CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

STUDY OF *VACCINIUM CORYMBOSUM* BLUEBERRIES:

CHARACTERIZATION OF EXTRACTS

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology – with specialization in Microbiology

By

Sara Nunes da Costa e Silva

December 2016

CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

STUDY OF *VACCINIUM CORYMBOSUM* BLUEBERRIES:

CHARACTERIZATION OF EXTRACTS

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology - with specialization in Microbiology

By

Sara Nunes da Costa e Silva

Under the supervision of Prof. Maria Manuela Estevez Pintado

Under the co-supervision of Prof. Rui Manuel Santos Costa de Morais and Prof. Maria da Conceição Calhau

December 2016

This thesis is dedicated to the memory of my father, Rui da Costa e Silva (1954-2010).

Till this day I hear your voice saying to be the best I can be. May this work do justice to your image of me.

"Try not. Do. Or do not. There is no try.

Master Yoda *in* Star Wars Episode V

"When you're curious, you find lots of interesting things to do"

Walt Disney

Resumo

Nos últimos anos, a área de produção de mirtilo em Portugal tem vindo a aumentar, pelo que se torna premente encontrar mercados alternativos para escoar o fruto. Considerando a preferência atual dos consumidores por alimentos que, para além de serem saudáveis, podem ter efeitos benéficos para a saúde e que o mirtilo é percebido pelos mesmos como reunindo ambos os requisitos, a utilização deste fruto para produzir extratos para incorporação em produtos pode ser algo vantajoso, particularmente se considerarmos as indústrias alimentar, cosmética e, possivelmente, farmacêutica. Tendo em conta estes argumentos, o principal objetivo deste projeto foi produzir e caracterizar (química e biologicamente) um extrato de mirtilo, rico em antocianinas, tendo em vista a sua potencial aplicação nas indústrias alimentar, cosmética ou farmacêutica.

A primeira etapa desta tese visou selecionar qual a cultivar de mirtilo mais rica em compostos fenólicos e antocianinas, bem como avaliar se a seleção da fase de maturação permitiria obter mirtilos mais ricos. Das quatro cultivares estudadas, os mirtilos Goldtraube foram identificados como possuindo os teores mais elevados (a mais variados) de antocianinas e compostos fenólicos, particularmente em fases mais tardias de maturação. Após a seleção dos mirtilos (Goldtraube numa fase mais tardia de maturação), o processo de extração foi otimizado tendo sido definidas as seguintes condições: extração com etanol acidificado (0.01% HCl) utilizando uma mistura de 10 g de mirtilo para 100 mL de solvente, incubados durante 1 h a 40 ºC e com um tratamento de ultrassons (35 kHz, 15 min).

A segunda etapa deste trabalho, visou caracterizar a atividade antioxidante do extrato produzido através da análise da capacidade da mesma para proteger o ADN de danos oxidativos. Da análise dos resultados verificou-se que o extrato foi eficaz na proteção do ADN contra dois sistemas de degradação distintos (H_2O_2 e $H_2O_2/FeCl_3$) numa gama de concentrações que varia entre 160 e 200 µg mL^{-1}, respetivamente.

Paralelamente, avaliou-se o efeito da interação do extrato com microrganismos para avaliar o seu efeito sobre microrganismos nosocomiais. Dos oito patogénicos avaliados, o extrato (a 1000 µg mL^{-1}) apenas foi capaz de inibir significativamente o crescimento de 3 deles (MSSA, MRSA e uma estirpe de *E. coli*). Todavia, quando se avaliaram os resultados relativos à atividade antiadesiva e antibiofilme, o extrato foi capaz de inibir ambos os processos em todos os microrganismos avaliados. Numa tentativa de compreender o papel

dos diferentes compostos na atividade antimicrobiana, foram desenvolvidas duas linhas investigativas: i) avaliar o efeito de ácidos fenólicos presentes no mirtilo, tendo-se verificado que estes apenas foram capazes de inibir a formação de biofilme por *Staphylococcus* (e não a de *E. coli*). ii) simular a composição do extrato por forma a comparar a inibição induzida pela antocianina mais abundante sobre *Staphylococcus* (os microrganismos mais suscetíveis à ação do extrato). Os resultados demonstraram que, embora os compostos isolados possuam alguma atividade esta é inferior à verificada para o extrato, o que demonstra a importância da sinergia entre os diferentes compostos.

Como o extrato exibiu uma atividade antimicrobiana interessante sobre agentes nosocomiais, surgiu a questão se seria capaz de inibir contaminantes alimentares ou bactérias potencialmente benéficas, como é o caso dos probióticos. Para responder a esta questão, determinou-se a concentração de extrato capaz de inibir o crescimento de quatro potenciais patogénicos e avaliou-se o efeito da mesma sobre 5 potenciais probióticos. O extrato não provocou inibição do crescimento dos probióticos mas induziu uma maior produção/acumulação de ácidos orgânicos no meio de cultura (ácidos acético, cítrico e lático) e antecipou a produção de ácido propiónico. Adicionalmente, considerando a inibição seletiva e o efeito antiadesivo verificado previamente, o efeito do extrato sobre a adesão de probióticos e potenciais patogénicos intestinais foi avaliado tendo os resultados demonstrado que, independentemente de alguma redução da adesão de probióticos, a presença de extrato aumenta a inibição da adesão dos patogénicos sendo de destacar que a combinação do extrato com o *B.* Bo possibilitou, em alguns casos, inibições quase completas da adesão de patogénicos.

O extrato produzido neste trabalho possui uma demostrada atividade antioxidante e antimicrobiana, factos que, quando combinados com a sua coloração inerente, o torna interessante para incorporação tanto em produtos alimentares como em produtos cosméticos.

Abstract

In Portugal, there has been a significant increase in blueberry production, which has led to an increasing need to find alternative markets in which to place the fruits, preferably through products with high commercial value. If one considers the consumers' preference for foods that are not only healthy but also 'health promoting' and that blueberries are perceived as being so, the incorporation of blueberries and their extracts into a product may be something that increases the product's intrinsic value and, therefore be an advantage when contemplating industrial application.

The first step of this thesis aimed to select the raw matter to be used throughout the work. A study on four different cultivars and different ripening stages was carried out to select the blueberries that contained the higher levels of anthocyanins and other phenolics. The results showed that Goldtraube blueberries were the richest in anthocyanins (possessing both a higher content and a wider array of these compounds) as well as other phenolics, with later ripening stages being richer in anthocyanins. Therefore, Goldtraube blueberries collected at a later ripening stage were selected as the raw matter to be used. Afterwards, a solid-liquid extraction was optimized with the following conditions being identified as the most interesting: extraction of 10 g of blueberries with 100 mL acidified ethanol (0.01 % HCl), followed by 1 h incubation at 40 °C and a 15 min exposure to ultrasounds (35 kHz).

The second stage of this thesis focused on the characterization of some of the biological potential of the extract starting with its antioxidant potential. In order to determine the extracts capacity to protect DNA from oxidative damage, an existing agarose gel electrophoresis method was improved to allow for quantitative measurement. The extract was able of reducing DNA degradation at a concentration range of 160 to 200 µg mL^{-1} (for an H_2O_2 and an H_2O_2/iron degradation system, respectively).

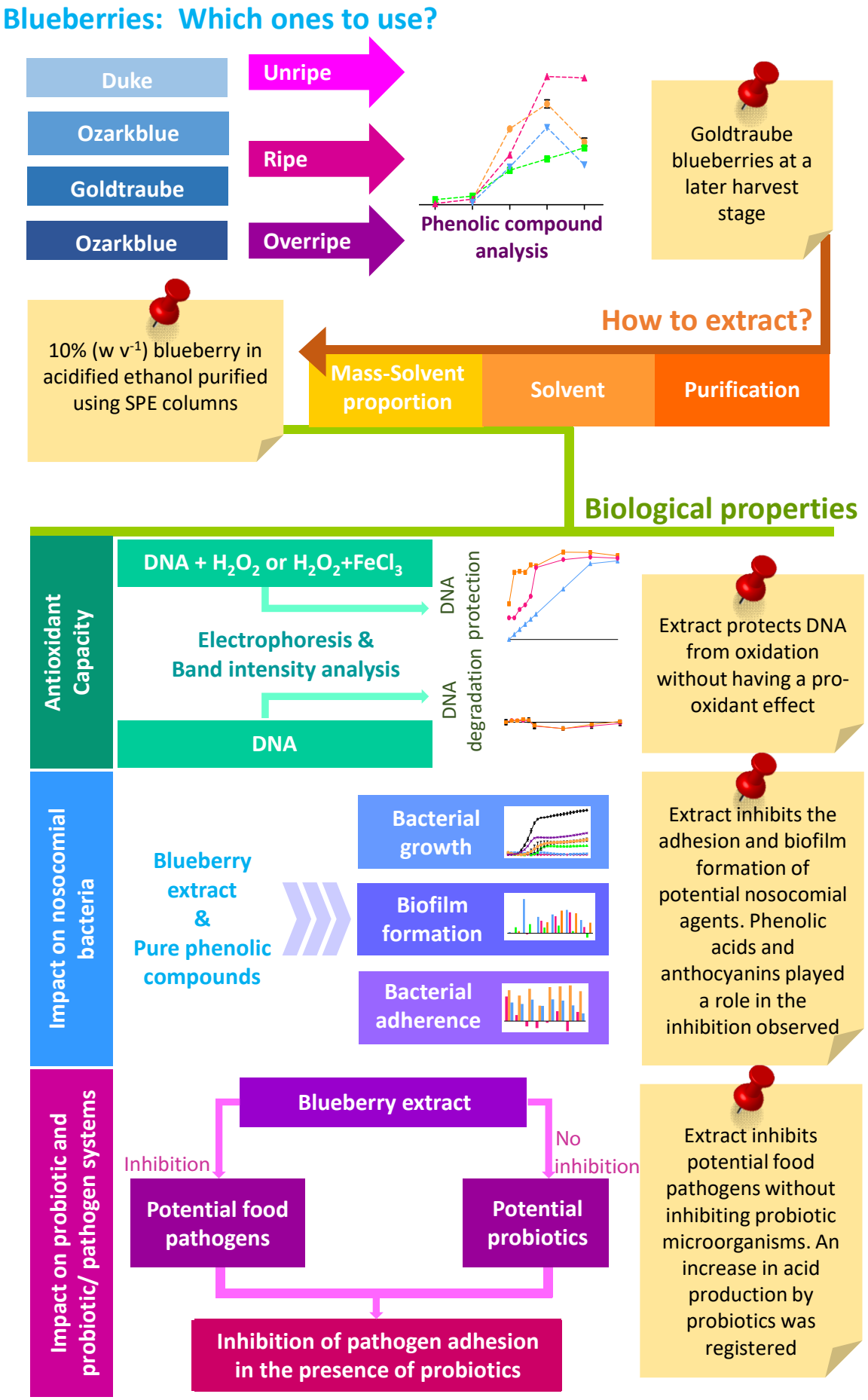
Simultaneously, the extract's impact upon nosocomial agents was evaluated. Out of the eight pathogens tested the extract was only capable of inhibiting the growth of three (MSSA, MRSA and one *E. coli* strain) at the highest concentration of extract tested. However, when considering the antibiofilm and antiadhesive assays, the extract proved to be more effective as it was capable of inhibiting all microorganisms at some level. In an attempt to better understand the role of the different compounds in the observed activity, two research lines were explored: i) the effect of phenolic acids, present in blueberries, was evaluated, with the

results demonstrating that they were only effective in inhibiting biofilm formation by *Staphylococcus* and had no effect upon *E. coli*. ii) the effect of a simulation of the extract, using the most abundant anthocyanin, against *Staphylococcus* (the most susceptible microorganisms to the extract). The results showed that, while the isolated compounds had some inhibitory effects, in most cases, the observed activity was significantly lower than that of the extract, hinting that synergies between the different compounds may play an important role in the observed inhibitions.

As the extract demonstrated an interesting effect upon potential nosocomial agents, its potential effect upon food pathogens/contaminants as well as its effect upon possible beneficial bacteria such as probiotic microorganism, came into question. To provide an answer to this question the concentration capable of inhibiting the growth of four potential food pathogens was determined and afterwards, five different probiotics were incubated with the extract at the same concentration. No inhibition of probiotic growth was observed in the presence of extract. However, an increase in production/accumulation of organic acids (acetic, lactic and citric acids) as well as an anticipation of propionic acid production was observed. As the extract appeared to have a selective effect and the previous work demonstrated that it possessed an interesting antiadhesive effect, a study on the extract's effect upon probiotics and potential pathogens' adhesion was carried out. The results showed that the combined presence of extract and probiotic was more effective in inhibiting pathogen adhesion than the probiotic alone. The apparent synergy established between the extract and *B.* Bo was a particular example of a successful partnership as their combined action allowed for, in some cases, an almost complete inhibition of pathogen adhesion.

Overall, the extract hereby proposed exhibited both antioxidant and antimicrobial capabilities properties that, when combined with their inherent colouring, makes it interesting to be used in food and cosmetic products.

Graphical abstract



Acknowledgements

Those we love don't go away, they walk beside us every day. Unseen, unheard, but always near. Still loved, still missed… so, first and foremost my thanks are to my father. He taught me the pleasure of learning and was one of my greatest supporters. As words fail in expressing my gratitude, I hope a simple heartfelt thank you will suffice.

I would like to acknowledge Escola Superior de Biotecnologia da Universidade Católica Portuguesa for suppling the necessary conditions to carry out my work and for teaching me that knowledge, imagination and resourcefulness are the pillars of scientific investigation. Similarly, I would like to acknowledge Mirtilusa S.A. for supplying the blueberries and for meeting my, somewhat strange demands and Fundação para Ciência e Tecnologia for providing the funding for my Ph.D.

I cannot forget to mention my supervisors, whose guidance made me a better scientist than I thought I could be: Professor Manuela Pintado, who was always there to encourage me along and pushing me to accomplish everything I could think of. Professor Rui Morais, always present whenever I needed to discuss a problem never failed to provide a good insight into whatever it was. Professor Conceição Calhau, who despite being slightly farther away was always quick in answering whenever called.

I would like to give a special thank you to Professor Ana Gomes, always there to provide an outsiders view and listen to me rant about any problem work related or not. To Professor Victor Freitas and Hélder Oliveira for their aid in the characterization of our extract. To Mariana, whose help in the final stages of my work proved an invaluable asset and to Sandra (my oldest duckling) whose helping hand, smile and snark made working that much fun.

Eduardo, we both now that, without you I would not have made it. You were my rock and my driving force. Always pushing me forward and never failing to catch me when I fell. This is not *my* work, it is *ours*. Mother, we both now you heard me complain more than you should and you probably know more about blueberries than you want, but hey! I'm saying thank you so that might help you cope with all the scientific mumbo-jumbo I sprouted out. Aunt 'Ni', always the voice of reason, you never failed in any (mental) calibration attempt. Thanks to you I never forgot the bigger picture and never failed to plan 2 steps ahead. You and Uncle Victor always helped me see straight, be correct and above all enjoy all the good

times. Thank you for that. Last but not least, to André, Mafalda and Carolina. The three small terrors, without knowing you gave me an amazing help… looking at things in wonderment is not something that comes easy to us adults but you sure helped.

Table of contents

Figure index

Chapter 1: Introduction

Chapter 1.4. Structure and thesis plan

Chapter 2: Raw matter selection

Chapter preamble

Chapter 2.1. Variation of anthocyanins and other major phenolic compounds throughout the ripening of four Portuguese blueberry (*Vaccinium corymbosum* L) cultivars

Chapter 3: Extract production

Chapter 3.1. Production of a food grade blueberry extract rich in anthocyanins: selection of solvents, extraction condition and a purification method

Chapter 4: In vitro antioxidant capacity

Chapter 4.1. DNA agarose gel electrophoresis for antioxidant analysis: development of a quantitative approach for phenolic extracts

Chapter 5: Effect on nosocomial bacteria

Chapter 5.1. Antimicrobial, antiadhesive and antibiofilm activity of ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction

Chapter 6.2. Impact of a purified blueberry extract on probiotic adhesion and its effect on probiotic/intestinal pathogen systems

Table index

Chapter 1: Introduction

Chapter 1.2. State of the art

Chapter 2: Raw matter selection

Chapter 2.2. Variation of anthocyanins and other major phenolic compounds throughout the ripening of four Portuguese blueberry (*Vaccinium corymbosum* L) cultivars

Supplemental Material

Chapter 4: In vitro antioxidant capacity

Chapter 4.1. DNA agarose gel electrophoresis for antioxidant analysis: development of a quantitative approach for plant extracts

Chapter 5: Effect on nosocomial bacteria

Chapter 5.1. Antimicrobial, antiadhesive and antibiofilm activity of ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction

Chapter 5.2. Antibiofilm potential of phenolic acids: The influence of environmental pH and intrinsic physicochemical characteristics

Chapter 6: Effect on probiotics & probiotic/pathogen systems

Chapter 6.1. Selective activity of a purified blueberry extract upon pathogenic and probiotic bacteria

Chapter 6.2. Impact of a purified blueberry extract on probiotic adhesion and its effect on probiotic/intestinal pathogen systems

List of abbreviations

1-9

8-OHdG	8-hydroxydeoxyguanosin

A

A. baumannii	*Acinetobacter baumannii*
AA	Ascorbic acid
ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
$ABTS^{\bullet+}$	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation
ACY	Total anthocyanin content
ADN	Ácido desoxirribonucleico
ASE	Accelerated solvent extraction
ATCC	American type culture collection

B

***B.* Bb12**	*Bifidobacterium animalis* Bb12
***B.* Bo**	*Bifidobacterium animalis* Bo
B. cereus	*Bacillus cereus*
BSA	Bovine serum albumin

C

C3Ara	Cyanidin-3-arabinoside
C3Gal	Cyanidin-3-galactoside
C3Glu	Cyanidin-3-glucoside
CA	Caffeic acid
Ca^{2+}	Divalent calcium cation
CCC	Countercurrent chromatography
CCUG	Culture collection of the university of Gothenburg
CFU	Colony forming unit
ChA	Chlorogenic acid
CI	Clinical isolate strain
cM3Glu/NChA	Malvidin-3-glucoside and neochlorogenic acid solution (500 and 125 µg mL^{-1}, respectively)

CO_2	Carbon dioxide
Cu^{2+}	Divalent copper cation
Cv.	Cultivar

D

D3Ara	Delphinidin-3-arabinoside
D3Gal	Delphinidin-3-galactose
D3Glu	Delphinidin-3-glucose
DF	Dilution factor
DHAA	Dehydroascorbic acid
DMBA	7,12-dimethylbenz[a]anthracene
DNA	Deoxyribonucleic acid

E

E. coli	*Escherichia coli*
***E. coli* CI**	*Escherichia coli* clinical isolate
***E. coli* R**	*Escherichia coli* reference strain
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide

