CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

IMPACT OF AN ANTHOCYANIN RICH EXTRACT UPON PROBIOTIC AND PROBIOTIC/HUMAN CELL SYSTEMS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to achieve the Master of Science level in Applied Microbiology

by
Mariana da Luz Cabral Veiga

[July 2018]

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IMPACT OF AN ANTHOCYANIN RICH EXTRACT UPON PROBIOTIC AND PROBIOTIC/HUMAN CELL SYSTEMS

IMPACTO DE UM EXTRATO RICO EM ANTOCIANINAS SOBRE PROBIÓTICOS E SISTEMAS PROBIÓTICOS/CÉLULAS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to achieve the of Master of Science degree in Applied Microbiology

by

Mariana da Luz Cabral Veiga

Place: CBQF/Escola Superior de Biotecnologia da Universidade Católica Portuguesa

Supervision: Professor Maria Manuela Estevez Pintado

Co-supervision: Sara Nunes da Costa e Silva, PhD

[July 2018]

To *Ester Sequeira,*

Lourdes Proença,

José Veiga,

Zacarias Cabral.

I carry you in my heart always.

"*If you don't like bacteria, you're on the wrong planet.*"

Stewart Brand

"*While one may encounter many defeats, one must not be defeated*."

Maya Angelou

Resumo

Devido à sua composição rica em compostos fenólicos, o mirtilo tem demonstrado diversos benefícios para a saúde humana, nomeadamente atividade antitumoral, anti-inflamatória e antimicrobiana, entre outras, e tendo sido mais recentemente associado à modulação da microbiota intestinal. Uma vez que anteriormente se demonstrou que um extrato de mirtilo rico em antocianinas era capaz de modular a adesão de microrganismos patogénicos num modelo *in vitro* de adesão intestinal, este trabalho procurou perceber o efeito do mesmo extrato sobre o metabolismo de microrganismos probióticos e como os metabolitos de fermentação dos mesmos poderiam afetar a viabilidade e modular a adesão bacteriana a células intestinais.

De um ponto de vista laboratorial, foram definidas 3 etapas para possibilitar a execução do trabalho experimental. Na primeira avaliou-se o efeito de um extrato de mirtilo sobre o processo fermentativo de quatro microrganismos probióticos, e de uma mistura dos mesmos, analisando-se o número total de colónias formadas, o consumo de açúcares e a produção de ácidos orgânicos. A segunda etapa focou-se na avaliação do efeito dos metabolitos de fermentação (ocorrida na presença e ausência de extrato) sobre a viabilidade celular da linhagem de células intestinais Caco-2. Posteriormente, e mantendo-se na mesma linha investigativa, analisou-se o impacto do extrato sobre a adesão das bactérias probiótica às linhagens intestinais Caco-2 e HT29-MTX, sendo esta última produtora de muco.

Os resultados relativos à fermentação do extrato, mostram que este não inibiu as contagens viáveis (embora as contagens de *Lactobacillus* fossem inferiores na presença de extrato relativamente às de *Bifidobacterium*) e que a produção de ácidos foi superior na presença do mesmo. Relativamente ao efeito dos sobrenadantes sobre a viabilidade de células Caco-2, verificou-se que estes tinham um impacto reduzido sobre o metabolismo celular, observando-se percentagens de inibição de metabolismo inferiores às dos controlos. Adicionalmente, os sobrenadantes resultantes das fermentações de *Bifidobacterium animalis* subsp. *lactis* BO na presença de extrato aparentaram promover o metabolismo celular.

Finalmente, no que se refere ao impacto do extrato sobre a adesão de duas bactérias probióticas (*Lactobacillus rhamnosus* R11 e *B. animalis* subsp. *lactis* BB12) aos modelos celulares selecionados, verificou-se que a presença de extrato e os maiores tempos de adesão testados (nomeadamente, 120 e 180 minutos) resultavam em maiores valores de percentagem relativa, sendo *Bifidobacterium* o microrganismo dos dois testados com maiores valores de adesão para ambas as linhagens.

Palavras-chave: microbiota intestinal, antocianinas, compostos fenólicos, bactérias probióticas.

Abstract

Blueberries have long been known to possess several biological properties, such as antimicrobial, antioxidant and anti-inflammatory activity, which are beneficial for human health. One of the latest additions to the list of blueberries known properties has been intestinal microbiota modulation. Considering that an anthocyanin rich blueberry extract has been previously shown to be capable of modulating pathogenic microorganisms adhesion in an *in vitro* adhesion model, this work sought to understand how the same extract would impact upon known probiotics' metabolism and how these microorganisms' metabolites would affect intestinal cell's viability and modulate bacterial adhesion to intestinal cells.

To do so, a three-step approach was drawn. In the first step, the extract's capability to interfere in the fermentative process of four different probiotic bacteria, as well as a mixture of those, was evaluated through determination of total viable counts, organic acid production and sugar consumption. Secondly, the supernatants resulting from the fermentation in the presence or absence of extract were recovered and studied to see if they exerted any negative impact upon the viability of a cell line resembling the intestinal epithelium, namely Caco-2 cells. Lastly, and keeping in this line of thought, the effect of the extract upon probiotic bacteria (*Lactobacillus rhamnosus* R11 and *B. animalis* subsp. *lactis* BB12) adhesion to intestinal cells, (namely Caco-2 and the mucus producing HT29-MTX) was assayed.

From a fermentation standpoint, the results obtained showed that the extract did not inhibit the growth of the bacteria tested (although the *Lactobacillus* tested had slightly lower viable counts in the presence of extract than the *Bifidobacterium*), and stimulated acid production which was significantly higher in its presence. When considering the fermentation supernatants' impact upon Caco-2's viability, the extract supernatants had an overall lower impact upon the cell's metabolism, with lower metabolic inhibitions being observed. Additionally, supernatants fermented by *Bifidobacterium animalis* subsp. *lactis* BO appeared to promote cellular metabolism.

Lastly, when evaluating the effect of the extract upon bacterial adhesion to the selected cellular models, it was possible to see that the presence of extract resulted in higher relative adhesion percentage values, and the *Bifidobacterium* tested had higher adhesion values for both cell lines in the presence of extract, which stands in accordance with reports that claim bifidogenic activity is enhanced in the presence of phenolic compounds.

Keywords: gut microbiota; anthocyanins; phenolic compounds; probiotic bacteria.

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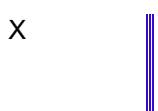
Many thanks and all my love.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List of Abbreviations

B. animalis BB12 ..... *Bifidobacterium animalis* subsp. *lactis* BB12
B. animalis Bo ..... *Bifidobacterium animalis* subsp. *lactis* Bo
$CaCl_2$ $2H_2O$ ..... Calcium chloride dihydrate
CFU ..... Colony forming units
CO_2 ..... Carbon dioxide
Cys ..... L-cysteine hydrochloride
ddH_2O ..... Deionized water
DMEM ..... Dulbecco's modified Eagle's medium
DMSO ..... Dimethyl sulfoxide
FBS ..... Fetal bovine serum
FOS ..... Fructooligosaccharide
H_2 ..... Hydrogen gas
H_2SO_4 ..... Sulfuric acid
HCl ..... Hydrochloric acid
HPLC ..... High performance liquid chromatography
HPLC-DAD ..... High performance liquid chromatography coupled with diode array detector
HPLC-RI ..... High performance liquid chromatography coupled with refractive index detector
IP ..... Inhibition percentage
iROS ..... Intracellular reactive oxygen species
$K_2HPO_4 \cdot 3H_2O$ ..... Dipotassium hydrogen phosphate trihydrate
$K_2S_2O_8$ ..... Potassium persulfate
LDH ..... Lactate dehydrogenase
L. plantarum ..... *Lactobacillus plantarum*
L. rhamnosus ..... *Lactobacillus rhamnosus*
$MgCl_2 \cdot 6H_2O$ ..... Magnesium chloride hexahydrate
MRS agar/broth ..... de Mann, Rogosa and Sharpe agar/broth
MTT ..... 3(4,5dimethylthiazol2yl)2,5diphenyltetrazolium bromide
$NaHCO_3$ ..... Sodium bicarbonate
NH_4Cl ..... Ammonium chloride
N_2 ..... Nitrogen gas
OD ..... Optical density
PBS ..... Phosphate-buffered saline
PMS ..... Phenazine methosulfate
PenStrep ..... Penicillin and streptomycin
pH ..... Cologarithm of the hydrogen cation concentration
ROS ..... Reactive oxygen species
rpm ..... Rotations per minute
SCFA ..... Short chain fatty acids

SPE ..... Solid phase extraction

TSB ..... Trypticase soy broth

upH_2O ..... Ultra-pure water

XTT ..... 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide

1. Introduction

Humanity has been using plants for centuries, and while various applications (food source, raw materials for clothing production, shelters construction or simply decoration) could be referenced, their application as herbal medicine is one of the oldest forms of healthcare known to mankind. In fact, the study of plants goes back hundreds of years, and it has permitted them to be explored not only for food, but also in order to obtain non-food products. From a technical standpoint, a medicinal plant is characterized as possessing pharmacologically active components that will allow it to be used, directly or indirectly, in therapeutic treatment to prevent or cure a certain disorder (Boscolo & de Senna Valle, 2008; Briskin, 2000; Djeridane et al., 2006). Nowadays, several works have focused on characterizing the potential medicinal properties of plants which may include anti-inflammatory, anti-tumoral, antidiabetic and antioxidant effect (Ekor, 2014; Jung et al., 2006; Roleira et al., 2015; Watt & Breyer-Brandwijk, 1962). In fact, several of the drugs commonly used today in medicine are of herbal origin, with their beneficial/ therapeutic effects thought to be due to the presence of several different compounds that can be found in numerous plants, such as terpenoids, alkaloids, flavonoids, phenolics, and some others. World Health Organization estimates that more than 80% of the world's population relies on traditional medicine resorting to plants for their primary healthcare needs (Ekor, 2014; Farnsworth, Akerele, Bingel, Soejarto, & Guo, 1985; Raskin et al., 2002; Who, 2013).

1.1. Plant extracts

Biological active compounds present in plants are not, normally, easily accessible. Therefore, plants have been the target of several works focusing on the extraction of these natural compounds, as well as in the removal of the fractions with lower biological relevance, thus originating extracts which may have several applications, such as medical (for example, extracts have been studied as cancer treatment alternative, treatment of diabetes and dermatological disorders, among others), agriculture (repellent effects and antifeedant, control of diseases in plants) and as a source of antimicrobial and antioxidant agents for the cosmetic and food industries (Armendáriz-Barragán et al., 2016; Djeridane et al., 2006; Hassan, Rahman, Deeba, & Mahmud, 2009; Hilou, Nacoulma, & Guiguemde, 2006; Ribeiro, Estanqueiro, Oliveira, & Sousa Lobo, 2015; Salem et al., 2015; Sasidharan, Chen, Saravanan, Sundram, & Latha, 2011; Seeram et al., 2006; Silva, Costa, Pereira, Costa, & Pintado, 2013).

The production of an extract allows for the removal of the compounds which showcase no relevant benefit to the host. In turn, this leads to the attainment of the bioactive components, in a concentrated form, whose incorporation into another product could be easier than the inclusion of the whole plant. However, these extractions usually require the use of organic solvents which may arise some concerns when considering their use/consumption by humans. Furthermore, the production of plant extracts may be an interesting alternative for waste reduction (particularly at the agro-food level) as these byproducts may function as a source of biologically relevant compounds and therefore contribute to the more efficient management of byproducts (Armendáriz-Barragán et al., 2016; Hassan et al., 2009; Ribeiro et al., 2015; K. A. Ross, 2014; Sasidharan et al., 2011).

Different extracts, obtained from different plants, can exhibit a vast array of potentially beneficial effects such as hepatoprotection, anti-tumoral, antioxidant, antimicrobial, antidiabetic, anti-inflammatory and antimalarial activity among several others. In these cases, it is perceived that the overall activity of an extract results from the synergistic action between its different constituents, particularly as tests performed with only the perceived "active principle" frequently yield poorer results than those observed for the complex chemical matrix which is the extract (Balasundram, Sundram, & Samman, 2006; Djeridane et al., 2006; Hassan et al., 2009; Hilou et al., 2006; Islam & Choi, 2009; Jung et al., 2006; Omoregbe, Ikuebe, & Ihimire, 1996).

1.1.1. Plant's biological potential

Plants have been associated with an abundance of epidemiological evidences supporting an array of potential health benefits (Asif, 2015; Graf, Milbury, & Blumberg, 2005; Hollman, 2001; Jung et al., 2006; Neto, 2007; Omoregbe et al., 1996; Osman, Adawi, Ahrné, Jeppsson, & Molin, 2008; Pandey & Rizvi, 2009; Ribeiro et al., 2015; Roleira et al., 2015; Runnie, Salleh, Mohamed, Head, & Abeywardena, 2004; Salem et al., 2015; Sasidharan et al., 2011; Vendrame et al., 2011; Y.-P. Wang et al., 2010). Therefore, it stands to reason that plant's constituents are, at least in part, responsible for the benefits observed. Dietary fiber and phenolic compounds, two classes of components abundant in plants, have been widely recognized as exerting a positive influence upon human health (J. W. Anderson et al., 2009; Cheynier, 2012; Giada, 2013; Kaczmarczyk, Miller, & Freund, 2012). For instance, phenolic compounds have been reported as contributing to the prevention of neurodegenerative diseases (*e.g.* Parkinson and Alzheimer) as well as with the amelioration and/or prevention of other neurological pathologies such as memory loss, posttraumatic stress disorder (PTSD) and ischemic brain damage. Furthermore, several epidemiological studies have also linked phenolic compound's ingestion to a reduction of the risk for developing diabetes, cancer, cardiovascular and inflammatory diseases (Albishi, John, Al-Khalifa, & Shahidi, 2013; Bulotta et al., 2014; Nohynek et al., 2006; Ranilla, Kwon, Apostolidis, & Shetty, 2010; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013; Selma, Espín, & Tomás-Barberán, 2009; Sevgi, Tepe, & Sarikurkcu, 2015; Valdés et al., 2015; Wallace, 2011). On the other hand, dietary fiber has also been associated with an array of potential health benefits, including a reduction of the risk of developing coronary heart disease, hypertension and of suffering a stroke (due to dietary fiber's potential to aid in the control of blood pressure),and it also helps to prevent obesity and certain gastrointestinal disorders, such as duodenal ulcer, constipation and hemorrhoids (J. W. Anderson et al., 2009; Eswaran, Muir, & Chey, 2013; S. Fuller, Beck, Salman, & Tapsell, 2016; Kaczmarczyk et al., 2012; J. Slavin, 2013; Wu et al., 2015; Ye, Arumugam, Haugabrooks, Williamson, & Hendrich, 2015). A concrete example of this biological potential is blueberry, as its fruit is known to exert many health benefits, with existing reports of its antioxidant, antimicrobial, antitumoral and anti-inflammatory activity, as well as gut modulating capacity (Bränning et al., 2008; Carey, Gomes, & Shukitt-Hale, 2014; Dulebohn et al., 2008; Guglielmetti et al., 2013; Molan, Lila, Mawson, & De, 2009; Neto, 2007; Piljac-Žegarac, Belščak, & Piljac, 2009; Vendrame, Daugherty, Kristo, Riso, & Klimis-Zacas, 2013).

It has been described the use of dietary fiber and phenolic compounds as ingredients capable of enhancing technological and nutritional properties of food, thus increasing the functionality of food

products. Furthermore, non-extractable polyphenols, which are not released from the food matrix and therefore manage to reach the colon nearly intact, have been shown to exhibit health promoting properties as well, namely in relation to gastrointestinal health. The gut microbiota is responsible for converting these compounds in small size phenolics that are better absorbed and that persist in the blood for more than 48 hours, showcasing antioxidant and anti-inflammatory activity (González-Sarrías, Espín, & Tomás-Barberán, 2017; J. Landete, 2012; Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013; Yan et al., 2018).

1.1.2. Plants' bioactive compounds

Diet has long been associated with positive, or negative, impacts upon the health and wellbeing, with foodstuff of plant origin being strongly associated with a healthier status. Fruits and vegetables are generally rich in water, sugar, fiber, several phytonutrients and vitamins, all of which are essential for body's the homeostasis. Most importantly, the potential benefits of their consumption have been frequently associated with their vitamin, phenolic and fiber contents.

1.1.2.1. Phenolic compounds

Phenolic compounds represent the primary source of phenolics in the human diet. They are secondary metabolites of plants which exert a photoprotective function and other crucial roles for the plants' metabolism and survival, such as prevention of oxidative stress, structural support and protection against pathogen's infection. Moreover, they are also responsible for the pigmentation and some of the organoleptic characteristics showcased by plants, such as flavor and color, and are sometimes found in other organisms, *e.g.* bacteria, fungi and algae (Balasundram et al., 2006; Djeridane et al., 2006; J. Landete, 2012).

In nature, *ca.* 8000 different phenolic compounds have been reported so far. According to their chemical structures, they can be divided into several groups, that range from the relatively simple phenolic acids to highly polymerized tannins. Overall, chemically wise, phenolic compounds may be defined as molecules comprised of at least one phenol unit (an aromatic ring with one hydroxyl substitution, as seen in Figure 1), with molecules containing more than one of these subunits being conventionally classified as polyphenols. In fruit and vegetables, phenolic compounds are frequently found in glycosylated forms (*i.e.* associated with a sugar moiety) as the free forms often present more toxicity that the glycosylated one (Das, Bhaumik, Raychaudhuri, & Chakraborty, 2012; Hollman & Arts, 2000; J. Landete, 2012; Vermerris & Nicholson, 2008a, 2008b).

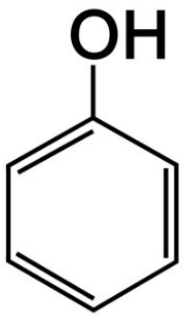

Figure 1.1 Basic structure of a phenolic compound.

