Universidade do Minho
Escola de Ciências

Maria Judite da Silva Barreto

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Dissertação de Mestrado
Mestrado em Ciências - Formação Contínua de Professores
Área de Especialização em Biologia e Geologia

Trabalho realizado sob a orientação da
Professora Doutora Ana Arminda Lopes Preto Almeida
e da
Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real

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DECLARAÇÃO

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Abstract

Colorectal carcinoma (CRC) is a common malignancy in the western world and is also regarded as one of the most preventable cancers. It is generally recognized that the type of diet is an important risk factor for CRC and tumor behavior. Interest in the short-chain fatty acids (SCFA) production by propionibacteria from dairy diet, on the human organism has increased rapidly in the last ten years due to the fact that gastrointestinal functions and beneficial effects are associated with these acids. SCFAs, namely butyrate, propionate and acetate are the major products of the propionibacteria fermentation and metabolism of undigested dietary fibers in the human large intestine. These SCFA have been reported as anti-proliferative and anti-neoplastic agents that induce differentiation, growth arrest and apoptosis in CRC cells lines. It is known that SCFAs protect against development of CRC and therefore, in this monographic review we focus on new aspects of cellular functions of SCFAs as a nutraceuticals in the prevention and/or treatment of CRC. In this context we also aimed at developing a simple, feasible and easily implemented protocols for undergraduate students in secondary school labs using protocols with baker´s yeast cells to illustrate the effect of acetic acid on two key cellular biological processes, namely on cell cycle and cell death.

Resumo

O carcinoma colorretal (CCR) é um tumor maligno comum no mundo ocidental, mas é também considerado um dos cancros mais evitáveis. É geralmente reconhecido que o tipo de dieta é um importante fator de risco para o desenvolvimento e o comportamento deste tipo de cancro. O interesse nos ácidos gordos de cadeia curta (AGCC), produzidos no organismo humano pelas propionibacterias presentes nos lacticínios, cresceu rapidamente nos últimos dez anos, uma vez que várias funções gastrointestinais e efeitos benéficos estão associados a estes ácidos. Os AGCC, nomeadamente o butirato, o propionato e o acetato, são os principais produtos resultantes da fermentação das propionibacterias e do metabolismo das fibras não digeridas no intestino grosso humano. Estes AGCC têm sido referidos como sendo agentes anti-proliferativos e anti-neoplásicos, induzindo a diferenciação, a paragem do crescimento e a apoptose em linhas celulares de CRC. Sabe-se que os AGCC protegem contra o desenvolvimento do CRC e, portanto, nesta revisão monográfica concentramo-nos em novos aspetos das funções celulares dos AGCC como nutracêuticos na prevenção e / ou tratamento do CRC. Neste contexto, foi também nosso objectivo desenvolver um protocolo simples e de fácil implementação para estudantes do ensino secundário, recorrendo à utilização de fermento de padeiro, de forma a ilustrar o efeito do ácido acético em dois processos biológicos celulares chave, nomeadamente, no ciclo celular e na morte celular.

Contents

List of abbreviations

APC – Adenomatous polyposis coli

Bak – Bcl-2-antagonist/killer

Bax – Bcl-2-associated X protein

Bcl-2 – *B-cell lymphoma 2* gene regulator of apoptosis

Bcl-W– Bcl-2-associated pro-survival protein

BRAF – v-RAF murine sarcoma viral oncogene homolog B

Cat D – Cathepsin D

CD95 (Fas/APO-1) – Cell surface receptor of the TNF receptor family

CRC – Colorectal carcinoma

DISC – Death-inducing signaling complex

DNA – Deoxyribonucleic acid

EGFR – Epidermal growth factor receptor

EPIC – European Prospective Investigation into Cancer and Nutrition

FFA2 – Free fatty acid receptor 2

GSTs – Glutathione S-transferases

HATs - Histone acetyltransferases

HDACs – Histone deacetylases

LAB – Lactic acid bacteria

MAPK – Mitogen-activated protein kinase

Muc1/3/4 – Membrane-bound mucins

NRP-1 – Isoform-specific receptor for VEGF

p21 (WAF1/CIP1) – Cyclin-dependent kinase inhibitor 1

pRb – Retinoblastoma protein

ROS – Reactive oxygen species

SCFA – Short chain fatty acids

TNF – Tumor Necrosis Factor

TRAIL – TNF-related apoptosis-inducing ligand

VEGF – Vascular endothelial growth factor

Chapter 1 – Bibliographic review

1. Hallmarks of cancer: the role of apoptosis

Cancer is a very heterogeneous disease, developing in different tissue types and displaying great genetic diversity. Every day our body produces potentially malignant cells. These, however, should not be considered cancerous, we only should name it cancer when there is clinical manifestation or evidence of increased cell proliferation and capability of metastasize in the organism (Simões, 2010).

There are several evidences which indicate that tumourigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Hanahan & Weinberg, 2000). However, recent insights suggest that the underlying etiology and progression of the disease can be reduced to two events, mutations that give rise to excessive proliferation and a compensatory disruption of survival signaling pathways that ensures the persistence of these hyperproliferative cells (Green & Evan, 2002).

Hanahan and Weinberg (2000) proposed that six capabilities acquired during tumor development are shared in common by most and perhaps all types of human tumors. These six essential alterations in cell physiology that collectively dictate malignant growth are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of proto growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Two enabling characteristics crucial to the acquisition of the six hallmark capabilities are the two new emerging hallmark capabilities, the metabolic alterations and signaling interactions of the tumor microenvironment crucial to cancer phenotypes (Figure 1) (Hanahan & Weinberg, 2011).

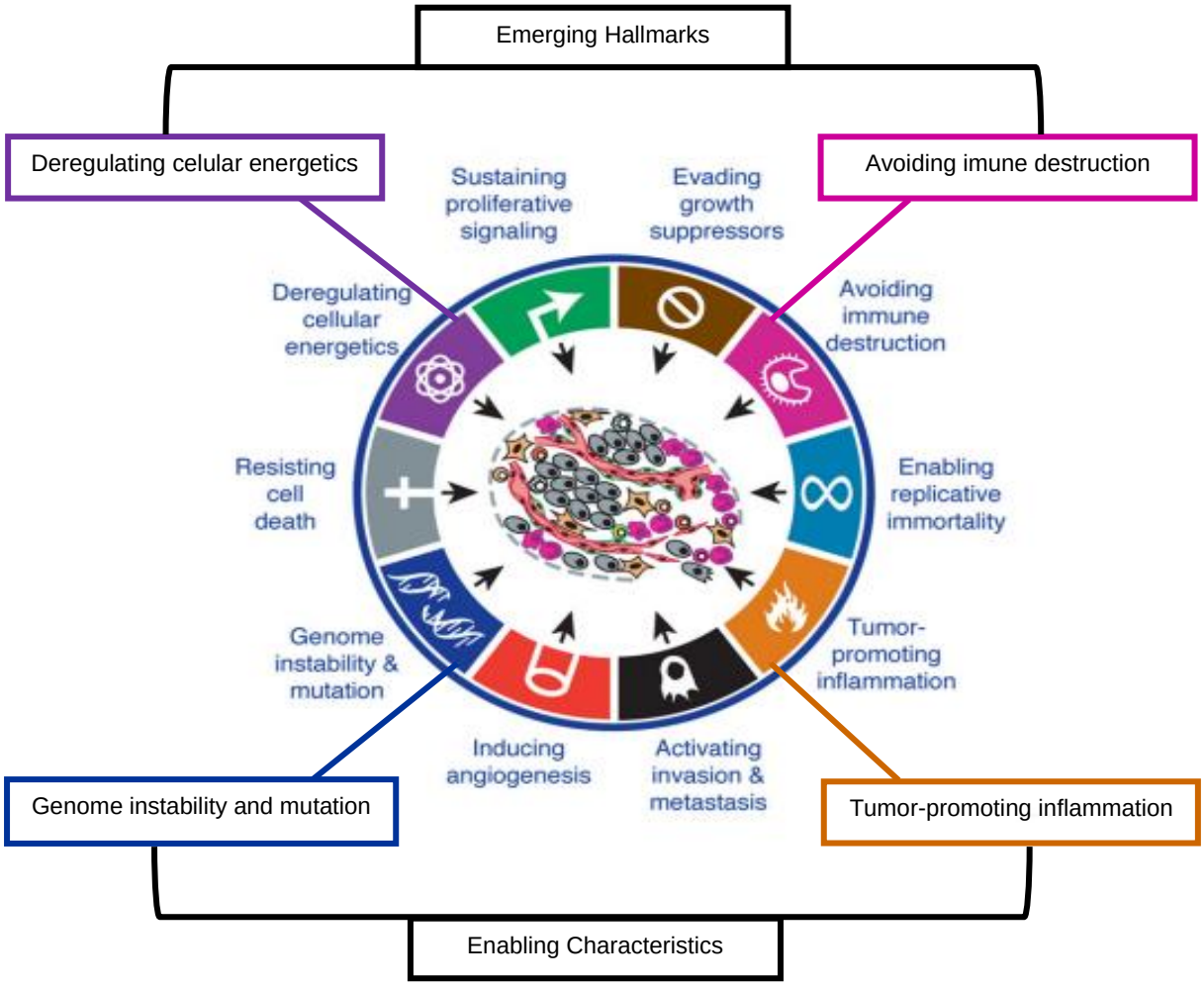


Figure 1 - Emerging Hallmarks and Enabling Characteristics (Adapted from Hanahan & Weinberg, 2011).

Normal tissues carefully control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth-and-division cycle, thereby ensuring a homeostasis of cell number and thus maintenance of normal tissue architecture and function. Cancer cells, by deregulating these signals, become "masters of their own destinies" (Claesson *et al.*, 2012). The ability of tumor cell populations to expand is determined not only by the rate of cell proliferation but also by the rate of cell death, being programmed cell death – apoptosis – the major source of this death. The evidence come mainly from studies in mouse models and cultured cells, as well as from analyses of human cancer tissue, that showed that cancer cells acquired resistance toward apoptosis which is a hallmark of most and perhaps all types of cancer (Hanahan & Weinberg, 2000).

Apoptosis is a genetically predetermined mechanism that may be elicited by several molecular pathways and organisms can trigger this process to remove unwanted and potentially

dangerous cells. The activation of apoptotic genes culminate in morphological and biochemical changes leading to chromatin condensation followed by nuclear condensation, DNA fragmentation and packing of nuclear fragments into multiple membrane-enclosed apoptotic bodies (Ramos, Rabelo, Duarte, Gazzinelli, & Alvarez-Leite, 2002). Apoptosis can be also regulated *via* the action of several oncogenes and subsequently oncoproteins (Salakou *et al.*, 2007).

There are two different apoptosis pathways, called the extrinsic and intrinsic pathways. In the extrinsic pathway (also known as "death receptor pathway"), apoptosis is activated by the cell-surface death receptors CD95 (Apo-1 or Fas)/TRAIL/tumor necrosis factor (TNF) receptor 1 family proteins which are located on the plasma membrane, and directly activates the caspase cascade via the recruitment of the "initiator" caspase-8 within a death-inducing signaling complex (DISC). The intrinsic pathway (also called "mitochondrial pathway"), leads to the release of cytochrome *c* from the damaged mitochondrion, which then binds to the adaptor molecule APAF-1 and an inactive "initiator" caspase, procaspase 9, within a multiprotein complex called the apoptosome. This leads to the activation of caspase 9, which then triggers a cascade of caspases activation (caspases 3 and 7) resulting in the morphological and biochemical changes associated with apoptosis (Tzifi *et al.*, 2012). Caspase activated cell death is regulated by genes of the Bcl-2 family, for instance by the pro- and anti-apoptotic genes, Bax and Bcl-2, respectively (Fauser, Prisciandaro, Cummins, & Howarth, 2011; Jin & El-Deiry, 2005). Currently, the intrinsic apoptotic pathway is more widely implicated as a barrier to cancer pathogenesis. Both apoptotic pathways are targets for cancer treatment (Shao, Gao, Marks, & Jiang, 2004).

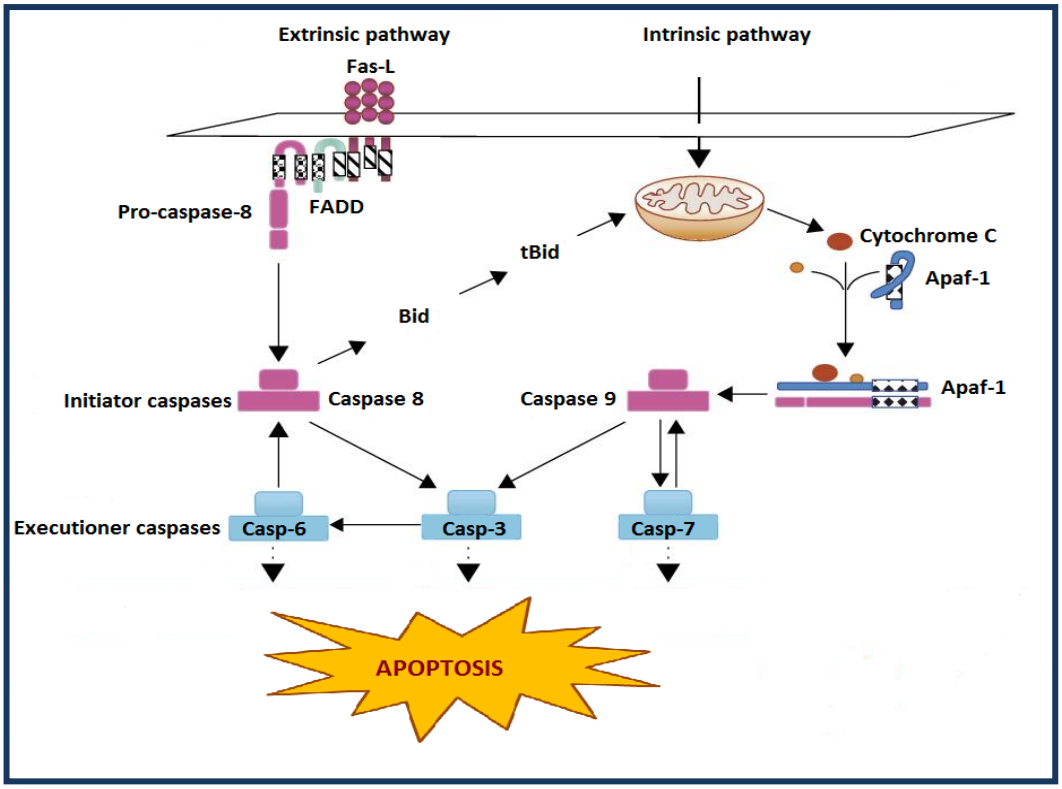


Figure 2 - Schematic representation of apoptotic pathways.

2. Colorectal carcinoma

Colorectal carcinoma (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million cancer cases in the world, accounting for 9.8% of total cancer cases, according to data from GLOBOCAN project (2008). In Europe, the incidence of CRC is 229.229 cases, accounting for 13.5% of total cancer cases. In Portugal, the CRC is the second most prevalent cancer (after prostate cancer) with an incidence number of 3951 cases and an incidence rate of 16.5% of total cancer cases (Globocan project, 2008, http://globocan.iarc.fr/). Cancer prevention is an essential component of cancer control strategies because about 40% of all cancer deaths can be prevented (World Health Organization, 2008, http://www.who.int/en/).

Studies reported that dietary patterns, lifestyle, physical inactivity and obesity increased CRC risks, especially in genetically predisposed populations (Doll & Peto, 1981; Potter, 1999; Qin *et al.*, 2010). CRC is thus causally related to both genes and environment. Environment is a risk factor that may cause mutations and initiate cancer or enhance growth by genetic and epigenetic mechanisms (Ferguson, 1999; Simões, 2010) .

2.1. Types of colorectal carcinoma

About 75% of patients with CRC have sporadic disease with no apparent evidence of having inherited the disorder. The remaining 25% of patients have a family history of CRC that suggests a hereditary contribution, common exposures among family members, or a combination of both. Genetic mutations have been identified as the cause of inherited cancer risk in some colon cancer-prone families; these mutations are estimated to account for only 5% to 6% of CRC cases overall. It is likely that other undiscovered genes and genetic factors contribute to the development of familial CRC in conjunction with nongenetic risk factors (National Cancer Institute, 2013, http://www.cancer.gov/cancertopics/pdq/genetics/colorectal). Among the non-sporadic CRC cases, 5 to 15% can be attributed to the following hereditary CRC syndromes: Lynch syndrome (also hereditary nonpolyposis CRC or HNPCC), familial adenomatous polyposis (FAP), and MUTYH-associated polyposis (MAP) (Castells, Castellvi-Bel, & Balaguer, 2009).

Two-thirds of CRCs are located in the rectum, rectosigmoid, or sigmoid colon with the other third distributed in the remainder of the colon. Adenocarcinomas are the most common type of CRC and have its origin in intestinal epithelial cells. The progression from adenoma to carcinoma occurs by the sequential accumulation of genetic changes, which is the more accepted model for understanding carcinogenesis process (presented in Figure 3) (Bruckner *et al.* 2000) Available from: http://www.ncbi.nlm.nih.gov/books/NBK20861/; Cancer Research UK).