F

FA	Ferulic acid
Fe^{2+}	Divalent iron cation
Fe^{3+}	Trivalent iron cation
$FeCl_3$	Iron chloride (III)
FRAP	Ferric reducing antioxidant power

G

GA	Gallic acid
GLP-1	Glucagon-like peptide 1
GRAS	Generally recognized as safe

H

H_2	Hydrogen gas
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
$HO^{\bullet}$	Hydroxyl radical

$HO_2^•$	Hydroperoxyl radical
HPLC	High pressure liquid chromatography
HPLC-DAD	High pressure liquid chromatography - Diode array detector
HPLC-MS	High pressure liquid chromatography coupled with Mass spectrometry
HPLC-RI-UV	High pressure liquid chromatography coupled with Refractive index detector and Ultra-violet detector
HSCCC	High speed countercurrent chromatography
I	
ICC	Interclass correlation coeficient
L	
L. acidophilus	*Lactobacillus acidophilus*
L. monocytogenes	*Listeria monocytogenes*
L. mucosae	*Lactobacillus mucosae*
L. plantarum	*Lactobacillus plantarum*
L. rhamnosus	*Lactobacillus rhamnosus*
log	Base 10 logarithm
log P	Partition coefficient (into octanol)
M	
M3Ara	Malvidin-3-arabinoside
M3Gal	Malvidin-3-galactoside
M3Glu	Malvidin-3-glucoside
M3Glu/NChA	Malvidin-3-glucoside and neochlorogenic acid solution (500 and 125 μg mL^{-1}, respectively)
MAE	Microwave assisted extraction
Mg^{2+}	Divalent magnesium cation
MIC	Minimum inhibitory concentration
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MRS agar/broth	de Mann, Rogosa and Sharpe agar/broth
MRS+CYS agar/broth	de Mann, Rogosa and Sharpe agar/broth supplemented with 0.5g L^{-1} L-cysteine-HCl
MRSA	Methicillin resistant *Staphylococcus aureus*

MRSA CI	Methicillin resistant *Staphylococcus aureus* clinical isolate
MRSA R	Methicillin resistant *Staphylococcus aureus* reference strain
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MSA	Molecular surface area
MSSA	Methicillin sensitive *Staphylococcus aureus*
MSSA CI	Methicillin sensitive *Staphylococcus aureus* clinical isolate
MSSA R	Methicillin sensitive *Staphylococcus aureus* reference strain
MTBE	Methyl *tert*-butyl ether
MW	Molecular weight

N

N_2	Nitrogen gas
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NChA	Neochlorogenic acid
NCTC	National collection of type cultures
NKC	Natural killer cells

O

OD	Optical density
OHM	Ohmic heating assisted extraction
ORAC	Oxygen radical absorbance capacity

P

p	p-value
p-CA	*p*-Coumaric acid
P. aeruginosa	*Pseudomonas aeruginosa*
***P. aeruginosa* CI**	*Pseudomonas aeruginosa* clinical isolate
***P. aeruginosa* R**	*Pseudomonas aeruginosa* reference strain
P. mirabilis	*Proteus mirabilis*
P3Ara	Peonidin-3-arabinoside
P3Gal	Peonidin-3-galactoside
Pb^{2+}	Divalent lead cation
PBS	Phosphate-buffered saline solution

PCA	Plate count agar
PEF	Pulsed electric field
PLE	Pressurized liquid extraction
pK_a	Cologarithm of the acidity constant
ppm	Parts per million
PS	Polystyrene
PS-rp	Polystyrene exposed to rabbit plasma
PS-rp-acy	Polystyrene exposed to rabbit plasma and then extract
PS-rp-ts	Polystyrene exposed to rabbit plasma and then a test solution
PSA	Polar surface area
Pt3Gal	Petunidin-3-galactoside
Pt3Glu	Petunidin-3-glucoside

Q

Q3Glu	Quercetin-3-glucoside
Q3Rut	Quercetin-3-rutinoside

R

R	Reference strain
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rp	Rabbit plasma
rpm	Rotations per minute

S

S1	Blueberry ripening stage: green fruits with a red crown (unripe fruit)
S2	Blueberry ripening stage: light pink blueberries with no observable green area (unripe fruit)
S3	Blueberry ripening stage: blueberries collected from bunches containing only 25% of ripe blueberries (ripe fruit)
S4	Blueberry ripening stage: blueberries collected from bunches containing 75% or more of ripe blueberries (ripe fruit)

S5	Blueberry ripening stage: blueberries that fell from the bush (overripe fruit)
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica*
S. enteritidis	*Salmonella enteritidis*
S. epidermidis	*Staphylococcus epidermidis*
S. typhimurium	*Salmonella typhimurium*
scCO$_2$	Supercritical carbon dioxide
SFE	Supercritical fluid extraction
SCFA	Short chain fatty acids
SinA	Sinapic acid
SLE	Solid liquid extraction
SO$_2$	Sulphur dioxide
SPE	Solid phase extraction
SyrA	Syringic acid

T

TAC	Total antioxidant capacity
TAE	Tris-Acetate EDTA buffer
TE	Tris EDTA buffer
TFA	Trifluoroacetic acid
TPC	Total phenolic content
TSB	Tryptone soy broth

U

UAE	Ultrasound assisted extraction
upH$_2$O	Ultra-pure water
US	Ultrasound
UV	Ultraviolet

V

V. angustifolium	*Vaccinium angustifolium*
V. ashei	*Vacciunium ashei*
V. cholerae	*Vibrio cholerae*
V. corymbosum	*Vaccinium corymbosum*
VEC	Vascular endothelial cell

VRSA	Vancomycin resistant *Staphylococcus aureus*
X	
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide

Scientific output

Through the execution of the several phases of this Ph. D. program several publications were generated, as follows:

Published works

Papers in international peer reviewed journals

Sara Silva, Eduardo M. Costa, Marta C. Coelho, Rui M. Morais e Manuela E. Pintado. Variation of anthocyanins and other major phenolic compounds throughout the ripening of four Portuguese blueberry (*Vaccinium corymbosum* L) cultivars. *Natural Product Research.* [In Press]
DOI: 10.1080/14786419.2016.1209668

Sara Silva, Eduardo M. Costa, Rui M. Morais, Manuela E. Pintado. Anthocyanin extraction from plant tissues: a review. *Critical reviews in food science and nutrition* [In Press]
DOI: 10.1080/10408398.2015.1087963

Sara Silva, Eduardo M. Costa, Marta Mendes, Rui M. Morais, Conceição Calhau, M. Manuela Pintado. 2016. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction. *Journal of Applied Microbiology* **121**:693-703
DOI: 10.1111/jam.13215

Sara Silva, Eduardo M. Costa, Bruno Horta, Conceição Calhau, Rui M. Morais, M. Manuela Pintado. 2016. Anti-biofilm potential of phenolic acids: the influence of environmental pH and intrinsic physicochemical properties. 2016. *Biofouling* **32** (8): 853-860
DOI: 10.1080/08927014.2016.1208183

Oral communications

Sara Silva, Eduardo M. Costa, Rui M. Morais, Conceição Calhau, Manuela Pintado. Production of anthocyanin rich, food grade, blueberry extracts: solvent selection; purification and antibacterial potential of the final extract *in* XIII Encontro de Química dos Alimentos, September 2016, Porto, Portugal

Sara Silva, Eduardo M. Costa, Rui M. Morais, M. Manuela Pintado. Antimicrobial activity of an anthocyanin rich blueberry extract purified using SPE *in* III International Conference on Antimicrobial Research - ICAR, October 2014, Madrid, Spain

Sara Silva, Marta Coelho, Eduardo M. Costa, Miguel F. Pereira, Manuela Pintado. Optimização e caracterização da fracção fenólica de extractos aquosos de mirtilo (folha e fruto seco) *in* CIBIA 9, January 2014, Valencia, Spain.

Sara Silva, Eduardo M. Costa, Liana Trovão, Rui M. Morais, Conceição Calhau e M. Manuela Pintado. Insights into the antimicrobial activity of ubiquitous phenolic acids *in* V International Conference on Environmental, Industrial, and Applied Microbiology - BioMicroWorld. October 2013, Madrid, Spain

Poster communications

Sara Silva, Eduardo M. Costa. Miguel F. Pereira, Maria R. Costa, Rui M. Morais, M. Manuela Pintado. Antimicrobial properties of blueberry infusions related to phenolic composition *in* V International Conference on Environmental, Industrial, and Applied Microbiology - BioMicroWorld. October 2013, Madrid, Spain

Sara Silva, Eduardo M. Costa, Marta C. Coelho, Rui M. Morais, Conceição Calhau, M. Manuela Pintado. Variation of the anthocyanin, phenolic content and antioxidant capacity in ripening blueberry fruit *in* 7th International Workshop on Anthocyanins, September 2013, Porto, Portugal.

Sara Silva, Eduardo M. Costa, Maria R. Costa, Miguel F. Pereira, Rui M. Morais, Manuela E. Pintado. A comparative analysis of the antibiofilm properties of crude blueberry extracts against MRSA and MSSA *in* Biofilms 5, December 2012, Paris, France.

Unpublished works

Papers submitted (or awaiting submission) to international peer-reviewed journals

Sara Silva, Eduardo M. Costa, Conceição Calhau, Rui M. Morais, Manuela E. Pintado. Production of a food grade blueberry extract rich in anthocyanins: selection of solvents, extraction condition and purification method. Submitted to: *Journal of Food Measurement & Characterization*

Sara Silva, Eduardo M. Costa, Sandra Vicente, Mariana Veiga, Conceição Calhau, Rui M Morais, Manuela E. Pintado. DNA agarose gel electrophoresis for antioxidant analysis: development of a quantitative approach for phenolic extracts. Submitted to: *Food Chemistry*

Sara Silva, Eduardo M. Costa, Sandra Vicente, Mariana Veiga, Rui M Morais, Conceição Calhau, Manuela E. Pintado. Antiadhesive and antibiofilm effect of malvidin-3-glucoside and malvidin-3-glucoside/neochlorogenic acid mixtures upon *Staphylococcus*. Submitted to: *Phytomedicine*

Sara Silva, Eduardo M. Costa, Hélder Oliveira, Mariana Veiga, Bruno Horta, Sandra Vicente, Vitor de Freitas, Rui M Morais, Conceição Calhau, Manuela E. Pintado. Selective activity of a purified blueberry extract upon pathogenic and probiotic bacteria. Submitted to: *Food Control*

Sara Silva, Eduardo M. Costa, Mariana Veiga, Bruno Horta, Sandra Vicente, Rui M. Morais, Conceição Calhau, Manuela E. Pintado. Impact of a purified blueberry extract on, *in vitro*, probiotic mucin-adhesion and its effect on probiotic/intestinal pathogen systems. [Awaiting submission].

Sara Silva, Eduardo M. Costa, Mariana Veiga, Rui M. Morais, Conceição Calhau, Manuela Pintado. Health promoting properties of blueberries: A review. [Awaiting submission]

Patent

Combined inhibitory effect of blueberry extract and probiotic BB12 against food pathogens. [*in writing*]

Chapter 1

Introduction

This chapter aimed to characterize the available knowledge as well as explain the relevance and objective of the present thesis

"We begin to learn wisely when we're willing to see the world from other people's perspective"

Toba Beta *in* ***Master of Stupidity***

Framework

The rise in life expectancy has led to an increase of the concern with the quality of life. Fruits and vegetables have been systematically associated with a reduction of the risk of chronic diseases, such as cardiovascular and neurodegenerative diseases (Gaziano et al., 2015, Morton et al., 2000, Wang et al., 2005, Krikorian et al., 2010). The consumption of fruits and vegetables has been systematically associated with a reduction of the risk of these pathologies (namely through the improvement of blood flow, protection against oxidative stress and exacerbated inflammatory responses) and, considering that phenolic compounds are present in high amounts in these food products it stands to reason that they have been associated with these benefits (Shukitt-Hale et al., 2008, Del Bo' et al., 2015, Wu et al., 2002). Furthermore, in later years, with the information flowing from the scientific community to the general population, the consumers' demand (and hence the industry's search) for healthier or health promoting foodstuffs has grown (Rossi et al., 2009, Gibson and Williams, 2000, Lee et al., 2002, Neto, 2007, Szajdek and Borowska, 2008, Wu et al., 2011).

Dubbed by the media as a "superfruit", blueberry is a prime example of a foodstuff that has gained a strong health promoting connotation, association that is supported by scientific literature where blueberries have been associated with several health benefits, namely their role in the maintenance of blood sugar levels, reduction of oxidative stress, anti-inflammatory effect, prevention of cardiovascular diseases, antimicrobial and antitumoral activity (Wood, 2009, Cassileth, 1999, Neto, 2007). This makes the incorporation of blueberries or their extracts, into foodstuffs a relatively easy way to grant them some functionality and increase their commercial value (Rossi et al., 2009, Gibson and Williams, 2000, Szajdek and Borowska, 2008).

In Portugal, blueberry production (typically using *Vaccinium corymbosum* bushes) has increased significantly in the two decades. In fact, according to FAOSTAT, since 2004, the total production more than doubled. This may mean an increase in the supply which, in turn may translate into a reduction of the selling price. Furthermore, with the increase in production, there is also an increase of the residues associated with blueberry production. The most substantial being the leaves that fall from the bushes in the autumn. Considering this, the study of new potential applications may aid in the valorisation of both blueberries

and blueberry leaves, possibly opening alternative commercial venues while exploiting health promoting formulations.

References

CASSILETH, B. R. 1999. Complementary and Alternative Cancer Medicine. *Journal of Clinical Oncology,* 17**,** 44-52.

DEL BO', C., FRACASSETTI, D., LANTI, C., PORRINI, M. & RISO, P. 2015. Comparison of DNA damage by the comet assay in fresh versus cryopreserved peripheral blood mononuclear cells obtained following dietary intervention. *Mutagenesis,* 30**,** 29-35.

GAZIANO, T. A., PRABHAKARAN, D. & GAZIANO, J. M. 2015. Global bruden of cardiovascular disease. *In:* MANN, D. L., ZIPES, D. P., LIBBY, P. & BONOW, R. O. (eds.) *Traunwald's Heart Disease - A textbook of Cardiovascular Medicine.* 10 ed. Philadelphia, USA: Elsevier Saunders.

GIBSON, G. R. & WILLIAMS, C. M. 2000. *Functional foods: Concept to product.,* Cambridge, England, Woodhead Publishing Limited.

KRIKORIAN, R., SHIDLER, M. D., NASH, T. A., KALT, W., VINQVIST-TYMCHUK, M. R., SHUKITT-HALE, B. & JOSEPH, J. A. 2010. Blueberry Supplementation Improves Memory in Older Adults. *Journal of Agricultural and Food Chemistry,* 58**,** 3996-4000.

LEE, J., DURST, R. W. & WROLSTAD, R. E. 2002. Impact of Juice Processing on Blueberry Anthocyanins and Polyphenolics: Comparison of Two Pretreatments. *Journal of Food Science,* 67**,** 1660-1667.

MORTON, L. W., CACCETTA, R. A.-A., PUDDEY, I. B. & CROFT, K. D. 2000. Chemistry And Biological Effects Of Dietary Phenolic Compounds: Relevance To Cardiovascular Disease. *Clinical and Experimental Pharmacology and Physiology,* 27**,** 152-159.

NETO, C. C. 2007. Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition & Food Research,* 51**,** 652-664.

ROSSI, M., NEGRI, E., BOSETTI, C., PELUCCHI, C. & LA VECCHIA, C. 2009. Epidemiology behind fruit and vegetable consumption and cancer risk with focus on flavonoids. *In:* FRAGA, C. G. (ed.) *Plant phenolics and human health:*

Biochemistry, nutrition and pharmacology. New Jersey, USA: John Wiley & Sons Inc.

SHUKITT-HALE, B., LAU, F. C., CAREY, A. N., GALLI, R. L., SPANGLER, E. L., INGRAM, D. K. & JOSEPH, J. A. 2008. Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutritional Neuroscience,* 11**,** 172-182.

SZAJDEK, A. & BOROWSKA, E. J. 2008. Bioactive Compounds and Health-Promoting Properties of Berry Fruits: A Review. *Plant Foods for Human Nutrition,* 63**,** 147-156.

WANG, Y., CHANG, C.-F., CHOU, J., CHEN, H.-L., DENG, X., HARVEY, B. K., CADET, J. L. & BICKFORD, P. C. 2005. Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage. *Experimental Neurology,* 193**,** 75-84.

WOOD, M. 2009. *The Earthwise Herbal: A Complete Guide to New World Medicinal Plants*, North Atlantic Books.

WU, L. H., XU, Z. L., DONG, D., HE, S. A. & YU, H. 2011. Protective Effect of Anthocyanins Extract from Blueberry on TNBS-Induced IBD Model of Mice. *Evidence-based Complementary and Alternaternative Medicine: eCAM,* 2011**,** 525462.

WU, X., CAO, G. & PRIOR, R. L. 2002. Absorption and Metabolism of Anthocyanins in Elderly Women after Consumption of Elderberry or Blueberry. *The Journal of Nutrition,* 132**,** 1865-1871.

State of the art

The state of the art has been partially published:
Critical Reviews in Food Science and Nutrition
doi:10.1080/10408398.2015.1087963

1. Blueberry

Blueberry production can be made using different *Vaccinium* species tough *Vaccinium corymbosum* (*V. corymbosum*, highbush blueberries), *Vaccinium ashei* (*V. ashei*; rabbiteye blueberries) and *Vaccinium angustifolium* (*V. angustifolium*, lowbush blueberries) are the most predominant species when considering commercial production (Zhao, 2007). While the most common species used for blueberry production are either highbush or lowbush blueberries, the selection of the species to be used is, to a large extent, made by considering the plants' need for low temperatures and winter cold hardiness with highbush blueberries requiring higher periods of cold than lowbush blueberries (Zhao, 2007, Godoy et al., 2008, Kalt et al., 2001, Trehane, 2009).

From a nutritional standpoint, blueberries are rich in water and sugars, particularly glucose and fructose though other sugars such as galactose and rhamnose may be found, frequently as sugar moieties associated with phenolic compounds. These berries also possess a relatively high amount of organic acids (e.g. citric and ascorbic acids), minerals (e.g. phosphorus, potassium and magnesium) and pectins as can be seen in Table 1 (Vrhovsek et al., 2012, Prior et al., 2001, Sousa et al., 2007).

As blueberries are reported as possessing both a high content and high variety of compounds, mostly phenolic in nature, their incorporation into foods may be a way to confer food products some functionality. Furthermore, as the consumers associate blueberries with healthy foods, their incorporation (either directly or through an extract) may increase the commercial value of a food product while also possibly aiding in the improvement of its flavour, colour or even shelf-life (e.g. through their antioxidant activity) (Gibson and Williams, 2000, Lee et al., 2002, Neto, 2007, Szajdek and Borowska, 2008, Wu et al., 2011, O'Brien et al., 2015).

Table 1. Nutritional profile of blueberries (adapted from Sousa et al. 2007).

