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Bioquímica Aplicada

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Professora Doutora Ana Preto

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RESUMO

O cólon humano possui diferentes espécies de microrganismos, capazes de modular o ecossistema intestinal, dado que desempenham um papel em vários processos fisiológicos essenciais para a homeostasia humana. Entre esses processos, a produção de ácidos gordos de cadeia curta (AGCC), nomeadamente o acetato, propionato e butirato, atraíram muito interesse principalmente devido às suas propriedades antineoplásicas, especialmente contra o cancro colorretal (CCR). O acetato, o AGCC mais abundante no cólon humano, tem sido associado a efeitos protetores em células CCR e o nosso grupo de investigação já reportou que esse metabolito inibe a proliferação e autofagia das células CCR, induz apoptose, promove permeabilização da membrana lisossomal e altera o fenótipo glicolítico.

Distúrbios na microbiota do cólon podem ser causados por uma ampla gama de xenobióticos, sendo os pesticidas um dos mais frequentes. Esses produtos químicos são introduzidos diariamente na nossa dieta e sabe-se que a exposição crónica alimentar a níveis inseguros de pesticidas está relacionada a uma vasta gama de disfunções, distúrbios e promoção de certos tipos de cancro, como o CCR. O tebuconazol, um dos fungicidas mais utilizados na agricultura, tem sido relatado como um possível agente cancerígeno. Sendo o cólon um dos principais alvos desse fungicida, o nosso grupo demonstrou que em células normais do cólon, este inibe o crescimento celular, induz morte celular por produção de espécies reativas de oxigénio (ERO) associada à apoptose numa linha celular de cólon normal. Dessa forma, o objetivo desta tese de mestrado foi entender se o acetato é capaz de proteger contra os efeitos do tebuconazol nas células normais do cólon.

Os nossos resultados sugerem uma possível tendência para um papel protetor do acetato através do aumento da proliferação, viabilidade celular e atividade mitocondrial. Este é o primeiro estudo a investigar um papel protetor do acetato na toxicidade de contaminantes alimentares no cólon humano, o que pode ajudar na descoberta de novas abordagens na prevenção do CCR.

Palavras-chave: acetato, microbiota, prevenção, tebuconazol.

ABSTRACT

The human colon harbours different species of microorganisms, which are capable of modulate the intestinal ecosystem since they play a role in several physiologic processes essential for the human homeostasis. Between these processes, the production of short chain fatty acids (SCFA), namely acetate, propionate and butyrate, have attracted a lot of interest mainly due to their antineoplastic capabilities especially against colorectal cancer (CRC). Acetate, the most abundant SCFA in the human colon, have been associated with protective effects against CRC cells and our research group have already reported that this metabolite inhibits CRC cell proliferation and autophagy, induces apoptosis, promotes lysosomal membrane permeabilization and changes the glycolytic phenotype.

Disruptions in colon microbiota can be caused by a wide range of xenobiotics, being pesticides one of the most frequent. These chemicals are daily introduced into our diet and chronic dietary exposure to unsafe levels of pesticides is known to be related with a wide range of organs dysfunctions, disorders and the promotion of certain types of cancer, such as CRC. Tebuconazole, is one of the most used fungicides in agriculture and has been pointed as a possible carcinogen. Being the colon one of the main targets of this fungicide, our group proved that in normal colon cells it inhibits cell growth, induce cellular death through apoptosis associated ROS production in normal colon cell line. The aim of this master thesis was to understand if acetate is able to protect against tebuconazole effects in normal colon cells.

Our results suggest a possible tendency for a protective role of acetate through the increasing of the cell proliferation, viability and mitochondrial activity. This is the first study uncovering a protective role of acetate in the toxicity of food contaminants in human colon, which may help in the finding new approaches in CRC prevention.

Keywords: acetate, colon microbiota, prevention, tebuconazole.

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LIST OF ABREVIATIONS

Ahr Aryl hydrocarbon receptors

AMPK (AMP)-activated protein kinase

Bax Bcl-2-associated X protein

Bcl-2 B-cell lymphoma 2

Caspase-3 Cysteine-aspartic acid protease 3

CRC Colorectal cancer

CYP19 Aromatase

CYP1A1 Cytochrome P450 1A1

CYP1A2 Cytochrome P450 1A2

CYP450 cytochrome P450

Cyp-51 Lanosterol 14α-demethylase

DAPI 4′,6-diamidino-2-phenylindole

DCF Dichlorofluorescein

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DPBS Dulbecco's Phosphate-Buffered Saline

dUTP Deoxy uridine triphosphate

EDTA Ethylenediamine tetra acetic acid

FBS Fetal bovine serum

FITC Fluorescein isothiocyanate

GI Gastrointestinal

HDACs Histone deacetylases

IC_{50} Half-maximal inhibitory concentration

LMP Lysosomal membrane permeabilization

MCT Monocarboxylate transporters

mRNA Messenger RNA

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PFA Paraformaldehyde

qPCR Quantitative polymerase chain reaction

ROS Reactive oxygen species

RPMI Roswell Park Memorial Institute

SCFA Short chain fatty acids

SMCT Sodium-coupled monocarboxylate transporters

SRB Sulforhodamine B

TD Toxicodynamic

TdT Terminal deoxynucleotidyl transferase

TK Toxicokinetic

Tris Tris(hydroxymethyl)aminomethane

TUNEL Terminal transferase dUTP nick end labelling

VGCC Voltage-gated calcium channels

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CHAPTER 1 – INTRODUCTION

1.1. The human colon

The human colon, also known as the large bowel, is an organ that makes part of the digestive system and plays an important role in water, fluids, vitamins and electrolytes reassert and conservation in human body (Junqueira and Carneiro, 2013).

This organ with 1.5 meters is divided into four parts: ascending, transverse, descending and sigmoid colon and it is histologically constituted by five layers: s*erosa*, *subserosa*, *muscularis*, *submucosa* and *mucosa* (**Figure 1**). These layers are coated by a epithelium rich in blood vessels and connective tissue, which promotes the absorption of substances, but also acts as a barrier to external agents (DeSesso and Jacobson, 2001; Junqueira and Carneiro, 2013; Boron and Boulpaep, 2015).

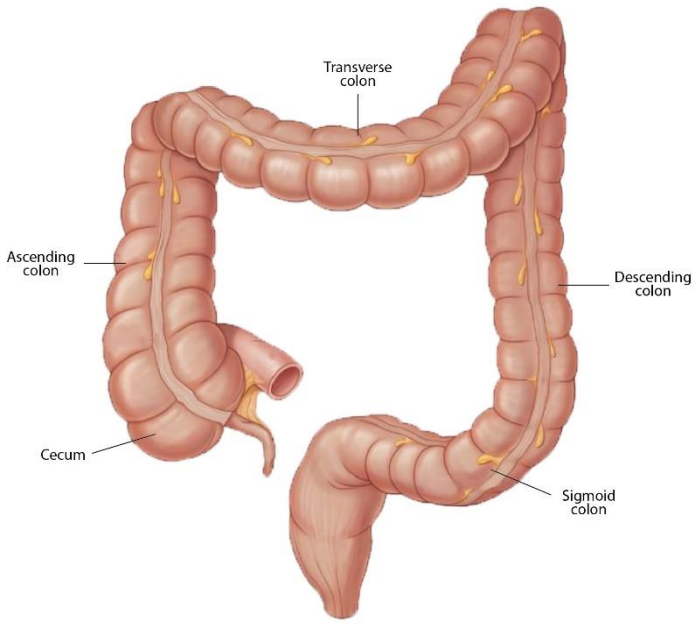

Figure 1: Colon anatomy and morphology. Adapted from Gray's Anatomy for Students.

The *mucosa*, specifically, is the innermost layer of the colon and it is the one that comes in contact with the gastrointestinal content. It is extremely well adapted and presents several structures like folds (*plicae*), depressions (*crypts)* and finger like projections (*vili)* that increase the secretory and absorptive capacity. Beyond these properties, it is in the *mucosa* that most of microorganisms localize (DeSesso and

Jacobson, 2001; Irving and Catchpole, 2009; Kim and Ho, 2010; Junqueira and Carneiro, 2013; Sellers and Morton, 2014).

Although in humans the liver is the main organ contributing to drug metabolism, various studies have demonstrated the importance of the colon in extrahepatic drug metabolism (Cummings, 1975). The gastrointestinal tract (GI-tract), namely colon, is considered the major path of entry for a wide variety of undesirable compounds including orally administered drugs and food contaminants but also compounds with neither nutrient or relevant functional value (Cummings, 1975; Boron and Boulpaep, 2015; Wilson and Nicholson, 2017).

Colon microflora presents a high metabolic capacity, capable to catalyse several reactions in drug metabolism. The interaction of these undesirable compounds and drugs with the colon microflora and the presence of numerous enzymes like cytochrome P450 enzymes (CYPs) may cause a biotransformation which can compromise intestinal barrier conditions and even lead to carcinogenesis (Cummings, 1975; Elefterios and Bezirtzoglou, 2012).

Overall, colorectal cancer ranks third in terms of incidence but second in terms of mortality. Over 90 % of CRC cases are sporadic, where a complex interplay between genetic and external factors determines neoplastic transformation to colorectal carcinogenesis (Vipperla and Keefe, 2016). Factors like dietary habits, obesity, smoking and alcohol are highly associated with the predisposition to CRC (Brenner, Kloor and Pox, 2014; Flint *et al.*, 2015; Vipperla and Keefe, 2016).

Thus, realizing how diet and other factors directly influence the colon microbiota is very important to clarify how colon is so susceptible to the development of certain kind of diseases.

1.1.1. Normal colon microbiota

Colon microbiota refers to a complex of microorganisms that colonizes the digestive tract and includes not just bacteria, but also other microbes, being the major reservoir of microorganisms the in human body (Cummings and MacFarlane, 1997; Hooper, 2004).

The human intestinal microbiota harbours 10^{12} microorganisms and it is composed by 500-1000 species and over of 3500 bacterial species (Gill *et al.*, 2006; Frank *et al.*, 2007; Jandhyala *et al.*, 2015). Colon ecosystem involves a stable community in normal symbiotic state (normobiosis) constituted predominantly by two major *phyla*: *Bacteroidetes*, such as *Prevotella* and *Bacteroides genera*; and *Firmicutes*, such as *Clostridium*, *Eubacterium* and *Streptococcus genera*. *Proteobacteria*, *Actinobacteria*,

Fusobacteria and *Verrucomicrobia* were also found in colon ecosystem, but in minor proportions **(Figure 2)** (Gill *et al.*, 2006; Liu, Cao and Cong, 2013; Jandhyala *et al.*, 2015; Gomes *et al.*, 2017).

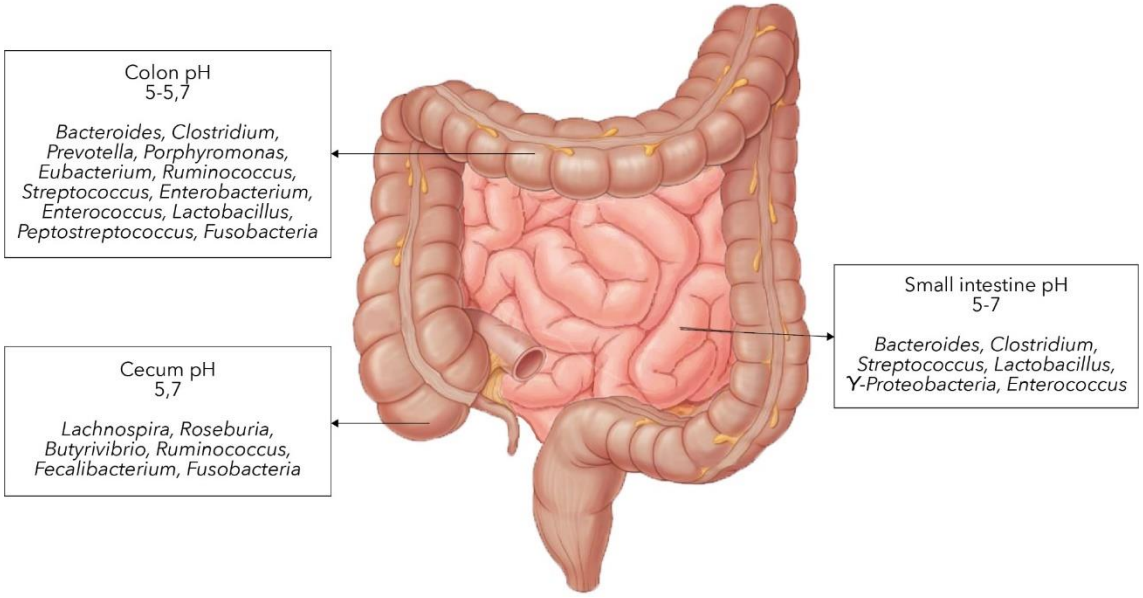

Figure 2: Normal distribution of human colon flora. Adapted from: Jandhyala *et al.*

This microbial profile is metabolic active using host-derived and diet-derived energy sources for growth, essentially by fermentative metabolism (Russell *et al.*, 2013) and plays an important role in the intestinal development and homeostasis (O'Hara and Shanahan, 2006; Marchesi and Shanahan, 2007). The colon microbiota maintains a symbiotic interaction with the gut mucosa. This mutual relationship between human health and colon microbiota is widely recognized. The intestinal microbiota is responsible for numerous important functions in human body like nutrient absorption and metabolism, degradation of indigestible substances, metabolization of drugs and xenobiotics, regulation and modulation of the immune system and protection against pathogens.

Thus, an healthy microbiota is essentially for health maintenance (Hooper, 2004; Gill *et al.*, 2006; Marchesi and Shanahan, 2007; Birt and Phillips, 2014; Jandhyala *et al.*, 2015; Landman and Quévrain, 2016).Moreover, these gut microorganisms are also responsible for protein digestion, yielding amines, carbon dioxide (CO_2), thiols, indoles, hydrogen (H_2), hydrogen sulphide (H_2S) and other metabolites which are potentially toxic for the colon contributing to its inflammation. However, these negative effects are balanced with the production of anti-proliferative and anti-inflammatory agents making the intestinal microbiota a very complex system (Macfarlane & Macfarlane, 2012; Birt and Phillips, 2014).

It is already reported the importance of microbiota in nutrients metabolism, and other important function

is the production of short chain fatty acids (SCFA) which are the main metabolites generated from the colonic microorganisms metabolism of carbohydrates and proteins and present an important role in stabilization of intestinal microbiota (Macfarlane & Macfarlane, 2012; Gomes *et al.*, 2017).

1.1.1.1. Short chain fatty acids

Short chain fatty acids, namely acetate, propionate and butyrate, can be produced naturally in the liver (Tan, McKenzie, *et al.*, 2014), but they are the main end products of carbohydrates, proteins, glycoproteins, polysaccharides and oligosaccharide fermentation made by colonic microorganisms, such as *Bifidobacterium*, *Bacteroides, Fecalibacterium*, *Roseburia* and *Enterobacteria* (Cummings *et al.*, 1987; Macfarlane and Macfarlane, 2003; Candela *et al.*, 2011; Jandhyala *et al.*, 2015). Caproate, valerate, formate and branched-chain fatty acids can also be formed but in minor concentrations (Macfarlane and Macfarlane, 2003).

The microflora composition affects directly the quantity of substrate available to bacterial fermentation, so, the SCFA production is dependent of the bacterial profile (Miller and Wolin, 1996; Macfarlane and Macfarlane, 2003; Allison and Macfarlane, 2009). Extrinsic factors like diet composition, age, diseases, exposure to drugs or xenobiotics and intrinsic factors like high or low pH, high bile salt concentrations or low micronutrient can influence the amount of SCFA in human intestine. However, their concentrations vary along the intestine according to the pH. Lower values of pH remain to higher rates of carbohydrates fermentation (**Figure 3**). In colon's normal conditions, acetate, propionate and butyrate are present at a molecular ratio of 60:25:15, respectively (Cummings *et al.*, 1987; Tan, McKenzie, *et al.*, 2014; Koh *et al.*, 2016).

As the principal anions of the intestine, SCFA are absorbed by non-ionic diffusion but also by active transport, mediated by a sodium-coupled transporter (Roy *et al.*, 2006). Butyrate is consumed by the gut epithelium, being the principal energy source for colonocytes. Propionate is metabolized in the liver by hepatocytes and can be found in its periphery in lower concentrations. Lastly, acetate is the only fatty acid that remains in the liver or can be released in peripheral venous system and it's the most detectable SCFA in blood. Although, propionate and butyrate can also affect indirectly the peripheral organs through hormonal and nervous systems activation (Hosseini *et al.*, 2011; Gonçalves and Martel, 2013; Russell *et al.*, 2013; Tan, McKenzie, *et al.*, 2014; Koh *et al.*, 2016).

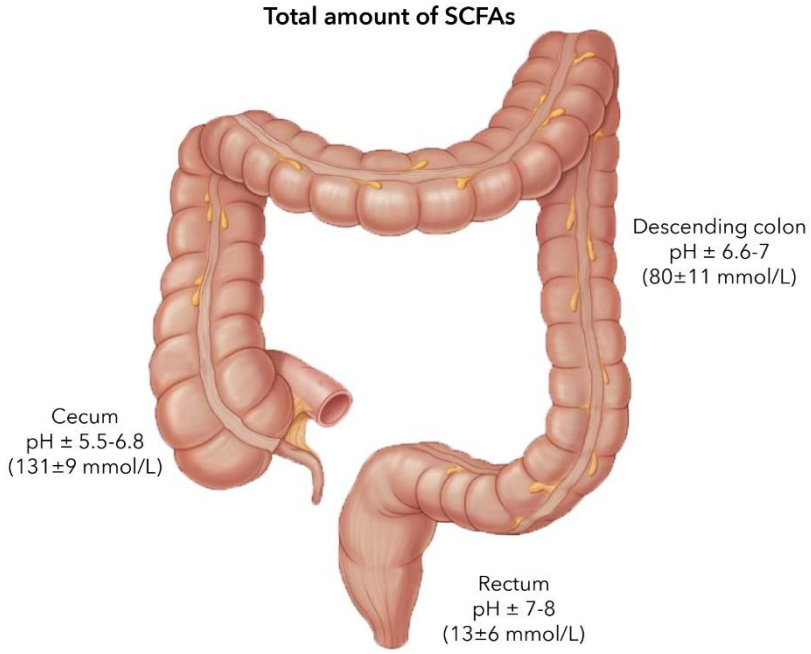

Figure 3: SCFA concentrations along the intestine according to pH. Adapted from: Gomes *et al.*

1.1.1.1. Impact of diet in SCFA production

The availability of non-digestible carbohydrates required for SCFA production varies with the type of diet and mealtimes. The ingestion of these compounds leads to an increased colonic fermentation, enhancing SCFA production. A high fiber diet enriched with whole grains and cereals is associated with a low risk of colorectal cancer in comparison with a poor fiber diet (Louis, Hold and Flint, 2014; Flint *et al.*, 2015; Trial *et al.*, 2015).

