: maltodextrin/casein/whey). The intervention products was 56g (containing 50 g protein) whey protein isolate (WPI90, commercially available, provided by Volac Int. Ltd.), calcium caseinate (commercially available, provided by Garret Ingredients Thornbury) and maltodextrin (commercially available, Myprotein). The study started with a two-week run-in period where no intervention was provided to any of the volunteers, but they consumed their habitual diet and collected dietary information. During this period subjects attended the Hugh Sinclair Unit of Human Nutrition for a 'familiarisation visit' in order to have their blood pressure measured and the elasticity of vessels determined by 3 non-

invasive techniques: Flow Mediated Dilatation (FMD), Pulse Wave Analysis (PWA) and Digital Volume Pulse (DVP). The aim of the two-week run-in period was to familiarise subjects with the techniques to minimise the impact of stress on the subsequent (baseline) assessments.

After the two-week run-in period participants were randomly assigned to one of the three feeding groups (G1: whey/cmaltodextrin/casein, G2: casein/whey/maltodextrin, G3: maltodextrin/casein/whey). At visit 1 (baseline) subjects were advised on how to take the protein powders. The participants were advised to replace a drink or snack from their habitual diet with the study protein to ensure the diets are isocaloric. This is necessary in order to avoid any changes in body weight. Participants were asked to consume the intervention products for a period of 8 weeks. Intervention periods were separated by a four-week wash-out period when participants did not consume any powders.



***Graph 1**: Chronic study design*

All powders are commercially available. The sachets of the studypowders were identical and provided in individual doses of 28g in powder form, 2 sachets were consumed a day.

At the end of each treatment, participants were asked whether they experienced any change in their health (side-effect) and how they tolerated the powders.

During intervention, subjects were asked to attend the Hugh Sinclair Unit of Human Nutrition for six intervention visits (on the first and the last days of each intervention period) over a period of 32 weeks. During these visits, fasting spot urine and fasting blood samples were taken and vascular reactivity was measured. Furthermore, subjects have worn 24-hour ambulatory blood pressure monitors on these days.

