Universidade de Aveiro Departamento de Biologia
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Ana Cristina Marques da Silva

Microbiota and colorectal cancer: a preliminary study in Portuguese patients

Microbiota e cancro colorectal: um estudo preliminar em pacientes portugueses

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Catarina Marques, Cientista Convidada do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Isabel da Silva Henriques
Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Juan Carlos Mellídez Barroso
Médico Oncologista do Centro Hospitalar do Baixo Vouga, E.P.E.

Catarina Pires Ribeiro Ramos Marques
Cientista Convidada do Departamento de Biologia da Universidade de Aveiro

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palavras-chave Comunidade bacteriana, mucosa intestinal, DGGE, genotipagem, atividade antimicrobiana, resistência a antibióticos, aplicações biotecnológicas, terapias complementares

resumo O cancro colorectal (CCR) é o terceiro tipo de cancro fatal no mundo e, nos últimos anos, Portugal tem vindo a assistir a um aumento da sua incidência. Dada a relevância da simbiose entre a microflora intestinal e o hospedeiro para a manutenção da homeostase do organismo, vários estudos têm-se focado na análise do microbiota associado a situações de saúde e doença, nomeadamente ao CCR. Cada vez mais é relevante conhecer a comunidade microbiana intestinal associada a CCR, pois pode constituir uma ferramenta para diferentes aplicações clínicas no âmbito desta patologia. Portanto, o presente estudo pretendeu realizar uma caracterização preliminar da comunidade bacteriana não cultivável e cultivável, extraída a partir de tecidos tumorais (TT) e tecidos adjacentes saudáveis (TN) da mucosa intestinal de pacientes portugueses que apresentem CCR. Adicionalmente testou-se o potencial antimicrobiano e a resistência a antibióticos das estirpes bacterianas isoladas no sentido de verificar como se comportam em situações de stresse (presença de bactérias patogênicas e antibióticos). Por fim, é apresentada uma revisão sumária acerca das aplicações de microrganismos como estratégias terapêuticas complementares para o combate do CCR. De um modo geral, observou-se alguma diferença na diversidade da comunidade bacteriana entre TN e TT de cada paciente, conforme os perfis genéticos obtidos por DGGE. No que concerne as bactérias isoladas foram identificados alguns géneros semelhantes em TN e TT (*e.g.*, *Escherichia*, *Klebsiella*, *Pseudomonas*), muito embora, outros tivessem sido registados apenas em TN (*e.g.*, *Citrobacter*) ou TT (*e.g.*, *Enterococcus*). Alguns dos isolados bacterianos revelaram resistência a bactérias Gram-positivas e Gram-negativas, apresentando todos eles resistência a, pelo menos, três antibióticos diferentes. Estas respostas auxiliam na compreensão da resposta do microbiota a agressões infeciosas em situações de CCR. Por outro lado, e tendo em conta a relevância do microbiota na evolução da doença, as potencialidades biotecnológicas das bactérias têm vindo a ser exploradas para terapias complementares ou adjuvantes no combate ao CCR. Estas envolvem a modelação do microbiota através de pro- e prebióticos, transplante de microbioma fecal e terapia bacteriofágica, para além de outras estratégias inovadoras basedas em sistemas CRISPR, genes bacterianos essenciais e de resistência, e sistemas de comunicação entre bactérias.

keywords Bacterial community, intestinal mucosa, DGGE, genotyping, antimicrobial activity, resistance to antibiotics, biotechnological applications, complementary therapies

abstract The colorectal cancer (CRC) is the third deadly cancer in the world, and in the last years its incidence rate has been increasing in Portugal. Given the relevance of the symbiosis between the intestinal microbiota and the host for body homeostasis, many studies have been focusing on the analysis of the microbiota associated with health and disease scenarios, namely with CRC. It is increasingly more important to know the microbial community associated with CRC, once it can be exploited as a tool for different clinical applications against CRC. As such, the present study intended to perform a preliminary characterization of the non-cultivable and cultivable bacterial community isolated from tumoral (TT) and adjacent healthy (TN) mucosa tissues of patients with CRC. Additionally, it was tested the antimicrobial potential and antibiotic resistance of cultivable bacterial isolates in order to verify how they behave under stressful conditions (*i.e.*, presence of pathogens and antibiotics). It is also presented a short review on the applications of microorganisms or their abilities to fight CRC. In a general view it was observed some difference between the diversity of bacterial community from TN and TT samples, according to the DGGE profiles. Identical genera of bacteria were identified in TN and TT samples (*e.g.*, *Escherichia*, *Klebsiella*, *Pseudomonas*), although some were only found in TN (*e.g.*, *Citrobacter*) or TT (*e.g.*, *Enterococcus*). Some bacterial isolates showed antimicrobial activity against Gram-positive and Gram-negative pathogens, and all of them were resistant to at least three different antibiotics. These responses help to understand the behavior of gut bacteria under infectious aggressions, which often occur in CRC-affected patients. On the other hand, given the relevance of gut microbiota on CRC development, the biotechnological abilities of bacteria have been explored as complementary or adjuvant therapeutics for controlling CRC. They mainly involve microbiota modulation through the consumption of pro- and prebiotics, and fecal microbiome transplantation, bacteriophage therapy, but also other groundbreaking strategies targeting CRISPR, essential and resistant bacterial genes, and quorum sensing systems.

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Abbreviations

A_{600}	Absorbance at λ600nm
BFT	*Bacteroides fragilis* Toxin
CDI	*Clostridium difficile* Infection
CEA	Carcinoma Embryonic Antigen
CRC	Colorectal Cancer
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats associated (Cas) genes
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
dNTPs	Desoxirribonucleotídeos Fosfatados
ErbB2/3	Receptors Tyrosine Kinase 2 and 3
ETBF	Enterotoxigenic *Bacteroides fragilis*
FMT	Fecal Microbiome Transplantation
IBD	Inflammatory Bowel Disease
IBS	Inflammatory Bowel Syndrome
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
$MgCl_2$	Magnesium chloride
p53	Protein acts as a Tumor Suppressor (molecular mass 53 kDa)
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pks	Polyketide Synthases
PMC	Pseudomembranous Colitis
QS	Quorum Sensing
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
SCFAs	Short-chain fatty acids
TAE	Tris-Acetate-EDTA
TEMED	Tetramethylethylenediamine
TN	Tissue Sample Not Affected to CRC
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TT	Tissue Sample Affected to CRC
VEGF	Vascular Endothelial Growth Factor

CHAPTER I

Chapter I. General Introduction

1.1 Colorectal Cancer

1.1.1 Incidence, Mortality and Survival Rates

Colorectal cancer (CRC) is the third cancer with higher incidence worldwide for men (746 thousand cases) and the second for women (614 thousand cases) (Globocan, 2012), being this ranking similar for the Portuguese context in 2010 (Miranda *et al*., 2015). In 2016, the United States estimated 70,820 and 63,670 new CRC cases in men and women, respectively (Marley and Nan, 2016).

During the past few years the CRC incidence rates have decreased more than 4% per year. This decline has been attributed to its earlier detection and removal of pre-cancerous polyps as a result of increased CRC screening. However, this trend is not in accordance for all geographic regions. For instance, CRC is still highly incident in North America and Europe, but other countries usually evidencing low incidence rates, like Japan and Thailand, are starting to present an increased risk level (Siegel and Jemal, 2016; Marley and Nan, 2016). Notwithstanding, CRC is the third deadliest cancer in the world, with 694 thousand deaths (Globocan, 2012). In the United States were reported 26,020 and 23,170 deaths respectively for males and females, in 2016 (Marley and Nan, 2016). In Portugal, though, this cancer provoked 3549 deaths in 2011 and in 2014 the numbers raised to 3760 deaths (Miranda *et al*., 2013; Miranda *et al*., 2015). As such, CRC still claims for a considerable research effort towards its early detection and efficient treatment. This is particularly relevant to enhance the survival rates, which are between 65% and 58% after 5 and 10 years of diagnosis, respectively (Siegel and Jemal, 2016).

1.1.2 Risk Factors

The CRC usually develops slowly, over a period of 10 to 20 years, through a series of progressive changes in the histological integrity of the mucosal epithelium in the colon, characterized as in other oncologic cases by the abnormal and uncontrollable development of cancer cells derived from abnormal changes in the cell and in genetic traits (Sears and Garrett, 2014; Siegel and Jemal, 2016). These changes can be associated with the coaction of internal and external factors (Figure 1) to which the host may be continuously exposed during his/her life, leading to alterations in the bowel microenvironment, genetic integrity and stability of microbiota (Candela *et al*., 2014; Dulai and Keku, 2015; Keku *et al*., 2015; Nistal *et al*., 2015).

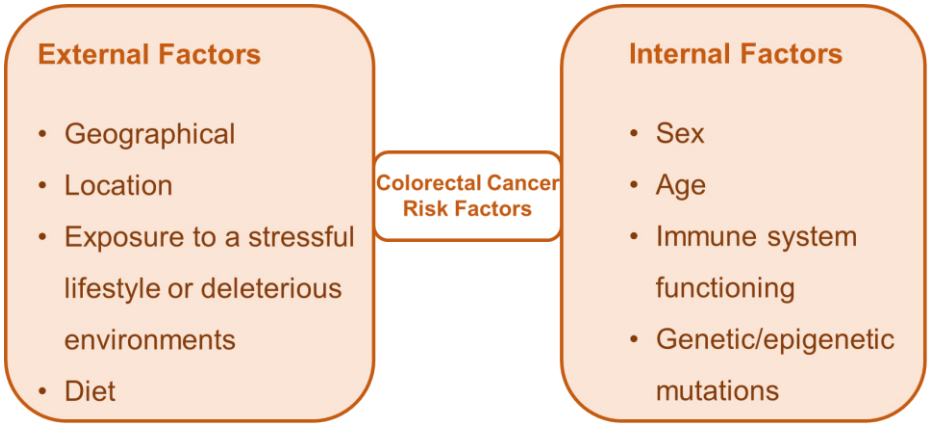


Figure 1. Scheme representing the factors associated with the development of colorectal cancer

Age and sex can have a relevant impact on the development of CRC. There is a CRC incidence in world of 10.1% for men and 9.2% for women. In American citizens it is estimated that 90% of new cases of CRC occur in people with 50 years old, being the median age of cancer diagnosis is 69 and 73 years for men and women, respectively (Globocan, 2012; Siegel and Jemal, 2016). As such, age and sex factors influence CRC occurrence.

Genetic mutations such as in oncogene KRAS and cellular tumor antigen p53 or epigenetic alterations, like DNA methylation or modification of antisense RNA were described as factors that trigger tumor initiation and progression (Markle *et al.*, 2010; Dulai and Keku, 2015; Nistal *et al.*, 2015).

The hereditary burden contributes as well for disease pathogenesis. The most common and well-known genetic syndrome that affects 1 in 35 CRC patients is the Lynch syndrome, also known as the hereditary nonpolyposis CRC. However, individuals with this syndrome are predisposed to many other types of cancer, though the risk for CRC is far more enhanced. Other example of predisposing genetic syndrome, is familial adenomatous polyposis. It is characterized by the development of hundreds to thousands of colorectal polyps in affected individuals that, without immediate intervention, approaches 100% of risk to develop cancer until the 40 years old age (Siegel and Jemal, 2016).

Among behavioral factors, a toxic lifestyle namely related with tobacco and alcohol consumption may induce great modifications in the normal immune function, bowel inflammation (Bilinski *et al.*, 2012), enhanced oxidative stress, genetic mutations (Kim *et al.*, 2012) and, ultimately, to CRC development (Øyri *et al.*, 2015; Wong *et al.*, 2016; Siegel and Jemal 2016). On the other hand, the geographical localization may also

interfere with the gut microenvironment and microbiota, leading to a varied incidence of bowel diseases and CRC. Previous studies demonstrated that people from regions with low CRC incidence like *Africa*, were more affected when they immigrated to high risk countries (*e.g.*, United States) (O'Keefe *et al.*, 2007; Ou *et al.*, 2013). Such evidence was explained by diet habits, mostly related with an increased consumption of fat-, meat-rich and processed foods (Ou *et al.*, 2013). Moreover, low fiber ingestion together with a more sedentary lifestyle are growing side by side with the number of obesity cases, particularly in western countries (Center *et al.*, 2009). These conditions have been mentioned as promoters of bowel pathologies and CRC (Kim *et al.*, 2012; Yang *et al.*, 2013). Some studies described the relevance of fiber consumption for controlling the evolution of inflammatory bowel disease (IBD) into CRC, which jointly with physical exercise and generally healthy diet can reduce the risk of cancer (Wong *et al.*, 2016). A concerning aspect is the increasing occurrence of CRC in young people presenting a unhealthier lifestyle (Bishehsari *et al.* 2014). Additionally, a high risk of CRC development is also associated with some diseases besides obesity, such as diabetes (Dominianni *et al.*, 2015; Dulai and Keku, 2015; Wong *et al.*, 2016). The use of pharmaceuticals can indeed alter, sometimes permanently, the taxonomic, genomic, and functional profiles of the human gut microbiota. This is particularly drastic whenever antibiotics are consumed, once they contribute to the development of bacterial resistance, by lowering the diversity of sensitive bacteria, increasing horizontal transfer of antibiotic-resistance genes and enabling the dominance of pathogenic and resistant strains. Therefore, the chronic ingestion of antibiotics may change bacterial community composition (Panda *et al.*, 2014; Mikkelsen *et al.*, 2016). Besides, the protective action of a balanced microbial community is likely to be disrupted, what brings negative consequences for the host (Modi *et al.*, 2014; Becattini *et al.*, 2016; Mikkelsen *et al.*, 2016).

The recent awareness of the huge relevance of intestinal microbiota in host homeostasis and response to the exposure to different factors, is pushing research forward to get a deeper understanding of host-microbiota symbiosis and how can it be explored and used in different clinical perspectives.

1.2 The Human Intestinal Microbiota

The human body sustains a vast and complex microbial ecosystem, which is usually referred as the human microbiome. The human microbiome concept was initially proposed by Joshua Lederberg as the "ecological community of commensal, symbiotic,

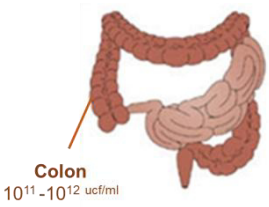
and pathogenic microorganisms that literally share our body space" (Lederberg and McCray, 2001), being associated to different body niches (*e.g.,* buccal cavity, vagina, skin, gut, lungs, urinary tract) with specific metabolic and microhabitat features (Vogtmann and Goedert, 2016). More specifically, the microbiome concept involves, besides species abundance and phylogenetic diversity (*i.e.*, microbiota), the whole genome of the microbial community inhabiting a certain body niche. Nevertheless, both microbiota and microbiome terms have been largely used synonymously in the literature.

Due to the role of the microbiota on the host metabolism and immune system functioning, different studies have been recently highlighting its relevance on the health and disease status of human individuals (Wang & Jia 2016).

The gut microbiome is complex, abundant and diverse, either taxonomically and functionally, and its genome greatly overlaps that of the host (Wu and Sherman, 2015; Vogtmann and Goedert, 2016). Along the gastrointestinal tract, the microbiota increases in abundance and diversity in order to ensure a structured microbial composition, representing more than 10 fold the number of human cells (Dominianni *et al.*, 2015; Nistal *et al.*, 2015) (Figure 2). The large bowel is the most microbially-populated human body niche, especially by bacteria. In a general way, the bacteria are present in two parts of the large intestine: the lumen (which may not comprise the same communities as in the crypts and epithelium) and/or mucosa. The mucosa-adherent bacteria are normally associated to polysaccharides surface and affect the host immune system at the mucosa level. Despite the changing environment in large bowel due to the excretion of debris and water circulation, some of these bacteria are mucosa-resident, being thereby more significant in colon diseases pathogenesis [*e.g.*, inflammatory bowel disease, irritable bowel syndrome (IBS), Crohn's disease and, at a malignant stage, colorectal cancer (CRC)] (Sun and Chang, 2014; Keku *et al.*, 2015; Wu and Sherman, 2015).