In addition to increasing the production of acetate, butyrate and propionate, a high fiber diet is also essential for colon maintenance and healthy. These non-digestible carbohydrates are fermented by host microorganisms in the microbiota providing the main energy source to support microbial growth in the colon (Duncan *et al.*, 2007; Louis, Hold and Flint, 2014).

Furthermore, some studies have demonstrated that a lower fiber intake of may cause alteration in microbiota composition (dysbiosis), decreasing the microbial activity (**Figure 4**). This type of diet have

been largely associated with some diseases such as diabetes, obesity and certain types of cancer, namely, CRC development (Gill and Rowland, 2002; Duncan *et al.*, 2007; Gomes *et al.*, 2017).

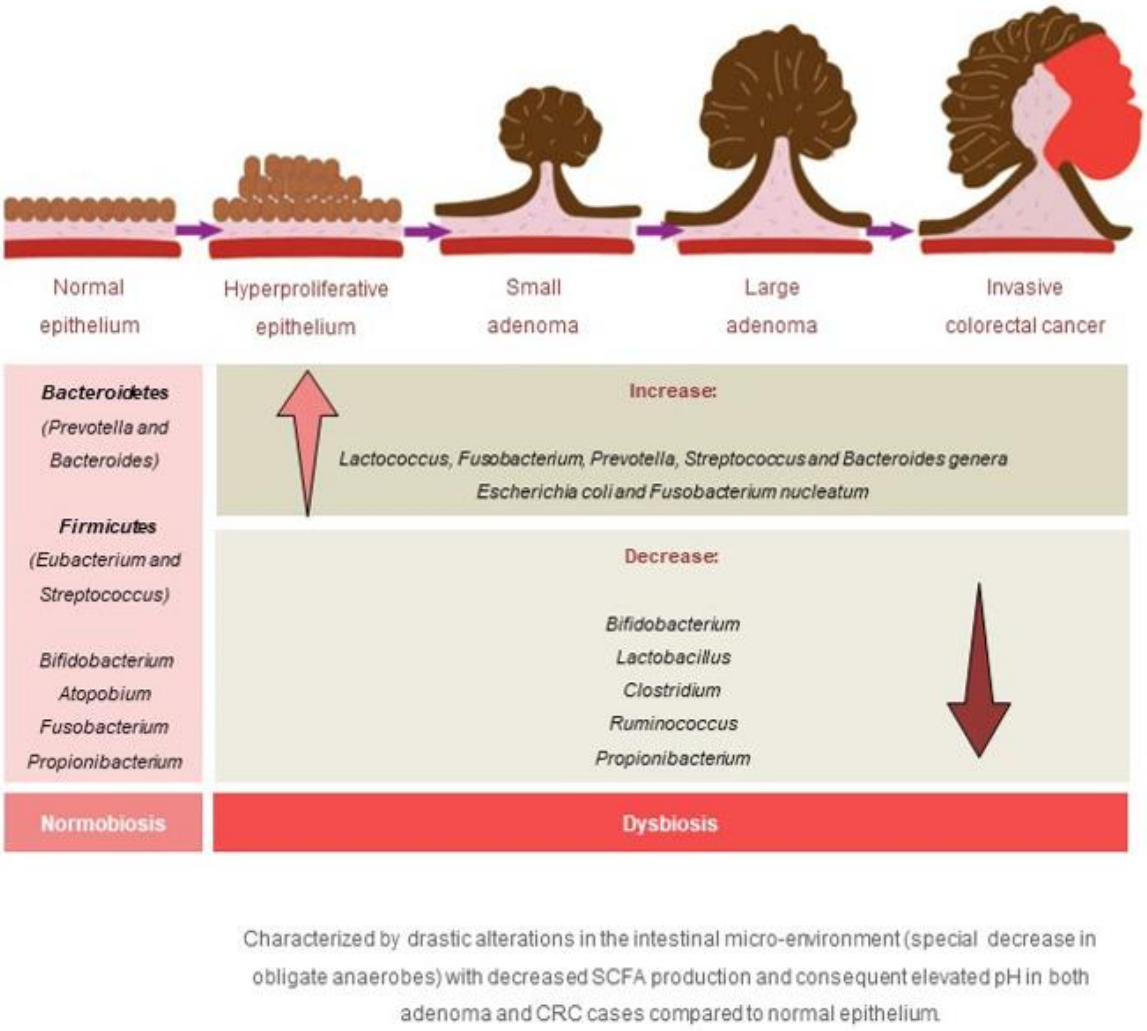

Figure 4: Differences in colon microbial composition in the different stages of CRC development. Adapted from: Gomes *et al.*

1.1.1.2. Physiological effects of SCFA

It was already mentioned that diet influences directly the microbiota composition and the substrate for bacterial metabolite formation. A rich fiber diet increases the substrate and consequently the production of SCFA (Candela *et al.*, 2011; Kasubuchi *et al.*, 2015).

The principal function of SCFA is maintaining the colon homeostasis. They play important roles in numerous processes such as regulation of intracellular pH, cell volume and other functions associated with ion absorption and gut motility (Louis, Hold and Flint, 2014). Acetate, butyrate and propionate work on cell membrane assembly and mucosal cell migration resulting in an increase of colonocytes proliferation and differentiation that contribute to a healthy intestinal barrier. This effect is of extreme importance since a strong intestinal barrier reduces the changes of infection by bacteria, namely, Gram-negative pathogens, decreasing the possibility of colon inflammation or tumour invasion (Van der Beek *et al.*, 2017; Tian *et al.*, 2018). SCFA also protect the epithelium through immune and oxidative stress modulation. In addition, they have multiple beneficial effects in human body and health maintenance affecting positively other organs like muscles, kidney, heart and brain, providing energy sources for their correct function **(Figure 5)** (Macfarlane & Macfarlane, 2012).

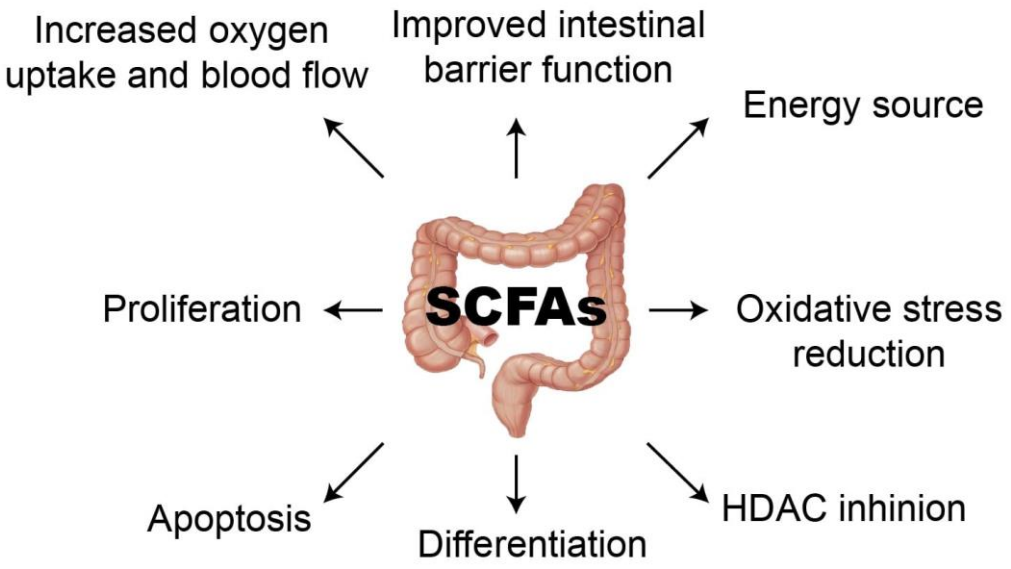

Figure 5: Schematic representation of SCFA functions in the human body. Adapted from: van der Beek *et al.*

SCFA have been described as potent anti-inflammatory and anti-cancer agents. They are associated with the induction of apoptosis mechanisms, cell cycling arrest, inhibition of cell migration, reducing invasiveness of cancer cells. Furthermore, they inhibit the activity of histone deacetylases (HDACs) in colon and immune cells and regulate colonic regulatory T cells which is particularly important for the anti-inflammatory response. Butyrate is the most well studied SCFA and it is very important for the colon health. It has been demonstrated as the most potent SCFA since it exerts multiple effects such as the inhibition and prevention of colon carcinogenesis. Propionate exhibits an antiproliferative effect similar to

butyrate, but weaker. However, various studies have demonstrated that acetate and propionate potentiate the effect of butyrate (Vecchia *et al.*, 1997; Canani *et al.*, 2011; Hosseini *et al.*, 2011; Macfarlane & Macfarlane, 2012; Tan, McKenzie, *et al.*, 2014; Boron and Boulpaep, 2015; Gomes *et al.*, 2017).

On the other hand, acetate is the less studied SCFA. So, our research group have been focused in understanding the role of acetate in colorectal cancer in order to discover new approaches to prevent or treat CRC.

1.1.1.3. Acetate

Acetate is the shortest monocarboxylic acid with only two-carbons (C2). Endogenously, it is mainly produced by acetogenic bacteria such as *Acetobacterium* species, *Clostridium aceticum* and *Propionibacterium.* It can also be produced via the Wood-Ljungdahln, which is a biochemical reaction that leads to the formation of acetate from carbon dioxide and hydrogen. (Miller and Wolin, 1996; Ragsdale and Pierce, 2008; Koh *et al.*, 2016).

Acetate, propionate and butyrate exhibits pKa values of 4.76, 4.88 and 4.82, respectively. At neutral pH, less than 1% of these fatty acids exist in the protonated form, meaning that these compounds do not cross the plasma membrane via simple diffusion (Roy *et al.*, 2006; van der Beek *et al.*, 2017). However, the transport of these metabolites requires the intervention of carrier transporter proteins. The main transport systems acting in the uptake of SCFA are monocarboxylate transporters (MCTs) mainly monocarboxylate transporter-1 (MCT-1), expressed in apical and basolateral membrane of colon epithelium and monocarboxylate transporter-4 (MCT-4), expressed only in the basolateral membrane. The expression of these monocarboxylate transporters is dependent upon the expression of the transmembrane glycoprotein CD147, which is a chaperone. Furthermore, sodium-coupled monocarboxylate transporters (SMCT) also intervenes, like SMCT-1 and SMCT-2 (**Figure 6**) (Cuff, Lambert and Shirazi-beechey, 2002; Roy *et al.*, 2006; Kim, Park and Kim, 2014; Ferro *et al.*, 2016). Our research group showed that the acetate transport occurs via SMCT-1 and by passive diffusion via aquaporins in CRC cells (Ferro *et al.*, 2016).

The formation of this SCFA occurs in higher quantities than butyrate and propionate. Acetate is the primary substrate for the cholesterol synthesis, so, it is rapidly absorbed in the proximal colon and transported to the liver for this purpose. In 2002, Duncan and co-workers published an article where they prove that acetate is largely utilized by bacteria to produce butyrate and, recently, Boets *et al.*

demonstrated that less than 1 % of acetate is absorbed colonically and less than 15 % is incorporated into fatty acids. This indicates that besides the fact that acetate is involved in SCFA *de novo* lipogenesis it also acts, not as a single component, but together with them (Duncan *et al.*, 2002; Boets *et al.*, 2017).

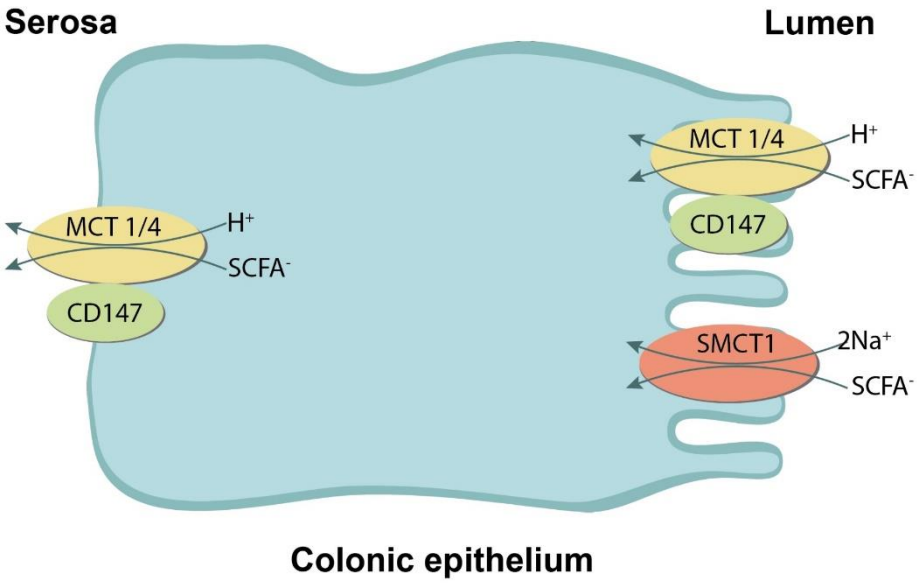

Figure 6: Schematic representation of short-chain fatty acids transporters in the colon epithelium. Adapted from: Sivaprakasam *et al*.

One of the most important beneficial effects exerted by SCFA is the role in the modulation of host defence responses and protection against infectious agents. In 2011, Fakuda *et al.* reported that acetate seems to be involved in the protection of enteropathogenic infection caused by *Escherichia coli.* In this publication they used germ-free BALB/c mice and mice monoassociated with bifidobacteria (producer of SCFA). The animals were orally inoculated with a single gavage of 10^4 CFU of *E. coli* O157:H7 strain 44^{Rf} and after 7 days the germ-free mice died and the mice with *bifidobacitirium* association survived. To understand the differences between germ free and bifidobacteria monoassociated mice they analysed the metabolic profile of feces and they observed that the concentration of acetate is significantly higher in the monoassociated mice in comparison with germ free mice. With these results they proposed that acetate produced by protective bifidobacteria improves intestinal protection mediated by epithelial cells and thereby protects the host against lethal infection (Fukuda *et al.*, 2011).

Besides the physiological effects in the colon, acetate is involved in several potentially important processes in colon carcinogenesis. Studies in cancer cell lines have demonstrated that acetate is capable to induce apoptosis mechanisms with mitochondrial alterations (Jan *et al.*, 2002). Furthermore, our group shown

that acetate decreased CRC cell proliferation followed by DNA fragmentation and caspase-3 activation, promotes lysosomal membrane permeabilization (LMP), induces mitochondrial dysfunctions, inhibits autophagy and changes CRC cell glycolytic phenotype (Marques *et al.*, 2013; Oliveira *et al.*, 2015).

Thus, based on this data, our research group hypothesized that beyond the effects on CRC cells, acetate could exert protective effects on normal colon cells against external factors like food contaminants, namely, pesticides.

1.2. The use of pesticides in food production

Over the years, it has been observed an increase in the world population, which can be closely associated with an increase in food production, being expected to double by 2050 (Silva *et al.*, 2019). Increased food production will require massive farming. However, this type of food production needs to be profitable, which makes the use of pesticides necessary in order to overcome problems such as plagues and pests. Since agriculture is the main consumer of these types of pesticides (about 80%), its introduction into the agro-livestock industry may cause instabilities in the ecosystem, thus contributing to large-scale environmental imbalance (Silva *et al.*, 2019).

To maintain the effectiveness of the agricultural production these substances are widely used to prevent the crops of the pests and vectors of plant diseases, yielding the quality of the product. Pesticides are also used to improve the weight and nutritional value of the food. Despite agriculture being the major consumer of these substances, they can be used in public health activities, for example, to control diseases like dengue or malaria (Boxal, 2001; Cooper and Dobson, 2007; Damalas and Eleftherohorinos, 2011).

Thus, from this point of view, these chemicals seems to be an easy and useful tool to handle the agricultural production. However, these substances are released deliberately into the ecosystem and their intensive application due to the agricultural massification can result in a bioenvironmental dispersity contamination. When a pesticide reaches the soil in the target area, it can disappear through degradation/dispersion or it can be volatile and leave residues in water or soil accessible to the population. The extensive use of these chemicals implies that humans are constantly exposed to them (Alavanja and States, 2009; Damalas and Eleftherohorinos, 2011; Silva *et al.*, 2019) consequently leading to some health hazards. The general population is exposed to these pesticides through indirect sources, including food, water or by inhalation of contaminated air (Parrón *et al.*, 2014).

1.2.1. Pesticides classification

Pesticides are substances, or mixtures of substances, used to prevent or reverse the damage caused by a particular pest, disease and other plant pathogens to maintain its high production and product quality. The generic term "pesticide" represents multiple subgroups according to their target organism and function, being classified into insecticides (used to kill certain insects that may cause diseases in animal and humans), herbicides (used to prevent the growth of invasive weeds) and fungicides (used to protect crops from various fungi). Beyond that, they can be a chemical substance (synthetic or naturally occurring), biological agent (virus or bacteria), antimicrobial or another substance used against any pest (Shibamoto and Bjeldanes, 2009; Damalas and Eleftherohorinos, 2011; Parrón *et al.*, 2014; Hernández, Gil and Lacasaña, 2017).

1.2.2. Pesticides in the food chain

During the food production, pesticides are used in several different steps, either pre- or post-harvest. The direct consumption of treated products is the principal route for the pesticides to enter in the food chain (Damalas and Eleftherohorinos, 2011). Moreover, some of them are insoluble or persistent and the constant application can contaminate the soil as well the water. In the last few years, water contamination by pesticides has been increasing, being a huge concern for the public health. This contaminated water is used in the processing or preparation of food and it is accessible to the consumption by animals, being possible to find pesticides residues in meat, milk, non-pastured products or in fish that live on this types of water (Taylor, Harris and Gaston, 2007; Damalas and Eleftherohorinos, 2011; Parrón *et al.*, 2014; Kim, Kabir and Jahan, 2017).

Diet appears to provide the easier source of multiples carcinogens that reach the DNA, challenging the human repair machinery. This type of exposure originating from pesticide residues in food or water commonly is associated with low doses and it is chronic, which means that the long-term exposition of low doses of these chemicals increases the long-term diseases, like cancer (Carpy, Kobel and Doe, 2010).

1.2.3. The impact of pesticides exposure in human health

Despite their benefits in controlling agricultural pests, plant diseases, harmful organisms to humans and vector-borne diseases, pesticides present some risks to public health. These substances or mixtures of substances are designed to be toxic against a specific organism, however, it can also present toxic effects in humans (Cooper and Dobson, 2007; Damalas and Eleftherohorinos, 2011).