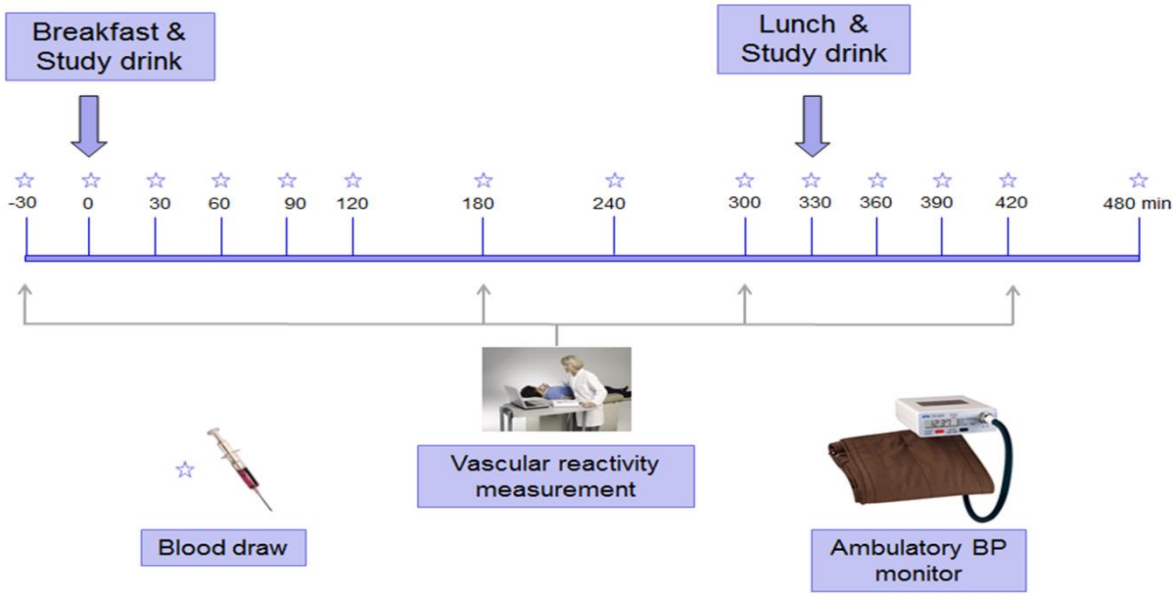
Acute study days

The acute study has been designed to test the hypothesis that ingestion of breakfast (croissant, butter, and short breads, with a milk shake which contains 28g protein powder) and lunch (Philadelphia cheese sandwich, with butter and a drink containing 28 g protein powder) containing whey protein (mixed into water with vanilla flavouring) result in a postprandial increase in vascular reactivity and a reduction in blood pressure, plasma lipids and markers of insulin resistance compared with the equivalent dose of casein and maltodextrin.

Subjects (n=24) for the acute study were the participants who were already involved in the chronic study. Subjects will be required to attend longer sessions at the Hugh Sinclair Unit of Human Nutrition on three of the chronic study visits days (the first day of each intervention).

Vascular measurements (see details below) were conducted after subjects have rested for 30 minutes. The non-invasive vascular reactivity measurements were conducted in the following order: FMD and PWA at baseline (at -30 mins, fasted) and at regular intervals (120, 300, 450 mins) following protein drink consumption (28 g protein).

After a cannula was inserted into the ante-cubital vein, blood samples has been taken at 30 min (fasted) before breakfast and 0, 30, 60, 90, 150, 210, 270, 330, 360, 390, 420, 480 min after breakfast at 0 min (**Graph 2**). Lunch was given at 330 min. Subjects were asked to finish their meals within 10-20 minutes.



***Graph 2**: Acute study design*

Physical examination

Waist circumference, height, weight and blood pressure were measured at the screening visit, and repeated on each of the visits performed.

Blood and urine samples

A 50 ml fasting blood sample were taken during each of the six chronic study visits to the nutrition unit. From the fasting blood samples, glucose, insulin, nitric oxide (NO), fasting lipids (total, LDL, HDL cholesterol, triacylglycerol and non−esterified fatty acids), inflammatory biomarkers (CRP, TNF−α and IL6), ACE activity, AcSDKP-activity were measured. Postprandial blood samples (taken during three acute study visits; total 173 ml) were tested for glucose, insulin, TAG, NEFA, CRP, TNF-α, IL6, and nitric oxide, ACE activity, AcSDKP-activity.

A fasting spot urine sample has been collected prior to the chronic intervention visits on each treatment arm (week 0 and 8; 6 times in total) to the nutrition unit. From the urine

collection, urinary microalbuminurea (marker of microvascular disease), creatinine and calcium excretion were measured.

Vascular measurments: Flow Mediated Dilatation (FMD)

Vascular function was assessed non-invasively using FMD, a technique which assesses the vasodilatory response of a vessel to elevations in blood flow-associated shear stress. Occlusion of the forearm is created by inflating a sphygmomanometric cuff (as used for measuring blood pressure) on the arm. After maintaining an inflation pressure of 200 mm Hg for 5 minutes, the pressure is released quickly to induce a brief high flow state and vasodilatation of the brachial artery. The brachial artery is imaged using ultrasound both before the application of the cuff (baseline) and for 5 minutes after release of the pressure cuff. Endothelium-dependent vasodilation is assessed by measuring the change in brachial artery diameter compared to baseline, and is defined as % FMD. This measurement reflects changes in elasticity of larger blood vessels.