Phenolics have been systematically reported as being beneficial for health and wellbeing. One of the properties most commonly associated with phenolics is antioxidant activity, as the position of the hydroxyl group bound to the aromatic ring are efficient electron donners. Moreover, phenol groups can also accept electrons resulting in the formation of relatively stable phenoxyl radicals. Consequently, this allows for the disruption of chain oxidation reactions that can be detrimental in biological systems. When in foods, they can help limit the oxidative damage of the matrix itself (allowing for longer shelf life) or, after ingestion, be absorbed by the body and act as local antioxidants. For instance, anthocyanins (water soluble flavonoid pigments that are abundant in red and purple fruits)

have been described as capable of protecting liver and red blood cells against in vitro and in vivo oxidative damage and several phenolic rich extracts have been associated with the reduction of plasmatic antioxidant levels and oxidation stress markers. There are evidences that the beneficial effects attributed to dietary polyphenols depend on their biotransformation by the gut microbiota. Additionally, these compounds have been used in order to increase functionality of some foods (Duda-Chodak, Tarko, Satora, & Sroka, 2015; W. Liu et al., 2013; Olas, 2017; Sevgi et al., 2015; Silva et al., 2017; Y.-P. Wang et al., 2010; Yan et al., 2018; Youdim, Martin, & Joseph, 2000).

1.1.2.2. Fiber

Dietary fiber, found in vegetables, fruits, grains and legumes, is described as a group of complex non-starch carbohydrates and lignin which includes polysaccharides, oligosaccharides, lignin, and associated plants substances. These compounds are not affected by the digestive process and are not absorbed in the small intestine. However, they may be metabolized, in the colon, by local microorganisms, resulting in several secondary products that can be absorbed into the bloodstream. The different types of dietary fiber may be categorized according to their sources, solubility (soluble and insoluble dietary fiber), fermentability and physiological effects (Adam et al., 2014; Kendall, Esfahani, & Jenkins, 2010).

The intake of fiber has gradually decreased for the past 200 years, regardless of the fact that its consumption has been associated with an array of important health benefits. It binds to substances (such as sugar and cholesterol) preventing or slowing their absorption into the blood, therefore contributing to the regulation of blood sugar levels and protecting against cardiovascular problems by lowering the levels of blood cholesterol. Soluble dietary fiber (*e.g.* non-cellulosic polysaccharides, oligosaccharides, β-glucans, pectins and gums) has been described as being responsible for an increase in gut content viscosity and is, therefore, frequently associated with the regulation of blood glucose levels, a reduction in serum cholesterol and a delay in bowel movements. Pectins for instance, are another example of a carbohydrate/fiber present in plants, which helps to regulate the gastrointestinal system, and also prevents cardiovascular diseases and cholesterol. On the other hand, it also diminishes some antibiotics' bioavailability. Additionally, fiber may contribute to the modulation of the gut microbiota due to its fermentability, contributing to an increase in beneficial genres, such as *Bifidobacteria*, and it has been associated with colon, stomach, breast and prostate cancer prevention. Furthermore, dietary fiber has been shown to significantly reduce the risk of gaining body weight and fat, notwithstanding the quantity of fat ingested or the amount of physical exercise performed, as it reduces the overall energetic intake. Rich sources of soluble fiber include fruits, vegetables, oats and pulses (Adam et al., 2014; Brown, Rosner, Willett, & Sacks, 1999; S. Fuller et al., 2016; J. L. Slavin, 2008; Smith & Tucker, 2011; Trinidad, Mallillin, Loyola, Sagum, & Encabo, 2010; Tucker & Thomas, 2009; Wu et al., 2015; Ye et al., 2015; Zinman et al., 2015).

Insoluble fiber is poorly broken down by the gut and absorbed into the bloodstream (such as cellulose, hemicellulose and lignin). Given their bulking capacity and their (although low) fermentability by the gut microbiota, insoluble fiber is often associated with a reduction of bowel transit time and improvement of laxation, while its fermentation may contribute to the modulation of the gut microbiota. Since soluble and insoluble fibers are present in different proportions in food, and that both have different

biological potentials, it is important to eat a variety of fiber rich foodstuff, in order to ingest both types of dietary fiber at sufficient levels (Brownawell et al., 2012; CHEN & Camire, 1997; Dreher, 2018; S. Fuller et al., 2016; Holscher, 2017; Kaczmarczyk et al., 2012; Mudgil & Barak, 2013; K. Ross, Ehret, Godfrey, Fukumoto, & Diarra, 2017; J. L. Slavin, 2005; Trinidad et al., 2010).

1.1.2.3. Other compounds

All plants exposed to the atmosphere are coated with layers of a lipidic material that helps to reduce water loss and functions as a barrier to prevent pathogens' entry. The main constituents of these coatings are fatty acids polymers (joined by ester linkages) such as cutin, suberin and waxes (Duke et al., 2016; Fich, Segerson, & Rose, 2016).

Moreover, plants produce a large array of organic, non-phenolic, compounds that have no connection to their development or function, but confer them some protection against herbivores, insects and microbes or function as attractants for pollinators and seed-dispersing animals. Terpenes represent the largest class of secondary metabolites in plants. The compounds belonging to this class are usually insoluble in water and are biosynthesized from acetyl-CoA or glycolytic intermediates. However, some terpenes have well defined roles in plant growth and/or development and can be considered primary metabolites (Daniel, 2016; Duke et al., 2016).

Carbohydrates are the basis of the formation of plants. Their complexity varies between a simple monosaccharide to homogeneous and heterogeneous polysaccharides. Some of the biological properties of heterogenous polysaccharides include a protective action of the mucosa and irritated skin, anti-inflammatory effect and immunomodulating activity (Duke et al., 2016; Hardy, Brand-Miller, Brown, Thomas, & Copeland, 2015; Hendrix, 1993).

Other compounds of interest that can be found in plants include phytosterols (promising agents in hormonal therapy), salicylates (are used for the development of aspirin), terpenoids (that have anti-inflammatory and antimicrobial activity) and alkaloids (compounds that, despite their pharmacological activity such as anesthetic, antitumoral, antiarrhythmic and antimalarial activity, showcase a high level of toxicity) (Aniszewski, 2015; Baye et al., 2017; Cushnie, Cushnie, & Lamb, 2014; Malakar, 2017; Racette, Lin, Ma, & Ostlund Jr, 2015; Ras et al., 2015; Tholl, 2015).

1.2. Blueberry, a fruit with potential

The past years have seen an increase of life expectancy and, therefore, the society's concern with the quality of life has also arisen. As mentioned previously, the connection between health and diet has been reported by several authors, with some foodstuffs being regarded as more beneficial than others and a balanced diet, such as the Mediterranean diet, being paramount for the proper functioning of the human organism (Appleton et al., 2016; Conlon & Bird, 2014; R. H. Liu, 2013; Reedy et al., 2014; Willcox, Scapagnini, & Willcox, 2014).

Blueberries are a prime example of a fruit considered to be beneficial. They are known as a "superfruit" thanks to its nutritional value and health promoting potential, which is believed to be related to its high content of phenolic compounds (especially anthocyanins). In fact, several studies have shown that this fruit possesses some bioactive properties (antioxidant, antitumoral and antimicrobial activity,

among others) (Asif, 2015; Quirós-Sauceda et al., 2014; Shahidi & Ambigaipalan, 2015; J. Slavin, 2013; Valdés et al., 2015).

Table 1.1 Nutritional profile of blueberries. Adapted from M. Sousa, Curado, Vasconcellos, and Trigo (2007).

Nutrients per 100 g of blueberries	
Water content	83000 – 87000 mg
Proteins	400 – 700 mg
Lipids	500 mg
Glucose	5000 – 7000 mg
Fructose	5000 – 7000 mg
Fiber	1000 – 1500 mg
Calcium	11.4 – 12.2 mg
Iron	0.6 mg
Magnesium	5.8 – 8.4 mg
Phosphorus	14 – 47 mg
Potassium	48 – 112 mg
Sodium	3.4 – 4.3 mg
Zinc	0.1 mg
Copper	0.1 mg
Manganese	0.4 – 1.2 mg
Ascorbic acid (Vitamin C)	22 – 62 mg
Tannins	270 – 550 mg
Pectins	300 – 600 mg
Anthocyanins	300 – 725 mg

Blueberries' antioxidant activity has been widely associated with their high phenolic compounds, particularly with their anthocyanin content. These compounds can interact with reactive oxygen species (ROS) and prevent the oxidation of biologically relevant molecules, such as proteins, consequently avoiding the alteration of metabolic pathways and helping the maintenance of homeostatic balance. Additionally, antioxidant compounds have also been shown to protect DNA against oxidation and to possess antimutagenic properties. On another subject, several authors have also studied the effect of blueberries intake in the preservation of brain function. It has been reported that blueberry phenolics may help to protect against damage induced by an array of neurotoxic agents (*i.e,* they exhibit a neuroprotective effect), and in some cases, they have also been reported as causing an improvement of memory skills (Burdulis et al., 2009; Carey et al., 2014; Dulebohn et al., 2008; Jo et al., 2015; W. Liu et al., 2013; Piljac-Žegarac et al., 2009; Silva et al., 2017; Subash et al., 2014).

Blueberry extracts in particular, have been shown to possess an antitumoral effect, namely through the inhibition of the proliferation of several tumor cell lines (including breast, skin and cervix tumor cell lines) and the attenuation of growth of tumors on rats (Diaconeasa, Leopold, Rugină, Ayvaz, & Socaciu, 2015; Seeram et al., 2006). The inhibition of tumorigenesis has also been linked with anthocyanins rich extracts. This activity is thought to be a consequence of anthocyanins' potential immunomodulatory effects, which may stimulate the activity of natural killer cells, a specific type of lymphocytes that are very important in the host rejection of abnormal cells (Neto, 2007; Y.-P. Wang et al., 2010).In fact, blueberries have also been described as possessing some anti-inflammatory potential,

with reports showing that their consumption is inversely related with the expression of 12 different inflammatory biomarkers, with this effect being believed to contribute to the prevention of other conditions such as diabetes, cardiovascular diseases and osteoporosis (Riso et al., 2013; Vendrame et al., 2013). For instance, blueberries have been shown to improve blood flow, endothelial function and to decrease myocardial infarction risk (Cassidy et al., 2013; Olas, 2017; Wallace, 2011). Additionally, blueberry's extracts have been cast as a possible alternative to the treatment of obesity (with results showing a decrease in fat accumulation), as well as a possible treatment for diabetes (anthocyanins attenuate insulin sensitivity and induce the production of glucagon-like peptide-1, which interacts with pancreatic cells responsible for the secretion of insulin) (Guo & Ling, 2015; Li, Zhang, Liu, Sun, & Xia, 2015; Tsuda, 2015; Vendrame, Daugherty, Kristo, & Klimis-Zacas, 2014; Vendrame et al., 2013).

On the other hand, phenolics compounds, and blueberry phenolics in particular, have been systematically reported as having relevant antimicrobial activity. Blueberry extracts have been reported to inhibit several potential pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Acinetobacter baumannii*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Salmonella thyphimurium*, among others (Burdulis et al., 2009; Nohynek et al., 2006; Shen et al., 2014; Silva et al., 2013; Silva et al., 2016).

1.2.1. Gut microbiota: function and modulation

On a different note, polyphenols have been studied for their microbiota modulating potential. The human gut harbors a complex microbial community denominated intestinal microbiota, composed of over 1000 different bacterial species. The development of the gut microbiota begins at birth and is strongly influenced by diverse factors which include immunological factors, antibiotic usage, host genetics and dietary habits. The most predominant phylum in the human gut are Firmicutes (which includes genera of bacteria such as *Lactobacillus*, *Enterococcus* and *Clostridium*) and Bacteroidetes (which includes *Prevotella* and *Bacteroides* genera), which together represent 90% of the known phylogenetic categories existing in the gut flora (Bernalier-Donadille, 2010; Boulangé, Neves, Chilloux, Nicholson, & Dumas, 2016; Claesson et al., 2012; Conlon & Bird, 2014; Marchesi et al., 2015).

Gut microbiota is essential for the normal functioning of the body and its health, besides contributing to maximize the absorption of nutrients. In fact, the connection between the composition of the intestinal microbiota and the overall health and wellbeing has been widely and systematically reported by the scientific community. The normal gut flora is constituted by several different genera and species, which range from bacteria known to be beneficial to the host (such as *Lactobacillus* and *Bifidobacterium*) to potentially harmful bacteria such as *Clostridium* and *Salmonella* (Alkasir, Li, Li, Jin, & Zhu, 2017; Boulangé et al., 2016; Carabotti, Scirocco, Maselli, & Severi, 2015; Claesson et al., 2012; Hu, Wang, & Jin, 2016; I Naseer et al., 2014; Mangiola et al., 2016; Zhao, 2013).

The gut microbiota is responsible for fermenting dietary polysaccharides and short chain fatty acids (SCFAs) (that have not been absorbed previously in the small intestine) which, in turn allows for the removal of calories from indigestible dietary compounds, such as fiber. Therefore, the gut microbiota results in the production of several metabolites that can be absorbed by the host. Certain species of bacteria present in the gut have been shown to improve the processing and absorption of nutrients (Boulangé et al., 2016; Carabotti et al., 2015; Conlon & Bird, 2014; Quigley, 2013).

An imbalance in the normal flora has been described to contribute to intestinal disorders such as chronic inflammatory bowel, cardiovascular diseases, as well as obesity and mental health problems. This imbalance is dubbed dysbiosis and it occurs due to a loss of diversity of the species present, occurring an increase of the presence of a specie that had not been predominant before or even due to the depletion of a specie. For instance, the depletion of *Faecalibacterium prausnitzii*, belonging to the Firmicutes phylum, has been associated with inflammatory bowel disorder even though it is just a single specie (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Conlon & Bird, 2014; Laparra & Sanz, 2010; Marchesi et al., 2015; Million, Lagier, Yahav, & Paul, 2013).

Modulation of the microbiota has been studied as an alternative treatment for some pathologies, including liver pathologies and inflammatory bowel disease, since the increase or reduction of certain species may be beneficial, as mentioned previously. This modulation may occur through probiotics, prebiotics or polyphenols, since these can influence the composition or metabolic activity of the intestine's normal flora, contributing to reduce pathogenic species such as *Clostridium* (whose reduction has been associated with ameliorating autistic related symptoms), while increasing the presence of others such as *Lactobacillus* and *Bifidobacterium* and also stimulating the production of beneficial compounds such as butyric acid from these bacteria, for example. This stimulation can be done, for instance, using prebiotics, which are food constituents that promote the growth of microorganisms that exert a positive effect on the host. Essentially, they are a specific type of dietary fiber that, when fermented by the gut microbiota, allows for both the selective growth of beneficial bacteria such as bifidobacteria, as well as an increase of the production/accumulation of beneficial compounds (Dueñas et al., 2015; Eswaran et al., 2013; A. Faria, I. Fernandes, S. n. Norberto, N. Mateus, & C. Calhau, 2014a; Guglielmetti et al., 2013; Pequegnat et al., 2013; J. Slavin, 2013).

1.2.2. Blueberries and gut microbiota

Not only prebiotics are associated with a potentially healthier lifestyle. Polyphenols have long been studied as one of the most likely class of compounds, present in whole plant foods, which are capable of affecting physiological processes that may grant some protection against chronic diet-associated diseases. The gut microbiota plays a very important part in the release of polyphenols that are bound to dietary fiber and that can be then absorbed into the blood serum. The majority of polyphenols are not absorbed at the upper gastrointestinal level, and therefore, are able to reach the colon and be metabolized by the microbiota, which alters these compound's bioactivity and consequently lets them be absorbed. This happens through the enzymatic activity of the gut microbial community (Cardona et al., 2013; Etxeberria et al., 2013; A. Faria, I. Fernandes, S. n. Norberto, N. Mateus, & C. a. o. Calhau, 2014b; Marchesi et al., 2015).

Recent studies have shown that the intake of polyphenol extracts, such as the ones obtained from the consumption of a blueberry powder drink, help to modulate the human gut microbiota, increasing the abundance of *Bifidobacterium* and *Lactobacillus* and diminishing the presence of *Clostridium histolyticum*, which considering that the latter has been associated with inflammatory bowel disease, contributes to a healthier profile of the gut. Moreover, different studies have shown that phenolics can alter the *Bacteroides*/*Firmicutes* balance, with *Firmicutes* being more predominant in people who suffer from obesity and *Bacteroides* being reported as significantly contributing to the

reduction of blood pressure and high-density lipoprotein cholesterol. Furthermore, it is thought that *Bacteroides* are the genera mostly involved in the reactions necessary for anthocyanins' metabolization, since they express all the enzymes needed. Likewise, in a 16 weeks long study with mice, the dietary administration of proanthocyanidin-rich extracts led to a shift in the predominance of *Bacteroides*, *Clostridium* and *Propionibacterium* spp. to a predominance of *Bacteroides*, *Lactobacillus* and *Bifidobacterium* spp. In another study, a proanthocyanidin-rich extract from grape seeds given to healthy adults for 2 weeks was shown to significantly increase the presence of Bifidobacteria in the gut. Thus, polyphenols may be capable of reforming the microbial community present in the gut and consequently improve the health of the host, through host-microbial interactions. One of the mechanisms involved in the modulation, are the microbial enzymes, which can alter phenolics and other compounds through hydrolysis, cleavage and decarboxylation, among others. For instance, the gut microbiota is responsible for hydrolyzing glycosides into aglycones and degrading them into simple phenolic acids. Other possible mechanism involved in the modulation of the gut microflora is thought to be associated with the fact that polyphenols act differently upon distinct microbial colonies, affecting their viability differently. For example, an anthocyanin rich blueberry extract has been shown to have antimicrobial activity against different food pathogens, but no action upon probiotic bacteria. Additionally, phenolic compounds, which are important components of blueberries, have been reported to exert selective antimicrobial activity against intestinal pathogens such as *Salmonella*, but also against *Staphylococcus*, by generating hydrogen peroxide and by altering the permeability of the cellular membrane. (Boto-Ordóñez et al., 2014; Boulangé et al., 2016; Cardona et al., 2013; Duda-Chodak et al., 2015; Dueñas et al., 2015; Etxeberria et al., 2013; Faria et al., 2014b; Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; M. Hidalgo et al., 2012; Laparra & Sanz, 2010; Marchesi et al., 2015; Million et al., 2013; Puupponen-Pimiä et al., 2005; Selma et al., 2009; Valdés et al., 2015; Vendrame et al., 2013).

