Universidade do Minho
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Marta Alexandra Rodrigues Casanova

Development of an improved propionibacterium for potential use as a nutraceutical towards the prevention/treatment of colorectal cancer

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Development of an improved propionibacterium for potential use as a nutraceutical towards the prevention/treatment of colorectal cancer

Dissertação de Mestrado
Mestrado Genética Molecular

Trabalho efetuado sob a orientação da
Professora Doutora Ana Arminda Lopes Preto Almeida
a da
Professora Doutora Lígia Raquel Marona Rodrigues

janeiro de 2015

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Título

Development of an improved propionibacterium for potential use as a nutraceutical towards the prevention/treatment of colorectal cancer

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A todos vocês, obrigado por tudo.

Dedico esta Tese á minha madrinha

Maria Arminda Gomes Rodrigues

Development of an improved propionibacterium for potential use as a nutraceutical towards the prevention/treatment of colorectal cancer

Abstract

Propionibacterium freudenreichii is a commercially important bacterium that is well-known for its role as ripening starter in the cheese industry and its probiotic potential. These bacteria may beneficially modulate the intestinal ecosystem and can exert anti-neoplastic effects, particularly against colorectal cancer (CRC), via the production of short chain fatty acids (SCFAs), namely acetate and propionate. Thus, propionibacteria can be envisaged as a potential nutraceutical towards the prevention/treatment of CRC. In that sense, the aim of this thesis was to develop strategies to enhance the production of SCFA by *P. freudenreichii* under the simulation of human colon environment, as well as to evaluate its effects on CRC cells. In order to optimize and characterize the production of SCFAs, acetate and propionate by *P. freudenreichii*, different culture media and different fermentation conditions were evaluated. Moreover, the SCFAs toxic concentrations for the bacterium were determined. Additionally, a digestive stress challenge and random mutagenesis of *P. freudenreichii* DSM 20271 were performed. Finally, the effect of the propionibacteria fermentation broth on CRC cells and the CRC cells conditioned medium on the growth and biotransformation performance of the bacteria were studied.

The basal medium (BM) was found to be the best to produce SCFA by *P. freudenreichii* with high amounts of acetate and propionate being produced, mainly when supplemented with glycerol. However, the results obtained with the medium "mimicking the content of the human colon" (MCHC) were not favorable regarding SCFAs production. The adapted *P. freudenreichii* to digestive stress lost the ability to produce high amounts of SCFAs in yeast extract-lactate (YEL) and BM media, in particular propionate. Moreover, partial inhibition of the bacteria growth and SCFAs production occurred at the following concentrations of pure SCFAs: 4 g L^{-1} acetate; 3 g L^{-1} propionate; 6 g L^{-1} propionate; 1 g L^{-1} acetate and 3 g L^{-1} propionate. Pure acetate and propionate, as well as the bacterial fermentation broth inhibited the CRC cells RKO proliferation and promoted their accumulation in the sub-G1 phase of the cell cycle. In conclusion, the results gathered in this work suggest that the co-culture of *P. freudenreichii* and CRC cells was found to be possible and favorable for the bacteria and that *P. freudenreichii* could potentially be used in the CRC prevention/treatment *via* their ability to produce SCFAs.

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Desenvolvimento de uma Propionibacterium melhorada para potencial uso como um nutracêutico para a prevenção / tratamento do cancro colorectal

Resumo

A *Propionibacterium freudenreichii* é uma bactéria comercialmente importante, conhecida pela sua utilização como cultura de arranque na produção de queijo, bem como pelo seu potencial probiótico. Estas bactérias podem modular beneficamente o ecossistema intestinal e exercer os efeitos antineoplásicos, em particular contra o cancro colorectal (CRC), através da produção de ácidos gordos de cadeia curta (AGCC), nomeadamente acetato e propionato. Assim, as propionibactérias podem ser vistas como potenciais nutracêuticos para a prevenção/tratamento do CRC. Nesse sentido, o objectivo desta tese foi desenvolver estratégias para aumentar a produção de AGCC pela *P. freudenreichii* em condições que simulam o cólon humano, bem como avaliar o seu efeito nas células de CRC. A fim de otimizar e caracterizar a produção dos AGCC, acetato e propionato, diferentes meios de cultura e diferentes condições foram avaliados. Além disso, as concentrações de AGCC tóxicas para a bactéria foram determinadas. Adicionalmente, realizaram-se ensaios de adaptação ao stresse digestivo e de mutação aleatória na *P. freudenreichii* DSM 20271. Finalmente, foi estudado o efeito do meio de fermentação da bactéria nas células de CRC, bem como o efeito do meio condicionado das células de CRC no crescimento e produção de AGCC pela bactéria.

O meio basal (BM) demonstrou ser o melhor para produzir AGCC pela *P. freudenreichii*, obtendo-se grandes quantidades de acetato e propionato, principalmente no meio BM suplementado com glicerol. No entanto, os resultados obtidos com o meio que "imita o conteúdo do cólon humano" (MCHC) não foram favoráveis relativamente à produção de AGCC. A *P. freudenreichii* adaptada ao stresse digestivo perdeu a capacidade de produzir grandes quantidades de AGCC, particularmente propionato, nos meios de extrato de levedura-lactato (YEL) e BM. Adicionalmente, observou-se uma inibição parcial do crescimento bacteriano e produção de AGCC para as seguintes concentrações de AGCC puros: 4 g L^{-1} acetato; 3 g L^{-1} propionato; 6 g L^{-1} propionato; 1 g L^{-1} acetato e 3 g L^{-1} propionato. O acetato e o propionato, bem como o meio de fermentação da bactéria inibiram a proliferação das células de CRC RKO e induziram um aumento de células na fase sub-G1 do ciclo celular. Em conclusão, os resultados deste trabalho sugerem que a co-cultura entre células de CRC e *P. freudenreichii* é possível e favorável para a bactéria e que a *P. freudenreichii* poderá potencialmente ser usada na prevenção/tratamento de CRC através da sua capacidade de produzir AGCC.

Scientific output

Poster presentation in conferences:

- Casanova M., Preto A., Rodrigues LR. "Improved production of acetate and propionate by *Propionibacterium freudenreichii"*. II Symposium of the PhD Programme on Molecular and Environmental Biology, Braga, November 2014.

- Casanova M., Preto A., Rodrigues LR. "Improved production of acetate and propionate by *Propionibacterium freudenreichii"*. XVIII Congress of the Portuguese Biochemical Society, Coimbra, December 2014.

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List of symbols and abbreviations

[A], Concentration of Acid

AA, Area Acid

Adapted *P. freudenreichii*, *Propionibacterium freudenreichii subsp. freudenreichii* DSM 20271 tolerant to digestive stress established by us in the laboratory (chapter 3)

AIS, Area Internal Standard

APC, Adenomatous polyposis coli

BM, Basal médium

Cat-D, Cathepsin D

CCM, Control with DMEM medium diluted in HEPES solution

CFB, *Cytophaga-Flavobacterium-Bacteroides phylum*

CLA, Conjugated linoleic acid

CM, 'Conditioned medium'

CM½, Conditioned medium diluted in fresh DMEM medium (50% v/v);

CRC, Colorectal cancer

DCW, Dry cell weight

DMEM, Dulbecco's Modified Eagle's Medium

ENU, N-nitroso-N-ethylurea; N-ethyl-N-nitrosourea or Ethylnitrosourea

FAO, Food and Agriculture Organization

FAP, Familial adenomatous polyposis

FBS, Fetal bovine serum

FOBT, Fecal occult blood testing

GERD, Gastroesophageal reflux disease

GI, Gastrointestinal

GRAS, Generally regarded as safe

HMP, Human microbiome project

HNPCC, Lynch syndrome

HPLC, High performance liquid chromatography

$IC_{30,}$ 30 % maximal inhibitory concentration

$IC_{50,}$ Concentrations of half maximal inhibitory concentration

[IS],- Concentration of Internal Standard

LMP, Lysosomal membrane permeabilization

MCHC, “Mimic the content of the human colon” medium

NTG, N-methyl-N'-nitro-N-nitrosoguanidine

OD, Optical density

P. freudenreichii, *Propionibacterium freudenreichii subsp. freudenreichii* DSM 20271

PI, Propidium iodide

QPS, Qualified presumption of safety

R, Correlation Coefficient

RT, Retention Time

SCFA, Short chain fatty acid

SEM, Standard error of the mean

VSL#3, Commercially available probiotic formulation

WHO, World health organization

YEL, Yeast extract-lactate medium

Chapter 1.|

Literature review

1.1. Cancer

Formerly humans died by natural causes or through violence, accidents and by an amazing variety of infectious diseases but with the years the main causes of death vary. In the past decade, the leading causes of death worldwide were ischaemic heart disease, stroke, chronic obstructive lung disease and lower respiratory infections. Actually, the leading causes of death are heart disease and cancer (World Health Organization 2014). Although changes in living conditions during the 20th century and advances in medicine have contributed to change morbidity and mortality standards, there is still a shadow over humanity, namely cancer, once that although it is the second leading cause of death after heart disease (World Health Organization 2014), the death rates have remained constant compared with death rates for heart disease which have been decreasing (Siegel et al. 2014).

Cancer is a public health problem worldwide that induce death every day and its incidence is increasing. The results of GLOBOCAN 2012 are worrying as according to these results, an estimated 14.1 million new cancer cases and 8.2 million cancer related deaths occurred in 2012 (Ferlay et al. 2013), compared with 12.7 million and 7.6 million respectively, in 2008 and predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population (Ferlay et al. 2010). In 2012, lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectal (1.4 million, 9.7%) cancers represented the most common diagnosed cancer worldwide and lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) cancers were the most common causes of cancer death (Ferlay et al. 2013).

In Portugal, cancer is also a serious health problem. In 2012, there was an estimated 49 thousands new cancer cases and 24 thousands cancer related deaths being colorectal, prostate and breast cancers the most diagnosed but also the most common causes of cancer death (See Figure 1.1)(Ferlay et al. 2013).

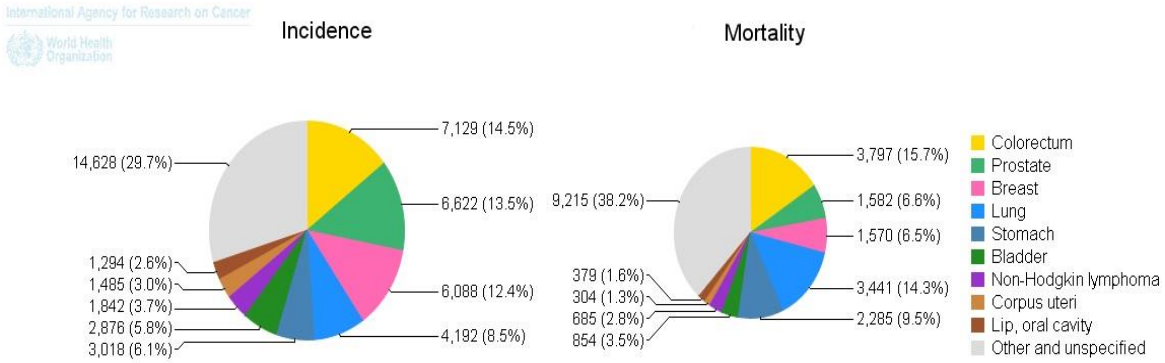


Figure 1.1. Estimated cancer incidence and mortality rates Portugal in 2012 (Adapted from (Ferlay et al. 2013)).

Cancer is a disease normally associated to a rapid cell proliferation and uncontrolled cell growth. However cancer is more complex than this simply characterization evolving survival or 'hardiness' strategies that allows the existence of this disease (Aktipis et al. 2013). Cancer is a multistep process that leads normal cells to acquire biological capabilities becoming malignant cells. These biological capabilities are designated by "hallmarks of cancer" which were proposed by Hanahan and Weinberg in 2000. Together constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases. The hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000). Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list: reprogramming of energy metabolism and evading immune destruction (See Figure 1.2)(Hanahan and Weinberg 2011).

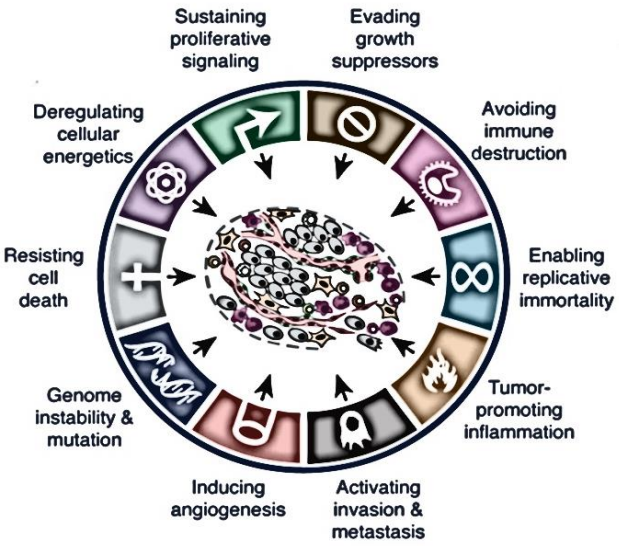


Figure 1.2. The Hallmarks of Cancer originally proposed by Hanahan and Weinberg (Adapted from (Hanahan and Weinberg 2011)).

All cancer are a result of multiple mutations (Loeb and Loeb 2000; Hahn and Weinberg 2002), but only 5-10% of all cancer cases can be attributed to genetic defects, whereas the remaining 90-95% have their roots in the environment and lifestyle (See Figure 1.3) (Anand et al. 2008). This data shown that the majority of cancers are not of hereditary origin and that lifestyle factors, such as dietary habits, smoking, alcohol consumption, and infection, have a profound influence on their development (Irigaray et al. 2007; Mucci et al. 2001). The hereditary factors cannot be modified, but we can prevent cancer by manipulating and modifying the lifestyle and environmental factors.

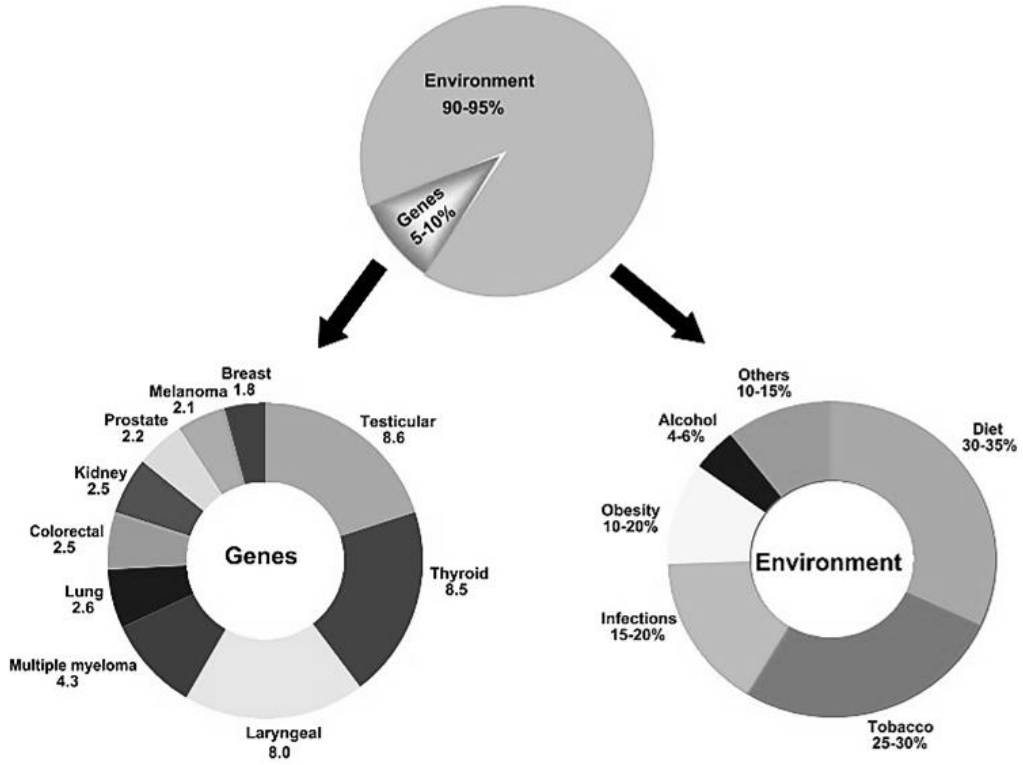


Figure 1.3. The role of genes and environment in the development of cancer (Adapted from (Anand et al. 2008)).

1.1.1. Colorectal cancer

Colorectal cancer (CRC) is a malignancy that affects the large intestine wall, and depending on the degree of invasion can compromise other organs, either directly or through metastasis (World Health Organization 2014).

In Portugal, CRC is a growing healthy problem, as it is incidence and mortality rates have been increasing since the 1980s (Portuguese society for digestive endoscopy 2012). According to the most recent estimates by the International Agency for Research on Cancer (Ferlay et al. 2013) CRC, in Portugal, is the most commonly diagnosed cancer with 7129 new cases and it is the most

common cause of cancer deaths with 3797 deaths reported in 2012 (See Figure 1.1)(Ferlay et al. 2013).

In the development of CRC, a strong genetic component but also environmental factors, including diet and lifestyle, have a major impact on its risk. These factors are strongly supported by epidemiological studies, which have shown that environmental factors have the potential to influence the normal structure and function of the colon, and various dietary components have been proposed as being either beneficial or potentially harmful to colonic health (Lipkin et al. 1999). For example, Azcarate-Peril and co-workers reviewed this issue and an increasing number of studies demonstrated that lifestyle-associated factors that increase the risks of CRC include elevated body mass index, obesity, and reduced physical activity (Azcarate-Peril et al. 2011). However, it has been suggested that diet regimens rich in fruit, vegetables and poor in meat might have a protective effect, reducing the incidence of colorectal adenomas by modulating the composition of the normal nonpathogenic commensal microbiota (Azcarate-Peril et al. 2011; Davis and Milner 2009; Hakansson et al. 2012).

CRC is thought to develop over many years in a multistep process that transform normal glandular epithelium into invasive adenocarcinoma as a result the accumulation of both acquired genetic and epigenetic changes (Vogelstein et al. 1988). The steps involved in this process were first described by Fearon and Vogelstein (1990) in the classic adenoma to cancer progression, 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 (Fearon and Vogelstein 1990). This model allows to understand the molecular pathogenesis of CRC and is based on the premise that tubular and tubulovillous adenomas are premalignant neoplasms that will progress to invasive adenocarcinoma (See Figure 1.4) (Lao and Grady 2011; Fearon and Vogelstein 1990). However it is known that there are more classes of premalignant polyps with potential for malignant transformation and there are multiple molecular pathways to CRC and it is important to distinguished well-defined inherited syndromes as Lynch syndrome (HNPCC) and familial adenomatous polyposis (FAP), which are unusual conditions that have a substantial cancer risk with a percentage of 2% to 5% of all CRCs (Lichtenstein et al. 2000).

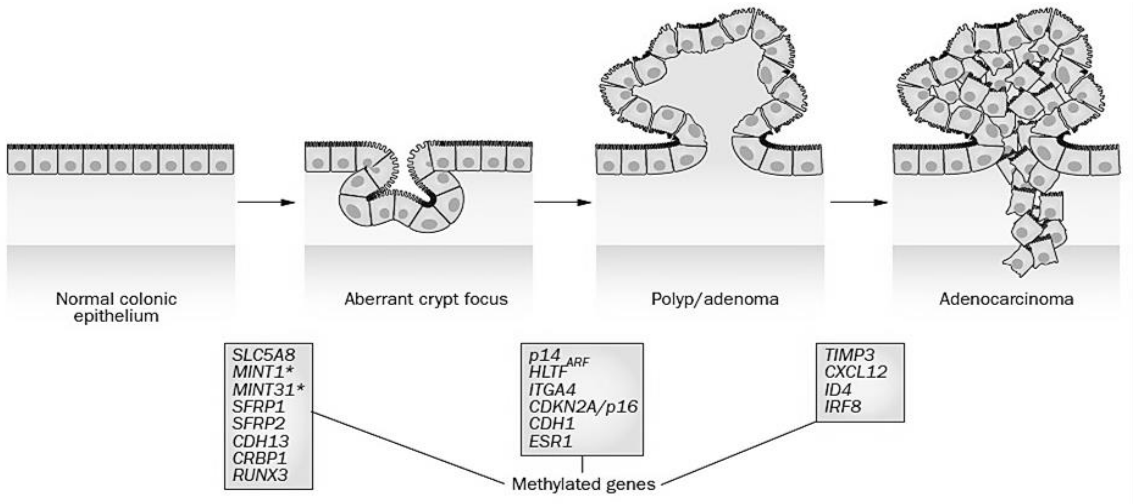


Figure 1.4. Histological steps of the colorectal cancer, polyp to adenocarcinoma sequence, associated with commonly methylated genes (and loci) involving in this process (Lao and Grady 2011).

Assessment of the CRC epigenome has revealed that virtually all CRCs have aberrantly methylated genes and that the average CRC methylome has hundreds to thousands of abnormally methylated genes (Ahnen 2011). These genes are implicated in either the initiation or progression of CRC (Barrow and Michels 2014). We can see in Figure 4 the common methylated genes (and loci) identified at the histological steps of CRC polyp to adenocarcinoma sequence. The genes listed between normal epithelium and aberrant crypt focus as well as those genes listed between aberrant crypt focus and polyp/adenoma might be involved in the initiation of colorectal cancer, as well as those genes listed between polyp/adenoma and adenocarcinoma could have a role in the progression and metastasis of CRC (Lao and Grady 2011).

APC, K-RAS and p53 genes play a critical role in development of CRC. APC regulate the entry of epithelial cells into adenoma-carcinoma progression, being proposed as a ‘‘gatekeeper’’ gene (Kinzler and Vogelstein 1996). A mutation of the gatekeeper leads to a permanent imbalance of cell division over cell death. The RAS oncogene promotes tumor formation through stimulation of cell proliferation, motility and regulation of apoptosis. Activating mutations in the RAS oncogenes mediates deregulated cell growth, evasion of apoptosis and malignant transformation (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 (Levine 1997). Mutations in p53 render the cells susceptible to failure of apoptosis and increased accumulation of DNA damage, allowing unregulated growth (el-Deiry et al. 1993).

In the onset of the disease, when CRC is early and highly curable, symptoms and signs are less common and less obvious being most common and prominent late in colon cancer. Advanced CRC is likely to be incurable, what difficult an early diagnostic and affects the effectiveness of treatment (Cappell 2005), thus, symptoms depend on cancer location, cancer size and presence of metastases.

There are a range of screening and diagnostic tests for CRC as fecal occult blood testing (FOBT), barium enema, flexible sigmoidoscopy, colonoscopy (Helm et al. 2003; Selby et al. 1992; Detsky 2001; Spinzi and Minoli 2001; Cappell 2005), which the general asymptomatic population should perform regularly for early detection and prevention of CRC.

The treatment depends on the staging of CRC being the surgery the main curative treatment. Chemotherapy and radiotherapy may be recommended depending on the staging of cancer and other medical factors. The drugs must often use for CRC include 5-Fluorouracil, capecitabine, irinotecan and oxaliplatin (American Cancer Society 2015). However, there are national CRC treatment guidelines that helps the physicians in the choice of treatment. This guidelines are annually adapted to take into account Portuguese clinical practice (Pinto et al. 2010).

It is generally agreed that, given the significant negative effect of CRC on people's quality of life and the associated high mortality rates, it is essential to develop primary and secondary strategies for the prevention of CRC. As a primary prevention strategy is very important to educate the general population to modify dietary risk factors and adopt healthy habits. As a secondary prevention strategy is essential the detection and reduction of premalignant adenomatous lesion and early detection of cancer in a curable stage should be the primary goal.

1.2. Digestive system

The digestive system is a group of organs working together to convert food into energy and basic nutrients to feed the entire body. Food passes through a long tube inside the body known as the gastrointestinal (GI) tract suffering mechanical and chemical action that allows the degradation of food (Seeley 2011). GI tract is made up of the oral cavity, pharynx, esophagus, stomach, small intestines and large intestines. There are others organs that help your body to digest food but do not have food pass through them as teeth, tongue, salivary glands, liver, gallbladder and pancreas (Gray and Lewis 1918; Seeley 2011).

The intake of food, its breakdown into nutrients and their absorption and elimination of the indigestible waste is essential for a healthy body. Actually, there are a variety of digestive problems such as gastroesophageal reflux disease (GERD), irritable bowel syndrome, celiac disease, food allergies, diverticulitis, ulcerative colitis and Crohn's disease (Cencic and Chingwaru 2010). Thus, it is essential that each organ of the digestive system preform their function correctly.

1.2.1. Colon

The large intestine or colon allows the lower GI tract and it is the portion of GI which extends from the end of the ileum to anus. It is about 1.5 meters long, being one fifth of the whole extent of the GI tract and their contents take about 18-24 hours to cover its entire extension (Seeley 2011; Gray and Lewis 1918). The large intestine is divided into the cecum, colon, rectum and anal canal (See Figure 1.5). The colon corresponding the major part of the large intestine being constituted by the ascending, transverse, descending and sigmoid colon (Seeley 2011).

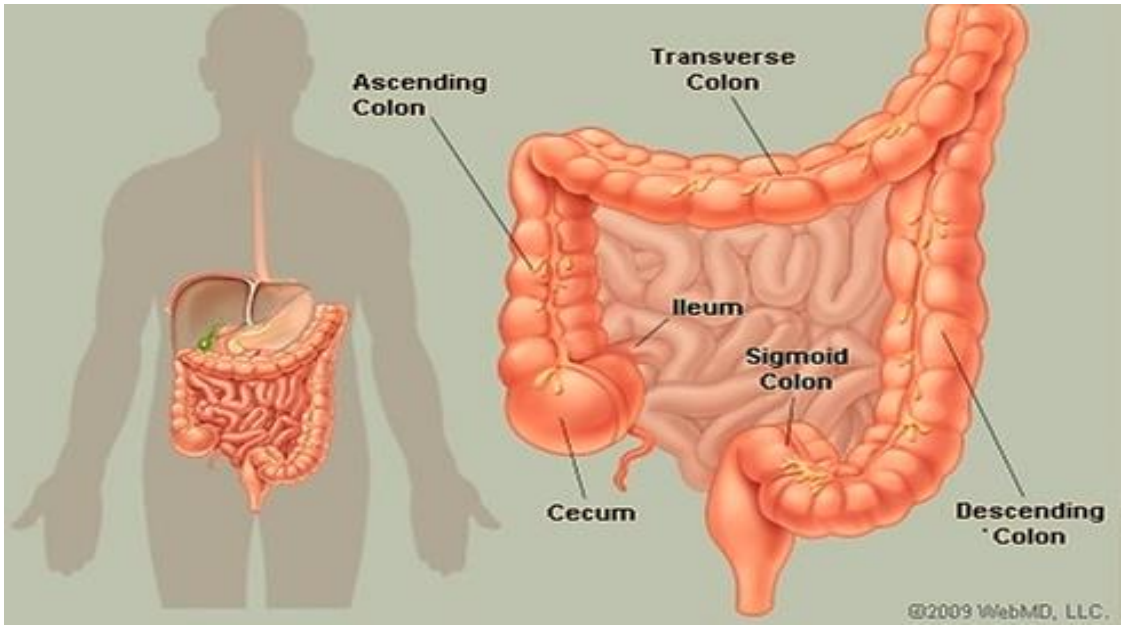


Figure 1.5. The Human large intestine anatomy (Adapted from (WebMD 2014)).

The main function of the large intestine are: reabsorption of water and mineral ions; formation and temporary storage of feces; maintaining a resident of population of over 500 species of bacteria and bacterial fermentation of indigestible materials (Seeley 2011).

The waste left over from the digestive process, namely chime, arrive to large intestine by cecum and it is passed through the colon by means of peristalsis, first in a liquid state and ultimately in a solid form. The feces passes through the colon, water and salts is absorbed as well occurs the

secretion of mucus by mucus glands present in the mucosa layer. During this transit the feces suffer the intensive action of microorganisms present in the lumen of colon. These bacteria perform several useful functions such as synthesizing various vitamins, processing waste products and food particles and protecting against harmful bacteria (Cencic and Chingwaru 2010).

1.3. Gastrointestinal microbiota

In the human GI tract resides the *microbiota*, a large and diverse community of microorganism which have a critical role in the evolution of the intestinal functions and in overall health of the host (Saavedra and Dattilo 2012).

The total amount of genes in the various species represented in our indigenous microbial communities is estimated about 2-4 million, exceeding the number of our human genes >100-fold (Backhed et al. 2005). The collective genomes of microbiota is termed "microbiome" and it was introduced in 2001 by Hooper and Gordon. Microbiome provide us genetic and metabolic attributes which we not have, including the ability to harvest otherwise inaccessible nutrients (Di Mauro et al. 2013).

In order to collect and integrate the genomic information from many diverse human microbiomes an international collaborative project, "the human microbiome project (HMP)", was launched in 2007 (Turnbaugh et al. 2007). Thus the Human Microbiome Project Consortium provided the first reliable estimates of the structure, function, and diversity of the healthy ("reference") human microbiome (Human Microbiome Project 2012b, 2012a) and all data are available at http://commonfund.nih.gov/hmp/publications.aspx.

Actually, microbiota is considered as an "organ within an organ" with its own functions: modulates expression of genes involved in mucosal barrier fortification, angiogenesis, postnatal intestinal maturation and a critical role in supporting normal digestions as well as affects energy harvest from the diet and energy storage in the host, fermenting unused energy substrates to short chain fatty acids (SCFAs) (Hooper et al. 2001; Saavedra and Dattilo 2012). These functions are intimately strain-related.

1.3.1. Composition of gastrointestinal microbiota

The composition of GI microbiota undergoes major modifications during our life being influenced by the host genotype and physiology, the colonization history, environmental factors, food and drugs (e.g. antibiotics) (Sharma et al. 2010).

Infants are born without GI microbiota, but rapidly after birth the infants GI is colonized by bacteria coming from the maternal vaginal and intestinal flora, and it is an important stage of development of intestinal functions (Hattori and Taylor 2009; Aagaard et al. 2013). The composition of the microbiota stays unstable until the age of approximately 3-4 years, when it becomes mature (den Besten et al. 2013).