Nutrients per 100 g of blueberries	
Water content	83 – 87 g
Proteins	400 – 700 mg
Lipids	500 mg
Glucose	5 – 7 g
Fructose	5 – 7 g
Fibre	1 – 1.5 g
Ashes	190 – 250 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
Pectins	300 – 600 mg

2. Health promoting potential of blueberries

There is a vast array of epidemiological evidences supporting the notion that a fruit and vegetable rich diet plays an important role in the prevention of several pathologies (e.g. reduction of the risk of cardiovascular disease and some forms of cancer, for example). As phenolic compounds and other antioxidants are abundantly present in fruits and vegetables, it stands to reason that they could be, at least in part, responsible for some of the potential beneficial health effects their consumption brings (Rossi et al., 2009, Graf et al., 2005, Neto, 2007, Szajdek and Borowska, 2008). Blueberries, widely recognized as possessing both an elevated content and variety of phenolic compounds (particularly anthocyanins), have been the focus of several works that aim to characterize their intrinsic health promoting potential.

2.1. Antioxidant activity

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are highly reactive compounds, both radical and non-radical, that can interact with several biologically relevant molecules (e.g. proteins and deoxyribonucleic acid (DNA)) potentially compromising their function, altering metabolic pathways and associated homeostatic balance (Borek, 2001, Milbury and Richer, 2008). Consequently, their presence has been associated with the development of several inflammatory conditions, degenerative diseases, cancer, etc. However, cells possess mechanisms that allow them to overcome the oxidative challenge posed by human metabolism (Sies, 2007). In some instances, due either to an excessive production, a deficient elimination of reactive species, imbalances between the amount of ROS/RNS present and the body's natural capacity to cope with therefore allowing oxidative

stress to occur. An increase in the consumption of antioxidants, exogenous compounds that are able to interact with the radicals originating stable compounds and prevent the oxidation of other molecules, may be a way to cope with oxidative stress (Yao and Vieira, 2007, Cooper et al., 2002, Mathew et al., 2015).

Blueberries are perceived as fruits with a strong antioxidant capacity (a property that has been ascribed to be a direct consequence of this fruit's richness in phenolic compounds) (Prior et al., 1998, Kalt et al., 1999, Burdulis et al., 2009, Szajdek and Borowska, 2008, Piljac-Žegarac et al., 2009). This is supported by the results of Wu et al. (2002) and Mazza et al. (2002), who reported a significant increase in human plasmatic hydrophilic and lipophilic antioxidant capacity after blueberry ingestion. On their own, anthocyanins (one of the major groups of blueberry phenolics) have been described as being not only inhibitors of lipid peroxidation but also as capable of protecting liver and red blood cells against *in vitro* and *in vivo* oxidative damage (Ramirez-Tortosa et al., 2001, Kong et al., 2003, Narayan et al., 1999, Youdim et al., 2000a, Youdim et al., 2000b, Heinonen et al., 1998).

2.2. Anti-mutagenic effect

Alterations in DNA, either the result of errors in DNA replication or of environmental stimuli, can lead to mutated proteins with a compromised/nullified activity which, in turn, may lead to an array of different diseases. As blueberries are known for their antioxidant capacity it stands to reason that they may possess some protective effect against DNA oxidation. This potential has been substantiated by the results observed by Del Bo' et al. (2010), who reported that the supplementation of rat's diet with blueberries allowed for an improvement in lymphocyte protection against oxidative damage (induced by H_2O_2), though it had no significant impact upon overall plasma antioxidant activity. Similarly, Pepe et al. (2013) reported that blueberries were capable of reducing the frequency of micronuclei appearance in mice exposed to 7,12-dimethylbenz[*a*]anthracene (DMBA) or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), two known mutagenic agents. Anthocyanin rich blueberry extracts have also been reported as possessing some anti-mutagenic effect, with Liu et al. (2013) reporting anthocyanin rich blueberry extracts as capable of reducing ultraviolet (UV) induced DNA damage in HepG2 cells, an effect that may be induced through the regulation of ROS scavenging systems.

2.3. Antitumoural effect

Blueberry, and its extracts, have been described as possessing interesting antitumoural/anticancer properties. Dried blueberry powder has been shown to reduce some of the toxicity of acrylamide (a known genotoxic and carcinogenic agent) by reversing acrylamide induced alterations in the liver's enzymatic levels and by reducing lymphocytes, liver and bone marrow cells' DNA damage (Zhao et al., 2015). Blueberry extracts have also been reported as possessing some antitumoural activity. Seeram et al. (2006), Diaconeasa et al. (2015) showed that blueberry extracts were capable of effectively inhibiting, *in vitro*, the proliferation of several tumour cell lines from the colon (HT-29 and HCT116), prostate (LNCaP), breast (breast), mouth (KB and CAL-27), cervix (HeLa), ovaries (A2780) and skin (B16F10). Similarly, Kou et al. (2016) reported that blueberry extracts were capable of reducing the cell viability of MDA-MB-231 breast cancer cells *in vitro*, while oral administration of the extracts to mice led to lower CD-1 tumour growth. Liu et al. (2016) stated that acylated blueberry anthocyanins not only exhibited a strong antitumoural effect on H22 murine tumours, but they were also able to function synergistically with cyclophosphamide (a known chemotropic agent) attenuating its toxic effect upon the liver while stimulating tumour necrosis.

2.4. Anti-inflammatory potential

Inflammatory processes, while important for the immunologic response, can also be an important factor in the development of several chronic diseases such as diabetes, cardiovascular diseases, arthritis and osteoporosis (Libby, 2007). Torri et al. (2007) reported that the consumption of a crude blueberry extract (ca. 300 mg kg^{-1} d^{-1}) by rats resulted in a positive anti-inflammatory effect, as it led to a reduction in paw oedema, myeloperoxidase activity (a maker for neutrophil infiltration) and formation of granulomatous tissue, while Ebenezer et al. (2016) described that the supplementation of the diet with 2% of freeze-dried blueberry powders, led to a decrease in inflammation in a post-traumatic stress disorder rat model. Furthermore, through a study containing 2375 participants, Cassidy et al. (2015) found that anthocyanin consumption was inversely associated with 12 different inflammatory biomarkers, while Giongo et al. (2011) reported that consumption of blueberries (either fresh or puréed) had a similar effect upon 24 obese children.

2.5. Immunomodulatory effect

One of the means through which anthocyanins are thought to inhibit tumour formation is linked with their potential immunomodulatory effects, possibly through the stimulation of natural killer cells (NKC; a group of lymphocytes responsible for the immune response to abnormal cells) (Seeram, 2008, Vivier et al., 2008, McAnulty et al., 2011). This possibility is supported by the work of McAnulty et al. (2011), who reported that chronic consumption of blueberries, by humans, led to higher basal levels of NKC and interleukin-10 (which downregulate the inflammatory response) (Sanjabi et al., 2009, McAnulty et al., 2011). This increase in the production of anti-inflammatory cytokines was also observed by Huang et al. (2014) when exposing VECs to malvidin-3-glucoside and malvidin-3-galactoside, two of the major anthocyanins of blueberries. Another example of the possible role of blueberry in immunomodulation is given by Shukitt-Hale et al. (2008). These authors reported that a blueberry supplemented diet allowed for an attenuation of kainic acid (a potential neurotoxic acid) induced production of inflammatory cytokines in rat hippocampus, which in turn resulted in a reduction of neurotoxicity (Shukitt-Hale et al., 2008, Zhang and Zhu, 2011).

2.6. Neuroprotection

Several authors have hinted that the consumption of blueberries may aid in the reversal of some age-related and oxidative stress induced decline in brain function (Wang et al., 2005, Krikorian et al., 2010). In fact, the neurotoxic effects of kainic acid, in rats, have been reported to be reduced after consumption of blueberry, which ameliorated the exacerbated inflammatory response in the hippocampus (Shukitt-Hale et al., 2008). In fact, blueberry consumption (either directly or through anthocyanin rich extracts) has been demonstrated to have an *in vitro* neuroprotective effect against damage induced by an array of neurotoxic agents (such as trimethyltin), while also exhibiting some *in vivo* effects in protecting and, in some cases, even enhancing the learning and memory capabilities of mice (Jo et al., 2015, Andres-Lacueva et al., 2005). An effect that Krikorian et al. (2010) showed to be expanded to humans, as the consumption of blueberries by older adults improved memory capabilities, which may be explained by an increase in synaptic plasticity resulting from a modulation of the microglia phenotype and microglia-neuron crosstalk (Meireles et al., 2016). On a different note, blueberry consumption by aged rats has also been linked with a reduction of ischemia induced apoptosis of brain cells as a possible result of their capacity to interact with ROS and RNS, which accumulate during the ischemic phase, particularly as

anthocyanins are known to accumulate in the central nervous system (Wang et al., 2005, Andres-Lacueva et al., 2005).

2.7. Cardiovascular disease prevention

Cardiovascular diseases have emerged as one of the leading causes of death in developed countries with the diet having been identified as one of the possible means to reduce the risk of developing these pathologies (Gaziano et al., 2015, Morton et al., 2000). Relatively recent data has suggested that the intake of specific fruits, among which are blueberries, may be more effective in managing cardiovascular diseases, as flavonoids have been associated with improved blood flow and endothelial function, and blueberry consumption, in particular, has been related to a reduction of the risk of myocardial infarction in women. (Cassidy et al., 2016, Cassidy et al., 2013). Additionally, considering that low grade chronic inflammation has been associated with higher risks of developing cardiovascular disease the anti-inflammatory potential associated with blueberry may also play an important role in reducing the risk of developing these pathologies (Danesh et al., 2000, Riso et al., 2013, Basu et al., 2010). Moreover, vascular endothelial cells (VEC), whose damage has also been linked with the development of vascular diseases, have been described as being capable of integrating anthocyanins into their membrane and cytosol. In turn, anthocyanins are thought to aid in the preservation of VEC function, either by aiding in the stabilization of the cellular membrane or by helping maintain oxidative balance (Ramirez-Tortosa et al., 2001, Youdim et al., 2000a). The results reported by Huang et al. (2016) lent further credibility to this theory as they reported that malvidins (a major class of blueberry anthocyanins) were capable of decreasing the concentration of ROS (intracellular) and xanthine oxidase-1 (intra- and extracellular) while leading to higher levels of superoxide dismutase in umbilical cord VECs (Cisowska et al., 2011).

2.8. Anti-obesity

Obesity is widely recognized as one of today's major health threats, as it is both a major risk factor in an array of health problems such as diabetes and cardiovascular diseases and considerably hard to treat (Caballero, 2007, Hill and Peters, 1998). As treating obesity with drugs is frequently associated with negative side effects and little to none long term efficacy, some authors have defended that using natural plant extracts could pose an interesting alternative for long term weight management and blueberries were proposed as one of the possible sources to be exploited (Song et al., 2013). Blueberries, when lyophilized or

processed unto a juice, had no significant impact in either weight gain or body fat accumulation in mice fed with a high-fat diet (Prior et al., 2008, Prior et al., 2010, DeFuria et al., 2009). However, when considering an anthocyanin extract, the same authors described a significant reduction in body weight and fat accumulation. However, Seymour et al. (2011), Vendrame et al. (2013) and Vendrame et al. (2014) reported that the supplementation of a high-fat diet with blueberry powder led to less intraperitoneal accumulation of fat, an increase of adiponectin levels, a decrease of inflammatory markers and an amelioration of dyslipidaemia.

2.9. Anti-diabetes

Diabetes are a group of diseases characterized by high blood glucose levels. Given its rising prevalence and potential harmful effects diabetes are one of the major concerns of modern medicine (Carvalho et al., 2003, Wild et al., 2004). Anthocyanin rich extracts have been demonstrated to attenuate insulin sensitivity and hyperglycaemia , while a diet supplemented with blueberry powder has been shown to enhance glucose tolerance in post-menopausal mice and insulin sensitivity in humans (Elks et al., 2015, Stull et al., 2010, Takikawa et al., 2010). Moreover, anthocyanins have been demonstrated to induce the production of glucagon-like peptide-1 (GLP-1), which interacts with pancreatic cells responsible for the induction of insulin secretion. The molecules that block GLP-1 degradation have been used for therapeutic purposes and thus this food mediated increase of GLP-1 production could pose an interesting new strategy for the treatment of diabetes (Herman et al., 2006, Vilsbøll et al., 2008, Tsuda, 2015).

2.10. Antimicrobial potential

Phenolic compounds have long since been associated with antimicrobial activity. Therefore blueberries, as a good source of these compounds, have been regarded as a potential source for antimicrobial agents for medicinal, pharmaceutical, cosmetic and food industries (Burdulis et al., 2009, Jaakola et al., 2004, Cisowska et al., 2011). Several authors have reported on the *in vitro* antimicrobial activity of blueberry extracts, having found them to be capable of inhibiting the growth of known potential pathogens such as *Escherichia coli, Vibrio cholerae, Vibrio parahaemolyticus, Acinetobacter baumannii, Salmonella thypimurium, Salmonella enteritidis, Pseudomonas aeruginosa, Shigella flexneri, Shigella sonnei, Listeria monocytogenes, Bacillus cereus, Staphylococcus epidermidis,* methicillin sensitive and methicillin resistant *Staphylococcus aureus* (Pertuzatti et al., 2016, Khalifa et

al., 2015, Shen et al., 2014, Lacombe et al., 2012, Zimmer et al., 2014). Furthermore, Khalifa et al. (2015) described blueberry extracts as being effective inhibitor of *V. cholerae* virulence factors. These results, which stand in line with those published in an earlier work regarding the effect of a blueberry extract upon *S. aureus* virulence factors, indicate that these blueberry extracts, even when present at concentrations below in which they are unable to inhibit bacterial growth they may still affect their metabolism in an advantageous way (Silva et al., 2015). Moreover, some authors have reported that blueberry extracts may be effective in reducing biofilm formation, bacterial resistance structures notorious for their imperviousness to traditional antimicrobial agents (Zimmer et al., 2014, Bjarnsholt, 2013, Bridier et al., 2005, Fux et al., 2005).

2.11. Prebiotic potential

In the last decade, the importance of the gut microbiota in human metabolism and health has become almost impossible to dispute. Given that a large fraction of anthocyanins are not absorbed in the upper gastrointestinal tract, most of ingested anthocyanins end up exposed to the intestinal microbiota which, in turn, ends up metabolizing them and can, therefore play an important role in its modulation (Kay, 2006, Bingham, 2006). Hidalgo et al. (2012) reported that the incubation of malvidin-3-glucoside (typically the most abundant anthocyanin in blueberries) with faecal slurry has caused an increase of beneficial bacteria (e.g. *Lactobacillus* spp. and *Bifidobacterium* spp.), with Vendrame et al. (2011) reporting similar observations, for humans, after a six-week consumption of a blueberry powder drink.

3. Blueberry phenolic compounds

Phenolic compounds play a myriad of roles in plants' physiology, metabolism and survival, from providing structural support to aiding in the management of environmental stresses (Vermerris and Nicholson, 2007). Blueberries have long since been recognized as a good source of phenolic compounds (Table 2). Anthocyanins are the most prevalent family of flavonoids in blueberries with authors reporting the presence of up to fifteen different anthocyanins (monoarabinosides, monoglucosides and monogalactosides of cyanidin, peonidin, delphinidin, petunidin and malvidin) (Barnes et al., 2009, Routray and Orsat, 2011, Gavrilova et al., 2011). In spite of being the most abundant family of compounds, and therefore the one most frequently associated with blueberries biological activity, several other phenolics have been reported. Brambilla et al. (2008), Kader et al. (1996) and Skrede et al. (2000) reported the presence of three types of phenolic acids (gallic, syringic and

vanillic) and five different cinnamic acids (chlorogenic, the major derivative present, caffeic, ferulic, *o*- and *p*-coumaric acids). Another group of phenolic compounds frequently associated with blueberries, are flavonoids. Though less studied, other phenolic compounds have been reported to be present in blueberries. Kader et al. (1996) and Taruscio et al. (2004) have reported the presence of other flavonoids, and their glycosylated forms (arabinosides, glucosides and galactosides), namely catechin, epicatechin, myricetin, kaempferol and quercetin. Vrhovsek et al. (2012) found an array of different flavonoid glycosides, though some appeared to be cultivar specific, such as pentosides, glucosides, galactosides of isorhamnetin, syringetin and laricitrin as well as their aglycone counterpart.

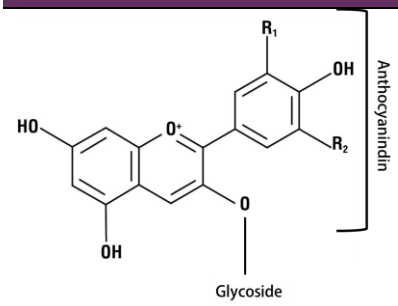
Table 2. Phenolic composition of blueberries (adapted from Gavrilova et al., 2011)

	Blueberry cultivar			
Compound family	Toro	Legacy	Duke	Bluecrop
Anthocyanins	56.35 ± 1.04	68.55 ± 2.35	83.64 ± 3.16	41.99 ± 0.25
Flavonols	2.28 ± 0.8	5.17 ± 0.03	3.51 ± 0.16	6.08 ± 0.45
Cinnamic acids	33.12 ± 1.78	62.27 ± 1.97	25.97 ± 3.21	67.54 ± 3.03

4. Anthocyanins and anthocyanin extraction

Anthocyanins are naturally occurring, water soluble, flavonoid pigments, structurally composed by the α- or β- linkage of an anthocyanidin to a sugar moiety (Table 3) (Castañeda-Ovando et al., 2009; Vermerris and Nicholson, 2007). In later years, they have gathered the attention of both the scientific and industrial communities due to their vast range of possible applications. Reports have been made about their potential for the development of better industrial colorants, health promoting foods, supplements and also on the improvement/development of solar based, renewable energies (Buchweitz et al., 2013; Chien and Hsu, 2013; Norberto et al., 2013; Stintzing et al., 2006; Wallace and Giusti, 2013; Wu et al., 2011). As anthocyanins are frequently found as secondary plant metabolites, it stands to reason that one of the main approaches used in their production is through extraction and isolation from plant tissues. However, the innate characteristics of these matrixes (e.g. uneven compound distribution and high enzymatic activity) may hinder the extraction process as they can difficult the diffusion of compounds, allow for simultaneous extraction of other compounds and even lead to artefactual changes in compounds. As such, the selection of an adequate extraction method gains particular relevance (Antolovich et al., 2000).