The excessive use of pesticides results in a chronic exposure to the low levels of their active substances, being the diet the principal route of contamination. This chronic exposure can cause cellular mutations and, in the last years, several studies have demonstrated the link between pesticides and different diseases like asthma, allergies, hypersensitivity, hormone disruption, neurodegenerative diseases (Parkinson Disease), diabetes and cancer (Parrón *et al.*, 2014; Rizzati *et al.*, 2016; Kim, Kabir and Jahan, 2017).

The Hallmarks of Cancer model argue that DNA damage is not the only event leading to malignancies, being other carcinogen-induced changes in some protective elements also needed (Hanahan and Weinberg, 2011). Exposure to xenobiotics, namely, pesticides have been described as a potent inducer of genomic damage, oxidative stress and a high expression of some cancer-related genes. These effects caused by pesticides have been associated with the induction of somatic genomic mutations and epigenetic changes (**Figure 7**). Genomically mutated cells may induce alterations in the tissue microenvironment providing their survival and expansion. In this way, new considerations about the carcinogenic or cancer-promoting effects of environmental chemicals must be taken into consideration (Uyemura *et al.*, 2017; Yumi *et al.*, 2017).

Párron T. *et al.* published a study in which they compare the risk of cancer development in populations of 10 different districts of Andalusia (south of Spain). The results have demonstrated that populations living in areas with high pesticide utilization present an increased risk of cancer. According to the statistical analysis, stomach, colorectal, liver and lung cancers are the most prevalent types and this data supports the evidence of pesticides as risk factor for cancer progression (Parrón *et al.*, 2014).

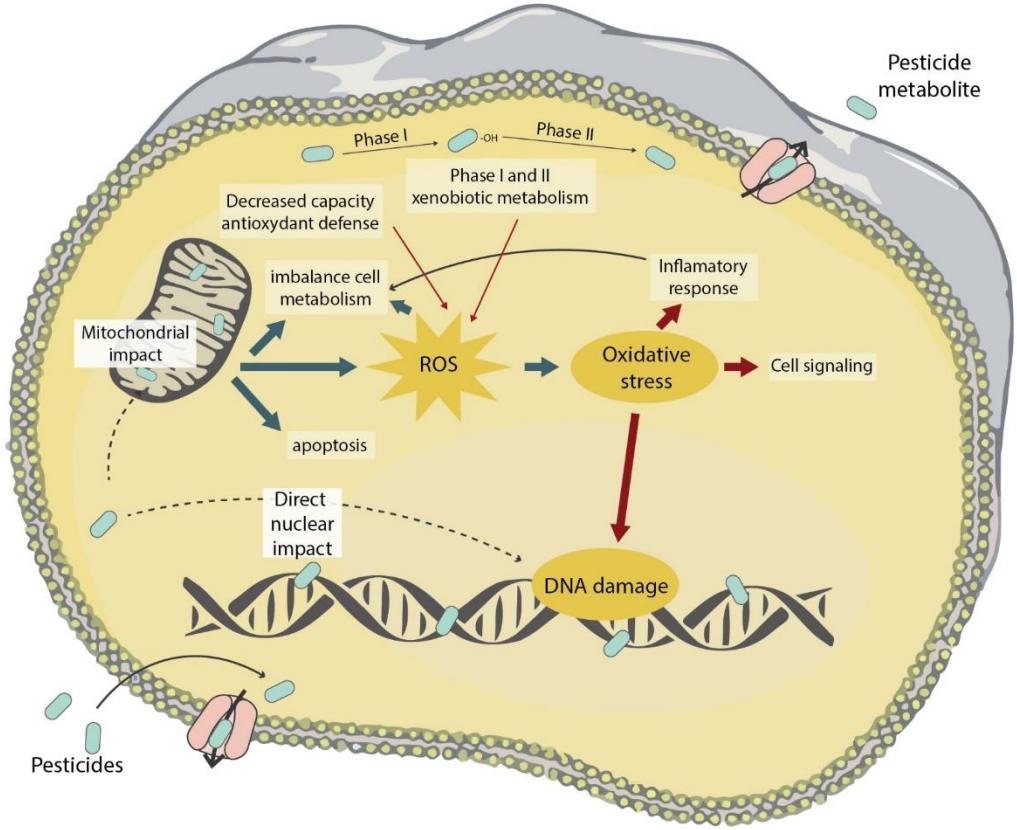

Figure 7: Illustration of various effects of pesticides in the cell. Adapted from: Rizzati *et al.*

1.2.4. Biochemical interactions of pesticides

Pesticides can enter the human body through oral, respiratory or dermal exposure. After being absorbed, they are conducted to the bloodstream and can reach distant tissues and organs. They can be excreted through skin, exhaled air and urine, but in some cases, they accumulate in specific tissues/organs (Kim, Kabir and Jahan, 2017). When accumulated, pesticides can suffer some biochemical reactions, namely, toxicokinetic (TK) and toxicodynamic (TD) reactions leading to long-term problems. Data about these reactions is important to the consideration of the risks induced by pesticides to humans (Gabsi, Zenker and Preuss, 2016; Hernández, Gil and Lacasaña, 2017).

TK reactions occur when pesticides interacts with other substances and changes their absorption, distribution, metabolism or elimination. This interaction may cause an increase in the internal dose of the pesticide concentration at its target site. It can also interfere with membrane transporters present in excretory organs, increasing or decreasing their expression, wich what results in alterations in their bioavailability and toxicity (Hernández *et al.*, 2013; Hernández, Gil and Lacasaña, 2017). When a

pesticide/substance acts as inhibitor or substrate of certain membrane proteins, it is expected an alteration of absorption and transport of the other pesticide/substance to its target sites. Furthermore, through TK reactions, pesticides can induce or inhibit CYP450 enzymes. This superfamily of enzymes is responsible for protecting the organism by processing chemicals through oxidative reactions to inactive metabolites, that can be eliminated from the body and play a role on the activation of protoxins and pro-carcinogens essential to the protection of chemical-induced diseases. So, with this interaction with CYP450, pesticides are capable to control their detoxification and the toxicity degree (Abass, Turpeinen and Rautio, 2000; Hodgson, 2003).

On the other hand, TD reactions may also occur, but with less frequency. Whereas TK reactions have their mechanisms well demonstrated, TD reactions are still poorly understood. They happen when one pesticide presents the same effect as the other substance/pesticide but act by different mechanisms, potentiating it (Hernández, Gil and Lacasaña, 2017). TD reactions involving pesticides can lead to the induction of mitochondrial dysfunction through mitochondrial respiratory chain complex damages or alterations in oxidative phosphorylation. They can also result in the generation of oxidative stress with the production of reactive oxygen species (ROS), by the decrease of antioxidant species or by the upregulation of xenobiotic enzymes like CYP450. Is important to notice that these effects are extremely harmful since they can alter cellular signalling enzymes involved in cell differentiation and survival, inhibit the cell growth, induced DNA damage and interfere with inflammatory responses (Rizzati *et al.*, 2016). A very common example is when a pesticide can interfere with DNA repair mechanisms and the other induce DNA damage, leading to genotoxicity problems (Hernández *et al.*, 2013; Hernández, Gil and Lacasaña, 2017).

1.2.5. The effect of pesticides in the human colon

The gastrointestinal (GI) tract is the principal route of entry for food contaminants, namely pesticides in the bloodstream. Colon, specifically, presents the greatest surface area for the absorption of these compounds. Moreover, factors like the blood supply, mucosal structure, intestinal motility, gut flora, enzymatic and cellular transport systems makes colon the preferential absorption site (National Research, 2004).

It is important to considered that the gut is the major endocrine organ in the human body, and once in the colon, pesticides can suffer biotransformation reactions which can generate undesirable metabolites. These generated compounds may cause morphological and functional changes of intestinal epithelial

cells increasing the intestinal permeability and bacterial translocation as well as the disruption of microorganism's environment leading to microbiota dysbiosis. In this way, this alteration in colon's microbiota, caused by pesticides metabolites, may promote chronic inflammation facilitating the cancer initiation and progression (Potter, 1999; Strickland, Potter and Joo, 2004; Won *et al.*, 2007; Parrón *et al.*, 2014).

It was already described the role of SCFA, namely acetate, in the colon microbiota and their importance for the host individual health and homeostasis. Indeed, these SCFA presents local and/or systemic effects. So, research on acetate's protective role in the colon against food contaminants like pesticides can be very useful to create preventive strategies to minimize the effects caused by these chemical compounds (Reygner *et al.*, 2016).

Based on these evidences, the pesticide chosen to evaluate if acetate presents or not a protective role against pesticides was tebuconazole, which is one of the most used fungicides in Portugal as well as in Europe.

1.2.6. Tebuconazole

Tebuconazole [(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol] is a triazole fungicide currently used worldwide (**Figure 8**) (Strickland, Potter and Joo, 2004). It is widely used in agriculture due to its effectiveness to control diseases in fruit, cereal, nut and vegetable crops (Strickland, Potter and Joo, 2004; Muñoz-Leoz *et al.*, 2011). In addition to agricultural applications, triazole fungicides are also used in industrial applications and as an active ingredient in numerous pharmaceutical products like fluconazole (Victoria Castelli, Gabriel Derita and López, 2017; Petricca *et al.*, 2019).

Tebuconazole presents some intrinsic properties, such as chemical and photochemical high stability, low biodegradability and due to its persistency is frequently founded in agricultural products, water and foods (Kahle *et al.*, 2008; Muñoz-Leoz *et al.*, 2011; Petricca *et al.*, 2019). In an investigation performed by Silva V. *et al.,* 317 soil samples in Europe were collected and analysed. Their results showed that tebuconazole is one of the most founded pesticides at high concentrations in the soil samples, and consequently responsible for the soil contamination (Silva *et al.*, 2019).

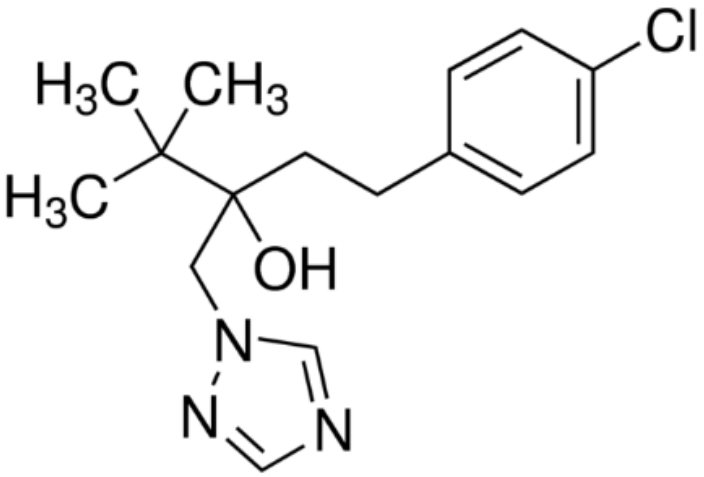

Figure 8: Tebuconazole chemical structure. Available from: https://www.sigmaaldrich.com/catalog/product/sial/32013, accessed on 08/06/2018.

This broad-spectrum fungicide acts by disruption of cell membranes and walls through the inhibition of fungal lanosterol-14R-demethylase which is a highly conserved cytochrome P450 enzyme encoded by the CYP51 gene (**Figure 9**) (Fischer *et al.*, 1991; Goetz *et al.*, 2007; Kahle *et al.*, 2008). That inhibition decreases the production of ergosterol which is fundamental for the integrity of fungal cell membrane, causing their disruption (Lamb, Kelly and Kelly, 1998).

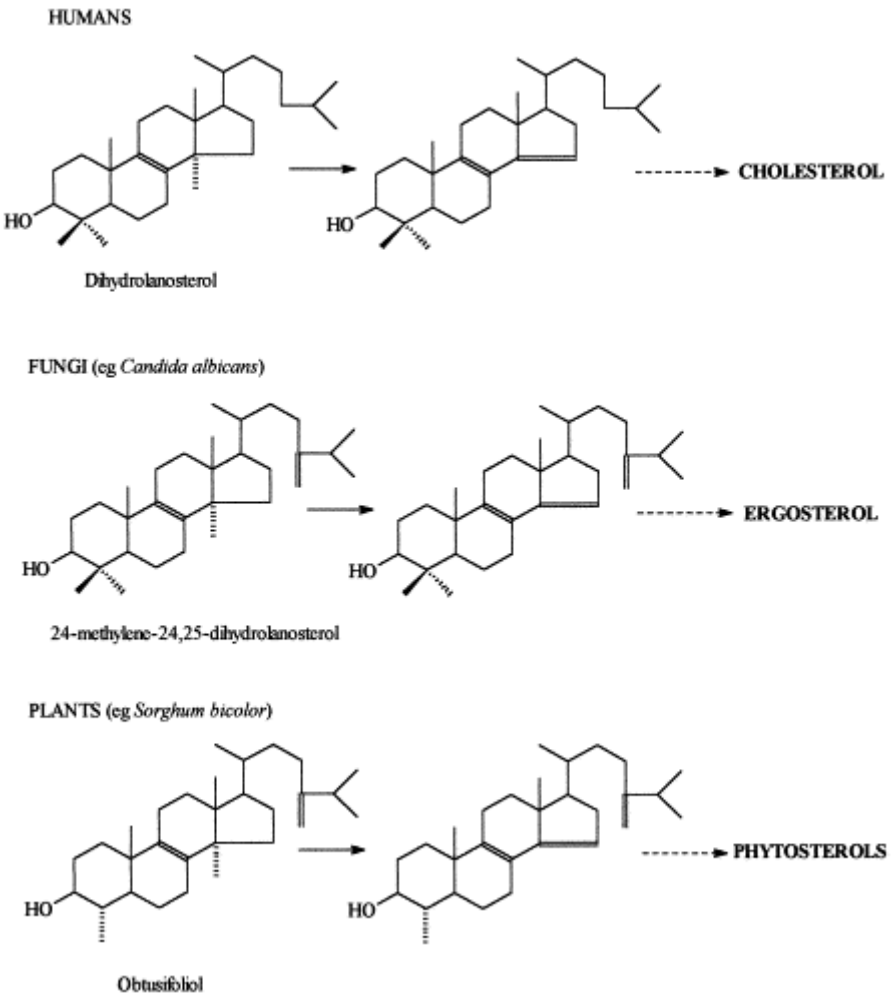

Figure 9: Schematic representation of CYP51 mechanism of action. Adapted from: Lamb *et al.*

CYP51A1 (Cytochrome P450 Family 51 Subfamily A Member 1) is the only family of P450's which is evolutionary conserved between animals, plants and fungi (Lepesheva and Waterman, 2004). However, in humans, the inhibition of this enzyme is not crucial, once the diet supplies the steroid intermediate with cholesterol (Tro *et al.*, 2004; Goetz *et al.*, 2007). In addition to CYP51, most of the triazoles can also inhibit the aromatase CYP19 which converts androgens into estrogens affecting the steroid homeostasis (Taxvig *et al.*, 2007; Petricca *et al.*, 2019). The inhibition of this enzyme may cause multiple endocrine-disrupting effects.

To evaluate the potential of endocrine-disrupting effects of tebuconazole Taxvig C. and co-workers assessed the endocrine-disrupting activity *in vivo*. Rats were exposed to 50 or 100 mg/kg of tebuconazole during pregnancy from gestational day 7 and continued during lactation until postnatal day 16. At the end of the exposure period, they observed a reduction of testosterone followed by an increase of progesterone levels. This hormone destabilization affects the reproductive development causing an increased nipple

retention (Taxvig *et al.*, 2007). Furthermore, Christen V. *et al.* studied the antiandrogenic effects caused by these pesticides. For this study, MDA-kb2 (mammary gland /breast epithelial cell line) was treated with different doses of tebuconazole and azole mixtures and the results suggested a strong antiandrogenic activity for tebuconazole but more evident in mixtures with other triazoles (Christen, Crettaz and Fent, 2014).

CYP450s are divided into two distinct and different groups according to their function. The first group includes the enzymes responsible for the metabolization of xenobiotics (drugs, pesticides, pollutants, etc.) and the other includes the enzymes which participate in key biosynthetic mechanisms, such as sterol biosynthesis and steroidogenesis (Lepesheva and Waterman, 2004). Besides CYP51 and CYP19, it is known that triazole pesticides are capable to modulate the expression of numerous CYP450's monooxygenases. The presence of the azole group in their structure makes many triazoles capable to inhibit several CYP450's isoforms, enzymes necessary for the metabolism and detoxification of lipophilic compounds, namely pesticides. Through the inhibition of these enzymes, they decrease their metabolism and consequently increase their internal concentration as well as their toxicity (Dvorak, 2011). Moreover, they act synergistically with other substances or pesticides, such as pyrethroids (insecticide) enhancing their toxic effect. Regarding tebuconazole, there are not enough studies to prove its behaviour, however, predicting the biological responses caused by this specific fungicide is important for the establishment of correct usage parameters (Hernández, Gil and Lacasaña, 2017).

Besides endocrine-disrupting effects, reproductive alterations have been described. In 2016, Zhou *et al.* have demonstrated the effects of tebuconazole in trophoblast cells, which are essential for successful placenta development. Trophoblast cell line HTR-8 was treated with 0, 5, 10, 20, 40 and 80 μM during 24 h. Firstly, they evaluate the effect of tebuconazole on cell viability observing a significant reduction of viability at 20-80 μM. After that, they could observe a change in cell cycle distribution of G1 and G2 phases of trophoblasts and an increase of necrotic cells, late apoptotic cells and early apoptotic cells, followed by a down-regulation of anti-apoptotic protein Bcl-2 and the increase of the apoptotic protein Bax. Lastly, they shown that tebuconazole is capable to decrease the invasive and migratory capacity of trophoblasts through the disrupting protease systems, angiogenic factors, hormones, cytokines and growth factors. These data evidence the adverse effects of tebuconazole in human pregnancy (Zhou *et al.*, 2016).

Additionally to endocrine and reproductive effects, nervous and immune systems have been reported as potential targets for triazole fungicides. Moser *et al.* have investigated the neurological and immunological

effects caused by tebuconazole exposure in rats. Initially rats were administrated with 0, 6, 20, and 60 mg/kg of tebuconazole from gestational day (14) to postnatal day (7). One group of rats was used for immunological and reproductive parameters evaluation and other for neurobehavioral tests, which include a series of functional and cognitive assays. At the level of the immunological system, no significant differences were observed. However, rats exposed to tebuconazole, mainly to 20 and 60 mg/kg presented alterations in acquisition of learning and neuropathological evaluations demonstrated pyknotic cells across hippocampal, which reveals tissue apoptosis and cells loss in specific areas of hippocampus and neocortex. Thus, these results confirm that tebuconazole causes neurobehavioral and neuropathological nefarious effects (Moser *et al.*, 2001). Corroborating these results, other investigators showed that exposure of 5.2 μM of tebuconazole in PC12 cells (adrenal phaeochromocytoma) inhibits depolarization-evoked calcium influx through inhibition of voltage-gated calcium channel (VGCC) reducing dopaminergic neurotransmission. This inhibition is very prejudicial to nervous system since the maintenance of the intracellular calcium homeostasis is of extreme importance for its correct functioning (Heusinkveld *et al.*, 2013).