The adult human GI tract contains all three domains of life: Bacteria, Archaea, and Eukarya, being the Bacteria the most dominant and most diverse group of microorganisms present in the human colon with 10^{14} citizens (98% of all species) (Hattori and Taylor 2009). Bacteria is usualy separated into two broad categories, namely as beneficial (e.g., *Bifidobacterium* and *Lactobacillus*) or harmful (e.g., *Enterobacteriaceae* and *Clostridium* spp.) which compete with each other to colonized the GI tract (Backhed et al. 2005).

In the composition of GI microbiota there may be between 500-1000 different species present, based on variation in 16S ribosomal RNA genes, which belong to more than 70 genera. The most abundant phyla in the gut is *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Hattori and Taylor 2009; Abubucker et al. 2012). The Figure 1.6 represent the diversity of bacteria in GI tract being CFB the designation of *Cytophaga-Flavobacterium-Bacteroides* phylum (Backhed et al. 2005).

The *Bacteroidetes* phylum manly produces acetate and propionate, whereas the *Firmicutes* phylum has butyrate as its primary metabolic end product. Most bacterial activity occurs in the proximal colon where substrate availability is highest, once the availability of substrate declines, extraction of free water reduces diffusion of substrates and thus microbial products (Di Mauro et al. 2013).

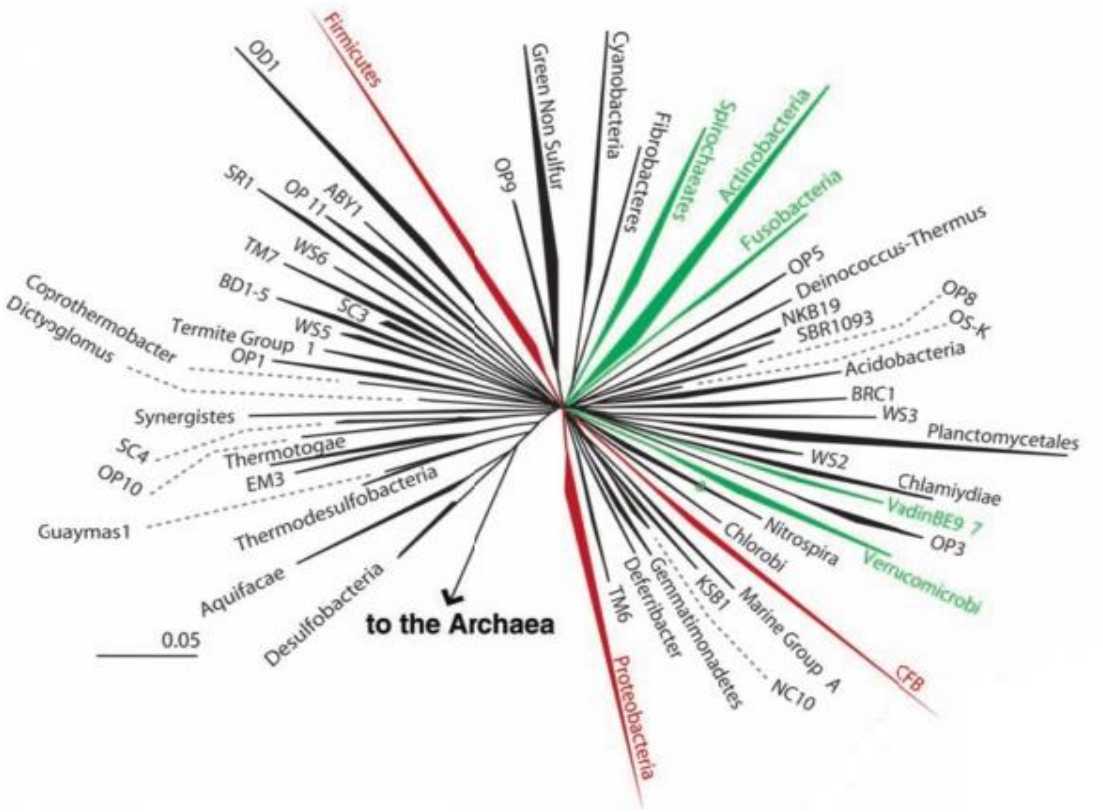


Figure 1.6. Representation of the diversity of bacteria in the human intestine. Wedges represent division: Those numerically abundant in the human gut are red, rare divisions are green, and undetected are black. Wedge length is a measure of evolutionary distance from the common ancestor (Adapted from (Backhed et al. 2005)).

The Human Microbiome Project (HMP) provided a reference collection of 16S ribosomal RNA gene sequences collected from the human gut that allow us to better associate changes in the microbiome with changes in health, but it is important to highlight that there is no single healthy microbiome. Each person harbors a unique and varied collection of bacteria that is the result of a life history as well as their interactions with the environment, diet and medication used (Ding and Schloss 2014). Thus it is important to understand the microbiome diversity and the mechanisms that are associated with this diversity to assess disease risk and personalize therapies. The second phase of the HMP, which is ongoing (2013-2016), will be a powerful feature to understand the role of the microbiome in human health and disease (Integrative 2014)

1.1.1.1. Colorectal cancer and gastrointestinal microbiota

The association between CRC and GI microbiota has been studied for many years and several of these studies have shown that patients with CRC have significant changes in GI microbiota, significant decreases in GI SCFAs concentrations and a significant increase in GI pH compared with

the healthy individuals (Scanlan et al. 2008; Sobhani et al. 2011; Ohara et al. 2010; Ohigashi et al. 2013).

These studies suggested that the changes in microbial community correspond to a decrease in bacteria species of *Firmicutes* and *Bacteroidetes*, demonstrating that microbial community composition changes collectively in CRC (Scanlan et al. 2008; Ohigashi et al. 2013; Ohara et al. 2010). It has been previously reported that specific bacterial species, such as *Streptococcus bovis* (Tjalsma et al. 2006; Ellmerich et al. 2000) or *Bacteroides* (Aries et al. 1969; Hill et al. 1971) are involved in CRC.

However some people have doubts if the change in microbiota in CRC patients is a result or a cause for the initiation of colon carcinogenesis. CRC itself, the symptoms of the disease, and its therapy have the potential to alter the GI microbial composition. Changes in the microbiome may precede or contribute to the development of factors related to cancer susceptibility (Lampe 2008). The results of Ohigashi and co-workers suggest that it is not the progression of CRC that causes changes in the GI environment but rather that cancer initiates and progresses in a GI environment that has changed (Ohigashi et al. 2013).

1.3.2. Microbiota modulation of gastrointestinal functions

The human GI microbiota is intrinsically linked with the organism health, since this endogenous microbiota form a symbiotic relationship with their eukaryotic host and this close partnership helps maintaining homeostasis by performing essential and non-redundant tasks (Zhu et al. 2011). This symbiotic relationship comprises a diverse communities of microbes, which include mutualists (symbiotically beneficial microbes), commensals (microbes that are neither harmful nor beneficial to the host), and pathogens (microbes that are detrimental to the host) (Aagaard et al. 2013).

The host has evolved to establish many processes that sustain unresponsiveness toward the commensal bacteria while at the same time maintaining responsiveness toward pathogens. These processes include the production of IgA and various antimicrobial peptides and epigenetic control of pro-inflammatory responses, all of which separate routes leading to excessive inflammatory response (Hattori and Taylor 2009). On the other hand, pathogens also have evolved to equip various virulence factors, including effectors that confer additional abilities for evading the host defense system, eventually inducing pro-inflammatory responses *via* change of the microbiota composition in favor of the pathogens (Raskin et al. 2006; Stecher et al. 2007). In contrast, commensal bacteria is evolved

in carbohydrate metabolism, energy production, cell maturation and proliferation toward intestinal homeostasis (Rawls et al. 2007).

Mutualist bacteria as *Propionibacterium freudenreichii* has a symbiotic beneficial relationship with host due to their end products of fermentation, which are essential mucosal nutrients including amino acids and SCFAs (O'Sullivan et al. 2005). Besides fermentation, the metabolic products of microflora includes vitamins K and B complex, secondary bile acid production, neutralization of dietary carcinogens such as nitrosamines, and conversion to active metabolites of some prodrugs (Cencic and Chingwaru 2010).

1.4. Nutraceuticals

The term "nutraceutical" was created from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice, MD, founder and chairman of the Foundation for Innovation in Medicine (FIM) (Cranford, NJ). According to DeFelice, nutraceutical can be defined as “a food (or a part of food) that provides medical or health benefits, including the prevention and or treatment of a disease” (Brower 1998). The food sources used as nutraceuticals are all natural and can be categorized as dietary fiber, probiotics, prebiotics, polyunsaturated fatty acids, antioxidant, vitamins, polyphenols or spices (Das et al. 2012).

According to a consensus definition, a probiotic is “a live microbial food ingredient that is beneficial to health” (Salminen et al. 1998) and a prebiotic is "a selectively fermented ingredient, or a fiber that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora, conferring benefits on the well-being and health of host " (de Vrese and Schrezenmeir 2008; Douglas and Sanders 2008).

Probiotics claimed benefits that include the improvement of lactose digestion, increased resistance to invasion by pathogenic bacteria in the gut, prevention of intestinal disturbances, treatment and prevention of antibiotic treatment and acute diarrhea, alleviation of irritable bowel syndrome and of inflammatory bowel disease, and possibly protection against cancer (Salminen et al. 1998; Kumar et al. 2010). In contrast, the specific effects of prebiotics on health are indirect (Cencic and Chingwaru 2010). However, probiotics and prebiotics share unique roles in human nutrition, largely centered on manipulation of populations or activities of the microbiota that colonize the human GI tract.

The regular consumption of probiotics or prebiotics has a positive impact in health that include enhanced immune function, improved colonic integrity, decreased incidence and duration of intestinal infections, down-regulated allergic response, and improved digestion and elimination (Douglas and Sanders 2008).

The Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) Expert Consultation develop guidelines, recommend criteria, and define the methodology for evaluation of probiotics, besides identifying which data are required to accurately substantiate health claims. The selection of probiotics must respect these standards that include safety, technological properties, survival to digestive stresses and at least one property that benefits human health (Sanders and Marco 2010).

Nutraceuticals can cover most of the therapeutic areas such as anti-arthritic, cold and cough, sleeping disorders, digestion and prevention of certain cancers, osteoporosis, blood pressure, cholesterol control, pain killers, depression and diabetes (See Figure 1.7) (Das et al. 2012).

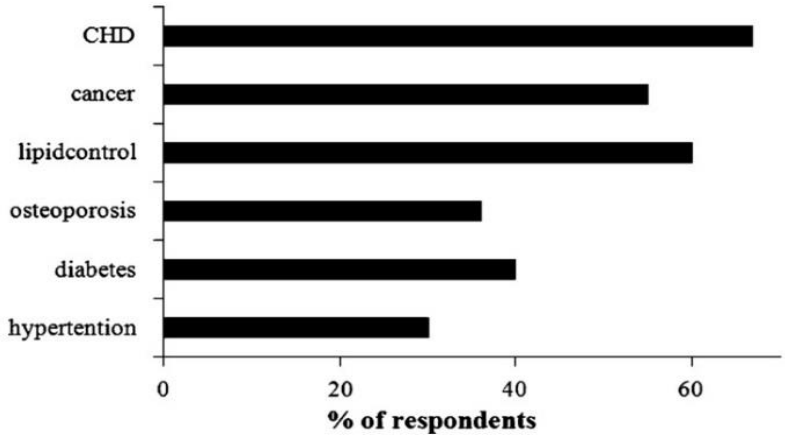


Figure 1.7. Therapeutic areas covered by nutraceutical products (Das et al. 2012).

1.4.1. Nutraceuticals role in colorectal cancer

Currently, there is a general consensus that probiotic, and particularly propionibacteria, play a role in the CRC development. This role has been experimentally demonstrated in the recent years.

Bassaganya-Riera and co-workers demonstrated the efficacy of VSL#3 (commercially available probiotic formulation) and conjugated linoleic acid (CLA) in suppressing colon carcinogenesis and also showed that CLA has a more pronounced anti-carcinogenic and anti-inflammatory activity than VSL#3 (Bassaganya-Riera et al. 2012).

Hakansson and co-workers demonstrated using a rat model that the consumption of blueberry husks and probiotics promotes a delay of colonic carcinogenesis and hepatic injuries (Hakansson et al. 2012). Others studies showed that propionibacteria possesses mechanisms of cancer prevention at a cellular level including the promotion of differentiation, induction of apoptosis and inhibition of proliferation of colon tumor cell lines but not on normal epithelial cells, via their metabolites, namely SCFAs (Jan et al. 2002; Marques et al. 2013; Bindels et al. 2012; Abrahamse et al. 1999; Emenaker et al. 2001).

Regarding tumor prevention, there are some nutraceuticals that have been well studied such as phytochemicals, including curcumin (turmeric), capsaicin (green chilies), epigallocatechin gallate (green tea), gingerol (ginger), genistein (soya beans), sulforaphane (cruciferous vegetables), tangeretin (citrus species), allicin (garlic), diallyl sulfide (garlic), anethole (fennel, camphor), and ß-carotene (Tripathi et al. 2005; Wargovich et al. 2010; Xavier et al. 2009; Ramos et al. 2013). Some of these nutraceuticals have been sold as food supplement, herbal products and processed foods that promise benefits health.

1.5. Propionibacteria as a probiotic

Various microorganisms have traditionally been employed for the manufacture of fermented milks and cheeses. Dairy propionibacteria have a long history of safe use in fermented food products and possess the Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) status (Thierry et al. 2011).

A number of publications have reviewed the probiotic properties of propionibacteria (Azcarate-Peril et al. 2011; Thierry et al. 2011; Salminen et al. 1998; Sanders and Marco 2010; Saikali et al. 2004). This health-beneficial properties is a result of the broad variety of functional metabolites which they produce, such as acetate, propionate, vitamins type B, CLA, trehalose, bifidogenic factors, among others (Cousin et al. 2012a; Dalmasso et al. 2011; Borowicki et al. 2011; Thierry et al. 2011; Ammar et al. 2013; Chen et al. 2012; Lan et al. 2007a). Although, many of the properties above-mentioned that have been reported for propionibacteria are highly strain-dependent (Cousin et al. 2012a; Lan et al. 2007a).

Dairy propionibacteria has low nutritional requirements and is able to survive and remain active in various environments, including cheese, but also in the GI tract (Herve et al. 2007). During the cheese manufacturing, *Propionibacterium* has to stand different successive stresses including

heating over 50 °C, acidification of the curd to pH 5.2, osmotic stress due the NaCl addition in the brining step, and low temperature (4 to 12 °C) during cheese ripening. Therefore, it also bears the acid- and bile-related stresses encountered in the digestive tract, which is a prerequisite for its use as probiotic (Lan et al. 2007a).

At present, the genus *Propionibacterium* is classified as Actinobacteria with a high G+C content, that make them more related to corynebacteria and mycobacteria than lactic acid bacteria. The current taxonomic position of propionibacteria is the following: Phylum *Actinobacteria*; Class *Actinobacteria*; Subclass *Actinobacteridae*; Order *Actinomycetales*; Suborder *Propionibacterineae*; Family *Propionibacteriaceae*; Genus *Propionibacterium*, which comprises 14 species such as *freudenreichii, acidipropionici, acnes* and others. In the more conventional and general way, propionibacteria are divided based on habitat of origin, in two main groups "*dairy* or *classical propionibacteria*" and "*cutaneous propionibacteria*" (Thierry et al. 2011; Zarate 2012).

Propionibacteria are Gram positive, catalase positive, high G+C%, non-spore forming and non-motile pleomorphic bacteria. In general, microorganisms of the genus Propionibacterium are anaerobic to slightly aerotolerant and morphologically heterogeneous including rod-shaped and filamentous branched cells that may occur singly, in pairs forming a V or a Y shape, or arranged in "Chinese characters" (Falentin et al. 2010; Thierry et al. 2011).

Propionibacteria has a peculiar metabolism characterized by the formation of acetate and propionate as the main fermentation end products, and also by several interconnected pathways that are used simultaneously (Poonam et al. 2012). This bacterium can metabolize a variety of substrates like carbohydrates, polyols (glycerol; erythritol; adonitol) and organic acids (lactic and gluconic acids) (Feng et al. 2011).

The production of acetate and propionate by these bacteria involves a complex metabolic cycle with several reactions in which substrates are metabolized to pyruvate via glycolysis, pentose phosphate or the Entner-Doudoroff pathways, generating ATP and reduced co-enzymes. Pyruvate is then oxidized to acetate and carbon dioxide or reduced to propionate (See Figure 1.8) (Zarate 2012). The latter transformation occurs via the Wood-Werkman cycle or transcarboxilase cycle which represents the key component of the central carbon metabolic pathway in propionibacteria (Crow 1987; Thierry et al. 2011). The Wood-Werkman cycle in propionibacteria is unique, function as a cyclic process, because the propionate production is coupled to oxidative phosphorylation and yields more ATP than in the other propionate-producing bacteria (Falentin et al. 2010).

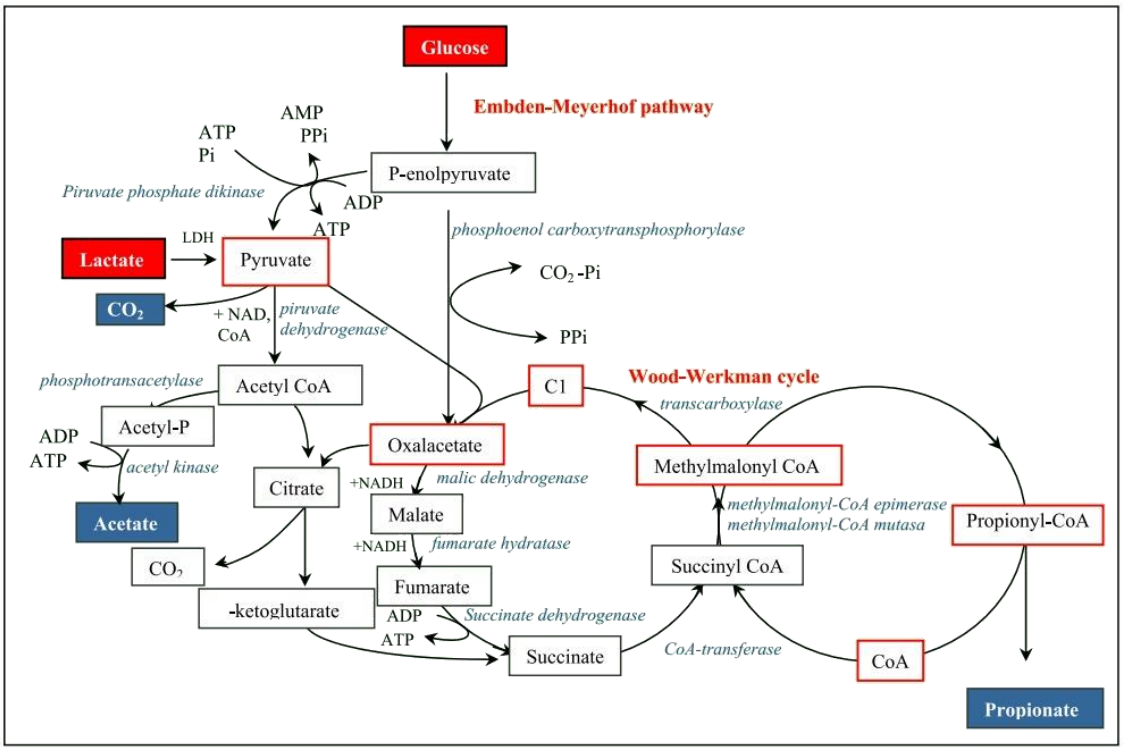


Figure 1.8. Schematic representation of main fermentation end products in propionibacteria (Zarate 2012).

Depending on the strains, the substrate used, and the environmental conditions, propionibacteria modulate the proportions of pyruvate either reduced to propionate, or oxidized to acetate and carbon dioxide, to maintain the redox balance (Thierry et al. 2011). For example, the disruption of acetate kinase gene in *P. acidipropionici* led to a 14% decrease of acetate production and a 13% increase of propionate yield using glucose as substrate (Suwannakham et al. 2006).

1.6. Short chain fatty acids

SCFAs are saturated aliphatic organic acids that consist of 1-6 carbons of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (95%) in the human gut (Cook and Sellin 1998). Acetate, propionate and butyrate are present in an approximate molar ration of 60:20:20 in the GI tract (Hijova and Chmelarova 2007; Binder 2010; Cummings et al. 1987). Depending on the diet, the total concentration of SCFAs decreases from 70-140 mM in the proximal colon to 20-70 Mm in the distal colon (Topping and Clifton 2001). In the cecum and colon 95% of the produced SCFAs are rapidly absorbed by colonocytes while the remaining 5% is secreted in feces (Topping and Clifton 2001; Ruppin et al. 1980; Hoverstad et al. 1982)

To the microbial community, SCFAs are a necessary waste product, required to balance redox equivalent production in the anaerobic environment of the gut. SCFAs also have many other activities including the regulation of host genes involved in maintenance of intestinal homeostasis (Comalada et al. 2006).

The amount and type of fiber consumed has dramatic effects on the composition of the gut microbiota and consequently on the type and amount of SCFAs produced (den Besten et al. 2013). The luminal pH in the colon is the result of the microbial SCFA production and the neutralizing capacity of bicarbonate, once most SCFAs are absorbed by the host in exchange for bicarbonate. As the concentration of SCFAs decline from proximal to the distal colon, the pH increases from cecum to rectum (Cummings et al. 1987; Annison et al. 2003; Ward and Coates 1987). This drop in pH from the ileum to the cecum is very important. Since the lower pH values change gut microbiota composition and it prevents overgrowth by pH-sensitive pathogenic bacteria (Duncan et al. 2009). Studies of human fecal microbial communities showed that at pH 5.5 the butyrate-producing *Firmicutes*-related bacteria comprised 20% of total population and when the luminal pH increases to 6.5 the butyrate-producing bacteria almost completely disappear and the acetate- and propionate-producing *Bacteroides*-related bacteria become dominant (Walker et al. 2005).

SCFAs produced by the microbiota in the human gut can be found in hepatic, portal and peripheral blood (Cummings et al. 1987; Murase et al. 1995). These SCFAs affect lipid, glucose and cholesterol metabolism in various tissues. These results indicate that SCFAs are transported from the intestinal lumen into the blood compartment of the host and are taken up by organs where they act as substrates or signal molecules (Gao et al. 2009; Fushimi et al. 2006; Demigne et al. 1995; Todesco et al. 1991)

It became apparent that SCFAs might play a key role in the prevention and treatment of the metabolic syndrome, bowel disorders and certain types of cancer (Donohoe et al. 2011; Blouin et al. 2011; Tang et al. 2011b). Some studies have demonstrated that an improvement of SCFA synthesis by colonic microbiota positively influenced the treatment of ulcerative colitis, Crohn's disease and antibiotic-associated diarrhea (Binder 2010; Cummings et al. 1987; Di Sabatino et al. 2005)

1.6.1. Short chain fatty acids antitumor activity

In the recent years, some authors have been demonstrated the antitumor activity of SCFAs. Butyrate in particular is the preferred energy source for the cells in the colonic mucosa and has been

demonstrated to induce apoptosis in CRC cells and its production is safe and without consequences for the normal epithelium growth (Fung et al. 2011). The mechanisms by which butyrate and others SCFAs regulate cell proliferation/differentiation and apoptosis are still unclear. In this sense, it has been described that butyrate is able to block cell proliferation, mainly in the G0-G1 and G2-M phases of cell cycle (Darzynkiewicz et al. 1981; Heerdt et al. 1997). This effect could be mediated by inhibition of the histone deacetylase activity (Fung et al. 2011), an increased cyclin D and $p21^{Waf1}$ expression (Hinnebusch et al. 2002; Hu et al. 2011) or a decreased expression of the proto-oncogenes *c-src* and *c-myc* (Foss et al. 1989; Souleimani and Asselin 1993). On the other hand, butyrate has also been described to reduce the levels of apoptosis inhibitors such as Bcl-2 and Bc-XL together with the upregulation of pro-apoptotic Bak and Bax, or the induction of caspase-3 protease activity in some tumor cell lines (Mandal et al. 2001). Butyrate and propionate can induce autophagy in cancer cells by inhibiting apoptosis, whereas inhibition of autophagy enhances the induction of apoptosis by SCFA (Donohoe et al. 2011; Tang et al. 2011a; Lee et al. 2012; Lee and Lee 2012).

Although the effect of acetate and propionate is less studied and their implications on the physiology of the colonocytes are not well documented, these are also known to regulate cell proliferation/differentiation and apoptosis (Lan et al. 2007b; Jan et al. 2002). Similarly to butyrate, propionate and acetate induce apoptosis through analogous events, including cell cycle arrest in the G2/M phase, mitochondrial depolarization, generation of reactive oxygen species, Bax translocation, caspase activation, chromatin condensation and nuclear degradation (Jan et al. 2002; Lan et al. 2008; Lan et al. 2007a; Lan et al. 2007b). Our group showed that in CRC-derived cell lines acetate *per se* inhibits proliferation and induces apoptosis, lysosomal membrane permeabilization (LMP) and cathepsin D (Cat-D) release from the lysosome (Marques et al. 2013). Our results led us to conclude that acetate induces LMP and subsequent release of Cat-D in CRC cells undergoing apoptosis, and suggest exploiting novel strategies using acetate as a prevention/therapeutic agent in CRC, through simultaneous treatment with Cat-D inhibitors.

Chapter 2.|

Rationale and aims

2.1. *Rationale* of the thesis project

Cancer is a public health problem worldwide with a high incidence and mortality, being a disease that compromises the normal function of colon. According to the most recent estimates by the International Agency for Research on Cancer, CRC in Portugal is the most commonly diagnosed cancer with 7129 new cases and it is the most common cause of cancer deaths with 3797 deaths reported in 2012 (Ferlay et al. 2013).

Emerging with this health problem are the new treatment approaches and the research of new solutions. Environmental factors, including diet and lifestyle, have a major impact in development of CRC. Several studies showed that a balanced diet enriched in fibers and a healthy lifestyle contributed for maintenance of a healthy body (Irigaray et al. 2007; Lichtenstein et al. 2000; Ohara et al. 2010). Probiotics appears in this context of healthy food with a new hope for maintenance of our body health and may prevent CRC.

Propionibacterium freudenreichii is well known to its long history of safe use in fermented food products (Thierry et al. 2011) and its probiotic properties. (Azcarate-Peril et al. 2011; Thierry et al. 2011; Salminen et al. 1998; Sanders and Marco 2010; Saikali et al. 2004). The propionibacteria health-beneficial properties is a result of the broad variety of its functional metabolites, such as acetate and propionate (Cousin et al. 2012a; Dalmasso et al. 2011; Borowicki et al. 2011; Thierry et al. 2011; Ammar et al. 2013; Chen et al. 2012; Lan et al. 2007a). SCFAs, namely acetate, propionate and butyrate, are naturally present in the human gut, and it is required to balance redox equivalent production in the anaerobic environment of the gut, in maintenance of intestinal homeostasis, as well affect lipid, glucose and cholesterol metabolism in various tissues.(Cencic and Chingwaru 2010; Das et al. 2012; Kumar et al. 2010).

Our group have recently demonstrated that acetate *per se* inhibits proliferation and induces apoptosis in CRC cells (Marques et al. 2013). Other studies also showed that acetate, propionate and butyrate induce apoptosis in cancer cells (Hague et al. 1995; Jan et al. 2002; Lan et al. 2007b) and its can also induce autophagy in cancer cells to dampen apoptosis (Donohoe et al. 2011; Tang et al. 2011a; Lee et al. 2012; Lee and Lee 2012).

Developing a strategy to maintain SCFA in the gut at levels that will protect normal colon mucosa cells and kill colorectal cancer cells is of major relevance for CRC prevention and/or therapy.

Inducing an enhanced SCFA production by propionibacteria in human colon environment could be of great impact as potential prevention and therapeutic agent against CRC.

2.2. Aims

The main aim of the current project was to develop an improved *P. freudenreichii* for potential use as a nutraceutical towards the prevention/treatment of colorectal cancer. This project was organized in three main approaches, with specific aims in order to achieve the proposed aim:

- *Propionibacterium freudenreichii* development and improvement

  Our specific aims were:

  - Optimization of the culture condition for the growth of *P. freudenreichii*;
  - Characterization of growth and biotransformation performance by *P. freudenreichii* in different media with different carbon source;
  - Development of an adapted *P. freudenreichii* to digestive stress;
  - Assessment of the toxic concentrations of acetate and propionate to *P. freudenreichii* cultures
  - Development of a robust *P. freudenreichii,* tolerant to digestive stress and to toxic concentrations of acetate and propionate.

- Effect of *Propionibacterium freudenreichii* conditioned medium in colorectal cancer cells

  Our specific aim was the evaluation of the fermentation broth medium produced by wild type and mutant *P. freudenreichii* on colorectal cancer cells survival *in vitro*.

- Effect of colorectal cancer cells conditioned medium in *Propionibacterium freudenreichii*

  Our specific aim was the characterization of growth and biotransformation performance of wild type and mutant *P. freudenreichii* in growth conditioned culture medium from colorectal cancer cells.

2.3. Thesis project outline

The work developed in this thesis can be divided in two major subjects; *Propionibacterium freudenreichii* and colorectal cancer derived cell lines.

During this thesis we evaluate the effect of *P. freudenreichii* on CRC cells as well the effect that CRC cells exert on *P. freudenreichii*, as represented in Figure 2.1.