Table 3. Structure of blueberry anthocyanins; R1, R2 and the glycoside vary according to the anthocyanin (adapted from Barnes et al., 2009)

Basic anthocyanin structure			
	Anthocyanidin		Glycoside
	Name	R1	R2	
	Delphinidin	-OH	-OH	Glucose Galactose Rhamnose
	Cyanidin	-OH	-H	
	Petunidin	$-OCH_3$	-OH	
	Peonidin	$-OCH_3$	-H	
	Malvidin	$-OCH_3$	$-OCH_3$	

Solid-liquid extraction (SLE) is the classic approach used to recover anthocyanins from plant tissues as their polarity facilitates dissolution into polar solvents, e.g. acidified methanol/ethanol and acetone (Antolovich et al., 2000; Barnes et al., 2009; Burdulis et al., 2009; Castañeda-Ovando et al., 2009; Galván D'Alessandro et al.; Naczk et al., 2006; Wallace and Giusti, 2013). With the advent of the green revolution and the increase in the demand for these compounds new, technologically advanced approaches are being pursued to better extract anthocyanins. Some of these emergent technologies include supercritical extraction, pulsed electric field assisted extraction, microwave assisted extraction, ultrasound assisted extraction and pressurized liquid extraction (Armenta et al., 2008; Galván D'Alessandro et al., 2014; Garofulić et al., 2013; Ghafoor et al., 2010; Golmohamadi et al., 2013; Huie, 2002; Junior et al., 2010; Liazid et al., 2011; Paes et al., 2014; Paula et al., 2014; Petersson et al., 2010; Puértolas et al.; Seabra et al., 2010; Segovia et al.; Truong et al., 2012; Vatai et al., 2009). However, it is difficult to compare the extraction yields of different methods since compound variation from matrix to matrix hampers comparisons. With that in mind, the present review seeks to analytically compile the available information in an effort to facilitate a better understanding regarding each methodology's potential.

4.1. Anthocyanin basic chemistry

One of the main issues with the manipulation of anthocyanins is that they are highly susceptible to degradation, particularly when isolated. Several physicochemical factors are known to interfere with anthocyanin stability, therefore knowledge of basic anthocyanin chemistry is required in order to better understand the limitations of a specific extraction protocol (Table 4) (Seeram et al., 2001, Adams, 1973, Zhang et al., 2007, Tanchev and

Joncheva, 1973, Kader et al., 1998, Furtado et al., 1993, Mori et al., 2007, Wesche-Ebeling and Montgomery, 1990, Fossen et al., 1998, Cabrita et al., 2000).

Though several factors that affect anthocyanins' chemical stability could be mentioned, pH and temperature stand as the most referred. Typically, anthocyanins are more stable under acidic conditions with pH values above 7 leading to their degradation (Seeram et al., 2001, Fleschhut et al., 2006, Castañeda-Ovando et al., 2009). This explains why most extraction protocols require the presence of an acidified environment, though strong acidic media may promote the hydrolysis of the glycoside bonds. Therefore, pH control stands as a relevant extraction variable with a considerable impact upon the quality of the extracted anthocyanins. Temperature, another determinant factor for anthocyanin stability, is frequently considered an extraction factor; therefore, this factor is discussed more in depth in the classic methods section.

Though relatively unstable when in solutions, anthocyanins may be stabilized through the addition of some compounds such as co-pigments, metallic ions or even other antioxidants. The protection granted by antioxidant compounds is the easiest to grasp; antioxidants that are more susceptible to oxidation than anthocyanins will be oxidized before them. Co-pigments (systems rich in π electrons) and metallic ions such as Al, Fe, Mg, Mo and Sn will interact directly with the anthocyanins hampering nucleophilic attack and the oxidation of the quinoidal base, respectively (Castañeda-Ovando et al., 2009, Shaked-Sachray et al., 2002). The decision to add any compound, despite their potential as stabilizers, must be made with its expected application in mind.

4.2. Anthocyanin extraction from plant tissues

Anthocyanins are found deposited inside the vacuole of cells in several different tissues such as leaves, petals, fruits, bulbs, rhizomes or stems (Stintzing and Carle, 2004, Gould et al., 2008). However, when looking at their presence within a given tissue, the accumulation of anthocyanins may vary. For instance, while some leaves are all red (fully coloured by anthocyanins) others exhibit this colour in restricted sections such as the margins distribution in a given organ. Similarly, in some plants leaves are red only when they are growing, stressed or senescent, while in others they are red throughout their lives. These variations (time, tissue, placement and inducibility) difficult the establishment of a unified explanation for the presence of anthocyanins in tissues (Gould et al., 2008).

Table 4. *Main advantages and disadvantages of the different extraction methods.*

	Advantages	Disadvantages
Solid-Liquid Extraction (SLE)	Simple protocol No need for specific equipment Can be food grade (Freedman, 1980a, Mussinan and Keelan, 1994)	Relatively long extraction times (Cacace and Mazza, 2002, Cacace and Mazza, 2003a, Cacace and Mazza, 2003b) High solvent consumption (Garcia-Viguera et al., 1998) Acid, air and light exposure may cause anthocyanin degradation (Vatai et al., 2009)
Supercritical Fluid Extraction (SFE)	Allows the removal of nonpolar interferents (Vatai et al., 2009, Seabra et al., 2010) Reduced exposure to light and air (Vatai et al., 2009) Extracts are Generally Recognized as Safe (GRAS) (Ghafoor et al., 2010) Inhibits enzymes that cause anthocyanin degradation (Seabra et al., 2010)	High associated costs (Junior et al., 2010) Mandatory use of a polar solvent (Seabra et al., 2010, Ghafoor et al., 2010) In some cases, similar yields may be achieved using SLE (Vatai et al., 2009) Requires CO_2 (Vatai et al., 2009, Seabra et al., 2010)
Ultrasound Assisted Extraction (UAE)	Low solvent consumption (Huie, 2002, Golmohamadi et al., 2013, Carabias-Martínez et al., 2005) Low energy consumption (Huie, 2002, Golmohamadi et al., 2013) Easy to scale up (Golmohamadi et al., 2013, Galván D'Alessandro et al., 2014, Vieira et al., 2013) Safe for consumption (Golmohamadi et al., 2013, Galván D'Alessandro et al., 2014, Vieira et al., 2013)	High associated costs (Vieira et al., 2013) Energy dissipation through heat may cause anthocyanin degradation (Golmohamadi et al., 2013, Tiwari et al., 2008)
Pressurized Liquid Extraction (PLE)	Low solvent consumption (Paes et al., 2014, Petersson et al., 2010, Carabias-Martínez et al., 2005, Santos et al., 2012) Low extraction time (Carabias-Martínez et al., 2005, Santos et al., 2012) Automation (Carabias-Martínez et al., 2005)	High temperatures used may cause anthocyanin degradation (Petersson et al., 2010)
Microwave Assisted Extraction (MAE)	Low extraction time (Armenta et al., 2008, Garofulić et al., 2013) Low solvent consumption (Armenta et al., 2008, Garofulić et al., 2013) Good reproducibility (Armenta et al., 2008, Garofulić et al., 2013)	Temperature may cause anthocyanin degradation (Armenta et al., 2008, Garofulić et al., 2013, Liazid et al., 2011)

4.2.1. Pre-treatment of tissues

Anthocyanins are frequently contained within intracellular organelles; therefore, their accessibility is dependent on the solvents' capacity to enter these structures and their integrity. Therefore, pre-treating the tissues in order to facilitate extraction could be an interesting approach to increase extraction efficiency, though any procedure must be made considering the limitations imposed by the chemical nature of the anthocyanins themselves.

4.2.1.1. Reducing particle size

Turning the samples into a powder/pulp is a common approach, as the reduction of particle size and consequent increase in contact area promotes the diffusion from the solid particles into the solvent, furthermore the sheer stress may induce some damage to the organelles that will allow for better solvent permeability (Dutta, 2007). Despite the fact that this is a relatively inert way to enhance the extraction yield, in a powder or in a pulp anthocyanins are significantly more exposed to oxidizing agents and therefore more prone to degradation. Thus, to avoid oxidation, several authors prefer to directly either homogenize the tissue with the solvent in the beginning of the extraction process or freeze the fruits (sometimes with liquid nitrogen, to avoid tissue decomposition) before turning them into a pulp (Antolovich et al., 2000, Burdulis et al., 2009, Barnes et al., 2009, Vrhovsek et al., 2012, Cacace and Mazza, 2002, Cacace and Mazza, 2003a, Cacace and Mazza, 2003b). As freezing induces some cellular damage, the addition of a freezing step may further aid in the extraction of the compounds (Pearce, 2001).

4.2.1.2. Pulsed electric field pre-treatment

Pulsed electric field (PEF) has gained increasing interest as a pre-treatment of samples, as it improves mass transfer without using high temperature (Segovia et al.). In its place, the matrix is exposed to external, moderate (1 to 10 kV/cm and 10 kJ/kg), pulsed (generally 5 to 50 pulses) electric fields that induce the electroporation of cell membranes, thus increasing membrane permeability and facilitating the extraction process, with authors describing yields up to 2.12 times higher for anthocyanins (Segovia et al., Puértolas et al., Gachovska et al., 2010, Corrales et al., 2009). However, despite the potential benefits of this approach (non-thermal treatment that is food safe), the pulsed field induces some degradation of anthocyanins, to chalcone and other pseudobases though, to the best of our knowledge, the reactions behind these transformations are not described (Odriozola-Serrano et al., 2009, Zhang et al., 2007). Zhang et al. (2007) reported that, for cyanidin-3-glucoside, higher intensity fields induced faster degradation rates than lower intensity fields and caused a

significant reduction in the half lives and D values of the compound when compared to exposure to 45 °C, thus demonstrating that the intensity of the electric field is a key factor to control when utilizing this approach.

Though several solvents can be used in the subsequent extraction, the most common is water, as this allows for the reduction of the cost and environmental issues associated with the usage of other solvents. This, combined with PEF's capacity to increases anthocyanin stability, enhances the appeal of this method (Puértolas et al., Segovia et al., Zhang et al., 2007).

4.2.1.3. Enzymatic assisted extraction

In plants, anthocyanins can be found inside the cellular vacuole (Gould et al., 2008). This implies that, in order to extract the pigments, the solvents must be able to transpose the cell wall, membrane, cytoplasm and the vacuolar membrane before reaching the anthocyanins. Therefore, the use of enzymatic cocktails that disrupt the cell wall network should facilitate the removal of anthocyanins from vegetable cells, particularly from matrixes that have thicker cell walls and higher amounts of pectin (Buchert et al., 2005). There are several, commercially available, blends of enzymes that can be used in the extraction of anthocyanins, most products are comprised of several enzymes, such as pectinases and cellulases, in various ratios (Buchert et al., 2005, Landbo and Meyer, 2001). Buchert et al. (2005) reported a significant increase in anthocyanin yield (13 – 41% in blueberry juice and 18 – 29% in black currant juice) when *Pectinex Ultra SP-L, Pectinex Smach, Pectinex BE 3-L* and *Biopectinase CCM* were used to produce juice. However, when using *Econase CE*, in blueberry juice, these authors reported a significant decrease in anthocyanin yield. Similar results have been reported by Landbo and Meyer (2001), who reported that the use of several enzymatic blends to extract anthocyanins from black currant residues either had no impact (*Pectinex BE* and *Novozym 89*) or caused a significant reduction (*Macer8 FJ* and *Macer8 R*) in anthocyanin yield. A possible explanation for this phenomena has been hypothesized by several authors; the enzymes may be hydrolysing the anthocyanins to their aglycone counterpart (Buchert et al., 2005, Wrolstad et al., 1994, Wightman and Wrolstad, 1995). This is supported by the results of Buchert et al. (2005), who found that, when using enzyme blends with high galactosidase activity, there is a considerable reduction in the amount of galactosides found when in comparison to the untreated counterpart (Buchert et al., 2005).

4.2.2. Classic approach to anthocyanin extraction

The classic approach used to extract anthocyanins from plant tissues is the same one that is used for other phenolics, i.e. the tissues (treated or not) are soaked with subsequent solvent

extraction, in a process known as solid-liquid extraction (SLE). Several parameters have been reported to affect SLE yield, the most commonly reported in literature are solvent type and temperature, though several others may be mentioned such as extraction time, particle size, solvent/mass ratio (Castañeda-Ovando et al., 2009, Vrhovsek et al., 2012). These last parameters vary mostly regarding general mass transfer principles and their manipulation yield is rarely associated with chemical changes. The smaller the particle the easier it will be for a solvent to be able to permeate it and extract the desired compounds (Dutta, 2007). With the increase of the contact time between solvent and tissue, the more complete shall be the diffusion from solid particle to liquid until partition equilibria is reached (Dutta, 2007). The time frame in which equilibria is reached varies according to several other conditions (e.g. temperature, solvent), therefore the extraction time should be determined for each extraction as the other extraction conditions are defined (Cacace and Mazza, 2002, Cacace and Mazza, 2003a, Cacace and Mazza, 2003b). As for the solvent/mass ratio the foremost thing that must be taken into account is the solubility of the desired compounds, ideally the amount of solvent added should be just enough to dissolve the desired compounds (Rostagno and Prado, 2013). However, as other compounds are extracted, frequently a higher amount of solvent is frequently necessary.

Another approach used by some authors to increase anthocyanin yield is to employ multiple extraction steps for a given tissue. Theoretically, the use of several extraction steps should allow for a more complete extraction though that is not always so (Revilla et al., 1998). Revilla et al. (1998) studied the effectivity of several extraction processes among which were four that considered several sequential extractions having found that, multiple extractions did not necessarily mean higher extraction yields.

4.2.2.1. Solvent

Most of the common solvents used in SLE are polar, employing methanol, ethanol, acetone and even water acidified with a vast array of both organic and inorganic acids (Metivier et al., 1980, Barnes et al., 2009, Vatai et al., 2009). In the 80's, Metivier et al. (Metivier et al., 1980) studied the effect of different solvent (methanol, ethanol and water) and acid (HCl, citric, acetic, propionic, tartaric and formic acid) combinations upon the extraction of anthocyanins from wine pomace having found that, on its own, methanol was the best performing solvent (extracting 20 and 73% more anthocyanins than ethanol and water, respectively). Though the type of acid used affected each solvent differently, methanol combined with citric acid appeared to be the best combination for extraction. Barnes et al.

(Barnes et al., 2009), when studying the extraction of anthocyanins from blueberry samples observed a somewhat different behaviour with non-acidified ethanol and acetone proving to be significantly more effective than methanol, isopropanol and acetonitrile in the extraction of anthocyanins. Overall, the acidification of the solvent led to the improvement of the extraction yield though the effect was, once again, acid dependent, the addition of trifluoroacetic acid (TFA) led to a significant increase of the anthocyanin yield for all solvents except acetone (Barnes et al., 2009). The ratio water:organic solvent is also an interesting variable to note as it varies with the solvent and the composition of the extraction tissue. For instance, in cabernet grapes decreasing the acetone:water ratio lead to lower yields of anthocyanins, while doing the same with ethanol had the opposite effect. On the other hand, for merlot grapes there was no apparent relationship between the water:solvent ratio and the extraction yield (Vatai et al., 2009). The differences observed between the efficacy of a given solvent/acid combination may be explained by the differences in anthocyanin composition in each source. Regardless, it is interesting to note that, while the most widely used extraction protocol uses methanol acidified with HCl, to the best of our knowledge, when different acids were tested, the combination methanol-HCl was not the best extractant (Metivier et al., 1980, Barnes et al., 2009).

Although acid addition appears to help the extraction process, the use of strong acids may cause the hydrolysis of the glycosidic bond, yielding an anthocyanidin and a sugar moiety (Castañeda-Ovando et al., 2009, Kapasakalidis et al., 2006). This was observed by Kapasakalidis et al. (Kapasakalidis et al., 2006) when, in an attempt to improve the phenolic content of black currant extracts, they employed an acid hydrolysis (using HCl 2M), and instead of obtaining some of the anthocyanins that were found in the original extracts, they found only their anthocyanidin counterpart. Besides the extraction protocol itself, the presence of strong acids may also pose a problem for the further processing of extracts. This is particularly true if a concentration step is required as, while the anthocyanin concentration in a solution may rise from the removal of the extraction solvent, so does the concentration of acid and thus the hydrolysis of anthocyanins may take place or be extended (Garcia-Viguera et al., 1998).

Given the possible acid mediated hydrolysis, some authors prefer to work with sulphured solutions using compounds such as SO_2 as extractants (Castañeda-Ovando et al., 2009, Cacace and Mazza, 2002, Cacace and Mazza, 2003a). In fact, this approach can even be more effective than the traditional alcohol/acid mix. Cacace and Mazza (Cacace and Mazza,

2003a, Cacace and Mazza, 2002, Cacace and Mazza, 2003b) reported that a concentration of 1000-1200 ppm of SO_2 allowed for the maximum yield of total phenolics and anthocyanins and that, at low temperatures, the extraction rates for anthocyanins are significantly higher than those observed for an ethanolic solution. However, despite the advantages presented this solution also poses some limitations that must be considered. For instance, in the production of edible extracts, the presence of SO_2 while not be critical from a food safety standpoint as it is a known preservative, it can induce adverse reactions in hypersensitive individuals (Freedman, 1980a, Mussinan and Keelan, 1994, Freedman, 1980b, Li and Zhao, 2006). Additionally, if seeking to produce antimicrobial extracts the activity observed may not be due only to the extracted compounds as sulphured compounds have been known to exhibit some antimicrobial activity (Kyung and Fleming, 1997).

4.2.2.2. Temperature

Heat sensitivity is an ubiquitous characteristic of anthocyanins. Therefore, when contemplating an extraction protocol this condition must be taken into account. Wang and Xu (Wang and Xu, 2007) and Aurélio et al. (Aurelio et al., 2008), when studying the impact of heat upon blackberry and hibiscus pulps, found that heating to 90 ºC lead to ca. 80% reduction of the half-life of anthocyanins thus yielding significantly lower levels of anthocyanins after a 6 h period (Vrhovsek et al., 2012, Cacace and Mazza, 2002, Cacace and Mazza, 2003a, Cacace and Mazza, 2003b, Castañeda-Ovando et al., 2009). The sensitivity of anthocyanins increases when they are present in extracts. Sadilova et al. (2007) found anthocyanin extracts to be much more susceptible to degradation as they detected no anthocyanins in purified strawberry and elderberry extracts after a 6h exposure to 95 ºC. This difference between extract and tissue stability is also evident in the work of Cacace and Mazza (Cacace and Mazza, 2002, Cacace and Mazza, 2003a, Cacace and Mazza, 2003b), who reported that, for blackcurrants, 30 – 35 °C appeared to be the best extraction temperature for anthocyanins, with higher temperatures resulting in lower anthocyanin contents, a likely consequence of heat induced degradation. Additionally, Vatai et al. (Vatai et al., 2008) reported that aqueous acetone solutions extraction procedures carried out at 20 ºC yielded significantly higher results than those carried out at 60 ºC, the exception being for pure acetone but, as 60 ºC is above the boiling point for this solvent it is possible that some artefacts occurred.