Adenocarcinomas start in the intestinal gland cells in the lining of the colon wall. There are one or two rare types of adenocarcinoma of the colon and rectum, called mucinous tumors and signet ring tumors and these terms refer to how the cells look under the microscope. Only about 1 to 2% of colorectal cancers are the signet ring type. Squamous cells cancers are the skin like cells that make up the bowel lining together with the gland cells. Carcinoid is an unusual type of slow growing tumor called a neuroendocrine tumor. Between 4 to 17% of every carcinoid tumors diagnosed start in the rectum and between 2 to 7% out of every carcinoid tumors diagnosed begin in the large bowel. Sarcomas are cancers of the supporting cells of the body, such as bone or muscle. Most sarcomas found in the colon or rectum are leiomyosarcomas, which account for less than 2% of colorectal carcinomas and have a high chance of metastasizing. Lymphomas are cancers of the lymphatic system. Only about 1 in 100 cancers

diagnosed in the colon or rectum (1%) are lymphomas (Cancer Research UK, 2013, http://www.cancerresearchuk.org/cancer-help/type/bowel-cancer/about/types-of-bowel-cancer).

2.2. Colorectal carcinoma: associated genetic alterations

The model of cell transformation and progression from adenoma to colorectal carcinoma is based on the concept that progression is accompanied by the accumulation of molecular alterations in which adenomatous polyposis coli (APC), K-RAS, and p53 genes play a central role (presented in Figure 3) (Fearon & Vogelstein, 1990). A number of data indicate that the simultaneous presence of alterations of these genes is not a frequent event. In particular, K-RAS and p53 mutations rarely co-exist in the same tumor, indicating that these alterations do not represent a synergistic evolutionary pathway (Smith *et al.*, 2002).

It was found that somatic mutations in APC gene are also found in the great majority of sporadic colorectal tumors (Miyoshi *et al.*, 1992). APC has been proposed to function as a ''gatekeeper'' gene, regulating the entry of epithelial cells into adenoma-carcinoma progression (Kinzler & Vogelstein, 1996), maintaining low levels of β-catenin in the absence of a Wnt signal, thus preventing excessive cell proliferation. Axis duplication and cell transformation are based on the activation of the canonical Wnt pathway that involves the multifunctional protein β-catenin (Rao & Kuhl, 2010). A mutation of the gatekeeper leads to a permanent imbalance of cell division over cell death (Kinzler & Vogelstein, 1996).

The RAS oncogene promotes tumor formation through stimulation of cell proliferation, motility and regulation of apoptosis. Activating mutations in the RAS oncogenes (H-, N-, and K-RAS) are found in approximately 20% of all human tumors (Shaw *et al.*, 2011) and mediates several key aspects of oncogenesis, including deregulated cell growth, evasion of apoptosis and malignant transformation, a consequence of the loss of GTPase activity (Shaw *et al.*, 2011; Smith *et al.*, 2002). The TP53 gene product, p53, functions as a transcription factor, exerting cell cycle control by binding to specific recognition sequences in a variety of genes including p21, Bax, and Bcl-2 in response to DNA damage or other cellular stress (A. J. Levine, 1997). About 70% of CRCs contain p53 mutations (Baker *et al.*, 1990), rendering them susceptible to failure of apoptosis and increased accumulation of DNA damage, allowing unregulated growth (el-Deiry *et al.*, 1993; Williams, Coxhead, & Mathers, 2003). These mutations in p53 are proposed to be relatively late events in the development of colorectal tumors, with the loss of p53-mediated

pathways of apoptosis considered to be an important determinant of progression from adenoma to malignant tumor (Smith *et al.*, 2002).

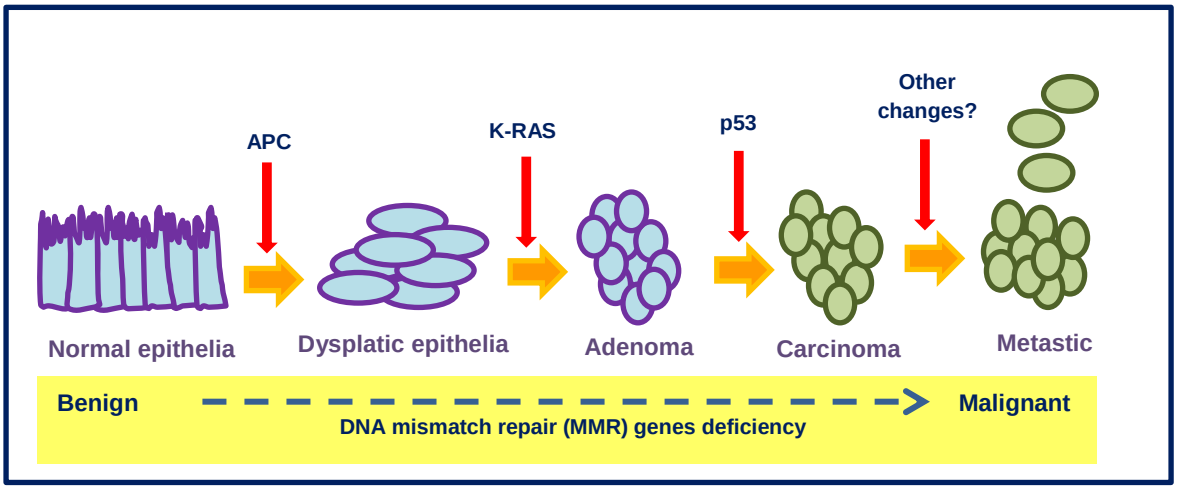


Figure 3 - Genetic changes associated with colorectal tumorigenesis (Adapted from Kinzler & Vogelstein, 1996; Smith *et al.*, 2002).

BRAF is one of the RAF genes involved in the important RAS/RAF/MEK/MAP kinase intracellular signaling pathway, which regulates different physiological processes including cell growth, differentiation, and apoptosis (Calistri *et al.*, 2005; Fang & Richardson, 2005). BRAF-activating gene mutations have been detected in many tumor types (Davies *et al.*, 2002). However, Calistri *et al.* (2005) observed a very low frequency of mutations in their series of sporadic colorectal carcinoma, and no evident association with other specific gene mutations, suggesting that BRAF could represent an alternative pathway to both p53 and K-RAS genes, mainly in hereditary CRC characterized by microsatellite instability (MSI) (Calistri *et al.*, 2005).

It is thought that at least 50% of colorectal cancers have a deregulation of the MAPK pathway (Fang & Richardson, 2005). Two major oncogenes are implicated in sporadic colorectal carcinogenesis, KRAS and BRAF mutations, which are alternative (Oliveira *et al.*, 2007). Mutations on KRAS and BRAF genes are frequently found in malignant and pre-malignant colorectal lesions (Rajagopalan *et al.*, 2002). Mutations of the KRAS proto-oncogene are an early event in development of CRCs (Bos *et al.*, 1987). KRAS oncogene is mutated in 21% of all human sporadic cancers, including in about 30% of CRCs cases (Fang & Richardson, 2005; Oliveira *et al.*, 2007), and BRAF mutations are found in 20% of all human cancers, including in about 10% CRCs cases (Ahlquist *et al.*, 2008; Oliveira *et al.*, 2007; Velho, Corso, Oliveira, & Seruca, 2010).

2.3. Classical chemotherapy for colorectal carcinoma

According to Sobrinho Simões (2010), there will never be possible for medicine to cure advanced cancers. However, there will have a percentage of cancers that are always curable, those who are localized "*in situ*" when diagnosed early that can be removed by surgical techniques that have evolved in the past years. Currently, over 50% of cancers can be cured by surgery and the other 50% have no cure; medicine only can keep them in a state of equilibrium with the immune defenses. In these cases cancer may be regarded as a chronic disease: the cancer exists, but the disease does not manifest or at least it is mitigated. Oddly enough, cancer is a disease extremely inefficient, hence the hope of turning it into a chronic disease (Simões, 2010).

The development of CRC often follows a defined pattern. The entire process frequently takes a long period, like decades, before a malignant tumor is finally formed, and is thought to develop in a multistep process, known as the "adenoma-carcinoma sequence" (Vogelstein *et al.*, 1988). Because colon epithelia are directly exposed to dietary compounds, elimination of precancerous or cancerous cells by nutritional or chemopreventive interventions, or both, represents an approach to the lowering of the incidence of colon cancer (Cai *et al.*, 2006; Dove-Edwin & Thomas, 2001) .

The current therapies are those in which a protein modification is identified in cancer. All modern drugs fall into one of two groups: either are small molecules which inhibit proteins action, which enter into the cell, or antibodies which are fixed on the cell surface, blocking, for example, receptors that are greatly increased in some cancer cells. These receptors stop working and the cell ceases to have the incentive to proliferate (Simões, 2010). In the last two decades several advances were achieved in the treatment of CRC. With more effective drugs, improved surgery, better radiotherapy and a strong randomized clinical trials evidence base, patients now have a higher chance of cure and, when cure is not achievable, they survive longer with their disease (Braun & Seymour, 2011). The optimum treatment strategy for patients with CRC depends on a large number of factors, such as age, performance status, the presence of other disorders or diseases and the treatment setting (adjuvant *versus* palliative *versus* neoadjuvant) (Braun & Seymour, 2011). The main types of treatment that can be used for colon and rectal carcinoma are surgery, radiation therapy, chemotherapy and targeted therapy. Surgery is the only curative treatment for CRC. Depending on the stage of the cancer, two or more of these

types of treatment may be combined at the same time or used after one another (American Cancer Society, 2013, http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-chemotherapy).

In recent years new different approaches to nutritional treatment have been used to correct the deficits observed in patients with colorectal cancer (de Oliveira & Aarestrup, 2012), such as the use of nutraceuticals.

3. Nutraceuticals in colorectal carcinoma prevention or therapy

In the last years several products have been commercialized in the form of pharmaceutical products, such as pills, tablets, solutions, etc., incorporating food extracts or phytochemical-enriched extracts to which a beneficial physiological function has been directly or indirectly attributed (Palthur, 2010). This variety of products cannot be truly classified as "food" or "pharmaceutical", so a combined term between nutrients and pharmaceuticals, "nutraceuticals", has been coined to designate them (Espin, Garcia-Conesa, & Tomas-Barberan, 2007).

According to Stephen Defelice (1995) "a nutraceutical is any substance that is a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, and processed foods such as cereals, soups and beverages" (DeFelice, 1995). However, there is often confusion in the use of this terminology as there is a slight difference between the functional foods and nutraceuticals. When food is being cooked or prepared using "scientific intelligence" with or without knowledge of how or why it is being used, the food is called "functional food". Functional food provides the body with the required amount of vitamins, fats, proteins, carbohydrates, etc. needed for its healthy survival. When functional food helps in the prevention and/or treatment of disease(s) and/or disorder(s) it is called a nutraceutical (Pandey, 2010).

The nutraceuticals revolution began in the early 1980s, sparked off when the actual or potential clinical benefits of calcium, fiber and fish oil were supported by clinical studies published in distinguished medical journals, and when physicians began to educate their colleagues and consumers about these substances via the media (DeFelice, 1995). Within

European Medicines law, a nutraceutical can be defined as a medicine for two reasons: it can be used for the prevention, treatment or cure of a condition or disease or it can be administered with a view to restoring, correcting or modifying physiological functions in human beings (Richardson, 1996). However, no specific regulation exists in Europe to control nutraceuticals (Espin *et al.*, 2007). The majority of the definitions indicate the health benefits of nutraceuticals and among these health benefits are prevention and treatment of diseases.

The major impact of eating habits on the prevalence of CRC has triggered efforts to design an optimal diet and/or to create food supplements specifically reducing the risk of cancer. Already in 1989 Fuller checked the growing interest in the use of live microbial agents for health maintenance and disease prevention or treatment (Fuller, 1989).

3.1. Prebiotics

A prebiotic was first defined by Gibson and Roberfroid as ‘‘a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health’’ (G. R. Gibson & Roberfroid, 1995). However many food components, especially many food oligosaccharides and polysaccharides (including dietary fiber), have been claimed to have prebiotic activity without due consideration to the criteria required.

In practical terms, prebiotics are short-chain carbohydrates (SCCs) that are nondigestible by human enzymes and that have been called resistant SCCs. They are sometimes referred to as nondigestible oligosaccharides (NDOs). Nonetheless, NDOs are not strictly oligosaccharides and their nondigestibility is largely assumed but not always proved (Cummings, Macfarlane, & Englyst, 2001). An oligosaccharide, according to the International Union of Pure and Applied Chemistry Joint Commission on Biochemical Nomenclature (IUPAC-IUB JCBN) definition, is a molecule containing a small number (2 to about 10) of monosaccharide residues connected by glycosidic linkages ("IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Abbreviated terminology of oligosaccharide chains. Recommendations 1980," 1982). Some of the carbohydrates that are named prebiotics often fall outside this definition because several of them have a degree of polymerization (DP) higher than 10 (Cummings *et al.*, 2001). Not all dietary carbohydrates are prebiotics, and clear criteria need to be established for classifying a food ingredient as a prebiotic. These criteria are, by Gibson and Roberfroid, (1) to resist gastric

acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (2) to be fermented by the intestinal microflora; (3) to stimulate selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing (G. R. Gibson, Probert, Loo, Rastall, & Roberfroid, 2004).

In 2004 the definition was updated when prebiotics were defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host wellbeing and health" (G. R. Gibson *et al.*, 2004). The definition considers microflora changes in the whole gastrointestinal tract and extrapolates the definition into other areas that may benefit from a selective targeting of bifidobacteria and lactobacilli (Bellei G., 2012). Any dietary material that is non-digestible and enters the large intestine is a candidate prebiotic. This includes polysaccharide-type carbohydrates such as resistant starch and dietary fiber, as well as proteins and lipids. However, current prebiotics are confined to non-digestible oligosaccharides, many of which seem to confer the degree of fermentation selectivity that is required (regarding bifidobacteria)(G. G. Gibson *et al.*, 2010)

This prebiotics compounds have been shown to be a source of SCFA both *in vitro* and *in vivo*. Pan *et al* (2009) demonstrated, using a rat model, that the intake of selected prebiotic oligosaccharides improved concentrations of feacal SCFA, including butyrate (Pan *et al.*, 2009). By producing a greater concentration of butyrate, the preferred energy source for colonocytes (Scheppach, 1994), a trophic effect may result within the gastrointestinal tract (Pan *et al.*, 2009). Hence, prebiotics can have an impact on gut health in general, and are believed to play an important role in the prevention of CRC, as it has been highlighted by a Fotiadis and co-workers review (Fotiadis, Stoidis, Spyropoulos, & Zografos, 2008). For example, Bindels *et al.* (2012) proposed that propionate production by propionibacteria could be one of the gut microbial functions responsible for the anti-tumor effect of prebiotic nutrients (Bindels *et al.*, 2012) .

Among the established prebiotics, inulin-type fructans, present in foods, have been studied widely in the setting of CRC (Pool-Zobel, 2005) and have been demonstrated to elevate the levels of bifidobacteria and to increase SCFA concentrations in the colon (Bouhnik *et al.*, 1999). Various studies have shown that these prebiotics prevent chemically induced preneoplastic lesions, aberrant crypt foci (ACF) and tumors in the colon of rats and mice (Reddy, Hamid, & Rao, 1997; Verghese, Rao, Chawan, & Shackelford, 2002).

3.2. Probiotics

The term probiotic was first used in 1965 in contrast to the word antibiotic and defined as "substances secreted by one microorganism, which stimulates the growth of another" (Schrezenmeir & de Vrese, 2001). These probiotics are nonpathogenic micro-organisms that, when ingested, exert a positive influence on the health or physiology of the host (Fuller, 1989). They can influence intestinal physiology either directly or indirectly through regulation of the endogenous microflora. This complex multicellular entity plays an important role in maintaining homeostasis in the body (Macfarlane & Macfarlane, 2012).

The mammalian intestinal tract contains a complex, dynamic, and diverse microbial community dominated by nonpathologic bacteria or "good bacteria" (Teitelbaum & Walker, 2002). The vast majority of bacteria in the human body reside in the large intestine, where the slow transit time, availability of nutrients, anaerobic conditions and pH are favorable for microbial growth (G. G. Gibson *et al.*, 2010). Colonic microorganisms have ample opportunity to degrade available substrates, which may be derived from either the diet or by endogenous secretions (Bergman, 1990; Cummings & Macfarlane, 1991; Miller & Wolin, 1979). Bacterial fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolizable energy for microbial growth and maintenance and other metabolic end products for use by the host (Cummings & Macfarlane, 1991; Wong, de Souza, Kendall, Emam, & Jenkins, 2006). In terms of end products, a variety of different metabolites arise, including short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, (G. R. Gibson, Willems, Reading, & Collins, 1996). Thus, carbohydrate fermentation generally leads to health promoting SCFA production (Figure 4).