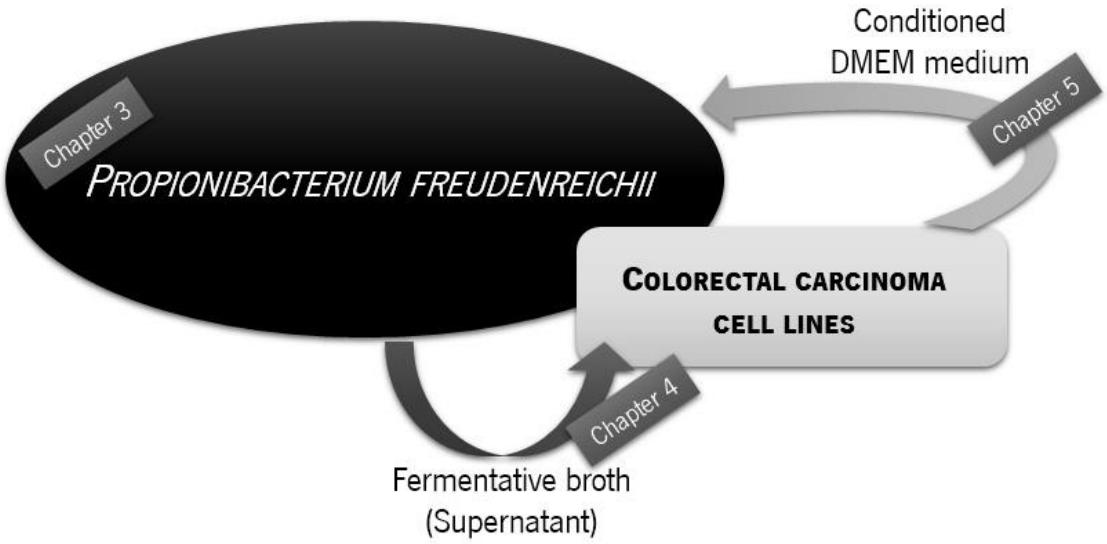


Figure 2.1. Thesis project outline

Briefly, in chapter 3 we will present the characterization and optimization of growth and biotransformation performance by *P. freudenreichii* in different media and culture condition as well as the evaluation of toxic concentrations of acetate and propionate. We will also show the digestive stress challenge and random mutagenesis of *P. freudenreichii* DSM 20271.

In chapter 4 we will present the effect of the fermentation broth produced by wild type and adapted *P. freudenreichii* on the CRC cells survival.

In chapter 5 we will present the characterization of growth and biotransformation performance by wild type and adapted P. freudenreichii in conditioned cell culture medium from CRC cells.

Chapter 3. | Strategies to improve acetate and propionate production by *Propionibacterium* and enhance resistance to digestive stress

3.1. Background

Propionibacterium freudenreichii subsp. *freudenreichii* DSM 20271 belongs to the genus of *Propionibacterium* routinely designed by dairy propionibacteria. It plays a very important role in human health and nutrition, being involved in a number of probiotic effects, such as increased levels of fecal bifidobacteria in humans (Bougle et al. 1999; Isawa et al. 2002), inhibition of undesirable flora (Lyon et al. 1993), beneficial modification of enzymatic activities within the gut (Saijo et al. 1978) and treatment of lactose intolerance (Zarate et al. 2000). More recently, *P. freudenreichii* was shown to induce *in vitro* cell death of human CRC by apoptosis (Jan et al. 2002).

Propionibacteria are an important class of organisms originally used for the production of Swiss cheese varieties and certain fermented foods. Fermented dairy products constitute 90% of the products in the probiotic market and a growing part in the functional food market (Cousin et al. 2012b). During the cheese making process, *P. freudenreichii* resists the harsh physical and chemical stresses, including heat and salt stresses (Lan et al. 2007a). Therefore, propionibacteria resistance to the digestive stresses is essential for their survival in the gut and also for exerting their beneficial effects. As such, Emmental cheese and yoghurt supplemented with dairy propionibacteria comprise efficient vectors to deliver live and metabolically active *Propionibacterium* stains to the human gut. Several authors have demonstrated that dairy propionibacteria can survive to the exposure to artificial gastric and intestinal fluids (Lan et al. 2007a; Jan et al. 2001; Leverrier et al. 2003; Cousin et al. 2012b). Additionally, Bougle and co-workers (1999) used *in vivo* models to demonstrate that some dairy propionibacteria can survive the passage through the digestive tract.

The industrial relevance of propionibacteria in the production of starters for the Swiss-type cheese manufacturing, of probiotics, vitamin B_{12} and SCFAs led to an increased number of reports on their capabilities. Additionally, new functions and potential applications of the dairy propionibacteria recently reported make them highly versatile organisms and easy to manipulate.

P. freudenreichii produces propionic acid, as well as acetic and succinic acids as the main by-products. Many studies reported the production of SCFAs by this bacterium from a variety of substrates (Cousin et al. 2012b; Poonam et al. 2012; Wang and Yang 2013; Zhu et al. 2012; Liu et al. 2012). Several carbon sources as glucose, fructose, maltose, sucrose, molasses, xylose, lactate, whey lactose and glycerol have been used for the production of SCFAs (Liu et al. 2012). All dairy

propionibacteria are able to metabolize lactose, except *P. freudenreichii* subsp. *freudenreichii* (Piveteau 1999).

Theoretically, 2 moles of glucose can yield 3 moles of propionate, 1 mole of acetate, 1 mole of carbon dioxide, and 1 mole of water. Three moles of lactate can be converted to 2 moles of propionate, 1 mole of acetate, 1 mole of carbon dioxide, and 1 mole of water; while 1 mole of glycerol can generate 1 mole of propionate and 1 mole of water (Wood 1981). Glycerol has been described as an ideal carbon source for *Propionibacterium* fermentation, providing a higher propionate yield as compared to glucose or lactic acid (Wang and Yang 2013; Himmi et al. 2000; Coral et al. 2008).

The microbial production process is self-inhibitory as the SCFAs produced can cause acid injury to bacteria and a high accumulation of by-products interferes with the downstream processes (Woskow and Glatz 1991). Attempts have been made to eliminate or reduce the end product inhibition through the development of propionic acid tolerant stains (Woskow and Glatz 1991) and by using extractive fermentation (Jin and Yang 1998).

Promising results have been reported on the use of chemical random mutagenesis as a possible approach to develop tolerant strains. Hermann and co-workers (1985) isolated a mutant strain of *C. acetobutylicum* by random mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Hermann et al. 1985). The conventional random mutagenesis process consists in exposing a microbial suspension to a selected mutagen. N-nitroso-N-ethylurea, also known as N-ethyl-N-nitrosourea or Ethylnitrosourea (ENU), is a potent monofunctional-ethylating agent that has been found to be mutagenic in a wide variety of mutagenicity test systems from viruses to mammalian germ cells. ENU possesses the dual action of ethylation and carbamoylation (Shibuya and Morimoto 1993). This mutagenic agent has been used in some studies with bacteria, being a good tool (Ohta et al. 2000; Richardson et al. 1987).

In this chapter, the optimization and characterization of *P. freudenreichii* DSM 20271 are performed in different culture conditions. Moreover, the SCFAs toxic concentrations are determined. Digestive stress challenge and random mutagenesis of *P. freudenreichii* DSM 20271 in solid medium using ENU are studied. Adapted or mutant bacteria are evaluated regarding their growth and biotransformation performance as compared to the parental strain in liquid medium.

3.2. Materials and methods

3.2.1. Culture media

P. freudenreichii strain was cultivated in anaerobic conditions. Different culture media were used in this work. The composition of yeast extract-lactate (YEL) medium was modified (Malik et al. 1968), as well as the "mimic the content of the human colon" (MCHC) medium (Gibson and Wang 1994) by the addition of sodium lactate. The basal medium (BM) was optimized from the YEL medium. The different compositions of these media are presented in Tables 3.1 to 3.3.

Table 3.1. Composition of YEL and BM media

Compound	YEL Medium	BM Medium
Resazurine	0.5 mg L^{-1}	0.5 mg L^{-1}
KH_2PO_4	0.5 g L^{-1}	0.5 g L^{-1}
$Na_2HPO_4.H_2O$	0.5 g L^{-1}	0.5 g L^{-1}
Trace elements H^+ solution	1 mL L^{-1}	1 mL L^{-1}
Trace elements OH^- solution	1 mL L^{-1}	1 mL L^{-1}
Salts + vitamins solution	50 mL L^{-1}	50 mL L^{-1}
Bicarbonate solution	50 mL L^{-1}	50 mL L^{-1}
Tryptone	10 g L^{-1}	—
Yeast extract	5 g L^{-1}	1 g L^{-1}
Sodium lactate	8.4 g L^{-1}	8.4 g L^{-1}

Table 3.2. Stock solution used in YEL and BM media

Trace elements H^+		Trace elements OH^-		Salts + vitamins		Bicarbonate	
HCl	1.8 g L^{-1}	NaOH	0.4 g L^{-1}	NH_4Cl	24 g L^{-1}	$NaHCO_3$	80 g L^{-1}
H_3BO_3	61.8 mg L^{-1}	Na_2SeO_3	17.3 mg L^{-1}	NaCl	24 g L^{-1}	$Na_2S.9H_2O$	
$MnCl_2$	61.3 mg L^{-1}	Na_2WO_4	29.4 mg L^{-1}	$MgCl_2.6H_2O$	8 g L^{-1}	5 g L^{-1}	
$FeCl_2$	1 g L^{-1}	Na_2MoO_4	20.5 mg L^{-1}	$CaCl_2.2H_2O$	8.8 g L^{-1}		
$CoCl_2$	64.5 mg L^{-1}			Biotin	20 mg L^{-1}		
$NiCl_2$	12.9 mg L^{-1}			Nicotinamid	0.2 g L^{-1}		
$ZnCl_2$	67.7 mg L^{-1}			p-Aminobenzoic acid	0.1 g L^{-1}		
				Thiamin	0.2 g L^{-1}		
				Panthotenic acid	0.1 g L^{-1}		
				Pyridoxamine	0.5 g L^{-1}		
				Cyanocobalamine	0.1 g L^{-1}		
				Riboflavin	0.1 g L^{-1}		
				Folate	50 mg L^{-1}		
				Lipoate	50 mg L^{-1}		

Table 3.3. Composition of MCHC Medium

Compound	MCHC Medium
Pectin	0.5 g L^{-1}
Xylan	0.5 g L^{-1}
Mucin	0.5 g L^{-1}
Starch	0.5 g L^{-1}
Peptone	0.5 g L^{-1}
Tryptone	2.5 g L^{-1}
Yeast extract	0.5 g L^{-1}
Bile salts	0.05 g L^{-1}
K_2HPO_4	2 g L^{-1}
$NaHCO_3$	0.2 g L^{-1}
NaCl	4.5 g L^{-1}
$MgSO_4.7H_2O$	0.5 g L^{-1}
$CaCl_2.2H_2O$	0.45 g L^{-1}
$MnCl_2.2H_2O$	0.2 g L^{-1}
Haemin	0.05 g L^{-1}
$FeSO_4.7H_2O$	5 mg L^{-1}
$CoCl_2.6H_2O$	0.05 g L^{-1}
Tween 80	2 mL L^{-1}
Sodium lactate	2.3 g L^{-1}
Thiamin HCl	4 µg L^{-1}
Calcium pantothenate	10 µg L^{-1}
Nicorinic acid	5 µg L^{-1}
4-Aminobenzoic acid	5 µg L^{-1}
Biotin	2 µg L^{-1}
Vitamin B_{12}	0.5 µg L^{-1}
Cysteine	0.8 g L^{-1}

Despite the different compositions of the culture media, these were prepared in a similar way. Briefly, the liquid medium was boiled and cooled down on an ice bath while nitrogen was passed through the liquid. Afterwards, the pH was adjusted whenever necessary and the culture medium was distributed (50 ml/bottle) in serum bottles of 125 mL that were then sealed with butyl rubber stoppers and aluminum crimps. Oxygen was removed from the headspace and the bottles were pressurized with a N_2-CO_2 gas mixture (80% - 20%) using a manifold. After autoclaving (121°C for 20 min), the serum bottles containing the medium were kept at room temperature. Before these media were used, several sterile solutions such as salts with vitamins solution, bicarbonate solution and sodium lactate solution.

YEL; BM and MCHC solid media were prepared in the same way as the liquid media. Briefly, liquid medium was prepared with the corresponding compounds depending on the medium envisaged; then it was boiled and cooled down on an ice bath while nitrogen was passed through the liquid. Afterwards, the medium was distributed in Erlenmeyer flasks and 1.5% of agar (BD, Agar,

Spain) was added. The pH was adjusted whenever necessary before autoclaving the media for 20 min. The culture media were left to cool down and all the other solutions as salts with vitamins solution, bicarbonate solution and sodium lactate solution were aseptically added before it became solid. The medium was dispensed aseptically in plates to become solid. As this process occurs in aerobic conditions, the plates have to be placed inside a vinyl anaerobic chamber (Coy Laboratory Products, Type C Vinyl Anaerobic Chamber, USA) at least for 10 hours before use to ensure the absence of oxygen.

The Dulbecco's Modified Eagle's Medium (DMEM; Biowest, DMEM High Glucose w/ Stable Glutamine w/Sodium Pyruvate, France) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Biowest, Fetal Bovine Serum Premium - South American Origin, France) was also used to cultivate the bacterial strain. In this culture medium, serum bottles of 70 mL were used. These serum bottles were washed in distilled water and some droplets were left inside the bottles. Next, the bottles were sealed with butyl rubber stoppers and aluminum crimps. Before autoclaving (121°C for 20 min), the total headspace was pressurized with a N_2 gas (100%) to remove the oxygen and subsequently the bottles were depressurized to atmospheric pressure. DMEM medium was then distributed, under the flame, into these empty serum bottles containing N_2. Furthermore, FBS was added to these bottles to a total volume up to 30 mL.

3.2.1.1. Stock solutions of carbon source

The carbon sources used were glucose (HIMEDIA, D-(+)-Glucose anhydrous, India) and glycerol (HIMEDIA, Glycerol, A.R., India). These stock solutions were prepared in 160 mL serum bottles with a final volume of 100 mL and a concentration of 113 g L-1. The bottles were sealed with butyl rubber stoppers and aluminum crimps and the headspace was pressurized with a N2 gas (100%) to remove the oxygen. Then, the stock solutions were autoclaved (121°C for 20 min). The BM medium was only supplemented with the corresponding carbon source before inoculation of P. freudenreichii.

3.2.2. Strain maintenance and reactivation

P. freudenreichii subsp. freudenreichii DSM 20271 was purchased from DSMZ (German collection of microorganisms and cell cultures). Freeze dried bacteria were reactivated and routinely cultivated on YEL medium.

The freeze dried pellet was re-suspended in reduced medium and the mixture was then transferred to a serum bottle containing the culture medium prepared as described above. The bacteria were incubated at 30°C, 99 rpm (Eppendorf, Excella® E24 incubator shake, Spain) until growth can be observed.

Stock cultures were prepared from this first culture and incubated at 37°C without agitation. After growth (3 days), the culture was kept at room temperature. In order to keep the culture active, these cells were transferred to fresh YEL medium each 1-2 months. Cells were also maintained as glycerol (25 – 30% v/v) stocks at -80°C, which were prepared after growing bacteria to an OD_{650nm} of 0.8 to 1.0.

3.2.3. Analytical methods

3.2.3.1. Bacterial Enumeration

Bacteria growth was monitored by measuring the absorbance of cell suspensions at 650 nm wavelength in a spectrophotometer (Hach, DR 2800 Spectrophotometer, Germany) using a plastic cuvette (Frilabo, Mico cuvettes of 1.5 mL, Portugal). To determine the dry cell weight (DCW), the bacteria were grown in different culture media to an $OD_{650\ nm}$ of approximately 1.0, i.e. in the early stationary phase. For each culture media, serial dilutions with distilled water were prepared to establish the calibration curve. The absorbance of each sample was read and the samples were filtered (GE Healthcare Life Science, Whatman™, Membrane Fiters, 0.2 µm, 47 mm, German) and further dried at 104° C for 24h. The calibration curves obtained for the different media were: $[(x - 0.1038)^{-1.3001}]$ g L^{-1} DCW ($x > 0$) for the YEL medium; $[(x + 0.0282)^{-1.7288}]$ g L^{-1} DCW ($x > 0$) for the BM medium; $[(x - 0.0209)^{-1.2376}]$ g L^{-1} DCW ($x > 0$) for the MCHC medium ; and $[(x - 0.1170)^{-0.8825}]$ g L^{-1} DCW ($x > 0$) for the DMEM medium. The "x" in all calibration curves represents the $OD_{650\ nm}$.

3.2.3.2. Short-chain Fatty Acids Quantification

SCFAs were measured through high performance liquid chromatography (HPLC) (Varian, Metacarb 67H column) equipped with UV detector (Jasco, UV-2070, Spain). The column was eluted isocratically with H_2SO_4 0.01N using a flow rate of 0.6 ml min^{-1} and detection was conducted at 210 nm. Standard solutions of formic acid, acetic acid, propionic acid, iso-butyric acid, n-butyric acid, valeric acid, hexanoic acid of known concentrations were used to establish the calibration curve of SCFAs, using the crotonic acid as an internal standard. The equations used to determine the SCFAs concentrations present in our samples are shown in Table 3.4.

Table 3.4. Equations for quantifications of SCFAs

	Average RT (min)	AA/AIS = a[A]/[IS] + b				R^2
Formic acid	10.444	AA/AIS =	0.0059	* [A]/[IS] +	-0.0055	0.9991
Acetic Acid	11.356	AA/AIS =	0.0034	* [A]/[IS] +	-0.0027	0.9988
Propionic Acid	13.301	AA/AIS =	0.0032	* [A]/[IS] +	-0.0027	0.9991
iso-Butyric Acid	15.029	AA/AIS =	0.0044	* [A]/[IS] +	-0.0021	0.9993
n-Butyric Acid	16.160	AA/AIS =	0.0033	* [A]/[IS] +	-0.0026	0.9992
Valeric Acid	22.028	AA/AIS =	0.0030	* [A]/[IS] +	-0.0013	0.9991
Hexanoic Acid	32.030	AA/AIS =	0.0027	* [A]/[IS] +	-0.0029	0.9988

RT, Retention Time; AA, Area Acid: AIS, Area Internal Standard; [A], Concentration of Acid; [IS], Concentration of Internal Standard; R, Correlation Coefficient.

Samples were centrifuged at 21130 g for 5 min (Eppendorf, Centrifuge 5424R, Spain), filtered with 0.2 µm filters (GE Healthcare Life Science, Whatman™, Spartan 12/0.2 RC, Germany) and added (volume = 0.8 mL) to a screw cap vial (Labbox, Srew vial for chromatography, Spain) containing 0.2 mL of internal standard.

3.2.4. Monitoring bacterial growth and SCFAs production

All the experiments regarding the evaluation of bacterial growth and SCFAs production were performed as described schematically in Figure 3.1. The first pre-inoculum was inoculated approximately 96 hours before T0 (initial time) and transferred to the second pre-inoculum after 64 hours. The second pre-inoculum was used with approximately 32 hours of growth, i.e. at the end of the exponential phase so it could be inoculated in different culture conditions. All experiments were run in triplicate, except for the ones using DMEM medium that were conducted in duplicate. Samples were taken at pre-defined time points (0, 16, 24, 40, 48, 64, and 72h) under aseptic conditions using a syringe to ensure the absence of oxygen inside the bottles. Optical density was measured and the samples were used to analyze the SCFAs production by HPLC. At the end of the experiment the fermentation broth was recovered to 50 ml falcons (Orange scientific, 50 mL Tube conical, Belgium), centrifuged at 10 000g for 20 min (HERAEUS, Megafuge 1.0R, Germany), filtered under aseptic conditions with sterile filters (0.2 µm) and were kept at -20° C until further use.

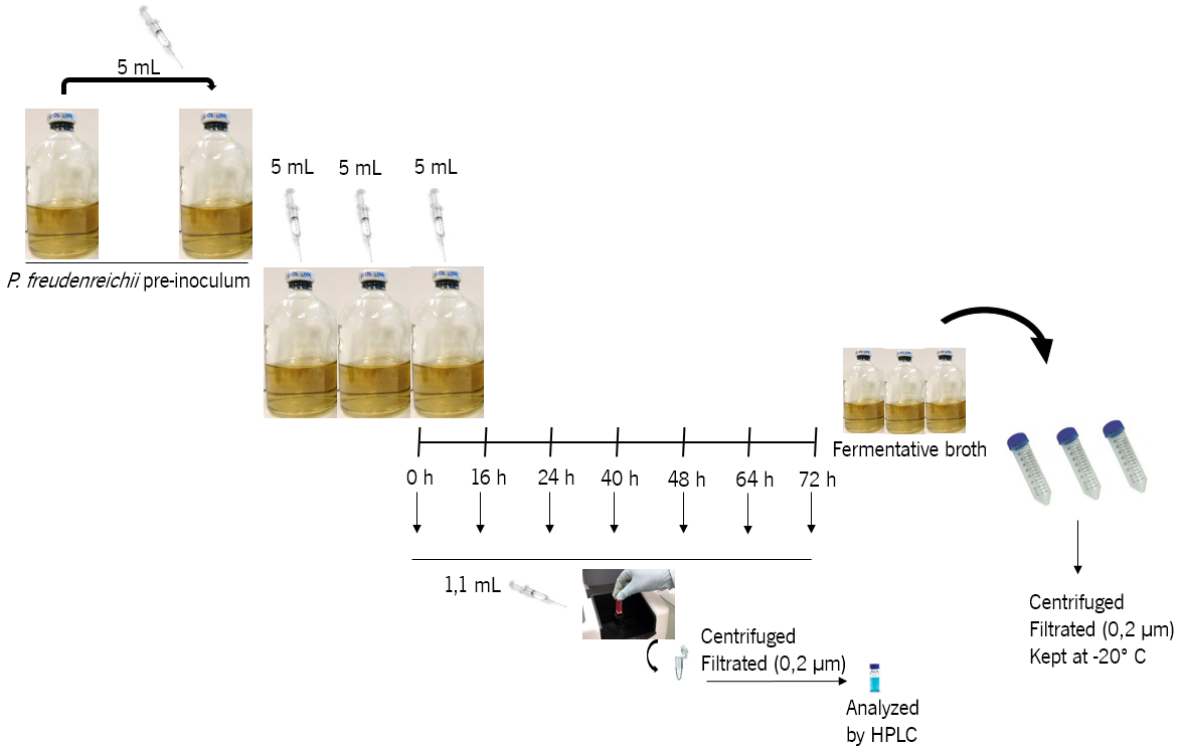


Figure 3.1. Monitoring of growth and SCFAs production by *P. freudenreichii*.

3.2.5. Digestive Stress Challenge

P. freudenreichii was subjected to acid and bile salts challenges as described by Lan and co-workers (2007) with some modifications. The conditions used in this protocol are explained in Table 3.5, as well the controls used to monitor the experiment. All experiments were conducted in triplicate. Moreover, the protocol was performed under anaerobic conditions using a vinyl anaerobic chamber without sterile condition. Thus, the chamber was cleaned with ethanol 70% before use to prevent contaminations.

Table 3.5. Conditions used to perform the digestive stress challenges

Conditions	Explanation
Negative Control A	Bacteria that were not subject to any stress;
Negative Control B	Bacteria that were subjected to all steps of the digestive stress challenge, using normal MCHC medium;
Acid Stress Control	Bacteria subjected to the acid stress steps (pH 5 and 2);
Bile Stress Control	Bacteria subjected to the bile stress steps (pH 5 and 2);
Digestive Stress Challenge	Bacteria subjected to all the steps of the protocol

The serum bottles containing MCHC medium were prepared as described above but in some cases the pH was adjusted to pH 5 and pH 2 using HCl 5M. In the case of the MCHC medium containing 0.1 % (w/v) dried ox bile, the concentration of bile salts was adjusted after supplementation using a stock solution of bile salts [50 g L^{-1}].

Early stationary-phase cultures were obtained as previously described and were diluted 10-fold in a pre-warmed (37° C) acidified (pH 5) MCHC medium. *P. freudenreichii* cells were acid-challenged at 37° C for 1 h before centrifugation (10 min; 10 000 g) and were then re-suspended in a pre-warmed (37° C) peptone water containing NaCl, in order to stabilize the bacteria. These bacterial suspensions were then diluted 5-fold in a pre-warmed acidified (pH 2) MCHC medium and challenged at 37° C for 30 min. In order to stop the acid stress, the cultures were subsequently centrifuged (10 min; 10 000g) and re-suspended in a pre-warmed peptone water containing NaCl. Then, the bile challenge was performed, bacterial suspensions were diluted 5-fold in a pre-warmed MCHC medium containing 0.1 % (w/v) dried ox bile and challenged at 37° C for 2 h. The bile challenge was stopped by centrifugation (10 min; 10 000g) and dilution on a peptone water containing NaCl.

All bacteria were seeded on solidified MCHC and DSMZ media, and also inoculated in liquid MCHC and YEL media to recover a robust culture adapted to the digestive stress. Bacterial plates were placed in an anaerobic box (BIOMÉRIEUX, GENbox Jar 7.0L, USA) and were incubated at 37° C without agitation. Also, the other cultures were left under these conditions until growth can be observed (approximately 4 days for liquid media and 14 days for solid media).

After growth could be observed, the cultures were transferred to fresh liquid YEL and MCHC media to reactivate its metabolism. Cultures obtained were then analyzed regarding their growth and SCFAs production in YEL and BM media. The culture with the best performance (growth and production) was then grown in DMEM medium and further evaluated regarding growth and SCFAs production.

3.2.6. Toxicity assay of Acetate and Propionate

P. freudenreichii adapted to the digestive stress (section 3.2.4) was used to further study the concentrations of acetate and propionate that are toxic for the bacteria. Under normal conditions, *P. freudenreichii* produced approximately 1 g L^{-1} of acetate and 3 g L^{-1} of propionate in YEL medium, therefore the concentrations of acetate and propionate used in this task were : 1 g L^{-1} acetate and 3 g L^{-1} propionate; 2 g L^{-1} acetate and 6 g L^{-1} propionate (2x the normal amounts produced); 4 g L^{-1}

acetate and 12 g L^{-1} propionate (3x the normal amounts produced); 8 g L^{-1} acetate and 24 g L^{-1} propionate (4x the normal amounts produced).

Two controls were included to monitor the experiment, namely a negative control (YEL medium) and a positive control (YEL medium with 10 g L^{-1} chloramine). The toxicity of acetate and propionate were also evaluated independently with the following concentrations: 1, 2, 4 and 8 g L^{-1} of acetate; and 3, 6, 12 and 24 g L^{-1} of propionate.

Before inoculation with *P. freudenreichii*, the serum bottles containing sterile YEL medium were supplemented with different concentrations of acetate and propionate depending the condition to be tested. The cultures were incubated at 37° C without agitation and bacterial growth and SCFAs production were evaluated.

3.2.7. Random Mutagenesis

Different conditions were used to obtain a robust *P. freudenreichii*. In order to recover bacteria resistant to the digestive stress the following conditions were used: 1 g L^{-1} bile salts, pH 2; 0.5 g L^{-1} bile salts, pH 4; and 0.5 g L^{-1} bile salts, pH 5. Moreover, the following conditions were tested to select bacteria presenting resistance to toxic SCFAs concentrations: 3 g L^{-1} acetate; 7g L^{-1} propionate; 2 g L^{-1} acetate and 6 g L^{-1} propionate; 3 g L^{-1} acetate and 7g L^{-1} propionate. Bacteria were also seed in a normal MCHC medium as control.

The MCHC medium was prepared as described in section 3.2.1 to ensure the absence of dissolved oxygen. The medium was dispensed in four individual 250 mL Erlenmeyer flasks and agar was added to each flask up to a final concentration of 20 g L^{-1} before autoclaving during 20 min. The culture media were let to cool down and afterwards acetate or/and propionate were aseptically added before the media became solid. In the case of the digestive stress conditions, the concentration of bile salts was adjusted when the experiment was prepared. The medium was plated as previously described. After solidification, 200 µL of a solution of MCHC medium acidified was added on the top, spread and let dry for 15 min. In order to obtain a given pH “x” on the plate, the acidified solution used had a pH “x”-1.

Four disposable Petri dishes were prepared for each condition above mentioned. The plates were placed inside a vinyl anaerobic chamber overnight before the mutagenesis experiment was conducted to ensure the absence of oxygen.

Random mutagenesis in *P. freudenreichii* was conducted as described by Hermann and co-workers (1985). An exponential-phase culture of *P. freudenreichii* adapted to digestive stress was

used to seed 200 µL and spread out on each plate. Two plates without mutagen were used as control for each condition, while the other two plates of each condition were used for the mutagenesis experiment. 15 min after seeding, a crystal of ENU was placed at the center of each plate. The plates were then incubated at 37° C inside the anaerobic box until growth was observed.

3.2.8. Statistical analysis

Results correspond to the mean ± standard error of the mean (SEM) at least three independent experiments. The data were analyzed using an analysis of variance (one-way ANOVA) followed by Tukey's Test to compare all to all experiments or Dunnett's test to assess the differences between control and different conditions tested. The confidence interval used was 95% and p-values ≤ 0.05 were regarded as statistically significant. All statistical tests were performed using the software Graphpad Prism 5.

3.3. Results and Discussion

P. freudenreichii is well-known for its capabilities to produce acetate and propionate (SCFA), which have been associated with the bacterium potential probiotic effect. Therefore, the goal of the current work was to optimize the production of those SCFA's (see Figure 3.2) and simultaneously improve the bacterium tolerance to higher concentrations of those fatty acids. Several culture media were evaluated in order to optimize the bacterium growth and production of acetate and propionate. Furthermore, the bacterium tolerance to the digestive stress and high concentrations of SCFA's was also studied.