Vascular measurments: Pulse Wave Analysis (PWA)/ Pulse Wave Velocity (PWV)

Aortic compliance was assessed from carotid to femoral pulse wave velocity (PWVc-f) using a non-invasive Sphygmocor system and the augmentation index is calculated from measurements of the carotid to radial wave form. PWVc-f is computed from the time delay between the upstroke of the arterial pressure wave at the carotid and femoral arteries and the anatomical carotid to femoral distance. On each occasion subjects are rested in the supine for 15 min and blood pressure is recorded using an automated sphygmomanometer (Omron 70CP). PWVc-f is measured using the SphygmoCor VW apparatus with Sphygmocor analysis software (SphygmoCor version 7.01 AtCor Medical Pty, Australia). A total of six measurements are made with the criteria that the coefficient of variation is less than 5% for three of the six

measurements for the results to be acceptable. Augmentation index is derived from measurements of the carotid to radial pulse wave form using the Sphymocor analysis software in the pulse wave analysis mode.

Ambulatory blood pressure (ABP)

Ambulatory blood pressure were measured over a 24h period at baseline (week 0) and week 8 of each intervention period using the ScanMed Oscillometric ABP device (www.scanmed.co.uk). This device has been validated independently according to the British Hypertension Society (BHS, www.bhsoc.org) Protocol (A/A grade).

Results

The obtained results will be submitted to a scientific journal after the complete data analysis and compared with the results of the *in vitro* investigation of the effects of proteins digested, reported in the chapter I of this thesis.

GENERAL CONCLUSIONS

This thesis focused on the study of milk proteins, antioxidant and other micronutrients that are likely to have significant influences as health-promoting molecules and to improve the nutritional quality of human and animal diets. Overall, predictive efficacy of the health-promoting components was determined by the use of *in vitro* models in all of the studies performed. Cell-based bioassays were found to be valuable screening tools for the evaluation of the cell-nutrient interaction effects.

Milk proteins are considered important sources of bioactive peptides with high nutritional and functional properties. The health-promoting effects of intact whey and casein proteins were evaluated after *in vitro* SGD. Permeate (absorbed fraction) and retentate (intestinal fraction) were obtained and used to study their health-effects *in vitro*. Soya protein was include as non-animal protein in all the experiments performed. Following SGD, the milk proteins exhibited antioxidant activity, ACE-inhibitory activity and tropho-functional properties at the intestinal cell level. The whey protein permeate exhibited higher ACE-inhibitory activity compared with the casein and soya protein permeate. SGD increased the ACE-inhibitory activity of whey protein and the antioxidant activity of all the protein tested. At specific concentrations, casein, whey and soya proteins were able to modulate intestinal cell viability and the production of intestinal mucus. Moreover, the proliferation of *Lactobacillus casei* was increased by specific concentrations of whey and casein proteins. Modulations of mucus production and probiotic bacteria growth were observed, and casein was the primary protein that was able to stimulate MUC5AC gene expression and promote *Lactobacillus casei* growth. *Lactobacillus casei* was chosen because it is physiologically present in the human gut. Additionally, previous studies have demonstrated the roles of milk peptides in the promotion of *Lactobacillus casei* growth (Pecorini et al., 2005). Altogether, the analyses of prebiotic bacteria growth and goblet cell proliferation may represent a complementary approach to the study of the bioactivities and functions of food proteins in the gut. Additionally, the comparison of the two major intact milk proteins performed in this thesis

could provide valuable information regarding which is efficacious in improving health. However, robust and well-designed *in vivo* studies will be required to confirm the potential beneficial influences of milk-derived proteins and peptides on human health. The interest in studying the functionality and health-benefiting potentials of milk bioactives in nutritional sciences is mainly related to medical research but also represents enormous benefits for health and food companies and for the animal production sector, in accordance with the increasing interest in the production of novel functional foods to improve the quality of food and feed. The results of this thesis support the presently available knowledge, which indicates that ACE-inhibitory peptides, caseino-phosphopeptides and intestinal health-promoting peptides from milk should be used for the formulation of healthy foodstuffs. The obtained results will stimulate both *in vitro* future studies aimed at the complete determination of the influences of milk proteins on the different target organs and their specific modes of action and *in vivo* studies with high predictive power, regarding human health. The *in vivo* human intervention study conducted at Reading University is currently addressing this topic and will help to define the roles of the intact milk proteins used in this thesis, in different human health parameters.