Each person has a distinct and highly variable microbiota, but a conserved set of gut colonizers (the core gut microbiota) and genes (the core microbiome) are shared among individuals (Qin *et al.*, 2010; Turnbaugh *et al.*, 2009) and may be required for the correct functioning of the gut (Tremaroli & Backhed, 2012). The human gut microflora differs in composition between infants and adults at various stages of life. Initial bacterial colonization of the human intestine begins at birth and on weaning *Bifidobacteria* decrease and a more "adult" profile of bacteria are present. In healthy elderly people, a decrease in the number of *Bacteroides* and *Bifidobacteria* has been reported (Roy, Kien, Bouthillier, & Levy, 2006). It is important that older people ingest sufficient amounts of dietary fiber to obtain the required amount of SCFA in

the gut lumen. Dietary supplements with defined food ingredients that promote particular components of the microbiota may also prove useful for maintaining health in older people (Claesson *et al.*, 2012; Macfarlane & Macfarlane, 2012) .

When probiotics are ingested, they are able to resist the physicochemical conditions prevailing in the digestive tract. The strains most frequently used as probiotics consist mostly of strains of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, bacterial types which have been used for centuries in the production of fermented dairy products (Fooks & Gibson, 2002; Heyman & Menard, 2002). Because of all their benefits, probiotics represent an emerging therapeutic option. There is accumulating evidence describing the capacity for probiotic strains to prevent CRC, and in some cases, treat established tumor (Geier, Butler, & Howarth, 2006).

The predominant species used as probiotic agents belong to the group of lactic acid bacteria (LAB). Due to their long history of safe use in foods, most species of LAB are considered as commensal microorganisms with no pathogenic potential (Chukeatirote, 2003). Within the LAB group, the genus *Lactobacillus* is the most widely encountered for probiotics (Chukeatirote, 2003). *L. acidophilus* NCFM strain exhibits ability to reduce levels of free amines in the intestine, leading to a low risk of colon carcinoma (Goldin & Gorbach, 1984). However, also here the controverse is present, for example, Shahani *et al.* (1980) observed that consumption of large quantities of fermented milk products containing *Lactobacillus* or *Bifidobacteria* were associated with a lower incidence of CRC (Shahani & Ayebo, 1980) although, other studies suggested that consumption of fermented dairy products had little influence or no protection (Kampman, Goldbohm, van den Brandt, & van 't Veer, 1994).

The mechanisms by which probiotics may inhibit CRC are not yet fully characterized. The production of SCFAs is one key mechanism by which probiotics and prebiotics may impart beneficial effects (Figure 4). Mattar *et al.* (2002) demonstrated that the addition of the probiotic *Lactobacillus casei GG* (LGG) to the Caco-2 cells induced MUC-2 expression (responsible for the production of mucins of the intestine mucus layer) that correlated with LGG dosage. They surmise that LGG may bind to specific receptor sites on the enterocyte and stimulate the up-regulation of MUC-2, resulting in increased inhibition of bacterial translocation (Mattar *et al.*, 2002). Ohkawara *et al.* (2005) investigated the bacterial strain *Butyrivibrio fibrisolvens* MDT-1 in the context of CRC treatment as it produces high amounts of butyrate, a SCFA with well-known apoptotic ability in cancer cells. In the 1,2-dimethylhydrazine-induced mouse model of colon carcinoma, administration of *Butyrivibrio fibrisolvens* MDT-1 led to a significant decrease in ACF,

and the number of mice with an increased proportion of aberrant crypts *per* foci was also reduced, indicating an inhibited progression of tumor development. This suggests that MDT-1 may be a potential new probiotic with the ability to reduce the incidence of CRC (Ohkawara *et al.*, 2005).

The genetic manipulation of probiotics is another area of research in the treatment of CRC, which can be a method to deliver important anti-neoplastic factors to the colon. Steidler *et al.* (2000) demonstrated that *Lactococcus lactis* genetically-engineered to produce the anti-inflammatory cytokine IL-10, reduced colonic inflammation in the dextran sulphate sodium model of colitis (Steidler *et al.*, 2000). This study highlighted the potential for probiotics to be used as a delivery system for anti-inflammatory or anti-tumorigenic substances which could help in the prevention or treatment of CRC (Geier *et al.*, 2006). This is an important advance in the field of treatment of CRC, as a probiotic strain could potentially be engineered to produce other cytokines. Castagliuolo *et al.* (2005) used a nonpathogenic strain of *E. coli* designed to deliver TGF-β genes to the colonic mucosa that successfully demonstrated to reduce the severity of experimental colitis in mice (Castagliuolo *et al.*, 2005). This strategy could also be beneficial in the prevention of CRC, since TGF-β could be administered as an anti-proliferative factor, thus suppressing tumor development (Geier *et al.*, 2006). The use of this technology provides a mean by which probiotic strains can be tailored to deliver a wide array of therapeutic genes or factors including other anti-proliferative factors, or pro-apoptotic factors including anti-inflammatory cytokines (Geier *et al.*, 2006).

3.2.1. Propionibacteria

Among different dietary bacteria, the *Propionibacterium* form a genus, which is found in specific dairy products (Mantere-Alhonen, 1995). Several experiments have shown that propionibacteria also possess probiotic characteristics when used alone or together with lactic acid and/or bifidobacteria (Mantere-Alhonen, 1995). Propionibacteria possess a peculiar fermentative metabolism which leads to the production of carbon dioxide and SCFA, such as propionate and acetate (Lan, Lagadic-Gossmann, Lemaire, Brenner, & Jan, 2007). In the last decades, several studies showed that the SCFA, namely acetate, propionate and butyrate, induces apoptosis in CRC cells but not in normal cells (Tang, Chen, Jiang, & Nie; Sakata 1987; Sauer, Richter, & Pool-Zobel, 2007). These results will be discussed later in this work.

The first time that the probiotic and growth promoting effect of pure propionibacteria was observed was in a study made with piglets by Mantere-Alhonen, in 1982 (Mantere-Alhonen, 1982). It was a rather large test material, thus the positive results of the feeding experiments should be considered as statistically significant, and they prove the ability of propionibacteria to act as probiotics (Mantere-Alhonen, 1995). In 2002 Jan and Balzacq proposed that propionibacteria could constitute probiotics efficient in digestive cancer prophylaxis via their ability to produce SCFA which induced apoptosis (Jan *et al.*, 2002). Propionibacteria can survive in the human intestine and was found to induce apoptosis in colorectal cancer cells but not in normal cells, at least in part, due to their specific property to produce propionate and acetate (Jan *et al.*, 2002).

Immerseel *et al.* (2010) suggested, that an ideal probiotic would be a colonizing bacterium that combines systemic anti-inflammatory and immunoregulatory effects with delivery of high butyrate levels at the site of action and that can be ingested in a stable form, such as spores (Van Immerseel *et al.*, 2010). These studies indicate that SCFA delivery via probiotic ingestion may be an exciting new prevention/treatment option for CRC (Geier *et al.*, 2006).

The possibility of using live propionibacteria in diet as preventive anti-cancer agents remains to be determined first by *in vivo* relevance of SCFA-based therapeutic strategy, and second by efficiently deliver SCFA to cancer cells (Lan *et al.*, 2007).

3.3. Symbiotics

When prebiotics and probiotics are administered simultaneously, the combination is termed symbiotics. Symbiotics have been proposed as a new preventive and therapeutic option.

The mechanisms by which pro-, pre- and symbiotics may inhibit colon carcinoma are beginning to be understood (Fotiadis *et al.*, 2008). The prebiotic in the symbiotic mixture improves the survival of the probiotic bacteria and stimulates the activity of the host's endogenous bacteria (Mugambi, Musekiwa, Lombard, Young, & Blaauw, 2012). Consumption of probiotics and prebiotics together can increase the beneficial effects of each, since the stimulus of probiotic strains leads to selection of ideal symbiotic pairs (de Oliveira & Aarestrup, 2012).

A human study has investigated the effect of an oligofrutose and inulin mixture together, a product called 'Synergy' that combines short-chain oligofrutose and long-chain inulin with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 on biomarkers of cancer (Rafter *et*

al., 2007). The study involved a twelve week double blind placebo-controlled trial in patients with cancer and polypectomised individuals. The symbiotic intervention resulted in significant alterations in the composition of the colonic bacterial ecosystem, which presumably have consequences for the metabolic activity of this organ. Colorectal cell proliferation and genotoxicity were significantly reduced, and the intestinal barrier function increased (Rafter *et al.*, 2007).

The most important conclusion from Brady *et al.* (2000) is that in animals it appears to be a synergistic effect of consumption of probiotic bacteria and prebiotics such as fructoligosaccharides on the attenuation of the development of CRC. The effect is often not large, but it could be beneficial, in combination with other ways to reduce risk (Brady *et al.*, 2000). For example, *in vitro* studies only comprising prebiotics, the increase in acetic acid was reported to be between two and six times higher as compared to controls and for butyric acid the highest concentrations observed were four times higher as compared to control (van Zanten *et al.*, 2012). These increases in concentrations of acetic and butyric acids were however lower than the increases observed for all symbiotic combinations investigated in van Zanten *et al.* (2012) study, where concentrations were three to eight times higher for both acetic and butyric acids as compared to control. These findings emphasize that a synergistic effect may be obtained when combining the prebiotic with the probiotic strains (van Zanten *et al.*, 2012).

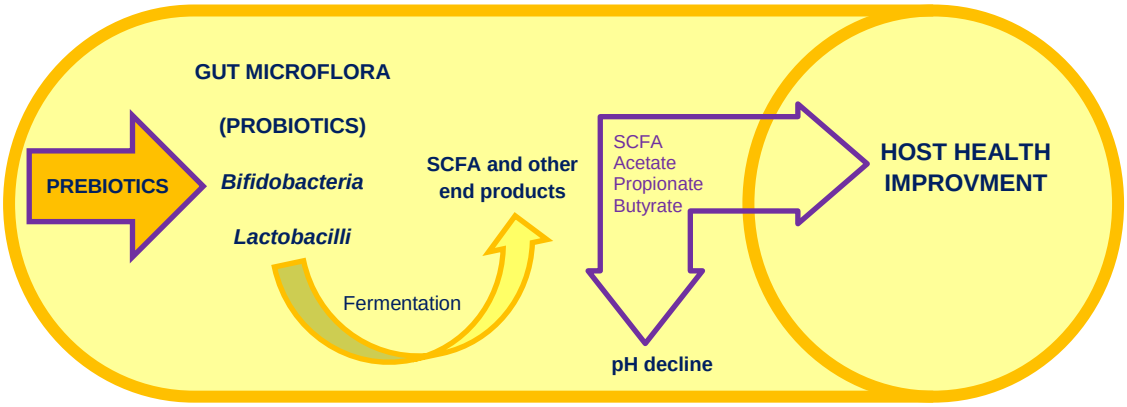


Figure 4 - General process of colonic fermentation by symbiotics. Prebiotics compounds are food source for probiotic microorganisms, which produce SCFA that contribute to host health improvement (Adapted from Huazano-García & López, 2013).

4. The association between diet, SCFA and colorectal carcinoma risk

Although there is a strong genetic component in the development of colorectal adenomas or carcinomas, it is generally accepted that environmental factors including diet and lifestyle have a major impact on risk (Azcarate-Peril, Sikes, & Bruno-Barcena, 2011). Diet and nutrition are estimated to explain as much as 30-50% of the worldwide incidence of CRC and a high intake of calories, fats, red meat and low consumption of fruits and vegetables are associated with the risk of CRC development (Cervi, 2005; Chan & Giovannucci, 2010). It is believed that a proper diet can prevent three to four million new cases per year (Garófolo, 2004).

Dietary fiber has been consumed for centuries and has been recognized as having health benefits. The consumption of foods rich in this dietary component such as fresh vegetables and fruits, whole grains and nuts is associated with gastrointestinal benefits, such as increasing stool bulk and improving laxation (Schneeman, 1999). Despite all the benefits, Scharlau *et al.* (2009) referred that human studies are not showing that fruit and vegetable intakes are associated with a reduced cancer incidence in general and in particular with reduced CRC risk (Fuchs *et al.*, 1999; Scharlau *et al.*, 2009). For example, Terry *et al.* (2001) results do not support the hypothesis that high consumption of cereal fiber decreases the risk of colon or rectal carcinoma, even based on a much broader range of cereal fiber intake than had been examined in previous cohort studies (Terry *et al.*, 2001). However, it is also likely that the frequency of fruit and vegetables consumption that is adequate to decrease cancer risk, taking into account other health consequences, probably varies with individual factors and, perhaps, with other cofactors in the population, such as multivitamin use and whether foods are fortified with other micronutrients. In contrast, the role of diet in cancer development is strongly supported by epidemiological studies, in particular in the case of cancers of the digestive tract (Vano, Rodrigues, & Schneider, 2009). Topping and Clifton (2001) observed that some studies showed that native East Africans, consuming a diet high in unrefined cereals, were at lower risk of CRC, diverticular disease, and constipation than Europeans who ate a diet low in such foods (Topping & Clifton, 2001). Another study by Bamia *et al.* (2013) allowed to conclude that adherence to Mediterranean diet may be associated with lower CRC risk (Bamia *et al.*, 2013) . The traditional Mediterranean diet is characterized by high intakes of vegetables, fruit/nuts, fish, cereals and legumes, moderate alcohol consumption (particularly wine during meals), low to-moderate consumption of dairy products (mainly cheese and yogurt) and low consumption of meat/meat

products (Trichopoulou & Lagiou, 1997) . The main source of lipids is olive oil consumed in large quantities, and it is the main source of monounsaturated fatty acids in Mediterranean populations (Bamia *et al.*, 2013). It has been suggested that up to 25% of colorectal carcinomas could be prevented by shifting to a Mediterranean diet (Trichopoulou, Lagiou, Kuper, & Trichopoulos, 2000).

Regarding diet, resistant starch, a type of dietary fiber, has been hypothesized to have specific anti-cancer properties since this form of fiber is preferentially fermented by microflora into potentially beneficial SCFA in the colon (Chan & Giovannucci, 2010). These SCFA produced from undigested dietary fibers in the human large intestine, have been extensively reported as antitumor agents that induce differentiation, growth arrest and apoptosis in colon carcinoma cells (Tang, Chen, Jiang, & Nie, 2011a). The most important role of SCFA in colonic physiology is their trophic effect on the intestinal epithelium. Sakata reported that the presence of SCFA in rat colon stimulates mucosal proliferation (Sakata, 1987). In human, SCFAs production from inulin-type fructan can increase the metabolic activity, pointing to trophic effects for normal colonocytes (Sauer, Richter, & Pool-Zobel, 2007).

SCFA are organic fatty acids with 1 to 6 carbon atoms, increasing from acetic (C2:0), propionic (C3:0), butyric (C4:0), valeric (C5:0) and caproic (C6:0) acids (Fauser *et al.*, 2011). These intermediate carboxylic acids at the physiological pH predominate in their dissociated form acetate, propionate, butyrate, valerate and caproate are the principal anions which arise from bacterial fermentation of polysaccharide, oligosaccharide, protein, peptide, and glycoprotein precursors in the colon (Bergman, 1990; Cummings & Macfarlane, 1991; Miller & Wolin, 1979). Among these, however, butyrate, acetate, and propionate have been mainly emphasized. In particular, butyrate was addressed to be more beneficial for promoting colonic health and more effective for stimulating the proliferation of intestinal mucosal cells than acetate and propionate (Sakata, 1987). These SCFA, especially butyrate, are recognized for their potential to act on secondary chemoprevention by slowing growth and activating apoptosis in CRC cells. Additionally, SCFA can also act on primary prevention by activation of different drug metabolizing enzymes. This can reduce the burden of carcinogens and, therefore, decrease the number of mutations, reducing cancer risk (Scharlau *et al.*, 2009).

Results from Takashi Sakata (1987) indicated that the stimulatory effect of SCFA on intestinal epithelial cell proliferation *in vivo* is substantial and highly reproducible, and that the effect of SCFA persists sufficiently long to be of nutritional significance (Sakata, 1987). When the

concentrations of SCFA used were compared with the lumen concentrations measured by Yang *et al.* (1970), it was clear that physiological doses of acetate had a trophic effect on colonic epithelium, and butyrate had a trophic effect on both jejunal and colonic epithelium. In contrast, propionate was effective only at superphysiological doses (Sakata, 1987; Yang *et al.*, 1970).

Several benefits can be obtained when the ingestion of dietary fibers are higher: increases in SCFA result in decreased pH, which indirectly influences the composition of the colonic microflora (eg, reduces potentially pathogenic clostridia when pH is more acidic), decreases solubility of bile acids, increases absorption of minerals (indirectly), and reduces the ammonia absorption by the protonic dissociation of ammonia and other amines (Wong *et al.*, 2006).

Because different types of dietary fiber produce varying amounts of the specific SCFA (Cummings, 1981), it is likely that the exact composition of fiber within the colonic lumen may determine its cellular effects, including its possible beneficial role in the prevention and/or treatment of colon cancer (McIntyre, Gibson, & Young, 1993).