Being the colon one of the first organs in contact with pesticides, namely in the involvement of their metabolism, its relevant the study of the effects caused by tebuconazole in this place. In this way, our research group have been focused in studying tebuconazole implications in the colon. Recently, it was showed that NCM460 (normal colon cell line) treated with 0.16 mM (IC_{50}) and 0.32 mM (double of IC_{50}) of tebuconazole lead to alterations in cell growth, cell death, induce DNA double strand breaks and oxygen reactive species (ROS) (Macedo, 2018).

Table 1: Negative effects of tebuconazole

Tebuconazole			
Model	Concentrations	Effect	Reference
Animal model Rat	50-100 mg/kg	Testosterone reduction and progesterone increase	(Taxvig *et al.*, 2007)
Cell line MDA-kb2	2.6-6.86 µM	Antiandrogenic effects	(Christen, Crettaz and Fent, 2014)
Cell line HTR-8	20-80 µM	Reduction of cell viability	(Zhou *et al.*, 2016)
Cell line HTR-8	20-80 µM	Induction of apoptosis	(Zhou *et al.*, 2016)
Cell line HTR-8	20-80 µM	Reduction of the invasive and migratory capacity in trophoblasts	(Zhou *et al.*, 2016)
Animal model Rat	20-60 mg/kg	Alterations in the acquisition of learning	(Moser *et al.*, 2001)
Cell line PC12	5.2 µM	Inhibition of VGCC and dopaminergic neurotransmission	(Heusinkveld *et al.*, 2013)
Cell line NCM460	50.7-100.14 µg/ml	Inhibition of cell growth	(Macedo, 2018)
Cell line NCM460	50.7-100.14 µg/ml	Induction of apoptosis	(Macedo, 2018)
Cell line NCM460	50.7-100.14 µg/ml	Induction of ROS	(Macedo, 2018)

CHAPTER 2 – RATIONALE AND AIMS

Every day we ingest food that, in some way, are contaminated with a huge number of toxic agents capable of promoting the development of several diseases or acting in the progression of pre-existing ones. As a result of the massive food production, pesticides are one of the most founded compounds in agriculture-derived products. Despite their benefits in crop protection and in the improvement of food quality, their excessive use results in serious environmental and health concerns (Parrón *et al.*, 2014; Silva *et al.*, 2019). Tebuconazole, one of the most used fungicide in agriculture, has been reported as a possible carcinogen. Furthermore, some studies also demonstrated that this chemical is implicated in hormone alterations, neurodegenerative diseases and immune system dysregulation (Taxvig *et al.*, 2007; Christen, Crettaz and Fent, 2014; Zhou *et al.*, 2016).

Being the gastrointestinal system, namely colon, one of the most affected systems of the human body, our research group have demonstrated that this chemical is capable to induce ROS production, activate autophagy and apoptosis mechanism and inhibit cell growth in normal colon cell line, which might demonstrate a potential involvement in colorectal cancer (CRC) development (Macedo, 2018).

The intestinal microbiota is responsible for several important functions in human body, assuming a significant role in intestinal homeostasis and helping in the prevention of intestinal carcinogenesis (Tan, Mckenzie, *et al.*, 2014). However, one of the most important function is the fermentation of non-digestible substrates in order to support the growth of specific microbes responsible to produce short chain fatty acids (SCFA), namely, acetate, propionate and butyrate, which has been associated with CRC prevention and therapy (Louis, Hold and Flint, 2014).

Acetate, is the most abundant SCFA in the colon and, our research group have already proved that it inhibits CRC cell proliferation, induces apoptosis and promotes lysosomal membrane permeabilization with cathepsin-D release which has a protective role in CRC cells (Oliveira *et al.*, 2015). Although, little is known about the influence of acetate in normal colon cells.

The increasing complexity of questions in cancer prevention, more approaches are required to understand other preventive pathways. In this project, **we aim to investigate the protective role of acetate in the toxicity of food contaminants** like tebuconazole **in human colon using *in vitro* models.**

Specifically, we aim at:

1- Evaluate the possible protection of acetate in the effect of tebuconazole in cell growth, viability, cell death and apoptosis.

2- Evaluate the impact of acetate and tebuconazole in the mitochondrial respiratory chain.

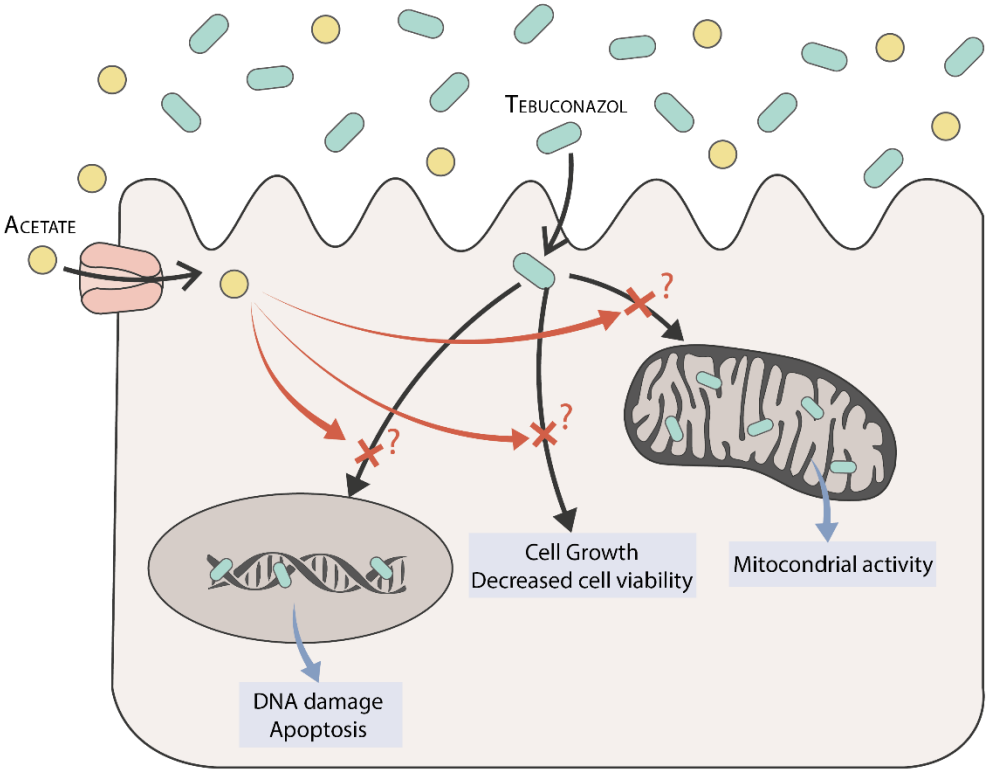

Figure 10: Schematic representation of the general aim of this work.

CHAPTER 3 – MATERIALS AND METHODS

3.1. Cell line and culture conditions

For this work, the cell line NCM460 obtained from INCELL, San Antonio, TX, USA was used. NCM460 is a normal human colon cell line, derived from normal colon epithelial cells (Moyer *et al.*, 1996).

Initially, one vial of frozen cells of NCM460 stored at -80 °C, was thaw. The mixture of cells and frizzing mixture was transferred to a 15 ml tube where it was resuspended in complete medium. The cell suspension was centrifuged at 1200 rpm for 5 minutes. The pellet was resuspended in fresh complete medium and transferred into a culture flask.

The cells grew in Roswell Park Memorial Institute (RPMI) 1640 medium (Biowest®) supplemented with 10 % FBS (v/v) (Biowest®) and 1 % penicillin/streptomycin (v/v) (Biowest®) and maintained in 25 cm^2 tissue culture flasks at 37 °C under a humidified atmosphere containing 5 % CO_2. Cells were subcultured once a week at a dilution of 1:5 and when they reach to 80 % of confluence, the culture medium was removed, and cells washed with PBS 1x buffer (Phosphate Buffered Saline). Washed cells were trypsinized (trypsin-EDTA 0.05 % (v/v)) for 5 min at 37 °C, in a 5 % CO_2 atmosphere. Fresh culture medium was added, in order to neutralize the trypsin effect and 1 ml of cell suspension was transferred to a new 25 cm^2 tissue culture flask with 4 ml of fresh culture medium.

All cells were manipulated in a flow chamber under aseptic conditions.

3.2. Mycoplasma detection

Mycoplasma contamination is one of the principal contaminations in cell cultures and can spread very quickly without detection. These organisms may interfere with several cellular characteristics and mechanisms compromising the results.

In order to test the presence of mycoplasma in cell culture, 200 µl of cell supernatant was collected from culture plates with almost 100% confluence for a microtube, which was heated in a dry bath at 95 °C for 10 minutes. Then, the samples were stored at -20 °C and the mycoplasma test was performed by polymerase chain reaction (PCR) using VenorTMGeM Mycoplasma Detection Kit (Sigma-Aldrich®).

Cells that showed positive results for mycoplasma contamination were discarded or treated with 2 µl of plasmocin treatment (Invivogen®) in every time they were manipulated during 3 weeks.

3.3. Cell treatments with acetate and tebuconazole

Cells were seeded and adhered into appropriate sterile plates for 24 hours before treatment in all experiments. Then, they were pre-incubated with 20 mM of acetate (a non-lethal concentration) and after 24 h of acetate treatment, cells were co-incubated with tebuconazole at a dose of IC_{50} (0.16 mM) and in some experiences with the double of the IC_{50} of tebuconazole (0.32 mM) which is dissolved in DMSO. The IC_{50} values are previously determined by sulforhodamine B (SRB) assay by our group (Macedo J, Master Thesis, 2018).

For all experiments two negative controls were used: one control only with cells and growth medium and the other with the highest concentration of DMSO and H_2O, to discard any influence of these solvents in the results. Controls just with acetate 20 mM and tebuconazole 0.16 mM were used too.

Furthermore, two main variables were tested. The first condition implies the refreshment of culture medium after 24h of acetate pre-incubation and the addition of a solution with acetate and tebuconazole. In the second condition tebuconazole was added directly into the wells, without the refreshment of the culture medium.

Table 2: List of treatments used in all experiments.

Treatments
Control negative with cells
Control negative with H_2O + DMSO
Acetate 20 mM
Tebuconazole $1.6x10^{-4}$ M (IC_{50})
Tebuconazole $3.2x10^{-4}$ M (2× IC_{50})
Acetate 20 mM + Tebuconazole $1.6x10^{-4}$ M (IC_{50})
Acetate 20 mM + Tebuconazole $3.2x10^{-4}$ M (2x IC_{50})

3.4. Sulforhodamine B assay

To determine the effect of acetate in cytotoxic effect caused by tebuconazole we performed SRB assay, which is a method widely used to evaluate the cell density through the measurement of cellular protein content (Skehan *et al.*, 1990).

NCM460 cells were seeded in 24-well plates with three wells per condition, at a final concentration of 2.5 x 10^5 cells/ml. In the following day, cells were exposed to the treatments for 72 h. After 72h of treatment, cells were washed with PBS 1× and fixed with fixing solution (ice-cold methanol containing 1 % of acetic acid (v/v)) at -20 °C for 90 minutes. Then, the fixing solution was carefully removed, and plates were left to dry at room temperature. When cells were completely dried, they were incubated with SRB solution (0.5 % (w/v) SRB dissolved in 1 % acetic acid (v/v)) at 37 °C in the dark for 90 minutes. After this incubation, the SRB solution was removed and the plates were washed with 1% (v/v) acetic acid and left to dry at room temperature. SRB was solubilised with 10 mM Tris, pH 10 and the absorbance was read at 540 nm in a microplate reader (SpectraMax® Plus 384 microplate reader from Molecular Devices).

The results were expressed relatively to the negative control (untreated cells), which corresponds at 100 % of cell proliferation.

3.5. Terminal transferase dUTP nick end labelling assay

To access the levels of apoptotic cell death it was performed the terminal transferase dUTP nick end labelling (TUNEL) assay using In Situ Cell Death Detection Kit, Fluorescein (Roche©). This assay is based on the recognition of apoptotic DNA through identification of blunt ends of double strand DNA breaks by terminal deoxynucleotidyl transferase (TdT) (Gavrieli, Sherman and Ben-Sasson, 1992).

Cells were seeded in 6-well plates for 24 h at a concentration of 2.5×10^5 cells/ml with two wells per condition, except for negative control where only one well was used. Then, cells were exposed to the treatments and 48 h before the end of treatment the positive control cells were treated with 250 µM of hydrogen peroxide (H_2O_2).

After 72 h of incubation, cells were washed with PBS 1x, trypsinized for 5 minutes and collected to 15 ml tubes. Then, plates were washed again with PBS 1x and the content was added to the respective 15 ml tube in order to minimize the cell losses. The suspension were centrifuged at 2000 rpm, at 4 °C for 10 minutes, the supernatant was discarded, and the pellet was resuspended with PBS 1× and centrifuged again under the same conditions. Samples were fixed with paraformaldehyde 4 % (PFA) for 15 minutes followed by another centrifugation with the same parameters. Finally, 500 µl of fixed cells were transferred to microtubes and storage at 4 °C where they could be stored for several weeks.

Cytospins of all samples were performed on Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific©). For that, 250 µl of cell suspension were subjected to a centrifugation cycle for 5 minutes at 500 rpm. After centrifugation, the cytospin slides were washed 3 times on an immunohistochemical box with PBS 1x and permeabilized with ice-cold 0.1 % Triton X-100 in 0.1 % sodium citrate for 2 minutes. Slides were washed again and then incubated with TUNEL reaction mix (dilution buffer, label solution and enzyme solution in a 10:9:1 ratio, respectively) in a lined container with wet paper under the slides to prevent dehydration, at 37 °C for 1 hour and protected from the light.

The slides were washed 3 times again in PBS 1x on a dark immunohistochemical box and mounted with 2 µl of VECTASHIELD Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories®) and maintained at -20 °C until visualization, in order to prevent the loss of fluorescence of the samples. The images were obtained in Olympus motorized BX63F Upright Microscope.

3.6. Trypan blue exclusion assay

In parallel with SRB and TUNEL assay, a sample of each condition was subjected to trypan blue exclusion assay to access the cellular viability. This is a dye exclusion method, based on the principle that nonviable cells with a dysfunctional membrane incorporate the dye, emitting a blue colour (Strober, 1997).

After the treatments, cells were washed with PBS 1x, trypsinized for 5 minutes and collected to 15 ml tubes. The suspension were centrifuged at 2000 rpm, at 4 °C for 10 minutes and the pellet was resuspended in 250 µl of PBS 1x. Then, 10 µl of cell suspension was mixed with 10 µl of trypan blue solution. 10 µl of this mixture were loaded in a Neubauer counting chamber and the number of stained and total cells was counted, being the dead cells the ones which present a blue colour. The percentage of cell death was determined according to the formula: (Number of blue cells ÷ Number of total cells) × 100.

3.7. MTT assay

In order to study the impact of both compounds in mitochondria, the MTT assay was accomplished. This technique allows the detection of live cells able to convert the soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to an insoluble purple formazan by the action of mitochondrial reductase (Mosmann, 1983).

Cells were seeded in 96-well plates for 24 h at a concentration of 2×10^5 cells/ml with four wells per condition. 72 h after all the treatments, the culture medium was removed and the cells were incubated with a solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml in PBS 1x) in the dark at 37 °C for 3 h. To dissolve the formed formazan crystals during MTT incubation, the MTT solution was removed and 200 µl of DMSO was added to each well, followed by 10 min of agitation in the dark, to solubilise the formazan crystals. Finally, the absorbance was read at 570 nm in a microplate reader (SpectraMax® Plus 384 microplate reader from Molecular Devices).

3.8. Statistical analysis

The results were obtained from at least three independent experiments and expressed as mean ± SD. All statistical analyses were performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

CHAPTER 4 – RESULTS

4.1. The influence of acetate in the reduction of cell proliferation caused by tebuconazole

To evaluate the effect of acetate on the cellular proliferation of a normal colon cell line (NCM460) exposed to tebuconazole it was performed an SRB assay. Initially, to infer the appropriate acetate concentration for this purpose, different concentrations of acetate (20 mM, 40 mM and 70 mM) below the acetate IC_{50} value (150,2 mM) were tested to find out which one induced less cell damage.

The results showed that, in NCM460 cells, the treatment with 20 mM of acetate is the one that has no effect on cellular proliferation **(Figure 11)**. Since the objective of this work is to evaluate the protective effect of acetate, the chosen concentration should not interfere with cell proliferation, in order to prevent an overlap with tebuconazole response, and thus being possible to evaluate the acetate effect in the response to tebuconazole. For that reason, acetate 20 mM was the concentration chosen to proceed with the experience.

Furthermore, it was tested the effect of 20 mM of acetate ($^1/_7$ IC_{50}) and 0.16 mM of tebuconazole (IC_{50}), separately to compare with the combined effect of both compounds in two independent approaches. (1) In the first approach the medium was changed after 24 hours of pre-incubation with acetate 20 mM while in the other (2) the medium refreshment after this pre-incubation period was not performed. The results suggested that, in the first variable the condition with the co-incubation with acetate 20 mM and 0.16 mM of tebuconazole seems to be more evident in the decreasing of cell proliferation (**Figure 12 A**). However, in the other, the co-incubation appears to increase the cell proliferation, although not reaching statistical significance (**Figure 12 B**).

Despite the cell line used be characterized as adherent, some of the cells can also grew in suspension (Moyer *et al.*, 1996). Because of that, in parallel with the SRB assay, it was also performed a trypan blue assay with the same conditions in order to include the non-adherent cells and confirm the previous data. The results obtained by Trypan Blue showed in both approaches a tendency of an increase of the percentage of viable cells (although not statistically significant) when the condition with 0.16 mM of tebuconazole is compared with the co-incubation with acetate 20 mM (about 65.59% *vs* 73.69% for the approach with medium change and 64.36% *vs* 76.38% for the approach without this variation, respectively) (**Figure 13 A/B**).