Among the bacterial groups found in the large bowel, Bacteroidetes and Firmicutes are the most represented phyla, followed by Actinobacteria, and Proteobacteria (Sun and Chang, 2014; Nistal *et al.*, 2015). Once the colon environment is characterized by low oxygen, the bacterial communities are essentially composed by strict anaerobes (*e.g.,* Gram-positive non-spore forming: Bacteroides, *Bifidobacterium*, *Eubacterium*, *Ruminococcus*; and spore-forming: *Clostridium*) (Figure 2). Notwithstanding, there is also facultative anaerobes and aerotolerant to aerobic bacteria, such as enterobacteria, lactobacilli, streptococci and enterococci (O'Hara and Shanahan 2006; Nistal *et al.* 2015). These bacteria present high proliferation in the colon, not only because of their adaptation to oxygen-depleted environments, but also because of the slow transit time that favors

microbial fermentation of diet elements and secretions, the low bile salt content, the release of pancreatic secretions into the colon and the increased pH (Nistal *et al.*, 2015).



Anaerobic Genera	Aerobic Genera
Bifidobacterium	*Escherichia*
Clostridium	*Enterococcus*
Bacteroides	*Streptococcus*
Eubacterium	*Klebsiela*

Proctective functions	Structural functions	Metabolic Functions
Nutrient competition	Barrier fortification	Synthesize vitamins
Production antimicrobial factors	Immune system development	Metabolize dietary carcinogens
Pathogen displacement		Salvage of energy
		Ion absorption

Figure 2. Representation of the most common anaerobic and aerobic genera of colon-colonizing commensal bacteria and supported functions on the intestinal mucosa (Adapted from O'Hara and Shanahan, 2006)

1.2.1 Intestinal Microbiota-Host Interactions

The intestinal microbiome plays a major role in the host physiology, metabolic regulation, detoxification, hormonal homeostasis and immune system response (Dulai and Keku, 2014; Burns *et al.*, 2015; Keku *et al.*, 2015; Vogtman and Goedert, 2016). More specifically, it influences the host:

● energy balance and nutrition through the absorption of ions, fermentation of non-digestible dietary components (*e.g.*, non-digestible carbohydrates) into more easily absorbed metabolites such as short-chain fatty acids (SCFAs), production of vitamins (*e.g.*, vitamin K and components of vitamin B), aminoacids, and transformation of bile acids (Macfarlene and Macfarlene 2003; Lefebvre *et al.*, 2009; Candela *et al.*, 2014; Tojo *et al.*, 2014; Keku *et al.*, 2015; Øyri *et al.*, 2015);

● postnatal immune system by leading its maturation from infancy up to the adult stages, guaranteeing its homeostasis and contributing to the formation of immunoglobulin A (IgA) and segregation of B cells, which are involved in innate and cell-mediated immunity (Tojo *et al.*, 2014; Nistal *et al.*, 2015; Øyri *et al.*, 2015);

● by acting as an antimicrobial barrier against pathogens through the production of harmful compounds, such as acetate and bacteriocins, and by competing for energy resources and colonization of pathogens (Candela *et al.*, 2014; Øyri *et al.*, 2015);

● nervous system, general behavior and cognitive function, being even possibly associated with nervous system disorders, because the microbiome is in contact with the second largest neuronal pool of cells in the human body that constitutes the enteric nervous system[1] (Candela *et al.*, 2014; Tojo *et al.*, 2014).

Therefore, the vital role of gut microbiota activity on the host metabolic homeostasis, uplifts the relevance of studying host-microbiota ecological relationships, both in health and unbalanced states.

1.3. Gut Microbiota Dysbiosis *versus* CRC

Since there is a host-microbiota bidirectional relationship, if the host is subjected and affected by certain risk factors, then in turn, the structure and function of the microbiota may be gradually modulated and transformed up to a dysbiotic situation. Considering that mucosa commensal bacteria contribute for the development of intestinal immune system, they help to control bowel inflammation through the stimulation of innate (*e.g.*, monocytes, macrophages) and adaptative (*e.g.*, lymphocites, regulatory cells) systems that mediate the homeostasis between pro-inflammatory (*e.g.*, IL-1B, interferon-γ, IL-8, TNF-α, IL-23, IL-12, IL-17, IL-6) and anti-inflammatory (*e.g.*, IL-10, TGF-β) molecules (Chow and Mazmanian, 2010). Thus, the disruption of such homeostasis degrades the host immune defenses, hence contributing to chronic inflammation and an increased risk of developing bowel diseases, which may end up in CRC.

Many studies have been lately devoted to unraveling the possible relation between these diseases and changes in the intestinal microbiome, and vice-versa (*e.g.*, Arthur *et al.*, 2011; Tojo *et al.*, 2014). Despite the existence of evidences of host-microbiome interrelation as a potential contributor for CRC carcinogenesis, a clear cause-effect is yet to be fully characterized (Cario, 2013; Soares, 2014). However, Sears and Garrett (2014) presented two major perspectives from the actual literature. In the 'microbiota perspective' the CRC pathogenesis is promoted either by individual microbes, a collective microbiota

[1] **Enteric nervous system** (ENS) is a part of the peripheral nervous system and a division of the autonomous nervous system which controls the gastrointestinal tract. This system is capable of autonomous function, controls the digestive system in the context of the physiological state locally and the body as a whole.

or an interaction of both (*i.e.,* individual microbes that trigger the appearance of a disease-generating microbiota), which possess virulence mechanisms that create pathologies. In the 'host perspective', the microbiota may alter tumor biology or, conversely, the tumor microenvironment may induce changes in the microbiota with ability to inhibit or promote tumor pathogenesis. Under a 'microbiota perspective', a dysbiosis scenario favors the proliferation of bacteria with pro-carcinogenic abilities (*i.e.*, can cause inflammation or produce DNA-damaging compounds) that initiate inflammation, which progression may turn into CRC. As such, understanding if and how the structure and functions of the microbiota affect or are affected by colon carcinogenesis has been the core of different research works.

Oke and Martin (2017) determined higher proportions of *Shigella*, *Citrobacter* and *Salmonella* at early stages of CRC, comparatively to the microbiota identified in healthy individuals. The dominance of *Fusobacterium nucleatum* in CRC-affected tissue comparatively to healthy mucosa has already been reported by several authors (*e.g.*, Keku *et al.*, 2015, Canha *et al.*, 2015). However, some authors found that *F. nucleatum* does not induce colitis nor it enhances colitis-associated CRC. Instead, it stimulates the immune system in order to promote the synthesis of pro-inflammatory mediators responsible for CRC pathogenesis. This way, it is possible to explain why patients with disease and a lower abundance of this bacterium can survive for longer periods than patients with higher *F. nucleatum* abundance (Gao *et al.*, 2017). Also it should be noted that studies with IBD patients, have proven that some isolates of *F. nucleatum* can be more invasive in inflamed parts of colon than the normal tissue. And this may be related to the variation of the number of copies of *F. nucleatum* pathogenic strains (Ohtani, 2014; Gao *et al.*, 2017).

The presence of *Escherichia coli* in the gut is quite common. However, some strains have been identified as a risk for CRC pathogenesis. Genotoxic *E. coli* strains harbor a polyketide synthase (*pks;* ≈54 kb) pathogenic island, which encoded enzymes are responsible for the synthesis of the genotoxin colibactin. When this *pks* gene is positive in CRC patients, higher levels of *E. coli* is present. The enterotoxigenic *Bacteroides fragilis* (ETBF) acts by the production of toxin BFT (*i.e.*, *B. fragilis* toxin), which binds to a specific colonic epithelial receptor and activates Wnt and NF-κB signaling pathways for increased cell proliferation, production of pro-inflammatory mediators and DNA damage. Although this toxin is not recurrently identified in CRC patients, recent genetic studies revealed an increased detection of its biosynthetic genes in colorectal biopsies. This outcome was often obtained in patients not subjected to antibiotic

pretreatment, presenting significantly higher occurrence of genetic damages in CRC cases (*e.g.*, double-strand breaks, gene mutations, chromosomal instability), highlighting its potential role in carcinogenesis (Keku *et al.*, 2015; Sun and Kato, 2016; Gao *et al.*, 2017). Although *Helicobacter pylori* causes non-cardia gastric adenocarcinoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma, some studies tried to figure out if *H. pylori* infections could increase CRC risk. The results did not support a positive and clear causality between both pathological scenarios. However, there may be indirect consequences deriving from gastric pathology with *Helicobacter* and the stimulation of colorectal tumor growth (Sun and Kato, 2016). Other relevant pro-carcinogenic bacterial species have been also considered potentially relevant for CRC development. *Streptococcus gallolyticus* has been indicated as an invader or contributor to CRC pathogenesis, by inducing abnormal crypt development (Keku *et al.*, 2015; Sun and Kato, 2016). *Clostridium leptum* and *C. coccoides* are frequently detected in CRC tissue samples (Tojo *et al.*, 2014; Gao *et al.*, 2017). *Enterococcus faecalis* strains produce reactive oxygen species capable of inducing DNA damage and genomic instability, which can both promote CRC initiation (Sears and Garrett, 2014; Lennard *et al.*, 2016; Zhou *et al.*, 2016).

1.4 Objectives

Despite the increased wealth of literature on human gut microbiome, either related with healthy or disease scenarios, some questions are yet to be fulfilled. A major gap concerns the coverage of different geographic areas as to understand if host-microbiota relationships assume similar profiles between countries. In the future, such knowledge will be quite valuable to develop efficient and improved tools for clinical diagnosis and therapeutics of CRC.

In this context, the present study is the first one ever conducted in Portugal, as far as I am aware. It intends to provide a preliminary insight on the composition and potentialities of gut bacteria associated with affected and non-affected mucosa tissues extracted from Portuguese patients diagnosed with CRC. Giving the great limitations for performing a representative culturing of the whole bacterial community inhabiting this body niche, besides making a qualitative analysis of cultivable aerobic/facultative anaerobic bacteria, it was also analyzed the diversity of the bacterial community, based on total DNA fingerprinting. Additionally, a short revision on the potential application of microbiota for CRC therapeutic purposes was also developed, in order to open new perspectives for upcoming research and needs in this topic.

In order to comply with the straightforward and clear description of the results obtained for the purposed goals, the dissertation was structured in different chapters:

Chapter I – General Introduction

Chapter II – Microbiota and colorectal cancer in a Portuguese population: bacterial composition and abilities

Chapter III – The microbiome as a complementary therapeutic tool against colorectal cancer

Chapter IV – Final considerations.

CHAPTER II

Chapter II. Microbiota and colorectal cancer in a Portuguese population: bacterial composition and abilities

2.1. Abstract

Gut microbiota contributes to the control of important metabolic and physiological functions of the human being. Colorectal cancer (CRC) has been having a higher incidence at the global level and from year to year. Thus, it is important to understand how alterations in the microbial community affect and are affected by CRC. Hence, the total community bacterial DNA extracted from normal (TN) and tumor (TT) mucosa tissues of sixteen patients was studied by PCR-DGGE to compare the microbial diversity between the two tissue types. The DGGE band profile revealed that the greatest part of the patients presented some differences between both tissues. Only in 4 samples there was a clear similarity between TN and TT (similarity >75%). The other part of the work intended to identify and characterize the antimicrobial activity and antibiotic susceptibility of cultivable aerobic/facultative anaerobic bacteria isolated from TN and TT mucosa tissues. Both tissues presented bacteria belonging to the genera *Morganella*, *Klebsiella*, *Pseudomonas*, *Streptococcus* and *Escherichia*. Only in TN was identified the genera *Citrobacte*r, *Proteus*, *Shewanella*, *Lactobacillus* and *Brevibacterium*; whilst *Enterococcus*, *Weisella* and *Cedecea* were solely detected in TT. In the antimicrobial tests it was possible to observe positive results against *Staphylococcus aureus*, *Micrococcus luteus* and *Pseudomonas aeruginosa* and negative results for the species *Klebsiella pneumoniae* and *Escherichia coli*. In the antibiotic susceptibility test, it was possible to emphasize that more than 50% of the tested isolates are resistant to at least half of the antibiotics tested.

Keywords*:* colon mucosa, DNA-based techniques, culture-dependent techniques, antimicrobial activity, antibiotic susceptibility

2.2. Introduction

At a global scale, the colorectal cancer (CRC) was the forth deadly cancer in men and third in women in 2012 (Ferlay *et al.*, 2015), being however the second deadly cancer for both sexes in Portugal (EUCAN, 2012), with 1,526 deaths for men and 1,161 for women in 2014 (Miranda *et al.*, 2015).

In the past decade, valuable studies have been focused on the association between the gut microbiota and/or microbiome profiles and CRC (*e.g.*, Sears and Garrett, 2014; Ohtani, 2014; Peterson *et al.*, 2014; Keku *et al.*, 2015). The clear relevance of a balanced host-microbiota symbiosis for the normal metabolism and immune system functioning of the host has been forcing scientists to understand the role of the gut microbiota on CRC biology (*e.g.*, Cario, 2013), pathogenesis (*e.g.*, Gao *et al.*, 2017), diagnosis (*e.g.*, Lennard *et al.*, 2016) and therapeutics (Belizário and Napolitano, 2015). The advent of next generation sequencing technologies has opened new opportunities and has been providing new insights over those different perspectives (Fontanges *et al.*, 2016). In particular, some studies have been devoted to the analysis of 16S rRNA gene sequencing from total community DNA extracted from different types of host samples (*e.g.*, stool, mucosa). One of the main goals has been to find out relevant trends on the intestine microbiota community structure associated with CRC, in order to ascertain how the microbiota can be affected or affect CRC carcinogenesis and proliferation, through host-microbiota relationship (Tojo *et al.*, 2014). Although recent studies have highlighted a clearer impact and a potential role-play of certain bacteria on CRC development, such linkage is still demanding further studies, given the great heterogeneity of gut microbial community profiles over varied factors, such as host age, clinical situation, lifestyle, behavior and family history, and geographic area (Candela *et al.*, 2014).

Many research works under this topic focus on culture-independent techniques (*i.e.*, on total community DNA) due to the limitation of culture-dependent methods to provide a reliable overview of the bacterial community associated with CRC. Notwithstanding, the isolation of representative bacterial strains from specific biological niches of CRC-affected hosts may offer a better understanding of their potential role on key metabolic pathways, as well as may be explored as biotechnological tools for CRC diagnosis and/or therapeutics. Therefore, as deep as the knowledge on the microbial and, more specifically, on the bacterial community traits along different geographic populations, the best will be this issue covered as to make available efficient and reliable alternatives for fulfilling certain clinical needs.

Thus, the present work describes a preliminary study conducted on a Portuguese population from the centre of Portugal, which has CRC, with the aim of characterizing the bacterial community structure (based on PCR-DGGE fingerprinting of total community DNA) on tumor-affected *versus* non-affected mucosa tissues. Additionally, culture-dependent methods were applied for the genotyping of cultivable aerobic bacteria isolated from these tissues towards their characterization in terms of bacterial specific richness, antibiotic susceptibility and antimicrobial activity. In summary, it is intended to identify potential shifts or changes on the bacterial populations determined in tumor *versus* normal mucosa tissues from Portuguese patients. This is the first approach ever performed in Portugal for CRC-associated microbiota, as far as I am aware. Covering populations from different geographical areas, especially regarding western countries, which present the highest CRC incidence rates, is very welcomed for improving the knowledge on host-microbiota symbiosis and CRC towards future clinical applications.

2.3. Materials and Methods

2.3.1 Collection and Processing of Samples and Culture of Bacteria

The study was approved by the Ethics Committee and the Administration of the Centro Hospitalar do Baixo Vouga, EPE, Aveiro (CHBV), and all the patients signed an informed consent form in which they agreed to participate as volunteers.

Affected (TT) and healthy (TN) tissue samples from colon mucosa were collected from 16 volunteer patients diagnosed with CRC that were subjected to surgery at the CHBV. After resection, the samples were immediately transported to the laboratory. The TN and TT tissue samples for total-community DNA analysis through PCR-DGGE were immediately frozen in liquid nitrogen and kept at -80ºC, until proceeding with DNA extraction and purification. Cultivable aerobic/facultative anaerobic bacteria were extracted from fresh TN and TT tissue samples through maceration, being the obtained extract diluted in a saline buffer before inoculation (100 µL) into plates containing different solid media (TSA and McConkey agar). The plates were incubated aerobically at 37ºC up to two weeks. After bacterial growth, the microbial colonies with apparently different morphology were isolated through several re-inoculation steps in the respective solid media. Pure bacterial isolates were stored in 25% glycerol at -80ºC for posterior genotyping and characterization studies.