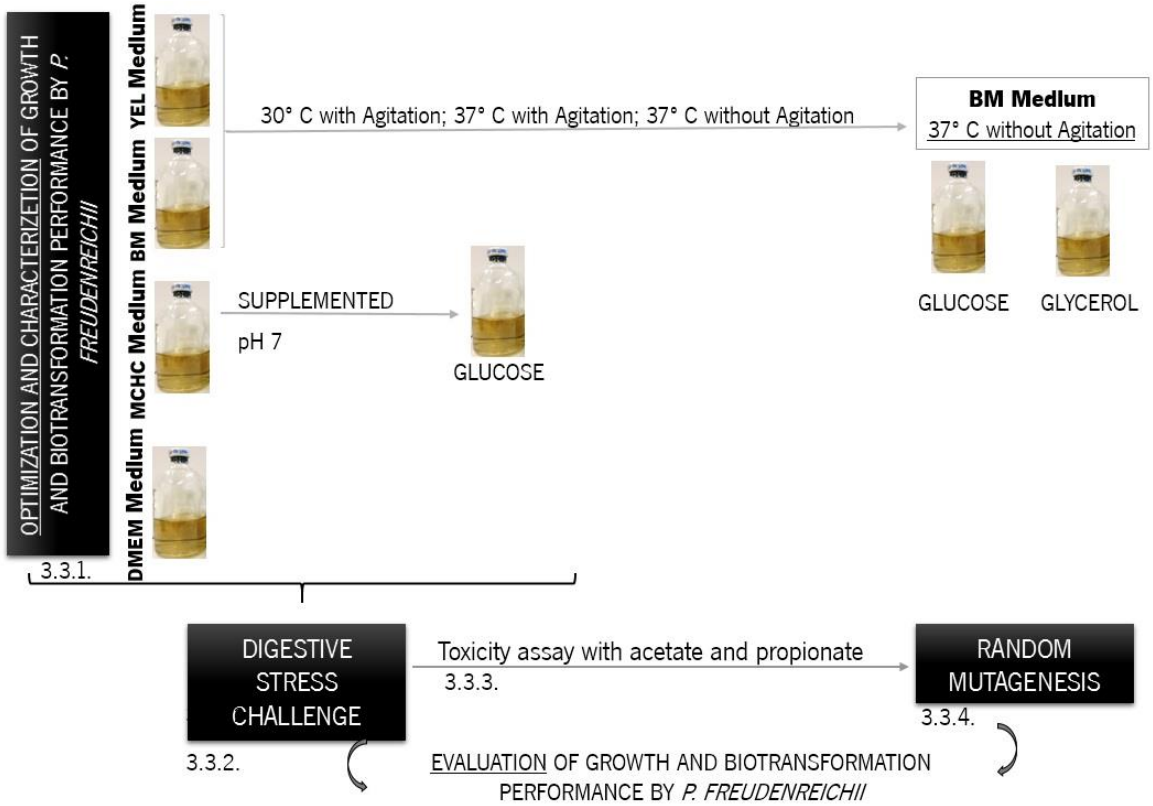


Figure 3.2. Assays performed to optimize and characterize *P. freudenreichii* and to study digestive stress challenge as well as random mutagenesis.

3.3.1. Optimization and Characterization of bacterial growth and SCFAs production in different media

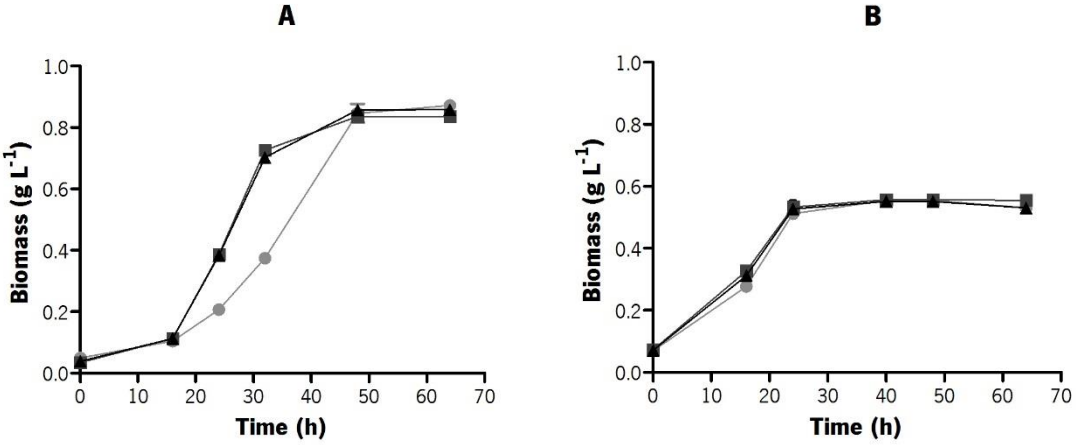
3.3.1.1. Yeast Extract-Lactate medium (YEL) / Basal Medium (BM)

P. freudenreichii has been routinely cultivated in YEL medium (Malik et al. 1968) and several studies showed its ability to grow in different operational conditions (e.g. temperature, agitation) (Lan et al. 2007a; Coral et al. 2008; Himmi et al. 2000; da Costa et al. 1999), thus our approach was to

test different temperatures and agitations, as well as to improve the YEL medium towards our goal (production of acetate and propionate) by changing its composition.

In order to develop a medium containing minimal amounts of the expensive compounds tryptone and yeast extract, the YEL medium was modified, namely tryptone was removed and different concentrations of yeast extract (5, 2.5, 1 , and 0 g L^{-1}) were evaluated (data not shown). Whenever growth and SCFA production could be obtained using simplest media, we used the herein designated Basal Medium (BM) that has no tryptone and contains 1 g L^{-1} of yeast extract.

Our first approach was to evaluate the differences between YEL and BM media (based on the YEL medium composition but simpler), as well as to examine different culture conditions (temperature and agitation). Figure. 3.3 A/B shows a typical fermentation pattern using YEL and BM media, respectively, under different culture conditions (30° C with agitation; 37° C with agitation and 37° C without agitation). Through the analysis of the growth and SCFAs production curves, apparently the three different conditions studied do not have a distinct effects on the growth and biotransformation performance, except for the case in which 30° C with agitation was used. Biomass production approximately reached 0.86 ± 0.01 g L^{-1} and 0.54 ± 0.01 g L^{-1} in YEL and BM media, respectively. Fermentation in both media was not very different. Acetate production was found to be around 1.58 ± 0.03 g L^{-1} in YEL medium and 1.69 ± 0.03 g L^{-1} in BM medium, while the propionate production was around 4.6 ± 0.04 g L^{-1} in YEL medium and 4.6 ± 0.07 g L^{-1} in BM medium.



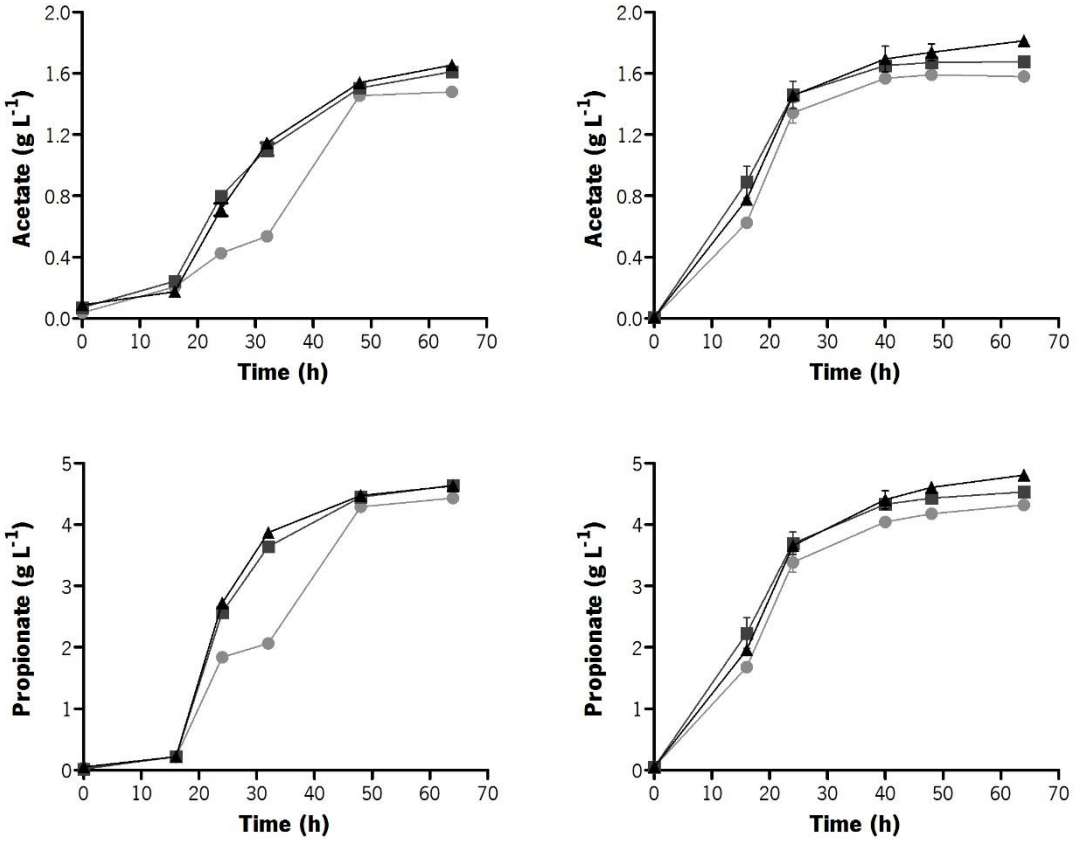


Figure 3.3. Biomass, acetate and propionate production by *P. freudenreichii* in YEL medium (A) and BM medium (B) at pH 7, 30 °C with agitation (—●—), 37 °C with agitation (—■—) and 37 °C without agitation (—▲—). Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate and the mean ± SEM are represented.

In order to choose among the studied media and culture conditions, the pair that results in the best performance of the bacterium (i.e. growth and SCFA production), several parameters were determined, including the growth rate, yield and productivity (Figure 3.4). The different temperatures and agitations studied did not affect the growth and SCFAs production by *P. freudenreichii* in YEL medium, since no significant statistical differences were observed in growth rate, yield and productivity. Nevertheless, in the BM medium slight differences could be observed, between 30° C with agitation and 37° C without agitation, in the acetate yield ($p<0.05$), propionate yield ($p<0.01$) and propionate yield ($p<0.05$), being 37° C the temperature that led to the best performance of the bacteria growing in BM medium. The results suggest that changing the YEL medium to a simpler composition, namely to the so-called basal medium (BM) did not affect the growth of *P.freudenreichii*, as no significant statistical difference was observed between the two media, except when using 37°C with agitation, condition for which a slight difference was observed ($p<0.05$). On the other hand, statistically significant differences were found between the two media regarding the yield and

productivity parameters of acetate and propionate production, thus indicating that modifications of the medium composition can affect the biotransformation performance but not the growth of *P. freudenreichii*. These statistical differences between the two media were not seen in all parameters equally, ranging from $p<0.05$ to $p<0.001$ or even no differences at all in some cases. The BM medium was found to have the best performance regarding SCFAs production (acetate yield and productivity) as compared to the YEL medium, in particular at 37 °C without agitation.

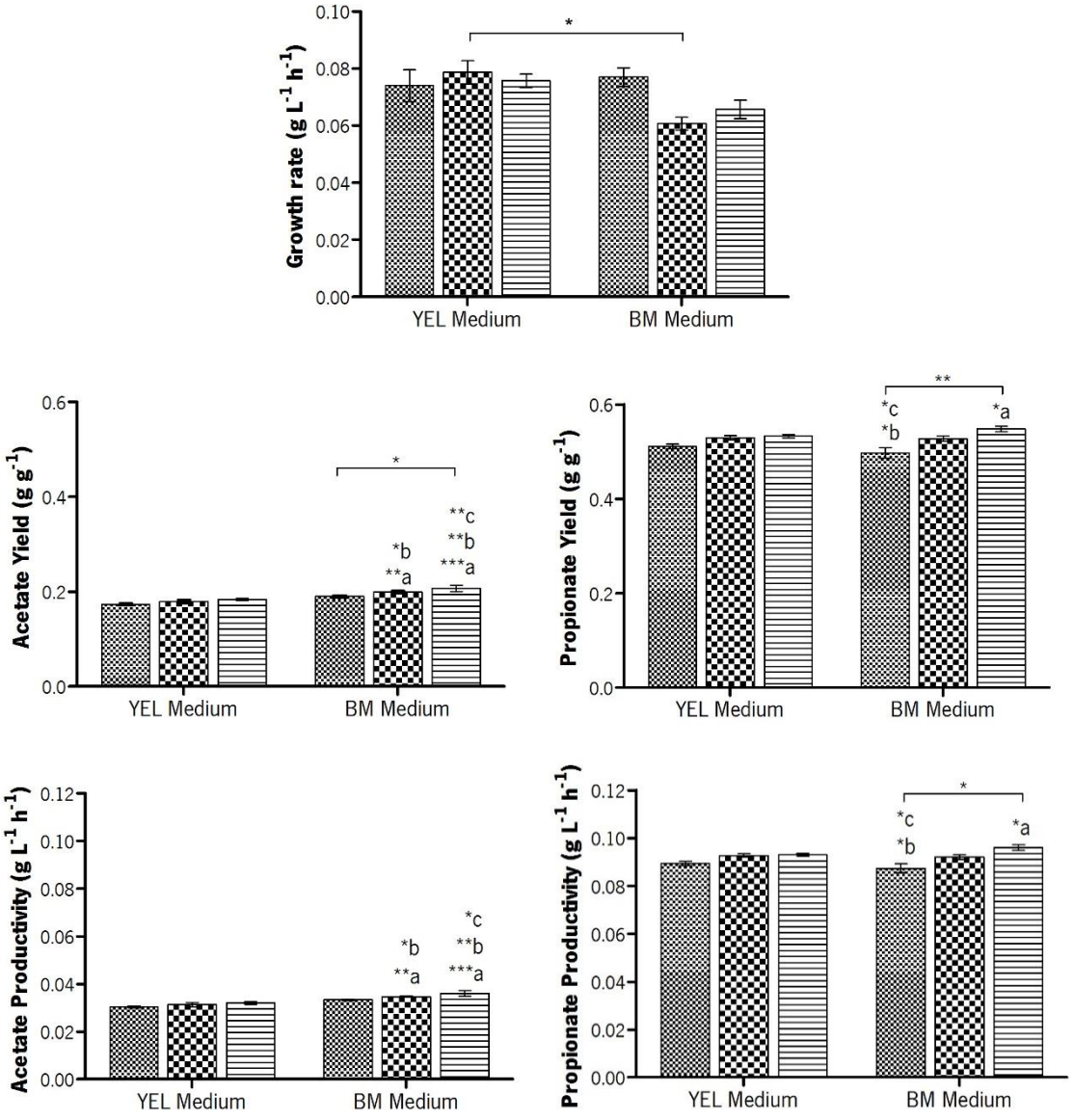


Figure 3.4. Bach fermentations kinetic parameters for *P. freudenreichii* in YEL medium and BM medium at pH 7, 30 °C with agitation (▩), 37 °C with agitation (▩) and 37 °C without agitation (▤). Concentration in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in triplicate and the mean ± SEM are represented. Values significantly different from 30 °C with agitation of YEL medium (a); 37 °C with agitation of YEL medium (b); 37 °C without agitation of YEL medium (c): * $p<0.05$; ** $p<0.01$; *** $p<0.001$. One-way ANOVA and Tukey's Test were used.

Seshadri and Mukhopadhyay (1993) reported an increase in biomass production and a decrease of propionate production by the strain ATCC 25562 with an increase of temperature from 30 to 37°C. Contrarily, Coral and co-workers (2008) found that both propionic acid production and biomass growth rate were higher at 30° C than at 36° C for the strain ATCC 4965. In our experiments with the strain DSM 20271, we found that the biomass growth rate was not affected by temperature increments, however the SCFAs production was found to be higher at 37° C than at 30° C using the BM medium. These divergent results can be explained by the fact that different strains were used in those experiments.

Considering the two fermentation media at the three operational conditions evaluated, a maximum concentration of acetate and propionate was obtained when using the BM medium, although this difference was not very pronounced as compared to the YEL medium. These results are in accordance with the ones from Coral and co-workers (2008). Those authors showed that a small amount of biomass using lactate at 30° C is able to produce a high propionic acid concentration. This conclusion has been also reported by several authors (Coral et al. 2008; da Costa et al. 1999; Himmi et al. 2000; Wang and Yang 2013), which studied the impact of different compounds in the culture medium, different carbon sources with different initial concentrations and other factors depending on the aim of the study.

Our first approach can help us to understand how *P. freudenreichii* behaves under anaerobic conditions, both regarding bacterial growth and SCFAs production, thus enabling the choice of the best conditions for the next experimental fermentation. In summary, the culture conditions selected were 37° C without agitation. These conditions are important and required for following experiments as in the future we aim to conduct co-culture assays with CRC cells lines towards the potential use of *P. freudenreichii* as a nutraceutical.

3.3.1.1.1. Basal Medium Supplemented with different carbon sources

Several authors reported that different carbon sources with different initial concentrations lead to distinct productions of acetate and propionate (Himmi et al. 2000; Coral et al. 2008; Wang and Yang 2013). Bearing in mind the goal of improving the SCFA concentrations, the BM medium was supplemented with two different carbon sources (glucose; glycerol). Glucose is an important carbon source as it is present in high levels in the animal organisms, while glycerol is relevant in industry. The impact of these carbon sources on the growth and biotransformation performance by *P. freudenreichii* is illustrated in Figure 3.5. The BM medium without supplementation was used as

a control. *P. freudenreichii* exhibited different performances depending on the carbon source used. The bacterial concentration obtained with glucose (0.94 ± 0.01 g L^{-1}) was higher than with glycerol (0.71 ± 0.01 g L^{-1}). Contrarily, higher levels of propionate production were obtained using glycerol (7.2 ± 0.1 g L^{-1}), thus indicating again that the production of SCFAs is not growth-associated.

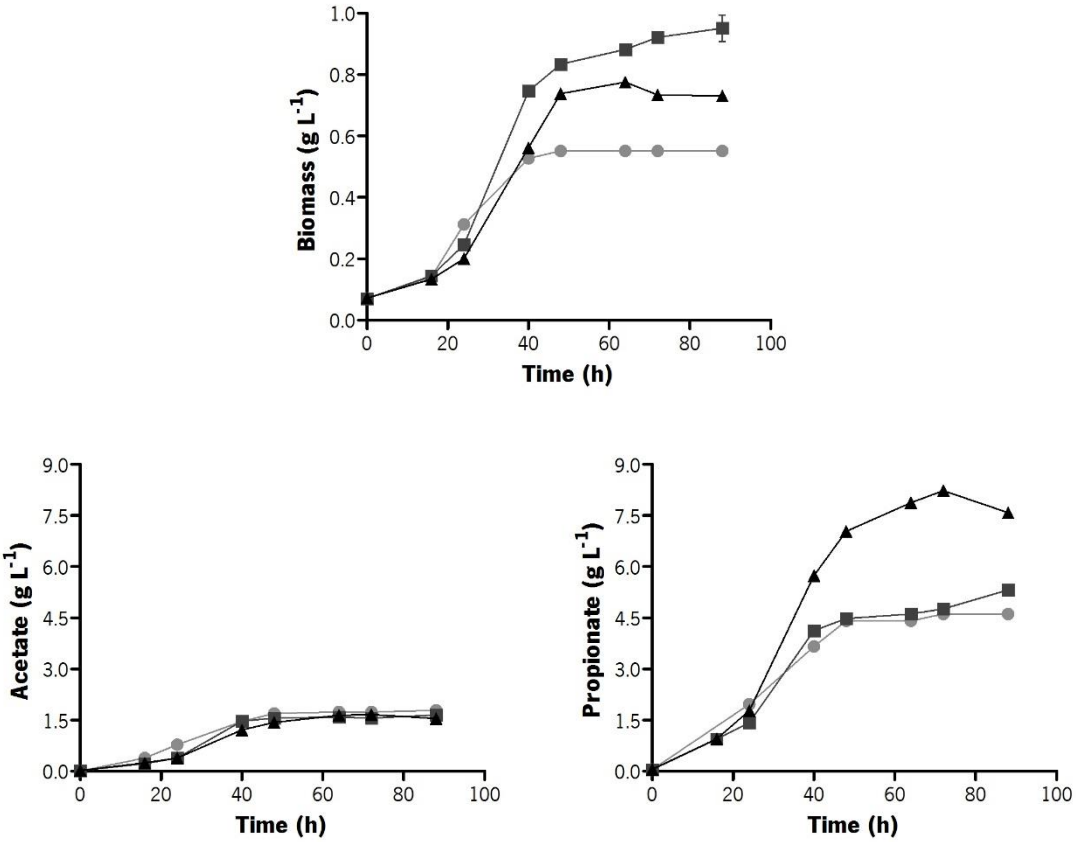


Figure 3.5. Biomass, acetate and propionate production by *P. freudenreichii* in BM medium (—●—) and supplemented with 3.7 g L^{-1} of glucose (—■—) and glycerol (—▲—) at pH 7, 37 °C without agitation Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate and mean ± SEM are represented.

Growth rates, yields and productivities obtained in the experiments using different carbon sources are shown in Figure 3.6. Although the biomass concentration was different among the control and media with glucose or glycerol, the growth rate was found to be statistically similar for all the conditions studied. Acetate production was found to be similar in all the experimental conditions tested, although yields and productivities for the two carbon sources were significantly different as compared to the control ($p<0.001$). Acetate productivity using the media with glucose also showed significant differences when compared to the media with glycerol ($p<0.01$). As can be observed in Figure 3.6 (propionate production), the yields and productivities of propionate also show

that glycerol supplementation led to the best performance and it was significantly different from the other conditions ($p<0.001$).

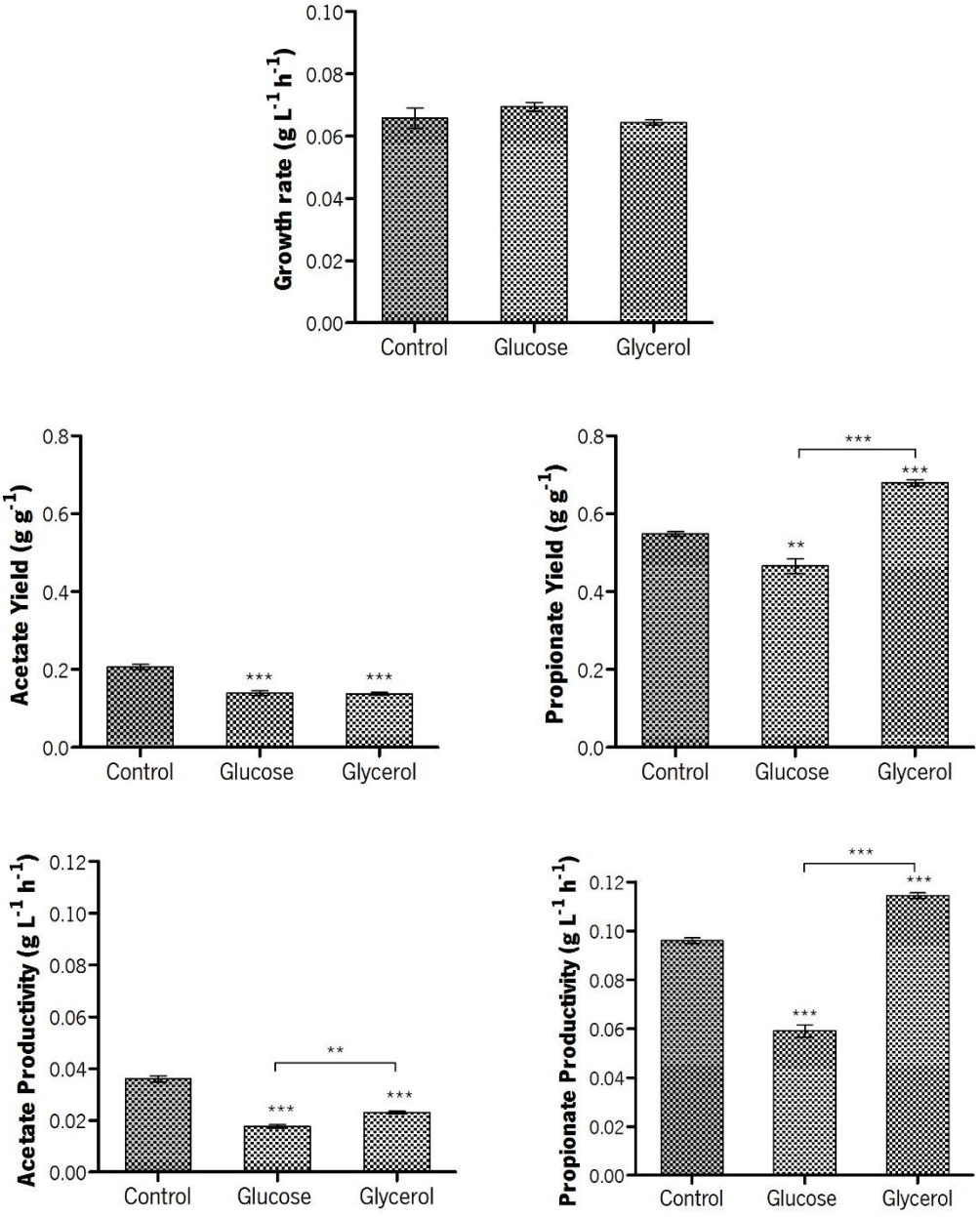


Figure 3.6. Bach fermentations kinetic of *P. freudenreichii* in BM medium as control and supplemented with 3.7 g L^{-1} of glucose and glycerol, 37 °C without agitation. Concentration in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in triplicate and the mean ± SEM are represented. Values significantly different from control and compare all with all conditions: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. One-way ANOVA and Tukey's Test were used.

These results are in good accordance with the general notion in the literature. Indeed, glycerol has been reported as the best carbon source for the production of propionate by *P. freudenreichii* followed by glucose (Coral et al. 2008; Himmi et al. 2000; Wang and Yang 2013).

Wang and Yang (2013) showed that propionate production from glucose by *P. freudenreichii* subsp. *shermanii* was relatively fast (0.19 g L^{-1} h) but led to a low product yield (0.39 g g^{-1}). In contrast, glycerol with a more reduced state led to a high propionate yield (0.65 g g^{-1}), but low productivity (0.11 g L^{-1} h). Coral and co-workers (2008) found that *P. acidipropionici* ATCC 4965 using an initial concentration of 20 g L^{-1} of glycerol led to a propionate yield of 0.703 ± 0.024 g g^{-1} and productivity of 0.037 ± 0.003 g L^{-1} h^{-1}.

The yields and productivities obtained in other studies can be slightly different from the ones herein reported and among them, since there are other factors that can affect these parameters, as previously mentioned. Moreover, our results are in accordance with the reports above mentioned, although we found higher productivities of propionate.

3.3.1.2. Medium mimicking the content of the human colon

Dairy propionibacteria have been identified as potential probiotics. However, to have a probiotic effects, *P. freudenreichii* have to survive and to remain metabolically active in the digestive tract. Since our main goal was to use this dairy propionibacteria as a nutraceutical agent to prevent/treat CRC, we evaluated how *P. freudenreichii* grows and produces SCFAs in a medium mimicking the content of human colon (Gibson and Wang 1994). The evaluation of *P.freudenreichii* performance in MCHC medium with pH 6; MCHC medium with pH 7; MCHC medium (pH 6) supplemented with glucose at 37 °C without agitation are represented in Figure 3.7.

The MCHC medium supplemented with glucose was found to be the best regarding biomass (0.98 ± 0.01 g L^{-1}) and propionate (2.09 ± 0.05 g L^{-1}) productions. These improvement of biomass, acetate and propionate productions have been reported in the literature (Wang and Yang 2013; Himmi et al. 2000). The change of the pH of the MCHC medium led to an improvement of the biomass, acetate and propionate concentrations, being the pH 7 the best for growth and SCFAs production by *P.freudenreichii*. As it is well-known, the pH of the gastrointestinal tract is different (Reis et al. 2008). Therefore, as we want the *P. freudenreichii* to have a good performance at the human colon (pH~6), further developments are required (most probably at the genetic level) to improve the bacteria robustness to these harsh conditions.

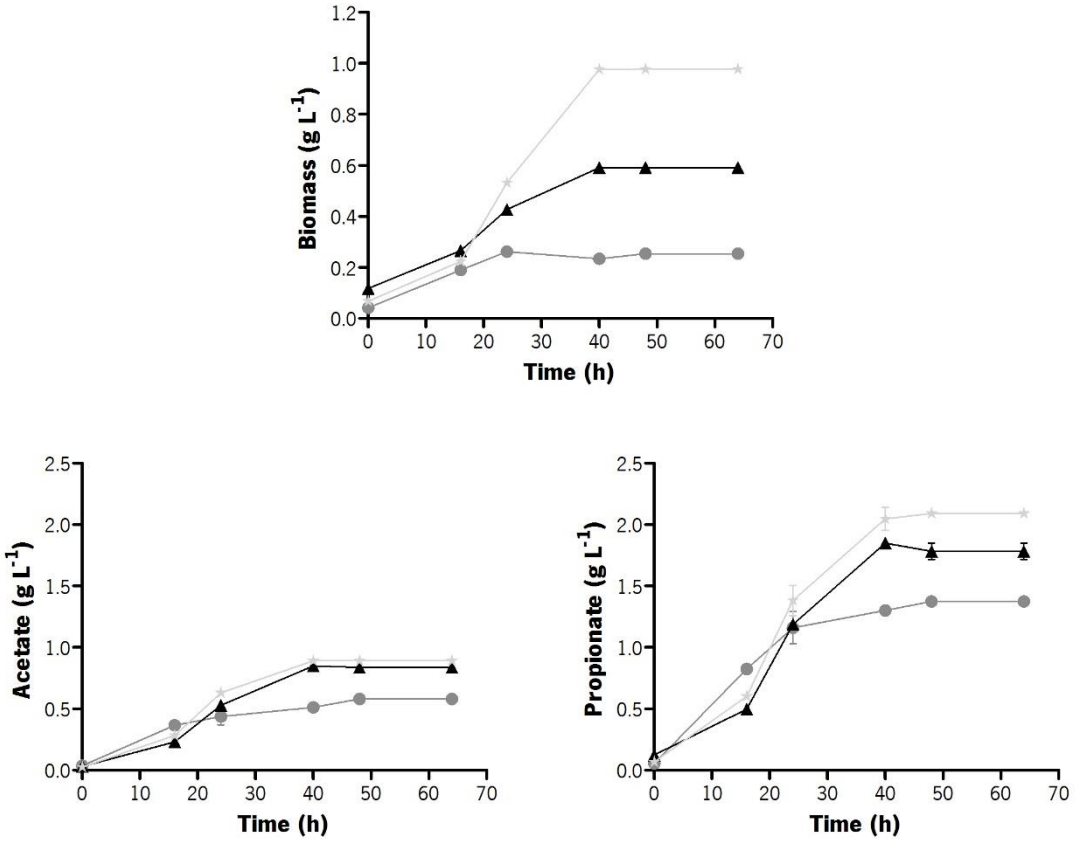


Figure 3.7. Biomass, acetate and propionate production by *P. freudenreichii* in MCHC medium at pH 6 (●); MCHC medium at pH 7 (▲) and MCHC medium (pH 6) supplemented with glucose (★) at pH 6, 37 °C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate and the mean ± SEM are represented.