1.3. Objectives

It has been previously shown that an anthocyanin rich blueberry, when in the presence of probiotic bacteria (particularly in the presence of *Bifidobacterium animalis* subsp. *lactis* Bo), was capable of inhibiting the adhesion of some potential food pathogens such as *L. monocytogenes* and *E. coli*. Thus, and considering the possible modulating potential of the extract, the main objective of this work was to assess the extract's impact upon Caco-2 cells, a cell line representative of the enterocytes coating the small intestine, viability and probiotics adhesion to it.

The first specific objective consisted on fermenting the extract, in order to evaluate the extract's impact upon the growth of probiotic bacteria (*Bifidobacterium animalis* subsp. *lactis* Bo, *Bifidobacterium animalis* subsp. *lactis* BB12, *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* R11 and a mix (1:1:1:1) of all the bacteria) and also, how its presence could affect organic acid production and sugar consumption. The resulting fermentation products were then analyzed to see if they possessed any negative effect upon Caco-2 and mucus producing HT29-MTX cell models viability. Finally, after concluding the previous objectives, we focused on the influence of the extract upon the adhesion of probiotic bacteria on a cell model, constituted of Caco-2 and HT29-MTX cells.

2. Materials and Methods

2.1. Samples

Blueberries (*Vaccinium corymbosum* L) from the cultivar Goldtraube, kindly provided by Mirtilusa, S.A. (Sever do Vouga, Portugal), were collected and stored at -20 °C until usage.

2.2. Extract production

The extraction protocol was performed as described by Silva et al. (2016). Briefly, blueberries were milled and the resulting pulp was mixed 1:9 with ethanol (Panreac, Spain) acidified with 0.01% (v v^{-1}) HCl (32% Merck, St. Louis, USA). This suspension was then homogenized using an Ultra-Turrax T18 (24 000 rpm, 1 min) (IKA, Staufen, Germany) and left to extract at 40 °C, for 1 h, in a light tight container. Afterwards, the mixture was placed in an ultrasound bath at 35 KHz (RK106 Bandelin Sonorex, Berlin, Germany) for 15 min. The resulting suspension was then centrifuged (6086 *g*, 4 °C, 15 min) and the supernatant filtered through a 4-7 µm filter (Prat Dumas, Couze St. Front, France).

2.3. Extract purification

The extract was purified using Solid Phase Extraction (SPE) columns in accordance with the protocol described by Silva et al. (2016). Briefly, ethanol was removed from the extracts using a rotary evaporator (175 bar, 40 °C, r-210, Buchi, Switzerland) and the resulting mass was suspended in deionized water (ddH_2O) acidified with 0.01% (v v^{-1}) 32% HCl. This solution was then loaded into Bond Elut Plexa SPE columns (Agilent Technologies, Santa Clara, USA), previously activated with one volume of ethanol and conditioned with two volumes of acidified ddH_2O. After loading the samples, the columns were washed with two volumes of acidified ddH_2O and the compounds were eluted using one volume of acidified ethanol. The resulting solution was evaporated using a rotary evaporator under the conditions referred previously. The resulting powder has been previously characterized as being comprised of ca. 637 mg g^{-1} anthocyanins (monoarabinosides, monoglucosides and monogalactosides of delphinidin, cyanidin, malvidin, peonidin and petunidin) with their aglycones, the anthocyanidins, also being present. Henceforth, whenever extract is mentioned it refers to the powder obtained in this step.

2.4. Chemical characterization

2.4.1. Total phenolic content and total anthocyanin content

The extract was dissolved in water at 1 mg mL^{-1} and its' total phenolic content was evaluated through the analysis of the area under the curve as described previously by (Silva et al., 2015). Briefly, separation was carried out using an injection volume of 40 µL in a High Performance Liquid Chromatography coupled with Diode Array detector (HPLC-DAD) system (Waters 600, Waters, Milford, MA, USA) measuring in 2-nm intervals, from 200 to 600 nm, equipped with a C18 Kromasil 100 column (25 x 0.46 cm; Teknokroma, Barcelona, Spain) and through the analysis of the spectra obtained at 320 nm (phenolic compounds) and 520 nm (anthocyanins). The total phenolic and anthocyanin contents

were then calculated considering a gallic acid (Sigma, St. Louis, USA) or cyanidin-3-glycoside (Extrasynthese, Lyon, France) standard curve, respectively. Samples were injected in duplicate.

2.5. Biological potential assessment

2.5.1. Microorganisms

The following bacteria were used throughout the present work: *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* R11, *Bifidobacterium animalis* subsp. *lactis* Bo and *Bifidobacterium animalis* subsp. *lactis* BB12. Lactobacilli inoculums were prepared using de Mann, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) and incubated at 37 °C, for 48 h, under aerobiosis. *Bifidobacterium* inocula were prepared using MRS broth supplemented with 0.05% (w v^{-1}) L-cysteine hydrochloride (Cys) (Merck, Darmstadt, Germany) and incubated for 48 h, at 37 °C, under anaerobic atmosphere (10% CO_2, 10% H_2 and 80% N_2; Whitley DG250 Anaerobic Workstation, Don Whitley Scientific, West Yorkshire, UK).

2.5.2. Impact upon probiotic growth

The extract's impact upon probiotic growth was evaluated as follows. Nutrient base medium was prepared as described in table 1 and it was used to dissolve the extract at a final concentration of 1 mg mL^{-1}. The resulting solutions were filtered using a 0.22 µm sterile filter (Millipore, Billerica, USA), inoculated at 2% (v v^{-1}) using a 48 h inoculum of each probiotic microorganisms (grown under the conditions described in section 2.5.1) or a 1:1:1:1 mixture of these inocula (mix) and incubated for 24 h at 37 °C under anaerobic conditions.

Samples were taken at the 0, 6, 12 and 24 h mark to assess organic acids' production, sugar consumption, measure the pH value and determine the total viable cells. Inoculated nutrient base medium was used as positive control, while plain nutrient base medium with and without extract were used as a negative control. Each condition was assessed in triplicate.

Table 2.1 Composition of nutrient base medium.

Component	**Concentration**	
Trypticase soy broth (TSB) without dextrose	5.0 g L^{-1}	Biokar Diagnostics, Beauvais, France
Bactopeptone	5.0 g L^{-1}	Amersham, Buckinghamshire, UK
L-cysteine hydrochloride	0.5 g L^{-1}	Merck, Darmstadt, Germany
Salt solution A (1.0% (v v^{-1}))		
NH_4Cl	100.0 g L^{-1}	
$MgCl_2 \cdot 6H_2O$	10.0 g L^{-1}	
$CaCl_2 \cdot 2H_2O$	10.0 g L^{-1}	
Trace mineral solution	1% (v v^{-1})	ATCC, Manassas, USA
Salt solution B (0.2% (v v^{-1}))		
$K_2HPO_4 \cdot 3H_2O$	200.0 g L^{-1}	
Resazurin solution	0.2% (v v^{-1}) of 0.5 g L^{-1}	

2.5.2.1. *Total viable cells determination*

Total viable counts were determined using decimal dilutions, which were plated in MRS agar supplemented with 0.05% (w v^{-1}) Cys and 0.02% bromophenol blue (Sigma-Aldrich, St. Louis, USA), a selective medium that permits to distinguish *Lactobacillus* from *Bifidobacterium*. Lactobacilli colonies grown in this media (48 h under anaerobiosis) exhibit a yellow/light blue color, while bifidobacteria's colonies are dark blue as described by H. Lee and Lee (2008). The dilutions were plated in quadruplicate using spread plate method and incubated, under anaerobic conditions, at 37 °C for 24 h. Results were expressed as the logarithm of colony forming units (CFU) as shown in the equation below in which CFU is the number of counted colonies, the volume corresponds to the plated volume and the dilution is the one in which was possible to count the grown colonies.

$$log\ CFU = log\ (CFU * \frac{1}{volume} * \frac{1}{dilution}) \quad (2.1)$$

2.5.2.2. *Organic acids determination, sugar consumption and pH measurement*

The pH value of the medium, at the different sampling times, was assessed using a potentiometer equipped with a Crison 52-08 electrode (Crison Instruments, Barcelona, Spain). Organic acid production and sugar consumption were evaluated using an High Performance Liquid Chromatography coupled with a refractive index (HPLC-RI) system (HPLC Pump K-1001, Knauer, Berlin, Germany) which consisted of an ion exchange Aminex HPX-87H Column (300 × 7.8mm) (Bio-Rad, California, USA), kept at 65 ℃ (L-7350 Column Oven; LaChrom, Merck-Hitachi, Germany); and a refractive index detector (L-7490 RI Detector; LaChrom, Merck-Hitachi, Germany) (S. Sousa et al., 2015). Samples were filtered using a 0.22 µm filter (Milipore, Billerica, USA) prior to injection. The injection volume was 50 µL and the mobile phase consisted on 13 mM H_2SO_4 at a rate of 0.8 mL min^{-1} All samples were injected in duplicate.

2.5.3. Cell lines and general growth conditions

Caucasian colon carcinoma (Caco-2) and mucous producing human colon (HT29-MTX-E12) cells were obtained from the European Collection of Authenticated Cells Cultures (ECACC 8601020 and 12040401, respectively) through Sigma-Aldrich (St. Louis, USA; ECACC) (references 09042001 and 12040401, correspondingly). Except when stated otherwise, the cells were grown using high glucose Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% (v v^{-1}) heat inactivated Fetal Bovine Serum (FBS; Biowest, France), 1% (v v^{-1}) Pen-Strep (Lonza, Basel, Switzerland) and 1% (v v^{-1}) of non-essential amino acids 100x (Lonza, Basel, Switzerland). All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.5.4. Cell viability determination

2.5.4.1. *Selection of the viability assay*

To obtain a better understanding of the cells response based on two viability assays, a comparison between the 3(4,5dimethylthiazol2yl)2,5diphenyltetrazolium bromide (MTT) and the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide (XTT) methods was performed. Briefly, 100 µL aliquots of cell suspensions (1 x 10^5, 1 x 10^4 and 1 x 10^3 cells mL^{-1}) were transferred into

96 well microtiters (Nucleon Delta Surface, Thermo Scientific, Roskilde, Denmark) and incubated to allow for cell adhesion. After 24 h, the viability of the cells was evaluated using both XTT and MTT assays. Plain culture media was used as a blank and cells with 100% dimethyl sulfoxide (DMSO) were used as negative control.

2.5.4.1.1. XTT assay

To assess the redox activity of mitochondrial oxidoreductases, the XTT assay was carried out as follows. Briefly, a 10 mM of Phenazine Methosulfate (PMS) solution (Sigma-Aldrich, St. Louis, USA) was prepared in phosphate buffered saline (PBS, 0.01 M; pH 7.4) and a 1 mg mL^{-1} XTT solution was prepared using the appropriate culture media, previously warmed to 37 °C. Both solutions were sterilized using a 0.22 µm sterile membrane filter (Millipore, Billerica, USA) and, immediately before being used, were mixed (2.5 µL of PMS per mL of XTT solution). Aliquots (25 µL) of mix were added to each well and the cells incubated, in the dark, for 2 h. The OD at 485 nm was then measured using a microplate reader (FLUOstar, OPTIMA, BMG Labtech, Ortenberg, Germany). All assays were performed in quintuplicate.

2.5.4.1.2. MTT assay

The mitochondrial succinate dehydrogenase redox activity was assessed through the reduction of the MTT as described by Coimbra et al. (2011). After the 24 h incubation, aliquots of a 12 mM MTT solution (prepared using PBS pH 7.4 and filtered through 0.22 µm sterile filters (Millipore, Bilerica, USA)) were added to each well (five replicates) in order to achieve a final concentration of 0.5 mg mL^{-1}. After 4 h of incubation (in the dark), the medium was aspirated, and the cells were treated with 100 µL of DMSO. The absorbance was read at 570 nm using a microplate reader (FLUOstar, OPTIMA, BMG Labtech, Ortenberg, Germany).

2.5.4.2. Test solution preparation

The extract was diluted at a 1 mg mL^{-1} concentration in ddH_2O and sterilized using a 0.22 µm syringe filter (Millipore, Billerica, USA). This solution was then mixed with high glucose DMEM (with 20% (v v^{-1}) FBS and 1% (v v^{-1}) Pen-Strep) to attain the following extract concentrations: 62.5, 125, 250 and 500 mg mL^{-1}. A fructooligosaccharide (FOS) solution was prepared at 6% (v v^{-1}) and filtered using 0.22 µm membrane filter, and then mixed with high glucose DMEM (with 20% FBS and 1% Pen-Strep) to attain a final solution with a 3% (v v^{-1}) concentration.

The extracts which were fermented by the different microorganisms (section 2.4.2) during 12 and 24 h, were filtered using 0.22 µm membrane filter and mixed with high glucose DMEM (with 20% FBS and 1% Pen-Strep) to prepare solutions with the same concentrations as those studied above.

Since autoclave could degrade the samples due to heat, these were filtered using 0.22 µm filters. This is a sterilization method used frequently for liquid samples, and particularly convenient to be applied here, since the phenolic rich extract being used is thermosensitive. Reports have shown how operations involving heat have led to a significant decrease in the content of anthocyanins present in the samples (Patras, Brunton, O'Donnell, & Tiwari, 2010).

2.5.4.3. *Extract impact upon cell viability*

The impact of the extract (before and after fermentation), upon CaCo-2 and HT29-MTX viability was determined using the XTT colorimetric method as described previously in section 2.5.4.1.1. Briefly, aliquots of 100 µL of a cell suspension ($1x10^5$ cell mL^{-1}) were seeded in 96 well microplates (Nucleon Delta Surface, Thermo Scientific, Roskilde, Denmark). After seeding, the culture medium was then carefully replaced with the different test solutions and incubated in the dark. Five replicates were used for each concentration tested. After 24 h, cell viability was assessed through the selected method.

2.5.5. Bacterial adhesion assessment

2.5.5.1. *Calibration curve*

A calibration curve relating OD of the microorganism and CFU mL^{-1} was made. Briefly, inoculums of *L. rhamnosus* R11 and *B. animalis* BB12 were made and incubated as described in section 2.4.1. After incubation, decimal dilutions of the inoculums were done and plated in MRS agar and the CFU were assessed. The OD of each dilution was measured using a spectrophotometer (UVmini 1240 UV-Vis spectrophotometer, Shimadzu, Japan) at 660 nm.

2.5.5.2. *Cell adhesion*

In order to assess the extract's impact upon lactic acid bacteria when in contact with intestinal cells, 48 h inoculum of *L. rhamnosus* R11 and *B. animalis* BB12 were made and then diluted to a density equivalent to $1x10^5$ log CFU in PBS. Briefly, aliquots of 100 µL of a cell suspension ($1x10^5$ cells mL^{-1}) were seeded in 96 well microplates (Nucleon Delta Surface, Thermo Scientific, Roskilde, Denmark) and after 24 h incubation, the medium was replaced with either 1:1 mix of medium and bacteria, 1:1:1 mix of medium, extract (500 mg mL^{-1}) and bacteria or 1:1:1 mix of medium, FOS (3 % v v^{-1}) and bacteria, with five replicates for each condition. The mixture was left in contact with the cells for 15, 30, 60, 120 and 180 minutes at 37 °C. After these periods, total viable counts were determined using decimal dilutions which were plated in quadruplicate in MRS agar supplemented with 0.02% bromophenol blue (Sigma-Aldrich, St. Louis, USA). The plates were then incubated for 48 h at 37 °C under aerobic conditions.

2.6. Statistical analysis

The statistical analysis of the results was performed using SPSS Statistics v24.0.0.0 (IBM, New York, USA) software. The normality of the distributions was evaluated using the Shapiro Wilk test. To analyze the differences between sample groups, when a normal distribution was observed, the One - Way ANOVA test was used in association with Scheffe's test, otherwise the non-parametric Mann-Whitney test was employed. The differences were considered statistically significant at a 5% confidence degree level though differences significant at a 1% level were also marked.

To evaluate the correlation between two continuous variables, exhibiting normal distributions, Person's test was employed (Pearson's R). However, when the distributions were not normal, Sperman's correlation, *i.e.* Sperman's Rho, was calculated instead. The correlations were considered statistically significant at a 5% confidence level but, correlations significant at a 1% confidence level were marked.

3. Results and discussion

3.1. Chemical characterization

Blueberries are rich in phenolic compounds and as such are known for their high antioxidant capacity. With that in mind, the extraction procedure proposed by Silva et al. (2013) seemed the most appropriate to be applied in this work, as the same species of blueberry was used (*V. corymbosum* L) and the extraction methodology aimed to obtain extracts rich in the phenolic and antioxidant compounds, thus valorizing the potentially bioactive components. The produced extracts' composition was analyzed through HPLD-DAD with two wavelengths being used: 310 nm for detection of phenolic compounds, and 520 nm for anthocyanins determination. These wavelengths are the closest to the maximum absorbance of these compounds that were available in the equipment.

When analyzing the composition of the produced extract, the most predominant anthocyanin found was malvidin. Additionally, delphinidin, cyanidins, petunidin-3-O-glucoside, petunidin-3-O-galactoside, peonidin-3-O-arabinoside and peonidin-3-O-galactoside were also present.

Table 3.1 Phenolic composition of the extract in µg of anthocyanin per mg of powder obtained after extraction and purification.