2.3.2 Mucosa-Associated Microbial Community analyzed by PCR-DGGE

Only 10 patients were considered for the study of microbial community diversity in tumoral and normal mucosa tissues. The microbial DNA extraction from tissues samples was performed with the QIAamp DNA Mini Kit, QIAGEN, following manufacturer's instructions. For the amplification of 16S rRNA gene fragments from total community genomic DNA it was used a nested PCR approach. In a first PCR the primers 27F and R1494 (Table 1) were applied at 100 nM in a 25 µL reaction consisting of 1µL template DNA, 2.5 mM MgCl2, Taq Polymerase buffer 1x, 200 µM dNTPs and 1U Taq polymerase. A pre-denaturation step of 5 min at 94ºC was followed by 30 cycles of denaturation (45 s at 94ºC), primer annealing (45 s at 56ºC) and extension (1.30 min at 72ºC), and a final extension step for 10 min at 72ºC (Pinheiro *et al.* 2013). In the second (nested) PCR, 1µL of 1st PCR amplicons was added with Taq polymerase buffer, $MgCl_2$, dNTPs, F984 and R1378 primers targeting the hypervariable regions V6-V7 (Table 1), and Taq polymerase to obtain the final concentrations described in Pinheiro *et al.* (2013). The nested PCR run was conducted as described: 10 min at 95ºC, 34 cycles of denaturation (1 min at 95ºC), primer annealing (1 min at 53ºC) and extension (1.5 min at 72ºC), and a final extension for 7 min at 72ºC. The PCR runs were carried out in a thermocycler C1000TM from Bio-Rad. After the PCR reaction, the amplified products were separated in a 1% (w/v) agarose gel electrophoresis made in 1x TAE for 1h and 100 V. For molecular weight comparisons a 1 Kb marker (GeneRulerTM DNA Ladder Mix, Fermentas) was loaded in the gels. The gel was observed and photographed in the Gel DocTM RX+ System with Image LabTM Software, Bio-Rad.

The denaturing gradient gel electrophoresis (DGGE) for the separation of DNA fragments was made in a polyacrylamide gel containing 8% of a 40% acrylamide/ bisacrylamide (37.5:1) solution and a 25%-65% chemical denaturing gradient. TEMED and ammonium persulfate (10%) were added to the gradient solutions in order to promote gel polimerization. The electrophoresis run was performed in TAE buffer (1x) at 75V and 60°C for 16h, using the Bio-Rad DCode™ Universal Mutation Detection System. The gel was loaded with 10 µL of PCR products mixed in 4 µL of loading dye 6x. Afterwards, the gel was stained in an ethidium bromide 5% solution for 5 min, washed with Milli-Q water and photographed in the Bio-Rad molecular imager (see above). The obtained fingerprint was analyzed with the software GelCompar II® (Applied Maths NV, V.6.4). The fingerprint profiles obtained for each sample was compared through the Dice similarity index, being constructed a similarity matrix. The dendrogram was built by applying the unweighted pair

group method with arithmetic mean (UPGMA) hierarchical clustering to the similarity matrix.

Table 1 - Sequence of the primers used in the different PCR amplifications performed

Primer	Sequence	Reference
BOX A1R	5'-CTACGGCAAGGCGACGCTGACG- 3'	Versalovic *et al.*, 1994
F27	5'-AGAGTTTGATC(A/C)TGGCTCAG-3'	Heuer *et al.*, 1997
R1494	5'-TACGG(C/T)TACCTTGTTACGACTT-3'	Heuer *et al.*, 1997
F985GC	5'-gc.-AACGCGAAGAACCTTAC-3'	Heuer *et al.*, 1997
R1378	5'-CGGTGTGTACAAGGCCCGGGAACG-3'	Heuer *et al.*, 1997

2.3.3 Genotyping and Identification of Cultivable Bacteria

The genomic DNA of isolated aerobic/facultative anaerobic strains was extracted by heat lysis of bacterial cell suspensions at 100ºC for 5 min. The DNA typing of the bacterial isolates was made through Box-PCR, which is based on a primer that targets sequences located between interspersed repetitive DNA elements. As a result, amplicons of different sizes generate species-specific and strain-specific genomic fingerprints, hence allowing the separation of different bacterial isolates (Versalovic *et al.*, 1994). Each Box-PCR master mix contained 1 µL template DNA, buffer 1x, 3 mM of $MgCl_2$, 200µM of dNTPs, 0.57 pmol of primer Box A1R (Table 1) and 5 U/µL Taq polymerase. The PCR program followed the conditions outlined by Versalovic *et al.* (1994). In brief, the mixtures were pre-denatured during 7 min at 95ºC, after which 30 cycles of denaturation (95ºC for 1 min), annealing (1 min at 53ºC) and extension (8 min at 65ºC), and a final extension for 16 min at 65ºC. The amplified products were electrophoretically separated as indicated in section 2.3.2. The band profiles originated for the bacteria were clustered in similarity dendrograms, which allowed the separation of different bacterial fingerprints (*i.e.,* bacterial isolates). The selected bacteria were then subjected to 16S rDNA amplification, using 25 µL reactions containing 1µL of template DNA, 2.5 mM MgCl2, Taq Polymerase buffer 1x, 200 µM dNTPs, 100 nM of each 27F and 1494R primers (Table 1), and 1U Taq polymerase. The PCR program consisted of a pre-denaturation step of 5 min at 94ºC, followed by 30 cycles of denaturation (45 s at 94ºC), primer annealing (45 s at 56ºC) and extension (1.30 min at 72ºC), and a final extension step for 10 min at 72ºC (Pinheiro *et al.* 2013). The 16S rDNA amplicons were electrophoretically checked and then subjected to

Sanger sequencing for the identification of the bacterial isolates. The nucleotidic sequences were compared with the deposited ones in the GenBank database through the use of the BLAST tool provided by the National Center for Biotechnology Information (NCBI). The nucleotide analysis, sequence alignment and phylogenetic trees were developed on the CLC Sequence Viewer 6.5 program.

2.3.4 Antimicrobial Activity of Bacterial Strains

(a) Spot-on-lawn Assay

All bacterial isolates were tested against pathogenic bacteria by the spot-on-lawn assay. The selected pathogenic bacteria corresponded to Gram-negative (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*) and Gram-positive species (*Micrococcus luteus*, *Staphylococcus aureus*). Positive results were revealed by the formation of a clear halo around the isolate being tested, demonstrating that it has antimicrobial activity against the pathogenic bacterium. Nine of the isolates presenting antimicrobial activity were further tested in agar diffusion assays (*cf.* following sub-section).

(b) Agar Diffusion Assay: Cell-Free Extract and Cell Wall Washes

In the agar diffusion assays it was tested the activity of cell-free extract and cell wall washes of the isolates evidencing antimicrobial activity in the 'spot-on-lawn' assays, against the same pathogenic bacteria. The fractions of cell-free extract and cell wall washes were obtained after growing the isolates at 37ºC overnight in TSB medium, followed by centrifugation of the inoculum suspension. The supernatant was filtered and stored at -80°C for posterior use in the agar diffusion assay as cell-free extracts. The pellet was resuspended in isopropanol, centrifuged and the supernatant stored at -80ºC. Both fractions were subjected to the agar diffusion assay against the bacterial pathogens (see section 2.3.4(a)). The test was run at 37 °C for 24 hours, and the positive results were recorded whenever a clear halo was formed.

2.3.5 Antibiotic Susceptibility of Bacterial Strains

The disk diffusion susceptibility test is simple, fast and practical. The test was performed by applying a layer inoculated with the target bacterial isolate to the surface of a Mueller-Hinton agar plate. Afterwards, paper disks impregnated with certain antibiotic concentrations were placed on the agar surface. The selected list of antibiotics is presented in Table 2, being included some antibiotics with broad spectrum of action. The test was run at 37 °C for 24 h. Positive results were revealed by the formation of a clear halo.

Table 2. List of the antibiotics tested and respective chemical class and mode-of-action

Antibiotic Class	Mode-of-action	Antibiotic
β-lactams	Inhibit cell wall synthesis	Amoxicillin (10 µg)
		Penicillin (10 U)
		Aztreonam (30 µg)
		Imipenem (10 µg)
		Cephalothin (30 µg)
Aminoglycosides	Inhibit protein synthesis	Gentamicin(30 µg)
		Streptomycin (300 µg)
Macrolides	Inhibit protein synthesis	Erythromycin (15 µg)
Phenocols	Inhibit protein synthesis - bacteriostatic	Chloramphenicol(30 µg)
Nonribossomal peptide	Inhibit cell wall synthesis	Bacitracin (10 U)

2.4 Results and Discussion

2.4.1 Culture-Independent Analysis

The DGGE profile and comparison dendrogram of the 16S rDNA genes amplified in the different samples for TN and TT mucosa tissues, from patients 1 to 10 is shown in Figure 3.

In the fingerprint obtained (Figure 3A) it is visible a common and intense band in all patients and tissue types (lateral blue arrow), suggesting the dominance of a bacterial group in the human colon, which is not affected by CRC pathogenesis. However, no clear patterns could be identified between patients for TN or TT tissues, thereby suggesting a heterogeneity in the microbiome composition, depending on the host-microbiome system. A general resemblance between TN and TT band patterns within patients was observed.

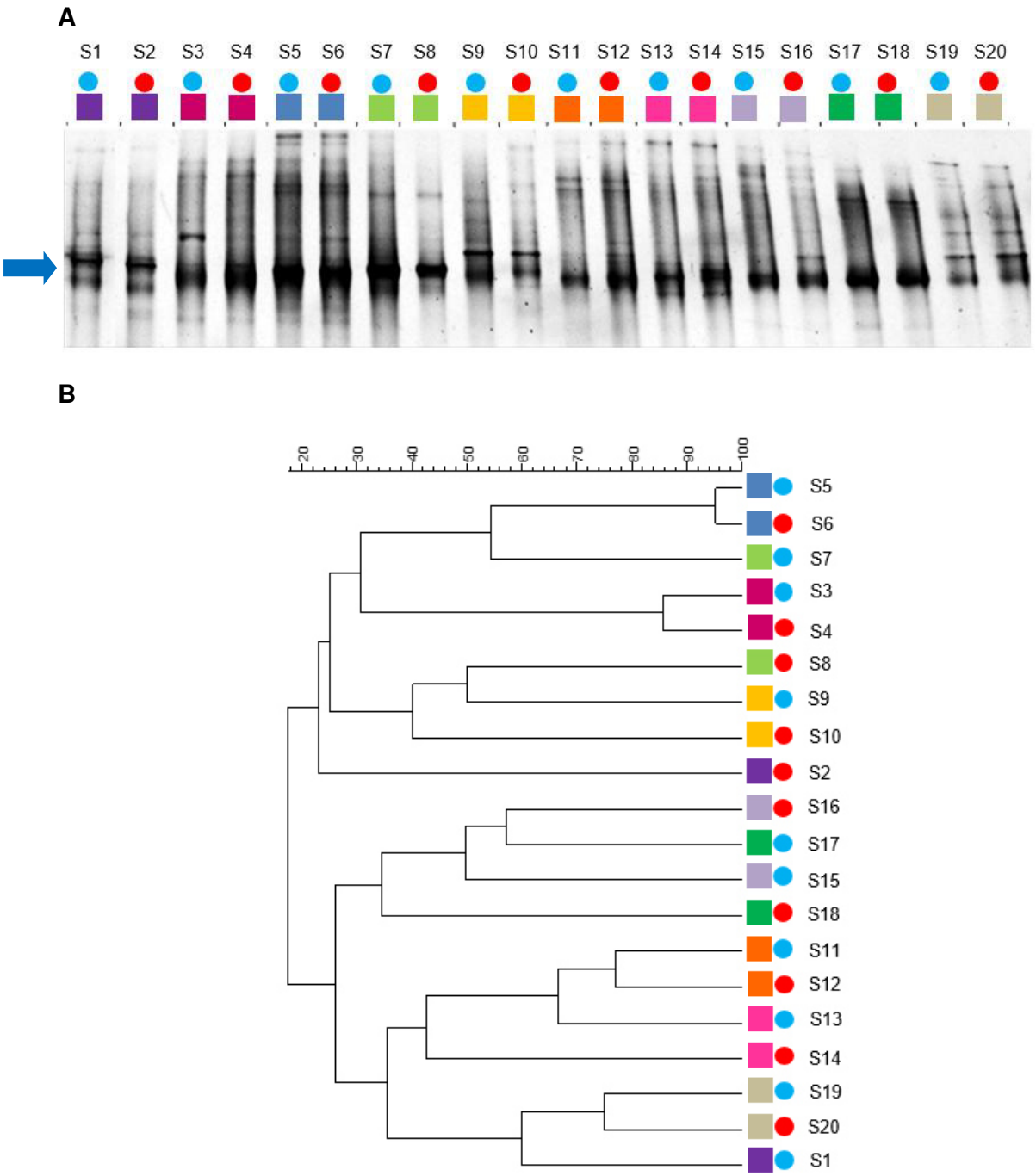


Figure 3. A. PCR-DGGE fingerprint of the bacterial 16S rRNA gene amplified from total DNA extracted from the different samples. **B**. Similarity cluster dendrogram of the bacterial 16S rDNA genes amplified from total microbial community DNA. The UPGMA dendrogram was built with Dice similarity index (outcome shown as similarity percentage). Different patients are indicated by different colored squares; TN and TT tissues are represented by blue and red circles, respectively.

Nevertheless, some shifts in the microbiome could be detected in the DGGE profile due to the appearance of a few different bands in TN or TT samples within the same patient. But

in a broad perspective, there was a tendency for higher bacterial diversity (*i.e.,* higher band number) in TT samples, irrespective of the patient. These outcomes are partly in agreement with the bacteria extracted from TN and TT, since similar species were identified in both, despite the occurrence of different and specific species in each one (*cf.* Figure 3A).

The DGGE fingerprint was further reinforced by the UPGMA dendrogram (Figure 3B). Two main clustered groups could be distinguished, which similarity (Dice index) was very low (17.4%). In a general view, only in 4 samples there was a clear similarity between TN and TT tissues from the same patient (similarity >75%), being the clustering of the remaining TN-TT samples *per* patient associated with lower similarity index values (<40%). In particular, the TN and TT samples of one patient (represented as violet squares) were even positioned in each of the main clusters identified, showing a clear separation between their respective microbiome.

Despite the efforts that have been devoted to the definition of a 'core microbiome' in healthy tissues (*e.g.,* Qin *et al.*, 2010), there is still a great gap since not all geographic areas have been covered for metagenomic analysis (culture-independent methods) of the human colon microbiome. This would be relevant since different interfering factors, as well as, the subsequent response of host-microbiome systems may modulate their behavior under or towards pathological scenarios (Arthur *et al.*, 2012; Sears and Garrett, 2014). Notwithstanding, there is an increased evidence for the active role (both positive and negative) of the microbiome on carcinogenesis (*e.g.*, Uronis and Jobin 2009; Sears and Garrett, 2014).

Thereby, it is expectable the occurrence of shifts in the microbiota structure associated with non-affected and affected tissues. Contrary to the outcomes herein obtained, Huipeng *et al.* (2014) recorded a decreased microbial diversity in colonic mucosa in CRC patients compared to healthy individuals, by PCR-DGGE. Similar results were obtained by Ahn *et al.* (2013) for fecal samples of CRC patients relatively to normal patients, based on the pyrosequencing of bacterial 16S rRNA genes extracted from fecal samples. This change in diversity has been usually related with the predominance of some specific *taxa*. Therefore, a potential future step within the present study will be to go deeper into this analysis, in order to have a closer overview of changes in the microbiota composition.

2.4.2 Genotyping and Identification of Aerobically-Cultured Bacteria

Based on their macroscopic morphologic differences, a total of 102 aerobic and/or facultative anaerobic bacterial strains were isolated from TN and TT tissue samples resected from 16 patients (Figure 4). As it is common knowledge, the greatest part of the microorganisms in the human colon are not cultivable. Notwithstanding, putting some efforts on their isolation and characterization may give relevant clues concerning the metabolism of specific bacterial groups inhabiting this biological niche (Eckburg *et al.*, 2005).