Growth rates, yields and productivities for all the conditions evaluated were determined and are presented in Figure 3.8. These parameters confirmed that the best conditions were the MCHC medium supplemented with glucose, followed by the MCHC medium at pH 7. Glucose supplementation showed the higher growth rate (0.109 ± 0.003 g L^{-1} h^{-1}), acetate productivity (0.022 ± 0.001 g L^{-1} h^{-1}) and propionate productivity (0.051 ± 0.002 g L^{-1} h^{-1}) with significant statistical differences as compared to control ($p<0.001$). The MCHC medium at pH 7 did not show significant differences as compared to glucose supplementation regarding the acetate and propionate productivities. However, it showed better results of acetate (0.369 ± 0.015 g g^{-1}) and propionate yields (0.804 ± 0.019 g g^{-1}) comparing both with the glucose supplementation with statistical differences of $p<0.001$ comparing to glucose supplementation.

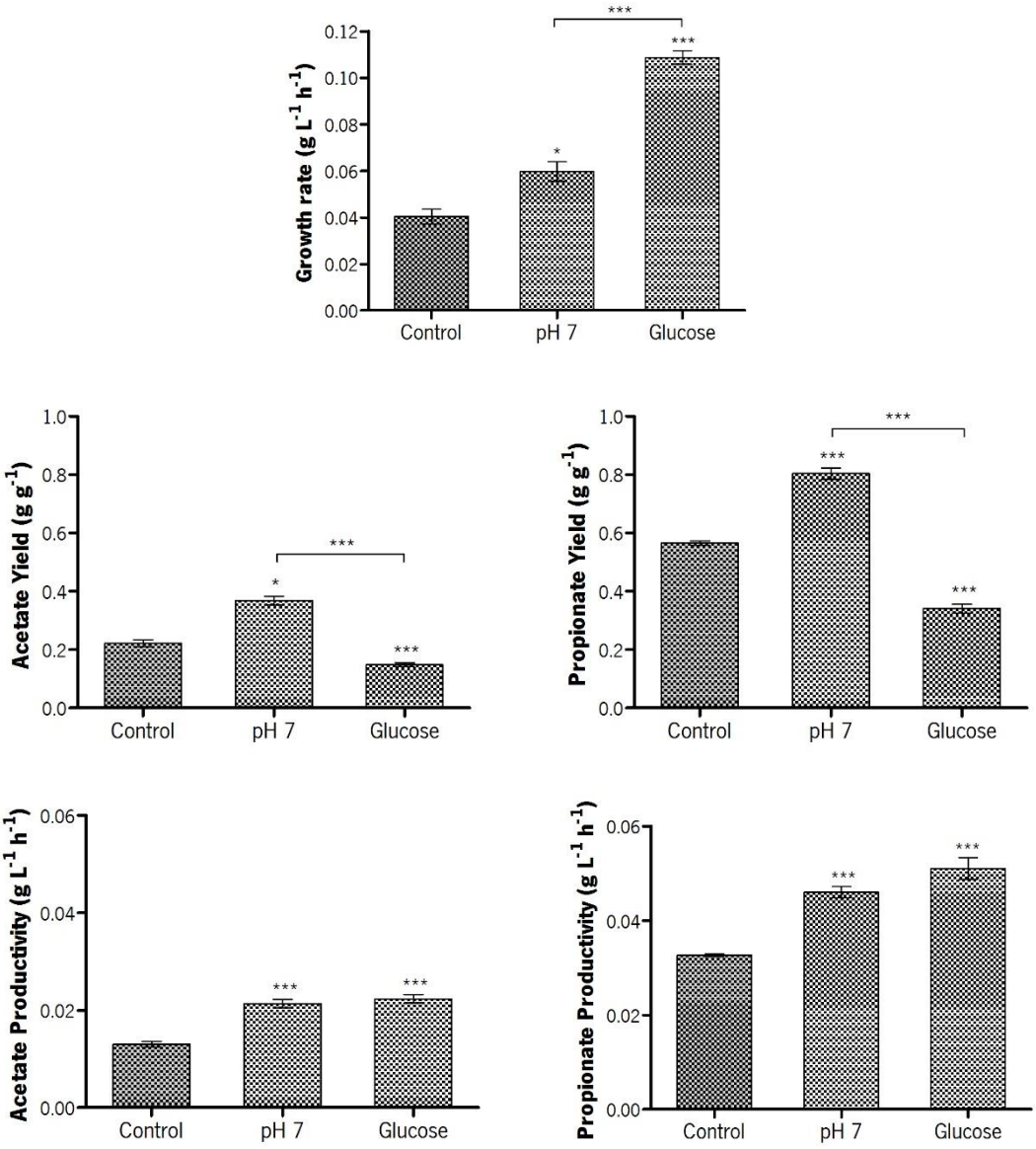


Figure 3.8. Bach fermentations kinetic of *P. freudenreichii* in MCHC medium (pH 6) as control, MCHC medium at pH 7 and MCHC medium supplemented with glucose at pH 6, 37 °C without agitation. Concentration in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in triplicate and the mean ± SEM are represented. Values significantly different from control and compare all with all conditions: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. One-way ANOVA and Tukey's Test were used.

Our results are in accordance with the results reported by Lan and collaborators (2007).Those authors cultured several strains of propionibacteria in Gibson modified medium (MCHC medium) at 37°C and evaluated the propionate production. They found that a set of strains produced propionate at concentrations around 1.32 g L^{-1} , while other strains produced lower amounts, thus suggesting that the propionate production rate is strain-dependent. *P. freudenreichii* subsp. *freudenreichii*, which the authors named TL3, produced 1.32 g L^{-1} of propionate, which is similar to

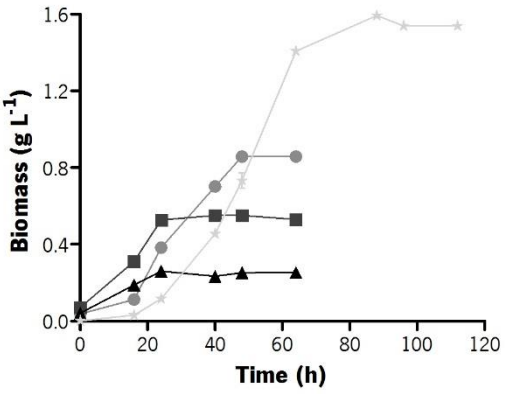
the propionate concentration that we obtained in our study (1.37 g L^{-1}) using *P. freudenreichii* subsp. *freudenreichii* DSM 20271.

3.3.1.3. Dulbecco's Modified Eagle's Medium

Dulbecco's Modified Eagle's Medium (DMEM) was used to culture *P. freudenreichii* in order to evaluate if the bacteria is able to grow and produce SCFAs in a culture medium that is commonly used by CRC cell lines, It is important to mention that in this study the antibiotics were removed from DMEM. Biomass, acetate and propionate productions using DMEM and the other culture media herein studied are presented in Figure 3.9.

P. freudenreichii exhibited a long culture time in DMEM exceeding the time that we previously set to monitor the fermentation experiments conducted with the other media. DMEM proved to be the best medium to grow *P. freudenreichii* with the highest concentration of biomass (1.539 ± 0.003 g L^{-1}). Contrarily to what we expected, DMEM was not the best medium for the production of SCFAs. However, it is important to mention that DMEM contains glucose (initial concentration of 4.05 g L^{-1}). Using this medium, *P. freudenreichii* was only able to produce 0.721 ± 0.002 g L^{-1} and 2.32 ± 0.01 g L^{-1} of acetate and propionate, respectively. These results are in good agreement with the results reported by Jan and collaborators (2002). Those authors showed, with the strain ITG18 (*P. freudenreichii* subsp. *freudenreichii*), that propionibacteria produced 2.7 g L^{-1} of propionate and 0.75 g L^{-1} of acetate under the same conditions as we used in the fermentation of DMEM (Jan et al. 2002).

Figure 3.9 illustrates the compilation of four important culture media that we studied along this work. *P. freudenreichii* showed different performances when grown in the different media. Our results revealed that although the BM medium did not lead to the best biomass production, it provided the highest yields of acetate (1.81 ± 0.01 g L^{-1}) and propionate (4.81 ± 0.02 g L^{-1}) followed by the YEL medium, DMEM and finally the MCHC medium.



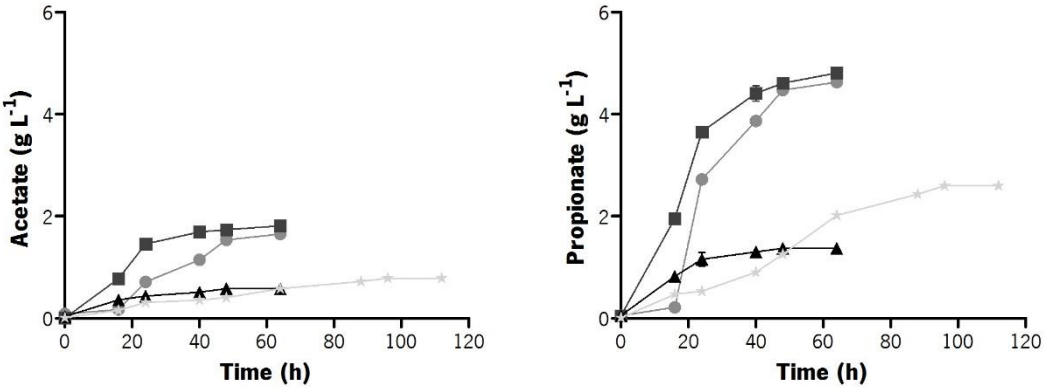
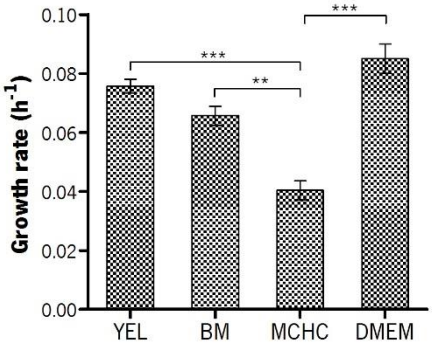


Figure 3.9. Biomass, acetate and propionate production by *P. freudenreichii* in YEL medium at pH 7 (—●—); BM medium at pH 7 (—■—); MCHC medium at pH 6 (—▲—); DMEM medium at pH 7.4 (—★—), cultivated at 37 °C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate, excluding DMEM medium (duplicate), the mean ± SEM are represented.

Growth rates, yields and productivities obtained for the bacteria grown on DMEM were determined and compared to the ones previously discussed for the other media, namely YEL, BM and MCHC media (see Figure 3.10). DMEM exhibited a higher growth rate (0.085 ± 0.005 g L^{-1} h^{-1}), but not statistically different from the other media. In contrast, DMEM showed the worst acetate and propionate productivities as compared to the other media presenting a statistical significant difference of $p<0.001$ to the YEL medium and BM medium, and $p<0.05$ to the MCHC medium regarding the acetate productivity; and $p<0.01$ to the MCHC medium regarding the propionate productivity.

Although the lower amounts of acetate and propionate production by *P. freudenreichii* in MCHC medium, its acetate and propionate yields showed high as compared to the other media presenting a statistical significant difference of $p<0.05$ to the YEL and DMEM media.



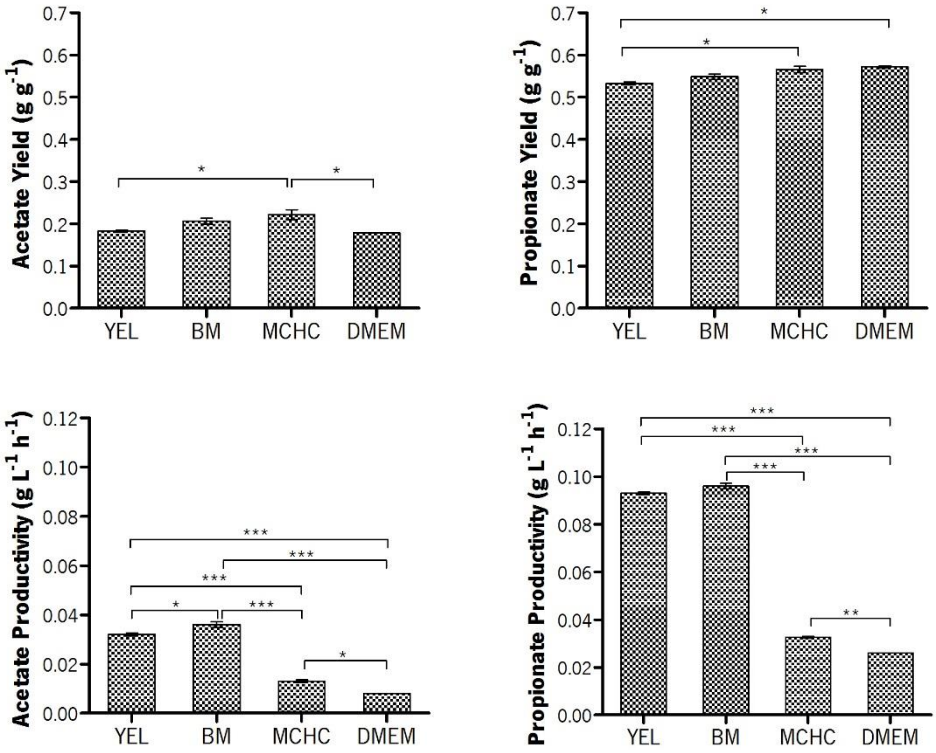


Figure 3.10. Bach fermentations kinetic of *P. freudenreichii* in YEL medium at pH 7; BM medium at pH 7; MCHC medium at pH 6; DMEM medium at pH 7.4, cultivated at 37 °C without agitation. Concentration in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in triplicate, excluding DMEM medium (duplicated), the average and standard error of the mean are represented. Values significantly different: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. One-way ANOVA and Tukey Test.

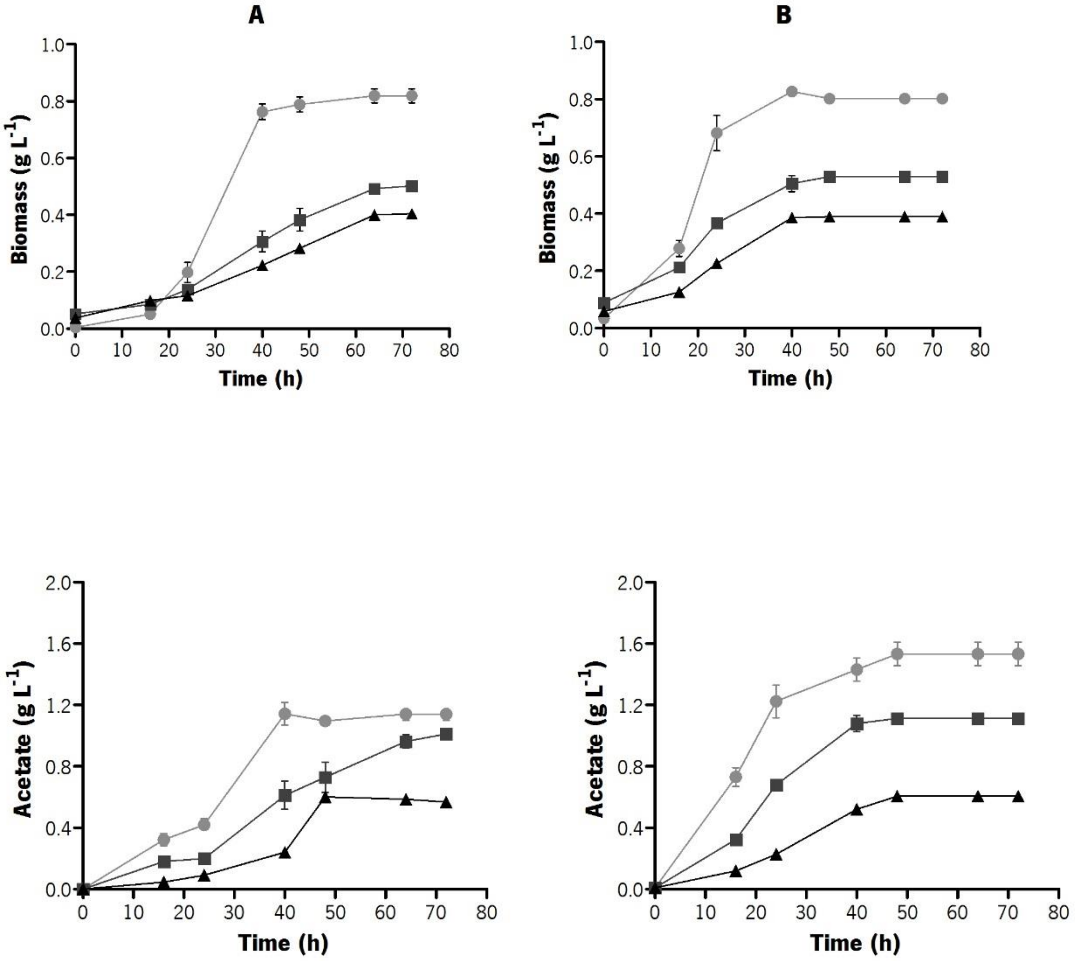
Our results clearly showed that the BM medium was the one that provided the best acetate and propionate productions, although it presented a yield and productivity on propionate similar to the YEL medium. Moreover, it was also clear that the MCHC medium was not appropriate the grow the bacteria, neither to produce SCFAs, however these results are the ones that better reflect a real scenario of the gastrointestinal tract and must therefore be considered in further developments and optimizations of *P. freudenreichii*.

3.3.2. Digestive Stress Challenge

The probiotic efficiency of *P. freudenreichii* depends on their capacity to withstand the digestive tract stresses and to remain metabolically active in the gut. The bacteria tolerance towards acidic pH and bile salts was performed as described in the section 3.2.5 aiming at the recovering a *P. freudenreichii* adapted to those stresses. Through these adaptation steps, bacteria were cultivated on solidified MCHC and DSMZ media, and in liquid MCHC and YEL medium. After recovery, in both

media conditions (solid and liquid), the evaluation of growth and biotransformation performance was conducted. The three different media (YEL, BM and MCHC) were used in this task.

Bacteria recovered in the two different ways (solid or liquid medium) were subjected to the same digestive stress challenge and presented different performances regarding the SCFAs production, as can be seen in Figure 3.11. The biomass concentrations were similar in both recovery procedures, but acetate and propionate productions were better using bacteria recovered from solid medium than from liquid medium independently of the medium (YEL, BM or MCHC) used.



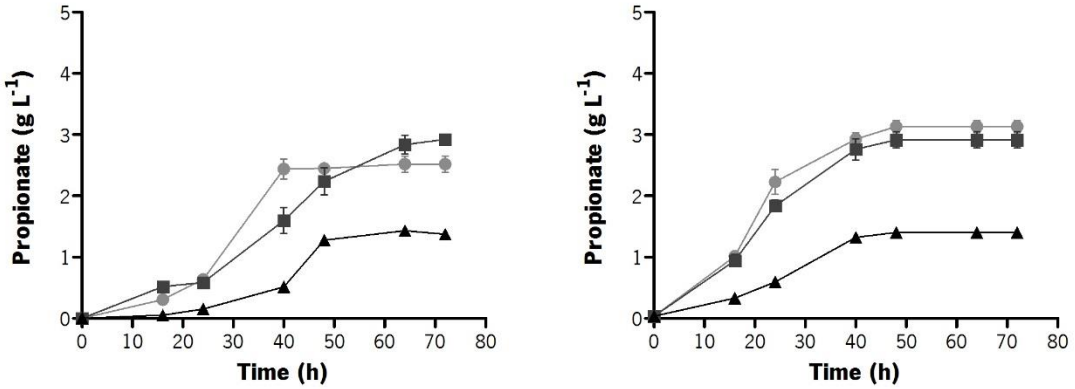
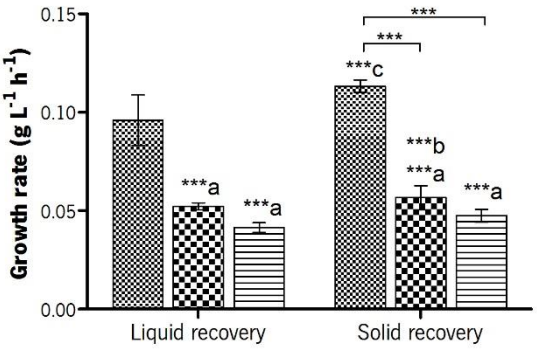


Figure 3.11. Biomass, acetate and propionate production by adapted *P. freudenreichii* recovered in liquid media (A) and solid media (B). Bacteria was cultivated in YEL medium (—●—), BM medium (—■—) MCHC medium (—▲—) at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in nine independent experiments and the mean ± SEM are represented.

In order to understand which way of recovery was more efficient to obtain a *P.freudenreichii* adapted to the digestive stress and with good performance regarding SCFA production, we determined the kinetic parameters of the fermentations (i.e. growth rate, yield and productivity) (see Figure 3.12). Adding to the significant statistical differences between the three culture medium that we discussed in the previous section, it can be seen that there are some cases in which the differences between the liquid and solid recovery of the adapted bacteria are significant, e-g- in YEL and BM medium, being the solid recovery the condition that provides the best results.



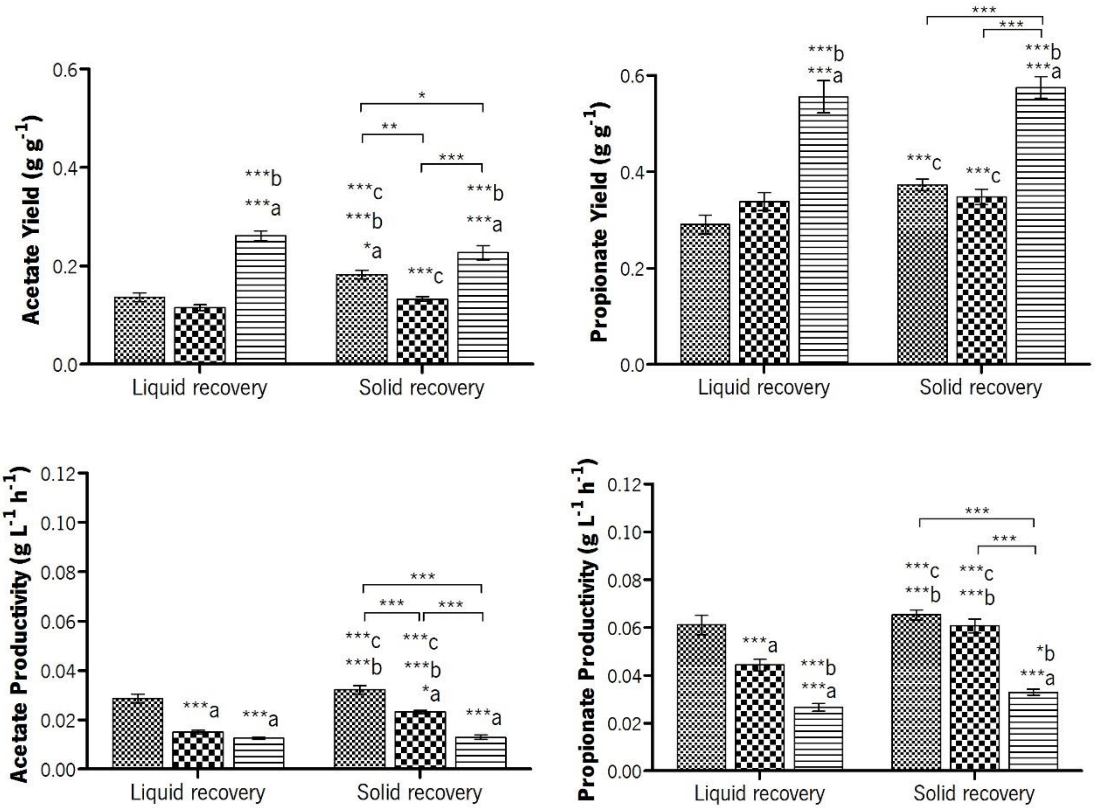


Figure 3.12. Bach fermentations kinetic of adapted *P. freudenreichii* recovered in liquid media and solid media. Bacteria was cultivated in YEL medium (▩), BM medium (▩) MCHC medium (▤) at 37° C without agitation. Concentration in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run nine times in independent experiments and the average and standard error of the mean are represented. Values significantly different from liquid recovery of YEL medium (a), BM medium (b) MCHC medium (c): * p<0.05; ** p<0.01; *** p<0.001. One-way ANOVA and Tukey Test.

The results obtained using the solid recovery showed higher acetate and propionate concentrations as compared to the liquid recovery. The growth rate, yield and productivity obtained with the adapted bacteria were similar for both recovery procedures, except for some particular cases for which the solid recovery was better for the growth and SCFA production by the bacteria. Therefore, we selected the adapted *P.freudenreichii* that was recovered from the solid medium.

The evaluation of growth and biotransformation performance by the adapted *P.freudenreichii* in DMEM was performed and the results are gathered in Figure 3.13. Production values in DMEM were similar to the ones obtained with non-adapted bacteria, namely 1.78 ± 0.01 g L^{-1}, 0.73 ± 0.01 g L^{-1} and 2.66 ± 0.10 g L^{-1} of biomass, acetate and propionate, respectively.

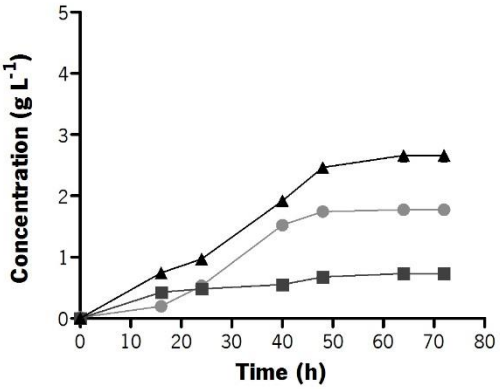


Figure 3.13. Biomass (—●—), acetate (—■—) and propionate (—▲—) production by adapted *P. freudenreichii* recovered in solid media. Bacteria was cultivated in DMEM medium at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in duplicated bottles and the average and standard error of the mean (SEM) are represented.

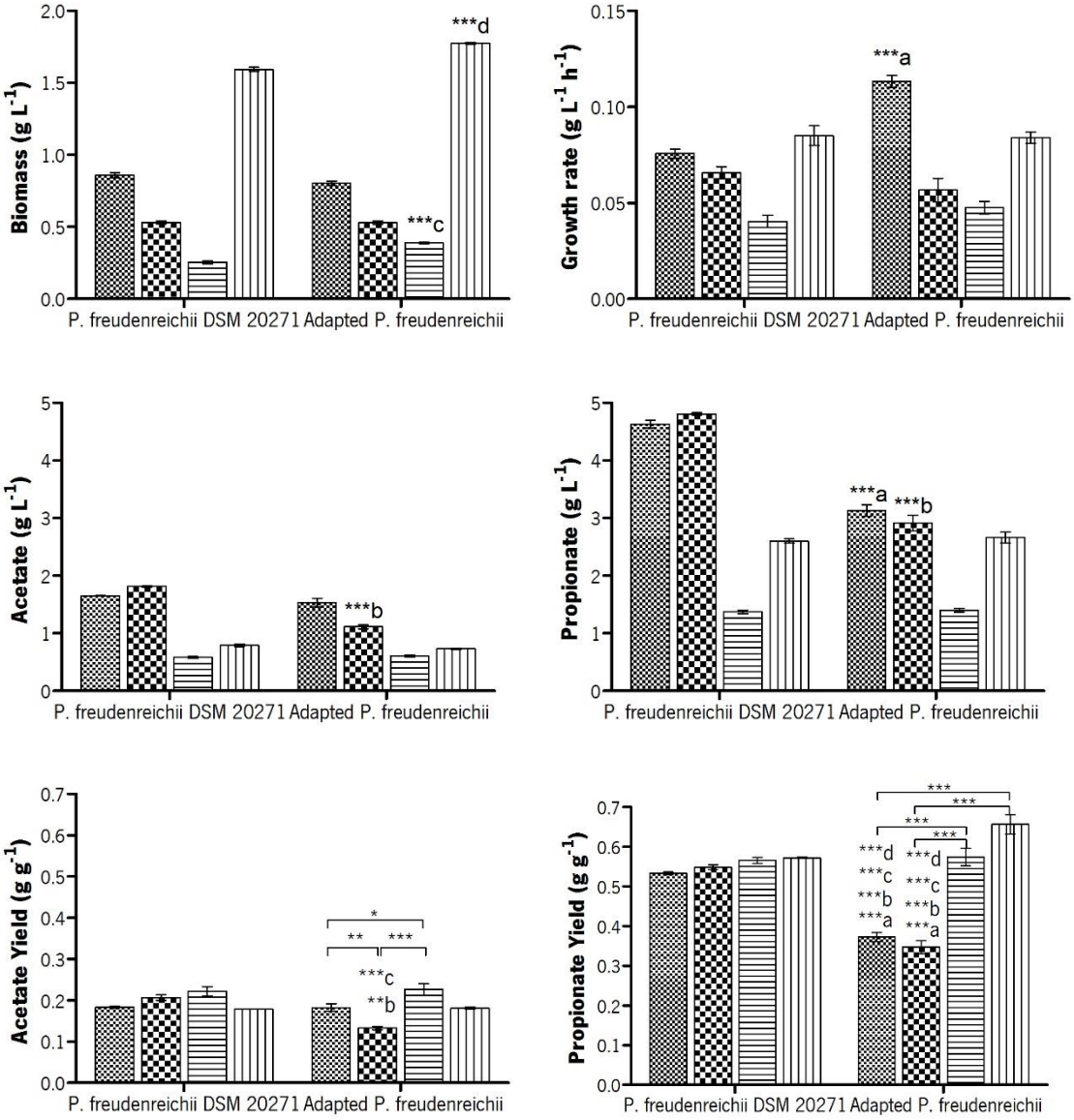
Several authors have performed digestive stress challenges to test the adaptation and tolerance of propionibacteria (Lan et al. 2007a; Jan et al. 2001; Leverrier et al. 2003; Cousin et al. 2012b). Being probiotics, these bacteria must be able to survive the acid stress imposed within the stomach in order to reach the intestine whereby they play their beneficial role. In all the studies reported, the authors could obtain an adapted propionibacteria to the digestive stress which was able to survive the challenge similarly to our findings. Moreover, these studies showed that the digestive stress tolerance is highly variable, depending on the strain, being our strain described as holding the best rate of survival during digestive stress challenges (Lan et al. 2007a).