5. Colonic SCFA concentrations and carcinogenesis

SCFA constitute approximately two-thirds of the colonic anion concentration (70-130 mmol/l), however, the rate and amount of SCFA produced depends on the species and amounts of microbiota present in the colon, the substrate source and gut transit time (Macfarlane & Macfarlane, 2012; Mortensen & Clausen, 1996). Total SCFA and local differences in SCFA concentration along the intestinal track are implicated in diseases of the colon, especially in cancer and gastrointestinal disorders, where disease often occurs distally. Therefore, increased SCFA production and a greater delivery of SCFA distally may have a role in preventing these diseases (Wong *et al.*, 2006).

In vivo, the study of SCFA is more difficult and relies mostly on determination of the concentrations in feces. The three main SCFA, butyrate, propionate and acetate, can be found in the gut in considerably high concentrations. These concentrations range from 40–80mM, 10–25mM and 10–20mM for acetate, propionate and butyrate, respectively (Alles *et al.*, 1999; Jenkins *et al.*, 1999; Topping & Clifton, 2001). The relative molar proportions range from 50–65% for acetate, from 10–25% for propionate and from 10–25% for butyrate, depending on the fiber consumed (Alles *et al.*, 1999; Jenkins *et al.*, 1999; Topping & Clifton, 2001). Nonetheless,

the *in vitro* production of total colonic SCFA is difficult to determine because more than 95% of the SCFAs are rapidly absorbed (Roy *et al.*, 2006) and metabolized by the host (Cook & Sellin, 1998; Topping & Clifton, 2001).

SCFA absorption rates are the same in all the regions of the colon, but as the concentrations of fermentation substrates are highest in the cecum and ascending colon, the concentration of SCFA decreases from the proximal to the distal colon (Roy *et al.*, 2006). In the gut, butyrate is the major energy source for colonocytes (Ahmad *et al.*, 2000), propionate is largely taken up by the liver and acetate enters the peripheral circulation to be metabolized by peripheral tissues (Wong *et al.*, 2006).

6. Effects of SCFA in CRC cells

A variety of biological effects of SCFA have been reported, and there is a vast number of experimental works showing new aspects of these molecules.

Although most studies focus on SCFA-induced apoptosis, Tang and co-workers demonstrated for the first time that butyrate and propionate are able to induce autophagy in human colon cancer cells (Tang *et al.*, 2011a). Autophagy is an evolutionarily conserved catabolic process in which the cytoplasmic contents and organelles are transferred into double membrane vesicles, called autophagosomes (Glick, Barth, & Macleod, 2010). Autophagosome ultimately fuses with a lysosome, where its contents are broken down by degradative enzymes and subsequently recycled (Glick *et al.*, 2010). This mechanism of type II Programed Cell Death (Tang *et al.*, 2011a), is responsible for the turnover of intracellular long-lived proteins and damaged organelles during cellular homeostasis. Autophagy plays also important roles in tissue development, differentiation and remodeling (B. Levine & Klionsky, 2004) and has been implicated in tumor development. In Tang *et al.* (2011a) *in vitro* study, human colon carcinoma cells were treated with propionate and butyrate at concentrations (1–3 mM) below their IC_{50} (half maximal inhibitory concentration) value toward the cancer cells and instead of inducing apoptosis, these SCFA induced extensive morphological alterations characteristics of autophagy. Consequently, the induced autophagy may provide tumor cells with an alternative energy supply to allow for adaptive protein synthesis and help overcome mitochondria defects causing a cellular energy crisis. Autophagic degradation of defective mitochondria could retard the occurrence of apoptosis by circumventing the release of proapoptotic factors such as cytochrome *c* from the mitochondria

and the activation of the apoptotic caspase cascade. The results presented in their study suggest that induced autophagy by SCFAs would increase the resistance and flexibility of colon carcinoma toward an adverse microenvironment and compromise the efficacy of SCFAs themselves in colon carcinoma prevention (Tang *et al.*, 2011a).

Therefore, SCFA may have opposing effects either inducing autophagy and hence increasing cell resistance, or inhibiting the proliferation of cancer cells through induction of apoptosis. In line of this latter effect we have recently demonstrated that lysosomal membrane permebilization (LMP) and the release of Cat-D is important in regulation of apoptosis by acetate (Marques *et al.*, 2013) (Figure 5). An extensive revision was performed concerning the precise role of SCFA: butyrate, propionate and acetate in colorectal carcinoma and all data was compiled in Table I, and will be described in brief.

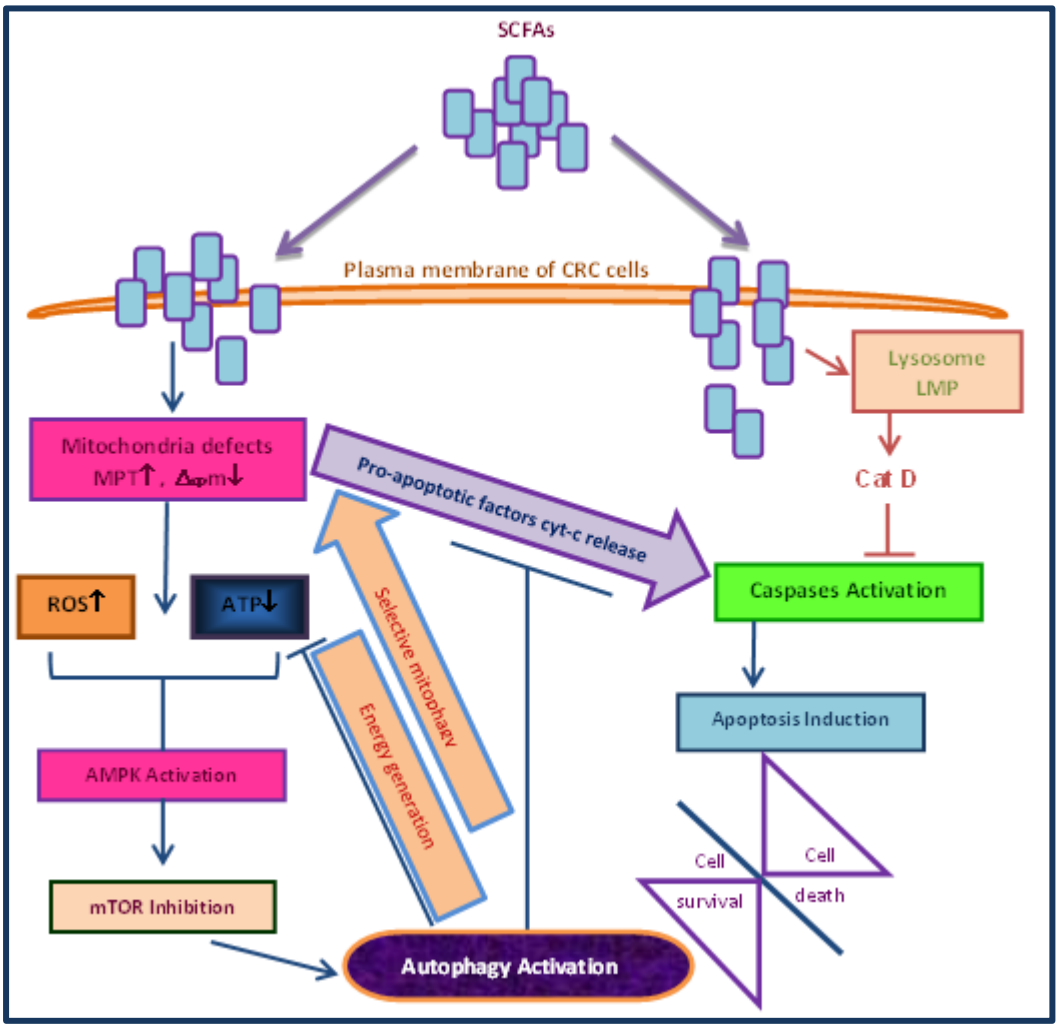


Figure 5 - Schematic representation of the proposed role of SCFAs in orchestrating two opposing cellular events: induction of autophagy which increases tumor resistance and contributes to its development or induction of apoptosis which increases tumor sensitivity and promotes elimination of colon cancer cells. **MPT**, mitochondrial membrane permeability transition; $\Delta\psi_m$, mitochondrial membrane potential; **ROS**, reactive oxygen species (Adaptated from Marques *et al.*, 2013; Tang, Chen, Jiang, & Nie, 2011b).

6.1. Butyrate

Of all SCFA butyrate is the most extensively studied because of its role in the prevention of CRC, inducing a variety of changes within the nucleus as a consequence of inhibition of cell differentiation, promotion of cell-cycle arrest and apoptosis of transformed colonocytes (Heerdt, Houston, & Augenlicht, 1997). Hinnebusch *et al.* (2002) demonstrated that apoptosis levels in human colon carcinoma cells were higher for treatment with butyrate than with other SCFA (Hinnebusch *et al.*, 2002). On the other hand, Marques *et al.* (2013) showed that acetate

induces high levels of apoptosis in CRC cells (Marques *et al.*, 2013). Butyrate presents the same effects in other types of malignant cells both *in vitro* and *in vivo* (Table 1).

Butyrate is a major energy source for colonic epithelial cells *in vivo*, accounting for about 70-90% of total energy consumption (Cook & Sellin, 1998; G. R. Gibson & Roberfroid, 1995; Scheppach, 1994), while most other cell types use glucose (Donohoe *et al.*, 2011), and is thought to stimulate cell proliferation (Scheppach *et al.*, 1992). Cells that metabolize butyrate at a higher rate are likely to be less susceptible to its apoptosis-inducing effects, which may explain why normal colonocytes are unaffected by the very high levels of this SCFA in the distal colon (Medina *et al.*, 1997). However, an important conclusion from Singh *et al.* (1997) study was that the response of colonic epithelial cells to butyrate may depend in part on the other energy sources available to the epithelium: in conditions of low energy availability, butyrate could stimulate growth, but in the presence of high levels of alternative high energy sources such as glucose, butyrate could switch from a growth stimulator to a growth inhibitor and/or an inducer of apoptosis, depending upon factors such as the level of exposure and the intracellular milieu (Sengupta, Muir, & Gibson, 2006; Singh *et al.*, 1997). Nevertheless, differentiation of colonocytes, either induced by butyrate or by other conditions, is reversible at early times of exposure. It has been hypothesized that less differentiated transformed cells are more sensitive to the apoptotic effects of butyrate than differentiated colonocytes (Matthews, Howarth, & Butler, 2006; Scharlau *et al.*, 2009). Thus, if a continuous *in vivo* exposure to high concentrations of butyrate is not attained, the irreversible commitment to differentiation might not occur and cells could return to their normal malignant phenotype and regain the capacity to proliferate (Cai *et al.*, 2006).

Boren and *et al.* (2003) observed that differences exist in the metabolism of butyrate between a butyrate-sensitive (HT29) and a butyrate-resistant (MIA) cell types. Incubation of MIA cells with butyrate had no effect on glucose utilization and was not associated with cellular differentiation (Boren *et al.*, 2003). It was, therefore, proposed that a cell will undergo apoptosis or differentiation depending on the ability of that cell to metabolize butyrate. However, cancer cells that are capable of metabolize butyrate have been identified (Lopez de Silanes *et al.*, 2004) and they are protected against its role as an apoptotic inducer. This finding suggests that the colonic butyrate-rich microenvironment may play a role in the selection of more aggressive colon carcinoma cells that maintain the ability to use butyrate as a carbon and energy source (Serpa *et al.*, 2010). Additionally, several studies showed that butyrate-resistant cancer cells may be

selected and give rise to more aggressive cancers (Chai, Evdokiou, Young, & Zalewski, 2000; Lopez de Silanes *et al.*, 2004; Mariadason, Corner, & Augenlicht, 2000).

Other studies propose a down regulation of butyrate transporters, such as MCT1, in human colon carcinoma tissue (Lambert, Wood, Ellis, & Shirazi-Beechey, 2002; H. Li *et al.*, 2003) which results in a reduced uptake and metabolism of butyrate in the colonocytes (Hamer *et al.*, 2008). A decline in the abundance of MCT1 in the membrane of colonic epithelial cells, and hence in butyrate uptake, would in turn reduce the availability of intracellular butyrate as a source of energy and as an important regulator of cellular homeostasis. In 2009 Thangaraju *et al.* showed that butyrate also elicits effects in colon cells extracellularly by serving as a ligand for GPR109A (Thangaraju *et al.*, 2009), a receptor in the lumen facing apical membrane of colonic epithelial cells that binds butyrate with low affinity (Clarke *et al.*, 2012). The expression of GPR109A is silenced in colon carcinoma by DNA methylation whereas its re-expression by butyrate exposure in cancer cells results in apoptosis without the involvement of histone deacetylases (HDAC) (Thangaraju *et al.*, 2009). Butyrate was also able to activate the receptor with an EC_{50} (concentration necessary for half-maximal activation of the receptor) of ≈1.6 mmol/L. Although the levels of butyrate in circulation are too low (≈5 μmol/L) to activate the receptor, butyrate is present at high levels (≈20 mmol/L) in colonic lumen (Hamer *et al.*, 2008). If GPR109A is expressed in the lumen-facing apical membrane of colonocytes, it might suggest that the ability of butyrate to prevent cancer and inflammation in the colon may also be mediated extracellularly via the receptor without entering into cells (Thangaraju *et al.*, 2009). Still regarding to transporters, the gene of the SMCT1 transporter is highly methylated in colon adenomas of African Americans, who are more likely to die from colon carcinoma than others, which points to its potential use as a marker for early detection (Brim *et al.*, 2011). Re-expression of SMCT1 in the presence of butyrate results in colon carcinoma cellular apoptosis (Brim *et al.*, 2011). Thangaraju (2008) observed that the induction of apoptosis in SW480 colon cancer cells by SMCT1/butyrate was associated with upregulation of pro-apoptotic genes (p53, Bax, Bad, Bak, FAS ligand, FAS receptor, TRAIL, and TRAIL receptors) and downregulation of anti-apoptotic genes (Bcl-2, Bcl-W, BclxL, Bfl-1, and survivin) (Thangaraju *et al.*, 2008). Butyrate causes cell death in colon carcinoma cells by two independent but complementary mechanisms: one through GPR109A independent of HDACs and other through SMCT1-mediated entry of butyrate into cells with subsequent inhibition of HDACs (Thangaraju *et al.*, 2009). By acting as an HDAC inhibitor, butyrate leads to histone hyperacetylation (Cousens, Gallwitz, & Alberts, 1979) and

enhances the accessibility of transcription factors to the DNA, causing biological effects in colon carcinoma cells (Wong *et al.*, 2006), including cell death (Shao *et al.*, 2004).