4.2.3. Modern technologies applied to anthocyanin extraction

Given the widespread interest in plant metabolites, there has been an effort to modernize the extraction protocols. With improvements from the classical approach ranging from a reduction of the amount of organic solvents used and less exposure to reducing agents to a decrease of the need for purification and concentration steps, overall improvement of the extraction yield, selectivity and/or kinetics (Huie, 2002). Several new methodologies have emerged and, though their potential benefits cannot be overlooked, it is important not to disregard the target compounds' characteristics in lieu of new technologies.

4.2.3.1. Supercritical fluid extraction

From a simplistic standpoint, supercritical fluid extraction (SFE) is a process in which supercritical fluids (at vapour-liquid critical point) are used to extract the components of interest from a solid or even liquid matrix. Several advantages can be listed in order to reason the usage of this approach: (i) the pre-treatment of samples with supercritical CO_2 ($scCO_2$) removes nonpolar components reducing the amount of interferents though, contrary to what happens for polar polyphenolics, it's usage doesn't increase the availability of anthocyanins (Vatai et al., 2009, Seabra et al., 2010); (ii) the absence of atmospheric O_2 and light during the extraction process reduces anthocyanin oxidation (Vatai et al., 2009); (iii) SFE extracts are generally recognized as safe (GRAS) and therefore their considered safe to use as food additives (Ghafoor et al., 2010); (iv) the use of $scCO_2$ and pressure, inhibits native enzymes that degrade anthocyanins (Seabra et al., 2010). The major drawback of this approach is related with its production costs. Currently, this method is associated mainly with the production of extracts that comply with strict environmental regulations or with high value products (Junior et al., 2010).

As traditional SFE is performed using only $scCO_2$, which extracts nonpolar compounds, to extract anthocyanins (polar molecules) a polar solvent must also be used. In fact, the choice of the solvent may be a key factor for the success of the extraction procedure. The most common approach uses a mix of CO_2, ethanol and in some cases water, with proportions varying according to the tissue and/or plant material (Seabra et al., 2010, Ghafoor et al., 2010). The ethanol/water fraction must be in proportions where they are soluble in $scCO_2$, at the pressure and temperature used, so the mixture can be considered supercritical. However, higher amounts may be used. In those cases two phases coexist, liquid and supercritical, but their presence may affect the yield and recovery of target compounds. (Paes et al., 2014). It is interesting to note that, contrary to what happens in the SLE, the

acidification of the solvents has been described as having no significant effect upon the extraction efficiency in SFE (Paes et al., 2014). As the water contained within the extraction matrix, when in contact with CO_2, results in the formation of carbonic acid (which lowers the pH) the addition of acid is, somewhat redundant (Paes et al., 2014, Junior et al., 2010).

Ghafoor et al. (2010) studied the extraction of anthocyanins from grape peel using SFE. They reported that, for this matrix, the optimal SFE conditions were 45 ºC and 16 MPa in the presence of 6-7% ethanol. These conditions were similar to those reported by Seabra et al. (2010) for the extraction of anthocyanins from elderberry pomace, and those reported by Paes et al. (2014) for blueberry residues, 40 °C and 20-21 MPa using aqueous ethanol as a co-solvent. As anthocyanins are sensitive to relatively high temperatures, it is interesting to note that most authors that focus on these compounds do not employ temperatures above 40 °C, though higher temperatures have been used in the extraction of other phenolic compounds (Paes et al., 2014, Ghafoor et al., 2010, Seabra et al., 2010, Vatai et al., 2009, Huie, 2002, Paula et al., 2014)

Vatai et al. (2009), compared the efficacy of conventional SLE against SFE both with and without a pre-treatment using supercritical CO_2 (to remove nonpolar compounds and purify the sample). They found that for Refosk and Cabernet grape pomace, similar results may be obtained when comparing the results of SLE using acetone or ethanol against SFE performed using ethanol as a co-solvent at a pressure of 15 or 30 MPa and a temperature of 40 °C.

4.2.3.2. Ultrasound assisted extraction

In ultrasound assisted extraction (UAE), the ultrasound frequencies are capable of facilitating the hydration of plant materials which leads to the enlargement of cell wall pores and occasionally, cause the rupture of the cell wall. This will promote mass transfer, therefore allowing for the increase of the extraction yield (Huie, 2002, Golmohamadi et al., 2013). The ultrasound assisted extraction has several advantages. It requires reduced amounts of solvents, it does not require CO_2 and has a relatively low energy consumption (Vieira et al., 2013, Galván D'Alessandro et al., 2014). The fact that it is a green approach, that it is relatively easy to scale up and that it is safe for Human intake, makes it a particularly interesting technique for the food industry (Galván D'Alessandro et al., 2014, Vieira et al., 2013, Golmohamadi et al., 2013). However, the production costs are relatively high, with other alternatives allowing for similar yields with lower costs (Vieira et al., 2013).

Ultrasound frequencies, a major extraction factor, can be divided into two bands, low power ultrasound (low amplitude and high frequency, 100-1000 kHz) or high power ultrasound (high amplitude and low frequency, 20-100 kHz) (Golmohamadi et al., 2013, Galván D'Alessandro et al., Galván D'Alessandro et al., 2014). The last is used in food processing and cleaning processes as it allows for the formation of cavitation bubbles, whose vibration creates fluid currents and disruptive forces on nearby cells and particles (Golmohamadi et al., 2013). However, the cavitational phenomena when coupled with the microjets of fluid and the asymmetrical collapse of air bubbles near the surface of the cell wall (all caused by the ultrasound) cause an increase in temperature that, in cooled reactors, may reach up to 70 °C (Golmohamadi et al., 2013). This may compromise anthocyanin stability as shown by the work of Tiwari et al. (2008), who demonstrated that exposure to an intermittent frequency of 20 kHz for a 10 min period reduced the anthocyanin content of strawberry juice by 3.2%. (Tiwari et al., 2008, Golmohamadi et al., 2013, Aurelio et al., 2008, Wang and Xu, 2007). As vibrational energy is dissipated as heat during the extraction procedure and anthocyanins are notorious for their sensitivity to heat, it stands to reason that the extraction time is a particularly important variable (Galván D'Alessandro et al., 2014, Golmohamadi et al., 2013). Golmohamadi et al. (2013) reported that, for 20 kHz, extraction times above 10 min caused a reduction of the total anthocyanin content in raspberry puree. However, Vieira et al. (2013) reported a steady increase of total monomeric anthocyanins throughout a 180 min period when extracting anthocyanins from jussara pulp at 40 kHz. This demonstrates the need for matrix contextualization and the development of specific protocols for each matrix.

4.2.3.3. Pressurized liquid extraction

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), allows for the fast extraction of compounds with little solvent consumption (Petersson et al., 2010, Paes et al., 2014, Carabias-Martínez et al., 2005, Santos et al., 2012). In this methodology, high pressure is used to maintain the solvents liquid at higher temperatures (frequently temperatures above solvent boiling point), this allows for the improvement of the solubility of the compounds, sample wetting and matrix penetration (Petersson et al., 2010, Huie, 2002). Immediately, the use of this technique for the extraction of anthocyanins raises concerns. In fact, as illustrated by the work of Petersson et al. (2010), degradation begins almost as the extraction itself begins. For it to be beneficial, a positive balance must be struck between the degradation and extraction kinetics. The sample composition, particularly anthocyanin profile, will be very important in the decision of whether this

methodology is of interest, particularly as the possibility of process automation makes this technique particularly appealing for industrial application (Petersson et al., 2010, Castañeda-Ovando et al., 2009, Carabias-Martínez et al., 2005). Despite its limitation regarding anthocyanin extraction, this technique has been used in several food matrixes such as blueberries, red onion and sweet potato, though to the best of our knowledge, few articles contemplate the degradation kinetics (Paes et al., 2014, Petersson et al., 2010, Truong et al., 2012) has been released.

4.2.3.4. Microwave assisted extraction

Microwave assisted extraction (MAE) is a process that uses microwaves to quickly, efficiently and evenly heat both solvent and target tissue. In hydrated tissues, the water present absorbs the energy with the resulting heat causing cell disruption (Garofulić et al., 2013). This technique allows for lower extraction times, requires less solvents and exhibits a good reproducibility (Armenta et al., 2008, Garofulić et al., 2013). On the other hand, the enhancement of compound diffusion form matrix to solvent, while potentially increasing the extraction yield, also facilitates the extraction of non-targeted compounds (Garofulić et al., 2013, Liazid et al., 2011, Armenta et al., 2008). Additionally, the usage of temperature in anthocyanins, as argued before, has significant limitations. Generally, and also to avoid overheating, low to moderate powers (coupled with longer extraction times) are used (Garofulić et al., 2013). However, this does not necessarily prevent anthocyanin degradation. Garofulić et al. (2013) reported that as temperature and/or irradiation time increases, the amount of anthocyanins detected on cherry extracts decreased significantly. While, for grape skin peels, Liazid et al. (2011) reported that, up to 100 °C, there was a significant increase in the amount of anthocyanins found in the extracts, with the values reducing only for temperatures above 100 °C. These authors report results that are somewhat contrary, but, in both cases, the need to find a suitable extraction temperature (where the increase in extraction kinetics compensates the degradation reactions) is clear, as is the importance of contextualizing it with the tissue that constitutes the matrix (Liazid et al., 2011, Pap et al., 2013).

4.2.3.5. Ohmic heating extraction

Ohmic heating assisted extraction (OHM), also known as electro-conductive heating, uses the inherent electrical resistance of a given foodstuff in order to turn electrical energy into thermal energy, which will increase membrane permeability (Loypimai et al., 2015). Though not extensively used for anthocyanin extraction, some work has been reported (Loypimai et

al., 2015). Though, the rapid heating process has been described as allowing for less thermal damage, Sarkis et al. (2013) reported that ohmic heating caused a similar degradation as conventional heating. As such, more work is needed to better ascertain whether ohmic heating is interesting when considering anthocyanin extraction (Sarkis et al., 2013).

4.2.4. Purification approaches

The extraction methodologies used to extract anthocyanins from plant tissues are not selective. In fact, the final product is, frequently, a solution that has large amounts of other compounds (e.g. sugars and organic acids) that, besides introducing a bias in functionality studies, can also be detrimental for anthocyanin stability (Jampani et al., 2014, He and Giusti, 2011, Castañeda-Ovando et al., 2009). Sugars are a prime example of this, as free sugars and their degradation products may lead to Maillard reactions (Jampani et al., 2014, Tsai et al., 2005). Therefore, after contemplating the extraction process, the removal of interferents stands as an important step, particularly in the study of anthocyanin properties as the high cost of standards usually are detrimental for their usage (He and Giusti, 2011, Castañeda-Ovando et al., 2009). Several techniques have been proposed to achieve anthocyanin separation from solid phase extraction (SPE) to sophisticated chromatographic techniques (Castañeda-Ovando et al., 2009).

4.2.4.1. Precipitation of anthocyanins

The use of bivalent lead (Pb^{2+}) to precipitate anthocyanins in aqueous solutions is one of the oldest approaches for anthocyanin purification. However, anthocyanins are not the only group of compounds that form low solubility lead salts, in fact other compounds with a carboxyl or other nucleophilic group (e.g. amino, phenolic, fatty and organic acids, tannins, flavonoids, etc.) are also precipitated by Pb^{2+} (Fuleki and Francis, 1968, Maekaji et al., 1963, Mattick et al., 1969). Therefore, this method stands more as a preliminary purification step, particularly as the most plentiful impurities, sugars, are not precipitated in this approach (Fuleki and Francis, 1968).

Given the amphoteric nature of the pigments, the reaction between anthocyanin and Pb^{2+} is pH dependent, with neutral and alkaline environments exhibiting better precipitation activity than their acidic counterparts (Fuleki and Francis, 1968, Mattick et al., 1969). Care must be taken when considering the pH value to be used as different anthocyanins exhibit different optimal precipitating pH and, therefore, some may remain in the original solution

Anthocyanin lead salts can be dissociated using an alcoholic solution (with either HCl or H_2SO_4) that will cause the precipitation of a lead salt that can be removed, yielding an alcoholic suspension of anthocyanins (Maekaji et al., 1963, Fuleki and Francis, 1968).

4.2.4.2. Solid phase extraction

Solid phase extraction is a separation process in which, dissolved compounds are separated according to their physicochemical characteristics. Traditional SPE uses adsorbent sorbents, such as C18, Sephadex, or Amberlite adsorption resins that establish bonds through their hydroxyl groups or hydrophobic bonds with the aromatic rings coupled with solvents with varying polarity (Castañeda-Ovando et al., 2009, Buran et al., 2014). Chandrasekhar et al. (2012) studied the efficacy of six different sorbent resins; nonpolar silica gel, weak acidic anion exchanger Amberlite IRC 80, weak acidic cation exchanger Amberlite IR 120, strong acidic cation exchanger Dowex 50WX8, non-ionic acrylic ester resins with moderate polarity Amberlite XAD4 and XAD7. Results showed that non-ionic acrylic ester resins showed the highest adsorption rates and highest elution capacity (ca. 93% recovery), while the silica nonpolar resin exhibited no detected adsorption of anthocyanins (Chandrasekhar et al., 2012).

This method is relatively cheap, easy and reproducible, thus explaining the preference it has been conceded. However, as non-selective interactions are established it is possible that using this approach, contaminants remain (Buran et al., 2014, He and Giusti, 2011, Denev et al., 2010, Socaciu, 2007). This is shown in the work of Buran et al. (2014) where, after purification of blueberry extract with an Amberlite resin, traces of chlorogenic acid and other flavonols were still found. As an alternative, He and Giusti (2011) developed an SPE system that takes advantage of the different charges that anthocyanins have at different pH values. The sorbent used in this new approach combines reversed-phase interactions with cation exchange interactions. This means that, if the anthocyanins are introduced into the system at a pH value of 2 (where they are in their flavylium cation form) they will interact with the negatively charged sorbent and the other compounds can be removed using solvents with various polarities (given that their pH values do not rise significantly) (He and Giusti, 2011). To remove the anthocyanins an alkaline eluent may be used, since it will shift the anthocyanins into a negatively charged particle (quinoidal base) that will no longer interact with the resin and can, therefore, be collected (He and Giusti, 2011). He and Giusti (2011) found that this new approach was superior to the other commonly used SPE methods in regards of anthocyanin purity and recovery, sorbent capacity, cost, simplicity and high

throughput. A similarly effective approach has been reported by Castañeda-Ovando et al. (2014). These authors exploited the tendency of the *o*-dihydroxy arrangement of anthocyanins to for metallic complexes with Fe^{3+}, Cu^{2+}, Fe^{2+}, Ca^{2+} and Mg^{2+} and the fact that these complexes, while stable under alkaline conditions, break when exposed to an acidic environment (Castañeda-Ovando et al., 2014, Yoshida et al., 2006). This approach, while allowing good purification rates and a relatively low cost must be first contextualized with the matrix as different anthocyanins exhibit different behaviours in response to the pH values and metallic (de)complexation, possibly leading to artefactual shifts in anthocyanin composition (Castañeda-Ovando et al., 2014).

4.2.4.3. Countercurrent chromatography

Countercurrent chromatography (CCC) is a chromatographic technique that uses two immiscible liquid phases, under gentle conditions, to separate relatively large amounts of sample (up to several hundred milligrams in a single run) (Schwarz et al., 2003, Degenhardt et al., 2000). High Speed CCC (HSCCC) has been demonstrated to be a potential mechanism to achieve large scale isolation of anthocyanins from various natural sources such as blackberries or elderberries. In HSCCC, the solvents are placed in a Teflon tube wound around a coil (frequently, connected in a series) that is then rotated. The movement causes mixing and settling zones between the two phases, inducing a continuous distribution of the sample (Schwarz et al., 2003, Degenhardt et al., 2000, Kostadinovik et al., 2013). The fact that this approach doesn't require expensive columns, uses gentle operating conditions and relatively cheap solvents (e.g. methyl tert-butyl ether (MTBE), *n*-butanol, acetonitrile, water, TFA) increases its interest from an economic standpoint, thus making it a good candidate for industrial production (Schwarz et al., 2003, Degenhardt et al., 2000, Kostadinovik et al., 2013). A system comprised of MTBE, *n*-butanol, acetonitrile and water acidified with TFA (2:2:1:5) has been used by several authors to purify anthocyanins from a range of sources such as wine, blueberries or red cabbage (Socaciu, 2007, Kostadinovik et al., 2013).

4.2.5. Method selection

Plant phenolics have definitively gathered the interest of the scientific community, with anthocyanins belonging to one of the groups that show the most biological and industrial potential. However, the pigments' complex chemistry raises several issues when contemplating the extraction protocol to use. From relatively simplistic approaches to more technological ones, the range from which to choose is vast, and given the lack of straightforward papers that compare all methodologies for a given sample, it is hard to

conclude which method is better given the extraction yield alone (different samples, have different amounts of anthocyanins to begin with, therefore variations in yield from paper to paper also reflect sample variation). Therefore, a clear understanding of the underling goal of the extraction, the potential use of the final extract and the sample itself plays an important role in the selection of the extraction/purification approach to be used (Table 5). Despite that, some overall guidelines can be perceived. Methods that imply the used of high temperatures may induce degradation while promoting the extraction therefore are less suitable for samples richer in methylated anthocyanins (more susceptible to degradation) (Sarkis et al., 2013). Similarly, methods that improve the extraction yield while using mostly water as a solvent (MAE, UAE, PEF), present interesting economic and ecological advantages though the reduction of production and waste management costs must be compared with the equipment cost. If pure extracts are needed, SFE is an interesting approach as it allows for the removal of nonpolar solvents, though, from a cost perspective a simpler (less expensive) extraction approach may be of interest if followed by an appropriate purification protocol (anthocyanin precipitation for lower purity levels, CCC or adsorbent and ion-exchange SPE resins for purer extracts).

Table 5. Summary of the most common extraction methods used for anthocyanin extraction for different matrixes.