	Concentration (µg mg^{-1})	
	Mean	Standard deviation
Delphinidin-3-glucoside	25.7	2.1
Delphinidin-3-galactoside	16.4	0.70
Cyanidin-3-glucoside	4.20	0.50
Cyanidin-3-galactoside	6.50	0.70
Malvidin-3-glucoside	62.0	2.4
Malvidin-3-galactoside	37.3	1.7
Petunidin-3-glucoside	22.5	2.2
Neochlorogenic acid	32.2	2.8
n.i.*	10.1	0.70

* n.i.: non-identified anthocyanin expressed in equivalents of cyanidin-3-glucoside

Blueberries have a particularly high concentration of phenolics, and in particular, anthocyanins. These compounds are responsible for the fruit's dark color and health benefits. In this work, the extract presented a total anthocyanin content of 37.4 mg per 100 g of fruit. Previously, Prior et al. (1998) reported, for *V. corymbosum* blueberries, concentrations of total phenolics ranging from 233 to 273 mg per 100 g of fruit and total anthocyanins ranging from 62 to 157 mg per 100 g of fruit, values which are 1.6 to 4 times higher than the ones found in this work. From a composition standpoint, reports by Hosseinian and Beta (2007) and Nicoue, Savard, and Belkacemi (2007) affirm that malvidin, delphinidin-, cyanidin-, petunidin-, and peonidin-glycosides are the most abundant anthocyanins found in wild blueberry. Similarly, Taverniti et al. (2014) reported that the main anthocyanins found in a wild blueberry extract were malvidin, delphinidin, cyanidin and petunidin, with malvidin 3-glucoside and 3-galactoside as well as delphinidin 3-glucoside being the ones present at higher concentrations (ranging from 224 µg

mL^{-1} to 251 µg mL^{-1} and being equivalent to 45% of all anthocyanins found in the extract). Last but not least, Faria et al. (2005) reported that a blueberry (*Vaccinium myrtillus*) extract possessed a total phenolics amount of 257.9 ± 8.5 mg per 100 g of fruit, with several anthocyanins such as delphinidin, cyanidin, malvidin, petunidin and peonidin (associated with sugars such as glucose, galactose and arabinose) being identified.

Overall, any differences in composition between the extracts produced and those described in literature, may be due to different factors. For instance, the anthocyanin profile of blueberries can vary between different cultivars, as well as being influenced by the ripening stage, harvest season and growing conditions. As reported by Giovanelli and Buratti (2009), total phenolics and total anthocyanins contents differ from cultivated blueberries to wild blueberries, with wild blueberries possessing a much higher anthocyanin to total phenolic ratio. Additionally, the extraction procedures also affect the obtention of phenolic compounds, with certain compounds being more susceptible to the extraction methodologies employed than others (He et al., 2016).

3.2. Biological potential assessment

3.2.1. Extract's impact upon probiotic's growth and fermentation

3.2.1.1. Analysis of total viable counts

In order to evaluate the impact of the extract upon probiotic growth, a basal medium was used. As this medium only allows for microbial survival, its usage permitted to better analyze if the microorganisms tested could grow or even incorporate in their fermentative process the blueberry extract. Furthermore, addition of the extract to MRS broth led to a decline in the amount of anthocyanins detected, possibly due to an interaction between the compounds of the medium and the extract (data not shown). This also contributed to the use of the basal medium, since analysis of a mix of the extract with this medium, showed no detectable decrease in the anthocyanins content. As such, the blueberry extract was dissolved in this medium and bacterial growth in the presence and absence of extract was controlled for a 12 and 24 h period.

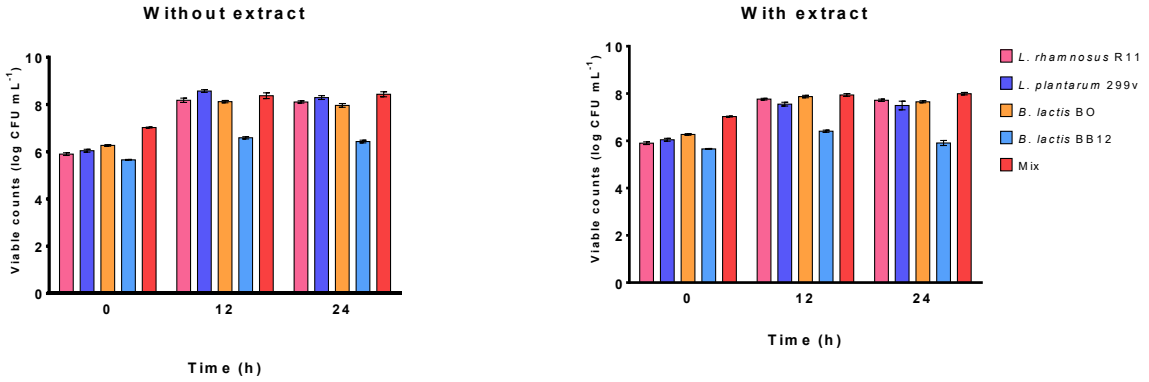

Figure 3.1 Total viable counts obtained in the presence and absence of extract (12 and 24 h) for each microorganism and mix. All values in log CFU mL-1, correspond to the average of four replicates.

As can be seen in Figure 3.1, there was a decrease in the values of viable counts for all microorganisms in the presence of the extract, being this difference more prominent for lactobacilli, namely *L. rhamnosus* R11 and *L. plantarum* 299v. On the other hand, the bifidobacteria and the Mix of bacteria tested were less susceptible to the presence of the extract.

The presence of extract impacted *L. rhamnosus* R11 total viable counts (Figure 3.2). When comparing the presence and absence of extract, the difference was of approximately 0.4 log CFU mL^{-1} for both sampling times (12 and 24 h), a reduction that, while small was statistically significant ($p < 0.05$). However, even though the extract seemed to exert a slightly inhibitory activity, since the decrease in viable counts in the final 12 h was lower in the presence of extract than in its absence. On the one hand, this dichotomy may be due to the probable depletion of the nutrients originally present in the basal medium and consequent consumption of the sugar moieties of anthocyanins present in the extract, which will then lead to a reduction in the decrease of total viable counts. On the other hand, while the decrease in total viable counts might be explained by the production and consequent accumulation of acids, the presence of the anthocyanin extract may ameliorate the toxic effect of the acids' presence, as organic acids have been shown to be captured by anthocyanins.

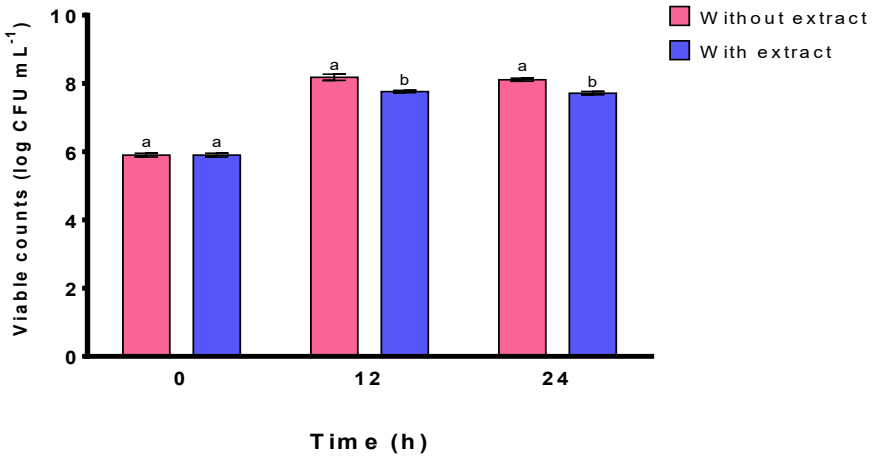

Figure 3.2 Total viable counts obtained for L. rhamnosus *R11 in the presence and absence of extract. All values in log CFU mL^{-1}, correspond to the average of four replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples at each sampling time.*

According to Biswas et al. (2012), *L. rhamnosus* was able to not only survive but grow in the presence of blueberry juice although with lower viable counts than in the presence of skim milk alone. This stands in line with the results obtained since, in the absence of extract (Figure 3.2) the values for the viable counts were higher. Additionally, Puupponen-Pimiä et al. (2001) showed that two strains of *L. rhamnosus*, namely VTT E-97800 and GG VTT E-96666, were the most sensitive out of the probiotic bacteria tested to some phenolic compounds from berries. However, Molan et al. (2009) reported that the addition of a water soluble blueberry extract on mixed fecal cultures, although from a different cultivar, resulted in an increase of *L. rhamnosus* ATCC 7469. These differences may be due to the genetic variations observed between the strains, even though they belong to the same species.

When it comes to *L. plantarum* (Figure 3.3) the differences in growth were more significant ($p < 0.05$), with viable counts being 1 log CFU mL^{-1} inferior in the presence of extract, after 12 h, and approximately 0.8 log inferior after 24 h. Once again, there was a slight decrease in viable counts in the

final 12 h. Reports have shown that certain anthocyanins reduce *L*. *plantarum*'s growth rate in a concentration dependent manner, with higher concentrations causing a stronger delay of the bacterial growth, which may also explain the results observed (J. M. Landete, Rodriguez, De Las Rivas, & Munoz, 2007; Marsilio & Lanza, 1998; Rodríguez et al., 2009; Rozès & Peres, 1998).

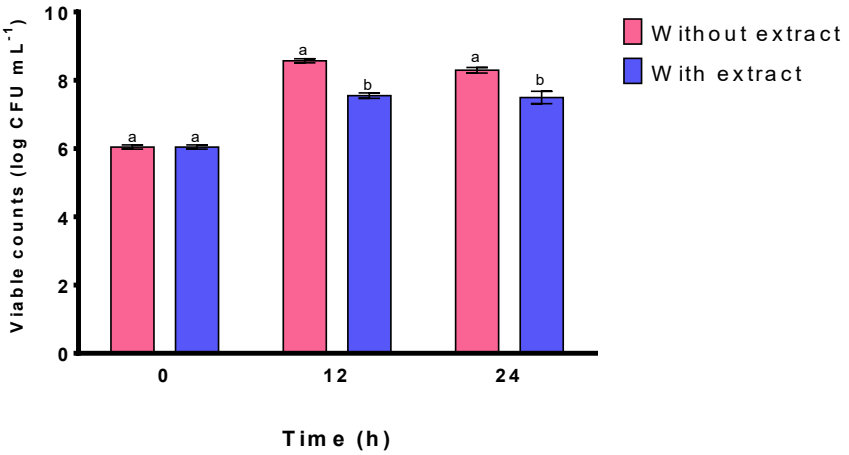

Figure 3.3 Total viable counts obtained for L. plantarum *299v in the presence and absence of extract. All values in log CFU mL^{-1}, correspond to the average of four replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples at each sampling time.*

For *B*. *animalis* subsp. *lactis* BB12 (Figure 3.4) while no statistical significant differences were observed at the 12 h mark ($p > 0.05$), the same cannot be said after 24 h, as the extract presence caused a statistically significant ($p < 0.05$) decrease in viable counts. When comparing the *B. animalis* BB12 positive control viable counts with those of the extract of the remaining microorganisms tested, it can be verified that this strain had poor growth in the basal medium, even when the extract was absent. For instance, when comparing with the results published by Silva et al. (2013), it can be seen that the viable counts for *B*. *animalis* BB12 are between 8 and 9 log CFU mL^{-1}, and thus higher than the values obtained in this work. Additionally, Tabasco et al. (2011) evaluated the effect of polyphenols on the growth of several bifidobacteria strains and concluded that *B. animalis* BB12 demonstrated the highest sensitivity to all phenolic extract tested. As such, this stands with accordance with the results obtained, in which a slight inhibition was exerted by the extract upon *B. animalis* BB12's growth.

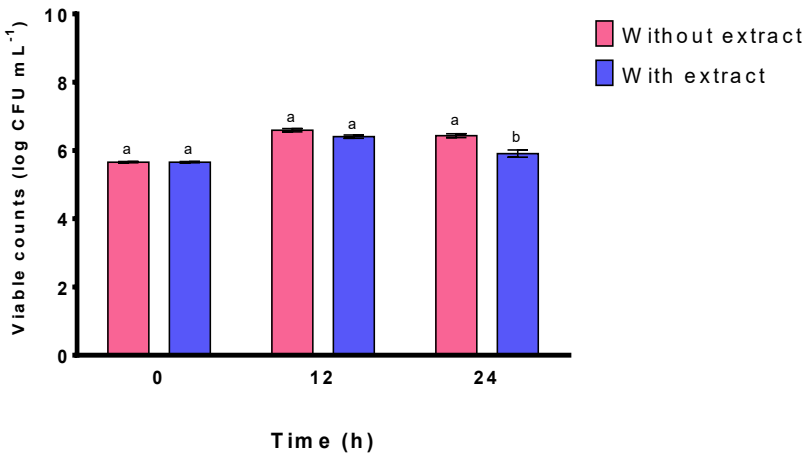

Figure 3.4 Total viable counts obtained for B. animalis *subsp.* lactis *BB12 in the presence and absence of extract. All values in log CFU mL^{-1}, correspond to the average of four replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples at each sampling time.*

For *B. animalis* subsp. *lactis* Bo (Figure 3.5), at 12 h the viable counts in the presence of extract are inferior to the counts in the absence of extract by 0.250 log CFU mL^{-1}, although not statistically significant ($p > 0.05$). This value increases to 0.311 log CFU mL^{-1} at 24 h, also not being statistically significant ($p > 0.05$). It is important to stand out that this was the microorganism who appeared to be less sensitive to the extract, since the viable counts presented the lowest difference between the presence and absence of extract.

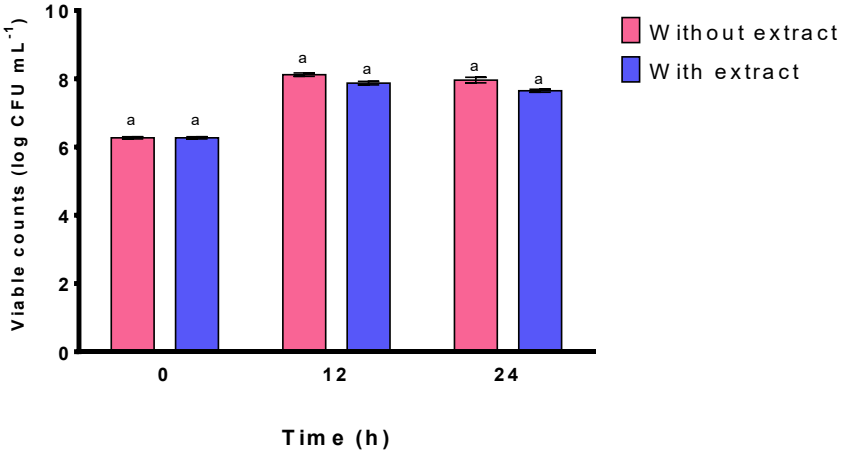

Figure 3.5 Total viable counts obtained for B. animalis subsp. lactis Bo in the presence and absence of extract. All values in log CFU mL^{-1}, correspond to the average of four replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples at each sampling time.

The impact observed of the blueberry extract upon both bifidobacteria strains viable counts is in disaccord with the work of Silva (2016) which reported how a blueberry extract did not have significant impact upon *B. animalis* Bo's growth and even led to higher viable counts for *B. animalis* BB12 at the 12 h mark. However, this difference may also be related to the medium used, since the basal medium used in this work does not possess any sugar, unlike the MRS medium used in the work referenced. Furthermore, from a statistical standpoint, and considering bifidobacteria only, the extract was not a significant factor ($p > 0.05$) in the differences observed between the 12 and 24 h mark, only time. However, through the analysis of the within subjects' contrasts, resulting of the ANOVA repeated

measures test, it was verified that time presents itself as a determining factor when comparing initial time with both the 12 and 24 h mark, which makes sense taking into account microbial growth throughout time. Nonetheless, the test evaluated that the differences between the 12 h and 24 h are not only associated with time, but also with the presence of extract ($p < 0.05$).

Previously, it has been reported that anthocyanins can be degraded by the gut microflora, through hydrolyzation by the intestinal enzyme glucosidase, an enzyme which is also expressed by *Lactobacillus* spp. and *Bifidobacterium* spp (Donkor & Shah, 2008; Morais, de Rosso, Estadella, & Pisani, 2016). Thus, even though a small inhibition may have occurred, it stands to reason that the extract would not possess any antimicrobial activity against these bacteria as they could metabolize the main bioactive components present in the extract: anthocyanins. This stands in line with the reports of Silva et al. (2013), in which an anthocyanin rich blueberry extract was capable of inhibiting different foodborne pathogens, but had no effect upon probiotic bacteria and of Biswas et al. (2012), who showed the antimicrobial effect of a pasteurized blueberry juice upon milk pathogens, but it did not inhibit probiotic bacteria. Moreover, Puupponen-Pimiä et al. (2001) reported that different berries extracts, including blueberry, did not display any inhibition against lactic acid bacteria, although the extracts had effect against foodborne pathogenic Gram positive bacteria.

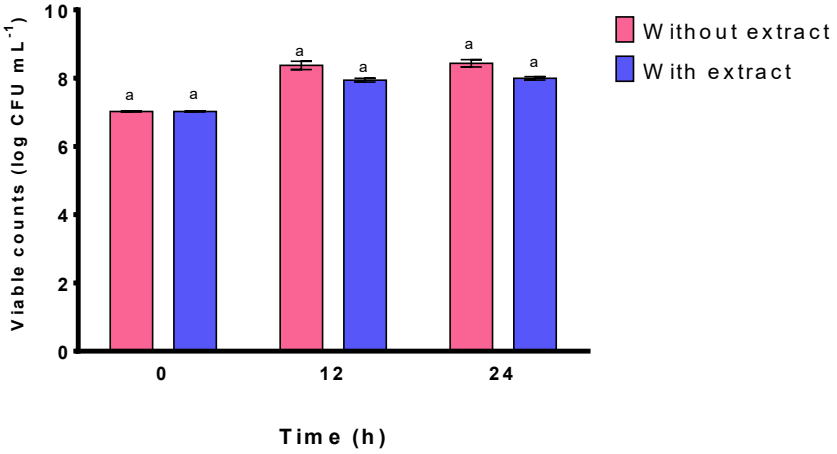

Figure 3.6 Total viable counts obtained for the Mix in the presence and absence of extract. All values in log CFU mL^{-1}, correspond to the average of four replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples at each sampling time.