For Donohoe and co-workers (2012) butyrate inhibits the growth of cancerous colonocytes because it is metabolized inefficiently due to the Warburg effect, described as a high degree of aerobic glycolysis. Consequently, butyrate accumulates in the nucleus, acts as an HDAC inhibitor and causes cell death (Shao *et al.*, 2004; Wong *et al.*, 2006). Robert Li and Cong Jun Li (2006) confirmed the accumulation of acetylated histone 3 (H3) due to butyrate treatment (R. W. Li & Li, 2006). In addition to this function, butyrate increases histone acetylation through conversion to acetyl-CoA and stimulating histone acetyltransferases (HATs) (Barshishat, Polak-Charcon, & Schwartz) activity (R. W. Li & Li, 2006). However, the metabolic state of the cell influences intranuclear butyrate and acetyl-CoA levels and determines whether butyrate functions to inhibit HDACs or stimulate HATs to epigenetically regulate the expression of different target genes (Donohoe *et al.*, 2012). Deregulation in the expression or activity of HATs and HDACs may lead to alterations in gene expression profiles, associated with the reactivation or silencing of genes critical for cancer progression, differentiation and apoptosis (Iacomino, Tecce, Grimaldi, Tosto, & Russo, 2001; Marchion & Munster, 2007; Sambucetti *et al.*, 1999). For example, hyperacetylation of histones can support chromatin opening and induction of p21 (WAF1/CIP1) gene expression (Davie, 2003), and increased histone acetylation in the promoter of $p21^{WAF1}$ gene has been reported after butyrate treatment. Activation of tumor-suppressor genes, like $p21^{WAF1}$, is claimed to be the major cause of growth arrest and/or apoptosis. On the other hand, down regulation of $p21^{WAF1}$ expression in colorectal carcinomas is associated with histone hypoacetylation of total chromatin (Lu *et al.*, 2008). It is possible, that nucleosome conformations are altered due to histone H3 hypoacetylation, and that the access to chromatin of transcriptional regulatory proteins may be reduced in colorectal carcinomas (Lu *et al.*, 2008). It has also been reported that butyrate can induce a hypermethylation of DNA (de Haan, Gevers, & Parker, 1986), contributing to repression of the transcription of a specific region of DNA. Still regarding to DNA molecules, alkaline phosphatase (ALP) is a hydrolase isoenzyme responsible for removing phosphate groups nucleotides, and their levels are frequently elevated in patients with metastatic CRC (Saif, Alexander, & Wicox, 2005). ALP activity and the dipeptidyl peptidase-IV have been used as markers of colonocyte differentiation in many studies with Caco-2 and HT29 colon carcinoma cells and were increased during culture of malignant cells in the presence of butyrate (Whitehead, Young, & Bhathal, 1986). Regarding transcription, it has been demonstrated that

transcriptional attenuation triggered by butyrate plays an important role in the down regulation of two key genes that regulate colonic cell maturation and transformation – c-myc and cyclin D1, and therefore eliminate their increased steady state levels, which might be expected from the elevation in Wnt signaling (Cai *et al.*, 2006; Maier *et al.*, 2009). Scharlau *et al.* (2009) also observed that butyrate treatment enhanced mRNA gene expression, protein expression and enzyme activity of GSTs, and other stress response genes in human primary colon cells, LT97 cells and HT29 cells (Scharlau *et al.*, 2009). In response to DNA damage, p53 is activated and turns on the transcription of one of its downstream genes, $p21^{WAF1}$ (Archer, Meng, Shei, & Hodin, 1998; el-Deiry *et al.*, 1993). For Harper *et al.* (1993) the $p21^{WAF1}$ product binds to cyclin complexes and inhibits the function of cyclin-dependent kinases (Harper *et al.*, 1993). Nakano *et al* (1997) found that butyrate markedly induces $p21^{WAF1}$protein and causes G1-phase arrest. They also observed that butyrate can strongly activate the WAF1/CIP1 promoter, and that the two p53-binding sites are not required for the transcriptional activation by butyrate (Nakano *et al.*, 1997). By inhibiting the HDAC activity recruited to the $p21^{WAF1}$ promoter by Sp1 or Sp3, butyrate induces the expression of $p21^{WAF1}$and thereby stops cell proliferation (Davie, 2003). Nakano *et al* (1997) suggested that butyrate-induced growth arrest in human colon carcinoma cells is due to the p53-independent activation of p21 promoter mediated through specific Sp1 sites in the promoter region (Nakano *et al.*, 1997). Siavoshian *et al.* (2000) showed that exposure to butyrate resulted in arrest in the G1 phase of the cell cycle, and that was associated with $p21^{WAF1}$ induction at the protein and mRNA level and overexpression of cyclin D3 (Siavoshian *et al.*, 2000). Regarding cell cycle progression, Robert Li and Cong Jun Li (2006) observed that MDBK cells (Madin-Darby bovine kidney epithelial cells) were arrested at the G1/S boundary and DNA replication was blocked after butyrate treatment (R. W. Li & Li, 2006). A different study showed that after 6 hours following treatment of CRC cells with 5 mM of butyrate, the percentage of cells in G1 phase of the cell cycle increased by 40% (Maier *et al.*, 2009). Another gene that is regulated by p53 and that could influence the decision to commit to an apoptotic pathway is Bax, which belongs to Bcl-2 family. It is known that members of the Bcl-2 family play important roles in regulating apoptosis by functioning as promoters (e.g. Bax, Bak, and Bok) or inhibitors (e.g. Bcl-XL, Bcl-w, A1, and Mcl-1) of cell death of transformed cells (Carpinelli *et al.*, 2012; Gewies, 2003; Tzifi *et al.*, 2012). It is also known that overexpression of Bcl-2 can block p53-mediated apoptosis (A. J. Levine, 1997). Bax binds to Bcl-2 and antagonizes its ability to block apoptosis so a p53-dependent Bax synthesis could tip the scales toward apoptosis (A. J. Levine, 1997). Hague *et al.* (1996)

suggested that Bcl-2 and Bak play a pivotal role in sodium-butyrate-induced apoptosis in colonic epithelial cell and that overexpression of Bcl-2 does not protect against Bak-mediated apoptosis (Hague *et al.*, 1996). Zhang *et al.* (2010) observed that there is a decrease of Bcl-2 (anti-apoptotic protein) expression in a dose-dependent manner following butyrate treatment in human colon carcinoma cells, and also a dose-dependent increase of Bax with increase of butyrate concentration (Zhang *et al.*, 2010). The ratio between Bax and Bcl-2 determines the cells' survival or death. A decrease in the levels of Bcl-2 and an increase in Bax leads to the loss of mitochondrial transmembrane potential, a key event in the induction of apoptosis. Their results indicate that the treatment of butyrate leads to a shift from an anti-apoptotic to a pro-apoptotic state (Zhang *et al.*, 2010). Chirakall *et al.* (2006) showed that butyrate induces apoptosis through upregulation of Bak. Their data strongly suggest that Bak upregulation is mediated through increased Sp3 binding at the promoter region rather than Sp1 binding, which decreased after treatment with butyrate. They also observed increased Bak and reduced Bcl-xL in response to sub-apoptotic concentrations of butyrate (Chirakkal *et al.*, 2006).

Concerning grow and spread of CRC, Neuropilin (NRP)-1 is a member of the VEGF receptor family in colon carcinoma, and high levels of NRP-1 staining in human colorectal carcinoma tissues is associated in tumor proliferation and angiogenesis and in decreased apoptosis (Ochiumi *et al.*, 2006). Yu *et al.* (2010) data show that different CRC cells exposed to butyrate down-regulate NRP-1 expression through decreased mRNA production leading to reduction in protein levels. Modification of Sp family activity by butyrate, and the potential of NRP-1 as a Sp1 target led Yu *et al.* to investigate the ability of butyrate to modulate NP-1 expression, with a view to providing an alternative therapy or chemopreventive strategy for colon carcinoma, as NRP-1 is involved in both angiogenesis and the prevention of apoptosis in CRC. Their data suggest two potential mechanisms for chemoprevention - through the apoptotic regulatory function of NRP-1 and through its pro-angiogenic role (Yu *et al.*, 2010) .

Colon carcinoma cells can also acquire mechanisms to escape CD95-mediated apoptosis (Fan *et al.*, 1999). Fan *et al.* (1999) demonstrated that butyrate exposure selectively induces expression of CD95-L and CD95, which means that butyrate stimulation of colonic apoptosis is mediated by this death receptor (Fan *et al.*, 1999). Because caspases are the executioners of apoptosis, they are considered the key players in apoptotic cell death (Park *et al.*, 2007). Butyrate treatment induced apoptosis by activation of caspase-9 (Zhang *et al.*, 2010) and

caspase-3 (Ramos *et al.*, 2002; Zhang *et al.*, 2010) through the mitochondria cytochrome *c*-mediated apoptotic pathway (Shao *et al.*, 2004).

A common characteristic of the majority of CRCs is the constitutive activation of the canonical Wnt signaling pathway. Bordonaro *et al.* (1999) reported that butyrate hyper-induces canonical Wnt transcriptional activity in CRC cells (Bordonaro *et al.*, 1999) and that there is a relationship between enhanced Wnt activity and higher levels of apoptosis (Bordonaro, Lazarova, & Sartorelli, 2008). Later (2007), Bordonaro *et al.* observed that colon carcinoma cells with mutations in components of the canonical Wnt signaling hyper-induce this pathway in the presence of butyrate (Bordonaro *et al.*, 2007). They had previously identified, on ten human CRC cell lines with different Wnt signaling mutations, two classes of CRC cell lines: those which respond to butyrate treatment with a high fold induction of canonical Wnt activity and apoptosis, and those which exhibit a relatively lower fold induction of canonical Wnt activity and apoptosis. This could happen also *in vivo*: there may be CRC subtypes that respond to butyrate with hyper-induction of canonical Wnt signaling and high levels of apoptosis, as well as CRC subtypes that respond with a lower fold induction of canonical Wnt activity and apoptosis (Bordonaro *et al.*, 2008; Lazarova, Bordonaro, Carbone, & Sartorelli, 2004). The fact that only a subtype of colonic carcinomas cells responds to butyrate with hyper-activation of Wnt signaling and enhanced apoptosis is probably the reason why some epidemiological studies are inconsistent about the association between fiber intake and colon carcinoma. Lazarova *et al.* (2004) suggested that exposure to butyrate may be most beneficial for those malignancies in which butyrate is able to induce relatively high levels of Wnt activity that lead to apoptosis (Lazarova *et al.*, 2004). Even though several studies of butyrate in animal models have demonstrated a protective effect of butyrate on colorectal carcinogenesis, the same role of butyrate on carcinogenesis in humans is still difficult to understand. Again, Bordonaro *et al.* (2008) believe that the inconsistent findings in the literature on the protective role of dietary fiber and its degradation product butyrate against colon carcinoma can be at least partially explained by: (a) the existence of different subtypes of colonic neoplasms that differ in the induction of canonical Wnt signaling and apoptosis in response to butyrate; (b) variations in colonic microflora that generate different levels of butyrate by fermentation of dietary fiber; and (c) the timing of exposure of colonic cells to fiber/butyrate in relation to the specific stage of colonic tumorigenesis (Bordonaro *et al.*, 2008).

Another pathway that has been shown to contribute to cancer proliferation and survival is the PI3K signaling pathway (Wang, Li, Wang, Kim, & Evers, 2002). Activation of PI3K, a down-

regulator of the RAS signaling pathway, is necessary for actin cytoskeletal rearrangement, which is associated with the transformed phenotype, and is increased in 86% of human CRCs (Phillips, St Clair, Munday, Thomas, & Mitchell, 1998). Wang *et al.* (2002) showed that PI3K inhibition enhances apoptosis induced by butyrate in the aggressive human colon carcinoma cell line, KM20, and in HCT116 colon carcinoma cells (Wang *et al.*, 2002).

Butyrate has also anti-inflammatory activity in colon adenocarcinoma cells mediated by inhibition of the NF-κB pathway (Tedelind, Westberg, Kjerrulf, & Vidal, 2007). NF-κB regulates genes involved in controlling cell proliferation, cell death, immune response, and inflammatory responses (Luhrs *et al.*, 2001; Segain *et al.*, 2000; Yin, Laevsky, & Giardina, 2001) and it has been suggested that blocking NF-κB can cause tumor cells to stop proliferating, die, or become more sensitive to the action of anti-tumor agents (Garg & Aggarwal, 2002). Kaler *et al.* (2008) showed that in intestinal epithelial cells, butyrate activity modulates signaling by a major pro inflammatory cytokine, the tumor necrosis factor α (TNFα). Epithelial cells with k-RAS sensitizes cells to HDACi induced apoptosis. Kaler *et al.* (2008) demonstrated that butyrate interfered with TNFα-induced NF-κB activity in colon carcinoma cell lines (HCT116 and Hke3 cells), and that this activity of butyrate was unaffected by the presence of a mutant K-RAS (Kaler, 2008)

Zhang *et al.* (2010) analyzed the roles of MAPKs - ERK1/2, JNK and p38 MAPKs, as they play central roles in the signaling pathways of cell proliferation, survival, and apoptosis. Butyrate induced apoptosis in the RKO human colorectal carcinoma cell line via activation of the JNK MAPK pathway. In addition, inactivation of ERK MAPK was involved in butyrate-induced growth inhibition of RKO cells (Zhang *et al.*, 2010).

Although the aforementioned studies demonstrate that butyrate has apoptotic properties, contradictory results exist. One important reason for the opposite effects of butyrate when tested *in vitro* vs *in vivo* may be the different conditions that exist in these two systems, such as: (a) differences between *in vitro* and *in vivo* experimental environments, (b) timing of butyrate administration with respect to tumor development, (c) amount of butyrate utilized in the experiments, (d) effects of fiber that are independent of butyrate production (e.g., fecal transit time, fecal bulk, and other bioactive agents present in the fiber), and (e) interaction of fiber with dietary fats (e.g., the complementary action of fish oil and pectin in suppressing intestinal tumorigenesis) (Bordonaro *et al.*, 2008; Lupton, 2004).

The global conclusion is that colonocyte-exposure to butyrate in the gut lumen of humans could be protective by reducing survival of transformed colon cells, while at the same time

promoting survival of non-transformed colonocytes (Scharlau *et al.*, 2009). It must be remembered that butyrate is not available as a sole compound but probably acts in unison with other metabolites of dietary fiber and of the faecal gut flora. In this context the new approaches to investigate complete gut fermentation samples are of importance, since they self-evidently better reflect *in vivo* exposure conditions than butyrate alone (Scharlau *et al.*, 2009).

Despite all findings of the benefic effects of butyrate, there is an unresolved paradox concerning the putative protective role of this SCFA in colon carcinoma: CRCs still develop and grow despite the high concentrations of butyrate in the colon (Serpa *et al.*, 2010).

6.2. Propionate

The number of published studies regarding propionate is by far lower than butyrate. Although propionate is less frequently studied it has the same health-promoting properties. Propionate like butyrate exerts an antiproliferative effect towards colon carcinoma cells (Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011). Propionate not only adds to butyrate's effect of suppressing cell growth, but also to butyrate's effect of modulating histone acetylation in human colon cells (Hosseini *et al.*, 2011) . Based on these observations it can be speculated that the products of complex *in vitro* fiber fermentation, like propionate, may contribute to *in vivo* tumour suppressor agent activities resulting in the inhibition of tumor progression (Kiefer, Beyer-Sehlmeyer, & Pool-Zobel, 2006).

Jan *et al.* (2002) showed that propionate induces typical signs of apoptosis in human colorectal carcinoma cell lines, with loss of mitochondrial trans-membrane potential, generation of reactive oxygen species, caspase-3-processing and nuclear chromatin condensation (Jan *et al.*, 2002).

Bindels *et al.* (2012) demonstrated that, the anti-proliferative effect of propionate was partially cAMP level-dependent and associated with the activation of free fatty acid receptor 2 (FFA2, a G-protein-coupled receptor, also known as GPR43) (Bindels *et al.*, 2012). The authors argue that the pharmacological activation of FFA2 may be of therapeutic interest to control cancer cell proliferation and sustain the idea of a role for gut microbiota in the control of systemic cancer. The mammalian target of rapamycin (mTOR) negatively regulates autophagy and Tang et al (2011) demonstrated that propionate-induced autophagy was associated with decreased mTOR activity and enhanced AMP kinase activity (Tang *et al.*, 2011a).

6.3. Acetate

A study by Barcenilla *et al.* (2000) revealed that 95% of intestinal isolated bacterial strains utilizing acetate were butyrate producers. This suggests that butyrate production by bacteria is heavily dependent on the availability of acetate (Barcenilla *et al.*, 2000). Hence the activities of acetate producing bacteria which influence the availability of acetate can play a significant role in colonic health as rate-limiting steps in butyrate formation (Abell, Conlon, & Mcorist, 2006). Acetate induces cell proliferation arrest of intestinal epithelial cells in a concentration and pH dependent manner (Matsuki *et al.*). Jan *et al.* (2002) showed that acetate, at levels of 9–16 mmol/L, induced typical signs of apoptosis in human colorectal carcinoma cell lines. Like with propionate, this effect included a loss of mitochondrial trans-membrane potential, the generation of reactive oxygen species (ROS), caspase-3-processing and nuclear chromatin condensation. Accordingly Marques *et al.* (2013) observed that acetate *per se* induces apoptosis in CRC-derived cell lines HCT-15 and RKO, by inducing DNA fragmentation, caspase activation, phosphatidylserine exposure to the outer leaflet of the plasma membrane and the appearance of a sub-G1 population (Marques *et al.*, 2013) .

Although cancer cells may block classical apoptotic pathways, cell death can still occur through the release of lysosomal enzymes (Kirkegaard & Jaattela, 2009). Recently, Marques *et al.* (2013) showed that Cat D was release to the cytosol in two CRC lines undergoing acetate-induced apoptosis. These results indicate that acetate induces a lysosomal apoptotic pathway (Marques *et al.*, 2013). Currently, lysosomal pathway of apoptosis is a widely accepted concept as partial lysosomal permeabilization with subsequent release of proteolytic enzymes into the cytosol, contributing to the death pathways, has been described in several models of apoptosis. The magnitude of lysosomal permeabilization determines the type of cell death mediated by lysosomal enzymes: a complete breakdown of the organelle with release of lysosomal enzymes attaining high cytosolic concentration results in unregulated necrosis, while partial, selective permeabilization triggers apoptosis (Guicciardi, Leist, & Gores, 2004). Among the released enzymes, cathepsins are of particular interest, since these molecules are often overexpressed in human cancers, and high expression levels have been associated with increased risk of relapse and poor prognosis. In contrast to their tumor promoting effects, there is also evidence that they function as tumor suppressors (Marques *et al.*, 2013). There are several human cathepsins identified, but the most relevant are the cysteine cathepsins B and L (Cat B and Cat L) and the

only lysosomal aspartic protease, cathepsin D (Cat D). Cat D has been implicated in apoptosis induced by staurosporine (Bidere *et al.*, 2003) (Johansson, Steen, Ollinger, & Roberg, 2003), interferon-g, Fas/CD95/APO-1 and TNF-a (Deiss, Galinka, Berissi, Cohen, & Kimchi, 1996) (Demoz *et al.*, 1999), oxidative stress (Roberg, Johansson, & Ollinger, 1999) sphingosine (Kagedal, Zhao, Svensson, & Brunk, 2001), and p53 (Wu, Saftig, Peters, & El-Deiry, 1998) and recently, as referred, by acetate (Marques *et al.*, 2013). In our model Cat D seems to have a protective role in acetate-induced apoptosis which can have important prevention/therapeutic implications. The protective role of Cat D demonstrated by our data might partly explain why Cat D is overexpressed in some CRC clinical cases in comparison to normal colon mucosa. We therefore hypothesize that increased expression of this protease might be beneficial to cancer cells and thus that Cat D might have an 'oncogenic-like effect', allowing CRC cells to survive in the presence of physiological levels of SCFA in the colon (Marques *et al.*, 2013). Accordingly, we also showed that inhibiting Cat D with PstA, a widely used specific inhibitor of Cat D enzymatic activity, increased acetate-induced apoptosis in CRC cells

Table 1 - Effects of SCFA butyrate, propionate and acetate in colorectal carcinoma cells.