Vitamin E supplementation is a common practice in animal nutrition due to its health-promoting effects as an antioxidant. Vitamin E seems to be involved in immune system improvements and the promotion of animal health and production (Vagni et al., 2011). Hung et al. (2004 & 2005) demonstrated an inverse relationship between antioxidant vitamin intake and CVD in humans. Vitamin E is considered to be one of the most important components of the cellular antioxidant system. In this thesis, the antioxidant roles of specific concentrations of α-tocopherol were demonstrated *in vitro* based on the ability to reduce oxidative damage induced by food toxicants, such as OTA. α-tocopherol was found to play an important role in reducing oxidative stress at the cellular level, in different cell models. Food toxicants, such as OTA are able to disrupt cell monolayers and damage DNA, which leads to cell death, as demonstrated in this thesis. Food toxicants reduce occludin membrane localization and influence cell-cell interactions, and these effects can be counteracted by the presence of antioxidants. In this

thesis, it was demonstrated that α-tocopherol supplementation may counteract short-term OTA toxicity at different cellular levels, which supports the defensive role of α-tocopherol in the cell membrane. Furthermore, the obtained results demonstrated that α-tocopherol is able to influence the functional interactions between tight junction proteins, which supports its protective roles in the cell membrane and cellular metabolism. The immunofluorescence analyses performed in this thesis revealed changes in the patterns of occludin and Zo1 in the presence of OTA. Pre-treatment with α-tocopherol blocked the loss of occludin protein in the tight junctions of kidney cells.

During my Ph.D., I have also investigated the *in vitro* roles of different micronutrients, specifically choline/methionine and different Zn formulations. Due to their physiological health-promoting effects, they are commonly used in animal nutrition and are parts of normal diets. Previous *in vivo* animal studies have indicated a potential relation between methyl groups and oxidative status and a direct effect of choline on antioxidant status. Choline and methionine have been identified as required nutrients and exhibit various biological functions in cells that include the regulation of growth and proliferation. The results of this thesis confirmed that choline and methionine have important roles in enhancing cell viability and counteracting oxidative stress in conditions of oxidative stress. Despite the wide use of Zn as animal feed supplement, few studies have focused on its effect *in vitro*. *In vitro* studies may be useful for improving Zn supplementation in animal feed and in human formulations. The intestinal cell lines INT-407 and IPI-2I were found to suitable models of the intestine and represent simple tools for investigations of the role of Zn supplements. Specific concentrations of both ZnO and $ZnCl_2$ are considered to be capable of maintaining the viability of intestinal cells, which underlines the beneficial role of Zn in on the human and swine intestinal epithelia.

Overall, these results contribute to the definition of the role of dietary health-promoting compounds in human and animal target tissues. The use of *in vitro* cell-based models to screen the biological activities of single compounds at specific concentrations and in strictly controlled environments could offer novel insights in the field of human

and animal nutrition; thus, *in vitro* models are an essential tool. However, cell-based models may not exactly reflect the *in vivo* conditions of the cells in their natural state in the intact organism in which bioavailability, metabolism and binding and transport proteins may influence the biological effects of dietary components. Therefore, further *in vivo* experiments are necessary to extend the *in vitro* results and to clarify the contributions of health-promoting components in animal feed and in human formulations.

The improved knowledge regarding milk protein bioactives, antioxidants and micronutrients represents a crucial point for future systematic efforts to improve food and feed quality. Finally, the dietary bioactive components with health -benefits supported by sufficient scientific substantiation have the potential to be increasingly important components of healthy lifestyles and to benefit the public and the food industry.

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