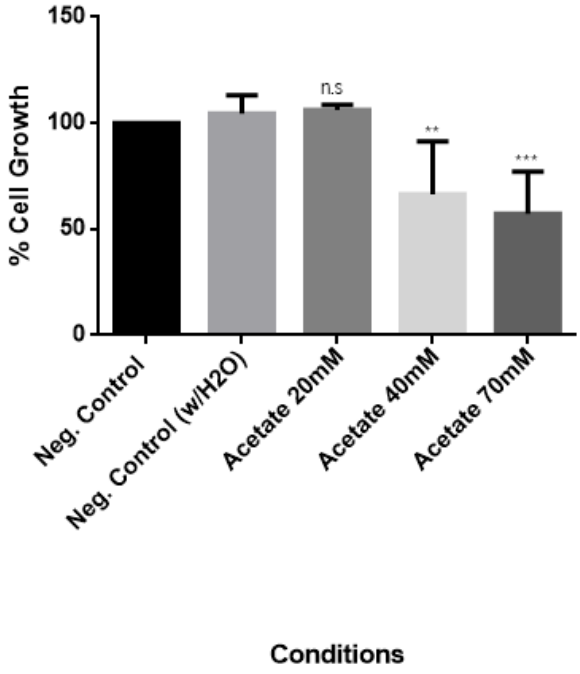

Figure 11: Test of different concentrations of acetate in NCM460 cell line. SRB assay analysis for NCM460 cells. Cells were treated with the highest concentration of H_2O present in the compound dilution, acetate 20 mM, 40 mM and 70 mM. The negative control was not subjected to any kind of treatment. SRB assay was performed 48 hours after treatments. Values represent mean ± SD of at least four independent experiments. ** P≤ 0.01; *** P ≤ 0.001; **** P≤ 0.0001 compared with negative control.

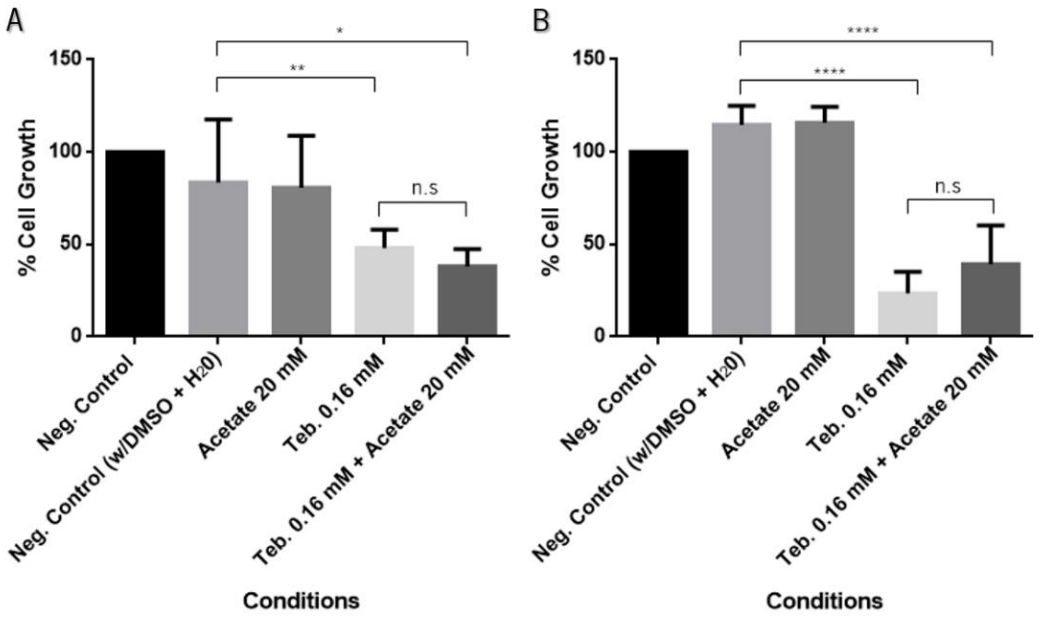

Figure 12: Effects of acetate in the decreasing of cell proliferation caused by IC_{50} of tebuconazole. (A) Statistical analysis of the effect of acetate on the cell proliferation in the approach with medium change after 24 hours of treatment with acetate and **(B)** without medium refreshment after the same time. Cells were exposed to 20 mM of acetate, 0.16 mM of tebuconazole and acetate 20 mM co-incubated with tebuconazole 0.16 mM. Cells exposed to DMSO + H_2O and cells without any treatment were used as negative control. After incubation period cells were subjected to an SRB assay to assess the differences in the cell proliferation. Values represent mean ± SD of at least four independent experiments. * P≤ 0.05; ** P≤ 0.01; **** P≤ 0.0001 compared with negative control (w/ DMSO + H_2O).

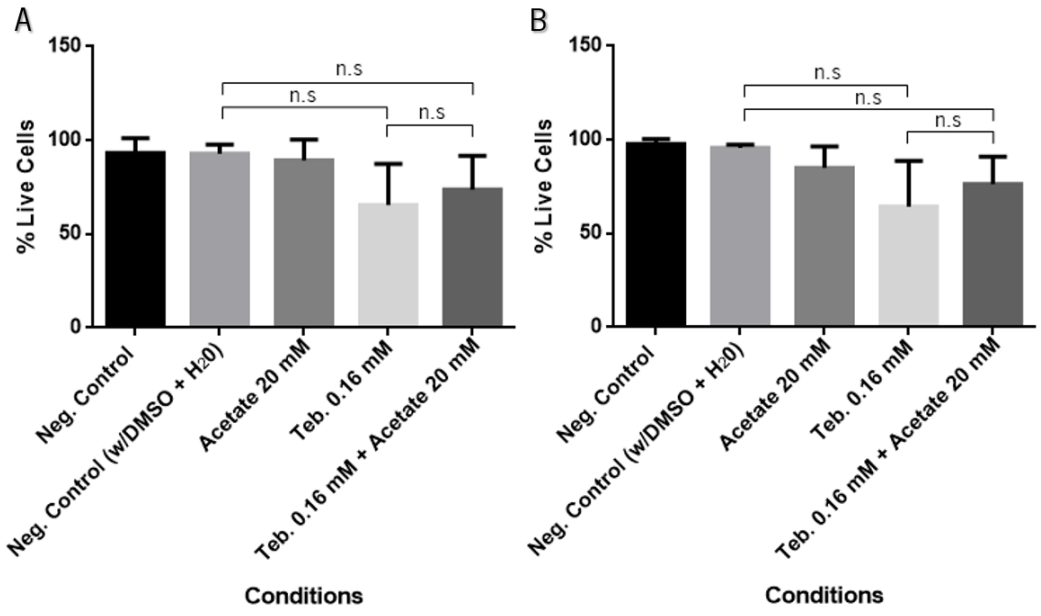

Figure 13: Acetate treatments alter the cell viability of NCM460p. Statistical analysis of trypan blue assay corresponding to the previous SRB assay in the approach with medium change **(A)** and without medium refreshment **(B)**. Cells were incubated with 0.16 mM of tebuconazole, acetate 20 mM and with acetate 20 mM conjugated with tebuconazole 0.16 mM. Cells treated with DMSO + H_2O and cells without treatment were used as negative controls. After the incubation period, treated cells were stained with trypan blue dye using a ratio of 1:1 to infer the number of viable and non-viable cells. Values represent mean ± SD of at least four independent experiments.

4.2. Both acetate and tebuconazole induce DNA strand breaks

After studying the conjugated effect of acetate and tebuconazole on cell proliferation, the next question was understanding the effects on DNA strand breaks. For this purpose, it was performed the TUNEL assay to identify DNA strand breaks. Initially, NCM460 cells were exposed to acetate 20 mM, 0.16 mM of tebuconazole and with the mixture of both compounds. As positive control, cells were treated with 250 μM of H_2O_2, a concentration that was reported to induce DNA strand breaks in these cells (Macedo, 2018). However, with these concentrations it was not possible to observe an increase in the TUNEL positive cells in the tested conditions compared with the negative control (**Figure 14**).

To clarify the effect of acetate in the DNA strand breaks caused by tebuconazole, it was decided to increase tebuconazole concentration for 0.32 mM which is the double of the IC_{50}. Again, the two approaches previously described were performed, being the cells pre-incubated with 20 mM of acetate during 24 hours and after this period cells were co-incubated with the 0.32 mM of tebuconazole (2× IC_{50}).

In the approach with medium change after 24 hours of pre-treatment with acetate we could observe a discrete increase of TUNEL positive cells in the condition with acetate 20 mM + 0.32 mM of tebuconazole in comparison with the condition with tebuconazole alone **(Figure 15)**, however, it is not a statistically significant difference (44.78% for 0.32 mM of tebuconazole and 45.69% for acetate 20 mM + tebuconazole 0.32 mM) **(Figure 17 A)**. On the other hand, the approach without medium change showed a decrease of TUNEL positive cells in the condition with acetate 20 mM + tebuconazole 0.32 mM in comparison with the condition with tebuconazole alone **(Figure 16)**, once again, this difference is also insignificantly (41.85% for 0.32 mM of tebuconazole and 34.31% for acetate 20 mM + tebuconazole 0.32 mM) **(Figure 17 B)**.

Despite these results, statistical analyses of these data revealed a very high standard deviation at the representative condition of 0.32 mM of tebuconazole (2× IC_{50}) for 3 independent experiences, therefore, it was unable to guarantee a 100% the reliability of the results for this condition **(Figure 17 B)**.

In parallel with this experiment, a trypan blue assay was performed again. One sample of each condition was taken and mixed with trypan blue dye at a 1:1 dilution to guarantee exactly the same conditions for each experiment. The statistical analysis of both approaches presented very similar results, highlighting a tendency for a lower percentage of live cells in the condition with acetate 20 mM + tebuconazole 0.32 mM comparing with the condition only with tebuconazole. This tendency is more obvious in the approach without medium change after 24 hours **(Figure 18 B)** in comparison with the other approach **(Figure 18**

A). Although the trypan blue results obtained with the first approach bet in agreement with the results obtained in the TUNEL assay, the same was not observed for the second approach.

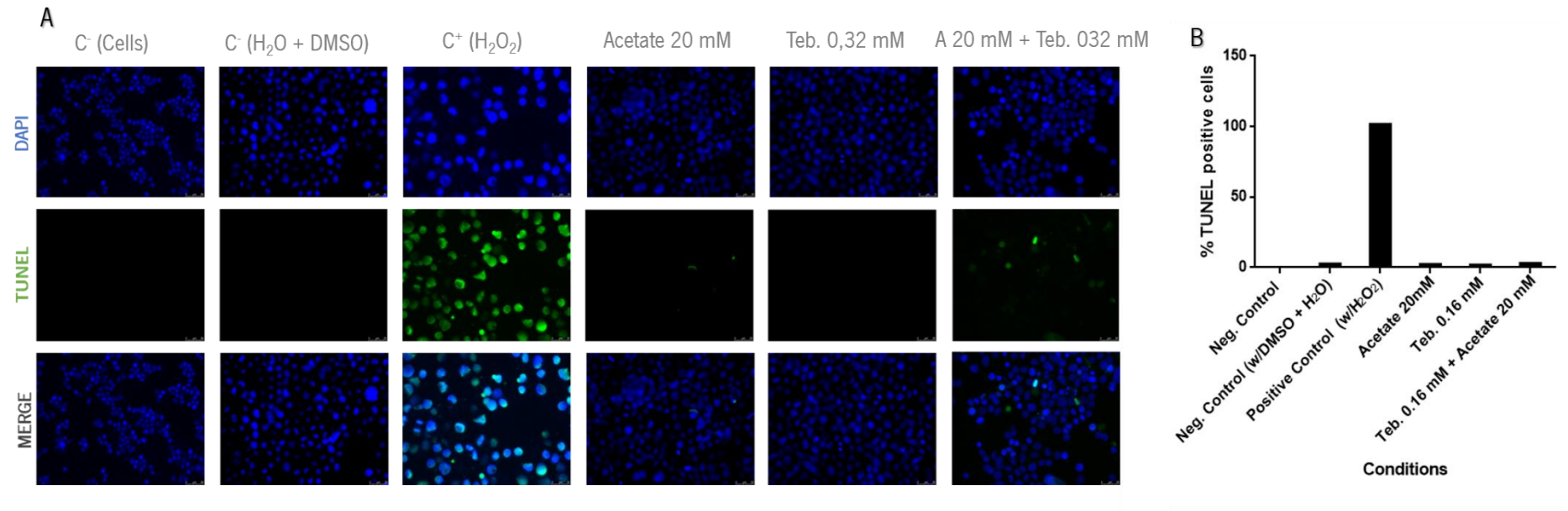

Figure 14: Acetate 20 mM and tebuconazole 0.16 mM (IC50) alone or in combination, did not induce DNA strand breaks. (A) Representative images of DAPI (4',6diamidino-2-phenylindole), TUNEL (FITC - fluorescein isothiocyanate) and merged were obtained by confocal microscopy (×400). (B) Analysis of TUNEL assay in NCM460 cells. Cells were treated with acetate 20 mM, tebuconazole 0.16 mM and acetate 20 mM + tebuconazole 0.16 mM. H_2O_2 and DMSO + H_2O was used as a positive and negative control, respectively. The other negative control was not subjected to any treatment.

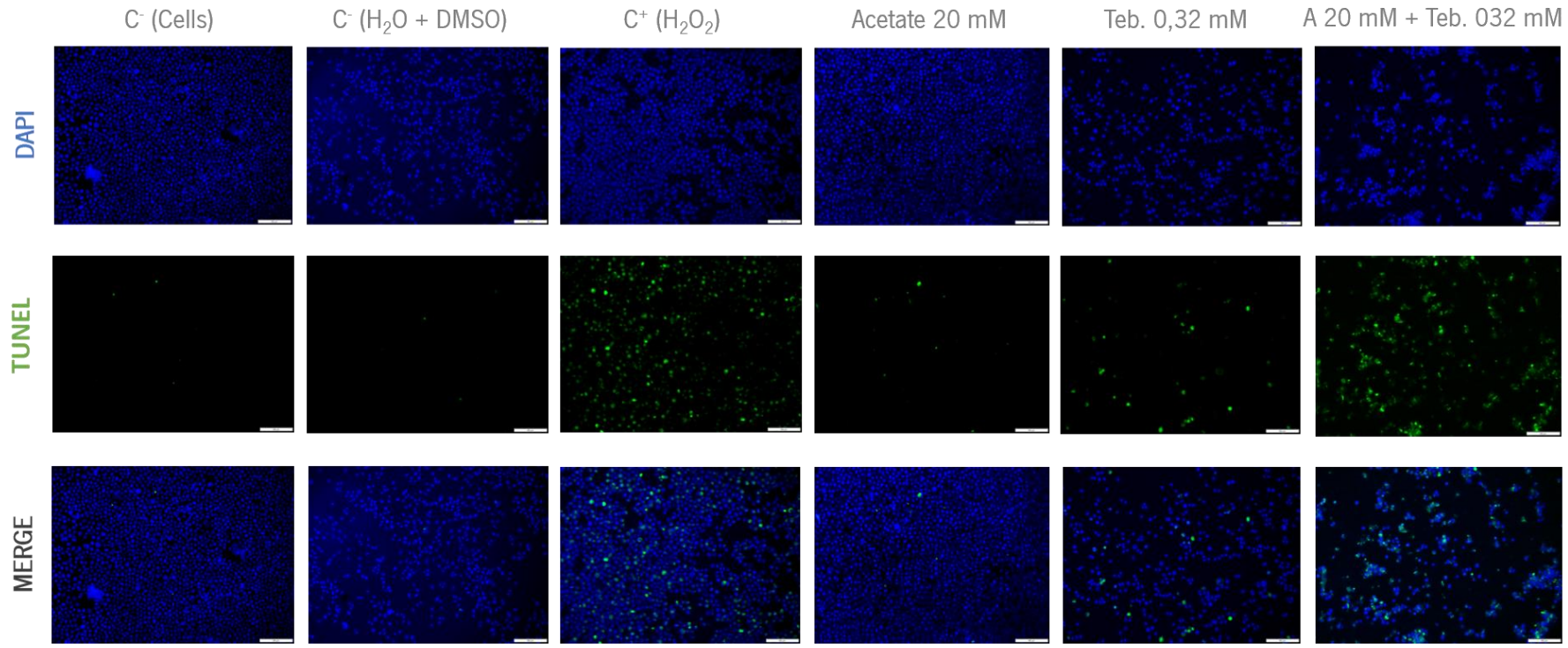

Figure 15: Acetate induces DNA strand breaks in the approach with medium refreshment. Representative images of DAPI (4',6diamidino-2-phenylindole), TUNEL (FITC - fluorescein isothiocyanate) and merged were obtained by confocal microscopy (×200). NCM460 cells were exposed to 20 mM of acetate, 0.32 mM of tebuconazole and 20 mM of acetate for 24 hours co-incubated with tebuconazole 0.32 mM during 48 hours. H_2O_2 and DMSO + H_2O were used as positive and negative control, respectively. The negative control was not subjected to any treatment. After treatments cells were subjected to a TUNEL assay to assess the induction of DNA strand breaks.

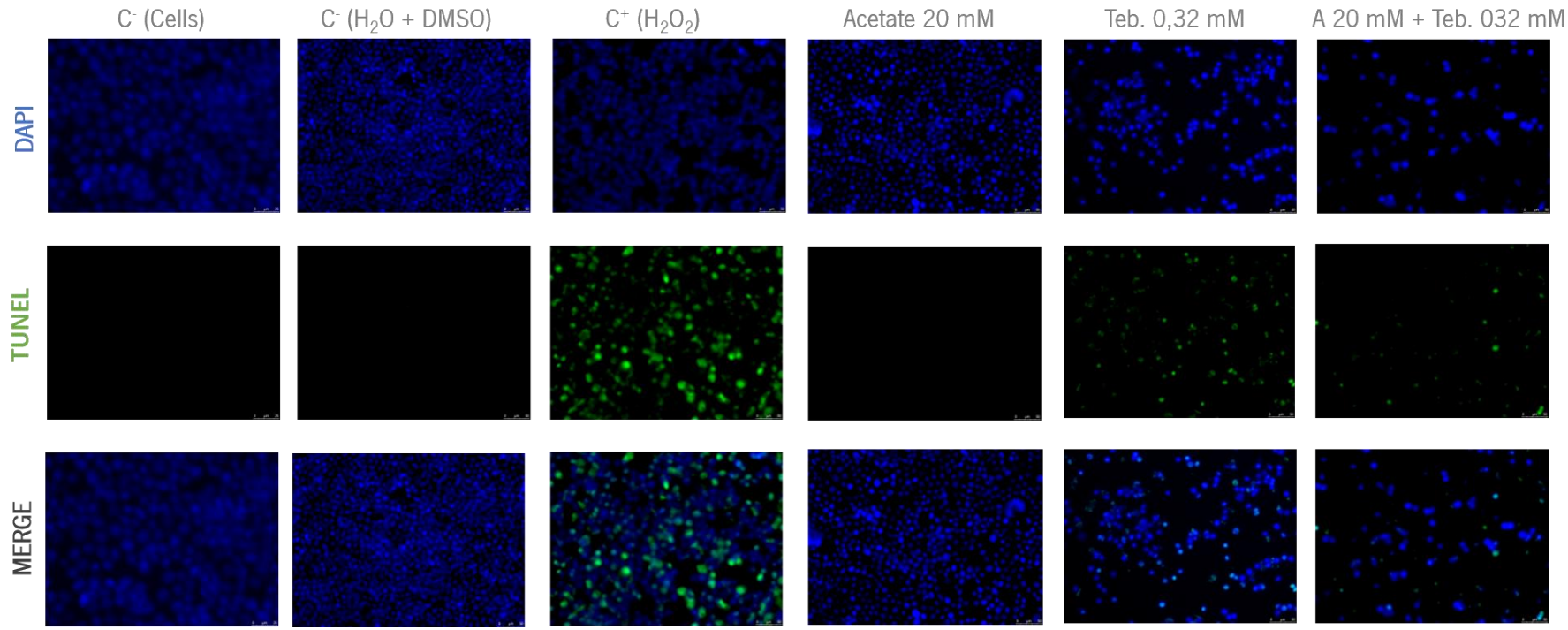

Figure 16: Acetate reduces DNA strand breaks in the approach without medium refreshment. Representative images of DAPI (4',6diamidino-2-phenylindole), TUNEL (FITC - fluorescein isothiocyanate) and merged were obtained by confocal microscopy (×200). NCM460 cells were exposed exactly to the same conditions, but there was no medium change. Cells were treated with 20 mM of acetate, 0.32 mM of tebuconazole and 20 mM of acetate for 24 hours co-incubated with tebuconazole 0.32 mM during 48 hours. H_2O_2 and DMSO + H_2O were used as positive and negative control, respectively. The negative control only with cells was not subjected to any treatment. After treatments, cells were subjected to a TUNEL assay to assess the induction of DNA strand breaks.