BUTYRATE	Study	Concentrations	Effects	Reference
	7 CRC cell lines (HRT18, HCT48, LS174T, SKCO-1, SW480, SW620 and SW1116)	2 mM	Induction of carcinoembryonic antigen (CEA) production was not a general phenomenon among treated cells	(Tsao, Shi, Wong, & Kim, 1983)
	WI-38 cell line (normal human embryonic lung fibroblast)	5 – 20 mM	Maximum inhibition of DNA synthesis, but not affected cell viability	(de Haan *et al.*, 1986)
	SV-40-transformed cell line (SVWI-38)		DNA synthesis did not affected transformed cells, but cell viability was decreased	
	γ-Irradiation-transformed cell line (CT-1)			
	Cell lines originating from 6 colorectal adenomas and 7 CRC	1 – 4 mM	Apoptosis occurred in colorectal adenoma and carcinoma cells	(Hague *et al.*, 1993)
	HT29 colon adenocarcinoma cells	5 mM	Induction of growth inhibition and differentiation	(Barnard & Warwick, 1993)
	WiDr (Human Colon Adenocarcinoma) cell line	0.625 – 10mM	Inhibition of proliferation	(Nakano *et al.*, 1997)
	S/RG/C2 (adenoma) cell line and HT29 cell line	0 – 10 mM	Growth inhibition and apoptosis	(Singh *et al.*, 1997)
	HT29 cells	0 – 20 mM	p21 is required for growth arrest by butyrate	(Archer *et al.*, 1998)
	YAMC (Young adult mouse colon cells)	1 mM	Induction of apoptosis	(Fan *et al.*, 1999)
	Caco-2 cells (Human CRC cells)	3 mM	Decrease of cell proliferation	Harrison et al, 1999
	Caco-2 cells	0.01 – 10 mM	Inhibition of proliferation	(Ruemmele *et al.*, 1999)
		0.01 – 100 mM	Induction of apoptosis	

HT-29 cells	1 – 8 mM	Inhibition of cell growth and proliferation	(Siavoshian *et al.*, 2000)
LS174T and HM7 colon carcinoma cells	2 mM	Activation of E-cadherin transcription through translocation of nuclear transcription factors bearing specific repressor activity	(Barshishat *et al.*, 2000)
LIM 1215 CRC cells	0 – 8 mM	Cell cycle arrest and apoptosis	(Chai *et al.*, 2000)
Caco-2 cells	2 – 10 mM	Stimulation of cell cycle arrest, apoptosis, alkaline phosphatase activity, transepithelial resistance, cell migration, urokinase receptor expression, and interleukin-8 secretion in undifferentiated Caco-2 cells; differentiated Caco-2 cells were essentially resistant to these effects	(Mariadason, Velcich, Wilson, Augenlicht, & Gibson, 2001)
HT29 cells	4 mM	Influences NF-κB in part by preventing the complete degradation of IκB-α by reducing proteasome activity in the cell and increases levels of the p100 IκB	(Yin *et al.*, 2001)
Rat model (F344 rats)	Pellets mixed into the diet – 1,5% (w/w)	No protection against azoxymethane-induced colon carcinogenesis	(Caderni *et al.*, 2001)
Caco-2 and RSB cells (Human colon carcinoma)	2 – 10	Inhibition of cell growth	(Avivi-Green, Polak-Charcon, Madar, & Schwartz, 2002)
KM20 cell line (Human colon carcinoma)	0 – 10 mM	DNA fragmentation and apoptosis	(Wang *et al.*, 2002)
HT29 cells	10^{-2} mM	Reduction of paracellular permeability	(Kinoshita, Suzuki, & Saito, 2002)
HT29 cells	2 mM	Increase in the level of methylation of retinoblastoma (RB1) gene	Gope, M. (2003)
Caco-2 cells	5 – 50 mM	Induction of apoptosis	(Ruemmele *et al.*, 2003)

HCT116 CRC cell line and HT29 cells	3 mM and IFN-γ cytokine	Inhibition signaling by IFN-g through the inhibition of STAT1 activation	(Klampfer, Huang, Sasazuki, Shirasawa, & Augenlicht, 2003)
HT29 cells	0 – 10 mM	Inhibition of proliferation	(Boren *et al.*, 2003)
Colo-320 (human colon carcinoma cell line) and SW1116 cells	5 mM	Activation of the expressions of p21WAF1 mRNA and protein	(Chen *et al.*, 2004)
HeLa cell line (Human cervical carcinoma)	10 – 50 mM	Induction of apoptosis	(Shao *et al.*, 2004)
Several CRC cell lines	0 – 5 mM	Increase in Wnt activity that contributes to apoptosis	(Bordonaro *et al.*, 1999) Bordonaro 2002 (Lazarova *et al.*, 2004) (Bordonaro *et al.*, 2008)
Caco2, HCT116 and HT29	0 – 10 mM	Induction of apoptosis through upregulation of BAK associated with increased Sp3 binding	(Chirakkal *et al.*, 2006)
HT29 cells	3 – 5 mM	Increased expression of HSFs (heat shock factors) and *hsps* (heat shock proteins), which might render colon carcinoma cells resistant to the chemopreventive effects of butyrate	(Cai *et al.*, 2006)
Colo320DM cells	64 μmol/L	Inhibition of the NF-κB pathway	(Tedelind *et al.*, 2007)
Fresh mice samples of tumors and their corresponding normal colorectal mucosa	NaBu dissolved in drinking water administered at a low (0.5% in drinking water) or high (1.5% in drinking water) dose	Average number of tumors found in mice receiving both NaBu and Folic Acid was significantly lower than the two components alone	(Lu *et al.*, 2008)
Rectal enemas of butyrate (rat model)	100 mM	Stimulation of the gene expression of both secreted (Muc2) and membrane-linked (Muc1, Muc3, Muc4) mucins	(Gaudier, Rival, Buisine, Robineau, & Hoebler, 2009)

	SW837 (CCL-235) rectal and Dld-1 (CCL-221) colon adenocarcinoma cells	5 mM	Transcriptional attenuation with important role in the down regulation of both c-myc and cyclin D1 genes	(Maier *et al.*, 2009)
	HCT116, HT29 and Caco-2 cell lines	0 – 20 mM	Down-regulation of NRP-1 and VEGF at the mRNA and protein level, through inhibition of Sp1 transactivation	(Yu, Waby, Chirakkal, Staton, & Corfe)
	RKO CRC cell line	0 – 40 mM	Induction of apoptosis	Yu Zhang *et al* (2010)
	HCT116 cells	0 – 20 mM	Induction of apoptosis and inhibition of proliferation	Kim Fung et al, 2011
PROPIONATE	**Study**	**Concentrations**	**Effects**	**Reference**
	HT29 cells	10 – 40 mM	Induction of apoptosis	(Jan *et al.*, 2002)
	Colo320DM cells	120 μmol/L	Inhibition of the NF-κB pathway	(Tedelind *et al.*, 2007)
ACETATE	**Study**	**Concentrations**	**Effects**	**Reference**
	HT29 cells	10 – 40 mM	Induction of apoptosis	(Jan *et al.*, 2002)
	Colo320DM cells	2,4 mM	Inhibition of the NF-κB pathway	(Tedelind *et al.*, 2007)
	HCT-15 and RKO CRC-derived cell lines	0 – 120 mM	Induction of apoptosis and inhibition of cell proliferation	(Marques *et al.*)

6.4. Combined effects of SCFA

There are very few studies with the combination of SCFA. In Matthews *et al.* (2012) study, butyrate alone, and the combination of butyrate and propionate treatment, reduced Caco-2 cell viability and induced significantly greater apoptosis than propionate alone. This suggests that distinct SCFA exert significantly different effects on cell viability that may be due to differences in their absorption, metabolism and/or ability to induce histone acetylation. Their study also showed that further separation of apoptosis into early and late apoptosis indicated that SCFA increase both phases of apoptosis, although butyrate alone and the combination of SCFA led to a greater extent compared to propionate alone (Matthews, Howarth, & Butler, 2012). Jan and co-workers observed that propionate and acetate-induced apoptosis in HT-29 cell occurred via the stereotyped biochemical events, including mitochondrial alterations, caspase activation and nuclear degradation (Jan *et al.*, 2002). Lan *et al.* (2007) observed that propionate and acetate produced by propionibacteria triggered apoptosis induced cell cycle arrest in G2/M phase prior to apoptosis at pH 7.5 in HT-29 human colon adenocarcinoma cell line (Lan *et al.*, 2007).

7. Colorectal Carcinoma – SCFA prevention and/or therapy?

The mainstay of CRC prevention is screening and detection of adenomatous polyps. CRC is an active area of scientific research, and studies range from cancer prevention and early detection to treatment. Cancer chemoprevention is characterized by the use of natural, synthetic, or biological (from a living source) substances to reverse, suppress, or prevent the development of cancer (Wattenberg, 1985). Epigenetic mechanisms by their potential reversibility represent interesting targets in CRC for chemopreventive approaches using dietary agents. Accumulating evidence suggests that natural molecules/nutrients present in our diet might modulate epigenetic events in humans (Schnekenburger & Diederich, 2012).

Jan *et al.* (2002) proposed that propionibacteria could constitute probiotics efficient in the prophylaxis of digestive cancer via their ability to produce apoptosis-inducing SCFA. They report that different strains and species of propionibacteria kill cancer cells via the metabolic production of two SCFA, propionate and acetate (Jan *et al.*, 2002). All three major components of short-chain fatty acids (butyrate, propionate and acetate) induce apoptosis and inhibit cell

proliferation. However, several studies show that butyrate is the most potent in inducing apoptosis and inhibition of cell proliferation in CRC (C. J. Li & Elsasser, 2005).

Determining which chemopreventive agents arise in the diet is fundamental to identify more effective strategies for cancer prevention and therapy (Tang *et al.*, 2011b). Schnekenburger and Diederich (2012) suggested that improved early cancer detection and dietary intervention are preventive approaches of choice to decrease CRC incidence (Schnekenburger & Diederich, 2012).

8. Conclusions

It is known that chemotherapy and radiotherapy operate based on, for example, a mechanism of induction of apoptosis in cancer cells by altering their metabolism. It is also known that if a cancer has an inoperative P53, which regulates the cell cycle and apoptosis, due to an inactivating mutation, it makes no sense to administer a drug to the patient whose mechanism of action is dependent on that molecule. Cancer should be analyzed for their molecular profile, identifying the main oncogenes and tumor suppressor genes mutated as the majority of patients are treated only based in the evaluation of cancer development, and following protocols established in the hospitals depending on the stage. Patients should be treated more based on the molecular profile of their cancers, and although in some cases this is already a current practise in others it would be very expensive and time consuming to be performed (Simões, 2010).

It is also well recognized that our lifestyle and dietary habits can prevent several diseases, including cancer. Among the several types of cancer, diet directly affects the gastrointestinal tract and thus can contribute to prevent or develop/accelerate tumors of gastrointestinal organs. It is known for decades that dietary fibers can contribute to health promoting, as they are indigestible in human tract and are fermented by colonic bacteria. The fiber types that are most amenable to fermentation are the soluble ones, found in foods such as berries, beans, flax seeds, plums, apples, and oats, and in some fiber supplements, such as those using psyllium and guar gum (Dharmarajan, Ravunniarath, & Pitchumoni, 2003a). Insoluble fibers (found in such foods as vegetables, the bran of grains e.g. wheat bran, nuts, and seeds) are not available for efficient fermentation, but it is still important in the colon. It provides

volume to the feces and its tendency to "speed things along" means that the fermentation will take place all along the length of the colon, including near the end, where the majority of colon carcinoma occurs (Dharmarajan, Ravunniarath, & Pitchumoni, 2003b).

Without insoluble fibers, most of the fermentation would take place in the top part of the colon and the top colon cells would get most of the benefit. As a result of this fermentation, SCFA are released and contribute to eliminate potential dangerous cells. It is difficult to get these in our food, so the body relies on the process going on in the colon to make these essential biomolecules. Among these SCFA, butyrate, propionate and acetate are gaining most attention in the last decades. Several studies proved that these compounds can induce cell death of various malignant cell types namely CRC cells through apoptosis, a mechanism used by organisms to maintain homeostasis and protection.

In the gastrointestinal tract, apoptosis is an important protective process eliminating cells with DNA damage that may otherwise progress to malignancy (Clarke *et al.*, 2012) . Some study confirms the pro-apoptotic actions of SCFA, further supporting their potential as important adjunctive therapies for the treatment of colonic neoplasms (Matthews *et al.*, 2012). Among the SCFA, butyrate seems to have the major effects in preventing malignant cells proliferation through apoptosis. Although many advances that have been made on the mode of action of these biomolecules, studies on these compounds are still needed, because despite of existing at high concentrations in the human intestine, transformed cells continue to arise in the colon and lead to the development of colorectal carcinomas.

Chapter 2 – Design of protocols for undergraduated students to illustrate the effect of the SCFA acetic acid on yeast cell cycle and cell death

1. Insights from *Sacharomyces cerevisiae* on the role of acetate in CRC cells - a practical application

One aim of this work was to develop a protocol for secondary school students, to illustrate the effect of the SCFA acetic acid on two key cellular biological processes, namely on, cell cycle and cell death. Most of the experimental protocols described in the literature to monitor these cellular processes involve expensive reagents, complex protocols and sophisticated equipment. In Portuguese secondary schools it is very difficult to implement the experimental protocols described in the literature. Indeed these protocols include the monitoring of different functional, structural and morphological alterations associated with quite intricate cellular events which required for its characterization, different fluorescent techniques and transmission electron microscopy. Since these experiments are not feasible in the labs of secondary schools, we aimed at exploiting very simple techniques and biological materials such as bright field microscopy associated with differential coloration of yeast cells to demonstrate the effect of a short chain fatty acid, like acetic acid on cell cycle progression and cell death.

Culturing yeast is simple, economical, and fast. Indeed yeast cells are characterized by a doubling time of approximately 90 min on a rich medium and are well adapted to both aerobic and anaerobic growth conditions. With few exceptions the yeast cell divides mitotically by forming a bud, which pinches off to form a daughter cell. The progression through the cell cycle can be monitored by the microscopic observation as described below.

Another advantage to work with yeast cells and in particular with the species *Saccharomyces cerevisiae*, the common baker´s yeast, is that it is a genetically well-characterized organism which provides useful and numerous genetic and molecular biology tools for researchers. *S. cerevisiae* has been therefore the most extensively studied one, and become progressively a preferred research cellular model system in several areas of cell biology. Though the easy handling and genetic tractability of *S. cerevisiae* resemble those of bacteria, yeast is additionally coupled to the functional advantage of being an eukaryotic cell. Moreover, the recognition that most basic cellular processes are conserved in *S. cerevisiae* led to its extensive use a preferred cellular model system in several areas of cell biology. It has become apparent that, among other cellular processes, the cell cycle and apoptotic core machinery are conserved in yeast to a degree that makes it a suitable model organism to approach pending questions on

cell cycle and apoptosis in human cells, and on its deregulation in the context of cancer, neurodegenerative diseases and aging (Carmona-Gutierrez *et al.*, 2010).

However, an intriguing question came rapidly to our mind: why does a unicellular organism commits suicide? Apoptotic cell death seemed to be a process that was absent in yeast. Not only did a cellular suicide program make no sense for an organism consisting of just one cell, plain homology searches indicated the absence of crucial regulators of apoptosis (Madeo *et al.*, 2004). However, discovery of an apoptotic phenotype in a yeast strain carrying a CDC48 mutation this idea (Madeo, Frohlich, & Frohlich, 1997). Yeast populations should not be interpreted just as a group of partitioned unicellular organisms that do not communicate among each other, but rather as a multicellular community of interacting individuals. Under certain circumstances, death of a single cell might be beneficial for the whole population, thus promoting the survival of the clone. When dying, aged yeast cells actively stimulate the survival of the clone by releasing defined substances into their surroundings. The death of older cells is advantageous for two reasons: first, because it spares nutrients for younger cells; and second, because the older cells release nutrients that can be metabolized by active proliferative younger cells. Several yeast physiological scenarios, in which altruistic death of single cells promotes survival of the population, strongly support this idea (Buttner *et al.*, 2006).