When mixing the four strains used in this work, the total viable counts obtained in the presence and absence of extract were not statistically significantly different ($p > 0.05$) (Figure 3.6), although the absence of extract led to higher values of viable counts. As such, taking into consideration the results obtained for the Mix of the probiotic bacteria, the impact of the extract was evaluated on the different genera of bacteria involved. As can be seen in Figure 3.7, in the absence of extract, the difference between the log CFU mL^{-1} values for *Lactobacillus* and *Bifidobacterium* was higher than in the presence of extract. This comes in accordance with the previous results, in which *Lactobacillus* species tested presented higher inhibitions than bifidobacteria. Bifidobacteria also presented higher values when it was inoculated with extract than without, with a statistically significant difference ($p < 0.05$) of 0.493 log CFU mL^{-1}, at 12 h, mark and 1.42 log CFU mL^{-1} after 24 h.

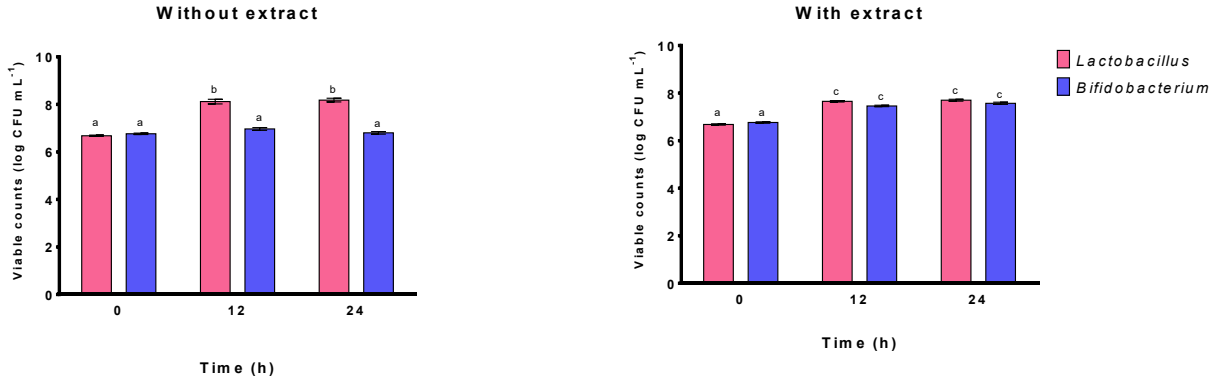

Figure 3.7 Total viable counts obtained in the presence and absence of extract for the genera of bacteria tested. All values in log CFU mL^{-1}, correspond to the average of four replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between all sets of data in both graphs.

Reports have shown that blueberries help increase bifidobacteria present in the gut and that these genera of bacteria are capable of interacting with anthocyanins, due to the presence of certain enzymes, as mentioned previously (Donkor & Shah, 2008; Donkor, Vasiljevic, & Gill, 2010; Morais et al., 2016). The increase of bifidobacteria levels in the gut has been widely associated with improving the health of the intestinal tract, (*e.g.*, bifidobacteria species help to reduce incidence of neonatal necrotizing enterocolitis, as well as ameliorate Inflammatory Bowel disease's symptoms and prevent gastrointestinal infections due to competitive exclusion of potentially pathogenic microorganisms) in addition to enhancing the immune system (Caplan & Jilling, 2000; R. Fuller & Gibson, 1997; Gueimonde, Margolles, Clara, & Salminen, 2007; Parvez, Malik, Ah Kang, & Kim, 2006; Venturi et al., 1999). Vendrame et al. (2011) reported that, after the continued consumption of a wild blueberry powder drink, the total counts of bifidobacteria in the microbiota were three times superior to the original amount, while Boto-Ordóñez et al. (2014) showed that the consumption of red wine, which is rich in anthocyanins and other phenolics, was associated with an increase in the counts of *Bifidobacterium* spp., with this variation being linked with the changes in anthocyanins and their metabolites, thus strengthening the hypothesis that phenolics function as potential bacterial substrates. Moreover, Guglielmetti et al. (2013) reported that blueberries could induce differential modulation of gut microbiota promoting some specific *Bifidobacterium* strains. This genre of bacteria is also responsible for microbial fermentations that result in the production of short chain fatty acids (SCFA), which contribute to the digestive process and have cancer preventing effects, namely due to their ability of interacting with compounds such as anthocyanins, which results in the inhibition of procarcinogenic enzymatic activities within the microbiota.

Taking into account the composition of the blueberry extract, which are mainly anthocyanins and no sugar, the lack of inhibition shown by the bifidobacteria tested stands with accordance with these reports, since not only do anthocyanins and phenolic compounds seem to increase the growth of bifidobacteria, but also there seems to be a positive correlation between the two, as mentioned before. In fact, Silva (2016) described how *B. animalis* Bo, in association with an anthocyanin rich blueberry extract, was able to fully inhibit the adhesion of pathogenic microorganisms (in the countable detection limit), such as *Escherichia coli* and *Listeria monocytogenes*. *B. animalis* BB12 also seemed to contribute

to a certain inhibition, but not as strong as *B. animalis* subsp. *lactis* Bo. The probiotic bacteria also suffered a slight inhibition due to the presence of the extract, even though this association led to a very strong inhibition of the potentially pathogenic bacteria.

On the other hand, for *Lactobacillus* some inhibition was present, with slightly lower viable counts being observed in the presence of extract (*ca*. 0.5 CFU mL^{-1} lower after 12 and 24 h ($p < 0.05$)). Most *Lactobacillus* spp. are unable to degrade polyphenols, but strains of *L. plantarum*, as well as *Lactobacillus pentosus* and *Lactobacillus paraplantarum*, have been shown to possess tannase activity and to metabolize phenolic acids. However, Cueva et al. (2010) demonstrated that several *Lactobacillus* species, including *L. plantarum,* were slightly inhibited by some phenolic acids at higher concentrations, which could help explain the decrease in viable counts observed in the present work. However, in this case, the phenolic acids were tested individually and in their pure form, leading to a higher inhibition than if they were integrated in a complex matrix, and moreover, the compounds used were not present in the extract tested in this work. Puupponen-Pimiä et al. (2001) demonstrated that a blueberry extract was capable of slightly inhibiting the growth of several *Lactobacillus* strains, including the ones used in this work, but at concentrations five times higher than the ones tested in our study.

To the best of our knowledge there is not enough reported data to allow for comparisons about the results obtained from the Mix of bacteria to be drawn. For instance, Tabasco et al. (2011) described the effects of a flavanol enriched grape seed extract upon different lactic acid bacteria and Bifidobacteria, and concluded that the strain of *L. plantarum* tested, not only reached its maximum growth, but was also capable of metabolizing the polyphenols tested, degrading flavanol monomers, while *Lactobacillus fermentum*, *L. acidophilus* and *B. animalis* subsp. *lactis* BB12 showed to be sensitive to the phenolic extracts assayed. On a different work, several lactobacilli and bifidobacteria were exposed to different phenolic acids. *L. rhamnosus* GG ATCC 53103 registered less than 10% of inhibition for all acids tested, while *Lactobacillus casei* 's inhibitions ranged from 20% to 62%. The Bifidobacteria tested had an increase in growth comparatively to the control in the presence of 4-OH phenylacetic acid (H. C. Lee, Jenner, Low, & Lee, 2006). Moreover, Molan et al. (2009) described how adding water extracts of two blueberry cultivars resulted in a significant increase of the growth of both *L. rhamnosus* and *B. breve*.

As such, it is possible to see that the effect of phenolic compounds upon lactobacilli and bifidobacteria varies depending upon the specie tested, as well as the concentration of the different compounds. Taking into account the strains used in this work, it was not possible to retrieve further conclusions, since the different *Lactobacillus* and Bifidobacteria used may interact differently than the ones used in the works referenced. However, in all of these works the bacteria were not mixed together. In the work described by Z. Li et al. (2015), pomegranate extract and pomegranate juice (both rich in phenolic compounds) were added to stool samples, which resulted in an increase of the viable counts of *Lactobacillus* and *Bifidobacterium*. However, higher concentrations of the juice led to an inhibition of both genres, while the extract in the same concentrations still showcased an increase in viable counts. Boto-Ordóñez et al. (2014) studied how red wine, also rich in phenolic compounds and anthocyanins, influenced microbial metabolites in a randomized clinical trial, and increased DNA quantity in the fecal concentration of *Bifidobacterium*. Furthermore, in a study described by Jakesevic et al. (2009), eight

LAB were given to mice in addition to rosehips. Higher values of phenolics were detected in the mice fed with the mixture of bacteria and the rose species. As such, these studies stand in accordance with the results obtained, in which the phenolic compounds present did not showcase any inhibition upon the microorganisms and even contributed to their metabolism, leading to an alteration of acid production.

3.2.1.2. Analysis of pH values during the fermentative process

When looking at table 3.1, which displays the acidification of the media throughout the fermentation, it is possible to verify that the pH values after 12 and 24 h were not statistically significantly different ($p > 0.05$) even though, when in the presence of extract, there was a slight decrease in pH values. The only exception to this pattern was observed for the bacteria mix, where statistically significant differences ($p < 0.05$) were observed between the presence and absence of extract (pH values were 1 unit higher in the absence of extract).

It is important to note that the extract has an intrinsically lower pH value than the basal medium used. Thus, even though the accumulation and/or consumption of organic acids may have played a factor in the inhibition of the viable counts, pH values did not appear to be a factor, since the values in the presence of extract did not differ significantly ($p > 0.05$) from those obtained in the absence of extract. This comes in accordance with the fact that acid tolerance is an important characteristic ascribed to lactic acid bacteria, as they need to survive fermentative processes know to lead to low pH values (Z. Mousavi, Mousavi, Razavi, Emam-Djomeh, & Kiani, 2011). Additionally, through statistical analysis, is possible to verify that the only factor relevant in the differences of pH values obtained is time, with the presence of extract not being a relevant factor to justify the discrepancies in the values presented. *Lactobacillus plantarum* is the only exception, with both time and the extract being important factors in the outcome.

Table 3.2 Variation of pH values during the fermentative process in the presence and absence of extract (12 and 24 h). All values correspond to four replicates.

		Extract		**Positive control**	
	h	*Mean*	*Standard deviation*	*Mean*	*Standard deviation*
L. rhamnosus R11	12	4.875	0.01607	5.100	0.01914
	24	4.803	0.007453	5.058	0.02544
L. plantarum 299v	12	6.143	0.02357	6.408	0.09855
	24	6.145	0.01258	6.173	0.01490
B. animalis Bo	12	4.880	0.01414	5.105	0.02986
	24	4.755	0.009574	4.952	0.01213
B. animalis BB12	12	6.167	0.4834	6.133	0.03399
	24	5.520	0.01732	5.930	0.01732
Mix	12	4.925	0.01258	5.817	0.04853
	24	4.950	0.01414	5.863	0.009428

3.2.1.3. Analysis of the production of organic acids and sugar consumption

The main products of bacterial fermentation are short chain fatty acids (SCFA), but mainly acetic, propionic and butyric acid. These SCFA can be used by the colonic mucosa, absorbed or excreted in the feces. As such, the acids present were identified and quantified. The results obtained regarding the sugars consumption/acid production during the fermentative process can be seen in table 3.3, and as can be seen, the acids detected were lactic, ascorbic, lactic, acetic, propionic and butyric, with their concentrations varying with the presence of extract and through sampling time. Sugar consumption displayed no significant differences ($p > 0.05$) between the presence and absence of extract, with only maltose and glucose being identified in small amounts.

Figure 3.8 Example of chromatogram for the detection of organic acids. The sample corresponds to the supernatant resulting from L. rhamnosus *R11 fermentation after 24 h in the presence of extract. Each injection was done in duplicate.*

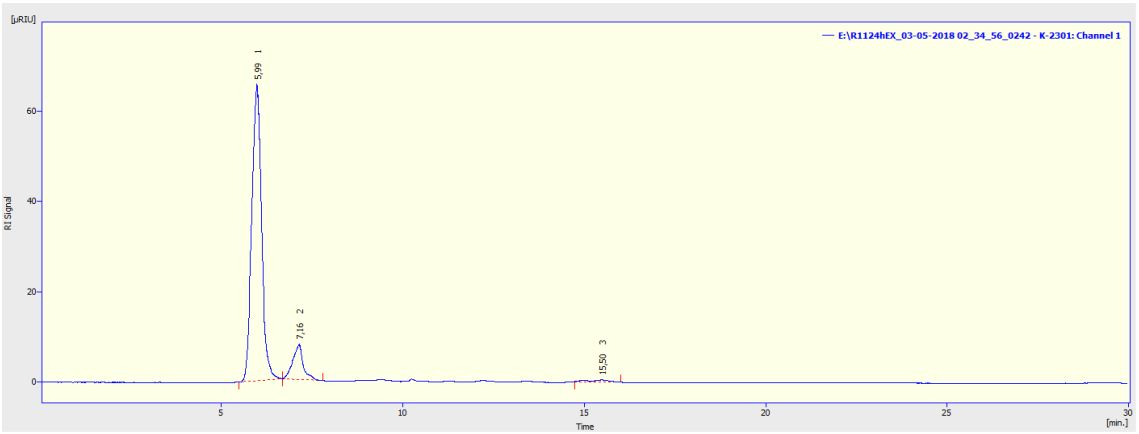

Figure 3.9 Example of chromatogram for the detection of sugars. The sample corresponds to the supernatant resulting from L. rhamnosus *R11 fermentation after 24 h in the presence of extract. Each injection was done in duplicate.*

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Table 3.3 Concentrations obtained for the organic acids detected after the extract's fermentation by the different microorganisms. All values in mg mL-1 correspond to the average of two injections of two replicates.

	12 h					24 h				
	B. animalis BB12	*L. plantarum* 299v	*B. animalis* Bo	*L. rhamnosus* R11	Mix	*B. animalis* BB12	*L. plantarum* 299v	*B. animalis* Bo	*L. rhamnosus* R11	Mix
Positive control										
Ascorbic	0.03±0.0019	0.00*	0.02±0.0021	0.02±0.0015	0.03±0.0024	0.01±0.0017	0.01±0.0031	0.01±0.0064	0.02±0.0054	0.01±0.0023
Malic	0.47±0.041	0.19±0.011	0.65±0.085	0.66±0.032	0.28±0.023	0.25±0.015	nd	0.42±0.021	0.57±0.013	0.28±0.031
Lactic	0.39±0.021	0.24±0.014	0.36±0.013	0.33±0.054	0.35±0.023	0.23±0.065	0.28±0.014	0.29±0.012	0.33±0.024	0.26±0.065
Acetic	0.12±0.0024	0.10±0.0091	0.23±0.012	0.18±0.033	0.20±0.012	0.17±0.0078	0.16±0.023	0.17±0.014	0.21±0.058	0.17±0.0021
Propionic	0.90±0.013	0.41±0.022	0.25±0.044	0.17±0.020	0.82±0.062	0.62±0.025	0.69±0.051	0.48±0.033	0.55±0.026	0.56±0.043
Butyric	0.20±0.035	nd	0.17±0.0028	0.12±0.023	0.14±0.0056	0.15±0.0019	0.20±0.032	0.15±0.027	0.18±0.045	0.19±0.015
Presence of extract										
Ascorbic	0.03±0.0015	0.03±0.0023	0.04±0.0017	0.02±0.0030	0.03±0.0024	0.04±0.0092	0.04±0.0054	nd	nd	0.02±0.0026
Malic	0.51±0.042	0.76±0.017	0.94±0.044	0.63±0.032	0.75±0.085	1.03±0.035	0.81±0.013	0.24±0.026	0.03±0.0049	0.46±0.028
Lactic	0.37±0.023	0.47±0.041	0.69±0.028	0.37±0.022	0.46±0.049	0.43±0.026	0.75±0.035	0.05±0.0028	nd	0.36±0.064
Acetic	0.27±0.034	0.33±0.022	0.45±0.042	nd	0.32±0.038	0.39±0.024	1.25±0.044	0.07±0.0032	nd	0.22±0.038
Propionic	0.75±0.018	0.30±0.035	0.38±0.058	0.24±0.010	0.31±0.057	nd	1.09±0.020	0.12±0.057	0.01±0.0058	0.57±0.084
Butyric	nd	0.17±0.0098	0.21±0.036	0.11±0.0051	0.15±0.022	0.35±0.026	1.02±0.0012	0.05±0.0096	nd	0.11±0.031

nd – not detected; * - detected but not quantifiable

Overall, in the presence of the extract, at both 12 and 24 h, the production of organic acids was higher, with certain acids, such as malic acid and propionic acid, only being detected in the presence of extract. This stands in accordance with the results of Z. E. Mousavi et al. (2013), who reported that the presence of an anthocyanin rich pomegranate juice during fermentation by probiotics, resulted in higher levels of acetic and propionic acid. Propionic acid and acetic acid are metabolized by the liver, and it has been suggested that they have effects on lipid and glucose metabolism. Propionic acid may inhibit hepatic cholesterol synthesis from acetic acid, and the higher the propionic: acetic acid ratio, the more pronounced the effects. Additionally, propionic acid has been reported to ameliorate the symptoms for atherosclerosis (Dibner & Buttin, 2002; Duda-Chodak et al., 2015; Kim et al., 2007).