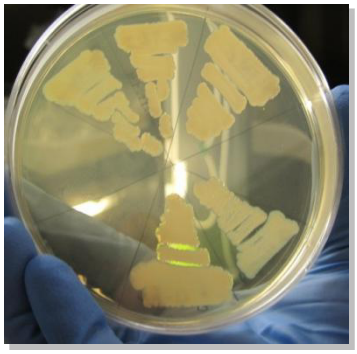


Figure 4. Individualized aerobic/facultative anaerobic bacteria from mucosa tissues

The typing fingerprints of the 102 aerobic/facultative anaerobic bacteria (46 from normal tissue and 56 from tumor tissue) were obtained upon separation of the Box-PCR products in agarose gel (see Figure 5A as an example). The dendrogram resulting from the analysis of these Box-PCR profiles allowed discriminating 79 dissimilar isolates (<80% homology) (*cf.* Annex A). These apparently distinct isolates, based on the molecular tool used, were then considered for 16S rDNA gene amplification and sequencing.

The analysis of the 16S rDNA sequences evidenced 57 matches to the 16S rDNA sequences deposited in the GenBank database (*cf.* Table 3). Among them, 48 presented 99 to 100% homology relatively to the deposited sequences indicating that are known species; 6 were 97 to 98% homologue, and 3 showed <97% homology, thereby suggesting that can be novel species or strains. In total, 17 different phylotypes were isolated from TN and TT tissues, which belong to the three phyla mostly identified in this type of samples (*i.e.*, Proteobacteria, Firmicutes and Actinobacteria) (Sears and Garrett, 2014; Peters *et al.*, 2016; Gao *et al.*, 2017).

Figure 6 provides the occurrence of the species in the normal *versus* tumoral tissue samples of different patients. According to these data, there are six species occurring in both TN and TT tissues, which are *Morganella morganii*, *Klebsiella*

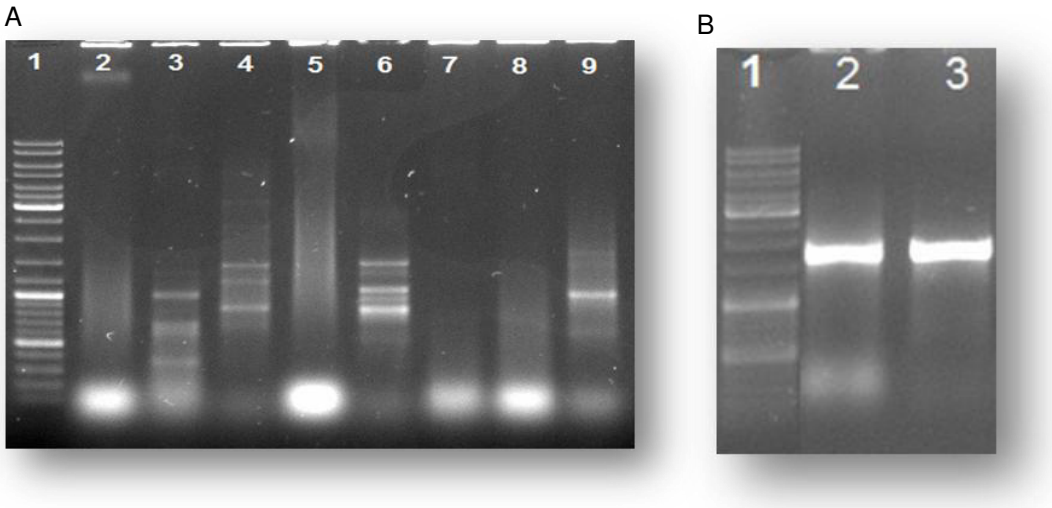


Figure 5. Electrophoresis profile in 1% agarose gel; A. line 1 - DNA marker "GeneRuler^{TM} DNA Ladder Mix"; lines 2 to 9 - the isolated bacteria profiles by Box-PCR; B. line 1 - DNA marker "GeneRulerTM DNA Ladder Mix"; lines 2 and 3 - 16S rDNA gene fragment

pneumonia, *Pseudomonas aeruginosa*, *Escherichia marmotae*, *Escherichia hermannii* and *Escherichia fergusonii*, being the latter the most represented one. Despite the existence of *Pseudomonas aeruginosa* in normal intestinal flora, its density is likely to enhance upon antibiotic treatment, hospitalization, immunosuppression conditions, and also under cancer pathogenesis, namely regarding CRC (Markou and Apidianakis, 2013). *E. marmotae* was isolated from *Marmota himalayana* feces (Liu *et al.*, 2015), but its identification in human samples has never been reported so far. *E. hermannii* and *E. fergusonii* have been recorded in patients with Crohn's disease (Sasaki *et al.*, 2007; Kaakoush *et al.*, 2012). Although this is not a cancer situation, microbial dysbiosis associated with Crohn's disease may ultimately drive to cancer pathogenesis. On the other hand, *M. morganii* makes part of the normal mucosa colonic flora in humans (Kim *et al.*, 2007), but species belonging to this genus were already described in the microbiome of a dysbiotic colon (Neu *et al.* 1989) as well. *K. pneumoniae* is, according to Saleh and Trinchieri (2011), a colitis-indicator associated with CRC in rats. Nevertheless, this species was also detected in the normal human flora (Conlan *et al.*, 2012).

Five bacterial species appeared only in TN: *Proteus mirabilis*, *Streptococcus pasteurianus*, *Citrobacter murliniae*, *Shewanella algae*, *Lactobacillus salivarius* and *Brevibacterium halotolerans*. *P. mirabilis* was observed in colitis-associated CRC (Saleh and Trinchieri, 2011), but the strain WGLW6 is common in healthy human microbiome (NIH Human Microbiome Project, 2015). Members of the *Citrobacter* genus were described as bacteria driver of CRC (Candela *et al.*, 2014), or as pathogens to the colon (Bailey *et al.* 2010); thus, its appearance in our TN samples is somehow surprising.

Table 3. Identification of the bacterial strains isolated from normal (N) and tumoral (T) intestine mucosa

Strain no.	Tissue type	Phylotype	Nearest relative in GenBank	Homology (%)	Phylum	Class	Order	Family	Aerobiosis	Gram staining
B2	N	*Shewanella algae*	*Shewanella algae* ATCC 51192	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Shewanellaceae	FA	Gram -
B3	N	*Morganella morganii*	*Morganella morganii* NBRC 3848	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B4	N	*Escherichia marmotae*	*Escherichia marmotae* HT07301	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B5	N	*Escherichia hermannii*	*Escherichia hermannii* CIP 103176	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B6	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B7	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B8	T	*Weissella confusa*	*Weissella confusa* JCM 1093	**91%**	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	FA	Gram +
B9	T	*Morganella morganii*	*Morganella morganii* subsp. *morganii* KT	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B10	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B11	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	**98%**	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B12	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B14	N	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* HS11286	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A	Gram -
B15	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B17	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	**97%**	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B18	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B18a	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B19	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B20	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B21	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B22	T	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A	Gram -
B23	T	*Escherichia hermannii*	*Escherichia hermannii* CIP 103176	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B24	T	*Escherichia hermannii*	*Escherichia hermannii* CIP 103176	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B25	N	*Lactobacillus salivarius*	*Lactobacillus salivarius* UCC118	99%	Firmicutes	Bacilli	Alteromonadales	Lactobacillaceae	A, FA	Gram +
B26	N	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A	Gram -
B27	N	*Escherichia hermannii*	*Escherichia hermannii* CIP 103176	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B30	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B31	N	*Escherichia marmotae*	*Escherichia marmotae* HT07301	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B32	N	*Escherichia marmotae*	*Escherichia marmotae* HT07301	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B33	N	*Streptococcus pasteurianus*	*Streptococcus pasteurianus* CIP 107122	**95%**	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	FA	Gram +

Table 3. (cont.)

Strain no.	Tissue type	Phylotype	Nearest relative in GenBank	Homology (%)	Phylum	Class	Order	Family	Aerobiosis	Gram staining
B34	T	*Escherichia marmotae*	*Escherichia marmotae* HT07301	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B35	T	*Escherichia hermannii*	*Escherichia hermannii* CIP 103176	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B36	T	*Escherichia hermannii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B37	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B38	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B39	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B40	N	*Citrobacter murliniae*	*Citrobacter murliniae* CDC 2970-59	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A	Gram -
B41	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B44	N	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* HS11286	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A	Gram -
B45	N	*Morganella morganii*	*Morganella morganii* NBRC 3848	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B46	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B48	T	*Enterococcus* sp.	*Enterococcus sp.*	99%	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	FA	Gram +
B49	N	*Proteus mirabilis*	*Proteus mirabilis strain* JCM 1669	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B51	N	*Cronobacter* sp.	*Cronobacter sp.*	**65%**	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B52	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B53	T	*Streptococcus parasanguinis*	*Streptococcus parasanguinis* FW213	99%	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	FA	Gram +
B54	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	**97%**	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B55	N	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	100%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B56	T	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A, FA	Gram -
B57	N	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* DSM 50071	99%	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	A, FA	Gram -
B58	N	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104	98%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	A	Gram -
B59	T	*Cedecea lapagei*	*Cedecea lapagei* DSM 4587	98%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B60	N	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* DSM 50071	**97%**	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	A, FA	Gram -
B61	N	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* DSM 50071	99%	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	A, FA	Gram -
B62	T	*Morganella morganii*	*Morganella morganii* M11	99%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	FA	Gram -
B63	N	*Brevibacterium halotolerans*	*Brevibacterium halotolerans* DSM 8802	99%	Actinobacteria	Actinobacteria	Actinomycetales	Brevibacteriaceae	A	Gram +
B64	N	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* DSM 50071	99%	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	A, FA	Gram -
B65	T	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* DSM 50071	99%	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	A, FA	Gram -

S. pasteurianus has also been detected in a normal intestinal flora (Wang *et al.* 2012). *S. algae* was described as a possible bacterium associated with obesity (Chiu *et al.* 2014), and it was found in the intestinal crypts of germfree BALB/c mice although the authors did not disregarded the hypothesis of being a possible contamination (Pédron *et al*. 2012).

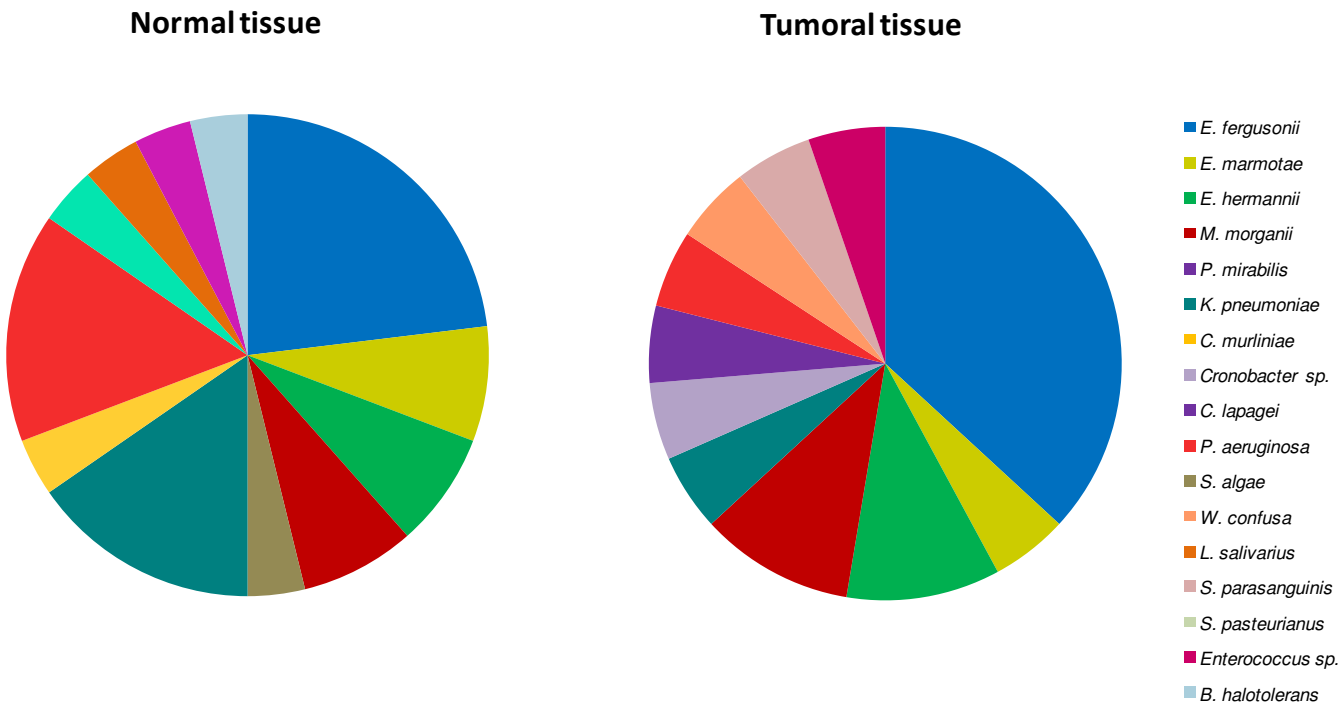


Figure 6. Representation of species relative occurrence in normal and tumoral tissue samples

In agreement with our outcome, Moore and Moore (1995) identified *L. salivarius* in the normal flora of the human gut, which has also been described as a potential probiotic against pathogenic bacteria (Dunne *et al*., 2001). The genus *Brevibacterium* has been associated with human infections (Gruner *et al*., 1994), but the species *B. halotolerans* has not been reported in colon samples.

From TT samples were isolated 4 bacterial species not inventoried in TN samples. They were *Weissella confusa*, *Enterococcus* sp., *Streptococcus parasanguinis* and *Cedecea lapagei*. According to what has been described particularly by Wang *et al.* (2012), Ahn *et al.* (2013) and Sears and Garrett (2014), there are bacterial genera that tend to be more abundant in CRC patients than in healthy intestinal microbiome, such as *Escherichia* and *Enterococcus* species. *Weissella confusa* was previously detected in the feces of both IBS and healthy patients (Ponnusamy *et al*., 2011). *Enterococcus* species

have been described by several authors as a possible probiotic that once administrated (*e.g.*, *E. faecium* CRL 183) in early stages of carcinogenesis can significantly inhibit CRC development (Sevieri *et al.*, 2008). On the other hand, a highly related species to *E. faecium*, *E. faecalis*, was also described by Candela *et al.* (2014) as CRC driver bacteria. *Streptococcus* genus is quite abundant in patients with CRC (Wang *et al.*, 2012) and Candela *et al.* (2014) reported that a species of this genus, *Streptococcus gallolyticus*, is considered to be a passenger bacteria. *C. lapagei* has been mentioned as a cause of bactereamia and it was already determined in a CRC patient (Akinosoglou *et al.*, 2012).

2.4.3 Antimicrobial Activity Tests

As previously mentioned, one of the key functions of the intestinal microbiome is to stimulate/inhibit host immune system and promote a defensive barrier against the invasion of pathogens, namely through the production of antimicrobial compounds. However, shifts in the normal microbial community colonizing the intestinal mucosa due to cancer pathogenesis, may greatly influence these mechanisms.

The isolated bacteria were tested for ascertaining their antimicrobial activity against pathogenic bacteria, by performing the spot-on-lawn assay. From the 57 strains identified, only 12 presented antimicrobial activity (Table 4) against two Gram-positive and/or one Gram-negative indicator bacteria. Nine showed activity against *M. luteus*, 6 against *S. aureus* and 2 against *P. aeruginosa*. Most of the positive bacterial strains were isolated from normal mucosa (B17, B57, B58, B60, B61, B63, B64). From these, only B17 (*E. fergusonii*) showed antimicrobial activity against the Gram-negative *P. aeruginosa*. Likewise, among the bacteria isolated from TT mucosa, only one (B35; *E. hermannii*) had slightly inhibited *P. aeruginosa* growth. All the remaining isolates (either from TN or TT tissue) presented inhibitory activity only against some Gram-positive bacteria species, being particularly noticeable the effect of B57 (*P. aeruginosa*), B61 (*P. aeruginosa*) and B62 (*M. morganii*) against *M. luteus*, and that of B61 (*P. aeruginosa*) and B63 (*B. halotolerans*) against *S. aureus*.

It is of common scientific and clinical knowledge that Gram-negative bacteria present more antimicrobial activity than Gram-positive, which is partly due to different membrane permeabilities and intrinsic metabolism. In particular, multi-drug resistant Gram-negative bacteria, namely belonging to the Enterobacteriacea family brings great therapeutic challenges (Bérdy, 2005), being quite limited the availability of effective drugs for controlling their proliferation. Therefore, the discovery of strains capable of

synthesizing new and more efficient antimicrobial compounds is of far most relevance. B17 and B35 isolates are hence promising bacteria for this purpose.