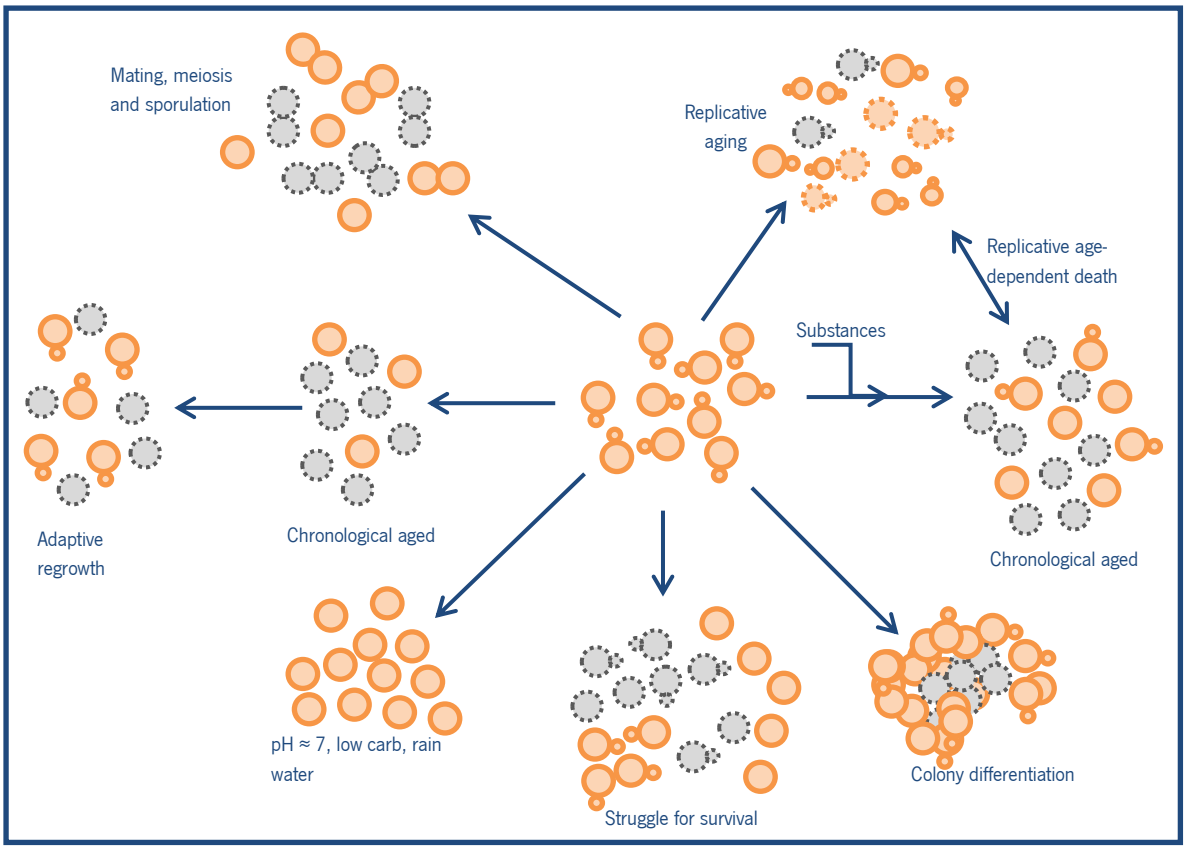


Figure 6 - Physiological scenarios of yeast apoptosis (Adapted from Buttner *et al.*, 2006).

Since 1997 numerous studies have shown that different stimuli can trigger a death process in yeast, which share with mammalian cells common apoptotic features. These stimuli can be provided externally in the form of chemical or physical stress, via heterologous expression of human proapoptotic proteins (exogenous triggers) or by the yeast cells themselves, as part of lethal signal transduction pathways (endogenous triggers) (Carmona-Gutierrez *et al.*, 2010).

Acetic acid acts as an exogenous trigger and has been extensively exploited by us and other groups as a compound commonly used to induce yeast apoptosis. Indeed we first showed in our laboratory that treatment with acetic acid at low concentrations could induce apoptotic cell death, while higher concentrations appeared to induce necrosis, a form of passive cell death (Ludovico *et al.*, 2002); (Ludovico, Sousa, Silva, Leao, & Corte-Real, 2001). Ludovico *et al.* (2001) observed concentration-dependent changes after treatment with acetic acid, which include chromatin condensation along the nuclear envelope, exposure of phosphatidylserine at the outer surface of the yeast cytoplasmic membrane and formation of DNA strand breaks

(Ludovico *et al.*, 2001). Treatment of yeast cells with acetic acid leads to mitochondrial cytochrome-*c* release (Ludovico *et al.*, 2002) and depends on the presence of the yeast orthologs of adenine nucleotide translocator. (Pereira, Camougrand, Manon, Sousa, & Corte-Real, 2007). Disruption of cytochrome-*c* partially prevents acetic acid-induced cell death, which is linked to enhanced mitochondrial membrane potential and loss of cytochrome-*c* oxidase activity. Consistently, ρ^0 cells, which lack mitochondrial DNA, and hence respiration-deficient cells, display resistance against acetic acid-induced cell death (Ludovico *et al.*, 2002). Valenti *et al.* (2008) demonstrated that acetic acid-induced apoptosis requires temporary activation of the proteasome (Valenti *et al.*, 2008), which suggests the existence of a cross-talk between the antioxidant defense and the proteolytic systems. We also found that the vacuolar protease Pep4p is translocated from the vacuole into the cytosol and has a role in mitochondria degradation in cells undergoing acetic acid induced apoptosis (Pereira *et al.*, 2010). Interestingly, as discussed above (see 6.3), our results on acetate induced apoptosis in CRC cells (see in 6.3) are in accordance with the results obtained in yeast. This reinforces the yeast cell as a good model to study basic mechanisms involved in cancer cells.

Taking into account all the aforementioned advantages on the use of yeast as a cell model system and the need to addresses the topics on cell cycle and cell death in the scope of the program of eleventh year, we considered interesting and adequate to develop a protocol based on very simple experimental approaches to demonstrate the effect of acetic acid on yeast cell cycle progression and cell death induction.

2. Experimental Procedures

2.1 Material and reagents

- 0,150 g of commercial baker's yeast (bought in the supermarket)

- 1 g of yeast extract

- 1 g of peptone

- 2 g of sacarose

- 100 ml of water (at ≈30°C)

- Optical Microscope

- Microscope slides and cover glass

- Balance

- Erlenmeyer flask

- Falcon tubes

- Watchglass

- Eyedropper

- Spatula

2.2. Culture Media

A liquid medium containing white flour (0.5 %, w/v) and sacarose (2%, w/v) was prepared and used to assess the effect of acetic acid on yeast growth and cell cycle. A more rich medium containing yeast extract (1%, w/v), peptone (1% p/v) and sacarose (2%, w/v) as carbon source was also prepared, and used in the assays to study effect of acetic acid in cell cycle progression and on cell death..

2.3. Yeast strain and incubation conditions

To perform the experiments commercial baker´s yeast was bought in the supermarket and used as inoculum. Yeast cells were incubated in the culture medium prepared as described above, at 30°C with orbital shaking (200 rpm) and a liquid/air ratio of 1:5 to assure a good aeration.

These culture media and incubation conditions were used in the protocols of analysis of cell cycle and cell death described below.

2.4. Protocol for the analysis of cell cycle

Cell proliferation is the process by which a cell of one type gives rise to two cells that are essentially identical. Given sufficient nutrients, yeasts cells double in number every 90 min approximately. The analysis of cell cycle in *S. cerevisiae* is a very simple experiment. Yeast grows by budding, which means that the original "mother" cell gives rise to an ellipsoidal daughter cell made of entirely new cell surface material (Herskowitz, 1988). The identification of the cell cycle phase of a budding yeast cell can be recognized by the cell morphology (bud size) and nuclear division. The size of the bud indicates approximately the position of the yeast cell in the cell cycle. For example, unbudded cells are in G1 (Herskowitz, 1988). To observe cell proliferation in the budding yeast we suggest the following experimental procedure, the same that was used by us in the laboratory.

2.4.1. Experimental Procedure

Procedure

Step 1: Distribute 5 ml of culture medium in each falcon tube and inoculate with a concentrated cell suspension to obtain an initial optical density at 640 nm (OD_{640}) of about 0.1.

Step 2: Add acetic acid from a stock solution (17.5 M) to obtain the following final concentrations:

- 0 mM → 5ml of the medium + yeast + 0 µl of acetic acid (negative control)
- 10 mM → 5ml of the medium + yeast + 2.8 µl of acetic acid
- 20 mM → 5ml of the medium + yeast + 5.6 µl of acetic acid
- 30 mM → 5ml of the medium + yeast + 8.57 µl of acetic acid

Step 3: Harvest a sample every 2 h to measure the $OD_{640,}$ and observe under the microscope to count approximately 200 cells, and determine the % of cells in the different phase of the cell cycle.

2. 5. Protocol for the analysis of cell death

The observation of cell death in *S. cerevisiae* is also a very simple experiment. To observe cell death cells were exposed to acetic acid at different concentrations (60, 80 and 120 mM), pH 3±0, and methylene blue, a dye that stains blue metabolic inactive cells ("death cells"). Cells metabolic active can also take the dye, but is processed by active enzymes and reduced. The reduced form of the dye is colorless and hence metabolic active cells ("viable cells") do not stain blue.

2.5.1. Experimental Procedure

Procedure

Step 1: Distribute 5 ml of culture medium in each falcon tube and inoculate with a concentrated cell suspension to obtain an initial optical density at 640 nm (OD_{640}) of about 0.1.

Step 2: Add acetic acid from a stock solution (17.5 M) to obtain the following final concentrations:

- 0 mM → 5ml of the medium + yeast + 0 µl of acetic acid (negative control)
- 60 mM → 5ml of the medium + yeast + 17.1 µl of acetic acid
- 80 mM → 5ml of the medium + yeast + 22.85 µl of acetic acid
- 120 mM → 5 mL of medium + yeast + 34.28 µl of acetic acid

Step 3: Place each falcon in the incubator at 30°C with shaking at 200 rpm.

Step 4: Harvest 10µl of the cell suspension and add 10 µl of methylene blue.

Step 5: Homogenize the mixture and transfer 10µl of the suspension to a Newbauer chamber. This is a special glass slide precisely divided into squares of 1 mm^2 area; the slide is covered with

a cover slip, leaving a volume of each square of 10.04 cm^3 or 0.1 mm^3, which is equivalent to 1 ml).

Step 6: Count the cells stained in blue and the colorless cells in a total of about 300 cells.

Step 7: To estimate the % of viable cells count 300 cells in the Newbauer chamber

- % viable cells = $\frac{\text{Colorless cells} \times 100}{\text{Total number of cells (colorless+blue)}}$

- % dead cells = $\frac{\text{Blue cells} \times 100}{\text{Total number of cells (colorless+blue)}}$

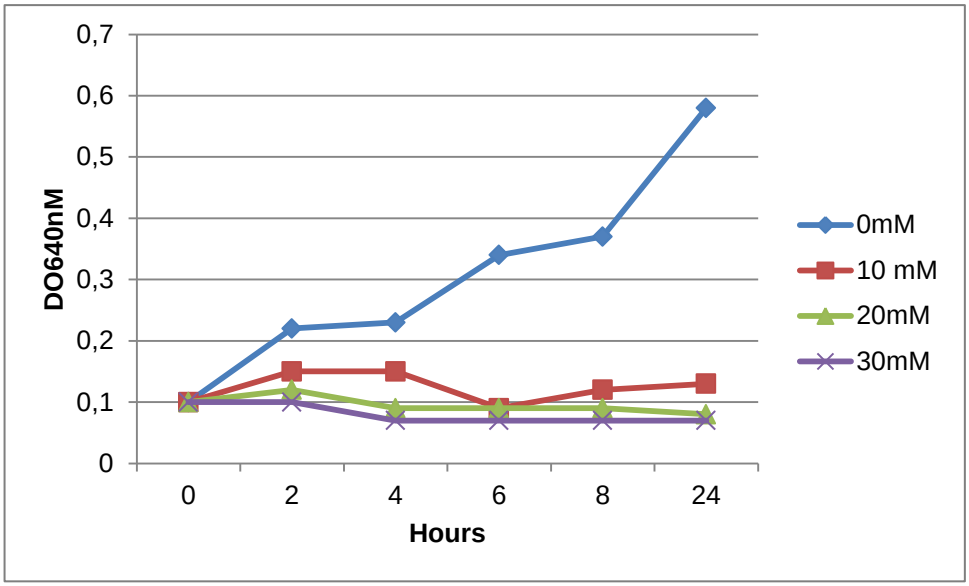
3. Results and discussion

3.1. Effect of acetic acid on cell cycle progression

Our first attempt was to growth the yeast cells in an inexpensive culture medium and of easy preparation. Therefore, we first tested a medium containing white flour and sacarose as carbon source. Though we observed cell growth and a negative effect of acetic acid in this medium the cell density achieved was quite low (figure 7.1 a) which could limit the observations under the microscope. Therefore in the following assays we used a rich medium with peptone, yeast extract and sacarose which allowed to achieve a higher cellular density figure 4.1 a) after 24 h (OD_{640} of about 3.5 in comparison with 0.6). The increase in acetic acid concentration led to an increase of the inhibition of yeast growth (Fig. 7b).

Cell samples from cultures in this medium in the absence and presence of different concentrations of acetic acid acetic were observed under the microscope to determine the % of cells in the different phases of the cell cycle (fig. 5c and 5d).