3.3.2.1. *Propionibacterium freudenreichii* DSM 20271 – Adapted *Propionibacterium freudenreichii* to digestive stress

Adapted *P. freudenreichii* to the digestive stress was obtained as the result of the digestive stress challenge, exposing the propionibacteria to acidic pH and increasing levels of bile salts. This stress can change the performance of the bacteria regarding growth and SCFA production, thus we compared the results obtained with both the adapted and non-adapted bacteria. The parameters used for this comparison were the final concentrations of biomass, acetate and propionate; growth rate; yields and productivities of acetate and propionate (Figure 3.14).

Regarding the growth of propionibacteria, it was found that the digestive stress challenge did not adversely affect the biomass production and growth rate. On the contrary, in DMEM and MCHC medium, an improvement of the biomass concentration and growth rate was observed.

The production of SCFA was affected by the digestive stress challenge, particularly the propionate production and consequently its yield and productivity. Significant statistical differences can be seen between YEL and BM medium ($p<0.001$) when comparing the results obtained for the non-adapted and adapted bacteria, indicating that the digestive stress challenge adversely affected the propionic acid production by the bacteria grown in both culture media. Also, this adverse effect was observed for the acetate production, thus acetate yield and productivity. Despite these differences, no statistical significance was observed between *P. freudenreichii* DSM 20271 and the adapted bacteria regarding acetate and propionate production, their yields and productivities.



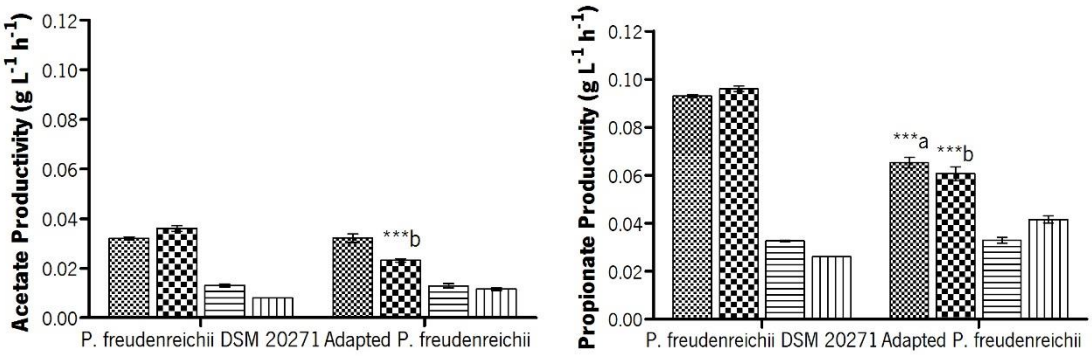


Figure 3.14. Bach fermentations kinetic of *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii* cultivated in YEL medium (▩), BM medium (▩) MCHC medium (▤) and DMEM medium (▥) at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate; in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in triplicate, excluding DMEM medium (duplicate), the mean ± SEM are represented. Values significantly different between the same medium: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. One-way ANOVA and Tukey's Test were used.

The digestive stress challenge was found to negatively affect the biotransformation performance of the propionibacteria in YEL and BM media, namely in what concerns the levels of propionate production. Despite these results, it is important to highlight that in the MCHC medium the performance of propionibacteria was not affected and indeed an improvement of biomass production was observed. Therefore, although we lost the best performance of the bacteria using YEL and BM media, we were able to obtain a great improvement using the MCHC medium and DMEM that are the media more related to the real scenario of the gastrointestinal tract.

3.3.3. Toxicity assay using acetate and propionate

The major factor that limits the SCFAs production by *P. freudenreichii* is the feedback inhibition by the high concentrations of the end-products present in the fermentation broth (Woskow and Glatz 1991). To gain a better understanding on the impact of the end-products on the bacterial growth and biotransformation performance, several concentrations of acetate and propionate were studied. The initial concentrations used were the ones that are commonly produced by *P. freudenreichii* in YEL medium, namely 1g L^{-1} of acetate and 3 g L^{-1} of propionate. Afterwards, increasing concentrations were also evaluated.

The impact of acetate toxic concentrations on biomass, acetate and propionate productions are presented in Figure 3.15. Feedback inhibition was observed at a 4 g L^{-1} acetate concentration in the fermentation broth, thus resulting in a long exponential growth phase with a lower biomass, acetate and propionate production. At an initial 8 g L^{-1} acetate concentration in the YEL medium, *P.*

freudenreichii was not able to grow or to produce SCFAs, being this concentration completely toxic to the bacterium.

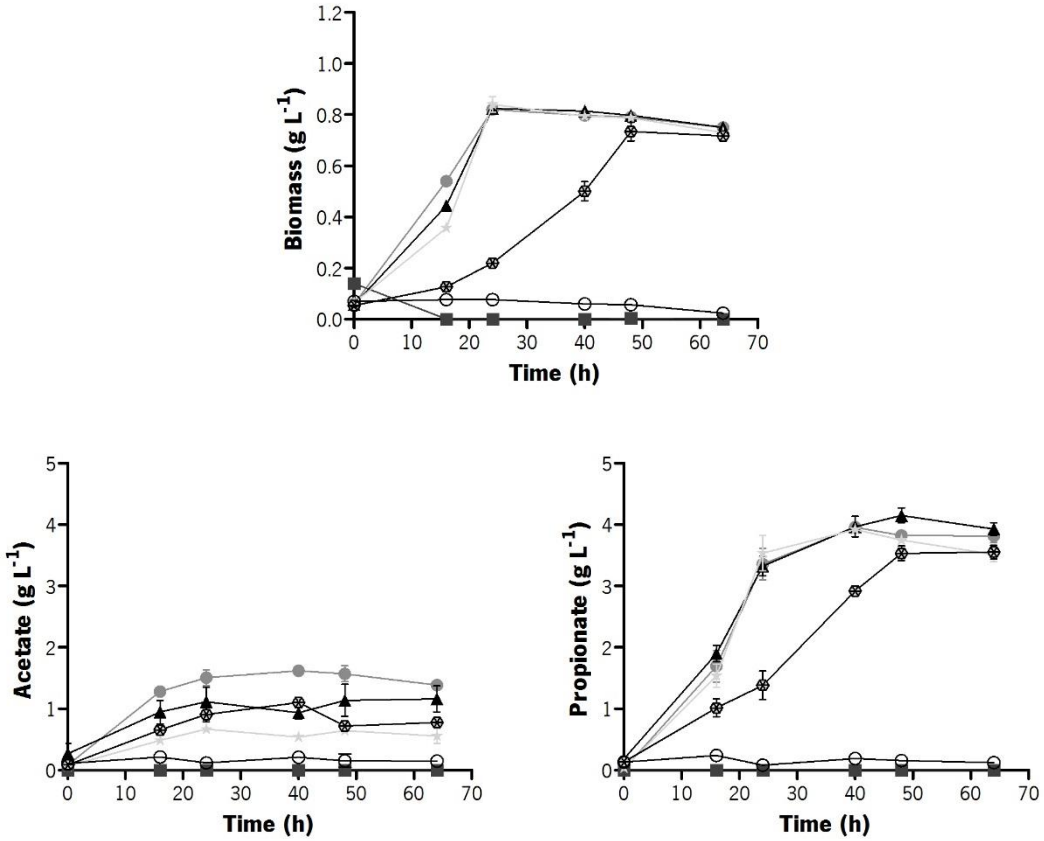


Figure 3.15. Batch fermentation kinetics of biomass, acetate and propionate production by adapted *P. freudenreichii* in YEL medium used as negative control (—●—), supplemented with 10 g L^{-1} of chloramine as positive control (—■—) and supplemented with an initial acetate concentration of 1 g L^{-1} (—▲—), 2 g L^{-1} (—★—), 4 g L^{-1} (—⊗—) and 8 g L^{-1} (—⊖—) at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate and mean ± SEM are represented.

Likewise, the propionate toxicity was also determined and the results are presented in Figure 3.16. At an initial 3 g L^{-1} and 6 g L^{-1} of propionate concentration in the YEL medium we observed the inhibition of growth and biotransformation by *P. freudenreichii*, particularly at 6 g L^{-1} of propionate. A total inhibition of growth and SCFAs production was observed for 12 g L^{-1} and 24 g L^{-1} of propionate.

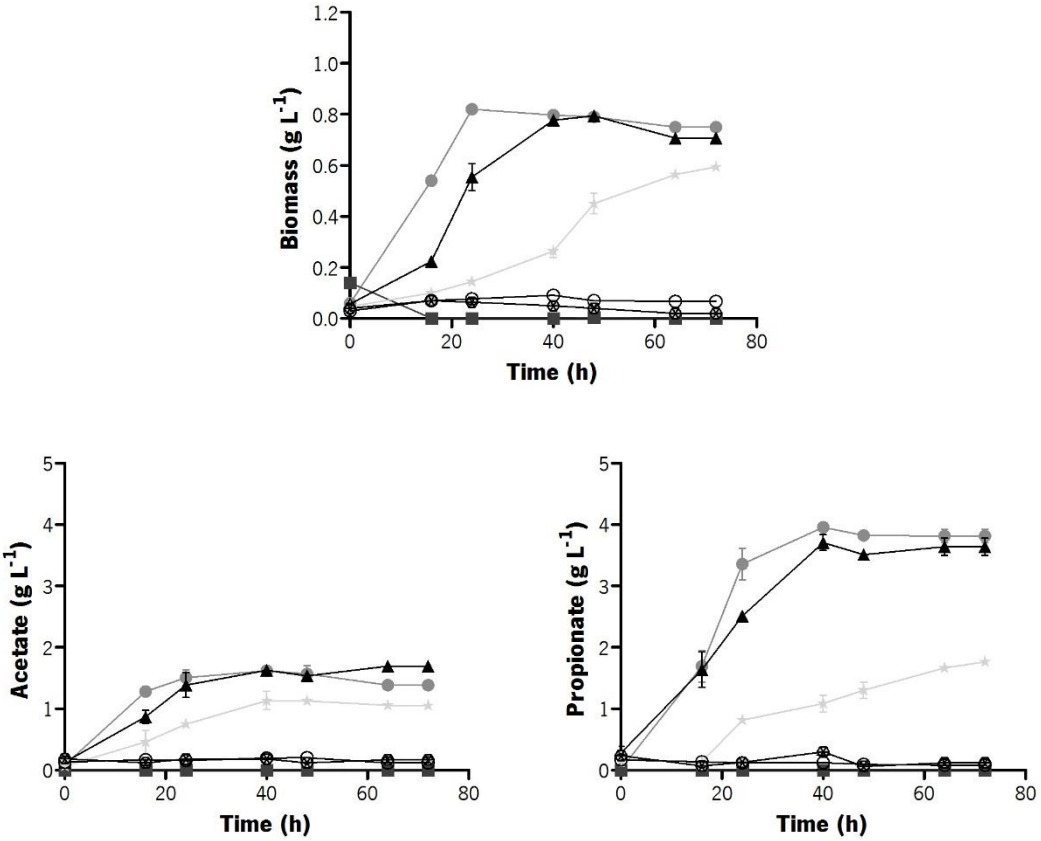


Figure 3.16. Batch fermentation kinetics of biomass, acetate and propionate production by adapted *P. freudenreichii* in YEL medium used as negative control (—●—), supplemented with 10 g L^{-1} of chloramine as positive control (—■—) and supplemented with an initial propionate concentration of 3 g L^{-1} (—▲—), 6 g L^{-1} (—★—), 12 g L^{-1} (—⊗—) and 24 g L^{-1} (—⊖—) at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate and the mean ± SEM are represented.

Combinations of different acetate and propionate concentrations were used to determine the toxic concentrations of both acids on the growth and SCFAs production by *P. freudenreichii* (see Figure 3.17). A toxic effect was observed at the initial concentration (1 g L^{-1} acetate and 3 g L^{-1} propionate), resulting in a lower biomass, acetate and propionate production. Other initial concentrations evaluated led to a total inhibition of the growth and SCFAs production.

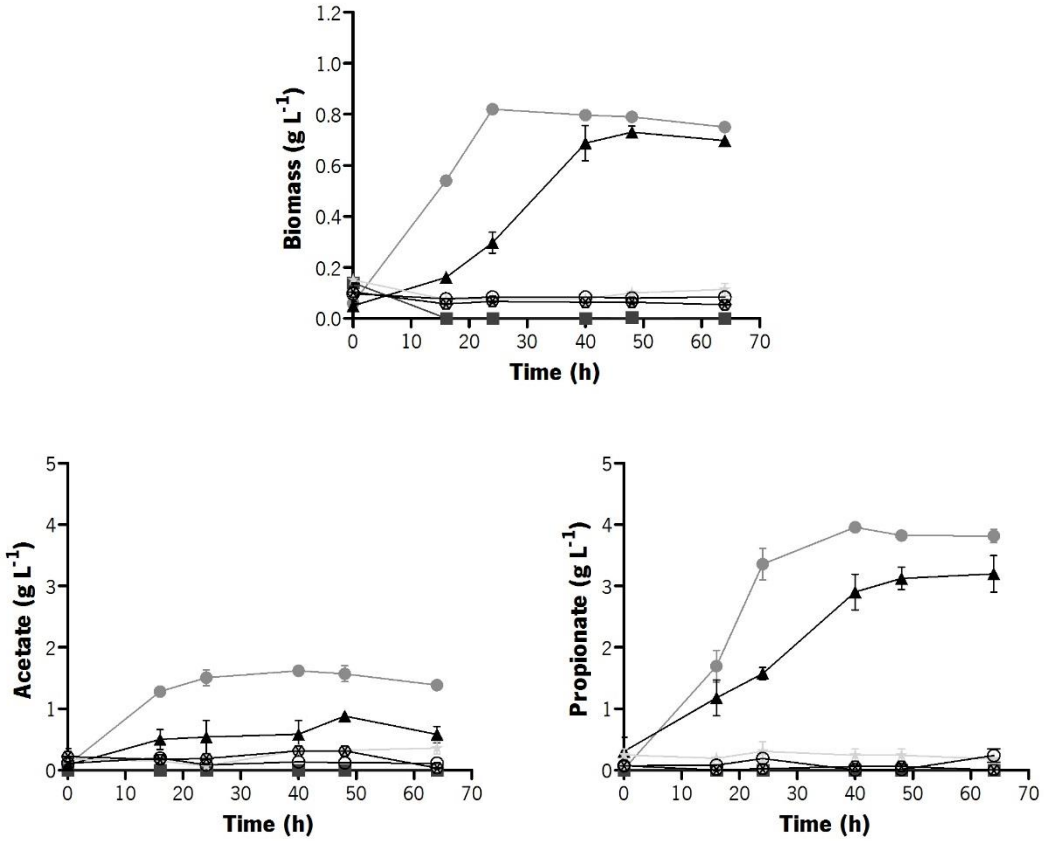


Figure 3.17. Batch fermentation kinetics of biomass, acetate and propionate production by adapted *P. freudenreichii* in YEL medium used as negative control (—●—), supplemented with 10 g L^{-1} of chloramine as positive control (—■—) and supplemented with an initial propionate concentration of 3 g L^{-1} (—▲—), 6 g L^{-1} (—★—), 12 g L^{-1} (—⊗—) and 24 g L^{-1} (—⊖—) at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in triplicate and mean ± SEM are represented.

A total inhibition of growth and SCFAs production by *P. freudenreichii* was found at 8 g L^{-1} acetate, 12 g L^{-1} propionate, and 2 g L^{-1} acetate plus 6 g L^{-1} propionate. However, a partial inhibition of the growth and SCFAs production was observed when the culture medium was supplemented with 4 g L^{-1} acetate; 3 g L^{-1} propionate; 6 g L^{-1} propionate; 1 g L^{-1} acetate and 3 g L^{-1} propionate. Growth rates, yields and productivities were determined for the experiments conducted under those conditions and are presented in Figure 3.18.

These parameters obtained for the different supplementations of the YEL medium were compared to the negative control. No significant statistical differences were observed between the supplementations with 2 g L^{-1} acetate and 3 g L^{-1} propionate and the control, except for the 2 g L^{-1} acetate supplementation conditions, for which statistic differences were found ($p<0.001$). These results demonstrated that an initial concentration of 2 g L^{-1} acetate inhibited the acetate production by *P. freudenreichii*, while an initial concentration of 3 g L^{-1} propionate had no inhibitory effect on the

P. freudenreichii performance. Other conditions evaluated (4 g L^{-1} acetate; 6 g L^{-1} propionate; 1 g L^{-1} acetate and 3 g L^{-1} propionate) led to a decrease of the *P. freudenreichii* performance.

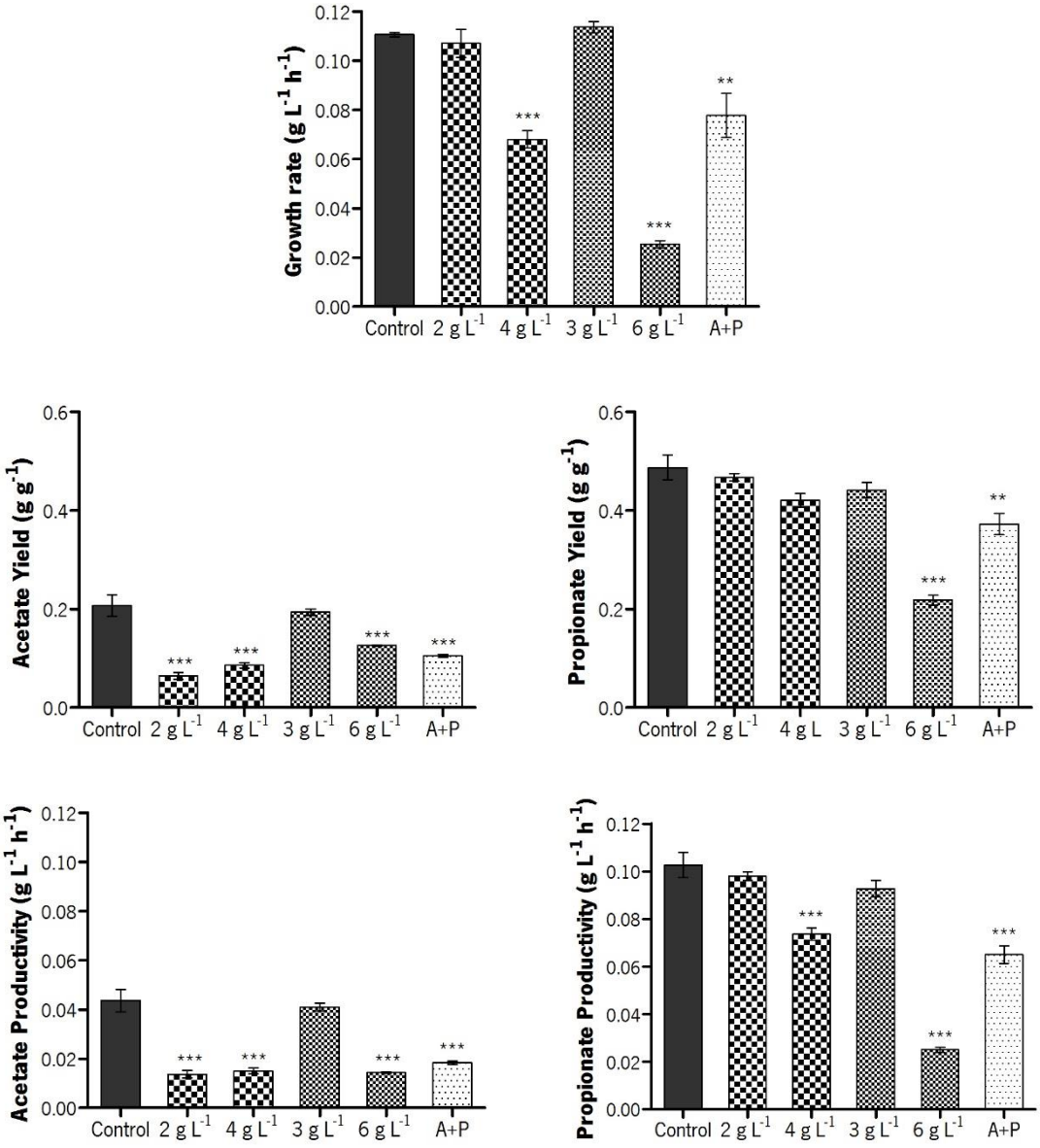


Figure 3.18. Bach fermentations kinetic of adapted *P. freudenreichii* cultivated in YEL medium used as control (■) and supplemented with an initial concentration of acetate (▩) propionate (▨) and 1 g L^{-1} acetate and 3 g L^{-1} propionate (▭) at 37° C without agitation. Concentration in gram per liter of biomass, acetate and propionate; in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in triplicate, the mean ± SEM are represented. Values significantly different between same medium: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. One-way ANOVA and Dunett`s test were used.

The toxic concentrations of acetate and propionate are strain-dependent. Moreover, there are a number of other factors that can also modulate this toxicity. For instance, Wang and collaborators (2012) found that 30.04 g L^{-1} propionic acid totally inhibited the growth of

propionibacteria, while our results revealed a total growth inhibition when using 12 g L^{-1} of propionic acid.

3.3.4. Random mutagenesis

To overcome the factors limiting the SCFA production by *P. freudenreichii*, we performed a random mutagenesis experiment. In order to recover a bacterium resistant to the digestive stress and higher amounts of SCFAs several conditions were used (described in section 3.2.7).

Unfortunately, we were not able yet to obtain a mutant bacteria that can resist to the digestive stress and simultaneously resistant to the toxic acetate and propionate concentrations used. We performed a second round of random mutagenesis experiments, and it was possible to observe the presence of some small colonies for two of the conditions studied (0.5 g L^{-1} bile salts, pH 4; and 0.5 g L^{-1} bile salts, pH 5), however more time is required for those colonies to grow and eventually to observe other colonies in the other conditions evaluated. This work is not completed and the experiments are still being conducted.

3.4 Conclusion

P. freudenreichii subsp. *freudenreichii* DSM 20271 growth and ability to produce acetate and propionate was evaluated using different culture media with different conditions. Moreover, the probiotic bacteria was subjected to a digestive stress challenge and its tolerance to high acetate and propionate concentrations was assessed.

Increasing temperatures were found to positively affect the growth and biotransformation performance of *P. freudenreichii*, being 37° C the best temperature. Although the BM medium was a simpler medium that yielded a lower biomass production, it was found to be the best culture medium evaluated. The DMEM medium was the best to grow *P. freudenreichii* leading to higher biomass concentrations and growth rates, but lower SCFAs production. The MCHC medium was the worst among all the culture medium evaluated exhibiting a low biomass concentration and SCFAs production. The pH of this medium was found to be the limiting factor.

The use of glucose in the culture media resulted in the higher amounts of biomass produced, leading to a relatively low propionate yield, whereas the glycerol fermentation showed a higher propionate yield and productivity, but exhibited a low biomass production.

P. freudenreichii colonies tolerant to digestive stress were recovered in solid medium and showed a better performance than when recovered in a liquid medium. However, the adapted *P. freudenreichii* lost the ability to produce high amounts of SCFAs in YEL and BM media, particularly propionate. This loss is not worrisome, as in other medium this was not observed, but contrarily an increase of biomass was obtained.

Acetate and propionate, alone or combined in different concentrations (4 g L^{-1} acetate; 3 g L^{-1} propionate; 6 g L^{-1} propionate; 1 g L^{-1} acetate and 3 g L^{-1} propionate) led to a partial inhibition of the bacteria growth and SCFAs production. Concentrations higher than the above mentioned were defined as the concentrations that lead to total inhibition of growth and SCFAs production, thus being completely toxic to the bacteria.

The results gathered in this work gave us a better knowledge on the behavior of *P. freudenreichii* under different culture and experimental conditions. Furthermore, this work constitutes a starting point towards an improved propionibacteria for applications in the functional food industry.

Chapter 4. | Effect of *Propionibacterium* fermentation broths on colorectal cancer cells

4.1. Background

Colorectal cancer constitutes a major concern in developed countries being associated to lifestyle such as obesity, physical activity, and diet which are emerging as critical elements for preventing colorectal cancer (Lee et al. 2015). Evidences have shown that low fat and higher dietary fiber diet could protect against colorectal cancer (Hinnebusch et al. 2002; Zhang et al. 2004).

The major impact of diet habits on the prevalence of colorectal cancers has triggered efforts to design an optimal diet and/or to create food supplements specifically reducing the risk of cancer. Therefore, a growing interest focuses on probiotics as cancer-preventing/treating agents. Propionibacteria is an important probiotic that is well-known for its long history of consumption in Swiss type cheeses and other fermented products (Piveteau 1999). They possess a peculiar fermentation metabolism which leads to the production of carbon dioxide and SCFAs, namely acetate and propionate (Jan et al. 2002). Indeed it has been reported a paradoxical effects on colonic epithelial proliferation. While the three major SCFAs stimulate proliferation of normal crypt cells, n-butyrate and, to a lesser degree, propionate and acetate inhibit growth of colon cancer cell lines (Hague et al. 1995; Scheppach et al. 1995; Marques et al. 2013).

Physiological concentration of acetate, propionate and butyrate present in the colon around the 60, 25 and 10 mM, respectively (Macfarlane and Macfarlane 2003; Cummings 1984). However, the colon concentration of SCFA can be modulated by food components as probiotics and depend on an equilibrium between microbial synthesis and colonic absorption. Moreover *P. freudenreichii* has been studied for its important role to produce enhanced concentration of SCFA which make this bacteria a useful probiotic in the context of colon cancer prevention- It has been shown that SCFA at physiological concentration acts on the survival of cancer cells and leads to cell cycle arrest in the G2/M phase, generation of reactive oxygen species, Bax translocation, mitochondrial depolarization, activation of caspases and chromatin condensation and fractionation (Lan et al. 2008; Marques et al. 2013).

Jan and co-workers (2002)demonstrated that the *Propionibacterium* strains, namely *Propionibacterium acidipropionici* strain CNRZ80, *P. freudenreichii* subsp*. freudenreichii* strain ITG18, and *P. freudenreichii* subsp. *shermanii* strain SI41, kill human cancer cell lines, such as HeLa, HT29 and Caco2 cells, apparently *via* SCFA produced. They also reported that SCFA induce apoptosis and acting on mitochondria membrane permeabilization. Lan and co-workers (2008) demonstrated, *in vivo* study, that *P. freudenreichii* TL133, via its metabolites, facilitated the

elimination of damaged cells by apoptosis in the rat colon after genotoxic insult and may play a protective role against colon cancer.

Several studies have showed a variety of biological effects of SCFA, and there is a vast number of experimental work showing new action mechanisms of these molecules (Tang et al. 2011a; Marques et al. 2013; Jan et al. 2002; Tedelind et al. 2007). Although most studies focus on SCFA-induced apoptosis, Tang and co-workers (2011a) demonstrated for the first time that butyrate and propionate induce autophagy in human colon cancer cells to dampen apoptosis whereas inhibition of autophagy potentiated SCFA induce apoptosis. SCFA may have opposing effects either inducing autophagy and hence inhibiting the proliferation of cancer cells or inducing apoptosis. In line of this latter effect, our group has recently demonstrated that acetate induces LMP and the release of Cat-D what is important in regulation of apoptosis in CRC cells, as mentioned in chapter 1 (Marques et al. 2013).

The aim of the present study was to study the effect of *Propionibacterium* fermentation broth containing SCFA (acetate and propionate) on colorectal cancer cells. For this purpose, we used fermentation broth of *Propionibacterium* optimized and characterized in chapter 3 to evaluate the *in vitro* effects on RKO a colorectal cancer derived cell line.

4.2. Materials and methods

4.2.1. Bacterial cultures

4.2.1.1. Bacterial strains

In this present work two *Propionibacterium* were used: *Propionibacterium freudenreichii subsp. freudenreichii* DSM 20271 (*P. freudenreichii*) and adapted *Propionibacterium freudenreichii subsp. freudenreichii* DSM 20271 to digestive stress established by us in the laboratory (chapter 3) (adapted *P. freudenreichii*). Bacterial cultivation was described in chapter 3 (sections 3.2.1. and 3.2.2)

Propionibacteria grow in different media and produced different concentration of SCFAs, namely acetate and propionate. Quantification of these SCFAs was performed by HPLC, as described in section 3.2.3.2, respective concentrations are represented in Table 4.1.

Table 4.1. Amounts of SCFAs concentration present in fermentation broth of *P. freudenreichii*

	***P. freudenreichii* DSM 20271**		**Adapted *P. freudenreichii***	
	Acetate (g L^{-1})	Propionate (g L^{-1})	Acetate (g L^{-1})	Propionate (g L^{-1})
DMEM	0.79 ± 0.02 [13 mM]	2.60 ± 0.04 [35 mM]	0.73 ± 0.01 [12 mM]	2.66 ± 0.10 [36 mM]
BM	1.81 ± 0.01 [29 mM]	4.81 ± 0.02 [64 mM]	1.11 ± 0.03 [19 mM]	2.91 ± 0.13 [40 mM]
YEL	1.65 ± 0.00 [27 mM]	4.63 ± 0.06 [63 mM]	1.53 ± 0.08 [25 mM]	3.13 ± 0.10 [42 mM]
MCHC	0.58 ± 0.02 [10 mM]	1.37 ± 0.03 [18 mM]	0.61 ± 0.02 [10 mM]	1.40 ± 0.03 [19 mM]

4.2.1.2. Supernatants preparation

Supernatants were collected after bacteria growth, when *P. freudenreichii* achieved the stationary phase approximately 10 hours after the end of exponential phase to ensure the a maximum of SCFAs were produced. As mentioned in section 3.2.4, fermentation broth were then recovered in 50 mL falcons (Orange scientific, 50 mL Tube conical, Belgium), centrifuged 10 000g for 20min (HERAEUS, Megafuge 1.0R, Germany), filtered in aseptic conditions with sterile filters of 0.2 µm and kept at -20° C with parafilm, the fermentation broth pH was adjusted to 7.10 – 7.40.

In order to obtain deproteinized supernatants the fermentation broth after being processed as described was autoclaved (121° C; 15 min; 1bar), centrifuged, the pH was adjusted, filtered and kept at -20° C protected with parafilm.