Table 4. Results of the spot-on-lawn antimicrobial activity test of bacterial isolates from normal (TN) and tumoral (TT) mucosa tissues. Values represent the average inhibition zone (halo) diameter (mm) ± standard deviation from three replicates

Strains	Tissue type	*Micrococcus luteus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
B17	TN	-	-	1.0 ± 0.0
B35	TT	-	-	1.0 ± 0.0
B46	TT	-	1.0 ± 0.0	-
B57	TN	15.0 ± 0.0	-	-
B58	TN	1.0 ± 0.0	-	-
B59	TT	2.0 ± 0.0	-	-
B60	TN	4.3 ± 4.9	1.7 ± 0.6	-
B61	TN	7.0 ± 0.0	2.5 ± 1.7	-
B62	TT	15.0 ± 0.0	-	-
B63	TN	2.0 ± 0.0	2.4 ± 1.9	-
B64	TN	2.8 ± 1.8	1.5 ± 0.6	-
B65	TT	2.0 ± 0.0	1.6 ± 0.9	-

The synthesis of bioactive compounds by *P. aeruginosa* and *Escherichia* spp. strains isolated from other sources was already reported, being demonstrated their inhibitory activity on the growth of Gram-positive but also of Gram-negative bacteria (*e.g.*, Cardozo *et al.*, 2013; Grewal *et al.*, 2014). *M. morganii* can cause opportunistic infections and some strains evidence resistance to multiple drugs due to the production of extended-spectrum β-lactamases (Liu *et al.*, 2016). Different virulence factors were identified in this species (Liu *et al.*, 2016), but no antimicrobial compounds were specifically described in the literature for a *M. morganii* strain, despite the significant antimicrobial activity of its phylotype B62 against *M. luteus* in the present study. Overall, an in-depth study is still required for the identification of the antimicrobial compound(s) that may elicit an inhibitory activity, as well as their synthesis should be optimized for future studies.

In order to obtain a more refined profile regarding the source of synthesis of the antimicrobial compound, the activity of cell-free extract and cell wall washes fractions (Figures 7 and 8) of nine of the isolates providing positive results in the spot-on-lawn

assays was then evaluated through agar diffusion tests, being the remaining ones still under test. The results obtained for these assays are summarized in Table 5.

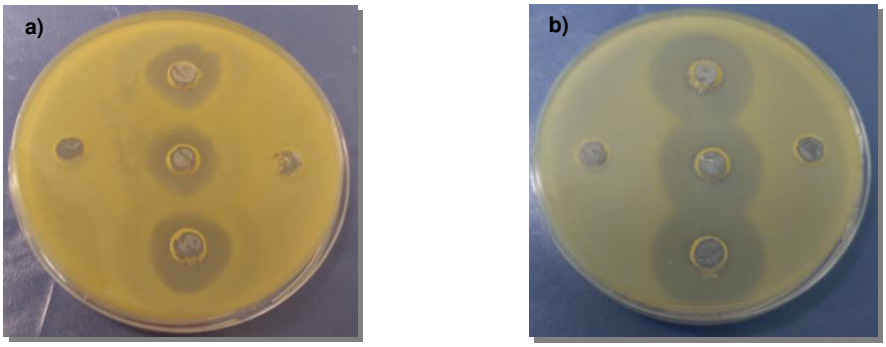


Figure 7. Antimicrobial activity of the cell wall washes of three isolates against *M. luteus* (a) and *S.aureus* (b)

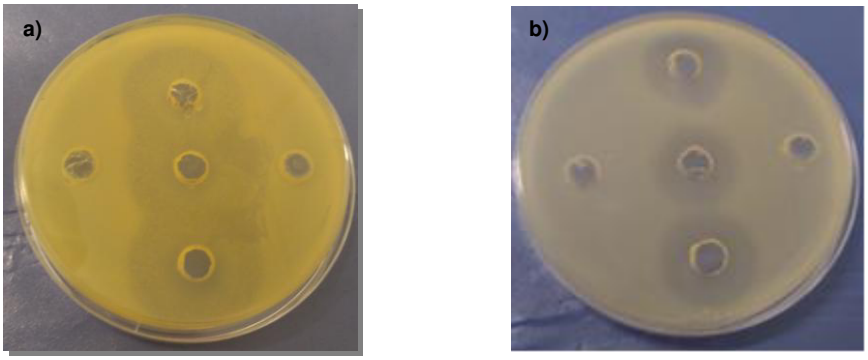


Figure 8. Antimicrobial activity of cell-free extracts from three isolates against *M. luteus* (a) and *S.aureus* (b)

Surprisingly, the strains B58, B59 and B62 did not present inhibitory activity in these assays, while B57 showed antimicrobial activity against *S. aureus* despite the negative outcome in the spot-on-lawn assays. The non-inhibitions observed can be explained by the conditions under which the isolates were maintained. For example, the use of nutritive media may affect the biosynthesis of antimicrobial substances, which usually occurs in deprivation of certain elements (Pingitore *et al*., 2007). Under less harsh conditions, the need for bacteria to produce secondary metabolites or bioactive compounds with a self-protective function is reduced (Nes *et al*., 2007). In turn, rich media may promote the adsorption of antimicrobial substances, hence limiting their bioavailability (Pingitore *et al*., 2007).

Table 5. Results of the antimicrobial activity of different bacterial fractions (*i.e.*, cell-free extract and cell wall washe). Values represent average inhibition zone (halo) diameter (mm) ± standard deviation of three replicates

Pathogenic Bacteria / Strains	Tissue type	*Micrococcus luteus*		*Staphylococcus aureus*	
		Cell-free extract	Cell Wall Wash	Cell-free extract	Cell Wall Wash
B57	TN	10.0 ± 0.0	3.0 ± 0.0	3.7 ± 2.5	5.5 ± 0.7
B58	TN	-	-	-	-
B59	TT	-	-	-	-
B60	TN	7.0 ± 0.0	7.0 ± 4.2	-	4.7 ± 2.1
B61	TN	1.0 ± 0.0	3.0 ± 0.0	-	-
B62	TT	-	-	-	-
B63	TN	11.0 ± 0.0	7.8 ± 4.0	7.3 ± 2.3	7.6 ± 2.5
B64	TN	11.0 ± 0.0	8.3 ± 4.1	4.7 ± 0.6	7.0 ± 2.7
B65	TT	10.0 ± 0.0	9.3 ± 3.6	8.7 ± 1.2	7.0 ± 2.8

Therefore, these assays should be fine-tuned taking into consideration different interfering factors on the substances activity, such as culture media, pH and temperature.

In general, the cell-free extracts presented higher activity against *M. luteus* comparatively to the cell wall washes for the isolates B57, B60, B61, B63, B64 and B65, whilst the contrary was observed for *S. aureus*, which growth was usually more affected by cell wall washes of B57, B60, B63, B64, B65.

2.4.4 Antibiotic Susceptibility Tests

In general, except for B25 (*Lactobacillus salivarius*; a probiotic bacterium with beneficial action) and B33 (*Streptococcus pasteurianus*; can induce infections), all the other bacterial isolates presented resistance to at least three of the tested antibiotics (Figure 9, Table 6). More than 75% of the isolates, and particularly Gram-negative bacteria were resistant to amoxicillin, penicillin, cephalothin, erythromycin and bacitracin. In turn, more than 78% were susceptible to the remaining antibiotics, especially to gentamicin for which 100% of the strains were susceptible. In fact, *Weissella confusa* (B8), *Lactobacillus salivarius* (B25), *Streptococcus pasteurianus* (B33), *Enterococcus* sp. and a strain of the phylotype *P. aeruginosa* (B60) were sensitive to a greater

Table 6. Results of the antibiotic susceptibility tests performed with Gram-negative and Gram-positive bacteria isolated from normal (TN) and tumoral (TT) mucosa tissues. R- Resistant; I – Intermediate; S- Susceptible; Amox. – amoxicillin; Aztreo. – aztreonam; Cephal. – cephalothin; Chloram. – chloramphenicol; Erythr. – erythromycin; Gentam. – gentamicin; Strepto. – streptomycin.

				Antibiotics									
Phylotype	Gram staining	Strain code	Tissue type	Amox. (10 µg)	Aztreo. (30 µg)	Bacitracin (10 U)	Cephal. (30 µg)	Chloram. (30 µg)	Erythr. (15 µg)	Gentam. (30 µg)	Imipenem (10 µg)	Penicillin (10 U)	Strepto. (300 µg)
Shewanella algae	-	B2	TN	R	S	R	R	S	S	S	R	R	S
Morganella morganii	-	B3	TN	R	S	R	R	S	R	S	S	R	S
		B9	TT	R	S	R	R	S	R	S	S	R	S
		B62	TT	R	S	R	R	S	R	S	S	R	S
Escherichia marmotae	-	B4	TN	R	S	R	R	S	R	S	S	S	S
		B32	TN	R	S	R	R	S	R	S	S	R	S
		B34	TT	R	S	R	R	S	R	S	S	R	S
Escherichia hermannii	-	B5	TN	R	S	R	R	S	R	S	S	R	S
		B24	TT	R	S	R	R	S	R	S	S	R	S
		B27	TN	R	S	R	R	S	R	S	S	R	S
		B35	TT	R	S	R	R	S	R	S	S	R	S
Escherichia fergusonii	-	B6	TN	R	S	R	R	S	R	S	S	S	S
		B11	TT	R	R	R	R	S	R	S	S	R	S
		B12	TN	S	S	R	R	S	R	S	S	R	S
		B17	TN	R	S	R	R	S	R	S	S	R	S
		B18	TN	R	S	R	R	S	R	S	S	R	S
		B20	TT	R	S	R	R	S	R	S	S	R	S
		B38	TT	R	S	R	R	S	R	S	S	R	S
		B39	TN	R	S	R	R	S	R	S	S	R	S
		B46	TT	R	S	R	R	S	R	S	R	R	S
		B54	TN	R	S	R	R	S	R	S	S	S	S
Weissella confusa	+	B8	TT	S	R	S	R	S	S	S	S	S	S
Klebsiella pneumoniae	-	B14	TN	R	S	R	R	S	R	S	S	R	S
		B22	TT	R	S	R	R	S	R	S	S	R	S
		B26	TN	R	S	R	R	S	R	S	S	R	S
		B58	TN	R	S	R	R	S	R	S	S	R	I
Lactobacillus salivarius	+	B25	TN	S	R	S	R	S	S	S	S	S	S
Streptococcus pasteurianus	+	B33	TN	S	R	S	S	S	S	S	S	S	S
Citrobacter murliniae	-	B40	TN	R	S	R	R	S	R	S	S	R	S
Enterococcus sp.	+	B48	TT	S	R	S	R	S	S	S	S	S	R
Proteus mirabilis	-	B49	TN	R	S	R	R	S	R	S	S	S	S
Cronobacter sp.	-	B51	TN	R	S	R	R	R	R	S	S	R	S
Streptococcus parasanguinis	+	B53	TT	R	S	R	R	S	R	S	S	S	S
Pseudomonas aeruginosa	-	B57	TN	R	S	R	R	S	R	S	S	R	S
		B60	TN	R	R	S	S	S	S	S	S	R	S
		B61	TN	R	R	R	R	I	R	S	S	R	S
		B64	TN	R	S	R	R	I	R	S	S	R	S
		B65	TT	R	S	R	R	I	R	S	S	R	S
Cedecea lapagei	-	B59	TT	R	I	R	R	S	R	S	I	R	S
Brevibacterium halotolerans	+	B63	TN	R	I	R	R	R	R	S	S	R	S

number of antibiotics. In regard to the distribution of the susceptibility of the bacteria according to the tissue type from which they were isolated, it was noticeable that the higher number of strains evidencing either resistance or susceptibility to antibiotics came originally from TN.

Amoxicillin is an antibiotic with a broad spectrum of action, being Gram-positive bacteria (*e.g.*, *Enterococcus* sp., *Streptococcus pneumoniae*), as well as Gram-negative bacteria (*e.g.*, *Escherichia coli*, *Proteus mirabilis, Klebsiella* spp., *Pseudomonas* spp.) susceptible to it (Abgueguen *et al.*, 2007; Tansarli *et al.*, 2013). Anderson (2017) had recently described some other amoxicillin resistant species, *Staphylococcus aureus* and *Staphylococcus epidermidis* (methicillin-resistant). Penicillin has a spectrum of action against several bacilli, and Gram-positive and Gram-negative cocci such as *Klebsiella* spp., *Proteus* spp. and *Enterobacter* spp. (Azevedo, 2014). Cephalothin is an antibiotic with a bactericidal action that inhibits the Gram-positive *Staphylococcus aureus*, *Staphylococcus epidermidis* and Gram-negative bacteria such as *Escherichia coli*, *Proteus mirabilis* and *Klebsiella* spp. (Azevedo, 2014). Relatively to bacitracin, its mode of action interferes with the protein synthesis of the microorganism and mainly affects Gram-positive cocci like *Staphylococci* and *Streptococci*, being however more inactive against Gram-negative bacteria (Glasser *et al.*, 2010). Some genera like *Streptococcus* have already been described as being resistant to penicillin (Passàli *et al.*, 2007). Gentamicin affects Gram-positive *Staphylococcus aureus* (Glasser *et al.*, 2010), but was also effective against Enterobacteriacea (Tansarli *et al.*, 2013) such as *Proteus mirabilis*, *Pseudomonas* spp., *Klebsiella* spp., and *Escherichia coli*, which are among of the cultivable bacteria identified in the mucosa samples herein studied.

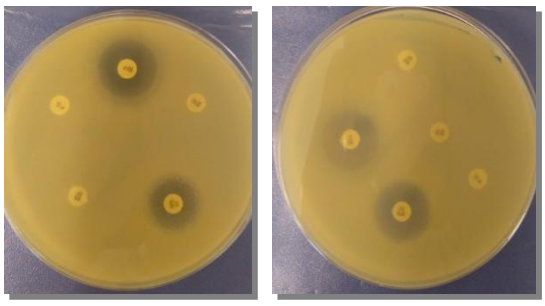


Figure 9. Antibiotic susceptibility assay with 10 antibiotic disks

In intestinal microbiota exists a large density of organisms sharing the same ecological niche giving the opportunity for the transference of genetic material, namely of antibiotic resistance genes (Schaik, 2015). Antibiotics disturb the balance in commensal

populations, and lead to a reduced or altered communication between the intestinal microflora and the underlying mucosa. A major consequence of shifts in the intestinal microflora to a more resistant microbiome is the enhanced host vulnerability to develop infections, antibiotic allergies, increase the predisposition to get metabolic syndrome, as well as the effectiveness of cancer therapeutics may then be strongly limited. Hence, the use of antibiotics should therefore be reduced to prevent the propagation of such resistance (Becattini *et al*., 2016).

2.5. Conclusions

The microbiome present in the intestine is quite diverse and complex, being an important key for maintaining the balance of host health (Ducan *et al*., 2007; Liang *et al*., 2014). When this balance is no longer present, situations of dysbiosis occur, which may lead to CRC development (Tojo *et al*., 2014). Thus, it is increasingly of common interest to identify the microbiome present in the colon and how this interferes with the development or control of CRC. This study made use of different molecular and qualitative techniques to perform a preliminary approach to unravel alterations of the microbiota community from normal to tumor colonic tissues. One relevant aspect of the present study regarded its application to Portuguese CRC patients, since our country was not considered in related studies, despite the great variations between geographic areas. The results showed some differences in the microbial composition of TN and TT tissues, and their potential influence on colon inflammation and CRC progression. Most cultivable aerobic/facultative anaerobic bacteria belonged to Proteobacteria phylum, though some belonged to Firmicutes and Actinobacteria. Twelve bacteria evidenced antimicrobial activity against Gram-positive and/or Gram-negative bacteria. The greatest part of these were isolated from TN tissue. Indeed, the bacteria with higher resistance and susceptibility to antibiotics were isolated from TN tissues. Notwithstanding future studies should bear on the use of third generation sequencing techniques to get a deeper understanding of changes in the microbiome of CRC patients. This will help perceiving how the microbiome could modulate CRC development, or be conversely modulated to counteract its evolution.