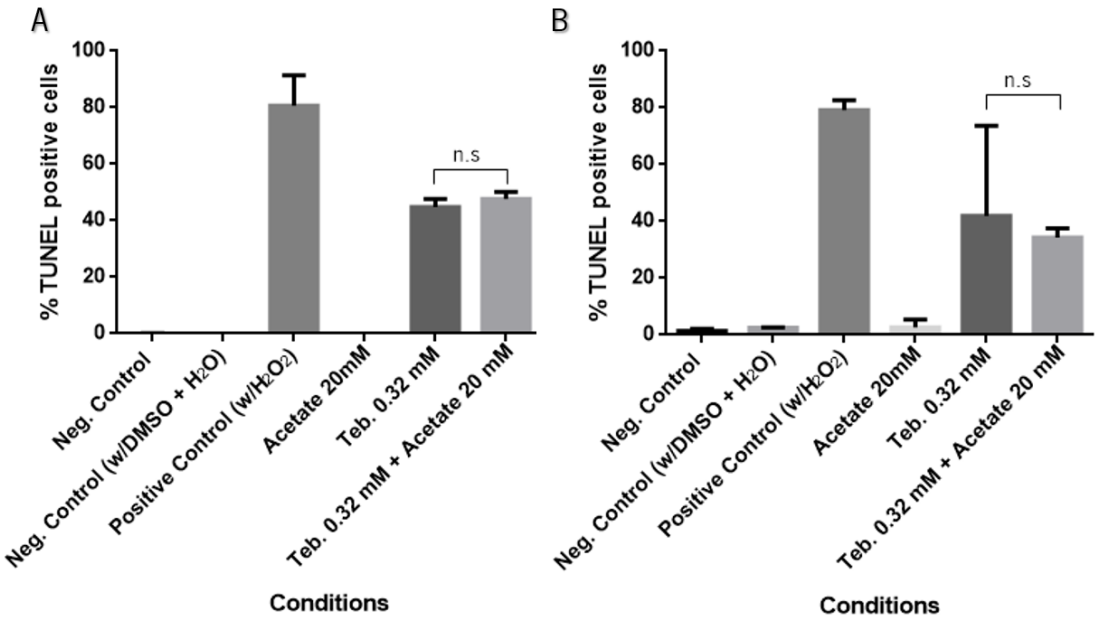

Figure 17: The influence of acetate in TUNEL positive cells. Statistical analysis corresponding to the previous representative imagens of TUNEL positive cells. NCM460 cells were exposed to 20 mM of acetate, 0.32 mM of tebuconazole and 20 mM of acetate for 24 hours co-incubated with tebuconazole 0.32 mM during 48 hours. H_2O_2 and DMSO + H_2O were used as positive and negative control, respectively. The negative control only with cells was not subjected to any treatment. (A) approach with medium refreshment and (B) approach without medium refreshment. Values represent mean ± SD of at least three independent experiments.

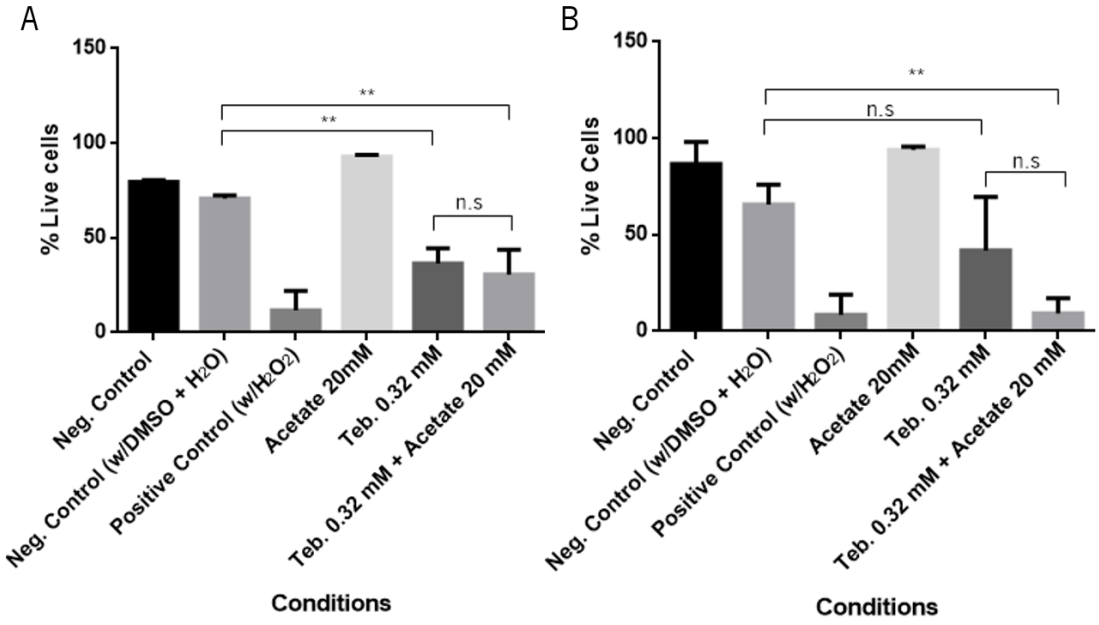

Figure 18: Acetate decreases the number of live cells. Statistical analysis of trypan blue assay after NCM460 cells being treated with 20 mM of acetate, 0.32 mM of tebuconazole and 20 mM of acetate for 24 hours co-incubated

with tebuconazole 0.32 mM during 48 hours. H_2O_2 and DMSO + H2O were used as positive and negative control, respectively. The negative control only with cells was not subjected to any treatment. The mixture of trypan blue solution with cell suspension was made using a ratio of 1:1. (A) Approach with medium refreshment and in (B) approach without medium refreshment. Values represent mean ± SD of at least three independent experiments. ** P≤ 0.01 compared with negative control (w/ DMSO + H_2O).

4.3. Acetate revels a tendency to increase the mitochondrial activity in cells treated with the IC_{50} of tebuconazole

Some studies have demonstrated that tebuconazole interacts with mitochondria and compromise their viability (Yang *et al.*, 2018). In order to understand if acetate is capable to increase the mitochondrial activity and consequently the cell viability reduced by tebuconazole it was performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

For this assay, NCM460 cells were exposed to acetate 20 mM, tebuconazole 0.16 mM (IC_{50}), tebuconazole 0.32 mM (2× IC_{50}) , acetate 20 mM + tebuconazole 0.16 mM and acetate 20 mM + tebuconazole 0.32 mM to compare the results of both concentrations, as in the previous tests the two approaches were kept.

In the first approach (with medium change) is possible to notice a slightly increase in the percentage of mitochondrial activity in the condition with cells treated with acetate 20 mM and tebuconazole 0.16 mM comparatively to the condition where cells were treated only with tebuconazole 0.16 mM (about 38.05% and 36.05% respectively), whereas when the tebuconazole concentration is doubled (0.32 mM) that increase on the percentage of mitochondrial activity was not observed. Statistical analyses of the results showed a non-significant decrease of mitochondrial activity from one condition to the other, about 9.01% for tebuconazole 0.32 mM and 4.44% for acetate 20 mM + tebuconazole 0.32 mM, not reflecting the same decrease observed in the previous concentrations (**Figure 19 A**).

Concerning the results of the second approach, they were quite contradictory. Absorbance values for the conditions with tebuconazole 0.16 mM, tebuconazole 0.32 mM; acetate 20 mM + tebuconazole 0.16 mM and acetate 20 mM + tebuconazole 0.32 mM were too low. These lower absorbance values accompanied with lower percentage of mitochondrial activity do not allow to infer conclusions accurately. Furthermore, the differences observed in the percentages of viable cells between each condition is minimal, contributing to a poor analysis of the results. Nevertheless, to the comparison between the condition only with tebuconazole 0.16 mM with the condition with tebuconazole 0.16 mM + acetate 20 mM have demonstrated

a slight increase in viable cells. However, between the conditions with tebuconazole 0.32 mM and acetate 20 mM + tebuconazole 0.32 mM, these differences were not observed. (**Figure 19 B**).

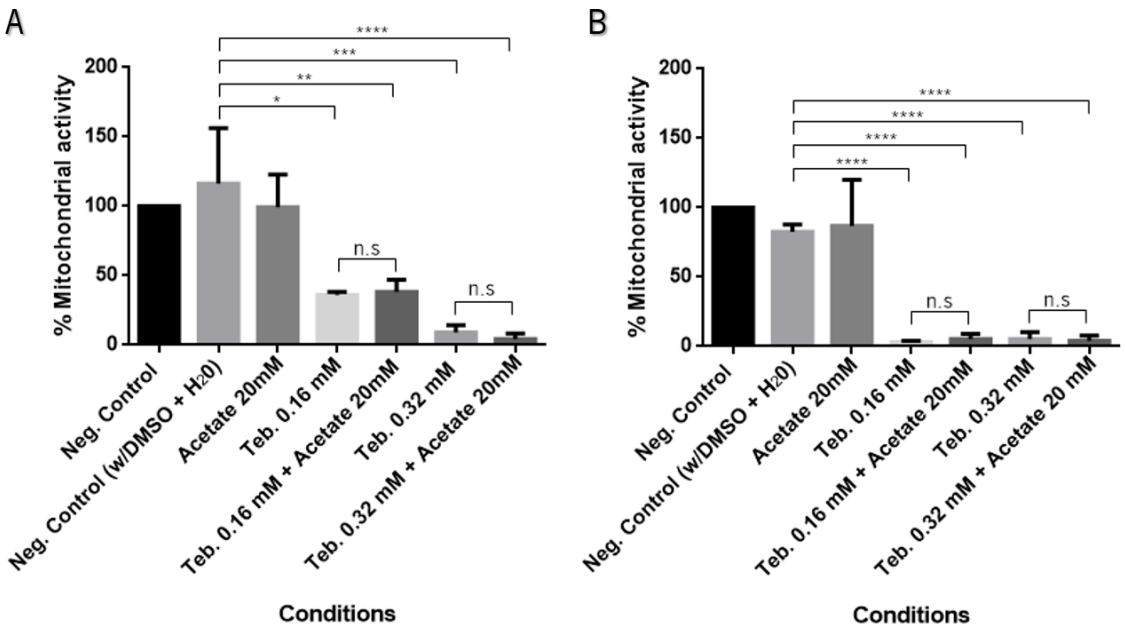

Figure 19: Tendency of acetate to increase the mitochondrial activity in cells treated with the IC_{50} of tebuconazole and, in contrast, to decrease the mitochondrial activity treated with the $2\times IC_{50}$ of tebuconazole. Statistical analysis of MTT assay in (A) approach with medium refreshment and (B) without medium refreshment. NCM460 cells were exposed to 20 mM of acetate for 24 hours and co-incubated with tebuconazole 0.16 mM and 0.32 mM during 48 hours. Conditions only with acetate 20 mM and tebuconazole 0.16 mM/0.32 mM were also evaluated. Cells treated with DMSO + H_2O and without treatment were used as negative control. After treatments cells were subject to MTT assay to estimate the mitochondrial activity. Values represent mean ± SD of at least four independent experiments. * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P≤ 0.0001 compared with negative control (w/ DMSO + H_2O).

CHAPTER 5 – DISCUSSION

The colon is considered one of the most susceptible organs in the human body, which may be partly explained by the fact that its epithelium is directly in contact with the external environment. In fact, this organ is one of the most likely to develop malignant processes, resulting both from intrinsic and extrinsic factors (DeSesso and Jacobson, 2001; Thomas *et al.*, 2016). Genetic factors, environmental exposure to mutagens (including contaminated food) or inflammatory conditions may be harmful and lead to genetic alterations that over time evolve to cancer-causing mutations. The progression of this cascade of events eventually culminates in the development of CRC. Constant exposition to both acute and chronic external perturbations, in an organ already predisposed to carry mutant clones, dramatically increases the rate of cancer-causing mutations, which results in enhanced vulnerability to carcinogenesis and metastatic invasion (Powers *et al.*, 2015; Weinberg and Zaykin, 2015).

Despite being a susceptible organ, the colon is also very complex and should be considered as an ecosystem that harbours a diverse cluster of microorganisms. Indeed, colon microbiota holds the most metabolically active microbial community which is essential for a healthy epithelium. Gut microbiota composition is directly affected by dietary intake, and various studies have demonstrated that a dysfunctional microbiota is related with some serious diseases and even CRC (Louis, Hold and Flint, 2014; Valdes *et al.*, 2018).

Normal intestinal microbiota can be disrupted by a wide range of food chemicals, namely pesticides (Blair *et al.*, 2014). Being the easiest and most effective way to control certain pests, plagues or other plants diseases and increase the nutritional value of food, these compounds play a significant role in the effectiveness of food production (Boxal, 2001; Damalas and Eleftherohorinos, 2011). However, they are daily introduced into our diet, being potentially toxic to humans. Moreover, these substances interact with the colon microbiota, which will metabolize them and, thus, disrupt the human homeostasis (Powers *et al.*, 2015). In this way, studying the interaction between microbiota – pesticide as well as their associated cellular response is extremely important to create new strategies to prevent certain pathologies and maintain a healthy microbiota (Defois *et al.*, 2018).

A healthy and functional microbiota is crucial for the homeostasis of the gastrointestinal tract and multiple distal organs and tissues. Moreover, the intestinal microbiota is also responsible for numerous physiological events such as protection against pathogens; production of nutrients and vitamins;

maintenance of the immune system and metabolism of undigested complexes to produce other essential metabolites, namely SCFA (Valdes *et al.*, 2018).

SCFA, namely acetate, propionate and butyrate, are the main source of energy of colon cells. They participate in several processes, playing a determining role in health and disease. Emerging data have shown that these metabolites are involved in anti-proliferative and pro-apoptotic mechanisms in a wide variety of cancer cell lines. Acetate, which is the most abundant SCFA in the blood flow, is also the less studied one (Gomes *et al.*, 2017). Our group have reported that acetate is involved in mechanisms capable of reducing the proliferation and invasiveness of CRC cells, specifically in decreasing cell proliferation and inducing apoptosis (Marques *et al.*, 2013; Oliveira *et al.*, 2015).

Based on these effects, the main objective of this work was to understand if acetate *per se* was capable to protect or minimize the cellular response in colon caused by pesticides, specifically tebuconazole, which is one of the most used fungicides in agriculture. To achieve this objective, it was used a normal colon mucosal cell line, NCM460. This cell line is characterized as being non-carcinogenic and this way, it being the most similar to the normal colon. However, during the immortalization process it can suffer some alterations, as any other cell line. Still, and for being the most similar to the normal colon, we consider it to be the best to evaluate the progression of the defects caused by the tebuconazole and to verify the impact of the acetate on them. Regarding the tebuconazole concentrations used for this work, it was decided to use the IC_{50} (0.16 mM) and the double of IC_{50} (0.32 mM) previously determined by our group. There is a lack of studies about tebuconazole metabolism as well as the concentrations of tebuconazole that can be found in food and in the human colon through dietary intake. Thus, it is difficult to choose a concentration that mimics what really happens in the colon. Despite this limitation, our research group have already performed some studies in order to determine the IC_{50} of tebuconazole in NCM460 cells and preliminary results have demonstrated that this concentration generates several cellular responses, such as inhibition of cell growth and induction of apoptosis followed by an increased intracellular ROS production (Macedo, 2018). In the case of acetate, during this study it was used 20 mM, because, within our range of concentrations below acetate IC_{50}, is the concentration that according with our results did not induce toxic effects on the used cell line.

In order to investigate different ways of cellular response to our treatments, it was decided to introduce two different approaches. The first one implies the medium change after 24h and the addition of fresh medium with acetate and tebuconazole, allowing the replacement of the nutrients needed for the cellular growth as well as the acetate levels. Contrary, in the second approach the medium is not removed and

the tebuconazole is directly added to the culture plates, keeping the initial conditions. During cellular growth, the consumption of the carbon source, vitamins, amino acids and other essential nutrients is fundamental for the correct cell development (Evie, Dickson and Elvin, 2014). However, the consumption of these products occurs in parallel with the release of other metabolites such as lactate and ammonium, that will change the medium conditions and consequently can alter the performance of the cells. Thus, with the introduction of these two approaches, it is possible to understand if the alteration of the cell culture microenvironment interferes with the cellular response to the treatments.

The results of the present work demonstrated a tendency for a possible protective role of acetate against tebuconazole. Despite this tendency, the data showed several fluctuations between the two approaches, not allowing to draw solid conclusions about the influence of the medium alterations in the cellular behaviour. The non-toxic concentration of acetate seems to exert a protective response, minimizing the inhibition of cell growth caused by tebuconazole in the approach without medium change as well as the reduction of DNA strand breaks in the approach. These differences between the two different approaches can be related with the sensitivity and dynamic of the cells to the treatments. Additionally, the trypan blue results performed at the same time of the SRB assay for both approaches demonstrated a slightly increase of cell viability in the cells treated with acetate.