	Matrix	References
Solid-Liquid Extraction (SLE)	Black Carrot	(Türker and Erdogˇdu, 2006)
	Blackcurrant	(Cacace and Mazza, 2002, Cacace and Mazza, 2003a, Cacace and Mazza, 2003b, Denev et al., 2010)
	Blackberry	(Denev et al., 2010, Oancea et al., 2013)
	Blueberry	(Ballinger et al., 1970, Barnes et al., 2009, Denev et al., 2010)
	Chokeberry	(Denev et al., 2010, Galván D'Alessandro et al., 2014)
	Elderberry	(Denev et al., 2010, Vatai et al., 2009)
	Grape	(Vatai et al., 2009)
	Hibiscus	(Cissé et al., 2012)
	Jamum	(Jampani et al., 2014)
	Jussara	(Borges et al., 2011)
	Purple Fleshed Sweet Potato	(Fan et al., 2008)
	Red Cabbage	(Chandrasekhar et al., 2012)
	Red Radish	(Patil et al., 2009)
	Strawberry	(Garcia-Viguera et al., 1998)
	Sweet Cherry	(Oancea et al., 2013)
	Wine Pomace	(Metivier et al., 1980)

	Matrix	References
Supercritical Fluid Extraction (SFE)	Blueberry	(Paes et al., 2014)
	Cricket Vine	(Paula et al., 2013, Paula et al., 2014)
	Elderberry	(Seabra et al., 2010, Vatai et al., 2009)
	Grape	(Ghafoor et al., 2010, Vatai et al., 2009)
	Jamobolan	(Maran et al., 2014)
Ultrasound Assisted Extraction (UAE)	Blackberry	(Oancea et al., 2013)
	Chokeberry	(Galván D'Alessandro et al., 2014)
	Grape	(Corrales et al., 2008, Ghafoor et al., 2009)
	Mangosteen	(Cheok et al., 2013)
	Sugar Beet Molass	(Chen et al., 2015)
	Sweet Cherry	(Oancea et al., 2013)
Pressurized Liquid Extraction (PLE)	Blueberry	(Paes et al., 2014)
	Grape	(Corrales et al., 2009, Corrales et al., 2008, Ju and Howard, 2003, Liazid et al., 2011)
	Jabuticaba	(Santos et al., 2012)
	Purple Fleshed Sweet Potato	(Truong et al., 2012)
	Red Cabbage	(Arapitsas and Turner, 2008)
	Red Onion	(Petersson et al., 2010)
Microwave Assisted Extraction (MAE)	Blackcurrant	(Pap et al., 2013)
	Blueberry	(Zheng et al., 2013)
	Grape	(Li et al., 2012)
	Mulberry	(Zou et al., 2012)
	Pomegranate	(Sinha et al., 2012)
	Red Raspberry	(Sun et al., 2007, Teng et al., 2013)
	Sour Cherry	(Garofulić et al., 2013)
Ohmic Heating Assisted Extraction (OHM)	Black Rice Bran	(Loypimai et al., 2015)
SLE with Pulsed Electric Field Pre-treatment	Grape	(Corrales et al., 2008)
	Purple Fleshed Potato	(Puértolas et al.)
	Red Cabbage	(Gachovska et al., 2010)
	Strawberry	(Odriozola-Serrano et al., 2009)

5. References

ADAMS, J. B. 1973. Thermal degradation of anthocyanins with particular reference to the 3-glycosides of cyanidin. I. In acidified aqueous solution at 100 °C. *Journal of the Science of Food and Agriculture,* 24**,** 747-762.

ANDRES-LACUEVA, C., SHUKITT-HALE, B., GALLI, R. L., JAUREGUI, O., LAMUELA-RAVENTOS, R. M. & JOSEPH, J. A. 2005. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutritional Neuroscience,* 8**,** 111-120.

ANTOLOVICH, M., PRENZLER, P., ROBARDS, K. & RYAN, D. 2000. Sample preparation in the determination of phenolic compounds in fruits. *Analyst,* 125**,** 989-1009.

ARAPITSAS, P. & TURNER, C. 2008. Pressurized solvent extraction and monolithic column-HPLC/DAD analysis of anthocyanins in red cabbage. *Talanta,* 74**,** 1218-1223.

ARMENTA, S., GARRIGUES, S. & DE LA GUARDIA, M. 2008. Green Analytical Chemistry. *TrAC Trends in Analytical Chemistry,* 27**,** 497-511.

AURELIO, D.-L., EDGARDO, R. G. & NAVARRO-GALINDO, S. 2008. Thermal kinetic degradation of anthocyanins in a roselle (Hibiscus sabdariffa L. cv. 'Criollo') infusion. *International Journal of Food Science & Technology,* 43**,** 322-325.

BALLINGER, W., MANESS, E. & KUSHMAN, L. 1970. Anthocyanins in ripe fruit of the highbush blue-berry, Vaccinium corymbosum L. *Journal of the American Society of Horticultural Science,* 95**,** 283-5.

BARNES, J. S., NGUYEN, H. P., SHEN, S. & SCHUG, K. A. 2009. General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography–electrospray ionization-ion trap-time of flight-mass spectrometry. *Journal of Chromatography A,* 1216**,** 4728-4735.

BASU, A., RHONE, M. & LYONS, T. J. 2010. Berries: emerging impact on cardiovascular health. *Nutrition Reviews,* 68**,** 168-177.

BINGHAM, M. 2006. The metabolism of polyphenols by the huma gut microbiota. *In:* OUWEHAND, A. C. & VAUGHAN, E. E. (eds.) *Gatsrointestinal microbiology.* New York, USA: Taylor & Francis Group.

BJARNSHOLT, T. 2013. The role of bacterial biofilms in chronic infections. *APMIS Suppl***,** 1-51.

BOREK, C. 2001. Antioxidant health effects of aged garlic extract. *J Nutr,* 131**,** 1010s-5s.

BORGES, G. D. S. C., VIEIRA, F. G. K., COPETTI, C., GONZAGA, L. V. & FETT, R. 2011. Optimization of the extraction of flavanols and anthocyanins from the fruit pulp of Euterpe edulis using the response surface methodology. *Food Research International,* 44**,** 708-715.

BRAMBILLA, A., LO SCALZO, R., BERTOLO, G. & TORREGGIANI, D. 2008. Steam-Blanched Highbush Blueberry (Vaccinium corymbosum L.) Juice: Phenolic Profile and Antioxidant Capacity in Relation to Cultivar Selection. *Journal of Agricultural and Food Chemistry,* 56**,** 2643-2648.

BRIDIER, A., SANCHEZ-VIZUETE, P., GUILBAUD, M., PIARD, J. C., NAÏTALI, M. & BRIANDET, R. 2005. Biofilm-associated persistence of food-borne pathogens. *Food Microbiology,* 45**,** 167-178.

BUCHERT, J., KOPONEN, J. M., SUUTARINEN, M., MUSTRANTA, A., LILLE, M., TÖRRÖNEN, R. & POUTANEN, K. 2005. Effect of enzyme-aided pressing on anthocyanin yield and profiles in bilberry and blackcurrant juices. *Journal of the Science of Food and Agriculture,* 85**,** 2548-2556.

BURAN, T. J., SANDHU, A. K., LI, Z., ROCK, C. R., YANG, W. W. & GU, L. 2014. Adsorption/desorption characteristics and separation of anthocyanins and polyphenols from blueberries using macroporous adsorbent resins. *Journal of Food Engineering,* 128**,** 167-173.

BURDULIS, D., SARKINAS, A., JASUTIENÉ, I., STACKEVICENÉ, E., NIKOLAJEVAS, L. & JANULIS, V. 2009. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (Vaccinium myrtillus L.) and blueberry (Vaccinium corymbosum L.) fruits. *Acta poloniae pharmaceutica,* 66**,** 399-408.

CABALLERO, B. 2007. The Global Epidemic of Obesity: An Overview. *Epidemiologic Reviews,* 29**,** 1-5.

CABRITA, L., FOSSEN, T. & ANDERSEN, Ø. M. 2000. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry,* 68**,** 101-107.

CACACE, J. E. & MAZZA, G. 2002. Extraction of Anthocyanins and Other Phenolics from Black Currants with Sulfured Water. *Journal of Agricultural and Food Chemistry,* 50**,** 5939-5946.

CACACE, J. E. & MAZZA, G. 2003a. Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering,* 59**,** 379-389.

CACACE, J. E. & MAZZA, G. 2003b. Optimization of Extraction of Anthocyanins from Black Currants with Aqueous Ethanol. *Journal of Food Science,* 68**,** 240-248.

CARABIAS-MARTÍNEZ, R., RODRÍGUEZ-GONZALO, E., REVILLA-RUIZ, P. & HERNÁNDEZ-MÉNDEZ, J. 2005. Pressurized liquid extraction in the analysis of food and biological samples. *Journal of Chromatography A,* 1089**,** 1-17.

CARVALHO, E. N. D., CARVALHO, N. A. S. D. & FERREIRA, L. M. 2003. Experimental model of induction of diabetes mellitus in rats. *Acta Cirurgica Brasileira,* 18**,** 60-64.

CASSIDY, A., BERTOIA, M., CHIUVE, S., FLINT, A., FORMAN, J. & RIMM, E. B. 2016. Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men. *The American Journal of Clinical Nutrition,* 104**,** 587-594.

CASSIDY, A., MUKAMAL, K. J., LIU, L., FRANZ, M., ELIASSEN, A. H. & RIMM, E. B. 2013. High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. *Circulation,* 127**,** 188-96.

CASSIDY, A., ROGERS, G., PETERSON, J. J., DWYER, J. T., LIN, H. & JACQUES, P. F. 2015. Higher dietary anthocyanin and flavonol intakes are associated with anti-inflammatory effects in a population of US adults. *The American Journal of Clinical Nutrition,* 102**,** 172-181.

CASTAÑEDA-OVANDO, A., GALÁN-VIDAL, C. A., CONTRERAS-LÓPEZ, E. & PÁEZ-HERNÁNDEZ, M. E. 2014. Purification of Anthocyanins with o-Dihydroxy Arrangement by Sorption in Cationic Resins Charged with Fe(III). *Journal of Chemistry,* 2014**,** 9.

CASTAÑEDA-OVANDO, A., PACHECO-HERNÁNDEZ, M. D. L., PÁEZ-HERNÁNDEZ, M. E., RODRÍGUEZ, J. A. & GALÁN-VIDAL, C. A. 2009. Chemical studies of anthocyanins: A review. *Food Chemistry,* 113**,** 859-871.

CHANDRASEKHAR, J., MADHUSUDHAN, M. C. & RAGHAVARAO, K. S. M. S. 2012. Extraction of anthocyanins from red cabbage and purification using adsorption. *Food and Bioproducts Processing,* 90**,** 615-623.

CHEN, M., ZHAO, Y. & YU, S. 2015. Optimisation of ultrasonic-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from sugar beet molasses. *Food Chemistry,* 172**,** 543-550.

CHEOK, C. Y., CHIN, N. L., YUSOF, Y. A., TALIB, R. A. & LAW, C. L. 2013. Optimization of total monomeric anthocyanin (TMA) and total phenolic content (TPC) extractions from mangosteen (Garcinia mangostana Linn.) hull using ultrasonic treatments. *Industrial Crops and Products,* 50**,** 1-7.

CISOWSKA, A., WOJNICZ, D. & HENDRICH, A. B. 2011. Anthocyanins as antimicrobial agents of natural plant origin. *Natural Products Communication,* 6**,** 149-56.

CISSÉ, M., BOHUON, P., SAMBE, F., KANE, C., SAKHO, M. & DORNIER, M. 2012. Aqueous extraction of anthocyanins from Hibiscus sabdariffa: Experimental kinetics and modeling. *Journal of Food Engineering,* 109**,** 16-21.

COOPER, C. E., VOLLAARD, N. B. J., CHOUEIRI, T. & WILSON, M. T. 2002. Exercise, free radicals and oxidative stress. *Biochemical Society Transactions,* 30**,** 280-285.

CORRALES, M., GARCÍA, A. F., BUTZ, P. & TAUSCHER, B. 2009. Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. *Journal of Food Engineering,* 90**,** 415-421.

CORRALES, M., TOEPFL, S., BUTZ, P., KNORR, D. & TAUSCHER, B. 2008. Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison. *Innovative Food Science & Emerging Technologies,* 9**,** 85-91.

DANESH, J., WHINCUP, P., WALKER, M., LENNON, L., THOMSON, A., APPLEBY, P., GALLIMORE, J. R. & PEPYS, M. B. 2000. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. *BMJ,* 321**,** 199-204.

DEFURIA, J., BENNETT, G., STRISSEL, K. J., PERFIELD, J. W., MILBURY, P. E., GREENBERG, A. S. & OBIN, M. S. 2009. Dietary Blueberry Attenuates Whole-Body Insulin Resistance in High Fat-Fed Mice by Reducing Adipocyte Death and Its Inflammatory Sequelae. *The Journal of Nutrition,* 139**,** 1510-1516.

DEGENHARDT, A., HOFMANN, S., KNAPP, H. & WINTERHALTER, P. 2000. Preparative Isolation of Anthocyanins by High-Speed Countercurrent Chromatography and Application of the Color Activity Concept to Red Wine. *Journal of Agricultural and Food Chemistry,* 48**,** 5812-5818.

DEL BO', C., MARTINI, D., VENDRAME, S., RISO, P., CIAPPELLANO, S., KLIMIS-ZACAS, D. & PORRINI, M. 2010. Improvement of lymphocyte resistance against H2O2-induced DNA damage in Sprague–Dawley rats after eight weeks of a wild blueberry (Vaccinium angustifolium)-enriched diet. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 703**,** 158-162.

DENEV, P., CIZ, M., AMBROZOVA, G., LOJEK, A., YANAKIEVA, I. & KRATCHANOVA, M. 2010. Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chemistry,* 123**,** 1055-1061.

DIACONEASA, Z., LEOPOLD, L., RUGINĂ, D., AYVAZ, H. & SOCACIU, C. 2015. Antiproliferative and Antioxidant Properties of Anthocyanin Rich Extracts from Blueberry and Blackcurrant Juice. *International Journal of Molecular Sciences,* 16**,** 2352.

DUTTA, B. K. 2007. *Principles of mass transfer and separation processes.,* New Delhi, India, Prentice-Hall of India.

EBENEZER, P. J., WILSON, C. B., WILSON, L. D., NAIR, A. R. & J, F. 2016. The Anti-Inflammatory Effects of Blueberries in an Animal Model of Post-Traumatic Stress Disorder (PTSD). *PLOS ONE,* 11**,** e0160923.

ELKS, C. M., TERREBONNE, J. D., INGRAM, D. K. & STEPHENS, J. M. 2015. Blueberries improve glucose tolerance without altering body composition in obese postmenopausal mice. *Obesity,* 23**,** 573-580.

FAN, G., HAN, Y., GU, Z. & CHEN, D. 2008. Optimizing conditions for anthocyanins extraction from purple sweet potato using response surface methodology (RSM). *LWT - Food Science and Technology,* 41**,** 155-160.

FLESCHHUT, J., KRATZER, F., RECHKEMMER, G. & KULLING, S. E. 2006. Stability and biotransformation of various dietary anthocyanins in vitro. *European Journal of Nutrition,* 45**,** 7-18.

FOSSEN, T., CABRITA, L. & ANDERSEN, O. M. 1998. Colour and stability of pure anthocyanins influenced by pH including the alkaline region. *Food Chemistry,* 63**,** 435-440.

FREEDMAN, B. J. 1980a. Sulphur dioxide in foods and beverages: its use as a preservative and its effect on asthma. *British journal of diseases of the chest,* 74**,** 128-34.

FREEDMAN, B. J. 1980b. Sulphur dioxide in foods and beverages: its use as a preservative and its effect on asthma. *Br J Dis Chest,* 74**,** 128-34.

FULEKI, T. & FRANCIS, F. J. 1968. Quantitative Methods for Anthocyanins. *Journal of Food Science,* 33**,** 266-274.

FURTADO, P., FIGUEIREDO, P., CHAVES DAS NEVES, H. & PINA, F. 1993. Photochemical and thermal degradation of anthocyanidins. *Journal of Photochemistry and Photobiology A: Chemistry,* 75**,** 113-118.

FUX, C. A., COSTERTON, J. W., STEWART, P. S. & STOODLEY, P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol,* 13**,** 34-40.

GACHOVSKA, T., CASSADA, D., SUBBIAH, J., HANNA, M., THIPPAREDDI, H. & SNOW, D. 2010. Enhanced Anthocyanin Extraction from Red Cabbage Using Pulsed Electric Field Processing. *Journal of Food Science,* 75**,** E323-E329.

GALVÁN D'ALESSANDRO, L., DIMITROV, K., VAUCHEL, P. & NIKOV, I. Kinetics of ultrasound assisted extraction of anthocyanins from Aronia melanocarpa (black chokeberry) wastes. *Chemical Engineering Research and Design*.

GALVÁN D'ALESSANDRO, L., DIMITROV, K., VAUCHEL, P. & NIKOV, I. 2014. Kinetics of ultrasound assisted extraction of anthocyanins from Aronia melanocarpa (black chokeberry) wastes. *Chemical Engineering Research and Design,* 92**,** 1818-1826.

GARCIA-VIGUERA, C., ZAFRILLA, P. & TOMÁS-BARBERÁN, F. A. 1998. The use of acetone as an extraction solvent for anthocyanins from strawberry fruit. *Phytochemical Analysis,* 9**,** 274-277.

GAROFULIĆ, I. E., DRAGOVIĆ-UZELAC, V., REŽEK JAMBRAK, A. & JUKIĆ, M. 2013. The effect of microwave assisted extraction on the isolation of anthocyanins and phenolic acids from sour cherry Marasca (Prunus cerasus var. Marasca). *Journal of Food Engineering,* 117**,** 437-442.

GAVRILOVA, V., KAJDŽANOSKA, M., GJAMOVSKI, V. & STEFOVA, M. 2011. Separation, Characterization and Quantification of Phenolic Compounds in

Blueberries and Red and Black Currants by HPLC−DAD−ESI-MSn. *Journal of Agricultural and Food Chemistry,* 59**,** 4009-4018.

GAZIANO, T. A., PRABHAKARAN, D. & GAZIANO, J. M. 2015. Global bruden of cardiovascular disease. *In:* MANN, D. L., ZIPES, D. P., LIBBY, P. & BONOW, R. O. (eds.) *Traunwald's Heart Disease - A textbook of Cardiovascular Medicine.* 10 ed. Philadelphia, USA: Elsevier Saunders.

GHAFOOR, K., CHOI, Y. H., JEON, J. Y. & JO, I. H. 2009. Optimization of Ultrasound-Assisted Extraction of Phenolic Compounds, Antioxidants, and Anthocyanins from Grape (Vitis vinifera) Seeds. *Journal of Agricultural and Food Chemistry,* 57**,** 4988-4994.

GHAFOOR, K., PARK, J. & CHOI, Y.-H. 2010. Optimization of supercritical fluid extraction of bioactive compounds from grape (Vitis labrusca B.) peel by using response surface methodology. *Innovative Food Science & Emerging Technologies,* 11**,** 485-490.

GIBSON, G. R. & WILLIAMS, C. M. 2000. *Functional foods: Concept to product.,* Cambridge, England, Woodhead Publishing Limited.

GIONGO, L., BOZZA, E., CACIAGLI, P., VALENTE, E., PASQUAZZO, M. T., PEDROLLI, C., IORIO, E. L. & COSTA, A. 2011. Short-term blueberry intake enhances biological antioxidant potential and modulates inflammation markers in overweight and obese children. *Journal of Berry Research,* 1**,** 147-158.

GODOY, C., MONTERUBBIANESI, G. & TOGNETTI, J. 2008. Analysis of highbush blueberry (Vaccinium corymbosum L.) fruit growth with exponential mixed models. *Scientia Horticulturae,* 115**,** 368-376.

GOLMOHAMADI, A., MÖLLER, G., POWERS, J. & NINDO, C. 2013. Effect of ultrasound frequency on antioxidant activity, total phenolic and anthocyanin content of red raspberry puree. *Ultrasonics Sonochemistry,* 20**,** 1316-1323.