CHAPTER III

Chapter III - The microbiome as a complementary therapeutic tool against colorectal cancer

3.1 Abstract

Bacteria-mediated therapy has been a promising alternative to cope with the conventional colorectal cancer (CRC) treatments, which efficacy is often limited to prevent cancer development or recurrence. In this mini-review it is intended to provide an overview on the recent research performed towards the application of bacteria as anticancer (adjuvant) agents. It is hence presented approaches regarding the modulation of microbial community colonizing the human intestine through probiotics and prebiotics, and fecal microbiome transplantation. The use of bacteriophage therapy and its potentialities regarding CRC control are also mentioned. Finally, some of the new research directions based on a more in-depth study of bacterial abilities are described, namely: the use of their defense mechanisms through the exploration of the clustered regularly interspaced short palindromic repeats (CRISPR) and the Cas family genes; target essential genes and quorum sensing systems for controlling the CRC-associated microbiota.

Keywords: bacterial-mediated therapy, CRISPR/Cas systems, microbiome, prebiotics, probiotics, quorum sensing systems, bacteriophage,

3.2 Introduction

Colorectal cancer is one of the deadly cancers affecting western population and ranked in the third position with more than 690,000 deaths worldwide in 2012 (Ferlay *et al.*, 2015). It is characterized by an abnormal proliferation of the epithelial cells in colon mucosa. The most frequent therapeutic strategies to counteract this proliferation are surgery, chemotherapy and radiotherapy. However, their efficacy and specificity tends to be limited depending on the disease staging, tumor mutations and microenvironment characteristics (American Cancer Society, 2017). For instance, the anoxic and hypoxia zones typical of the tumor core may severely constrain the delivery of chemoterapeutants, as well as, it may reduce their ability to provoke cell death due to the requirement of oxygen to induce apoptotic mechanisms (Shannon *et al.*, 2003). Consequently, they may fail to destroy all cancer cells and promote recurrence, due to their difficulty in reaching the whole necrotic area and possible toxicity to normal tissues (Belizário & Napolitano, 2015).

Alternatively, bacteria-based therapies have been studied as a potential adjuvant treatment for controlling the proliferation of cancer, namely colorectal cancer (CRC). There are consistent evidences of a significant role of microbiome on CRC progression or regression associated with the straight interplay between the host and gut-colonizing symbiotic microbial community (Wang & Jia, 2016). Besides, the unique abilities featured by bacteria also make them promising anticancer (adjuvant) agents: they are motile, can synthesize anticancer compounds, can proliferate and accumulate in the anoxic microenvironments, and stimulate the host immune system (Sears & Garrett, 2014). As such, this mini-review aims to describe the most recent approaches involving the use of bacteria, their derivatives, metabolic and/or molecular features in colon cancer therapy, highlighting as well new opportunities in bacteria-based treatments bearing on the recently evolved next-generation tools.

3.3 Alternative and adjuvant microbially-based therapies

Some studies have been describing the advantages of using bacteria for preventing CRC progression through different approaches. Part of them focus on gut microbiome modulation for the control of pathogenic microbes and enhancement of beneficial ones. This can be done through the ingestion of probiotics and prebiotics, but also through fecal transplantation and bacteriophage therapy. Moreover, the recent application of next-generation tools together with the development of increasingly more robust bioinformatic softwares has provided new insights for discovering potential adjuvant therapeutic agents against CRC.

3.3.1 Probiotics and Prebiotics

Probiotics are live bacteria that once administrated at adequate amounts confer or promote host health by the colonization of beneficial microorganisms (FAO/WHO, 2001). The associated benefits are mostly related with antimicrobial activity against pathogens (by reducing inflammation and colitis), immune-modulatory effects, regulation of the host metabolism and prevention of cancer progression (Belizário & Napolitano, 2015; Ambalam *et al*., 2016). Some examples are described below:

● modulation of gut microflora - a study reported by Uccello *et al.* (2012) suggested that after ten days of *Lactobacillus acidophilus* [N-2 and NCFM strains] ingestion, the activity of fecal enzymes (β-glucuronidase, nitroreductase and azoreductase) that facilitate the release of pro-carcinogenic substances from glucoronides deconjugation, was decreased, despite the need for a continuous probiotic ingestion as to maintain the enzymatic levels low;

● regulation of intestine pH - an alkaline intestinal pH promotes the proliferation of pathogenic bacteria that can drive to CRC. Hence the combination of fructo-oligosaccharides enriched with inulin[2] and probiotics resulted in an increase of some groups of lactic acid bacteria (*Bifidobacterium* and *Lactobacillus*) on the intestinal flora of CRC patients and a reduction in pH values (Rafter *et al*., 2007);

● immune-modulatory effects - *Lactobacillus casei* Shirota stimulates type 1 helper T cells and cellular immune system, reducing the incidence of tumor and IgE production (Yasui *et al*., 1999);

[2] **FOS enriched with inulin** is made by combining two substances that occur naturally in plants (chicory root, wheat, bananas, onion, and garlic) and helps healthy bacteria proliferation in the intestine.

● induction of apoptosis - certain probiotics interfere with different stages of signaling pathways, for example *Lactobacillus reuteri* promotes the death of stimulated immune cells by enhancing pro-apoptotic NF-κB and mitogen-activated protein kinase [MAPK][3] signaling (Iyer *et al*., 2008);

● antimutagenic effect - one study demonstrated that *Lactobacillus rhamnosus* 231 bounds N-methyl-N0-nitro-N-nitrosoguanidine and 2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline (carcinogens), hence exhibiting antimutagenic activities because probiotics bind mutagens and lead to biotransformation, and other data showed that the binding of mutagens can occur at bacterial (*Lactobacillus plantarum* KLAB21) cell wall by extracellular glycoproteins (Rhee & Park, 2001).

● inhibition of tyrosine kinase signaling pathway - this path controls the cellular proliferation and differentiation. An *in vitro* study revealed that the probiotic *Bacillus polyfermenticus* suppressed colon cancer tumor growth, by inhibiting two epidermal growth factor receptors that promote cancer development, ErbB2 and ErbB3, and their downstream signaling molecules E2F-1 and cyclin D1 (Ma *et al*., 2010).

Overall, it should be emphasized that the administration of probiotics in patients with CRC not only has an anti-carcinogenic power, but also allows the functional recovery of the bowel, as well as it prevents infections like bacteremia or septicemia after surgical intervention (Yang *et al*., 2016).

Prebiotics are substances that promote the development of beneficial gut microbiota (*i.e*., stimulate probiotics), down-regulate the expression levels of NF-κB, contribute to immunomodulation, fermentative production of SCFAs and activation of apoptosis (Gourineni *et al*., 2011; Belizário & Napolitano, 2015; Ambalam *et al*., 2016). Natural prebiotics may be obtained through the consumption of a diet rich in fiber, which boost the bowel transit. More recently, fructan mixtures such as Prebio 1® , which has 70% fructo-oligossacharides and 30% inulin (Wong *et al*., 2016). Inulin and fructo-oligossacharides, which occur naturally in plants like *Cichorium intybus* L., are linear fructose units (2 to 60) linked by β (2-1) bonds with terminal glucose unit. Inulin is known for stimulating the production of SCAFs (*e.g*., acetate, propionate, butyrate), promoting the decrease of intestinal pH and, consequently, stimulate the growth of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria*, and increase the absorption of calcium, magnesium and iron. Plus, it can retard the proliferation of tumor cells and activate apoptosis; however, it is more effective in premature adenoma cells than advanced stages of CRC (Bartolomeo

[3] **NF-κB** and **MAPK** key factors that regulate inflammation, cell proliferation and apoptosis by intracellular signaling pathways.

et al., 2013; Gualtieri *et al.*, 2013). Unlike probiotics, prebiotics are able to regulate the bacterial cell cycle and the metabolic activity of beneficial bacteria that exist in a healthy gut or in tumor cells. Hence, the conjugation of probiotic and prebiotic (*i.e.*, synbiotic) has been an excellent combination for homeostasis regulation purposes. For example, the probiotic *Bifidobacterium longum* and fructo-oligossacharides enriched with inulin are used in individuals with ulcerative colitis (Bartolomeo *et al.*, 2013; Gualtieri *et al.*, 2013; Belizário & Napolitano, 2015).

3.3.2 Fecal microbiome transplantation (FMT)

FMT consists in the transplantation of liquid suspension of intestinal flora from a healthy donor (like a healthy family member) into diseased individuals with the aim of reestablishing their intestinal microbiota (Gough *et al.*, 2011). This treatment has been used successfully in cases of CDI, but also in pseudomembranous colitis (PMC), IBD and irritable bowel syndrome (IBS) (Gough *et al.*, 2011; Peterson *et al.*, 2014). In fact, this transplantation can transfer a healthy microbiota, but also the pathogens carried in donor intestine. This and other problems, like the lack of knowledge on the exact mode of action except that it will restore the function and composition of intestinal flora, may constrain the use of this therapy (Gough *et al.,* 2011; Belizário & Napolitano, 2015). Even so, it can be a treatment option for diseases related with dysbiosis, though it is yet to be optimized (Peterson *et al.*, 2014).

3.3.3 Bacteriophage Therapy

Bacteriophages are phages or virus that can affect bacteria through surface receptors and have the ability to disturb their metabolism by causing their death, what may be used as a potential therapy for controlling infectious diseases (Bárdy *et al.*, 2016; Orndorff, 2016). The bacteriophage therapy can be an alternative to antibiotics, thereby helping to overcome antibiotic resistance problems. Phages may present two modes of action. The lytic cycle consists in the attachment of phage to the bacterial cell with subsequent replication inside it, causing cell lysis and liberation of new phages. The lysogenic cycle can be similar to the lytic cycle, or promote phage DNA integration into host chromosome, therefore resulting in cell replication for many generations (Sulakvelidze *et al.*, 2001). The application of phage therapy can have a phenomenal impact in diseases such as CRC. Recent investigations in mice demonstrated that the toxic *E* gene from the bacteriophage

ϕX174, lowered carcinoma embryonic antigen (CEA)[4] and repressed the growth of tumor cells. This study proved the efficacy of the association of bacteriophage therapy with CRC *CEA*-promoter to control the disease (Rama *et al.*, 2015).

3.4 Recent approaches to uncover new bacteria-mediated therapeutics

With the development of genetic and 'omics' tools either directed to genetic engineering, metagenomics, proteomics, metatranscriptomics or metabolomics, new perspectives have evolved in cancer research and, more specifically, on bacteria-mediated therapies. Under this context, it is hereinafter presented some of the recently evolved research lines regarding the application of bacteria as anticancer (adjuvant) agents to treat CRC.

3.4.1 CRISPR/Cas Systems

The bacteria evolve defense mechanisms to avoid phage infections. These mechanisms can elicit changes in membrane receptors or blockage of essential proteins for the integration of phage DNA into the host cell. Alternatively, they develop an innate immunity system by sequestering phage short DNA fragments and integrating them into their chromosome as repeated sequences – Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), which guarantee infection memory (Belizário & Napolitano, 2015). These repeated sequences are arrayed by proteins encoded by the Cas family genes[5]. CRISPR-Cas systems are classified according to the proteins and mechanisms of action, being divided into types I, II, III and IV (Hille & Charpentier, 2016). The type II of CRISPR system and protein Cas9 are often described in studies of genome editing applied to tumors. For example, the identification of genes associated with tumor growth or organoids culture used for CRC research can be performed by utilization of this system (Hille & Charpentier, 2016; Young & Reed, 2016). Matano *et al.* (2015) used CRISPR/Cas9 in genetically engineered organoids, to unravel the sequential formation of driver mutations of CRC reconstructed from intestinal samples of healthy humans. The authors verified that these specific mutations could be selected by changing niche factors in culture media, hence representing a successful disease model system. To further study the efficiency of this model system, the authors implanted the mutation-carrying organoids into mice as to perceive which could drive invasion and metastasis of CRC. However, and

[4] **Carcinoma embryonic antigen** is produced in gastrointestinal tissue during fetal development, but the production stops before birth. In adult, the expression of CEA is characterized by a oncofetal tumor marker and is overexpressed in over 90% of CRC cells.
[5] **Cas genes** encode for a family of proteins, typically nucleases, helicases, polymerases and polynucleotide binding proteins.

contrary to what happened with the organoids of disease model system, the engineered organoids did not produce tumors or metastasis. Even so, CRISPR/Cas9 has enormous potential for the isolation of genetic mutations promoting cancer (Matano *et al.*, 2015; Young & Reed, 2016).

3.4.2 Targeting essential genes

For recognizing possible treatment targets it is crucial to know the essential genes that stimulate the growth and survival of bacteria (Belizário & Napolitano, 2015). This knowledge can be obtained namely by the construction of a transposon library and the use of suitable software (*e.g.,* "ESSENTIALS"), which enables the rapid and sensitive transposon insertion sequencing analysis through data filtering, normalization and appropriate statistical tests for discovering many possible essential genes (Zomer *et al.*, 2012). The essential genes are directly involved in the metabolism of amino acids, carbohydrates, methanogenesis, xenobiotics and production of vitamins (Belizário & Napolitano, 2015). In fact, nearly 20% of the essential genes have a great interest towards drug discovery (Christen *et al.*, 2011). For example, enzymes of the folate biosynthesis pathway have been the target for the production of pharmaceuticals that inhibit folate synthesis, since its accumulation enhances CRC progression (Kim, 2007). Two pharmaceuticals were already produced to inhibit enzymes involved in the production of short-chain fatty acids by microbes (Belizário & Napolitano, 2015), which have been shown to repress intestine inflammation and CRC by promoting anti-inflammatory responses, apoptosis and cell differentiation (Smith *et al.*, 2013; Keku *et al.*, 2015).

3.4.3 Quorum Sensing Systems

Quorum sensing (QS) is the intracellular communication between bacteria that is mediated by small molecules extracellularly excreted, the autoinducers. There have been identified various autoinducers belonging to different chemical classes. These small molecules either diffuse (passively or actively) across cell membrane into the extracellular environment or are incorporated into vesicles and transferred between cells (Hense & Schuster, 2015). This communication system is more common in bacteria, being responsible for the control of diverse functions, such as the regulation of the production of substances, metabolites and virulence factors that for instance may stimulate the attack of enteric pathogenic *E. coli* or inhibit the immune system (Hsiao *et al.*, 2008; Belizário & Napolitano, 2015). QS molecules are expected to play a role in the host gut mucosa colonization by bacteria, what may in turn be connected to the promotion of certain

diseases. For example, some quorum sensing peptides (*e.g.*, vascular endothelial growth factor, VEGF) can activate the receptors at the endothelial cells, which are involved in the angiogenesis and pro-angiogenic cytokines SDF-1α and HGF (detent the Ras/raf/MEK/MAPK, PI3K/Akt and STAT intracellular signaling cascades over CXCR4 and MET receptor) signpost metastasis indorsing characteristics for colon cancer, and often necessitating quorum sensing mediated biofilm development (Wynendaele *et al.*, 2015).

Thus, it can be of great therapeutic value to target the molecular cues, receptors and signal transduction pathways in QS communication systems. Getting knowledge on how bacterial cells communicate can help avoiding gut colonization by pro-carcinogenic bacteria and further prevent the development of cancer (Belizário & Napolitano, 2015).

3.5 Conclusions

The influence of intestine microbiota on dysbiosis as well as the microbiota-host interplay in CRC pathogenesis, makes bacteria promising anticancer agents for the development of fine-tuned and efficacious adjuvant treatments to fight CRC. Hence, focusing the research on new strategies towards anticancer therapies has been very welcomed. The modulation of intestine microbiome through the use of probiotics and prebiotics as well as the transplantation of fecal microbiome and bacteriophage therapy may bring good outcomes regarding the prevention and inhibition of CRC progression. Still, more groundbreaking and precision therapies are required given the heterogeneity in CRC. As such, a new trend is arising considering the use of CRISPR-Cas9 systems, essential genes control and manipulation of quorum sensing pathways, for modulating intestine microbiome as to inhibit CRC progression or recurrence. Nevertheless, a great research effort is still required in a near future as to bring the promising outcomes obtained at disease, organoid and mouse models levels to the clinical level.

CHAPTER IV

Chapter IV. Final Considerations

The impact of intestinal human microbiota on host homeostasis starts early at birth and constrains its evolution and immune system through the rest of his/her life. Notwithstanding, it undergoes several modifications in response to different environmental factors such as lifestyle, diet, exposure to medicines and diseases. Under certain circumstances, a instability at the intestinal microbiome can come into a dysbiotic situation, which causes repercussions on host health and may lead to pathologies with different severity levels that may ultimately end up in CRC. In order to increase the generation of efficient diagnostic tools and therapeutic treatments for CRC, it is important to get a deeper knowledge on the CRC-associated microbiome, especially because of the great heterogeneity occurring between individuals (Oke and Martin, 2017; Pope *et al*., 2017). Thus, it is very important to study the diversity of the microbiota community and uncover its capabilities in a situation of CRC, so that later can be studied new methods of screening and treatment of the disease.