Some publications reported that triazole fungicides, such as tebuconazole, reduce the mitochondrial activity in trophoblasts (Zhou *et al.*, 2016). In this work, we analysed if acetate could protect against the reduction of mitochondrial activity caused by tebuconazole. The results showed that tebuconazole IC_{50} and 2× IC_{50}, reduce the mitochondrial activity of the cell, as it was already described in the literature. Concerning the combined effect of tebuconazole and acetate, the results showed a slightly increase in the mitochondrial activity upon the conjugated incubation of tebuconazole IC_{50} and acetate compared with the tebuconazole IC_{50} alone in both approaches. This could be explained by a possible increase of the mitochondrial metabolism. The results obtained for the double of IC_{50} of tebuconazole conjugated with acetate were the opposite of the previous ones. This result suggest that the response exerted by acetate is only protective at the IC_{50} concentration of tebuconazole, and probably, this effect may be even more evident at lower concentrations.

It is preliminary to draw concrete conclusions about the protective role of acetate against tebuconazole, as the representativeness of the results is not significant. Some results seem to show a predisposition for a protective role for acetate, while others contradict that tendency and several scenarios might explain these contradictory findings.

Ahr are sensors of chemicals or xenobiotics which are associated with the regulation of enzymes responsible for metabolizing these substances (Rothhammer and Quintana, 2019). Once activated, they recruit enzymes, such as CYP1A1, which is highly active in the intestinal cells (Larigot *et al.*, 2018). In 2009, Sergent *et al.* demonstrated in Caco-2 cells that tebuconazole is capable to modulate CYP1A1 activity and exert its toxicological response through interaction with this enzyme (Sergent *et al.*, 2009). Tebuconazole is able to control CYP1A1 metabolism by increasing its toxic concentration within the cell. However, how tebuconazole interact with this enzyme remained unknown for several years. Recently, Knebel *et al.* published an article where they demonstrated that tebuconazole interact with CYP1A1 and CYP1A2 by increasing Ahr expression in HepG2 and HepaRG human liver cell lines. Thus, tebuconazole or its metabolites act as ligands of these receptors, thereby inducing CYP1A1, controlling its degree of detoxification (Knebel *et al.*, 2019). A possible explanation for our results, is to hypothesize that acetate may be playing a protective role by decreasing the toxicological response of tebuconazole through the increasing of aryl hydrocarbon receptors (Ahr) responsiveness.

Un-Ho Jin and his co-workers demonstrated that approximately 20 mM of acetate (the same concentration as used in this work) is able to increase the Ahr expression and response, contributing to the maintenance of cellular homeostasis (Jin *et al.*, 2017). Thus, acetate and tebuconazole may be competing for the same ligand sites of Ahr. If acetate can significantly increase the expression of these receptors by binding to their active sites, it will prevent tebuconazole from binding to them, thereby inhibition the tebuconazole interaction with CYP1A1. Considering that the majority of tebuconazole's toxicological response is due to its interaction with this enzyme, by decreasing this interaction there is a consequently decrease of the individual effect of tebuconazole, minimizing the harmful effects of this chemical on cells.

Another possible scenario, as some of the results have been pointed to, is that acetate is potentiating tebuconazole toxicity. Studies demonstrated that the enzyme aryl hydrocarbon has more preference and affinity for planar and hydrophobic ligands (Knebel *et al.*, 2019). Although acetate is more planar, tebuconazole has a larger hydrocarbon chain and is therefore more hydrophobic, so, may have a higher affinity than acetate for the active site of these receptors. Another plausible explanation is that acetate and tebuconazole have a synergistic response in the cells, making their joint response more toxic than the individual effect of each one. By increasing the responsiveness of these receptors, acetate is indirectly increasing their affinity to tebuconazole, ultimately making tebuconazole response more potent. In addition, when the concentration of tebuconazole is doubled, there are also being increased the probability of tebuconazole occupying most of the active sites, avoiding the binding of acetate.

As mentioned above, by binding to these receptors, tebuconazole will act as a transcription factor for Ahr responsive genes, such as the cyp1a1/CYP1A1 gene, allowing the modulation of these enzymes. In this way, tebuconazole has the capacity of controlling its bioavailability within the cell, increasing the concentration on its target site. As a result of this interaction, dysfunctions in the mitochondrial pathway may occur. In addition, induction of these enzymes may have influence in signalling pathways involved in the cell cycle and cell growth (Yang *et al.*, 2018).

Summing up, the way that acetate might interact with tebuconazole as well as with other xenobiotics is still unknown. To test this hypothesis, it would be necessary to analyse the Ahr inducible potential by both tebuconazole and acetate, as well as the two of them combined in NCM460 cell line, and lastly the measurement of CYP activity, to fully confirm the previous conjectures.

CHAPTER 6 – CONCLUSIONS AND FUTURE PERSPECTIVES

In humans, the intestinal microbiota is considered vital, since it is responsible for several physiological processes, such as the production of SCFA, like acetate. However, the microbiota homeostasis can be easily disrupted by external agents, namely pesticides, which are daily introduced into our diet. Tebuconazole appears as one of the most founded pesticides in agriculture-derived food and, because of this, the main goal of this work was to understand if acetate is able to minimize and protect against the harmful effects caused by tebuconazole.

Previous results obtained by our group have shown that tebuconazole decreases cell proliferation and induces colon cell death through apoptosis, which is associated with increased production of ROS (Macedo, 2018). Also, our research group and others have already reported the role of SCFAs in the maintenance of the colon microbiota homeostasis in CRC cells (Gomes *et al.*, 2017) so, taking this into account, acetate would be expected to exert a protective response against tebuconazole in normal colon cells. Indeed, the results herein presented demonstrated that acetate was capable to interact with tebuconazole, slightly increasing the cell proliferation, viability and mitochondrial activity and decreasing the levels of DNA strand breaks in one of the approaches performed. Our data showed a tendency for a protective role of acetate, however, the magnitude of the response exerted by acetate was not significant enough to draw solid conclusions and validate acetate as a protective agent against tebuconazole.

Although part of the presented results has shown a tendency for a protective response, it must be taken into consideration that were also obtained some contradictory results, pointing to acetate as an enhancer of the cellular response exerted by tebuconazole. Throughout our experiments, it was possible to observe that the increase in the tebuconazole toxicological response always occurred when its concentration was doubled (from the IC_{50} (0.16 mM) to twice of the IC_{50} (0.32 mM)). Therefore, it is possible to conclude that the response exerted by acetate is dependent on the tebuconazole's concentration and *vice versa*.

Regarding the influence of the two different approaches performed in this work, the results are also inconclusive. The presence of other metabolites in the medium would be expected to interact with acetate and tebuconazole and influence their performance. However, the results obtained did not allow to establish a correlation between the approaches and the assays. In fact, while some assays evidenced a protective role of acetate for the approach with medium refreshment after 24 hours, others showed this positive response for the approach without medium change. These fluctuations of behaviour in both

approaches for all the assays did not allow us to understand what influence the medium change and nutrients resetting could have in the possible protective effect played by acetate.

We are still far from entirely understanding the interaction between acetate and tebuconazole and it is essential to consider further studies to better elucidate the role of acetate against pesticides and validate it as a possible protective agent against microbiota disruptors. Following this idea, in addition to the suggestions mentioned in the discussion chapter, it is also important in the future to consider other research ideas such as:

- **Adjust acetate and tebuconazole concentrations**

Throughout the intestine, the concentration of SCFA's, namely acetate, varies according to the pH. Presenting a higher concentration in the cecum, it decreases throughout the descending colon and finally reaches its lower concentration in the rectum (Koh *et al.*, 2016). Therefore, in order to mimic what happens in the gut, it would be important to test for the different concentrations of acetate present in this organ and with different pH's.

In addition, it would also be interesting to study tebuconazole's distribution and absorption along the colon through *in vivo* tests, and thus, adjust its concentrations accordingly.

- **Evaluate the individual protective role of butyrate and propionate as well the different mixtures of the three SCFA**

The health-promoting functions of all the three SCFA are well recognized, however, it is evident that their individual effects on the host differ considerably. In this way, and considering the potential of each metabolite, studying the protective role of propionate and butyrate alone is extremely important. For that, repeat all the tests that were done with acetate, but using these two metabolites, would be a good approach.

After understanding the individual response exerted by acetate, butyrate and propionate, the next step would be studying the combined effect of the three SCFA. Due to their biological effects, seems plausible that their joint effect might play a more significant response. Thus, testing mixture of acetate, propionate and butyrate mimicking physiological ratios and pre-incubate normal colon cells with these mixtures and access

their effect against tebuconazole, could be a good strategy to classify and validate SCFA as protective agents against food contaminants.

- Complementary tests

Apart from the tests that were performed during this work, other complementary tests should be taken into consideration.

Developing the hypotheses addressed in the discussion chapter, it would be important to determine the tebuconazole inducing potential in the CYP enzymes, as well as to evaluate the differences in Ahr expression in NCM460 cell line. Mass spectrometry techniques and gene expression analysis (through quantitative polymerase chain reaction- qPCR) would be adequate to analyse the mentioned parameters. If the results were like the ones obtained by Knebel *et al.* in hepatocytes cell lines, showing that tebuconazole affects CYP1A1 and CYP1A2 expression through Ahr pathway, the next step would be the introduction of the acetate treatments and the analysis of differences in gene expression and in the induction of CYP enzymes, in order to evaluate the influence of acetate on tebuconazole's metabolism (Knebel *et al.*, 2019).

Furthermore, exploring the publications about tebuconazole, Zhou *et al.* demonstrated that this pesticide induces changes in Bcl-2 protein expression level by decreasing it, which is accompanied by an increase in the expression of Bax protein in human trophoblast cell line HTR-8 (Zhou *et al.*, 2016). On the other hand, Emenaker *et al.* demonstrated in human colonocytes that acetate significantly increases Bcl-2 expression as well as decreases Bax expression (Emenaker *et al.*, 2001). Expression of these proteins by western blot could be performed in order to elucidate this question. The study of these pro and anti-apoptotic mechanisms may provide further indications of the level of acetate protection against induction of apoptotic mechanisms.

- *In vivo* studies

In an article published by Fukuda and co-workers, it was proved that acetate production can protect from enteropathogenic infections. For this purpose, they used a germ-free mice mono-associated with *Bifidobacterium* (SCFA's producer) and a germ-free mice without this mono-association, which were subsequently inoculated with an *E. coli* strain. Therefore, they analysed and plotted the metabolic profile by Nuclear Magnetic Resonance and concluded that there was an extra acetate production in the mice's mono-associated with *Bifidobacterium* compared to those that were not inoculated. In addition, they found that mices without mono-association died while the others survived. These results suggests that acetate promote the defence functions of host epithelial cells (Fukuda *et al.*, 2011).

Following this idea, if we adapted their protocol, but instead of inoculating with *E. coli*, we administer different doses of tebuconazole and then trace the metabolic profile as they did, analysing acetate and other SCFAs production, we would be able to infer whether acetate and other SCFAs can protect against this compound or not.

In summary, and despite the heterogeneity between approaches, the results presented in this Master thesis reveal a possible protective role for acetate against the effects caused by tebuconazole. Moreover, further studies are needed to consolidate our findings.

BIBLIOGRAPHY

Abass, K., Turpeinen, M. and Rautio, A. (2000) 'Metabolism of Pesticides by Human Cytochrome P450 Enzymes In Vitro – A Survey'.

Alavanja, M. C. R. and States, U. (2009) 'Introduction : Pesticides Use and Exposure Extensive Worldwide', *REVIEWS ON ENVIRONMENTAL HEALTH*, 24(4), pp. 303–310.

Allison, C. and Macfarlane, G. T. (2009) 'Effect of Nitrate on Methane Production and Fermentation by Slurries of Human Faecal Bacteria', *Microbiology*, 134(6), pp. 1397–1405. doi: 10.1099/00221287-134-6-1397.

van der Beek, C. M. *et al.* (2017) 'Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing', *Nutrition Reviews*, 75(4), pp. 286–305. doi: 10.1093/nutrit/nuw067.

Birt, D. F. and Phillips, G. J. (2014) 'Diet, genes, and microbes: Complexities of colon cancer prevention', *Toxicologic Pathology*, 42(1), pp. 182–188. doi: 10.1177/0192623313506791.

Blair, A. *et al.* (2014) 'Pesticides and human health', *Pesticides and human health. Occupational and Environmental Medicine*, 72(2), pp. 81–82. doi: 10.1136/oemed-2014-102454.

Boets, E. *et al.* (2017) 'Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study.', *The Journal of Physiology*, 592(2), pp. 541–555. doi: 10.1113/JP272613.This.

Boron, W. F. and Boulpaep, E. L. (2015) *Medical physiology :a cellular and molecular approach*. 2nd edn. Philadelphia, PA: Saunders/Elsevier.

Boxal, R. A. (2001) 'Post-harvest losses to insects- A World overview', *International Biodeterioration & Biodegradation*, pp. 137–152.

Brenner, H., Kloor, M. and Pox, C. P. (2014) 'Colorectal cancer', *The Lancet*. Elsevier Ltd, 383(9927), pp. 1490–1502. doi: 10.1016/S0140-6736(13)61649-9.

Canani, R. B. *et al.* (2011) 'Potential beneficial effects of butyrate in intestinal and extraintestinal diseases', *World Journal of Gastroenterology*, 17(12), pp. 1519–1528. doi: 10.3748/wjg.v17.i12.1519.

Candela, M. *et al.* (2011) 'Human intestinal microbiota: Cross-talk with the host and its potential role in colorectal cancer', *Critical Reviews in Microbiology*, 37(1), pp. 1–14. doi: 10.3109/1040841X.2010.501760.

Carpy, S. A., Kobel, W. and Doe, J. (2010) 'Journal of Toxicology and Environmental Health , Part B : Critical Reviews HEALTH RISK OF LOW-DOSE PESTICIDES MIXTURES : A Review of the 1985-1998 Literature On Combination Toxicology and Health Risk Assessment', (April 2013), pp. 37–41.

Christen, V., Crettaz, P. and Fent, K. (2014) 'Additive and synergistic antiandrogenic activities of mixtures of azol fungicides and vinclozolin', *Toxicology and Applied Pharmacology*. Elsevier Inc., 279(3), pp. 455–466. doi: 10.1016/j.taap.2014.06.025.

Cooper, J. and Dobson, H. (2007) 'The benefits of pesticides to mankind and the environment', *Crop Protection*, 26(9), pp. 1337–1348. doi: 10.1016/j.cropro.2007.03.022.

Cuff, M. A., Lambert, D. W. and Shirazi-beechey, S. P. (2002) 'Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1', *Journal of Physiology*, pp. 361–371. doi: 10.1013/jphysiol.2001.014241.

Cummings, J. H. (1975) 'The colon: Absorptive, secretory and metabolic functions', *Digestion*, 13(4), pp. 232–240. doi: 10.1159/000197712.

Cummings, J. H. *et al.* (1987) 'Short chain fatty acids in human large intestine, portal, hepatic and venous blood', *Gut*, 28(10), pp. 1221–1227. doi: 10.1136/gut.28.10.1221.

Cummings, J. H. and MacFarlane, G. T. (1997) 'Role of intestinal bacteria in nutrient metabolism', *Clinical Nutrition*, 16(1), pp. 3–11. doi: 10.1016/S0261-5614(97)80252-X.

Damalas, C. A. and Eleftherohorinos, I. G. (2011) 'Pesticide exposure, safety issues, and risk assessment indicators', *International Journal of Environmental Research and Public Health*, 8(5), pp. 1402–1419. doi: 10.3390/ijerph8051402.

Defois, C. *et al.* (2018) 'Food Chemicals Disrupt Human Gut Microbiota Activity And Impact Intestinal Homeostasis As Revealed By In Vitro Systems', *Scientific Reports*, 8(1), pp. 1–12. doi: 10.1038/s41598-018-29376-9.

DeSesso, J. M. and Jacobson, C. F. (2001) 'Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats', *Food and Chemical Toxicology*, 39(3), pp. 209–228. doi: 10.1016/S0278-6915(00)00136-8.

Duncan, S. H. *et al.* (2002) 'Acetate Utilization and Butyryl Coenzyme A (CoA): Acetate-CoA Transferase in Butyrate-Producing Bacteria from the Human Large Intestine', 68(10), pp. 5186–5190. doi: 10.1128/AEM.68.10.5186.

Duncan, S. H. *et al.* (2007) 'Reduced Dietary Intake of Carbohydrates by Obese Subjects Results in Decreased Concentrations of Butyrate and Butyrate-Producing Bacteria in Feces ', 73(4), pp. 1073–1078. doi: 10.1128/AEM.02340-06.

Dvorak, Z. (2011) 'Drug – drug interactions by azole antifungals : Beyond a dogma of CYP3A4 enzyme activity inhibition', *Toxicology Letters*. Elsevier Ireland Ltd, 202(2), pp. 129–132. doi: 10.1016/j.toxlet.2011.01.027.

Elefterios, E. and Bezirtzoglou, V. (2012) 'Intestinal cytochromes P450 regulating the intestinal microbiota and its probiotic profile', *co-action*, 1, pp. 1–11.

Emenaker, N. J. *et al.* (2001) 'Research Conference on Diet , Nutrition and Cancer Short-Chain Fatty Acids Inhibit Invasive Human Colon Cancer by Protein Expression in an In Vitro Cell Culture Model 1 , 2', *Journal of nutrition*, 131(March), pp. 3041S-3046S.

Evie, I. M., Dickson, A. J. and Elvin, M. (2014) 'Metabolite Profiling of Mammalian Cell Culture Processes to Evaluate Cellular Viability', 1601, pp. 137–152. doi: 10.1007/978-1-4939-6960-9.

Ferro, S. *et al.* (2016) 'Characterization of acetate transport in colorectal cancer cells and potential therapeutic implications', *Oncotarget*, 7(43).

Fischer, R. T. *et al.* (1991) 'Lanosterol 14a-Methyl Demethylase', *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 266(10), pp. 6124–6132.

Flint, H. J. *et al.* (2015) 'Links between diet, gut microbiota composition and gut metabolism', *Proceedings of the Nutrition Society*, 74(1), pp. 13–22. doi: 10.1017/S0029665114001463.

Frank, D. N. *et al.* (2007) 'Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States of America', 104(34), pp. 13780–13785. Available at: www.pnas.org%02cgi%02doi%0210.1073%02pnas.0706625104.

Fukuda, S. *et al.* (2011) 'Bifidobacteria can protect from enteropathogenic infection through production of acetate', *Nature*. Nature Publishing Group, 469(7331), pp. 543–547. doi: 10.1038/nature09646.