GOULD, K., DAVIES, K. M. & WINEFIELD, C. 2008. *Anthocyanins: Biosynthesis, Functions, and Applications*, Springer New York.

GRAF, B. A., MILBURY, P. E. & BLUMBERG, J. B. 2005. Flavonols, Flavones, Flavanones, and Human Health: Epidemiological Evidence. *Journal of Medicinal Food,* 8**,** 281-290.

HE, J. & GIUSTI, M. M. 2011. High-purity isolation of anthocyanins mixtures from fruits and vegetables – A novel solid-phase extraction method using mixed mode cation-exchange chromatography. *Journal of Chromatography A,* 1218**,** 7914-7922.

HEINONEN, I. M., MEYER, A. S. & FRANKEL, E. N. 1998. Antioxidant Activity of Berry Phenolics on Human Low-Density Lipoprotein and Liposome Oxidation. *Journal of Agricultural and Food Chemistry,* 46**,** 4107-4112.

HERMAN, G. A., BERGMAN, A., STEVENS, C., KOTEY, P., YI, B., ZHAO, P., DIETRICH, B., GOLOR, G., SCHRODTER, A. & KEYMEULEN, B. 2006. Effect of single oral doses of sitagliptin, a dipeptidyl peptidase-4 inhibitor, on incretin and plasma glucose levels after an oral glucose tolerance test in patients with type 2 diabetes. *The Journal of Clinical Endocrinology & Metabolism,* 91**,** 4612-4619.

HIDALGO, M., ORUNA-CONCHA, M. J., KOLIDA, S., WALTON, G. E., KALLITHRAKA, S., SPENCER, J. P. & DE PASCUAL-TERESA, S. 2012. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J Agric Food Chem,* 60**,** 3882-90.

HILL, J. O. & PETERS, J. C. 1998. Environmental Contributions to the Obesity Epidemic. *Science,* 280**,** 1371-1374.

HUANG, W.-Y., LIU, Y.-M., WANG, J., WANG, X.-N. & LI, C.-Y. 2014. Anti-Inflammatory Effect of the Blueberry Anthocyanins Malvidin-3-Glucoside and Malvidin-3-Galactoside in Endothelial Cells. *Molecules,* 19**,** 12827.

HUANG, W., ZHU, Y., LI, C., SUI, Z. & MIN, W. 2016. Effect of Blueberry Anthocyanins Malvidin and Glycosides on the Antioxidant Properties in Endothelial Cells. *Oxidative Medicine and Cellular Longevity,* 2016**,** 10.

HUIE, C. 2002. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry,* 373**,** 23-30.

JAAKOLA, L., MÄÄTTÄ-RIIHINEN, K., KÄRENLAMPI, S. & HOHTOLA, A. 2004. Activation of flavonoid biosynthesis by solar radiation in bilberry (Vaccinium myrtillus L.) leaves. *Planta,* 218**,** 721-728.

JAMPANI, C., NAIK, A. & RAGHAVARAO, K. S. M. S. 2014. Purification of anthocyanins from jamun (Syzygium cumini L.) employing adsorption. *Separation and Purification Technology,* 125**,** 170-178.

JO, Y. N., JIN, D. E., JEONG, J. H., KIM, H. J., KIM, D.-O. & HEO, H. J. 2015. Effect of anthocyanins from rabbit-eye blueberry (Vaccinium virgatum) on cognitive function in mice under trimethyltin-induced neurotoxicity. *Food Science and Biotechnology,* 24**,** 1077-1085.

JU, Z. Y. & HOWARD, L. R. 2003. Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red Grape Skin. *Journal of Agricultural and Food Chemistry,* 51**,** 5207-5213.

JUNIOR, M. R. M., LEITE, A. V. & DRAGANO, N. R. V. 2010. Supercritical fluid extraction and stabilization of phenolic compounds from natural sources—review (supercritical extraction and stabilization of phenolic compounds). *Open Chem Eng J,* 4**,** 51-60.

KADER, F., HALUK, J.-P., NICOLAS, J.-P. & METCHE, M. 1998. Degradation of Cyanidin 3-Glucoside by Blueberry Polyphenol Oxidase: Kinetic Studies and Mechanisms. *Journal of Agricultural and Food Chemistry,* 46**,** 3060-3065.

KADER, F., ROVEL, B., GIRARDIN, M. & METCHE, M. 1996. Fractionation and identification of the phenolic compounds of Highbush blueberries (Vaccinium corymbosum, L.). *Food Chemistry,* 55**,** 35-40.

KALT, W., FORNEY, C. F., MARTIN, A. & PRIOR, R. L. 1999. Antioxidant Capacity, Vitamin C, Phenolics, and Anthocyanins after Fresh Storage of Small Fruits. *Journal of Agricultural and Food Chemistry,* 47**,** 4638-4644.

KALT, W., RYAN, D. A., DUY, J. C., PRIOR, R. L., EHLENFELDT, M. K. & VANDER KLOET, S. P. 2001. Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (Vaccinium section cyanococcus spp.). *Journal Agricultural Food Chemistry,* 49**,** 4761-7.

KAPASAKALIDIS, P. G., RASTALL, R. A. & GORDON, M. H. 2006. Extraction of Polyphenols from Processed Black Currant (Ribes nigrum L.) Residues. *Journal of Agricultural and Food Chemistry,* 54**,** 4016-4021.

KAY, C. D. 2006. Aspects of anthocyanin absorption, metabolism and pharmacokinetics in humans. *Nutr Res Rev,* 19**,** 137-46.

KHALIFA, H. O., KAMIMOTO, M., SHIMAMOTO, T. & SHIMAMOTO, T. 2015. Antimicrobial Effects of Blueberry, Raspberry, and Strawberry Aqueous Extracts and their Effects on Virulence Gene Expression in Vibrio cholerae. *Phytotherapy Research,* 29**,** 1791-1797.

KONG, J.-M., CHIA, L.-S., GOH, N.-K., CHIA, T.-F. & BROUILLARD, R. 2003. Analysis and biological activities of anthocyanins. *Phytochemistry,* 64**,** 923-933.

KOSTADINOVIK, S., MIRHOSSEINI, H. & BOGEVA, E. 2013. Isolation of Anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to different varieties of red grape pomace from Macedonia. *Journal of Nutrition and Food Science,* 3.

KOU, X., HAN, L., LI, X., XUE, Z. & ZHOU, F. 2016. Antioxidant and antitumor effects and immunomodulatory activities of crude and purified polyphenol extract from blueberries. *Frontiers of Chemical Science and Engineering,* 10**,** 108-119.

KRIKORIAN, R., SHIDLER, M. D., NASH, T. A., KALT, W., VINQVIST-TYMCHUK, M. R., SHUKITT-HALE, B. & JOSEPH, J. A. 2010. Blueberry Supplementation Improves Memory in Older Adults. *Journal of Agricultural and Food Chemistry,* 58**,** 3996-4000.

KYUNG, K. H. & FLEMING, H. P. 1997. Antimicrobial Activity of Sulfur Compounds Derived from Cabbage. *Journal of Food Protection,* 60**,** 67-71.

LACOMBE, A., WU, V. C. H., WHITE, J., TADEPALLI, S. & ANDRE, E. E. 2012. The antimicrobial properties of the lowbush blueberry (Vaccinium angustifolium) fractional components against foodborne pathogens and the conservation of probiotic Lactobacillus rhamnosus. *Food Microbiology,* 30**,** 124-131.

LANDBO, A.-K. & MEYER, A. S. 2001. Enzyme-Assisted Extraction of Antioxidative Phenols from Black Currant Juice Press Residues (Ribes nigrum). *Journal of Agricultural and Food Chemistry,* 49**,** 3169-3177.

LEE, J., DURST, R. W. & WROLSTAD, R. E. 2002. Impact of Juice Processing on Blueberry Anthocyanins and Polyphenolics: Comparison of Two Pretreatments. *Journal of Food Science,* 67**,** 1660-1667.

LI, Y., HAN, L., MA, R., XU, X., ZHAO, C., WANG, Z., CHEN, F. & HU, X. 2012. Effect of energy density and citric acid concentration on anthocyanins yield and solution temperature of grape peel in microwave-assisted extraction process. *Journal of Food Engineering,* 109**,** 274-280.

LI, Y. & ZHAO, M. 2006. Simple methods for rapid determination of sulfite in food products. *Food Control,* 17**,** 975-980.

LIAZID, A., GUERRERO, R. F., CANTOS, E., PALMA, M. & BARROSO, C. G. 2011. Microwave assisted extraction of anthocyanins from grape skins. *Food Chemistry,* 124**,** 1238-1243.

LIBBY, P. 2007. Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutr Rev,* 65**,** S140-6.

LIU, W., CHEN, J., LI, Q. & SUN, A. 2016. Inhibitory effects of acylated blueberry anthocyanin on H22 murine tumors. *Food and Agricultural Immunology*, 27**,** 509-522.

LIU, W., LU, X., HE, G., GAO, X., LI, M., WU, J., LI, Z., WU, J., WANG, J. & LUO, C. 2013. Cytosolic protection against ultraviolet induced DNA damage by blueberry anthocyanins and anthocyanidins in hepatocarcinoma HepG2 cells. *Biotechnology Letters,* 35**,** 491-498.

LOYPIMAI, P., MOONGNGARM, A., CHOTTANOM, P. & MOONTREE, T. 2015. Ohmic heating-assisted extraction of anthocyanins from black rice bran to prepare a natural food colourant. *Innovative Food Science & Emerging Technologies,* 27**,** 102-110.

MAEKAJI, K., SUNAGAWA, M. & IMAI, H. 1963. The Anthocyanins in the Petals of White Peach (Prunus percica SlBE et ZUCC var. vulgaris MAXIM.). *Agricultural and Biological Chemistry,* 27**,** 165-169.

MARAN, J. P., PRIYA, B. & MANIKANDAN, S. 2014. Modeling and optimization of supercritical fluid extraction of anthocyanin and phenolic compounds from Syzygium cumini fruit pulp. *Journal of Food Science and Technology,* 51**,** 1938-1946.

MATHEW, S., ABRAHAM, T. E. & ZAKARIA, Z. A. 2015. Reactivity of phenolic compounds towards free radicals under in vitro conditions. *Journal of Food Science and Technology,* 52**,** 5790-5798.

MATTICK, L. R., WEIRS, L. D. & ROBINSON, W. B. 1969. The Effect of pH on the Precipitation of the Anthocyanins of Wine as Their Lead Salts. *American Journal of Enology and Viticulture,* 20**,** 206-212.

MAZZA, G., KAY, C. D., COTTRELL, T. & HOLUB, B. J. 2002. Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Human Subjects. *Journal of Agricultural and Food Chemistry,* 50**,** 7731-7737.

MCANULTY, L. S., NIEMAN, D. C., DUMKE, C. L., SHOOTER, L. A., HENSON, D. A., UTTER, A. C., MILNE, G. & MCANULTY, S. R. 2011. Effect of blueberry ingestion on natural killer cell counts, oxidative stress, and inflammation prior to and after 2.5 h of running. *Applied Physiology, Nutrition, and Metabolism,* 36**,** 976-984.

MEIRELES, M., MARQUES, C., NORBERTO, S., SANTOS, P., FERNANDES, I., MATEUS, N., FARIA, A. & CALHAU, C. 2016. Anthocyanin effects on microglia M1/M2 phenotype: Consequence on neuronal fractalkine expression. *Behav Brain Res,* 305**,** 223-8.

METIVIER, R. P., FRANCIS, F. J. & CLYDESDALE, F. M. 1980. Solvent Extraction of Anthocyanins From Wine Pomace. *Journal of Food Science,* 45**,** 1099-1100.

MILBURY, P. E. & RICHER, A. C. 2008. *Understanding the Antioxidant Controversy: Scrutinizing the "fountain of Youth"*, Praeger.

MORI, K., GOTO-YAMAMOTO, N., KITAYAMA, M. & HASHIZUME, K. 2007. Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany,* 58**,** 1935-1945.

MORTON, L. W., CACCETTA, R. A.-A., PUDDEY, I. B. & CROFT, K. D. 2000. Chemistry And Biological Effects Of Dietary Phenolic Compounds: Relevance To Cardiovascular Disease. *Clinical and Experimental Pharmacology and Physiology,* 27**,** 152-159.

MUSSINAN, C. J. & KEELAN, M. E. Sulfur compounds in foods: an overview. ACS symposium series, 1994.

NARAYAN, M., NAIDU, K. A., RAVISHANKAR, G., SRINIVAS, L. & VENKATARAMAN, L. 1999. Antioxidant effect of anthocyanin on enzymatic and non-enzymatic lipid peroxidation. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA),* 60**,** 1-4.

NETO, C. C. 2007. Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition & Food Research,* 51**,** 652-664.

O'BRIEN, M. C., MCCONNON, A., HOLLYWOOD, L. E., CUSKELLY, G. J., BARNETT, J., RAATS, M. & DEAN, M. 2015. Let's talk about health: shoppers' discourse regarding health while food shopping. *Public Health Nutrition,* 18**,** 1001-1010.

OANCEA, S., GROSU, C., KETNEY, O. & STOIA, M. 2013. *Conventional and Ultrasound-Assisted Extraction of Anthocyanins from Blackberry and Sweet Cherry Cultivars*.

ODRIOZOLA-SERRANO, I., SOLIVA-FORTUNY, R. & MARTÍN-BELLOSO, O. 2009. Impact of high-intensity pulsed electric fields variables on vitamin C, anthocyanins and antioxidant capacity of strawberry juice. *LWT - Food Science and Technology,* 42**,** 93-100.

PAES, J., DOTTA, R., BARBERO, G. F. & MARTÍNEZ, J. 2014. Extraction of phenolic compounds and anthocyanins from blueberry (Vaccinium myrtillus L.) residues using supercritical CO2 and pressurized liquids. *The Journal of Supercritical Fluids,* 95**,** 8-16.

PAP, N., BESZÉDES, S., PONGRÁCZ, E., MYLLYKOSKI, L., GÁBOR, M., GYIMES, E., HODÚR, C. & KEISKI, R. 2013. Microwave-Assisted Extraction of Anthocyanins from Black Currant Marc. *Food and Bioprocess Technology,* 6**,** 2666-2674.

PATIL, G., MADHUSUDHAN, M. C., RAVINDRA BABU, B. & RAGHAVARAO, K. S. M. S. 2009. Extraction, dealcoholization and concentration of anthocyanin from red radish. *Chemical Engineering and Processing: Process Intensification,* 48**,** 364-369.

PAULA, J. T., PAVIANI, L. C., FOGLIO, M. A., SOUSA, I. M. O. & CABRAL, F. A. 2013. Extraction of anthocyanins from Arrabidaea chica in fixed bed using CO2 and CO2/ethanol/water mixtures as solvents. *The Journal of Supercritical Fluids,* 81**,** 33-41.

PAULA, J. T., PAVIANI, L. C., FOGLIO, M. A., SOUSA, I. M. O., DUARTE, G. H. B., JORGE, M. P., EBERLIN, M. N. & CABRAL, F. A. 2014. Extraction of anthocyanins and luteolin from Arrabidaea chica by sequential extraction in fixed bed using supercritical CO2, ethanol and water as solvents. *The Journal of Supercritical Fluids,* 86**,** 100-107.

PEARCE, R. S. 2001. Plant Freezing and Damage. *Annals of Botany,* 87**,** 417-424.

PEPE, G., GROSSI, M. R., BERNI, A., FILIPPI, S., SHANMUGAKANI, R. K., PAPESCHI, C., MOSESSO, P., NATARAJAN, A. T. & PALITTI, F. 2013. Effect of blueberries (BB) on micronuclei induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz(a)anthracene (DMBA) in mammalian cells, assessed in in vitro and in vivo assays. *Mutation Research,* 758**,** 6-11.

PERTUZATTI, P. B., BARCIA, M. T., REBELLO, L. P. G., GÓMEZ-ALONSO, S., DUARTE, R. M. T., DUARTE, M. C. T., GODOY, H. T. & HERMOSÍN-GUTIÉRREZ, I. 2016. Antimicrobial activity and differentiation of anthocyanin profiles of rabbiteye and highbush blueberries using HPLC–DAD–ESI-MSn and multivariate analysis. *Journal of Functional Foods,* 26**,** 506-516.

PETERSSON, E. V., LIU, J., SJÖBERG, P. J. R., DANIELSSON, R. & TURNER, C. 2010. Pressurized Hot Water Extraction of anthocyanins from red onion: A study on extraction and degradation rates. *Analytica Chimica Acta,* 663**,** 27-32.

PILJAC-ŽEGARAC, J., BELŠČAK, A. & PILJAC, A. 2009. Antioxidant Capacity and Polyphenolic Content of Blueberry (Vaccinium corymbosum L.) Leaf Infusions. *Journal of Medicinal Food,* 12**,** 608-614.

PRIOR, R. L., CAO, G., MARTIN, A., SOFIC, E., MCEWEN, J., O'BRIEN, C., LISCHNER, N., EHLENFELDT, M., KALT, W., KREWER, G. & MAINLAND, C. M. 1998. Antioxidant Capacity As Influenced by Total Phenolic and Anthocyanin Content, Maturity, and Variety of Vaccinium Species. *Journal of Agricultural and Food Chemistry,* 46**,** 2686-2693.

PRIOR, R. L., E. WILKES, S., R. ROGERS, T., KHANAL, R. C., WU, X. & HOWARD, L. R. 2010. Purified Blueberry Anthocyanins and Blueberry Juice Alter Development of Obesity in Mice Fed an Obesogenic High-Fat Diet. *Journal of Agricultural and Food Chemistry,* 58**,** 3970-3976.

PRIOR, R. L., LAZARUS, S. A., CAO, G., MUCCITELLI, H. & HAMMERSTONE, J. F. 2001. Identification of Procyanidins and Anthocyanins in Blueberries and Cranberries (Vaccinium Spp.) Using High-Performance Liquid Chromatography/Mass Spectrometry. *Journal of Agricultural and Food Chemistry,* 49**,** 1270-1276.

PRIOR, R. L., WU, X., GU, L., HAGER, T. J., HAGER, A. & HOWARD, L. R. 2008. Whole Berries versus Berry Anthocyanins: Interactions with Dietary Fat Levels in the C57BL/6J Mouse Model of Obesity. *Journal of Agricultural and Food Chemistry,* 56**,** 647-653.

PUÉRTOLAS, E., CREGENZÁN, O., LUENGO, E., ÁLVAREZ, I. & RASO, J. Pulsed-electric-field-assisted extraction of anthocyanins from purple-fleshed potato. *Food Chemistry,* 136**,** 1330-1336.

RAMIREZ-TORTOSA, C., ANDERSEN, O. M., CABRITA, L., GARDNER, P. T., MORRICE, P. C., WOOD, S. G., DUTHIE, S. J., COLLINS, A. R. & DUTHIE, G. G. 2001. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biology & Medicine,* 31**,** 1033-7.