Osman et al. (2008) studied the anti-inflammatory activity of blueberry with some probiotic bacteria in a rat model and reported that the concentration of SCFAs was higher when blueberry was given with *L. plantarum*, which also concurs with the results obtained. Moreover, acid production was higher when probiotics were exposed to the extract, thus mixing the probiotics with the extract might have led to a metabolic shift, which in turn increased the production of acids, also explaining the difference in pH values presented in table 3.1. This hypothesis finds purchase with the works of Tabasco et al. (2011) who showed that a *L. plantarum* strain metabolized polyphenols and produced gallic acid; of M. Hidalgo et al. (2012), who showed that anthocyanin metabolized by fecal bacteria led to the production of gallic, syringic and *p*-coumaric acids, and of Ávila et al. (2009), who demonstrated that *Lactobacillus* and *Bifidobacterium* metabolism of anthocyanins produced several organic acids, including propionic acid. It is also interesting to note that for *B. animalis* BB12, the production of propionic acid was anticipated 12 h when the extract was involved. This stands in accordance with the findings of Silva et al. (2016), which showed that the interaction between an anthocyanin rich extract and *Bifidobacterium* led to an earlier production of this acid, and with the work of Ávila et al. (2009), who showed that anthocyanin metabolization by a *B. animalis* BB12 strain produced propionic acid.

Butyric acid was produced by all bacteria used in this work, in the presence and absence of extract, and this acid has been associated with potential health benefits, being an important energy substrate for colonic epithelial cells and affecting proliferation and differentiation. The metabolism of butyric acid occurs mainly in the colonic mucosa, but during increased production, butyric acid may be distributed to cells not in direct proximity to the gut causing systemic effects. Moreover, it has been associated with cancer prevention and better gastrointestinal function, since it helps to protect against pathological changes in the gut. This last protective effect is not only attributed to butyric acid, but to propionic and lactic acid as well, which were also detected (Duda-Chodak et al., 2015; German, 1999; Parvez et al., 2006; Roberfroid et al., 2010).

Lactic acid, succinic acid, branched-chain fatty acids and small amounts of valeric, heptanoic and caproic acid may also be formed during fermentation. However, only lactic acid was detected in this work. The physiological effects of these are less well-known; however, the pH is lowered by all SCFA, which may be beneficial per se. *Bifidobacterium* spp. and *Lactobacillus* spp. have been shown to exert a positive effect upon the host's health, partly due to the production of these SCFA. These benefits include antimicrobial activity against pathogenic microorganisms present in the gut, such as *Clostridium* spp, due to the production of short chain fatty acids, and also due to competition for growth substrate

and adhesion sites (Dibner & Buttin, 2002; Dicks & Botes, 2009; Duda-Chodak et al., 2015; Gilliland, 1990; Kim et al., 2007).

When it comes to sugar consumption, the only sugars detected were maltose and glucose. Glucose was only detected at the initial time and at the 12 h mark for both lactobacilli in the presence of extract, and thus, the presence of extract may have led to a reduction in the consumption of glucose. Furthermore, higher amounts of acids were produced from the same amount of sugar. Thus, it is likely other substrate might have been used by the bacteria, and studies such as those by Cheng, Liu, Chen, Zhang, and Zhang (2016) have shown that anthocyanins are possible carbon sources, which might have happened in this case.

3.2.2. Cell viability determination

To gain a better understanding of the fermented extract properties, its impact upon the intestinal cell line Caco-2 was evaluated. This cell line derives from human adenocarcinoma colon cells, and, if grown under the right conditions, is capable of mimicking the intestinal epithelium (for instance, they are an important asset to predict *in vivo* human absorption) and are thus broadly used (I. J. Hidalgo, Raub, & Borchardt, 1989; Yee, 1997).

The most important substrates for the gut microbiota are carbohydrates, including dietary fiber and oligosaccharides. Protein, fat and polyphenols may also reach the colon, but in smaller quantities. The carbohydrates which are not digested or absorbed are available to the colon and therefore, are able to become substrates for fermentation by the gut microflora. As such, the fermented basal medium (with and without blueberry extract) supernatants were put into contact with Caco-2 cells to see how they impacted their metabolism.

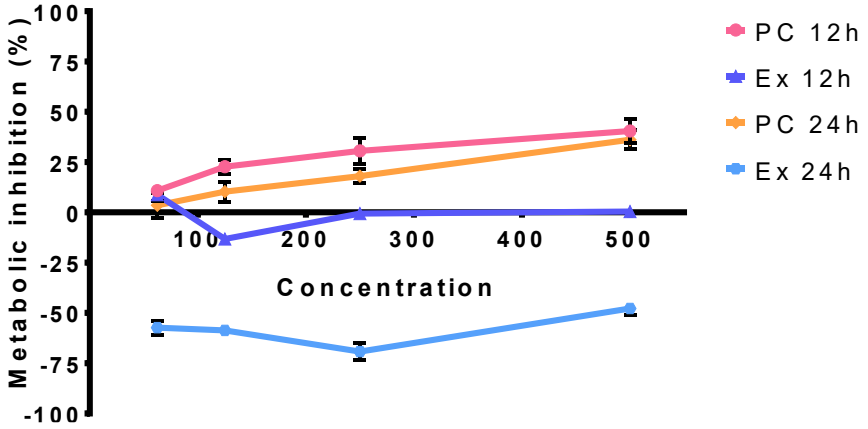

Figure 3.10 Percentage of metabolic inhibition exerted by B. animalis *subsp.* lactis *BO fermentation's supernatant upon Caco-2 cells' metabolism. PC – fermentation in the absence of extract; Ex – fermentation in the presence of extract. All values correspond to the average of five replicates.*

For *B. animalis* subsp. *lactis* Bo supernatants (Figure 3.10), statistical significant differences ($p < 0.05$) could be observed between the supernatants produced in the presence and absence of extract. In fact, the supernatants obtained from the extract fermentation had no deleterious effect upon the metabolic process of Caco-2 cells, while the supernatants obtained without extract showcased metabolic inhibitions which ranged from 10 to almost 50% (without statistically significant differences ($p > 0.05$) being observed). Moreover, the supernatant corresponding to the 24 h point, did not seem to

create any metabolic inhibition in any of the concentrations tested. On the contrary, it seemed to increase the cells' metabolic activity.

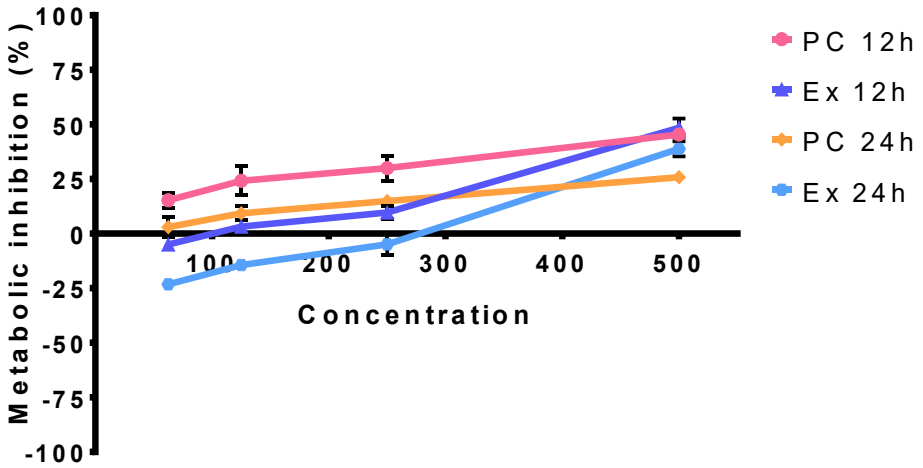

Figure 3.11 Percentage of metabolic inhibition exerted by B. animalis *subsp.* lactis *BB12 fermentation's supernatant upon Caco-2 cells' metabolism. PC – fermentation in the absence of extract; Ex – fermentation in the presence of extract. All values correspond to the average of five replicates.*

When it comes to the results obtained for *B. animalis* subsp. *lactis* BB12 (Figure 3.11), all the supernatants had a similar behavior, with slight metabolic inhibition growing with the increase in supernatant concentration. Additionally, it can be seen that for the two higher concentrations, there was a more accentuated increase in Caco-2 metabolism inhibition for the extract supernatant than for the positive control. For the lowest concentration, both extract supernatants solutions did not exert any negative impact, with the 24 h supernatant showcasing a promotion of the metabolic activity of *ca.* 25%.

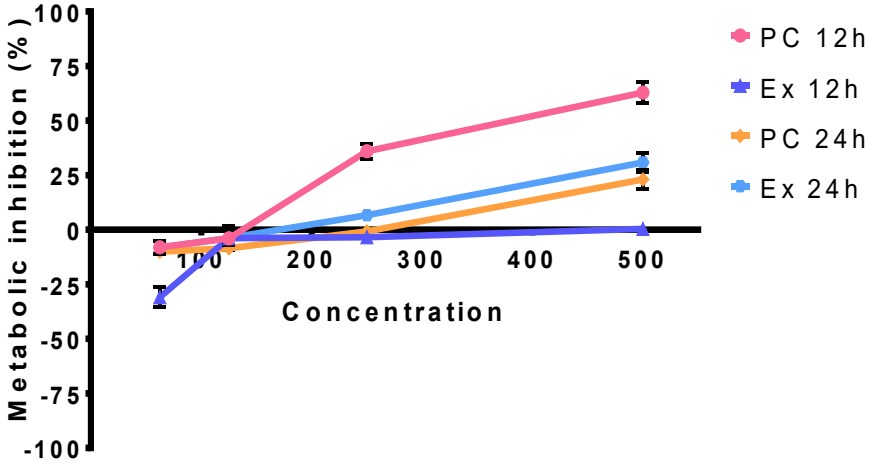

Figure 3.12 Percentage of metabolic inhibition exerted by L. rhamnosus *R11 fermentation's supernatant upon Caco-2 cells' metabolism. PC – fermentation in the absence of extract; Ex – fermentation in the presence of extract. All values correspond to the average of five replicates.*

When it comes to *L. rhamnosus* R11 (Figure 3.12), and unlike what happened with the remaining microorganisms in which the results were similar for both times, amongst the extract and positive control, the fermented extract supernatant and the positive control, both at 24 h, presented similar values of Caco-2 metabolism inhibition for all concentrations used. On the other hand, the extract supernatant at

the 12 h mark did not showcase any inhibition, with the lowest concentration tested appearing to cause metabolism promotion (approximately 30%).

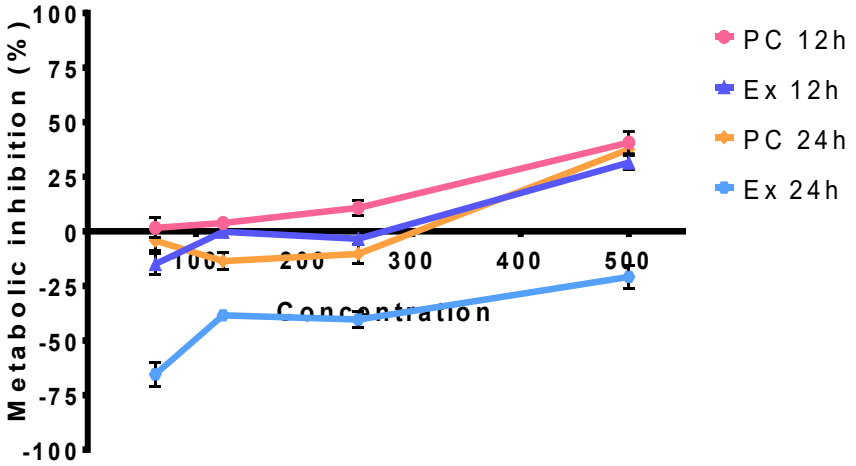

Figure 3.13 Percentage of metabolic inhibition exerted by L. plantarum *299v fermentation's supernatant upon Caco-2 cells' metabolism. PC – fermentation in the absence of extract; Ex – fermentation in the presence of extract. All values correspond to the average of five replicates.*

On the other hand, the other lactobacilli tested, namely *L. plantarum*, portrayed different results as can be seen in Figure 3.13. The supernatants corresponding to the positive control for both times and the extract supernatant for the 12 h point, had no inhibition for the two lowest concentrations and an inhibition of roughly 40% for the two highest concentrations. The 24 h fermented extract supernatant was significantly different ($p < 0.05$) for all concentrations assayed, as a 60% to 20% promotion of the cells' metabolism, from the lowest to the highest concentration respectively, was observed.

In these assays, it was interesting to denote the differences among the bacteria belonging to the same genera, particularly when it comes to the bifidobacteria tested, since they belong to the same species and only differ at the sub-species level. López, Gueimonde, Margolles, and Suárez (2010) reported how different strains of Bifidobacteria belonging to the same species led to strain-specific immune reactions *in vitro*. Such differences are in agreement with our work.

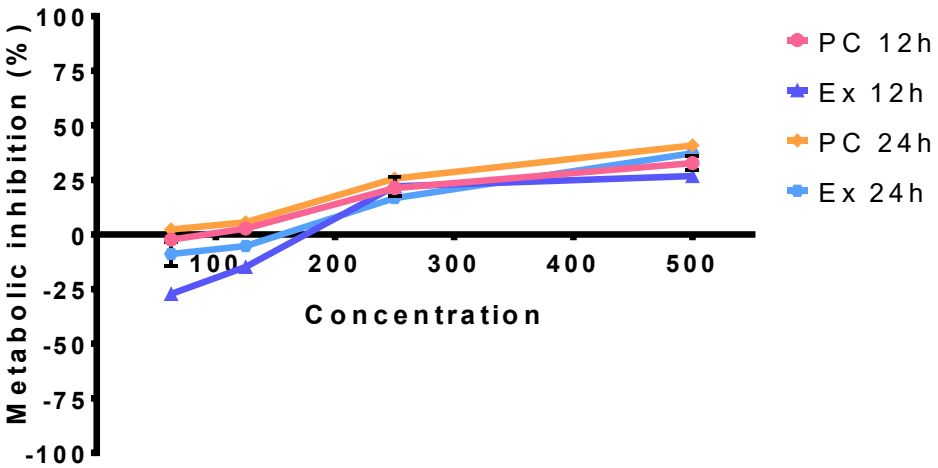

Figure 3.14 Percentage of metabolic inhibition exerted by the mix of bacteria fermentation's supernatant upon Caco-2 cells' metabolism. PC – fermentation in the absence of extract; Ex – fermentation in the presence of extract. All values correspond to the average of five replicates.

Finally, as shown in figure 3.14, the results obtained for all the supernatants tested from the fermentations with the mix of bacteria were very similar for all concentrations, with the same variation pattern being observed, despite the presence or absence of extract. The only value that stands out is that of the 12 h fermented extract supernatant, which showcased some promotion of metabolism (around 25%) and was the only statistically different ($p < 0.05$) value of all tested.

Generally, both positive controls (12 and 24 h) showcased higher inhibition percentages of the cells' metabolism than the end products of the fermentation in the presence of fermented extract supernatant. When in the presence of extract, the bacteria produced higher amounts of organic acids, which would in turn acidify the medium, and thus damage the cells. Blueberries contain high amounts of phenolic compounds, mainly anthocyanins, which may have beneficial effects either on the metabolism or on intestinal function. As such, the lack of inhibition shown by the resulting organic acids in the presence of extract, which include butyric, propionic, lactic and acetic acid, is most likely due to the composition of the extract, which probably exerted a protective effect upon the cells. Manna et al. (1997) reported how two olive oil phenolic compounds exerted a protective effect upon Caco-2 cells when exposed to xanthine oxidase and S. Wang et al. (2016) demonstrated how a phenolic extract reduced the induced production of intracellular Reactive Oxygen Species (iROS) and helped to prevent oxidative damage to Caco-2 cells. Additionally, the positive control (PC) at the 12 h mark appears to showcase a higher inhibition of the cells' metabolism than the same condition after 24 h. As bacteria not only produce these acids but also consume them, it is possible that at 12 h mark the amount of organic acids present was superior than the amount present after 24 h of fermentation. This may also apply when the extract supernatant was present, since 24 h mark exhibited, in general, better compatibility with the Caco-2 cells than the 12 h one.

When considering the results for both bifidobacteria strains, very different results were observed, with the supernatants corresponding to the fermentation of the extract by *B. animalis* Bo showing a positive effect upon the cells (metabolism promotion), while *B. animalis* BB12 did not show any significant differences ($p > 0.05$) between the presence and absence of extract. Mokkala, Laitinen, and Röytiö (2016) reported that the administration of *B. lactis* 420 to a Caco-2 cell model to study intestinal permeability, led to the release of factors during its growth that improved intestinal barrier integrity, as this bacterium in contact with Caco-2 cells increased transepithelial electrical resistance (TEER) values, with this response shown to be dose related. Additionally, Fukuda et al. (2011) demonstrated how *B. longum* subsp. *longum* JCM 1217 was capable of protecting germ- free mice against the Shiga-like toxin produced by *E. coli* O157:H7, probably due to the production of acetate, as well as inhibiting the translocation of the toxin from the gut lumen to the blood. The other strain tested, *B. adolescentis* JCM 1275 was unable to exert the same effect. As such, we can infer that the effect either positive or negative exerted by bifidobacteria is species related, and in the case of this work, subspecies related, since *B. animalis* BB12 and *B. animalis* Bo showcase very different results. Additionally, it is not unheard of the positive association between Bifidobacteria and phenolic compounds, and how these are capable of increasing the amount of these bacteria in the gut, as well as allowing for a positive effect upon health, as mentioned previously in this work.

The *Lactobacillus* tested also had differing results, with *L. plantarum* 299v exerting lower inhibitions upon Caco-2 metabolic activity than *L. rhamnosus* R11. The supernatant of the extract fermented by *L. plantarum* during 24 h had the highest promotion of cells' metabolism of all the supernatants tested. Karczewski et al. (2010) demonstrated that *L. plantarum* induced the activation of Toll-like receptor 2, which contributes to the epithelial integrity and consequently to the gut homeostasis.

Salovaara, Sandberg, and Andlid (2002) reported the influence of organic acids upon iron uptake and concluded that the effect obtained was dose-dependent and that it differed from acid to acid. For instance, propionic and acetic acid increased the absorption of Fe(II), while citric and oxalic acids inhibited it. Anthocyanins have also been shown to stimulate chemopreventive mechanisms, due to effects on signal transduction, apoptosis and epidermal growth factor receptor (Neto, 2007). On the other hand, Forester and Waterhouse (2010) demonstrated the inhibitory effect of an anthocyanin extract upon Caco-2 proliferation, with gallic acid being responsible for some inhibition. However, syringic acid and protocatechuic acid had no apparent toxicity upon the cell line tested and vanillic acid seemed to increase cell proliferation at the lowest concentration.