This study used different approaches to reveal the alterations of the microbial community from normal and affected mucosa tissues of CRC-affected patients undergoing surgery at the Centro Hospitalar do Baixo Vouga, E.P.E., and which participated in the study as volunteers. Furthermore, it was also studied how the responses of the cultivable microorganisms towards situations of stress (antimicrobial susceptibility and exposure to antibiotics). A relevant aspect of the present study is its application to Portuguese CRC patients, since there is gap on data availability for our country, despite the great percentage of population that has been deeply affected by this disease in Portugal.

Given the relevance of the intestine microbiome on gut homeostasis and CRC, bearing on the microbiome-host interplay to generate complementary therapies through the use of bacterial features and abilities, has been an appealing alternative within the drug discovery world. Different strategies are hence being explored in this direction, and several bacteria have been highlighting their value as anticancer agents.

Despite the valuable outcomes obtained in this work, future study approaches, should be performed to get a deeper understanding on the microbial community shifts in normal and tumoral tissues, together with the stability of the antimicrobial activity and susceptibility to antibiotics of the cultivable bacteria.

References

Abgueguen P, Azoulay-Dupuis E, Noel V, Moine P, Rieux V, Fantin B, Bedos JP. 2007. Amoxicillin is effective against penicillin-resistant *Streptococcus pneumoniae* strains in a mouse pneumonia model simulating human pharmacokinetics. Antimicrob Agents Chemother 51: 208-14 doi:10.1128/AAC.00004-06

Ahn J, Shina R, Pei Z, Dominianni C, Wu J, Shi J, Goedert J J, Hayes R B, Yang L. 2013. Human gut microbiome and risk for colorectal cancer. Journal of the National Cancer Institute 105: 1907–1911 doi: 10.1093/jnci/djt300

Akinosoglou K, Perperis A, Siagris D, Goutou P, Spiliopoulou I, Gogos CA, Marangos M. 2012. Bacteraemia due to *Cedecea davisae* in a patient with sigmoid colon cancer: a case report and brief review of the literature. Diagnostic Microbiology & Infectious Diseases 74: 303-6 doi: 10.1016/j.diagmicrobio

Ambalam P, Raman M, Purama R K, Doble M. 2016. Probiotics, prebiotics and colorectal cancer prevention. Best Practice & Research Clinical Gastroenterology 30: 119-131 doi: 10.1016/j.bpg.2016.02.009

American Cancer Society. Treatment of colon cancer by stage [Internet]. 2017. Available from: http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-by-stage-colon#

Anderson L. 2017. Antibiotic resistance: a global threat. Drugs.com [Internet] Available at https://www.drugs.com/article/antibiotic-resistance.html [accessed on June of 2017]

Arthur J C, Jobin C. 2011. The struggle within: microbial influences on colorectal cancer. Inflamm Bowel Diseases 17: 396–409 doi: 10.1002/ibd.21354

Azevedo S M M. 2014. Farmacologia dos antibióticos beta-lactâmicos. Universidade Fernando Pessoa e Faculdade de Ciências da Saúde 1-70

Bailey M T, Dowd S E, Parry N M, Galley J D, Schauer D B, Lyte M. 2010. Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium*. Infection and Immunity 78: 1509–1519 doi: 10.1128/IAI.00862-09

Bárdy P, Pantucek R, Benesík M, Doskar J. 2016. Genetically modified bacteriophages in applied microbiology. Journal of Applied Microbiology 121: 618-633 doi: 10.1111/jam.13207

Bartolomeo F, Startek J B, Van den Ende W. 2013. Prebiotics to fight diseases: reality or fiction?. Phytotherapy Research 27: 1457-1473 doi: 10.1002/ptr.4901

Belizário J E, Napolitano M. 2015. Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches. Frontiers in Microbiology 6: 1-16 doi: 10.3389/fmicb.2015.01050

Becattini S, Taur Y, Pamer E G. 2016. Antibiotic-induced changes in the intestinal microbiota and disease. Trends in Molecular Medicine 22: 458-478 doi: http://dx.doi.org/10.1016/j.molmed.2016.04.003

Bérdy J. 2005. Bioactive microbial metabolites, a personal view. The Journal of Antibiotics 58: 1–26 doi: 10.1038/ja.2005.1

Burns M B, Lynch J, Starr T K, Knights D, Blekhman R. 2015. Virulence genes are a signature of the microbiome in the colorectal tumor microenvironment. Genome Medicine 7: 1-12 doi:10.1186/s13073-015-0177-8

Candela M, Turroni S, Biagi E, Carbonero F, Rampelli S, Fiorentini C, Brigidi P. 2014. Inflammation and colorectal cancer, when microbiota-host mutualism breaks. World Journal Gastroenterology 20: 908-922 doi: 10.3748/wjg.v20.i4.908

Canha M B, Cidade J P P, Ribeiro, Moreira A F L,Nunes P P N. 2015. Role of colonic microbiota in colorectal carcinogenesis: A systematic review. Revista Española de Enfermedades Digestivas 107: 659-671 doi: S1130-01082015001100003

Cardozo V F, Oliveira AG, Nishio EK, Perugini MR, Andrade CG, Silveira WD, Durán N, Andrade G, Kobayashi RK, Nakazato G. 2013. Antibacterial activity of extracellular compounds produced by a *Pseudomonas* strain against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Ann Clin Microbiol Antimicrob. 17:12 doi: 10.1186/1476-0711-12-12.

Chan A T, Giovannucci E L. 2010. Primary prevention of colorectal cancer. Gastroenterology 138: 2029 – 2043 doi: 10.1053/j.gastro.2010.01.057

Chiu C-M, Huang W-C, Weng S-L, Tseng H-C, Liang C, Wang W-C, Yang T, Yang T-L, Weng C-T, Chang T-H, Huang H-D. 2014. Systematic analysis of the association between gut flora and obesity through high-throughput sequencing and bioinformatics approaches. Hindawi Publishing Corporation, BioMed Research International 2014:1-10 doi: 10.1155/2014/906168

Christen B, Abeliuk E, Collier J M, Kalogeraki V S, Passarelli B, Coller J A, Fero M J, McAdams H H, Shapiro L. 2011. The essential genome of a bacterium. Molecular Systems Biology 7: 1-7 doi: 10.1038/msb.2011.58

Conlan S, Kong H H, Segre J A. 2012. Species-level analysis of DNA sequence data from the NIH human microbiome project. Plos One 7: 1-7 doi: 10.1371/journal.pone.0047075

Dominianni C, Sinha R, Goedert J J, Pei Z, Yang L, Hayes R B, Ahn J. 2015. Sex, body mass index, and dietary fiber intake influence the human gut microbiome. Plos One 10: 1-14 doi: 10.1371/journal.pone.0124599

Dulai S, Keku T O. 2014. Gut microbiome and colorectal adenomas. Cancer Journal 20: 225-231 doi:10.1097/PPO.0000000000000050

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan G C, Shanahan F, Collins J K. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. The American Journal of Clinical Nutrition 73: 386S–3892S

Eckburg P B, Bik E M, Bernstein C N, Purdom E, Dethlefsen L, Sargent M, Gill S R, Nelson K E, Relman D A. 2005. Diversity of the human intestinal microbial flora. National Institutes of Health 308: 1635–1638 doi: 10.1126/science.1110591

EUCAN. 2012. [Internet] Estimated mortality for men and women in Portugal, 2012. EUCAN. Available at http://eco.iarc.fr/eucan/Country.aspx?ISOCountryCd=620 [accessed on October of 2017]

FAO/WHO. 2001. Evaluation of Health and Nutritional Properties of Powder Milk and Live Lactic Acid Bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report, pp. 1–34.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin D M, Forman D, Bray F. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 136:E359-86 doi: 10.1002/ijc.29210

Fontanges Q, Mendonca R, Salmon I, Mercier M, D'Haene N. 2016. Clinical application of targeted next generation sequencing for colorectal cancers. International Journal of Molecular Sciences 17: 1-10 doi: 10.3390/ijms17122117

Gao R, Gao Z, Huang L, Qin H. 2017. Gut microbiota and colorectal cancer. European Journal of Clinical Microbiology & Infectious Diseases 36:757-769 doi: 10.1007/s10096-016-2881-8

Glasser J S, Guymon C H, Mende K, Wolf S E, Hospenthal D R, Murray C K. 2010. Activity of topical antimicrobial agents against multidrug-resistant bacteria recovered from burn patients. Burns 36:1172-84 doi: 10.1016/j.burns.2010.05.013

GLOBOCAN. 2012. [Internet] Colorectal cancer estimated incidence, mortality and prevalence worldwide in 2012. GLOBOCAN. Available at http://globocan.iarc.fr/Pages/fact_sheets_population.aspx [accessed on June of 2017]

Gough E, Shaikh H, Manges A R. 2011. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. Clinical Infectious Diseases 53: 994-1002 doi: 10.1093/cid/cir632

Gourineni V P, Verghese M, Boateng J, Shackelford L, Bhat N K, Walker L T. 2011. Combinational effects of prebiotics and soybean against azoxymethane-induced colon cancer *in vivo*. Journal of Nutrition and Metabolism 2011: 1-9 doi:10.1155/2011/868197

Grewal S, Bhagat M, Vakhlu J. 2014. Antimicrobial protein produced by pseudomonas aeruginosa JU-Ch 1, with a broad spectrum of antimicrobial activity. Biocatalysis and Agricultural Biotechnology 3:332–337 doi: 10.1016/j.bcab.2014.04.006

Gruner E, Steigerwalt A G, Hollis D G, Weyant R S, Weaver R E, Moss C W, Daneshvar M, Brown J M, Brenner D J. 1994. Human infections caused by *Brevibacterium casei*, formerly CDC groups B-1 and B-3. Journal Of Clinical Microbiology 32: 1511-1518

Hense B A, Schuster M. 2015. Core principles of bacterial autoinducer systems. Microbiology and Molecular Biology Reviews 79: 153-169 doi: 10.1128/MMBR.00024-14

Heuer H, Krsek M, Baker P, Smalla K, Wellington E M H. 1997. Analysis of *Actinomycete* communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Applied and Environmental Microbiology 63:3233-3241

Hille F, Charpentier E. 2016. CRISPR-Cas: biology, mechanisms and relevance. Philosophical Transactions of the Royal Society B 371: 1-12 doi: 10.1098/rstb.2015.0496

Hsiao W W L, Metz C, Singh D P, Roth J. 2008. The microbes of the intestine: an introduction to their metabolic and signaling capabilities. Endocrinology Metabolism Clinics of North America 37: 857-871 doi: 10.1016/j.ecl.2008.08.006

Huipeng W, Lifeng G, Chuang G, Jiaying Z, Yuankun C. 2014. The differences in colonic mucosal microbiota between normal individual and colon cancer patients by polymerase chain reaction-denaturing gradient gel electrophoresis. Journal of Clinical Gastroenterology 48: 138-144 doi: 10.1097/MCG.0b013e3182a26719

Illumina. 2013. [Internet] 16S metagenomic sequencing library preparation. Illumina. Available at http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html [accessed on June 2017]

ISAPP. 2016. [Internet] Probiotics by Dr. Mary Ellen Sanders. ISAPP. Available at http://isappscience.org/probiotics/ [accessed on October 2016]

Iyer C, Kosters A, Sethi G, Kunnumakkara A B, Aggarwal B B, Versalovic J. 2008. Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-kB and MAPK signaling. Cellular Microbiology 10: 1442-1452 doi: 10.1111/j.1462-5822.2008.01137.x.

Kaakoush N O, Day A S, Huinao K D, Leach S T, Lemberg D A, Dowd S E, Mitchell H M. 2012. Microbial dysbiosis in pediatric patients with Crohn's disease. Journal of Clinical Microbiology 50: 3258–3266 doi: 10.1128/JCM.01396-12

Keku T O, Dulal S, Deveaux A, Jovov B, Han X. 2015. The gastrointestinal microbiota and colorectal cancer. American Journal Physiology Gastrointestinal Liver Physiology 308: 351-363 doi:10.1152/ajpgi.00360.2012

Kim Y-I. 2007. Folate and colorectal cancer: An evidence-based critical review. Mol. Nutr. Food Res. 267: 267–292 doi: 10.1002/mnfr.200600191

Kim J H, Cho C R, Um T H, Rhu J Y, Kim E S, Jeong J W, Lee H R. 2007. *Morganella morganii* sepsis with massive hemolysis. Journal of Korean Medical Science 22: 1082–1084 doi: 10.3346/jkms.2007.22.6.1082

Lennard K S, Goosen R W, Blackbum J M. 2016. Bacterially-associated transcriptional remodelling in a distinct genomic subtype of colorectal cancer provides a plausible molecular basis for disease development. Plos One 11: 1-18 doi:10.1371/journal.pone.0166282

Liu H, Zhu J, Hu Q, Rao X. 2016. *Morganella morganii*, a non-negligent opportunistic pathogen. Int J Infect Dis 50: 10-7 doi: 10.1016/j.ijid.2016.07.006

Ma E L, Choi Y J, Choi J, Pothoulakis C, Rhee S H, Im E. 2010. The anti-cancer effect of probiotic *Bacillus polyfermenticus* on human colon cancer cells is mediated through ErbB2 and ErbB3 inhibition. International Journal of Cancer 127: 780-790 doi: 10.1002/ijc.25011

Marley A R, Nan H. 2016. Epidemiology of colorectal cancer. International Journal of Molecular Epidemiology and Genetics 7:105-114 ISSN:1948-1756/IJMEG0036410

Markle B, May E J, Majumdar A P N. 2010. Do nutraceutics play a role in the prevention and treatment of colorectal cancer?. Cancer and Metastasis Reviews 29: 395-404 doi:10.1007/s10555-010-9234-3

Markou P, Apidianakis Y. 2014. Pathogenesis of intestinal *Pseudomonas aeruginosa* infection in patients with cancer. Frontiers in Cellular and Infection Microbiology 3: 1-5 doi:10.3389/fcimb.2013.00115

Matano M, Date S, Shimokawa M, Takano A, Fujii M, Ohta Y, Watanabe T, Kanai T, Sato T. 2015. Modeling colorectal cancer using CRISPR-Cas9–mediated engineering of human intestinal organoids. Nature Medicine 21: 256-262 doi: 10.1038/nm.3802

Mikkelsen K H, Allin K H, Knop F K. 2016. Effect of antibiotics on gut microbiota, glucose metabolism and body weight regulation: a review of the literature. Diabetes, Obesity and Metabolism 18: 444-453 doi:10.1111/dom.12637

Miranda N. 2013. Portugal, doenças oncológicas em números-2013. Direção-Geral de Saúde

Miranda N. 2015. Portugal, doenças oncológicas em números-2015. Direção-Geral de Saúde

Modi S R, Collins J J, Relman D A. 2014. Antibiotics and the gut microbiota. The Journal of Clinical Investigation 124: 4212-4218 doi:10.1172/JCI72333

Moore W E C, Moore L H. 1995. Intestinal floras of populations that have a high risk of colon cancer. Applied and Environmental Microbiology 61: 3202–3207

Nes I F, Yoon S-S, Diep D B. 2007. Ribosomally synthesized antimicrobial peptides (Bacteriocins) in lactic acid bacteria: a review. Food Science and Technology 16: 675-690 doi: https://doi.org/10.1016/S0734-9750(03)00077-6

Nistal E, Fernández-Fernández N, Vivas S, Olcoz J L. 2015. Factors determining colorectal cancer: the role of the intestinal microbiota. Frontiers in Oncology 5: 1-10 doi:10.3389/fonc.2015.00220

O'Hara A M, Shanahan F. 2006. The gut flora as a forgotten organ. EMBO Reports 7: 688–693 doi: 10.1038/sj.embor.7400731

Oke S, Martin A. 2017. Insights into the role of the intestinal microbiota in colon cancer. Therapeutic Advances in Gastroenterology 10: 417-428 doi:10.1177/1756283X17694832

Orndorff P E. 2016. Use of bacteriophage to target bacterial surface structures required for virulence: a systematic search for antibiotic alternatives. Curr Genet 62: 753-757 doi: 10.1007/s00294-016-0603-5

Øyri S F, Muzes G, Sipos F. 2015. Dysbiotic gut microbiome: A key element of Crohn's disease. Comparative Immunology, Microbiology and Infectious Diseases 43: 36–49 doi:http://dx.doi.org/10.1016/j.cimid.2015.10.005

Panda S, El khader I, Casellas F, Vivancos J L, Cors M G, Santiago A, Cuenca S, Guarner F, Manichanh C. 2014. Short-term effect of antibiotics on human gut microbiota. Plos One 9: 1-7 doi:10.1371/journal.pone.0095476

Passàli D, Lauriello M, Passàli G C, Passàli F M, Bellussi L. 2007. Group A *Streptococcus* and its antibiotic resistance. ACTA Otorhinolaryngologica Italica 27: 27-32

Pédron T, Mulet C, Dauga C, Frangeul L, Chervaux C, Grompone G, Sansonetti PJ. 2012. A cryptic-specific core microbiota resides in the mouse colon. mBio 3: e00116-12. doi: 10.1128/mBio.00116-12

Peters B A, Dominianni C, Shapiro J A, Church T R, Wu J, Miller G, Yuen E, Freiman H, Lustbader I, Salik J, Friedlander C, Hayes R B, Ahn J. 2016. The gut microbiota in conventional and serrated precursors of colorectal cancer. Microbiome 4: 1-14 doi:10.1186/s40168-016-0218-6

Peterson C T, Sharma V, Elmén L, Peterson S N. 2015. Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota. *Clinical and Experimental Immunology* 179: 363-377 doi: 10.1111/cei.12474

Pingitore E V, Salvucci E, Sesma F, Nader-Macías M E. 2007. Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB). In: Méndez-Vilas A (Ed.), Communicating Current Research and Educational Topics and Trends in Applied Microbiology.