4.2.1.3. Sterile bacterial media preparation

The bacteria media, namely BM; YEL and MCHC media, were elaborated as described in chapter 3 and stored at room temperature in sterile bottles. 24 hours before using, this medium were supplemented. In sterile condition the bottles were opened and the medium was filtered with sterile filters (0.2 μm) to a 50 mL falcon and kept at -20° C.

4.2.2. Cell culture

In the present work the human CRC cell line RKO (CRL-2577) was used. RKO is a poorly differentiated CRC cell line developed by Michael Brattain in 1984 (Ruan et al. 2010) and was kindly given by IPATIMUP that acquired it at ATCC. This cell line harbors several mutations including $BRAF^{V600E}$ (Oikonomou et al. 2011) which is an important mutation on the development of colorectal cancer.

RKO cell line was cultivated in DMEM medium supplemented with 10% (v/v) FBS and 1 % (v/v) penicillin-streptomycin. Cell lines were cultivated in 25 cm^2 tissue culture flasks (TPP, Tissue culture flask 25 cm2, German) and maintained in an incubator under 37°C in a humidified, 5% CO_2 controlled atmosphere. When achieving a density between 80-100%, the cell cultures were subcultivated. Briefly, the medium was removed from the flasks, cells were washed with PBS 1x and subsequently incubated with 0.5 mL of 0.05% trypsin/EDTA for 5 minutes at 37°C in a humidified, 5% CO_2 controlled atmosphere. After cells detachment 4.5 mL of medium containing serum was added to the flask in order to inhibit trypsin activity. To determine cell number 20 μL of cell suspension was introduced into a hemocytometer and live cells were counted. Cells were then reseeded in a 25 cm^2 tissue culture flasks at an appropriated density in order to reach 80-100% confluence within one week. The cell culture media were replaced by routine, every 3-4 days.

4.2.3. DMEM medium “consumed”

The fermentation broth of *P.freudenreichii* has no nutrients, thus we had to perform a control for this condition. We used a DMEM medium “consumed” by RKO cell line. We used a 25 cm^2 tissue culture flask with RKO without replacing the culture media for a week, assuring that all the nutrients in the medium were consumed. After one week in culture the “consumed” medium was removed to a 15 mL falcon and centrifuged by 1000g for 10 min in order to separate cells in the medium.

4.2.4. Preparation of carboxylic acid solution

Stock solution of acetate and propionate was performed by sodium acetate trihydrate and sodium propionate purchased from Sigma, The solutions were prepared in ultrapure water at the concentrations of 3 M and pH was adjusted to pH 7.4. All the solutions were sterilized by filtration (0.22 µm syringe filter units) before use and their proportion in the culture medium never exceeded 10% of the final volume. Solution of acetate and propionate were stored at 4° C.

4.2.5. Cell treatment conditions

For all treatment performed in this work, cells in exponential growth phase were cultured in 24-well plates at a cellular density of 100 000 cell mL^{-1} at a final volume of 0.5 mL per well, excluding to cell cycle analysis that cells were cultured in 6-well plates at a cellular density of 150 000 cell mL^{-1} at a final volume 1.5 mL per well and incubated at 37° C in a 5% CO_2 atmosphere during 24 hours, to allow cell adhesion. After 24 hours, culture medium was removed and replaced by different treatment conditions as described in Table 4.2. After treatment addition, cells were incubated for further 48 hours at 37° C, 5% CO_2 atmosphere.

Table 4.2. Assays and treatments performed in RKO cell line

Assay	Conditions
Pure SCFAs (Acetate and Propionate)	- Fresh DMEM complete medium (C. Negative); - Hydrogen peroxide _ 1Mm (C. Positive - IC_{50} Acetate (110 mM); - IC_{30} Acetate (74 mM); - Acetate (12 mM); - IC_{50} Propionate (60 mM); - IC_{30} Propionate (40 mM); - Propionate (35 mM); - IC_{50} Acetate (110 mM) & IC_{50} Propionate (60 mM); - IC_{30} Acetate (74 mM) & IC_{30} Propionate (40 mM); - Acetate (12 mM) & Propionate (35 mM);
Culture media of *P. freudenreichii*	- Fresh DMEM complete medium (C. Negative); - Hydrogen peroxide _ 1Mm (C. Positive); - BM Medium; - YEL Medium; - MCHC Medium pH 6; - MCHC Medium pH 7;
	- Fresh DMEM complete medium diluted in HEPES solution (C. Negative); - Hydrogen peroxide _ 1Mm (C. Positive); - BM Medium diluted in Fresh DMEM complete medium; - YEL Medium in Fresh DMEM complete medium; - MCHC Medium pH 6 in Fresh DMEM complete medium; MCHC Medium pH 7 in Fresh DMEM complete medium;
BM Supernatant	- Fresh DMEM complete medium diluted in BM medium (C. Negative); - Hydrogen peroxide _ 1Mm (C. Positive); - Control with pure acetate and propionate of P. freudenreichii DSM 20271 _ (14 mM - - 32 mM); - BM supernatant diluted in fresh DMEM medium by P. freudenreichii DSM 20271; - BM supernatant deproteinized diluted in fresh DMEM medium by P. freudenreichii DSM 20271;

	- Control with pure acetate and propionate of adapted P. freudenreichii _ (10 mM – 20 mM); - BM supernatant diluted in fresh DMEM medium by adapted P. freudenreichii; - BM supernatant deproteinized diluted in fresh DMEM medium by adapted P. freudenreichii.
DMEM Supernatant	- "consumed" DMEM medium by RKO cell line (C. Negative) - Hydrogen peroxide _ 1Mm (C. Positive); - Control with pure acetate and propionate of P. freudenreichii DSM 20271 _ (13 mM - - 35 mM); - DMEM supernatant by P. freudenreichii DSM 20271; - DMEM supernatant deproteinized by P. freudenreichii DSM 20271; - Control with pure acetate and propionate of adapted P. freudenreichii _ (12 mM – 3 mM); - DMEM supernatant by adapted P. freudenreichii; - DMEM supernatant deproteinized by adapted P. freudenreichii.
	- Fresh DMEM complete medium diluted in "consumed" DMEM medium by RKO cell line (C. Negative); - Hydrogen peroxide _ 1Mm (C. Positive); - Control with pure acetate and propionate of P. freudenreichii DSM 20271; - DMEM supernatant diluted in fresh DMEM medium by P. freudenreichii DSM 20271 _ (6.5 mM – 17.5 mM); - DMEM supernatant deproteinized diluted in fresh DMEM medium by P. freudenreichii DSM 20271; - Control with pure acetate and propionate of adapted P. freudenreichii 20271 _ (6.5 mM – 17.5 mM); - DMEM supernatant diluted in fresh DMEM medium by adapted P. freudenreichii; - DMEM supernatant deproteinized diluted in fresh DMEM medium by adapted P. freudenreichii.

4.2.6. Cell proliferation assay

In this present work the sulforhodamine B (SRB) assay was used to access the cell proliferation. This method was developed in 1990 for Skehan and their co-workers. The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates. SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to cell mass (Skehan et al. 1990).

After treatments, the medium was removed and washed with PBS 1x. Then cells were fixed in ice-cold methanol containing 1% acetic acid, and incubated with SRB. After washed with acetic acid, SRB were solubilized and absorbance were read at 540 nm in a microplate reader (SpectraMax 340PC Molecular Devices). Results were expressed relatively to the negative control (untreated cells), which were considered as 100% of cell proliferation.

4.2.7. Cell cycle analysis

Flow cytometry allows a quantitative characterization of multiple parameters of individual cells and their populations. Cell cycle analysis performed by flow cytometry allows a precise measurement of cellular DNA content and assessing the percentage of cells in the individual cell-cycle phases of interphase determined by peaks in a DNA content frequency histograms.

After treatments, cells harvest by scraping and the medium were collected to the same centrifuge tube, and centrifuged at 500 g for 3 minutes. The pellet was ressuspended in 500 µL PBS 1x and incubated on ice for 15 minutes. After this period, 1.5 mL of 100% cold ethanol (stored at -20° C) was added to pellet and incubated on ice during 15 minutes, to allow cell fixation. Cells were then washed with 3 ml PBS 1x, centrifuged at 500 g for 3 minutes at 4°C and the pellet was washed with PBS. The final pellet was ressuspended in 500 µL PBS 1x and incubated with 50 µL of RNase A solution (200 µg mL^{-1} in sodium citrate (1% w/v)) at 37° C for 15 minutes, to eliminate RNA. After 15 minutes incubation, 50 µL propidium iodide (PI) staining solution (0.5 mg mL^{-1} in sodium citrate (1% w/v)) was added and cells were mixed by vortex and incubated at room temperature for 30 minutes in the dark. Cells with red fluorescence (FL-3 channel (488/620 nm)) were analyzed in Epics XL flow cytometer (Beckman Coulter) with an average of 20 000 counts per sample. Data were analyzed with the FlowJo software to generate DNA content frequency histograms and quantify the amount of cells in each phases of interphase, including sub-G1 population assumed as death cells (cells with less than normal amount of DNA content).

4.2.8. Statistical analysis

As described in chapter 3, section 3.2.8.

4.3. Results and discussion

4.3.1. Acetate and propionate inhibits cell proliferation in CRC cell line

Acetate and propionate have been described in the literature to induce cell proliferation arrest of intestinal epithelial cells in concentration and pH dependent manner (Jan et al. 2002; Matsuki et al. 2013; Marques et al. 2013). In order to evaluate the effect of acetate and propionate on cell proliferation, CRC-derived cell line RKO were treated with different concentration of pure acetate and propionate: IC_{50} acetate (110 mM); IC_{30} acetate (74 mM); IC_{50} propionate (60 mM); IC_{30} propionate (40 mM); IC_{50} acetate & IC_{50} propionate; IC_{30} acetate & IC_{30} propionate; 12 mM acetate; 35 mM propionate and 12 mM acetate & 35 mM propionate (Figure 4.1). The last concentration of acetate and propionate are the concentration quantified in the fermentation broth when DMEM medium is used to cultivate *P. freudenreichii*. Concentrations of half maximal inhibitory concentration (IC_{50}) (110 mM; 60 mM) and 30 % maximal inhibitory concentration (IC_{30}) (74 mM; 40 mM) of acetate and propionate were determined in Ana Preto laboratory by Suellen Ferro (data not shown).

The IC_{50} of acetate and propionate reduced cell proliferation by approximately 53% and 46%, respectively. IC_{30} of acetate and propionate induced a reduction in cell proliferation of 19% and 17% respectively. 12 mM of acetate led to cell proliferation decrease of 7% and 35 mM of propionate a decrease in 12% but the condition of 12 mM of acetate as compared to the negative control no presenting statistical difference. These results are in accordance to the IC_{50} and IC_{30} of acetate and propionate determined in the laboratory as well as with the results already published by Ana Preto group (Marques et al. 2013).

Combination of acetate and propionate resulted in a higher effect in cell proliferation assed by SRB. The IC_{50} of acetate and propionate together inhibited almost totally of cell proliferation and the IC_{30} of acetate and propionate together reduced cell proliferation by approximately 80%, being a higher effect compared to the effect of IC_{30} of acids alone. Concentrations determined in broth fermentation medium (12 mM of acetate and 35 mM of propionate) when incubated the RKO cell reduced cell proliferation by approximately 21%.

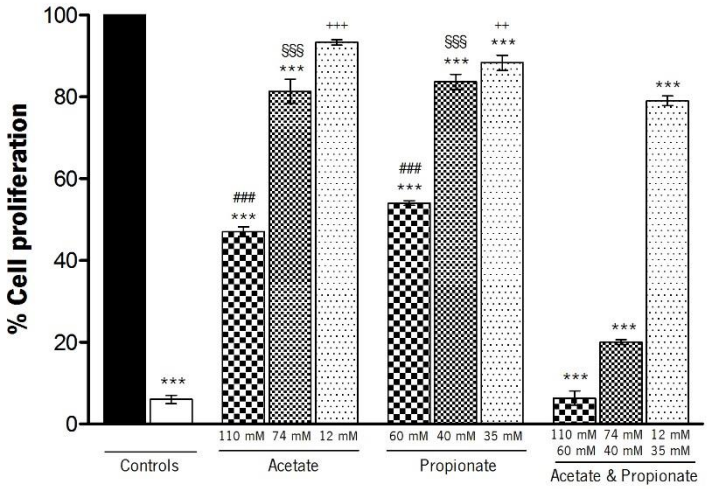


Figure 4.1. Cell proliferation analysis by SRB in CRC-derived cell line RKO treated with acetate and propionate, separately and together. Cells were incubated with IC_{50} (▩), IC_{30} (▩) and concentration of acetate and propionate detected in fermentation broth by *P. freudenreichii* (▭) for 48 h. Cells were incubated with fresh complete medium or hydrogen peroxide (1mM) as a negative (■) and positive (▭) control, respectively. Values represent mean ± S.E.M. of at least three independent experiments. *** $p<0.001$, compared with negative control cells. ### $p<0.001$, compared with IC_{50} Acetate & Propionate. §§§ $p<0.001$, compared with IC_{30} Acetate & Propionate. ++ $p<0.01$; +++ $p<0.001$ compared with Acetate & Propionate detected in fermentation broth of *P. freudenreichii*. One-way ANOVA and Tukey`s Test were used.

Lan and co-workers (2007b) showed in HT-29 colon cancer cells with approximately the same concentration of pure acetate and propionate (15 mM and 30 mM respectively) at pH 7 cell death reached 50% at 48h while with 12 mM and 35 mM of pure acetate and propionate, respectively, we obtained 21% of cell death using RKO cells. These results might reflect the importance of the cell line used in the assays, showing that different cell lines may have different sensitivity to SCFAs thus explaining that our results obtained with the RKO cell line are different of the results of Lan (Lan et al. 2007b). Lan and co-workers (2007 b) also reported that at 100 h after incubation the SCFA induce total cell death, meaning that the capability of SCFA induce death is time depend.

Our results led us to conclude that colorectal cancer cells death depend on the SCFAs concentrations and on the time of exposure to SCFA. Thus the use Propionibacteria producing SCFA might be a good tool to maintain this important role of in intestinal tract.

4.3.2. Effect of *P. freudenreichii* media in colorectal cancer cells proliferation

The CRC-derived cell line RKO was routinely cultivated in DMEM Medium supplemented with 10% FBS with antibiotics, being the medium containing all the nutrients needed for its growth. As we

aimed to evaluate the effect of *Propionibacterium* fermentation broth in RKO proliferation it was important to evaluate whether the media used to perform the fermentation broth (supernatant) satisfied the nutrition needs of RKO cells not affecting cell proliferation. We performed the incubation of *Propionibacterium* media (BM, YEL, MCHC (pH 6) and MCHC (pH 7) media) in total volume and diluted 50% (v/v) with fresh DMEM complete medium for 48h. Controls used were hydrogen peroxide (1mM) as a positive control and as a negative control fresh DMEM complete medium to assay with conditions not diluted and fresh DMEM complete medium diluted 50% (v/v) with HEPES solution (20 mM; pH 7.4) to assay with conditions diluted with fresh DMEM medium.

Propionibacterium media (BM, YEL, MCHC (pH 6) and MCHC (pH 7)) led to 90% of cell proliferation inhibition in RKO cells, what could be due to nutrient deficiency in the media (Figure 4.2A). We could observe that the dilution of *Propionibacterium* media with fresh DMEM complete medium can overcome the nutrition deficiency problem, showing that in BM medium no significant differences in cell proliferation was observed compared to the control. MCHC medium showed a cytotoxic effect in either at pH 6 and pH 7. YEL medium also showed a cytotoxic effect.

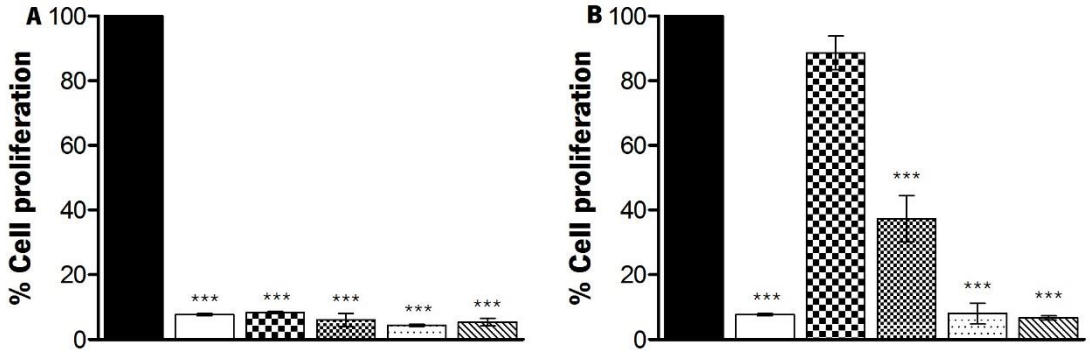


Figure 4.2. Cell proliferation analysis by SRB in CRC-derived cell line RKO treated with *Propionibacterium* media: BM medium (▩), YEL medium (▩), MCHC medium with pH 6 (▭) and pH 7 (▨). **A**) Cells were incubated with a total volume of different sterile media from *Propionibacterium* and with fresh DMEM complete medium as negative control (■A). **B**) Cells were incubated with a different sterile media of *Propionibacterium* diluted (50% v/v) in fresh DMEM complete medium. Fresh DMEM complete medium diluted (50% v/v) in HEPES solution (20 mM; pH 7.4) was used as negative control (■B). As a positive control was used hydrogen peroxide (1mM) (▭). Values represent mean ± S.E.M. of at least three independent experiments. *** $p<0.001$, compared with negative control cells. One-way ANOVA and Dunnett`s Test were used.

According with the literature (Boyd et al. 1988; Smith et al. 1995), RKO cell lines need to be cultivated in DMEM medium supplemented with 10% of FBS, being unbaled to proliferate in others media. BM medium when diluted with complete fresh DMEM medium, has no effect in cell proliferation thus BM medium is the only *Propionibacterium* medium that can be used to determine the effect for the metabolites produced by *Propionibacterium* in BM and DMEM fermentation broth.

4.3.3. Fermentation broth of *Propionibacterium freudenreichii* inhibits colorectal cancer cells proliferation

Propionibacterium produce a high amounts of acetate and propionate in BM supernatant, being the *P. freudenreichii* (29 mM acetate – 64 mM propionate) better producer than adapted *P. freudenreichii* (19 mM acetate – 40 mM propionate) as discussed in chapter 3. We could not evaluate the effect of the supernatant alone because we had to dilute the supernatant in fresh DMEM complete medium in order to overcome the nutritional deficiency problem observed previously, as BM medium *per se* has a cytotoxic effect on RKO cells. The concentration of SCFAs presented in the diluted supernatant were 14 mM – 10 mM of acetate and 32 mM – 20 mM of propionate in *P. freudenreichii* and adapted *P. freudenreichii*, respectively. The controls used were hydrogen peroxide (1mM) as a positive control, fresh DMEM complete medium diluted (50% v/v) in sterile BM medium as negative control and DMEM complete medium diluted (50% v/v) in sterile BM medium containing the pure SCFAs at the same concentrations presented in the supernatants. In order to eliminate the effect of proteins present in supernatants, the supernatants were deproteinized.

We evaluated the effect of fermentation broth obtained from *P. freudenreichii* and adapted *P. freudenreichii* grown in BM medium and diluted in fresh DMEM before RKO cells treatment the, results are presented in Figure 4.3. We could observed inhibition of cell proliferation in RKO cells although only reaching statistical significance compared to control in BM supernatant of *P. freudenreichii* ($p<0.01$) using tukey`s test. The BM supernatants show more effect in inhibiting cell proliferation in comparison with the BM diluted in DMEM medium containing the pure SCFAs, indicating that the effect of the supernatants might not be only a result of SCFA but other metabolites produced by the bacteria, once as BM supernatants were deproteinized the cytotoxicity effect is similar with the BM diluted in DMEM medium containing the pure SCFAs, although no significant difference between them were observed.

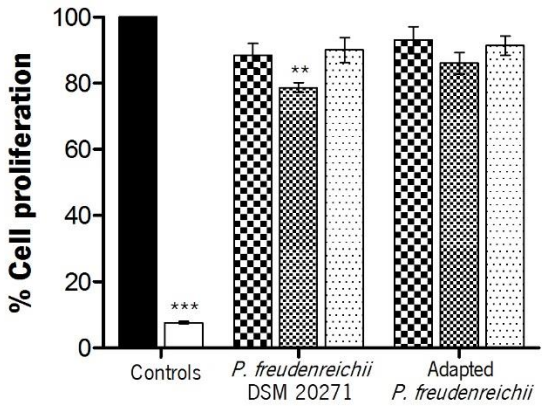


Figure 4.3. Cell proliferation analysis by SRB in CRC-derived cell line RKO treated with pure acetate and propionate (▩), BM supernatant (▩), BM supernatant deproteinized (▭) by *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii*. Cells were incubated with a supernatants diluted (50% v/v) in fresh DMEM complete medium and with fresh DMEM complete medium diluted (50% v/v) in sterile BM medium as negative control (■). Concentration presented corresponding the dilution being, in condition of *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii*, 14 mM – 10 mM of acetate and 32 mM – 20 mM propionate, respectively. As a positive control was used hydrogen peroxide (1mM) (▭). Values represent mean ± S.E.M. of at least three independent experiments. ** $p<0.01$, compared with negative control cells. No significant difference between different conditions. One-way ANOVA and Tukey`s Test were used.

In previous work, we showed that *Propionibacterium* fermented the DMEM medium, producing 13 mM of acetate and 35 mM of propionate by *P. freudenreichii* and 12 mM of acetate and 36 mM of propionate by adapted *P. freudenreichii*. In order to evaluate the effect of the DMEM supernatant, RKO cells were incubated with different supernatants *per se* or diluted with fresh DMEM complete medium for 48 h. The DMEM supernatants were deproteinized to eliminate cytotoxicity effect of proteins presented. Hydrogen peroxide (1mM) were used as a positive control, DMEM medium "consumed" by RKO cell line as negative control to condition not diluted and DMEM medium "consumed" diluted 50% (v/v) with fresh DMEM to condition diluted also with fresh DMEM medium. Pure SCFAs at the same concentration of fermentation broth were used too.

Figure 4.4A shows that both DMEM supernatants inhibit cell proliferation by 90% and this inhibition was more significant than the inhibition with DMEM "consumed" medium with pure SCFAs, indicating that the effect of DMEM fermentation broth resulted not only from the SCFAs. The deproteinization of supernatants, what decreased the cytotoxicity but the inhibition of cell proliferation was still significantly different when compared to DMEM "consumed" medium with pure SCFAs.

The results obtained with DMEM supernatant when added in the total volume could be due to the deficit in nutrients. Thus, we decide to study the effect of diluting the supernatants (see Figure 4.4B), ensuring the nutrients for RKO cells in the supernatants, consequently the concentration of SCFAs were also diluted. We observed an effect in the inhibition of cell proliferation and all the conditions showed significant difference compared to the negative control. DMEM supernatants inhibit cell proliferation between 10% - 20% although no significantly differences were observed between supernatant, supernatant deproteinized and respective control of "consumed" DMEM with pure SCFA (see Figure 4.4B).

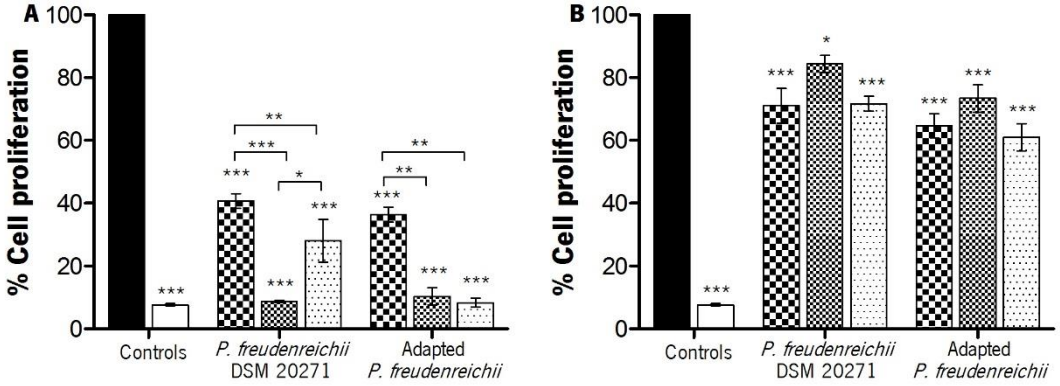
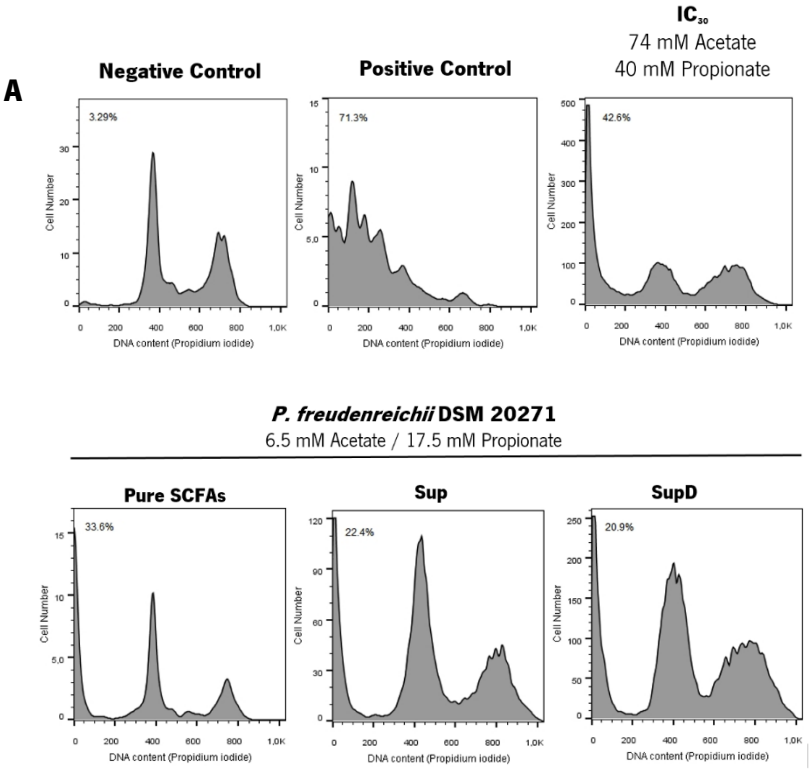


Figure 4.4. Cell proliferation analysis by SRB in CRC-derived cell line RKO treated with pure acetate and propionate (▩), DMEM supernatant (▩), DMEM supernatant deproteinized (▭) by *P. freudenreichii* and adapted *P. freudenreichii*. **A**) Cells were incubated with a total volume of fermentation broth of *P.freudenreichii* and with DMEM medium "consumed" by RKO cell line as negative control (■A). Concentration presented in condition of *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii* were 13 mM – 12 mM of acetate and 35 mM – 36 mM propionate, respectively. **B**) Cells were incubated with a supernatants diluted (50% v/v) in fresh DMEM complete medium and with fresh DMEM complete medium diluted (50% v/v) in DMEM medium "consumed" by RKO cell line as negative control (■B). Concentration presented corresponding the dilution being, in condition of *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii*, 6.5 mM – 6 mM of acetate and 17.5 mM – 18 mM propionate, respectively. As a positive control was used hydrogen peroxide (1mM) (▭). Values represent mean ± S.E.M. of at least three independent experiments. * $p<0.05$; ** $p<0.01$*** $p<0.001$, compared with negative control cells and between some conditions. One-way ANOVA and Tukey`s Test.

Both pure SCFA and propionibacteria culture supernatants from the dairy species *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici* induce apoptosis in CRC cells *in vitro* (Lan et al. 2007b; Jan et al. 2002; Hague et al. 1995; Lan et al. 2008). Lan and co-workers (2008) showed that pure SCFA produced by *P. freudenreichii* (15 mM of acetate and 30 mM of propionate) trigger apoptosis in HT-29 cells at pH 7.5 but necrosis at pH 5.5. Jan and co-workers reported that bacterial DMEM supernatant of *P. freudenreichii* subsp. *freudenreichii* strain ITG18 (12.5 mM of acetate and 36.3 mM of propionate at pH 5.6) was able to kill HT29 colon carcinoma cells and proved that this cytotoxic effect corresponding to SCFA concentration present in supernatant. Our result are according with all studies mentioned, but it is important to highlight that for the first time we can evaluate the effect fermentation broth of a *Propionibacterium* medium (BM) on CRC cell line as well as the asses to really cytotoxicity effect of *Propionibacterium* supernatant using supernatant diluted in fresh DMEM medium to eliminate the nutrient deficiency effect.

4.3.4. DMEM fermentation broth of *Propionibacterium freudenreichii* induce cell cycle arrest of CRC cells

The effect of supernatants diluted in fresh DMEM complete medium and respective controls on RKO cell cycle distribution was studied by flow cytometry measurement of DNA content of cells stained with propidium iodide (PI) and results are present in Figure 4.5. Treatment with pure SCFAs and supernatants of *P. freudenreichii* and adapted *P. freudenreichii* caused an accumulation of cells in sub-G1 phase between 20 to 35% compared to negative control cells (6.6 ± 3.3 %), but just the IC_{30} condition had a significant difference ($p<0.05$). Cells in sub-G1 phase are an indicative of cell death that could be due to necrosis or apoptosis, but in order to clarify the death process we will need to perform more specific protocols like annexin V staining. Proliferation inhibition was also observed in all conditions as shown by the decreased proportion of cells in S phase Supernatants of both strain evaluated showed similar results. These results were expected since the concentration of SCFA reported by HPLC analysis in supernatants of *P. freudenreichii* and adapted *P.freudenreichii* were similar. Supernatants of *P. freudenreichii* have 13 mM of acetate and 35 mM of propionate while supernatants of adapted *P. freudenreichii* have 12 mM of acetate and 36 mM of propionate, but to assess the cell cycle phase were used diluted concentration.