As shown before, the fermentation led to the production of several organic acids, including butyric and propionic acid. As such, it stands to reason that while the inhibition observed for the positive controls was probably due to the acids, as their presence also caused a lowering of the normal pH of the cells' surroundings, not all acids seem to have a negative effect upon the cells, and the anthocyanins present in the extract may have protected the membrane of the cells of being damaged. On the other hand, Yi, Fischer, Krewer, and Akoh (2005) reported that both Caco-2 and HT-29 cell lines' growth and proliferation was significantly inhibited when exposed to polyphenols at a concentration of approximately 1 mg mL^{-1}. However, these were anthocyanins purified fractions, and did not possess the remaining compounds present in the fermented extract tested, which may help explain these differences in results.

3.2.3. Impact upon probiotic's adhesion to intestinal cells

In order to evaluate the impact of the extract upon the adhesion of probiotic bacteria to the intestinal epithelium, the adhesion of the bacteria to Caco-2 and HT29-MTX cell lines was analyzed, without any compounds (control), with a known prebiotic (FOS) and with the anthocyanin rich extract.

To the best of our knowledge, there has been no study where the contact between cells and probiotics had such a short-term contact. The purpose of such short-spaced contact arises from this experiment working as an initial screening, allowing to see whether or not such short contact would be able to replicate data for longer contact.

When comparing the values of the inoculum with the total viable counts obtained, there was a statistically significant decrease ($p < 0.05$) for all microorganisms tested. However, adhesion values for all microorganisms ranged from at least 60% to 95%, which are considered high adhesion values. This stands in line with reports by Parkar, Stevenson, and Skinner (2008), in which the total bacteria adhering to a Caco-2 monolayer, including a lactic acid bacteria (*L. rhamnosus*), was significantly inferior to the initial bacterial concentration present in the inoculum.

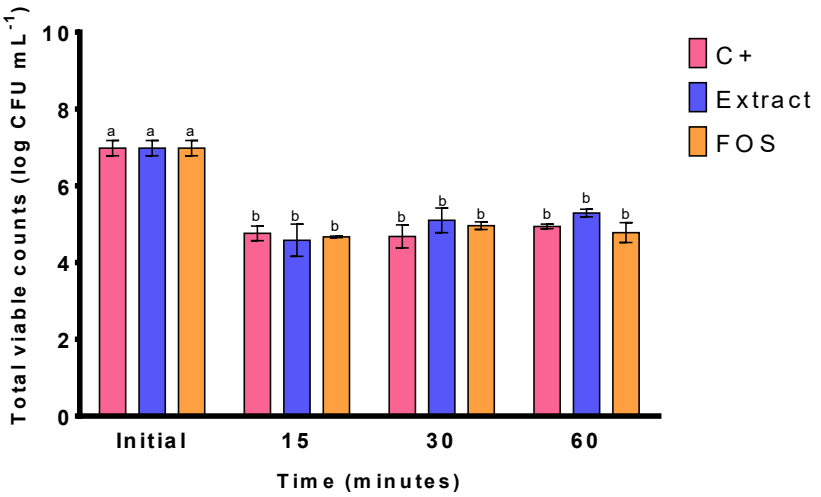

Figure 3.15 Total viable counts resulting of the adhesion to Caco-2 cells by B. animalis *subsp.* lactis *BB12 for the short adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

Bifidobacterium animalis BB12, in the Caco-2 short-term assay (Figure 3.15), registered lower adhesion values than those attained for the long-term assay. As can be seen in Tables 3.4 and 3.5, while the first ones ranged from 64% to 86%, the latter ranged from 66% to 95%. As such, a shorter contact between the bacteria and the cells leads to smaller adhesion values, as reported by Persat et al. (2015) and Dufrêne (2015), which showed that as time progressed, bacterial adhesion forces seemed to increase.

Table 3.4 Percentage values for B. animalis *subsp.* lactis *BB12 adhesion to Caco-2 cells for the short-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	15 min	*30 min*	*60 min*
Positive control	64%	67%	71%
Extract	76%	78%	86%
FOS	67%	71%	75%

It is interesting to denote that when the cells were in the presence of extract during a larger period of time (Figure 3.16) (two and three hours), the adhesion values were significantly higher ($p < 0.05$) than the ones obtained for the control and FOS. In fact, for *B. animalis* BB12 at the two hours mark, the presence of FOS led to values which were statistically significantly inferior ($p < 0.05$) than those obtained for the extract, but similar to those of the control. At the three hours mark however, the presence of FOS had statistically significantly ($p < 0.05$) higher values than those of the control (which stands in accordance with reports by Bezkorovainy (2001), who reported that FOS promoted the adhesion of probiotic bacteria), but still significantly lower ($p < 0.05$) values than the ones obtained by the presence of extract. Moreover, after two and three hours, the adhesion percentages obtained were around 95% in the presence of extract, which is an extremely high value and accounts for an average difference of 17% when in comparison with the presence of FOS. These results also showed that the studied microorganisms, although capable of adhering on their own to the intestinal epithelium, may

adhere more efficiently to the intestinal epithelium in the presence of a prebiotic, as previously shown by Marco Candela et al. (2005).

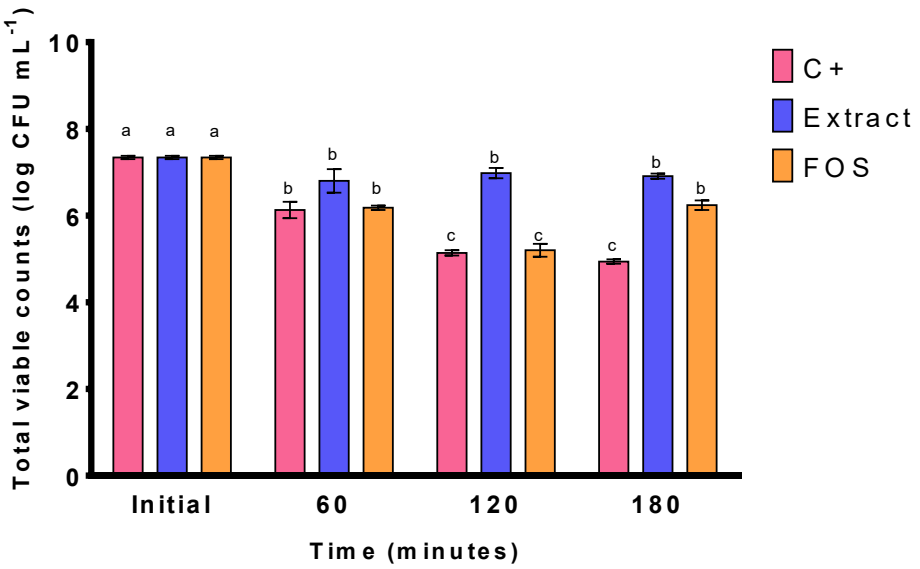

Figure 3.16 Total viable counts resulting of the adhesion to Caco-2 cells by B. animalis *subsp.* lactis *BB12 for the long adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

Riedel, Foata, Goldstein, Blum, and Eikmanns (2006) reported how several bifidobacterial strains, including *B. lactis* NCC 362, *B. breve* MB226 and *B. longum* NCC 490, showed strain-dependent adhesion to differentiated Caco-2 cells, with some strains showcasing high adhesion, while others had a very poor adhesion to the cells. The same was noted by Crociani, Grill, Huppert, and Ballongue (1995), who reported how different species of Bifidobacteria presented heterogeneous values of adhesion to Caco-2 cells, even between strains of the same species. However, in none of these works were phenolic compounds involved. As mentioned throughout this work, Bifidobacteria associated with phenolic compounds have been linked with increased health benefits and thus, the presence of the anthocyanin rich extract may help these bacteria adhere to the intestinal mucosa, which would explain the results obtained in this work (O'Callaghan & van Sinderen, 2016; Vendrame et al., 2011).

Table 3.5 Percentage values for B. animalis *subsp.* lactis *BB12 adhesion to Caco-2 cells for the long-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	60 min	*120 min*	*180 min*
Positive control	66%	71%	67%
Extract	90%	93%	95%
FOS	74%	70%	85%

When it comes to the adhesion of *B. animalis* BB12 to a HT29-MTX cell line, there were no significant differences ($p > 0.05$) between the control and the presence of extract for most of the conditions assayed (Figure 3.17 and 3.18). The HT29-MTX cell line has as a distinguishing characteristic the production of mucus. As such, it simulates the intestinal mucus, which is an influential factor in the adhesion of microorganisms (beneficial or not) since it plays a protective role on the intestinal cells.

Mucin glycoproteins polymerize, allowing certain microbes to adhere, including lactobacilli and bifidobacteria species. If the cells are washed before putting them in contact with the testing solutions, there will be a removal of the previously produced mucus. As such, a proper handling of the cell line is important, in order to replicate correctly what may happen in the gut (Gagnon, Berner, Chervet, Chassard, & Lacroix, 2013; Van Tassell & Miller, 2011).

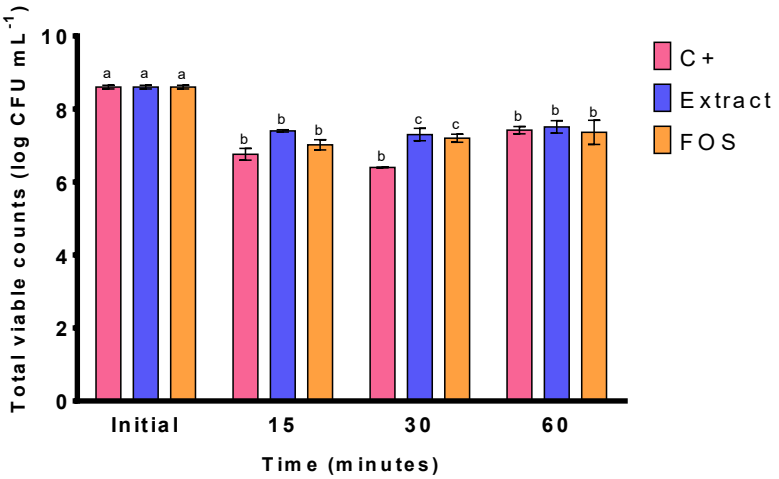

Figure 3.17 Total viable counts resulting of the adhesion to HT29-MTX cells by B. animalis *subsp.* lactis *BB12 for the short adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

For the short time adhesion to HT29-MTX cells (Table 3.6), while the presence of extract and FOS resulted in very similar adhesion percentages, with no statistically significant ($p > 0.05$) differences being observed, the values obtained for the control, up to the 30 minutes mark, were significantly lower ($p < 0.05$). Overall, adhesion values fluctuated between 74% to 87% of relative adhesion, with the highest value corresponding to the 60 minutes mark in the presence of extract. The short space between the testing times may help explain the lack of differences among the times tested.

Table 3.6 Percentage values for B. animalis *subsp.* lactis *BB12 adhesion to HT29-MTX cells for the short-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	15 min	*30 min*	*60 min*
Positive control	79%	74%	86%
Extract	86%	85%	87%
FOS	82%	84%	86%

When it comes to the long adhesion assay (Figure 3.18), there were differences registered among the three times tested. The absence of both extract and FOS led to lower adhesion values, in all sampling times, with these values, being significantly different ($p < 0.05$) than the ones obtained in the remaining conditions at the one and three hours' mark. Taking into account that FOS is considered to be a prebiotic, and thus may contribute to the adhesion of probiotic bacteria to the intestinal epithelium, it is interesting to denote that the presence of extract led to similar results in most of the times tested, showcasing a possible prebiotic effect, although more studies are needed to validate this hypothesis.

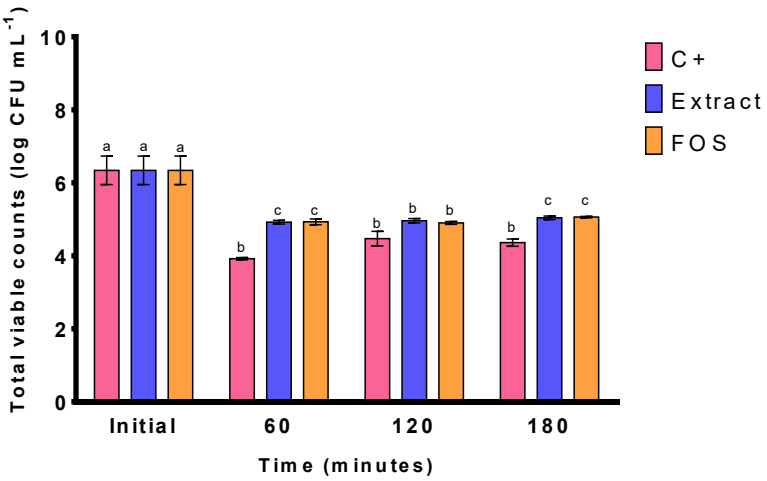

Figure 3.18 Viable counts resulting of the adhesion to HT29-MTX cells by B. animalis *subsp.* lactis *BB12 for the long adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

As can be seen on Table 3.7, the long-term adhesion values ranged from 62% to 80%, with the lowest values being always registered for the positive control. As mentioned previously, it is more challenging for probiotic bacteria to adhere without prebiotics (M Candela et al., 2008). These results are also in accordance with those obtained in this work for the same bacteria's adhesion to Caco-2 cells, with both the extract and FOS propelling higher adhesion values during the long adhesion assay.

Table 3.7 Percentage values for B. animalis *subsp.* lactis *BB12 adhesion to HT29-MTX cells for the long-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	60 min	*120 min*	*180 min*
Positive control	62%	71%	69%
Extract	78%	78%	80%
FOS	78%	77%	80%

Additionally, comparing the results for both cell lines, it can be seen that the adhesion values ranged among the same percentages, even though for the Caco-2 cell line a higher relative adhesion percentage was obtained in the presence of extract (with percentages reaching 95% of relative adhesion). The lower values obtained for the HT29-MTX cell line might be explained by the production of mucus, which could interfere with the bacterial adhesion (Gagnon et al., 2013; Van Tassell & Miller, 2011). Moreover, *Bifidobacterium*'s adhesion to Caco-2 in the presence of FOS produced lower adhesion values than those of the presence of extract (and was statistically significantly ($p < 0.05$) lower at the two hours mark), which did not happen with the HT29-MTX cell line, where values for the presence of extract and of FOS were similar. It has been reported that some probiotics' growth may not be influenced by certain prebiotics (Adebola, Corcoran, & Morgan, 2014). Nevertheless, since FOS presented the desired effect in the HT29-MTX cell line, this does not seem to be the case. As such, more studies are necessary to further evaluate these adhesion processes and ascertain to the differences between adhesion mechanisms.

Besides *B. animalis* BB12, another bifidobacteria (*B. animalis* subsp. *lactis* Bo) was used in this work under the same conditions but during a longer period of time, namely four hours. Even though these bacteria only differ in the sub-strain, the adhesion values obtained were significantly different ($p < 0.05$), with *B. animalis* Bo expressing higher relative adhesion values (Table 3.8). The differences registered for the different strains of the same bacterial species was previously reported in this work and also in the works of Silva et al. (2016) and López et al. (2010). Since these were distinct strains and only the HT29-MTX cell line was tested, the differences encountered may also be due to the presence of mucus, with *B. animalis* Bo having a better adaption to it than *B. animalis* BB12. However, as this was only a preliminary assay, further experiments, mimicking the work performed for *B. animalis* BB12 for both cells lines, are still required to gain a better understanding of the adhesion processes inherent to two bacterial strains.

Table 3.8 Relative percentage values for B. animalis *subsp*. lactis *BB12 and* B. animalis *subsp.* lactis *Bo adhesion to HT29-MTX cells for four hours. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	Positive control	*Extract*	*FOS*
B. animalis subsp. *lactis* Bo	81%	89%	89%
B. animalis subsp. *lactis* BB12	76%	74%	73%

Besides *Bifidobacterium*, *Lactobacillus* have been associated with potential health benefits and are usually a part of the normal gut microbiota. The *Lactobacillus* used in this experiment was *L. rhamnosus* R11, a known probiotic. Although this experiment works as a model for an adhesion screening, it was important to test a *Lactobacillus* in addition to the bifidobacteria already tested, in order to stablish a comparison between the two and to assess whether the *Lactobacillus* would also be able to adhere to the cell lines used. Furthermore, *Lactobacillus* have a higher tolerance to oxygen than bifidobacteria, and have been reported to express high adhesion values in the presence of intestinal mucus (Van Tassell & Miller, 2011).

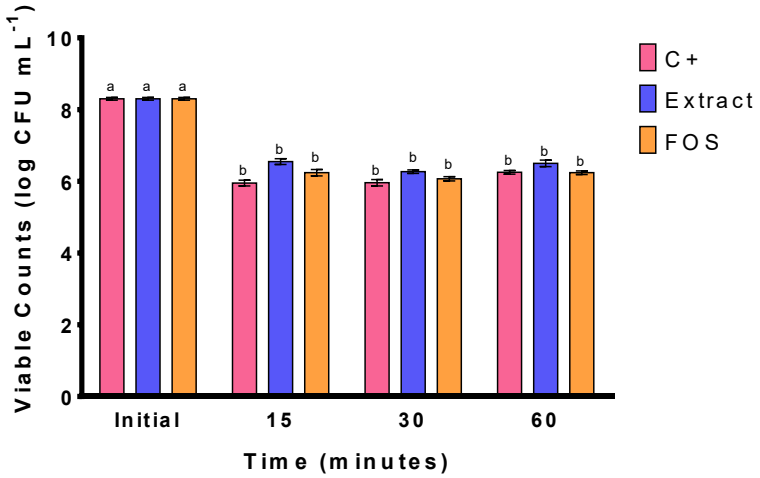

Figure 3.19 Total viable counts resulting of the adhesion to Caco-2 cells by L. rhamnosus *R11 for the short adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

When it comes to the short adhesion (Figure 3.19), no significant differences ($p > 0.05$) were found among the compounds tested and the control, nor among the times tested. As was the case with *B. animalis* BB12, this lack of differences may have been due to the short intervals between the tested times. As can be seen in Table 3.9, the relative adhesion percentage values for the short-term assay are in line with the values obtained for the total viable counts, with no statistically significant ($p > 0.05$) differences being registered, with the values ranging from 72% to 79%. Interestingly enough, despite the lack of differences observed, the highest relative adhesion value was, once again, obtained in the presence of extract.