Pinheiro J C, Marques C R, Pinto G, Bouguerra S, Gomes N C, Gonçalves F, Rocha-Santos T, Duarte A C, Roembke J, Sousa J P, Ksibi M, Haddioui A, Pereira R. 2013. The performance of *Fraxinus angustifolia* as a helper for metal phytoremediation programs and its relation to the endophytic bacterial communities. Geoderma 202–203: 171-182 doi:http://dx.doi.org/10.1016/j.geoderma.2013.03.014

Ponnusamy K, Choi J N, Kim J, Lee S-Y, Lee C H. 2011. Microbial community and metabolomic comparison of irritable bowel syndrome faeces. Journal of Medical Microbiology 60: 817–827 doi: 10.1099/jmm.0.028126-0

Pope J, Tomkovich S, Yang Y, Jobin C. 2017. Microbiota as a mediator of cancer progression and therapy. Translational Research 179: 139-154 doi:http://dx.doi.org/10.1016/j.trsl.2016.07.021

Qin J, Li R, Raes J, Arumugam M, Burgdorf K S, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende D R, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto J, Hansen T, Paslier D L, Linneberg A, Nielsen H B, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Dore´ J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Bork P, Ehrlich S D, Wang J. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59-67 doi: 10.1038/nature08821

Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson P C, Klinder A, O'Riordan M, O'Sullivan GC, Pool-Zobel B, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Van Loo J, Watzl B, Collins JK. 2007. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr 85: 488-496

Rama A R, Hernandez R, Perazzoli G, Burgos M, Melguizo C, Vélez C, Prados J. 2015. Specific colon cancer cell cytotoxicity induced by bacteriophage *E* gene expression under transcriptional control of carcinoembryonic antigen promoter. International Journal of Molecular Sciences 16: 12601-12615 doi: 10.3390/ijms160612601

Rhee C-H, Park H-D. 2001. Three glycoproteins with antimutagenic activity identified in *Lactobacillus plantarum* KLAB21. Applied and Environmental Microbiology 67: 3445-3449 doi: 10.1128/AEM.67.8.3445-3449.2001

Sasaki M, Sitaraman S V, Babbin B A,A Gerner-Smidt P, Ribot E M, Garrett N, Alpern J A, Akyildiz A, Theiss A L, Nusrat A and Klapproth J M A. 2007. Invasive Escherichia coli are a feature of Crohn's disease. Laboratory Investigation 87: 1042–1054 doi:10.1038/labinvest.3700661

Schaik W. 2015. The human gut resistome. Philosophical Transactions of the Royal Society B 370: 1-9 doi:http://dx.doi.org/10.1098/rstb.2014.0087

Sears C L, Garrett W S. 2014. Microbes, microbiota, and colon cancer. Cell Host & Microbe 15: 317-328 doi:http://dx.doi.org/10.1016/j.chom.2014.02.007

Shannon A M, Bouchier-Hayes D J, Condron C M, Toomey D. 2003. Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treat. Rev. 29: 297–307

Siegel R, Jemal A. 2016. Colorectal cancer facts & figures 2014-2016. American Cancer Society, 2014

Smith P M, Howitt M R, Panikov N, Michaud M, Gallini C A, Bohlooly-Y M, Glickman J N, Garrett W S. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341: 569-73 doi: 10.1126/science.1241165

Sulakvelidze A, Alavidze Z, Morris J G. 2001. Bacteriophage therapy. Antimicrobial Agents And Chemotherapy 45: 649-659 doi: 10.1128/AAC.45.3.649-659.2001

Sun J, Kato I. 2016. Gut microbiota, inflammation and colorectal cancer. Genes & Diseases 3: 130-143 doi:http://dx.doi.org/10.1016/j.gendis.2016.03.004

Tansarli G S, Athanasiou S, Falagas M E. 2013. Evaluation of antimicrobial susceptibility of *Enterobacteriaceae* causing urinary tract infections in Africa. Antimicrob. Agents Chemother. 57:3628–39.

Tojo R, Suárez A, Clemente M G, Reyes-Gavilán C G, Margolles A, Gueimonde M, Ruas-Madiedo P. 2014. Intestinal microbiota in health and disease: role of bifidobacteria in gut homeostasis. World Journal Gastroenterology 20: 15163-15176 doi:10.3748/wjg.v20.i41.15163

Uccello M, Malaguarnera G, Basile F, D'agata V, Malaguarnera M, Bertino G, Vacante M, Drago F, Biondi A. 2012. Potential role of probiotics on colorectal cancer prevention. BioMed Central Surgery 12: 1-8

Uronis J M, Jobin C. 2009. Microbes and colorectal cancer: is there a relationship?. Current Oncology 16:22-24 doi: 10.1186/1471-2482-12-S1-S35

Versalovic J, Schneider M, Bruijn F J, Lupski J R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in Molecular and Cellular Biology 5:25-40

Vogtmann E, Goedert J J. 2016. Epidemiologic studies of the human microbiome and cancer. British Journal of Cancer 114: 237–242 doi:10.1038/bjc.2015.465

Wang X, Yang Y, Moore D R, Nimmo S L, Lightfoot S A, Huycke M M. 2012. 4-hydroxy-2-nonenal mediates genotoxicity and bystander effects caused by *Enterococcus faecalis*-infected macrophages. Gastroenterology 142:543–551 doi: 10.1053/j.gastro.2011.11.020

Wynendaelea E, Verbekea F, D'Hondta M, Hendrixb A, Van De Wielec C, Burvenichd C, Peremanse K, Weverb O, Brackeb M, Spiegeleera B. 2015. Crosstalk between the microbiome and cancer cells by quorum sensing peptides. Peptides 64: 40-48 doi: 10.1016/j.peptides.2014.12.009

Wong C, Harris P J, Ferguson L R. 2016. Potential benefits of dietary fibre intervention in inflammatory bowel disease. International Journal of *Molecular Sciences* 17: 1-22 doi:10.3390/ijms17060919

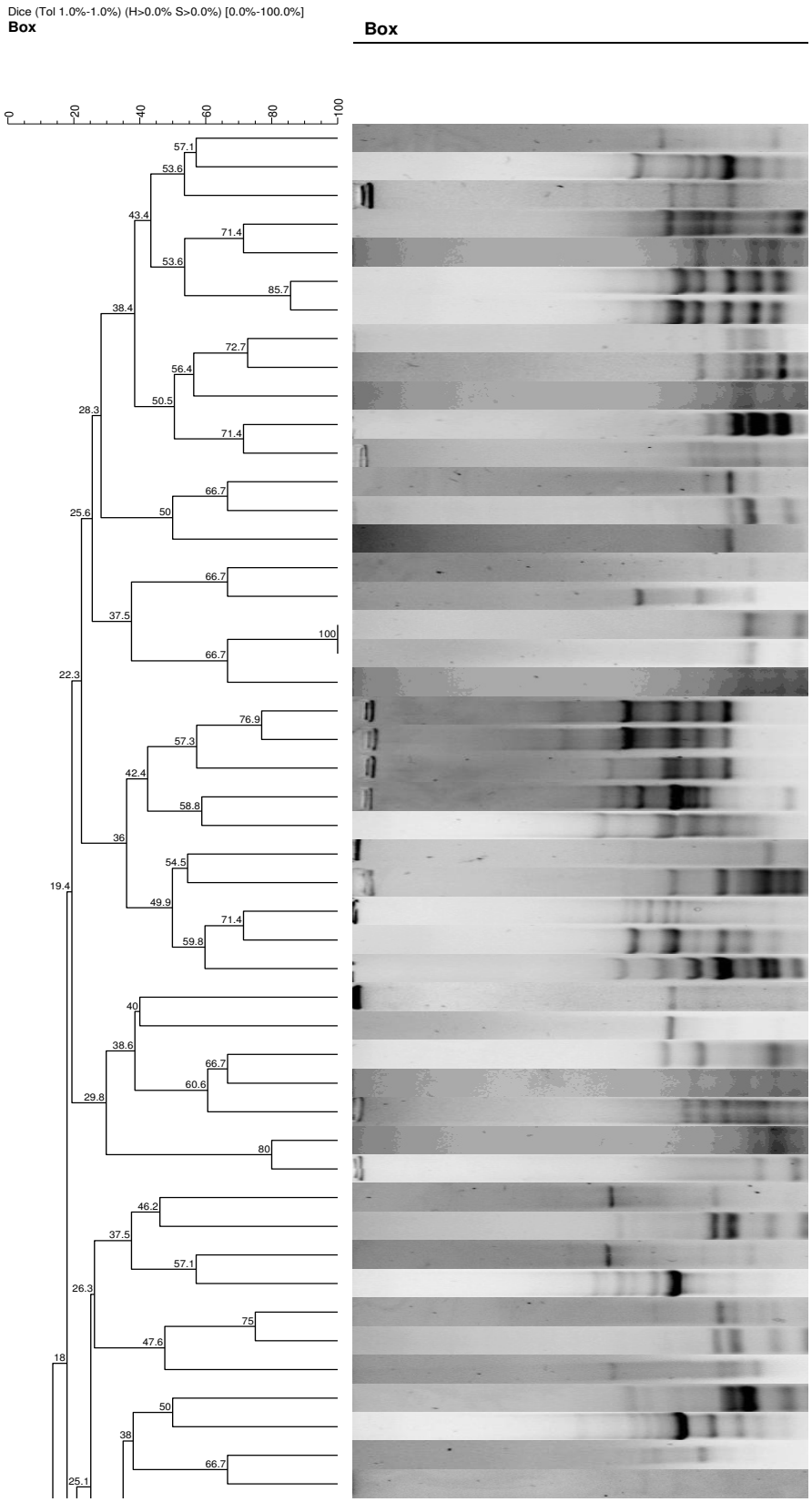
Yasui H, Shida K, Matsuzaki T, Yokokura T. 1999. Immunomodulatory function of lactic acid bacteria. Antonie Van Leeuwenhoek 76:383-9.

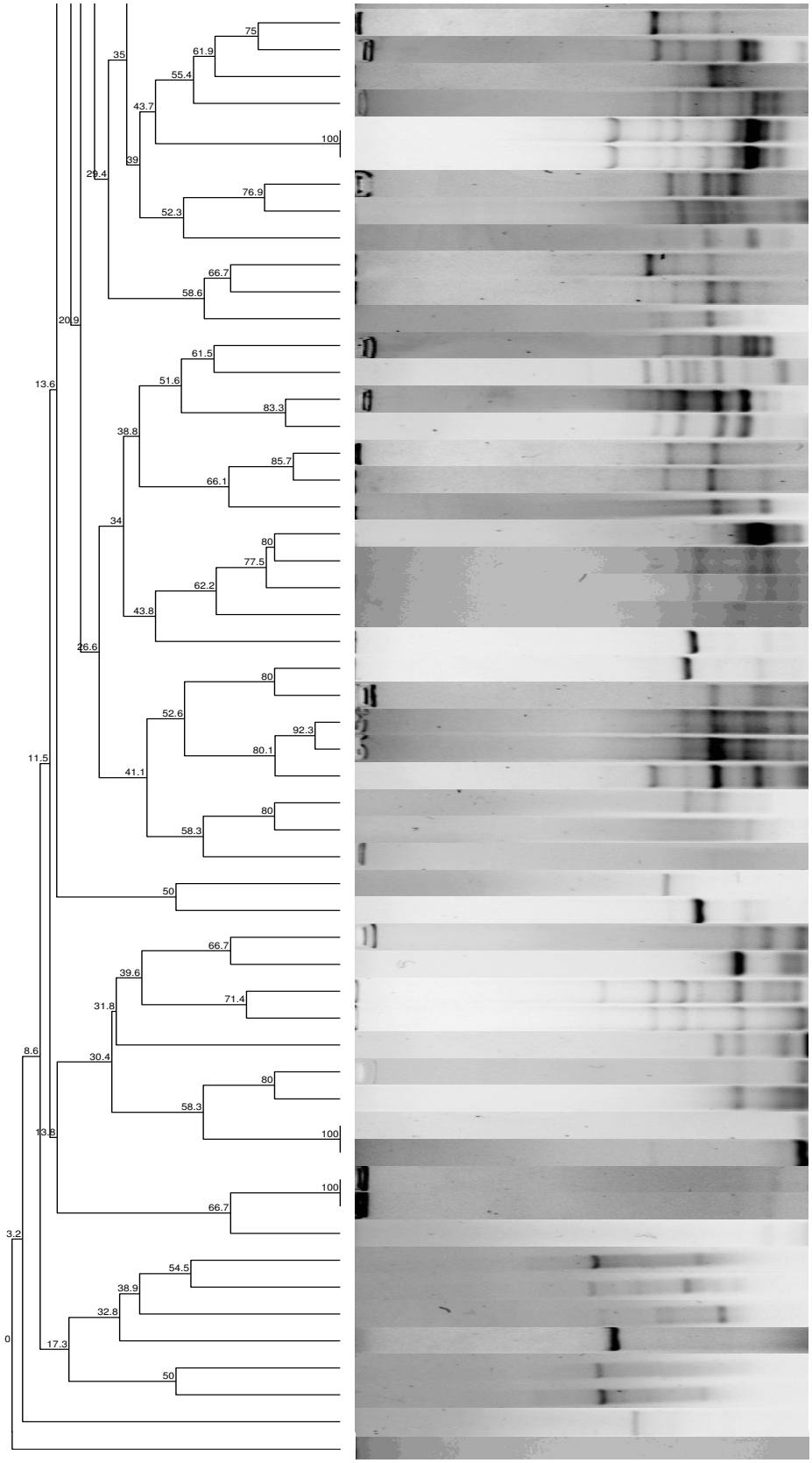
Young M, Reed K R. 2016. Organoids as a model for colorectal cancer. Current Colorectal Cancer Reports 12: 281-287 doi: https://doi.org/10.1007/s11888-016-0335-4

Zhou Y, He H, Xu H, Li Y, Li Z, Du Y, He J, Zhou Y, Wang H, Nie Y. 2016. Association of oncogenic bacteria with colorectal cancer in South China. Oncotarget 7: 80794–80802 doi: 10.18632/oncotarget.13094

Zomer A, Burghout P, Bootsma H J, Hermans P W M, Hijum S A F T. 2012. ESSENTIALS: software for rapid analysis of high throughput transposon insertion sequencing data. Plos One 7: 1-9 doi: https://doi.org/10.1371/journal.pone.0043012

Annex A. Dendrogram built by GelCompar II® with Box-PCR obtained profiles.




75
61.9
55.4
43.7
100
39
29.4
76.9
52.3
66.7
58.6
20.9
61.5
51.6
83.3
13.6
38.8
85.7
66.1
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62.2
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52.6
92.3
80.1
11.5
41.1
80
58.3
50
66.7
39.6
71.4
31.8
8.6
30.4
80
58.3
13.8
100
100
66.7
3.2
54.5
38.9
32.8
0
17.3
50