a


0,7
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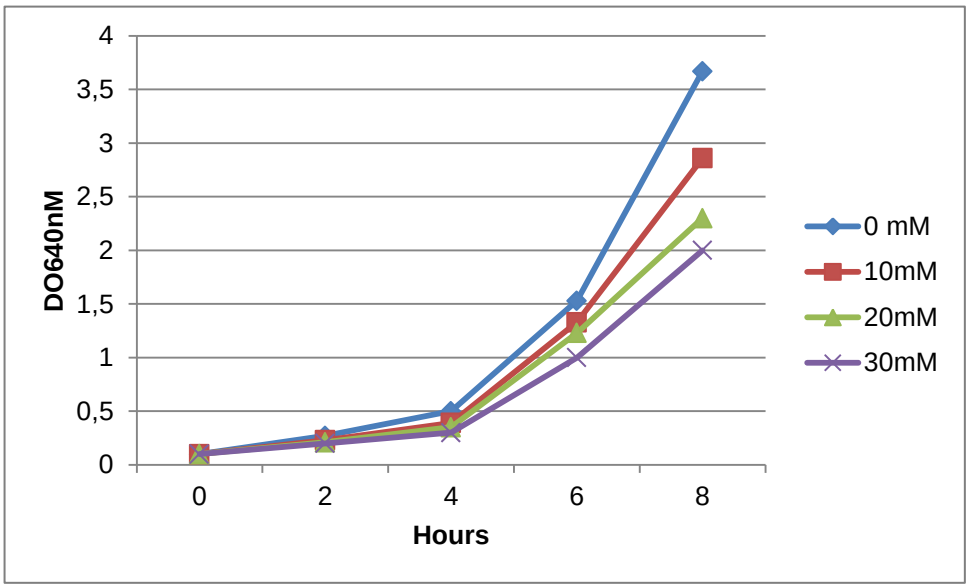
b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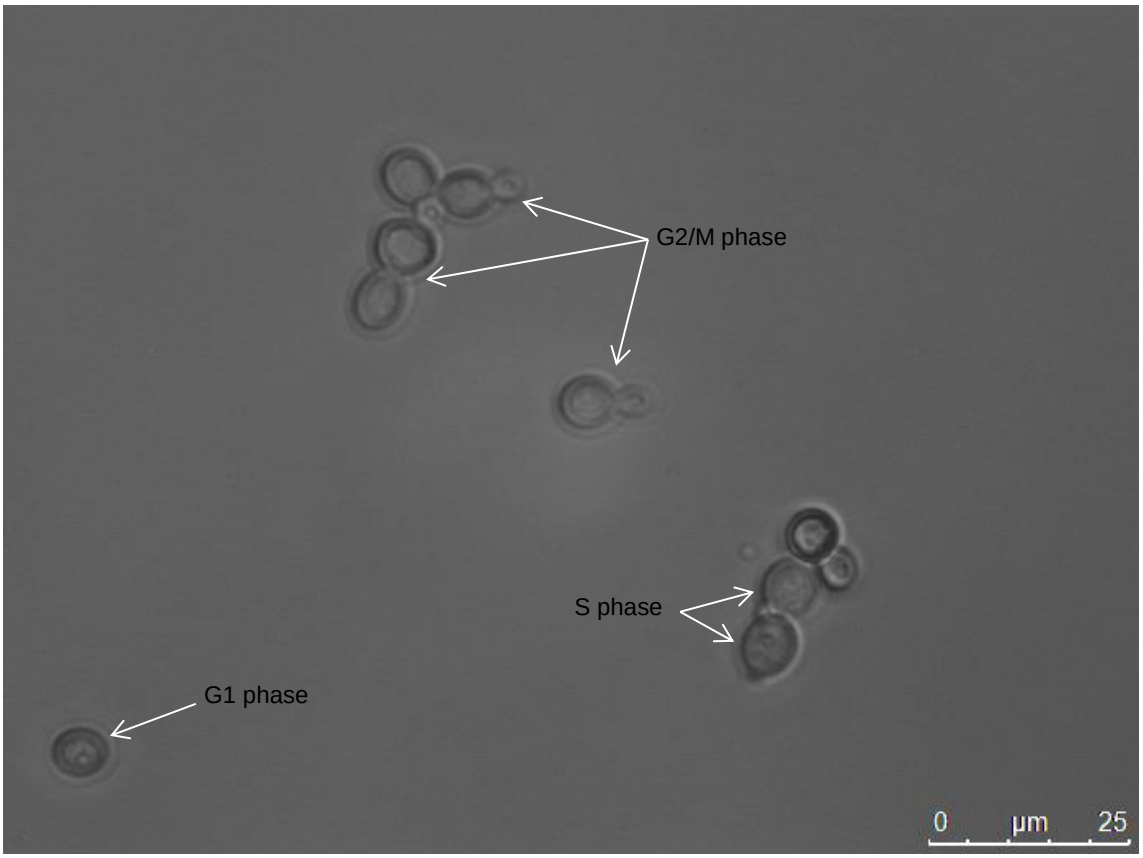
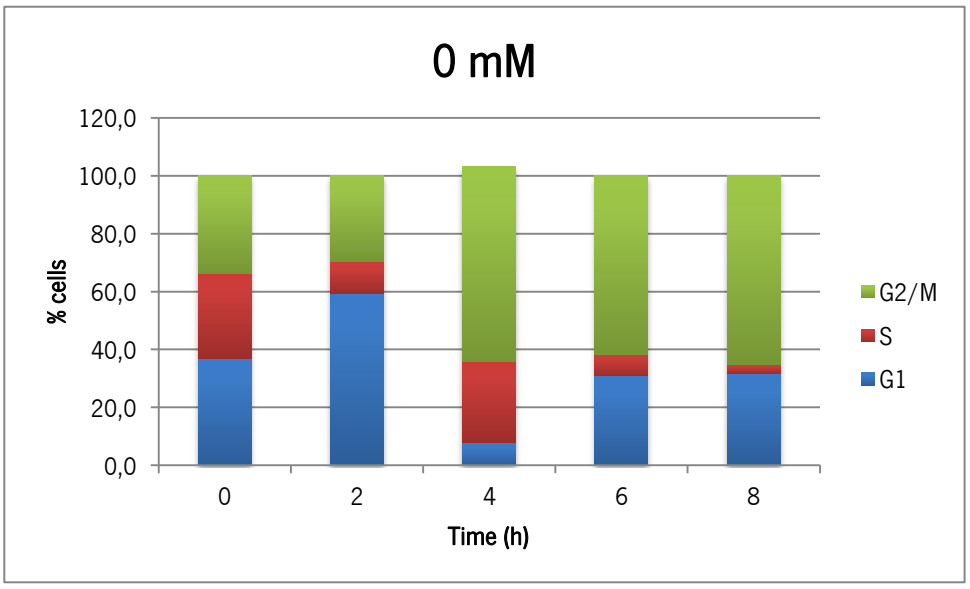

G2/M phase
S phase
G1 phase
0 µm 25

d


0 mM
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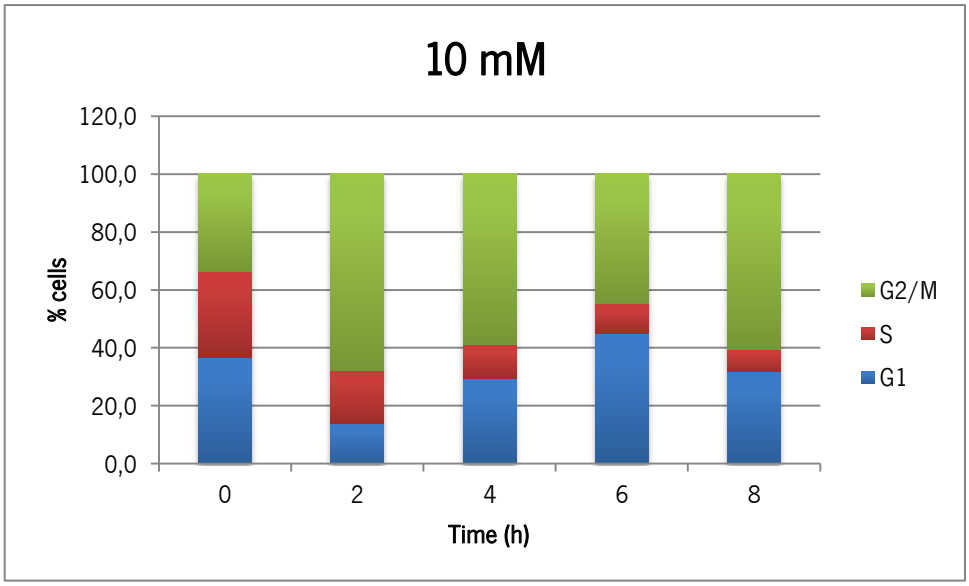
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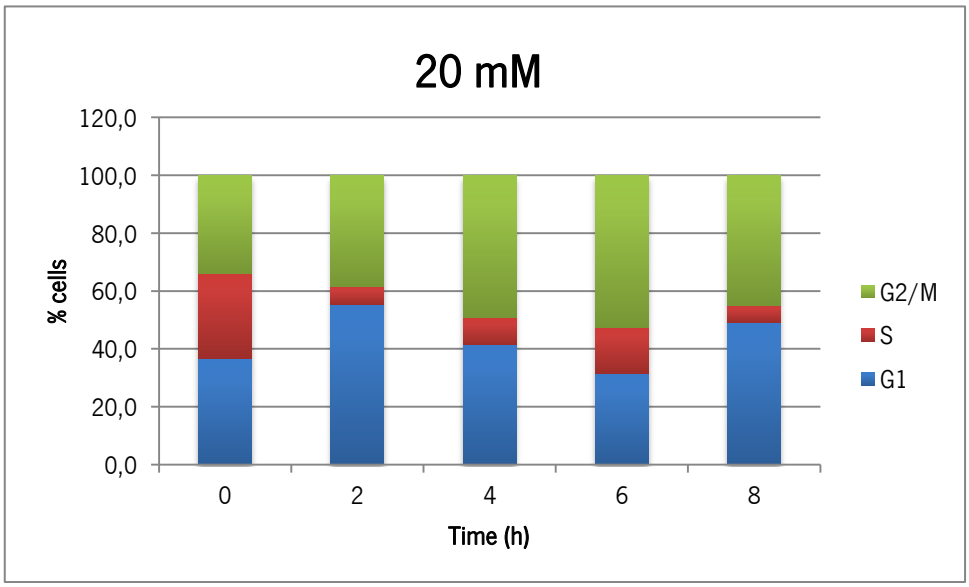
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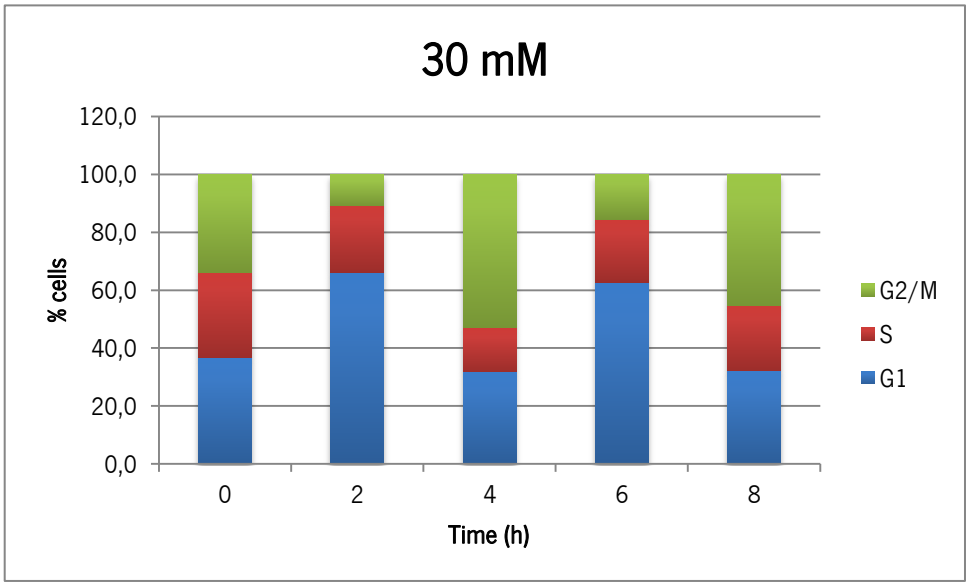


Figure 7 - Effect of acetic acid on yeast growth and cell cycle progression. Yeast cells were incubated in the absence or presence of acetic acid (10mM, 20mM and 30mM) in culture medium with flour (a) or in rich medium (b) and OD 640 were measured after 2, 4, 6 and 8 hours; (c) Microscope images of cells after 2 hours treatment with 10 mM acetic acid. (400X) showing yeast cells in different phases of the cell cycle; (d) Effect of 10, 20 and 30 mM of acetic acid on yeast cell cycle progression.

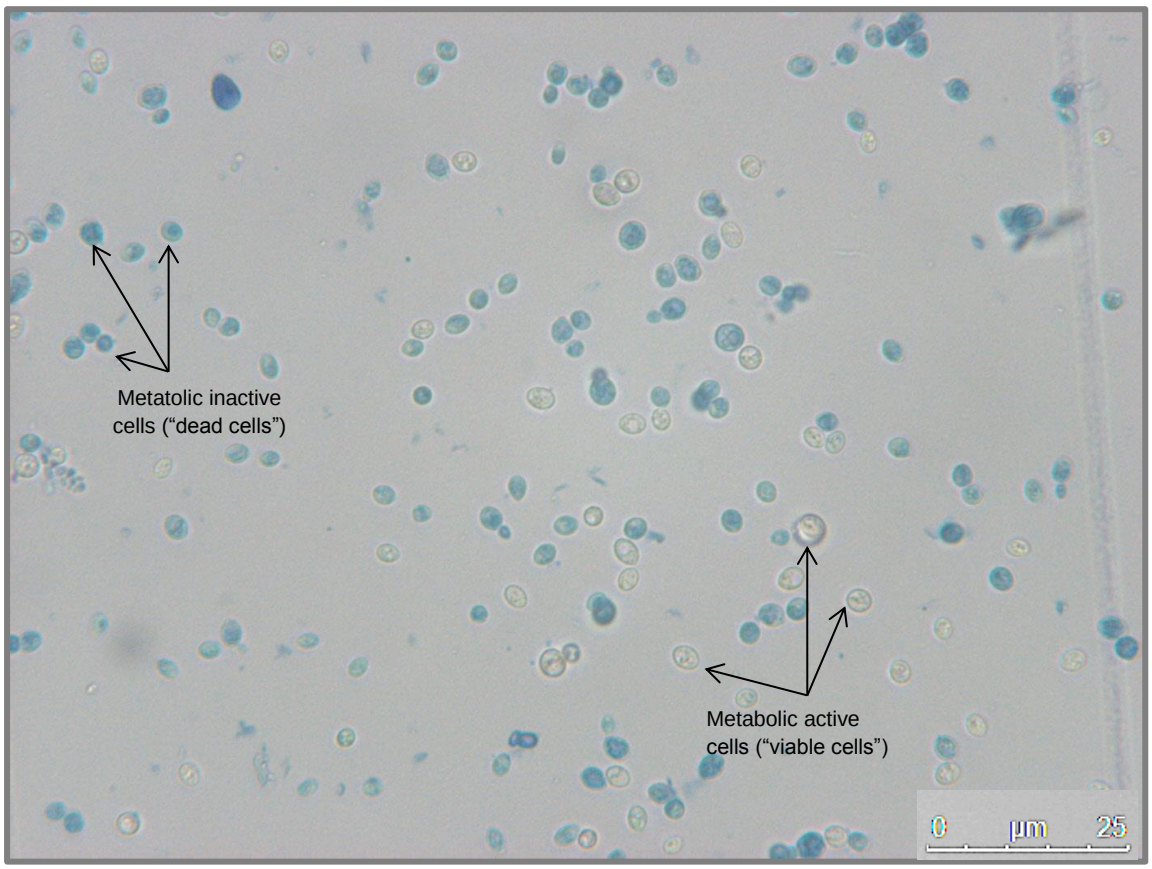
The analysis of the cell cycle progression of samples exposed to increasing concentrations of acetic acid along 8 h incubation, suggests that growth inhibition by acetic acid is associated with a slight perturbation in the progression of cell cycle. This is more apparent in the culture exposed to 30 mM of acetic acid which displays a higher percentage of cells in the S phase. This may indicate that cells actively involved in duplication of their cellular contents are more sensitive to acetic acid, and therefore do not proceed as efficiently to the G2/M phase as untreated cells.

3.2. Effect of acetic acid on cell death

Exposure of yeast cells to increasing concentrations of acetic acid led to a decrease in number of colorless cell (“viable cells”) and an increase in the number of blue cell (“dead cells”) after staining with methylene blue (Figure 8a). The percentage of viable cells decreased from 100% to 97,1% to 75.5 % and 16%, after 2 hours treatment with 60, 80 and 120 mM of acetic

acid, respectively. This indicates that acetic acid is inducing cell death, as assessed by the loss of metabolic activity (Figure 8b).

a



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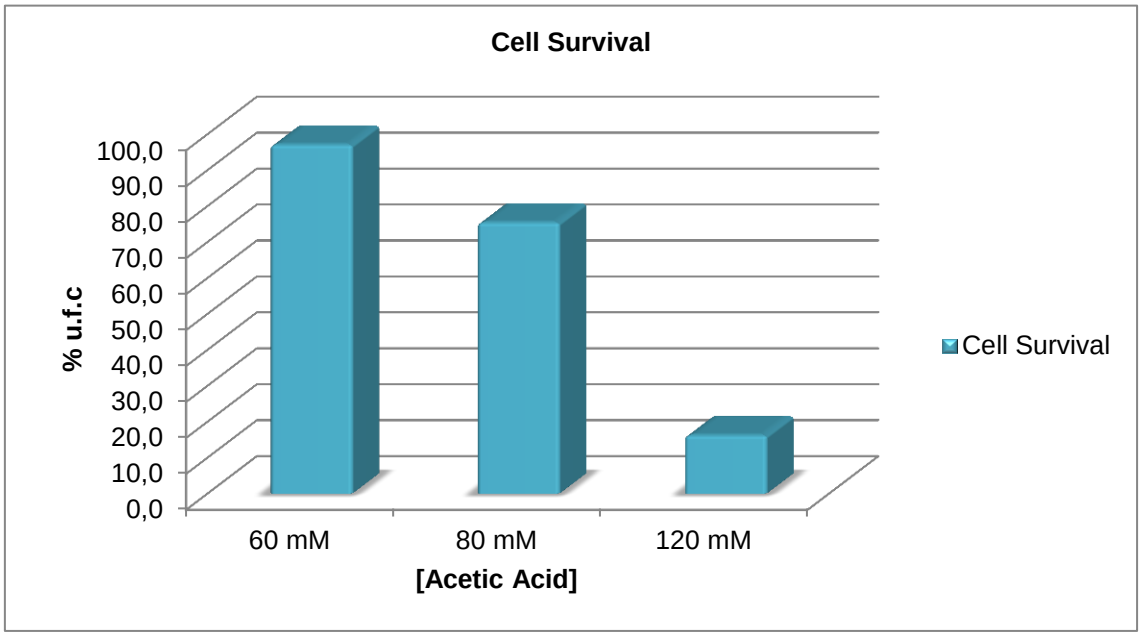

Figure 8 - Cell viability of yeast cells after exposure to acetic acid assessed by methylene blue staining. (a) Microscope image showing metabolic inactive cells ("dead cells") (blue) and metabolic active cells ("viable cells") (colorless) after 2 hours treatment with 60 mM acetate (400X). (b) Percentage of metabolic active cells ("viable cells") after acetic acid treatment (60 mM, 80 mM and 120 mM).

4. Final conclusions

In the second part of this thesis we intended to develop protocols to illustrate key biological processes that are discussed in biology classes for undergraduate students. The difficulty to accomplish this objective was essentially due to the need to adapt the protocols and techniques used in research laboratories, to the scarce facilities available in secondary school labs. Laboratories in secondary schools are not equipped with the appropriate instrumentation that allow the use of more advanced experimental approaches required to study complex cellular processes.

We demonstrate that very simple protocols associated with the use of yeast cells and of a basic optical microscope may be applied to illustrate two basic cellular processes, namely the cell cycle and cell death, in experimental biology at the secondary school level.

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