Table 3.9 Percentage values for L. rhamnosus *R11 adhesion to Caco-2 cells for the short-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	15 min	30 min	60 min
Positive control	72%	72%	75%
Extract	79%	76%	78%
FOS	75%	73%	75%

For the long-term adhesion assay (Figure 3.20) to Caco-2 cells, there was a consistent increase in bacterial viable counts throughout the assay in the presence of extract, resulting in adhesion values that by the end of the assay were significantly higher ($p < 0.05$) than those registered for FOS and the control. The adhesion values in the presence of extract were significantly different from the control ($p < 0.05$), but not from the presence of FOS ($p > 0.05$), at the one and two hours mark. Additionally, relative adhesion percentage values, as seen on Table 3.10, ranged from 56% to 81%, with the lowest values corresponding to the positive control. In the presence of the same cell line and the extract, the bifidobacteria tested reached relative adhesion percentage values of 95%, significantly higher than those obtained for *L. rhamnosus* R11.

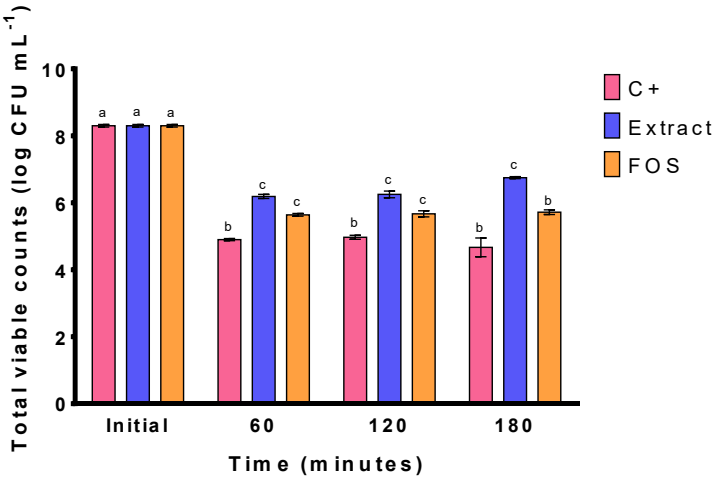

Figure 3.20 Total viable counts resulting of the adhesion to Caco-2 cells by L. rhamnosus *R11 for the long adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

The fact that *Lactobacillus* can adhere to Caco-2 stands in accordance with the findings of Hirano et al. (2003), who showed that *L. rhamnosus* demonstrated a high potential to adhere to a human

colon epithelial cell line, C2BBe1, which is a Caco-2 clone cell line. An interaction between this bacterium and the cell line was suggested, which may result in the modulation of intracellular events. Furthermore, our results also stand in line with those by Duda-Chodak et al. (2015), who showed that naringenin and phloridzin, both phenolic compounds, enhanced the adherence of *L. rhamnosus* to Caco-2 cells, a similar effect as the one presented by the extract used in this work. Nevertheless, this report also indicates that not all phenolic compounds have the same effect upon probiotic bacteria adhesion.

Table 3.10 Percentage values for L. rhamnosus *R11 adhesion to Caco-2 cells for the long-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	60 min	*120 min*	*180 min*
Positive control	59%	60%	56%
Extract	75%	75%	81%
FOS	66%	68%	67%

For the HT29-MTX cell line, as was the case with the Caco-2 cell line, in the short-term adhesion (Figure 3.21), for the first two times assayed (15 and 30 minutes), there were no statistically significant ($p > 0.05$) differences found, most likely due to the proximity of the times tested. The positive control at 60 minutes was significantly different ($p < 0.05$) than the presence of extract and FOS, probably due to the presence of these compounds contributing to the bacterial adhesion.

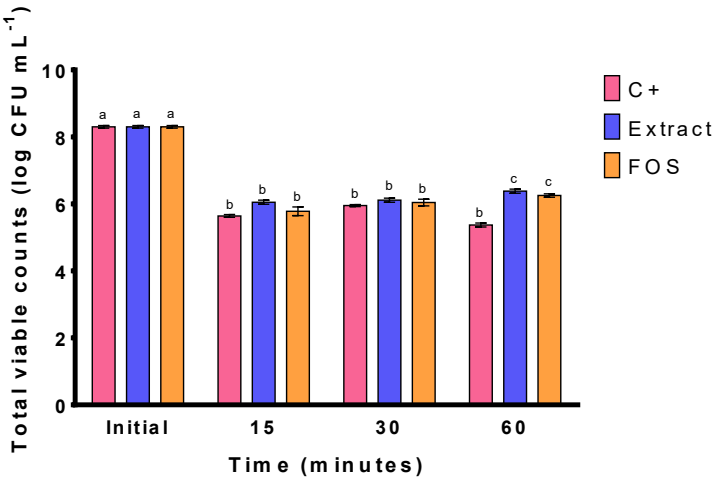

Figure 3.21 Total viable counts resulting of the adhesion to HT29-MTX cells by L. rhamnosus *R11 for the short adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

The adhesion percentages for the short contact (Table 3.11) ranged between 68% to 77%, with the highest percentage corresponding once again to the presence of extract, while the lowest referred to the positive control. The only statistically significant difference was obtained at the 60 minutes mark for the positive control which presented relative adhesion values which were significantly ($p < 0.05$) lower than the ones registered in the remaining conditions.

Table 3.11 Percentage values for L. rhamnosus *R11 adhesion to HT29-MTX cells for the short-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	15 min	*30 min*	*60 min*
Positive control	65%	68%	72%
Extract	73%	74%	77%
FOS	70%	73%	75%

When it comes to the long-term adhesion (Figure 3.22), no significant differences ($p < 0.05$) were found among the times tested, except after 180 minutes in which the presence of extract and FOS led to significantly ($p > 0.05$) higher relative adhesion values than the positive control. The maximum percentage attained was in the presence of extract (85%) and after the 180 minutes of contact. (Table 3.12). In fact, the highest relative adhesion values encountered for both cell lines were after 180 minutes, which stands in accordance with previous reports of how bacterial adhesion forces are stronger after longer exposure times, as previously mentioned (Dufrêne, 2015).

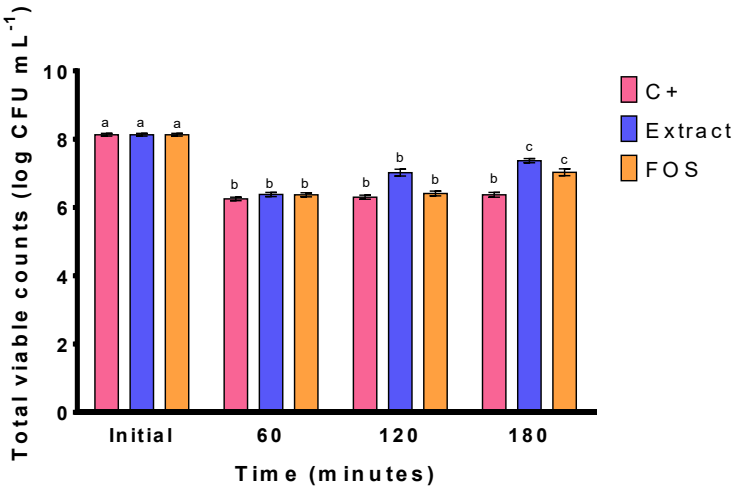

Figure 3.22 Total viable counts resulting of the adhesion to HT29-MTX cells by L. rhamnosus *R11 for the long-term adhesion assay. C+ - Positive control, adhesion in the absence of any compound. Extract – adhesion in the presence of the blueberry extract. FOS – adhesion in the presence of fructooligosaccharide. All values in log CFU mL^{-1} correspond to the average of five replicates. Different letters represent the statistically significant differences ($p < 0.05$) found between samples for each sampling time.*

Previously, Duda-Chodak et al. (2015) reported that flavonols can modulate the gut microbiota through the adhesion of bacteria to the intestinal epithelium, and how this adhesion is greatly influenced by different factors, among which are the compounds being used, the bacterial strains and the level of differentiation of the intestinal cells. The results showed how some flavanols inhibited adhesion of the different *Lactobacillus* tested to both Caco-2 and HT-29, while procyanidins increased *Lactobacillus casei*'s adhesion to HT-29 cells and epigallocatechin increased *L. casei* and *L. acidophilus* La-5's adhesion to Caco-2 cells. As such, the promotion of adhesion of *Lactobacillus* to Caco-2 in the presence of phenolic compounds stands in line with some of the findings of this work, since not all phenolics seem to exert the same effect.

Table 3.12 Percentage values for L. rhamnosus *R11 adhesion to HT29-MTX cells for the long-term adhesion assay. All values correspond to the average of five replicates.*

	Relative adhesion (%)		
	60 min	*120 min*	*180 min*
Positive control	70%	71%	74%
Extract	73%	80%	85%
FOS	74%	75%	78%

Nevertheless, our results do not stand in line with those by Parkar et al. (2008), in which the adhesion of a *L. rhamnosus* was reduced by the presence of phenolics. In the results obtained in this work, the bacteria alone were never capable of having statistically significant ($p > 0.05$) higher relative adhesion values than those registered in the presence of FOS or extract. Additionally, Gagnon et al. (2013) suggested HT29-MTX cells to be more accurate to study bacterial interactions with the intestinal epithelium due to its mucus layer formation, which the authors report as being more physiologically relevant. However, our results do not showcase significant differences ($p > 0.05$) between the adhesion values obtained for *L. rhamnosus* R11, when in the presence of Caco-2 cell line and HT29-MTX cell line.

Overall, the adhesion values for *L. rhamnosus* were slightly lower than those of the bifidobacteria tested for both cell lines (56% to 81% vs 62% to 95%). This does not stand in line with the works of M Candela et al. (2008), which reported that in the adhesion of different probiotic strains to a Caco-2 monolayer (including *Bifidobacterium longum*, *B. animalis* subsp *lactis*, *L acidophilus* and *L. plantarum)*, while the lowest adhesion values were obtained for *L. acidophilus*, the overall highest adhesion values were obtained for *L. plantarum.* However, these differences are most likely due to the variances in the species of *Lactobacillus* tested, as well as in the species of *Bifidobacterium* as shown by several studies (M Candela et al., 2008; Crociani et al., 1995; Laparra & Sanz, 2009; Tuomola & Salminen, 1998).

Similarly, the results obtained by Silva et. al (2016) do not support our data. In this work, the authors reported that a blueberry extract led to lower adhesion percentages of all probiotics tested on an *in vitro* probiotic mucin adhesion system, although it did not cause any significant differences ($p > 0.05$) for neither *B. animalis* BB12 nor *L. plantarum.* This system is an analogue system to the one used in this work, since mucin is an important component of mucus produced by intestinal cells. In our work, the presence of the anthocyanin rich extract led to higher relative adhesion values. Additionally, the association of a probiotic bacteria with a prebiotic has been vastly studied, with this relation being dubbed as synbiotic and acknowledged as playing a critical role in the modulation of the gut microbiota. For instance, *L. rhamnosus* GG and *B. animalis* BB12, in association with a specific oligofructose enriched inulin, led to an increase in the total counts of *Bifidobacterium* and *Lactobacillus*, and to a reduction in the numbers of *Clostridium perfringens* in the gut (Davis & Milner, 2009; Gibson & Roberfroid, 1995). As such, the bifidobacteria and the lactobacilli in association with the extract used in this work, may have a symbiotic function, with the extract propelling higher adhesion values than FOS or the control.

When regarding the mechanisms through which the bacteria are capable of adhering have yet to be totally unveiled, but there are more works demonstrating lactobacilli mechanisms to adhere to intestinal cell lines than bifidobacteria. Greene and Klaenhammer (1994) reported that Lactobacillus most likely were capable of adhering to Caco-2 cells through mechanisms that involve combinations of carbohydrate and protein factors on the bacterial cell surface, while Adlerberth et al. (1996) demonstrated how *Lactobacillus plantarum*'s ability to adhere to HT-29 cells was due to the protein structures on the bacterial cell surface and a mannose specific adhesin was identified. Additionally, Buntin, de Vos, and Hongpattarakere (2017) reported how, although mannose specific adhesion seems to be involved in the *Lactobacillus* adhesion to the intestinal epithelium, the ability to adhere to mucin seems to be strain dependent. These reports support our data in which they demonstrate *Lactobacillus* ability to adhere to cell lines resembling the intestinal epithelium.

All of the bacteria tested have been shown to contribute to a healthier gut and its impact upon intestinal cell lines has been studied. For instance, R. C. Anderson, Cookson, McNabb, Kelly, and Roy (2010) reported that *L plantarum* DSM 2648, *B. animalis* subsp *lactis* BB12 and *L. rhamnosus* HN001 showed an increase in TEER for Caco-2 cells, enhancing tight junction integrity. Moreover, Karczewski et al. (2010) reported how *Lactobacillus plantarum* had a protective effect upon the intestinal barrier through the production of certain proteins. Furthermore, the association of probiotic bacteria with different compounds, not exclusively phenolic compounds, is not unheard of, with certain works showcasing potentially beneficial results for human health. For instance, Mokkala et al. (2016) demonstrated how *Bifidobacterium lactis* 420 in association with fish oil helped to increase intestinal epithelial integrity in Caco-2 cells.

The mechanism through which phenolic compounds, including anthocyanins, are capable of influencing bacterial adhesion to the intestinal epithelium is not yell fully comprehended and is also outside of the scope of this work. However, it has been demonstrated the capacity of certain phenolic compounds or plant derived extracts to contribute to the adhesion and/or proliferation of probiotic bacteria. As such, increasing the adhesion of probiotic bacteria may be helpful to restore homeostasis of the gut microbiota, contributing to ameliorate symptoms of chronic diseases (i.e. inflammatory bowel disease), since the presence of these bacteria in association with phenolics has been associated with enhanced immunological defenses, antitumoral properties (i.e. prevention of colon cancer), as well as a protective effect of the gut mucosa against potentially pathogenic bacteria (Cardona et al., 2013; Hollman, 2001; Kechagia et al., 2013; Plummer et al., 2005).

4. Conclusions

Overall, this work allowed us to shed further light into blueberry anthocyanins' interaction with probiotic microorganisms and to validate the produced extract safety and capacity to modulate microbial adhesion to gastrointestinal tract cellular models.

First and foremost, the blueberry extract did not have a significant impact upon probiotic's growth and fermentation. In fact, while a slight decrease in total viable counts for *Lactobacillus* could be observed, the overall organic acids' production was significantly increased. Among these acids butyric and propionic acids were found, acids which have been shown to be beneficial for human health. In general, the anthocyanin rich extract was unable to significantly inhibit the growth, and fermentation, of the probiotic bacteria tested and it appeared to play a role in promoting their metabolism.

When it came to the impact of the fermentation's supernatants upon Caco-2 cells, results demonstrated that, overall, the extract's supernatant resulted in lower cellular metabolism inhibitions and in some cases, it appeared to stimulate it. Additionally, when comparing the results observed for the supernatants resulting from the fermentation in the presence of extract with the correspondent controls (fermentations without extract), it can be seen that the presence of extract led to lower metabolic inhibition percentages. *Bifidobacterium animalis* subsp. *lactis* Bo presented the most interesting activity, having the lowest metabolic inhibition values while, in fact, appearing to induce an increase of the overall metabolic rate.

Lastly, the results obtained for the cellular adhesion assays, both to Caco-2 and HT29-MTX, showed that the extract's presence helped modulate the selected microorganisms' adhesion to the cell lines. In fact, relative adhesion percentages registered where higher for all studied microorganisms in the presence of the extract for both cell lines, with particular relevance being given to the significantly higher relative adhesion values attained for bifidobacteria adhesion to Caco-2 and HT29-MTX, in all tested conditions. This is particularly important as *Bifidobacterium* are known probiotics associated with positive benefits for human health, and these results fully showcase its synergic relationship with phenolic compounds and anthocyanins, in a simulated gastrointestinal tract adhesion scenario.

In conclusion, this work allowed us to gain a better understanding of blueberry's anthocyanins interaction with probiotic microorganisms in simulated gut conditions, and to show that their role is multifaceted as they are a food source for probiotic bacteria. Additionally, their metabolites pose no harm to intestinal cells and they may be able to actively modulate microbiota constitution, as they are capable of potentiating probiotic adhesion, and bifidobacteria in particular, to intestinal cells.

5. Future Work

The study of the extract's impact upon probiotic adhesion hereby described was an exploratory approach to the topic and therefore still needs to be validated using other microorganisms, longer adhesion times and more complex cell models. Furthermore, considering the results obtained, and taking into account the elsewhere described antioxidant activity of phenolic compounds, it would be interesting to explore if the extract possesses any immunomodulatory/anti-inflammatory activity upon intestinal cells as well as to ascertain if the extract, fermented or not, could prevent the production/accumulation of intracellular ROS. From a different perspective, it would also be interesting to gather further insight into the overall metabolic impact of the extract upon probiotics, namely through the use of a metabolomic/genomic approaches.

Another interesting approach would be to further characterize the extracts' (fermented or not) cytotoxicity employing different approaches, namely through the analysis of cytolysis (e.g. measurement of lactate dehydrogenase) and DNA damage (e.g. through fluorescent labelling coupled with flow cytometry). This would allow us to draw a parallel between the capacities of the extract before and after fermentation by probiotic bacteria.

Furthermore, since this work focused solely on single layer and monocultures of the tested cell lines, it could be interesting to explore the impact of the extract upon differentiated and co-culture models, in terms of metabolism, cytotoxicity and probiotic adhesion.

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