Gabsi, F., Zenker, A. and Preuss, T. G. (2016) 'Demographic Toxicokinetic – Toxicodynamic Modeling of Lethal E ff ects', *Environmental Science & Technology*, 50(11), pp. 6017–6024. doi: 10.1021/acs.est.6b01113.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992) 'Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation', *Journal of Cell Biology*, 119(3), pp. 493–501. doi: 10.1083/jcb.119.3.493.

Gill, C. I. R. and Rowland, I. R. (2002) 'Diet and cancer : assessing the risk', *British Journal of Nutrition*, 88(1), pp. 73–87. doi: 10.1079/BJN2002632.

Gill, S. R. *et al.* (2006) 'Metagenomic Analysis of the Human Distal Gut Microbiome\r10.1126/science.1124234', *Science*, 312(5778), pp. 1355–1359. Available at: http://www.sciencemag.org/cgi/content/abstract/312/5778/1355.

Goetz, A. K. *et al.* (2007) 'Disruption of Testosterone Homeostasis as a Mode of Action for the Reproductive Toxicity of Triazole Fungicides in the Male Rat', *Toxicological Sciences*, 95(1), pp. 227–239. doi: 10.1093/toxsci/kfl124.

Gomes, S. *et al.* (2017) 'The Role of Diet Related Short-Chain Fatty Acids in Colorectal Cancer Metabolism and Survival : Prevention and Therapeutic Implications .', *Current Medicinal Chemistry*, 1.

Gonçalves, P. and Martel, F. (2013) 'Butyrate and Colorectal Cancer : The Role of Butyrate Transport Butyrate and Colorectal Cancer : The Role of Butyrate Transport', *Current drug metabolism*, (November). doi: 10.2174/1389200211314090006.

Hanahan, D. and Weinberg, R. A. (2011) 'Review Hallmarks of Cancer : The Next Generation', *Cell*. Elsevier Inc., 144(5), pp. 646–674. doi: 10.1016/j.cell.2011.02.013.

Hernández, A. F. *et al.* (2013) 'Toxic effects of pesticide mixtures at a molecular level : Their relevance to human health', *Toxicology*. Elsevier Ireland Ltd, 307, pp. 136–145. doi: 10.1016/j.tox.2012.06.009.

Hernández, A. F., Gil, F. and Lacasaña, M. (2017) 'Toxicological interactions of pesticide mixtures: an update', *Archives of Toxicology*. Springer Berlin Heidelberg, 91(10), pp. 3211–3223. doi: 10.1007/s00204-017-2043-5.

Heusinkveld, H. J. *et al.* (2013) 'Azole Fungicides Disturb Intracellular Ca 2 + in an Additive Manner in Dopaminergic PC12 Cells', *Toxicological Sciences*, 134(2), pp. 374–381. doi: 10.1093/toxsci/kft119.

Hodgson, E. (2003) 'In Vitro Human Phase I Metabolism of Xenobiotics I : Pesticides and Related Compounds Used in Agriculture and Public Health , May 2003', *Journal of Biochemical and Molecular Toxicology*, 17(4), pp. 201–206. doi: 10.1002/jbt.10080.

Hooper, L. V. (2004) 'Bacterial contributions to mammalian gut development', *Trends in Microbiology*, 12(3), pp. 129–134. doi: 10.1016/j.tim.2004.01.001.

Hosseini, E. *et al.* (2011) 'Propionate as a health-promoting microbial metabolite in the human gut', *Nutrition Reviews*, 69(5), pp. 245–258. doi: 10.1111/j.1753-4887.2011.00388.x.

Irving, M. H. and Catchpole, B. (2009) 'ABC of colorectal diseases. Anatomy and physiology of the colon, rectum, and anus.', *Bmj*, 304(6834), pp. 1106–1108. doi: 10.1136/bmj.304.6834.1106.

Jan, G. *et al.* (2002) 'Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria', *Cell Death and Differentiation*, pp. 179–188. doi: 10.1038/sj/cdd/4400935.

Jandhyala, S. M. *et al.* (2015) 'Role of the normal gut microbiota', *World Journal of Gastroenterology*, 21(29), pp. 8836–8847. doi: 10.3748/wjg.v21.i29.8787.

Jin, U. H. *et al.* (2017) 'Short Chain Fatty Acids Enhance Aryl Hydrocarbon (Ah) Responsiveness in Mouse Colonocytes and Caco-2 Human Colon Cancer Cells', *Scientific Reports*, 7(1), pp. 1–12. doi: 10.1038/s41598-017-10824-x.

Junqueira, L. C. and Carneiro, J. (2013) *Histologia Básica*. 12th edn.

Kahle, M. *et al.* (2008) 'Supporting information for: Azole fungicides: Occurrence and fate in wastewater and surface waters', *Environmental Science and Technology*, 42(19), pp. 7193–7200. doi: 10.1021/es8009309.

Kasubuchi, M. *et al.* (2015) 'Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation', *Nutrients*, 7(4), pp. 2839–2849. doi: 10.3390/nu7042839.

Kim, C. H., Park, J. and Kim, M. (2014) 'Gut Microbiota-Derived Short-Chain Fatty Acids, T Cells, and Inflammation', *Immune Network*, 14(6), p. 277. doi: 10.4110/in.2014.14.6.277.

Kim, K. H., Kabir, E. and Jahan, S. A. (2017) 'Exposure to pesticides and the associated human health effects', *Science of the Total Environment*. Elsevier B.V., 575, pp. 525–535. doi: 10.1016/j.scitotenv.2016.09.009.

Kim, Y. S. and Ho, S. B. (2010) 'Intestinal goblet cells and mucins in health and disease: Recent insights and progress', *Current Gastroenterology Reports*, 12(5), pp. 319–330. doi: 10.1007/s11894-010-0131-2.

Knebel, C. *et al.* (2019) 'The azole fungicide tebuconazole affects human CYP1A1 and CYP1A2 expression by an aryl hydrocarbon receptor-dependent pathway', *Food and Chemical Toxicology*. Elsevier, 123(November 2018), pp. 481–491. doi: 10.1016/j.fct.2018.11.039.

Koh, A. *et al.* (2016) 'From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites', *Cell*, 165(6), pp. 1332–1345. doi: 10.1016/j.cell.2016.05.041.

Lamb, D. C., Kelly, D. E. and Kelly, S. L. (1998) 'Molecular diversity of sterol 14α-demethylase substrates in plants, fungi and humans', *FEBS Letters*, 425(2), pp. 263–265. doi: 10.1016/S0014-5793(98)00247-6.

Landman, C. and Quévrain, E. (2016) 'Le microbiote intestinal : description, rôle et implication physiopathologique', *Revue de Medecine Interne*. Elsevier Masson SAS, 37(6), pp. 418–423. doi: 10.1016/j.revmed.2015.12.012.

Larigot, L. *et al.* (2018) 'AhR signaling pathways and regulatory functions', *Biochimie Open*. Elsevier Ltd, 7, pp. 1–9. doi: 10.1016/j.biopen.2018.05.001.

Lepesheva, G. I. and Waterman, M. R. (2004) 'CYP51 — the omnipotent P450', 215, pp. 165–170. doi: 10.1016/j.mce.2003.11.016.

Liu, Z., Cao, A. T. and Cong, Y. (2013) 'Microbiota regulation of inflammatory bowel disease and colorectal cancer', *Seminars in Cancer Biology*. Elsevier Ltd, pp. 1–10. doi: 10.1016/j.semcancer.2013.09.002.

Louis, P., Hold, G. L. and Flint, H. J. (2014) 'The gut microbiota , bacterial metabolites and colorectal cancer', *Nature Publishing Group*. Nature Publishing Group, (September). doi: 10.1038/nrmicro3344.

Macedo, J. (2018) *Toxicity assessment of products for the agri-food sector potentially implicated in human wellbeing*.

Macfarlane & Macfarlane (2012) 'Bacteria, Colonic Fermentation, and Gastrointestinal Health', *Journal of AOAC International*, 95, pp. 50–60. doi: 10.5740/jaoacint.SGE.

Macfarlane, S. and Macfarlane, G. T. (2003) 'Regulation of short-chain fatty acid production', *Proceedings of the Nutrition Society*, 62(1), pp. 67–72. doi: 10.1079/pns2002207.

Marchesi, J. and Shanahan, F. (2007) 'The normal intestinal microbiota', *Current Opinion in Infectious Diseases*, 20(5), pp. 508–513. doi: 10.1097/QCO.0b013e3282a56a99.

Marques, C. *et al.* (2013) 'Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release', *Cell Death and Disease*, pp. 1–11. doi: 10.1038/cddis.2013.29.

Miller, T. L. and Wolin, M. J. (1996) 'Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora', *Applied and Environmental Microbiology*, 62(5), pp. 1589–1592.

Moser, V. C. *et al.* (2001) 'The Effects of Perinatal Tebuconazole Exposure on Adult Neurological , Immunological , and Reproductive Function in Rats', *Toxicological Sciences*, 352(62), pp. 339–352.

Mosmann, T. (1983) 'Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays', *Journal of Immunological Methods*, 65(1–2), pp. 55–63. doi: 10.1016/0022-1759(83)90303-4.

Moyer, M. P. *et al.* (1996) 'NCM460, a normal human colon mucosal epithelial cell line', *In Vitro Cellular & Developmental Biology - Animal*, 32(6), pp. 315–317. doi: 10.1007/BF02722955.

Muñoz-Leoz, B. *et al.* (2011) 'Tebuconazole application decreases soil microbial biomass and activity', *Soil Biology and Biochemistry*. Elsevier Ltd, 43(10), pp. 2176–2183. doi: 10.1016/j.soilbio.2011.07.001.

National Research, C. (2004) *Intentional Human Dosing Studies for EPA Regulatory Purposes*. Washington, DC: The National Academies Press. doi: 10.17226/10927.

O'Hara, A. M. and Shanahan, F. (2006) 'The gut flora as a forgotten organ.', *EMBO reports*, 7(7), pp. 688–93. doi: 10.1038/sj.embor.7400731.

Oliveira, C. S. F. *et al.* (2015) 'Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy- independent degradation of damaged mitochondria', *Cell Death and Disease*, pp. 1–11. doi: 10.1038/cddis.2015.157.

Parrón, T. *et al.* (2014) 'Environmental exposure to pesticides and cancer risk in multiple human organ systems', *Toxicology Letters*. Elsevier Ireland Ltd, 230(2), pp. 157–165. doi:

10.1016/j.toxlet.2013.11.009.

Petricca, S. *et al.* (2019) 'Tebuconazole and Econazole Act Synergistically in Mediating Mitochondrial Stress, Energy Imbalance, and Sequential Activation of Autophagy and Apoptosis in Mouse Sertoli TM4 Cells: Possible Role of AMPK/ULK1 Axis', *Toxicological Sciences*. doi: 10.1093/toxsci/kfz031.

Potter, J. D. (1999) 'Colorectal Cancer : Molecules and Populations', *Journal of the National Cancer Institute*, 91(11).

Powers, S. *et al.* (2015) 'Substantial contribution of extrinsic risk factors to cancer development', *Nature*. Nature Publishing Group, pp. 1–15. doi: 10.1038/nature16166.

Ragsdale, S. W. and Pierce, E. (2008) 'Biochimica et Biophysica Acta Acetogenesis and the Wood – Ljungdahl pathway of CO 2 fi xation', 1784, pp. 1873–1898. doi: 10.1016/j.bbapap.2008.08.012.

Reygner, J. *et al.* (2016) 'Changes in Composition and Function of Human Intestinal Microbiota Exposed to Chlorpyrifos in Oil as Assessed by the SHIME ® Model', pp. 1–18. doi: 10.3390/ijerph13111088.

Rizzati, V. *et al.* (2016) 'Chemico-Biological Interactions Effects of pesticide mixtures in human and animal models : An update of the recent literature', *Chemico-Biological Interactions*. Elsevier Ltd, 254, pp. 231–246. doi: 10.1016/j.cbi.2016.06.003.

Rothhammer, V. and Quintana, F. J. (2019) 'The aryl hydrocarbon receptor: an environmental sensor integrating immune responses in health and disease', *Nature Reviews Immunology*. Springer US, 19(3), pp. 184–197. doi: 10.1038/s41577-019-0125-8.

Roy, C. C. *et al.* (2006) 'Short-Chain Fatty Acids: Ready for Prime Time?', *Nutrition in Clinical*, 21(4), pp. 351–366. doi: 10.1177/011542650602100435 1.

Russell, W. R. *et al.* (2013) 'Colonic bacterial metabolites and human health', *Current Opinion in Microbiology*. Elsevier Ltd, 16(3), pp. 246–254. doi: 10.1016/j.mib.2013.07.002.

Sellers, R. S. and Morton, D. (2014) 'The colon: From banal to brilliant', *Toxicologic Pathology*, 42(1), pp. 67–81. doi: 10.1177/0192623313505930.

Sergent, T. *et al.* (2009) 'CYP1A1 induction and CYP3A4 inhibition by the fungicide imazalil in the human intestinal Caco-2 cells-Comparison with other conazole pesticides', *Toxicology Letters*, 184(3), pp. 159–168. doi: 10.1016/j.toxlet.2008.11.009.

Shibamoto, T. and Bjeldanes, L. (2009) *Introduction to Food Toxicology*. 2nd edn. Elsevier.

Silva, V. *et al.* (2019) 'Pesticide residues in European agricultural soils – A hidden reality unfolded', *Science of the Total Environment*. The Authors, 653, pp. 1532–1545. doi: 10.1016/j.scitotenv.2018.10.441.

Skehan, P. *et al.* (1990) 'New Colorimetric Cytotoxicity Assay for', *Journal of the National Cancer Institute*, 82(13), pp. 1107–1112.

Strickland, T. C., Potter, T. L. and Joo, H. (2004) 'Tebuconazole dissipation and metabolism in Tifton loamy sand during laboratory incubation', *Pest Management Science*, 60(7), pp. 703–709. doi: 10.1002/ps.860.

Strober, W. (1997) 'Trypan Strober, W. 1997. Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology, 1–2. doi:10.1002/0471142735.ima03bs21Blue Exclusion Test of Cell

Viability', *Current Protocols in Immunology*, pp. 1–2. doi: 10.1002/0471142735.ima03bs21.

Tan, J., McKenzie, C., *et al.* (2014) *The role of short-chain fatty acids in health and disease.* 1st edn, *Advances in immunology*. 1st edn. Elsevier Inc. doi: 10.1016/B978-0-12-800100-4.00003-9.

Tan, J., Mckenzie, C., *et al.* (2014) 'The Role of Short-Chain Fatty Acids in Health and Disease'. Elsevier Inc., 121, pp. 91–119. doi: 10.1016/B978-0-12-800100-4.00003-9.

Taxvig, C. *et al.* (2007) 'Endocrine-disrupting activities In Vivo of the fungicides tebuconazole and epoxiconazole', *Toxicological Sciences*, 100(2), pp. 464–473. doi: 10.1093/toxsci/kfm227.

Taylor, P., Harris, C. A. and Gaston, C. P. (2007) 'Effects of refining predicted chronic dietary intakes of pesticide residues : a case study using glyphosate Effects of refining predicted chronic dietary intakes of pesticide residues : a case study using glyphosate', (July 2013), pp. 37–41. doi: 10.1080/02652030412331282385.

Thomas, Frederic *et al.* (2016) 'Evolutionary Ecology of Organs : A Missing Link in Cancer Development ?', *TRENDS in CANCER*. Elsevier Inc., (August). doi: 10.1016/j.trecan.2016.06.009.

Tian, Y. *et al.* (2018) 'Short-chain fatty acids administration is protective in colitis-associated colorectal cancer development', *Journal of Nutritional Biochemistry*. Elsevier Inc, 57, pp. 103–109. doi: 10.1016/j.jnutbio.2018.03.007.

Trial, C. S. *et al.* (2015) 'Dietary fiber intake and risk of colorectal cancer and incident and recurrent adenoma in the Prostate , Lung , Colorectal , and Ovarian', (C). doi: 10.3945/ajcn.115.113282.

Tro, E. R. *et al.* (2004) 'Comparative Assessment of the Inhibition of Recombinant Human CYP19 (Aromatase) by Azoles Used in Agriculture and as Drugs for Humans', 30(3), pp. 387–394. doi: 10.1081/ERC-200035093.

Uyemura, S. A. *et al.* (2017) 'A Perspective Discussion on Rising Pesticide Levels and Colon Cancer Burden in Brazil', 5(October), pp. 1–8. doi: 10.3389/fpubh.2017.00273.

Valdes, A. M. *et al.* (2018) 'Role of the gut microbiota in nutrition and health', *BMJ (Online)*, 361, pp. 36–44. doi: 10.1136/bmj.k2179.

Vecchia, M. G. *et al.* (1997) 'Acetate and Propionate Potentiate the Antiproliferative Effect of Butyrate on RBL-2H3 Growth', *General Pharmacology*, 29(5), pp. 725–728.

Victoria Castelli, M., Gabriel Derita, M. and López, S. N. (2017) 'Novel antifungal agents: a patent review (2013 - present)', *Expert Opinion on Therapeutic Patents*. Taylor & Francis, 27(4), pp. 415–426. doi: 10.1080/13543776.2017.1261113.

Vipperla, K. and Keefe, S. J. O. (2016) 'Diet, microbiota, and dysbiosis: a "recipe" for colorectal cancer', *Food & Function*. Royal Society of Chemistry, 7(4), pp. 1731–1740. doi: 10.1039/c5fo01276g.

Weinberg, C. R. and Zaykin, D. (2015) 'Is Bad Luck the Main Cause of Cancer ?', 107, pp. 1–4. doi: 10.1093/jnci/djv125.

Wilson, I. D. and Nicholson, J. K. (2017) 'Gut microbiome interactions with drug metabolism, efficacy, and toxicity', *Translational Research*. Elsevier Ltd, 179, pp. 204–222. doi: 10.1016/j.trsl.2016.08.002.

Won, J. L. *et al.* (2007) 'Pesticide use and colorectal cancer risk in the Agricultural Health Study', *International Journal of Cancer*, 121(2), pp. 339–346. doi: 10.1002/ijc.22635.

Yang, J. Di *et al.* (2018) 'Effects of tebuconazole on cytochrome P450 enzymes, oxidative stress, and endocrine disruption in male rats', *Environmental Toxicology*, 33(8), pp. 899–907. doi: 10.1002/tox.22575.

Yumi, J. *et al.* (2017) 'A critical discussion on diet , genomic mutations and repair mechanisms in colon carcinogenesis', *Toxicology Letters*. Elsevier Ireland Ltd, 265, pp. 106–116. doi: 10.1016/j.toxlet.2016.11.020.

Zhou, J. *et al.* (2016) 'Triazole fungicide tebuconazole disrupts human placental trophoblast cell functions', *Journal of Hazardous Materials*. Elsevier B.V., 308(294–302). doi: 10.1016/j.jhazmat.2016.01.055.