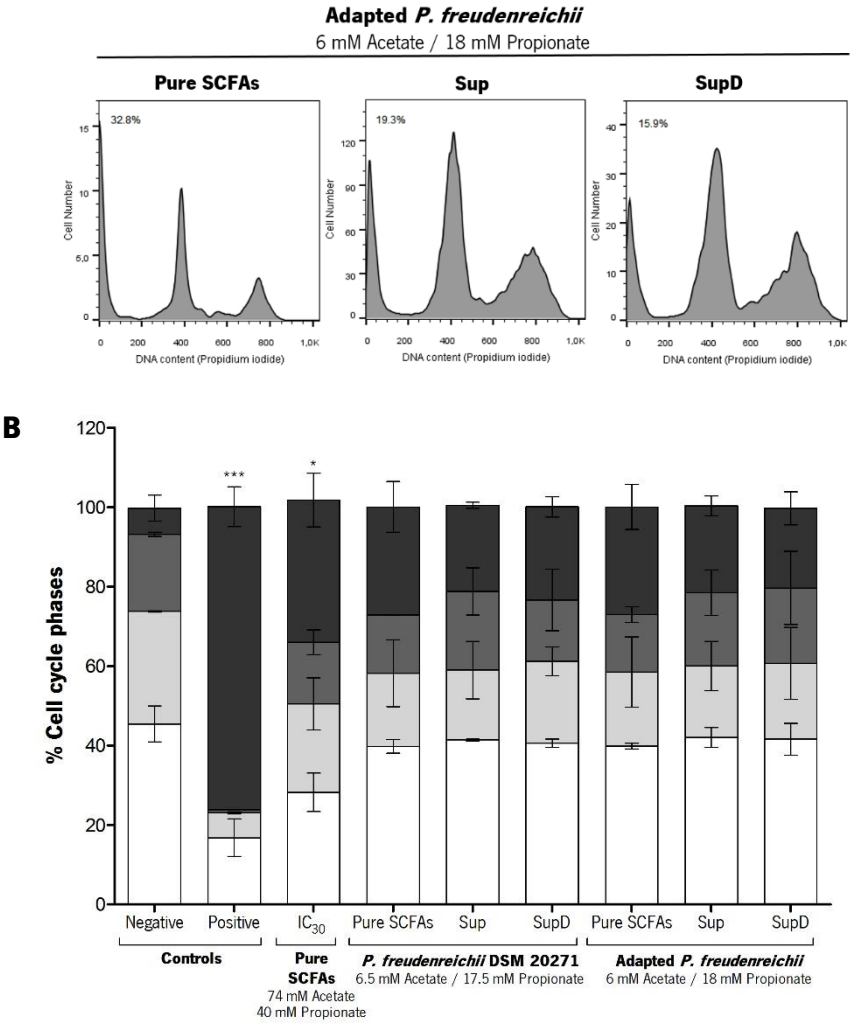


Figure 4.5. Impact of fermentation broth on cell cycle distribution. (A) Analysis of the treatment effect on the sub-G1 subpopulation of RKO cells by flow cytometry. Percentage of sub-G1 cells are shown. (B) Analysis of the distribution of cell-cycle phases: G0/G1(▭); S(▭); G2/M(▭) and SUB-G1(▬) in CRC-derived cell line RKO treated with pure acetate and propionate, DMEM supernatant, DMEM supernatant deproteinized by P. freudenreichii DSM 20271 and adapted P. freudenreichii, these conditions were diluted (50% v/v) in fresh DMEM complete medium. Fresh DMEM complete medium diluted (50% v/v) in DMEM medium "consumed" by RKO cell line and hydrogen peroxide (1mM) were used as negative and positive control, respectively. Values represent mean ± S.E.M. of at least two independent experiments. * $p<0.05$; *** $p<0.001$, comparing the percentage of sub-G1 population of treated cells with negative control and respective control of pure SCFAs. One-way ANOVA and Tukey`s Test were used.

Our results of cell cycle distribution are in concordance with previous reports from the literature (Marques et al. 2013; Lan et al. 2007b). Lan and co-workers (2007b) reported that HT-29 cells treatment with pure SCFAs (15 mM of acetate and 30 mM of propionate) caused an accumulation of cells in G2/M phase, cell cycle arrest and proliferation inhibition, as well as the cells in sub-G1 increased from 24h to 48h, being reported approximately 20% of cells in sub-G1 phase,

while our results also showed a cell cycle arrest and proliferation inhibition in all condition evaluated (fermentation broth and pure SCFA) but we cannot observed an accumulation of cells in G2/M phase.

4.4. Conclusion

In conclusion, our results show that when pure acetate and propionate are incubated together show a higher response than when used separately. These results highlight the importance of all SCFAs in the digestive tract. We could observe a cytotoxicity effect in different *Propionibacterium* media being the BM medium diluted with DMEM medium the only condition tested as fermentation broth of BM medium due to the BM toxicity *per se*. Fermentation broth of *P. freudenreichii* and adapted *P. freudenreichii* showed similarly results, and the difference observed might be related to the different concentration of acetate and propionate presented. For the first time we showed that the diluted BM *Propionibacterium* fermentation broth led to the inhibition of cell proliferation even with lower amounts of acetate and propionate. We also reported that DMEM *Propionibacterium* fermentation broth led to the inhibition of cell proliferation, cell cycle arrest and increase in sub-G1 phase, being with agreement with the literature (Jan et al. 2002; Lan et al. 2008; Marques et al. 2013), but our results not showed an accumulation of cells in G2/M phase. The results obtained in this present work demonstrated that *Propionibacterium* can be used as potential prebiotic by preventing or treating CRC.

Chapter 5. | Effect of colorectal cancer cells conditioned medium in growth and biotransformation performance of *Propionibacterium*

5.1. Background

Microorganisms reside symbiotically in the gut benefiting the host. Microbiota, a large and diverse community of microorganism, have a critical role in the evolution of the intestinal functions and in the overall health of the host (Saavedra and Dattilo 2012). Changes in microbiota composition can have major consequences, both beneficial and harmful, for human health (Guinane and Cotter 2013). Indeed, it has been suggested that disruption of the gut microbiota can be significant in response to pathological intestinal conditions such as obesity (Ley et al. 2006), malnutrition (Kau et al. 2011), systematic diseases such as diabetes (Qin et al. 2012) and chronic inflammatory diseases such as ulcerative colitis and Crohn's disease (Frank et al. 2007).

Relation between colorectal cancer (CRC) and gastrointestinal microbiota has been studied. These studies reported that patients with CRC have significant changes in gut microbiota, significant decreases in gut short chain fatty acids (SCFAs) concentrations and a significant increase in gut pH compared with healthy individuals (Scanlan et al. 2008; Sobhani et al. 2011; Ohara et al. 2010; Ohigashi et al. 2013).This changes in microbiota can be a result or a cause of the CRC, this is a uncertainty that several researchers want to clarify, thus this is an issue that is poorly understood.

Probiotics are part of our diary food being presented in fermentation food as yogurts, cheese, bread and others. Probiotics are live microbial food ingredient which play an important role in the maintenance of health, mainly in maintenance of healthy microbiota (Salminen et al. 1998). *Propionibacterium freudenreichii* is a probiotic bacteria that is considered safe for their long history of consumption in Swiss type cheeses and other fermented products. Several studies have reported its probiotics beneficial effects on health as a result of its functional metabolites like acetate and propionate (Cousin et al. 2012a; Dalmasso et al. 2011; Borowicki et al. 2011; Thierry et al. 2011; Ammar et al. 2013; Chen et al. 2012; Lan et al. 2007a).

Propionibacteria have been reported as a probiotic bacteria that possesses mechanisms of cancer prevention at a cellular level including the induction of apoptosis and inhibition of proliferation of colon tumor cell lines but not on normal epithelial cells, via their metabolites, namely SCFA (Jan et al. 2002; Marques et al. 2013; Bindels et al. 2012; Abrahamse et al. 1999; Emenaker et al. 2001). To develop *P. freudenreichii* with this anti-neoplastic effects we need to be able to grow the bacteria and assure that the bacteria remain metabolic active in co-culture with colorectal cancer cells. Thus our aim was to evaluate the effects of colorectal cancer cells conditioned medium in the

growth and biotransformation performance by *Propionibacterium freudenreichii* DSM 20271 and digestive stress adapted *Propionibacterium freudenreichii*.

5.2. Materials and methods

5.2.1. Cell Culture

As described in chapter 4, section 4.2.2.

5.2.2. Conditioned medium by RKO cells

RKO cell line was cultivated in 75 cm^2 tissue culture flasks (TPP, Tissue culture flask 75 cm^2, German) at a density of $20x10^4$ cell mL^{-1}, using DMEM medium supplemented with 10% FBS without antibiotic. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 72 hours to achieve a density between 80-100% in order for the medium being partially consumed without causing cell stress. The medium was removed to 50 mL falcons (Orange scientific, 50 mL Tube conical, Belgium) in aseptic conditions. Supernatant were stored at 20° C with parafilm. This supernatant are hereafter referred to as 'conditioned medium' (CM).

Six 75 cm^2 tissue culture flasks of RKO cell line were cultured to obtain a final volume of 90 ml. This procedure was performed in duplicate.

5.2.2.1. Quantification of glucose concentration in the medium

Determination of glucose concentration in conditioned medium was measured through high performance liquid chromatography (Varian, Metacarb 87H column) equipped with RI detector (KNAUER, RI Detector K - 2300, German). The column was eluted isocratically with H2SO4 0.01N using a flow rate of 0.7 ml min-1.

Standard solution of glucose of known concentration was used to elaborate the calibration curve of glucose quantification. An area of "x" represent (0.0026x + 36.1222) mg L-1 glucose (x > 0). The samples were centrifuged at 21130 g for 5 min (Eppendorf, Centrifuge 5424R, Spain), filtered with 0.2 µm filters (GE Healthcare Life Science, Whatman™, Spartan 12/0.2 RC, Germany) and it added 0.8 mL in a screw cap vial (Labbox, Srew vial for chromatography, Spain) containing 0.2 mL of Cellobiose as internal standard.

5.2.3. Bacteria Culture

As described in chapter 4, section 4.2.1.1.

5.2.4. Biomass and short-chain fatty acids production assays

We perform the sterilization of serum bottles of 70 mL, these serum bottles were prepared as described in chapter 3, section 3.2.1. The condition presented in Table 5.1 were added to serum bottles in aseptic conditions.

Conditioned medium was also 50 % (v/v) diluted in fresh DMEM medium supplemented with 10% FBS. The controls were performed with DMEM medium supplemented with 10% FBS diluted in HEPES solution (20 mM; pH 7.4) to perform final glucose concentration correspondent to conditioned medium.

Table 5.1. Conditions used to evaluate the effect of conditioned medium in growth and SCFAs production by Propionibacterium freudenreichii.

Bacteria	Conditioned medium	Control
Propionibacterium freudenreichii DSM 20271	CM (1 g L^{-1} Glucose) CM½ (2.7 g L^{-1} Glucose)	CCM (1 g L^{-1} Glucose) CCM½ (2.7 g L^{-1} Glucose)
Adapted *Propionibacterium freudenreichii*	CM (1.8 g L^{-1} Glucose) CM½ (3.2 g L^{-1} Glucose)	CCM (1.8 g L^{-1} Glucose) CCM½ (3.2 g L^{-1} Glucose)

CM, Conditioned medium; CM½, Conditioned medium diluted in fresh DMEM medium (50% v/v); CCM, Control with DMEM medium diluted in HEPES solution.

P. freudenreichii and adapted *P. freudenreichii* were firstly inoculated in YEL medium at 96 hours before the initial of experience and transferred to a pre-inoculum in YEL medium after 64 hours. We insure that bacteria strains were metabolically active. These pre-inoculums were used to inoculate the condition mentioned in Table 5.1.

All experience were run in duplicated bottles and samples were taken at several time points (0; 16; 24; 40; 48; 64; 72h) in aseptic conditions using a syringe to ensure the absence of oxygen inside the bottles. Optical density was measured (650 nm) and samples were analyzed for SCFAs production by HPLC as explained in chapter 3, section 3.2.4.

5.2.4.1. Bacteria biomass determination

As described in chapter 3, section 3.2.3.1.

5.2.4.2. Short-chain fatty acids quantification

As described in chapter 3, section 3.2.3.2.

5.2.5. Statistical analysis

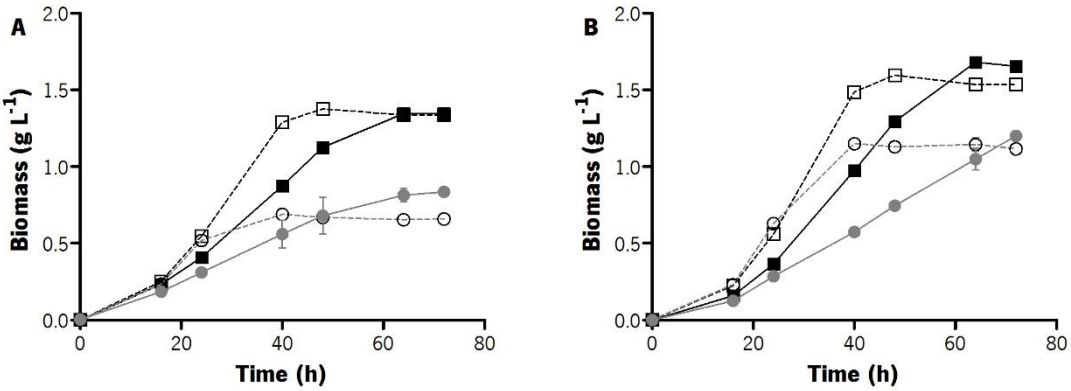
As described in chapter 3, section 3.2.8.

5.3. Results and discussion

In order to evaluate the effect of colorectal cancer in growth and biotransformation performance of the probiotic bacteria, *Propionibacterium freudenreichii* conditioned medium of RKO colorectal cancer cell line was used. Conditions tested were: conditioned medium (CM) and conditioned medium diluted (50% v/v) in fresh DMEM medium (CM½) and respective controls which are prepared with fresh DMEM diluted in HEPES solution at glucose concentration in respective conditioned medium. These conditions were tested in two strains; *P. freudenreichii* DSM 20271 and digestive stress adapted *P. freudenreichii*.

Conditioned medium was collected from RKO cells and glucose was quantified by HPLC. As we collected conditioned medium in two different times, we have different glucose concentrations, 1 g L^{-1} and 1.8 g L^{-1} glucose and the corresponding dilution in fresh DMEM medium were 2.7 g L^{-1} glucose and 3.2 g L^{-1} glucose. We used conditioned medium with 1 g L^{-1} glucose to evaluate the effect of CRC in *P. freudenreichii* DSM 20271 and the other conditioned medium to adapted *P. freudenreichii*, as describe in Table 5.1.

Figure 5.1 show the effect of colorectal cancer conditioned medium in the growth and biotransformation performance by *P. freudenreichii*. Our results showed that colorectal cancer cells conditioned medium affect the growth and biotransformation performance of *P. freudenreichii*. The growth of *P. freudenreichii* was negatively affected, having a slower growth with a long stationary phase, but the final biomass concentration was not affected, having similar biomass when compared to the control even at higher SCFAs concentrations. The results in the two strains, *P. freudenreichii* and adapted *P. freudenreichii* were similar leading us to conclude that they behave similarly.



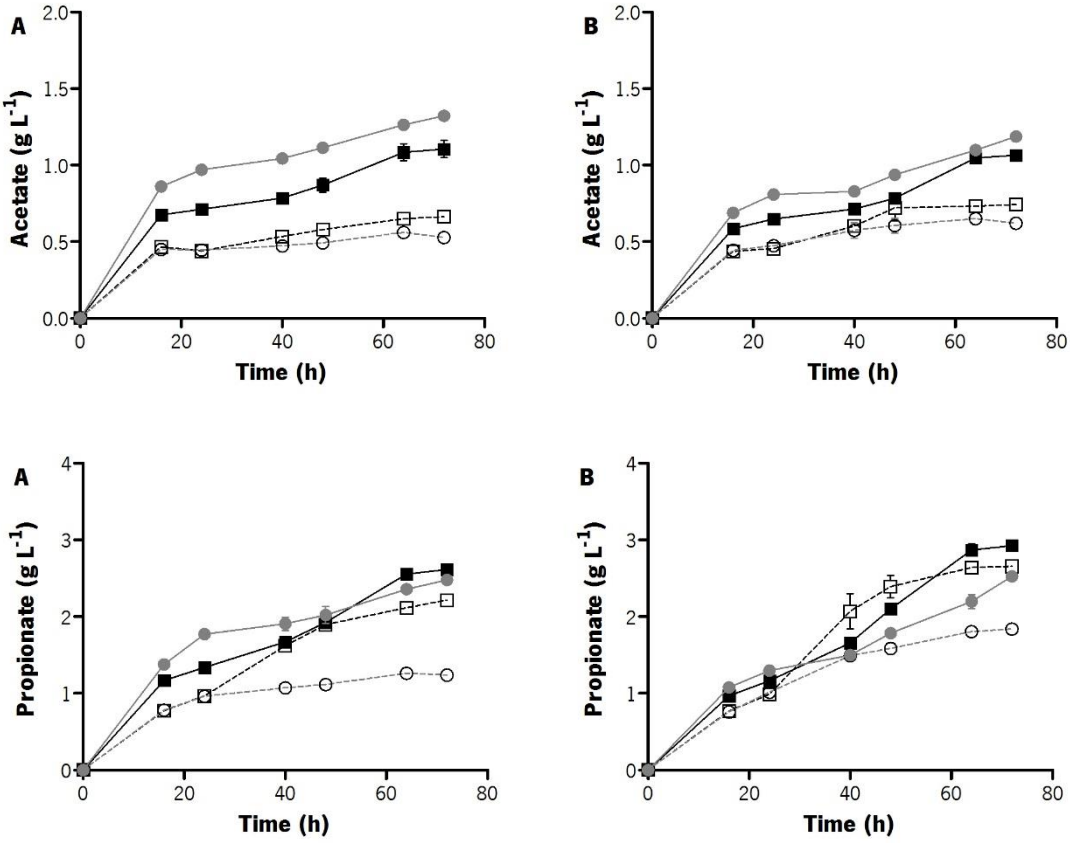


Figure 5.1. Biomass, acetate and propionate production by *P. freudenreichii* DSM 20271 (A) and adapted *P. freudenreichii* (B) at pH 7, 37 °C without agitation. Conditioned medium (-●-); Control of conditioned medium (-○-); Conditioned medium diluted 50% (v/v) in fresh DMEM medium (-■-) and Control of conditioned medium diluted (-□-). Concentration in gram per liter of biomass, acetate and propionate over time in hours. Each condition was run in duplicate and the mean ± SEM are represented.

Conditioned medium showed higher SCFAs production when compared with respective control, being this improvement more pronounced in acetate production. These results are very interesting as in the literature it is reported a microbiota disruption in patients with CRC and there is an uncertainty if microbiota is a result or a cause of CRC disease (Scanlan et al. 2008; Sobhani et al. 2011; Ohara et al. 2010; Ohigashi et al. 2013). Our results may help in decode this doubt, as we could not observe a negative effect in the capabilities of propionibacteria as despite the slower growth there was an improvement in the biotransformation performance, meaning that *P. freudenreichii* is able to grow in co-culture with colorectal cancer cells lines with a biotransformation performance improved. This biotransformation performance induced cell death of colorectal cancer cells as we showed in chapter 4.

In order to access the differences between conditioned medium and respective controls and also evaluate the performance of *P. freudenreichii* DSM 20271 compared to adapted *P.*

freudenreichii, parameters as growth rate, yield and productivity were calculated (see Figure 5.2). As mentioned the growth was affected by CRC cells conditioned medium and the results of growth rate shows statistic significant different between conditioned medium and respective controls. As well as yield and productivity parameters shows statistic significant different between conditioned medium and respective controls being the conditioned medium the condition with better performance.

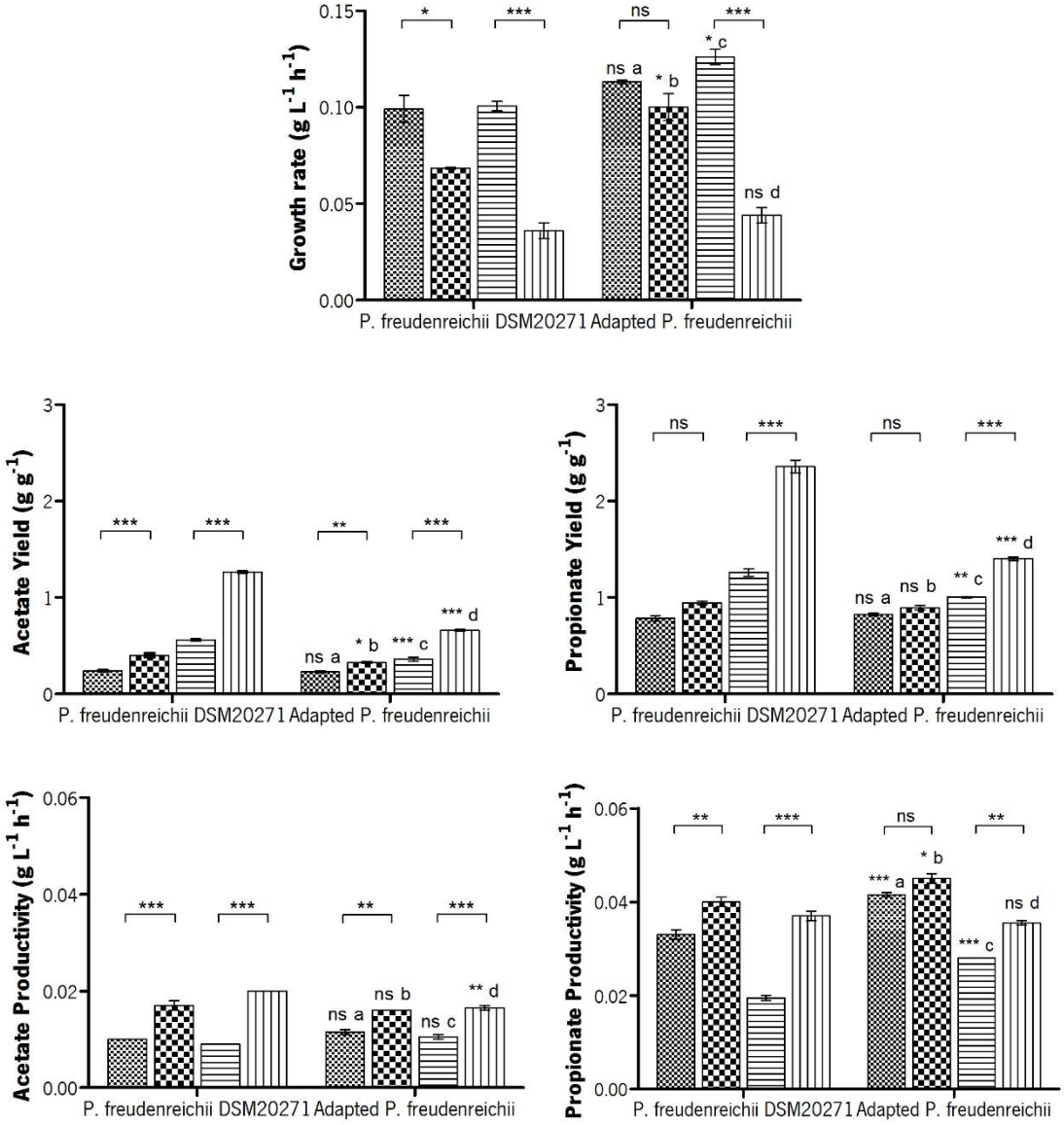


Figure 5.2. Bach fermentations kinetic of *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii* at pH 7, 37 °C without agitation. Conditioned medium (▤); Control of conditioned medium (▥); Conditioned medium diluted 50% (v/v) in fresh DMEM medium (▩) and Control of conditioned medium diluted (▩). Concentration in gram per liter per hour of growth rate and productivity; in gram per gram of yield. Each condition was run in duplicate and the mean ± SEM are represented. Values significantly different between conditioned medium conditions and respective controls as well as conditioned medium diluted 50% (v/v) in fresh DMEM medium (a); control of conditioned medium diluted (b); conditioned medium (c) and control of

conditioned medium (d): * $p<0.05$; ** $p<0.01$; *** $p<0.001$. “ns” report that no significant different. One-way ANOVA and Tukey`s Test were used.

The improvement of SCFAs production in conditioned medium may result due to metabolites excretes by RKO cell lines. Lactate is considered a waste product of glycolysis in cancer cells and it plays an important role in cancer development, maintenance, and metastasis (Doherty and Cleveland 2013). Our previous results showed the higher efficient of *P. freudenreichii* to ferment lactate with higher yield of SCFAs production. Thus the possible presence of lactate in CRC cells condition medium may be the reason of SCFAs production improvement in CRC cells conditioned medium conditions.

Is important to report that the difference in behavior between *P. freudenreichii* DSM 20271 and adapted *P. freudenreichii* were not evident, the *P. freudenreichii* DSM 20271 had a better performance than adapted *P. freudenreichii*, but this difference and behavior has already been reported in chapter 3.

5.4. Conclusion

Propionibacteria play an important role in maintenance of healthy body as well as its probiotics beneficial effects against the colorectal cancer. To development this function the propionibacteria has to survive to digestive stress and remain metabolic active to growth in the intestinal tract. Also it is important that in the presence of cancer cells, propionibacteria remain metabolic active to produce SCFAs in order to induce cancer cells death.

Our results showed that the CRC cells conditioned medium do not negative affected the performance of *P. freudenreichii*. Moreover we showed that CRC cells conditioned medium improve the SCFAs production by both *P. freudenreichii*. With these results we can conclude that *P. freudenreichii* is able to grow and to remain metabolically active in conditioned medium by colorectal cancer cells, leading to an improvement of the amount of SCFAs produced.

Chapter 6. | General conclusions and future perspectives

6.1. Main conclusions

P. freudenreichii subsp. *freudenreichii* DSM 20271, as well as the adapted *P. freudenreichii* to digestive stress herein developed, showed a great potential regarding the production of acetate and propionate as end-products. Moreover, the fermentation broth of these bacterial cultures inhibited the proliferation of the colorectal cancer cells RKO and led to cell cycle arrest. Although the main goal of the current thesis was not fully accomplished, our findings established the basis for further manipulations and optimizations of the bacterial strain towards an increased production of SCFA.

The main aim was to improve the *P. freudenreichii* features in order to further use it as a nutraceutical towards the prevention/treatment of colorectal cancer, thus it must be able to grow and produce acetate and propionate under defined conditions, namely 37° C, without agitation, in a medium that mimics the content of the human colon (MCHC) and in a medium that is commonly used by the colorectal cancer cells (DMEM). Moreover, these bacteria must resist the harsh conditions that occur during the digestion, so-called digestive stress. In the first part of this work we optimized these conditions. *P. freudenreichii* produced biomass, acetate and propionate in DMEM with higher yields and productivities as compared to the other media evaluated. However, the results obtained with the MCHC medium were not favorable regarding SCFAs production. Among all the media studied, the BM medium was found to be the best concerning the bacteria biotransformation performance, mainly when this medium was supplemented with glycerol. Furthermore, it was found that *P. freudenreichii* is not capable of metabolizing lactose. Different concentrations of acetate and propionate were produced by propionibacteria depending on the carbon source used, being glycerol the one that led to higher yields and productivities, followed by glucose.

Colorectal cancer cells, RKO, treated with pure acetate and propionate, as well as treated with the fermentation broth of *Propionibacterium* were found to change their behavior. Indeed, the exposure to the pure compounds or fermentation broth resulted in the inhibition of cell proliferation and the accumulation of cells in the sub-G1 phases. These results are in good agreement with the literature, although additional experiments are required to unequivocally demonstrate that that the fermentation broth is able to induce cell death of CRC cells by apoptosis.

The co-culture of *P. freudenreichii* and CRC cell lines has been reported as possible and favorable for the bacteria. In the current work we evaluated the impact on the propionibacteria

performance of its growth in a conditioned medium from colorectal cancer cells. *P. freudenreichii* not only exhibited a better growth, but also an improvement in the acetate and propionate production.

The adapted *P. freudenreichii* to the digestive stress developed in this work showed a little decrease in its performance as compared to the normal bacteria, however this decrease was more pronounced in the YEL and BM media. Moreover, it is important to mention that the most important asset to improve is the bacteria ability to survive the *in vivo* conditions even if a slight decrease in the SCFA production is observed.

Altogether our results suggest that *P. freudenreichii* promotes a cytotoxic effect on CRC cells, *via* their metabolites, and that the CRC cells increases the acetate and propionate production by *P. freudenreichii* which in turn will lead to an increase cytotoxic effect on the CRC cells.

6.2. Suggestions for future work

Based on the results gathered in the current thesis, it is clear that there is still a great amount of work required in order to accomplish our major objective. Firstly, the ongoing random mutagenesis experiments must be concluded. Currently, none of the cultures using different conditions/challenges has grown. Thus, whenever we will get some colonies, these cultures will have to be further characterized regarding their growth and ability to produce increased amounts of SCFA. Furthermore, the fermentation broths of those mutant strains will have to be tested against the RKO cells. Even if we can develop robust *P. freudenreichii*, resistant to acid pH, high bile salts concentration and high acetate/propionate concentrations, the issue related with the improvement of acetate/propionate production using the MCHC medium still needs to be addressed.

Since the optimal culture conditions were established, further optimization of the process should be focused on the strain improvement by genetic manipulation, which is an emergent area on the CRC research, as it can be an approach to deliver important anti-neoplastic factors to the colon. As future work, we suggest three different approaches to manipulate the propionibacteria, namely based in the metabolic pathways, butyrate and transporters. The enzymes that are known to have a critical role in the production of SCFA could be overexpressed towards an increased production of acetate and propionate. Moreover, butyrate has been reported as an important SCFA leading to the death of malignant cells and the promotion of healthy colonocytes. Therefore, *P. freudenreichii* could be manipulated to produce butyrate which would improve its beneficial effects. Another approach that could be implemented concerns the use of acetate and propionate

transporters. As discussed along the thesis, acetate and propionate can be toxic to the bacteria at given concentrations, thus limiting the production of these SCFAs. However, the effect can be contoured by the use/design of adequate transporters in the bacteria.

Finally, additional assays *in vitro* with colorectal cancer cells should be conducted to assess the type of cell death that is occurring. Moreover, other colorectal cancer cell lines and a "normal" colon cell line, such as NCM460 should be used to evaluate the effect of the *P. freudenreichii* fermentation broth in different cells for comparison purposes, as well as to assess the effect of the fermentation broths on "normal" colon cells. Furthermore, co-culture experiments with colorectal cancer cells and *P. freudenreichii* are required to support the findings reported in this thesis.

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