REVILLA, E., RYAN, J.-M. & MARTÍN-ORTEGA, G. 1998. Comparison of Several Procedures Used for the Extraction of Anthocyanins from Red Grapes. *Journal of Agricultural and Food Chemistry,* 46**,** 4592-4597.

RISO, P., KLIMIS-ZACAS, D., DEL BO', C., MARTINI, D., CAMPOLO, J., VENDRAME, S., MØLLER, P., LOFT, S., DE MARIA, R. & PORRINI, M. 2013. Effect of a wild blueberry (Vaccinium angustifolium) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors. *European Journal of Nutrition,* 52**,** 949-961.

ROSSI, M., NEGRI, E., BOSETTI, C., PELUCCHI, C. & LA VECCHIA, C. 2009. Epidemiology behind fruit and vegetable consumption and cancer risk with focus on flavonoids. *In:* FRAGA, C. G. (ed.) *Plant phenolics and human health: Biochemistry, nutrition and pharmacology.* New Jersey, USA: John Wiley & Sons Inc.

ROSTAGNO, M. A. & PRADO, J. M. 2013. *Natural Product Extraction: Principles and Applications,* Royal Society of Chemistry.

ROUTRAY, W. & ORSAT, V. 2011. Blueberries and Their Anthocyanins: Factors Affecting Biosynthesis and Properties. *Comprehensive Reviews in Food Science and Food Safety,* 10**,** 303-320.

SADILOVA, E., CARLE, R. & STINTZING, F. C. 2007. Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Molecular Nutrition & Food Research,* 51**,** 1461-1471.

SANJABI, S., ZENEWICZ, L. A., KAMANAKA, M. & FLAVELL, R. A. 2009. Anti- and Pro-inflammatory Roles of TGF-β, IL-10, and IL-22 In Immunity and Autoimmunity. *Current opinion in pharmacology,* 9**,** 447-453.

SANTOS, D. T., VEGGI, P. C. & MEIRELES, M. A. A. 2012. Optimization and economic evaluation of pressurized liquid extraction of phenolic compounds from jabuticaba skins. *Journal of Food Engineering,* 108**,** 444-452.

SARKIS, J. R., JAESCHKE, D. P., TESSARO, I. C. & MARCZAK, L. D. F. 2013. Effects of ohmic and conventional heating on anthocyanin degradation during the processing of blueberry pulp. *LWT - Food Science and Technology,* 51**,** 79-85.

SCHWARZ, M., HILLEBRAND, S., HABBEN, S., DEGENHARDT, A. & WINTERHALTER, P. 2003. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochemical Engineering Journal,* 14**,** 179-189.

SEABRA, I. J., BRAGA, M. E. M., BATISTA, M. T. & DE SOUSA, H. C. 2010. Effect of solvent (CO2/ethanol/H2O) on the fractionated enhanced solvent extraction of anthocyanins from elderberry pomace. *The Journal of Supercritical Fluids,* 54**,** 145-152.

SEERAM, N. P. 2008. Berry fruits for cancer prevention: current status and future prospects. *Journal of Agricultural Food Chemistry,* 56**,** 630-5.

SEERAM, N. P., ADAMS, L. S., ZHANG, Y., LEE, R., SAND, D., SCHEULLER, H. S. & HEBER, D. 2006. Blackberry, Black Raspberry, Blueberry, Cranberry, Red Raspberry, and Strawberry Extracts Inhibit Growth and Stimulate Apoptosis of Human Cancer Cells In Vitro. *Journal of Agricultural and Food Chemistry,* 54**,** 9329-9339.

SEERAM, N. P., BOURQUIN, L. D. & NAIR, M. G. 2001. Degradation Products of Cyanidin Glycosides from Tart Cherries and Their Bioactivities. *Journal of Agricultural and Food Chemistry,* 49**,** 4924-4929.

SEGOVIA, F. J., LUENGO, E., CORRAL-PÉREZ, J. J., RASO, J. & ALMAJANO, M. P. Improvements in the aqueous extraction of polyphenols from borage (Borago officinalis L.) leaves by pulse electric fields: Pulsed electric fields (PEF) applications. *Industrial Crops and Products*.

SEYMOUR, E. M., TANONE, I. I., URCUYO-LLANES, D. E., LEWIS, S. K., KIRAKOSYAN, A., KONDOLEON, M. G., KAUFMAN, P. B. & BOLLING, S. F. 2011. Blueberry Intake Alters Skeletal Muscle and Adipose Tissue Peroxisome Proliferator-Activated Receptor Activity and Reduces Insulin Resistance in Obese Rats. *Journal of Medicinal Food,* 14**,** 1511-1518.

SHAKED-SACHRAY, L., WEISS, D., REUVENI, M., NISSIM-LEVI, A. & OREN-SHAMIR, M. 2002. Increased anthocyanin accumulation in aster flowers at elevated temperatures due to magnesium treatment. *Physiol Plant,* 114**,** 559-565.

SHEN, X., SUN, X., XIE, Q., LIU, H., ZHAO, Y., PAN, Y., HWANG, C.-A. & WU, V. C. H. 2014. Antimicrobial effect of blueberry (Vaccinium corymbosum L.) extracts against the growth of Listeria monocytogenes and Salmonella Enteritidis. *Food Control,* 35**,** 159-165.

SHUKITT-HALE, B., LAU, F. C., CAREY, A. N., GALLI, R. L., SPANGLER, E. L., INGRAM, D. K. & JOSEPH, J. A. 2008. Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutritional Neuroscience,* 11**,** 172-182.

SIES, H. 2007. Total antioxidant capacity: appraisal of a concept. *J Nutr,* 137**,** 1493-5.

SILVA, S., COSTA, E. M., COSTA, M. R., PEREIRA, M. F., PEREIRA, J. O., SOARES, J. C. & PINTADO, M. M. 2015. Aqueous extracts of Vaccinium corymbosum as inhibitors of Staphylococcus aureus. *Food Control,* 51**,** 314-320.

SINHA, K., SAHA, P. D. & DATTA, S. 2012. Response surface optimization and artificial neural network modeling of microwave assisted natural dye extraction from pomegranate rind. *Industrial Crops and Products,* 37**,** 408-414.

SKREDE, G., WROLSTAD, R. E. & DURST, R. W. 2000. Changes in Anthocyanins and Polyphenolics During Juice Processing of Highbush Blueberries (Vaccinium corymbosum L.). *Journal of Food Science,* 65**,** 357-364.

SOCACIU, C. 2007. *Food Colorants: Chemical and Functional Properties,* CRC Press.

SONG, Y., PARK, H. J., KANG, S. N., JANG, S.-H., LEE, S.-J., KO, Y.-G., KIM, G.-S. & CHO, J.-H. 2013. Blueberry Peel Extracts Inhibit Adipogenesis in 3T3-L1 Cells and Reduce High-Fat Diet-Induced Obesity. *PLoS ONE,* 8**,** e69925.

SOUSA, M. B., CURADO, T., NEGRÃO E VASCONCELOS, F. & TRIGO, M. J. 2007. Mirtilo - Qualidade pós-colheita. *Divulgação AGRO,* 8.

STINTZING, F. C. & CARLE, R. 2004. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology,* 15**,** 19-38.

STULL, A. J., CASH, K. C., JOHNSON, W. D., CHAMPAGNE, C. M. & CEFALU, W. T. 2010. Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. *The Journal of nutrition,* 140**,** 1764-1768.

SUN, Y., LIAO, X., WANG, Z., HU, X. & CHEN, F. 2007. Optimization of microwave-assisted extraction of anthocyanins in red raspberries and identification of anthocyanin of extracts using high-performance liquid chromatography – mass spectrometry. *European Food Research and Technology,* 225**,** 511-523.

SZAJDEK, A. & BOROWSKA, E. J. 2008. Bioactive Compounds and Health-Promoting Properties of Berry Fruits: A Review. *Plant Foods for Human Nutrition,* 63**,** 147-156.

TAKIKAWA, M., INOUE, S., HORIO, F. & TSUDA, T. 2010. Dietary Anthocyanin-Rich Bilberry Extract Ameliorates Hyperglycemia and Insulin Sensitivity via Activation of AMP-Activated Protein Kinase in Diabetic Mice. *The Journal of Nutrition,* 140**,** 527-533.

TANCHEV, S. & JONCHEVA, N. 1973. Kinetics of the thermal degradation of cyanidin-3-rutinoside and peonidin-3-rutinoside. *Zeitschrift für Lebensmittel-Untersuchung und Forschung,* 153**,** 37-41.

TARUSCIO, T. G., BARNEY, D. L. & EXON, J. 2004. Content and Profile of Flavanoid and Phenolic Acid Compounds in Conjunction with the Antioxidant Capacity for a Variety of Northwest Vaccinium Berries. *Journal of Agricultural and Food Chemistry,* 52**,** 3169-3176.

TENG, H., LEE, W. Y. & CHOI, Y. H. 2013. Optimization of microwave-assisted extraction for anthocyanins, polyphenols, and antioxidants from raspberry (Rubus CoreanusMiq.) using response surface methodology. *Journal of Separation Science,* 36**,** 3107-3114.

TIWARI, B. K., O'DONNELL, C. P., PATRAS, A. & CULLEN, P. J. 2008. Anthocyanin and Ascorbic Acid Degradation in Sonicated Strawberry Juice. *Journal of Agricultural and Food Chemistry,* 56**,** 10071-10077.

TORRI, E., LEMOS, M., CALIARI, V., KASSUYA, C. A. L., BASTOS, J. K. & ANDRADE, S. F. 2007. Anti-inflammatory and antinociceptive properties of blueberry extract (Vaccinium corymbosum). *Journal of Pharmacy and Pharmacology,* 59**,** 591-596.

TREHANE, J. 2009. *Blueberries, Cranberries and Other Vacciniums*, Timber Press, Incorporated.

TRUONG, V. D., HU, Z., THOMPSON, R. L., YENCHO, G. C. & PECOTA, K. V. 2012. Pressurized liquid extraction and quantification of anthocyanins in purple-fleshed sweet potato genotypes. *Journal of Food Composition and Analysis,* 26**,** 96-103.

TSAI, P. J., DELVA, L., YU, T. Y., HUANG, Y. T. & DUFOSSÉ, L. 2005. Effect of sucrose on the anthocyanin and antioxidant capacity of mulberry extract during high temperature heating. *Food Research International,* 38**,** 1059-1065.

TSUDA, T. 2015. Possible abilities of dietary factors to prevent and treat diabetes via the stimulation of glucagon-like peptide-1 secretion. *Molecular Nutrition & Food Research,* 59**,** 1264-1273.

TÜRKER, N. & ERDOGˇDU, F. 2006. Effects of pH and temperature of extraction medium on effective diffusion coefficient of anthocynanin pigments of black carrot (Daucus carota var. L.). *Journal of Food Engineering,* 76**,** 579-583.

VATAI, T., ŠKERGET, M. & KNEZ, Ž. 2009. Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. *Journal of Food Engineering,* 90**,** 246-254.

VATAI, T., ŠKERGET, M., KNEZ, Ž., KARETH, S., WEHOWSKI, M. & WEIDNER, E. 2008. Extraction and formulation of anthocyanin-concentrates from grape residues. *The Journal of Supercritical Fluids,* 45**,** 32-36.

VENDRAME, S., DAUGHERTY, A., KRISTO, A. S. & KLIMIS-ZACAS, D. 2014. Wild blueberry (Vaccinium angustifolium)-enriched diet improves dyslipidaemia and modulates the expression of genes related to lipid metabolism in obese Zucker rats. *British Journal of Nutrition,* 111**,** 194-200.

VENDRAME, S., DAUGHERTY, A., KRISTO, A. S., RISO, P. & KLIMIS-ZACAS, D. 2013. Wild blueberry (Vaccinium angustifolium) consumption improves inflammatory status in the obese Zucker rat model of the metabolic syndrome. *The Journal of Nutritional Biochemistry,* 24**,** 1508-1512.

VENDRAME, S., GUGLIELMETTI, S., RISO, P., ARIOLI, S., KLIMIS-ZACAS, D. & PORRINI, M. 2011. Six-Week Consumption of a Wild Blueberry Powder Drink Increases Bifidobacteria in the Human Gut. *Journal of Agricultural and Food Chemistry,* 59**,** 12815-12820.

VERMERRIS, W. & NICHOLSON, R. L. 2007. Chapter3: Biosynthesis of phenolic compounds. *Phenolic Compound Biochemistry.* Springer.

VIEIRA, G. S., CAVALCANTI, R. N., MEIRELES, M. A. A. & HUBINGER, M. D. 2013. Chemical and economic evaluation of natural antioxidant extracts obtained by ultrasound-assisted and agitated bed extraction from jussara pulp (Euterpe edulis). *Journal of Food Engineering,* 119**,** 196-204.

VILSBØLL, T., BROCK, B., PERRILD, H., LEVIN, K., LERVANG, H. H., KØLENDORF, K., KRARUP, T., SCHMITZ, O., ZDRAVKOVIC, M. & LE-THI, T. 2008. Liraglutide, a once-daily human GLP-1 analogue, improves pancreatic B-cell function and arginine-stimulated insulin secretion during hyperglycaemia in patients with Type 2 diabetes mellitus. *Diabetic Medicine,* 25**,** 152-156.

VIVIER, E., TOMASELLO, E., BARATIN, M., WALZER, T. & UGOLINI, S. 2008. Functions of natural killer cells. *Nature Immunology,* 9**,** 503-510.

VRHOVSEK, U., MASUERO, D., PALMIERI, L. & MATTIVI, F. 2012. Identification and quantification of flavonol glycosides in cultivated blueberry cultivars. *Journal of Food Composition and Analysis,* 25**,** 9-16.

WANG, W.-D. & XU, S.-Y. 2007. Degradation kinetics of anthocyanins in blackberry juice and concentrate. *Journal of Food Engineering,* 82**,** 271-275.

WANG, Y., CHANG, C.-F., CHOU, J., CHEN, H.-L., DENG, X., HARVEY, B. K., CADET, J. L. & BICKFORD, P. C. 2005. Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage. *Experimental Neurology,* 193**,** 75-84.

WESCHE-EBELING, P. & MONTGOMERY, M. W. 1990. Strawberry Polyphenoloxidase: Its Role in Anthocyanin Degradation. *Journal of Food Science,* 55**,** 731-734.

WIGHTMAN, J. D. & WROLSTAD, R. E. 1995. Anthocyanin analysis as a measure of glycosidase activity in enzymes for juice processing. *Journal of food science,* 60**,** 862-867.

WILD, S., ROGLIC, G., GREEN, A., SICREE, R. & KING, H. 2004. Global Prevalence of Diabetes. *Estimates for the year 2000 and projections for 2030,* 27**,** 1047-1053.

WROLSTAD, R. E., WIGHTMAN, J. D. & DURST, R. W. 1994. Glycosidase activity of enzyme preparations used in fruit juice processing. *Food Technology,* 48**,** 90-98.

WU, L. H., XU, Z. L., DONG, D., HE, S. A. & YU, H. 2011. Protective Effect of Anthocyanins Extract from Blueberry on TNBS-Induced IBD Model of Mice. *Evidence-based Complementary and Alternaternative Medicine: eCAM,* 2011**,** 525462.

WU, X., CAO, G. & PRIOR, R. L. 2002. Absorption and Metabolism of Anthocyanins in Elderly Women after Consumption of Elderberry or Blueberry. *The Journal of Nutrition,* 132**,** 1865-1871.

YAO, Y. & VIEIRA, A. 2007. Protective activities of Vaccinium antioxidants with potential relevance to mitochondrial dysfunction and neurotoxicity. *NeuroToxicology,* 28**,** 93-100.

YOSHIDA, K., KITAHARA, S., ITO, D. & KONDO, T. 2006. Ferric ions involved in the flower color development of the Himalayan blue poppy, Meconopsis grandis. *Phytochemistry,* 67**,** 992-8.

YOUDIM, K. A., MARTIN, A. & JOSEPH, J. A. 2000a. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radical Biology & Medicine,* 29**,** 51-60.

YOUDIM, K. A., SHUKITT-HALE, B., MACKINNON, S., KALT, W. & JOSEPH, J. A. 2000b. Polyphenolics enhance red blood cell resistance to oxidative stress: in vitro and in vivo. *Biochimica et Biophysica Acta,* 1523**,** 117-22.

ZHANG, X.-M. & ZHU, J. 2011. Kainic Acid-Induced Neurotoxicity: Targeting Glial Responses and Glia-Derived Cytokines. *Current Neuropharmacology,* 9**,** 388-398.

ZHANG, Y., LIAO, X., NI, Y., WU, J., HU, X., WANG, Z. & CHEN, F. 2007. Kinetic analysis of the degradation and its color change of cyanidin-3-glucoside exposed to pulsed electric field. *European Food Research and Technology,* 224**,** 597-603.

ZHAO, M., LIU, X., LUO, Y., GUO, H., HU, X. & CHEN, F. 2015. Evaluation of Protective Effect of Freeze-Dried Strawberry, Grape, and Blueberry Powder on Acrylamide Toxicity in Mice. *Journal of Food Science,* 80**,** H869-H874.

ZHAO, Y. 2007. *Berry Fruit: Value-Added Products for Health Promotion,* CRC Press.

ZHENG, X., XU, X., LIU, C., SUN, Y., LIN, Z. & LIU, H. 2013. Extraction characteristics and optimal parameters of anthocyanin from blueberry powder under microwave-assisted extraction conditions. *Separation and Purification Technology,* 104**,** 17-25.

ZIMMER, K. R., BLUM-SILVA, C. H., SOUZA, A. L. K., WULFFSCHUCH, M., REGINATTO, F. H., PEREIRA, C. M. P., MACEDO, A. J. & LENCINA, C. L. 2014. The Antibiofilm Effect of Blueberry Fruit Cultivars Against Staphylococcus epidermidis and Pseudomonas aeruginosa. *Journal of Medicinal Food,* 17**,** 324-331.

ZOU, T., WANG, D., GUO, H., ZHU, Y., LUO, X., LIU, F. & LING, W. 2012. Optimization of Microwave-Assisted Extraction of Anthocyanins from Mulberry and Identification of Anthocyanins in Extract Using HPLC-ESI-MS. *Journal of Food Science,* 77**,** C46-C50.

Objectives

In Portugal, the area dedicated to the production of blueberries has suffered a significant increase in the last decade, with a similar trend being observed in other countries. Therefore, any attempt to circumvent a loss of value resulting from a rising supply of blueberries may be important to the economy in coming years. One of the possible ways to accomplish this uses the consumer's perception of the fruit itself. Sold by the media as a "superfruit", any product with added blueberries or blueberry products has an almost automatic healthy connotation, a perception that automatically raises the products value. Blueberry extracts may pose an interning alternative, as they are easier to incorporate into products than full blueberries. Furthermore, anthocyanin rich blueberry extracts pose as an interesting, natural alternative to traditional food colorants and antioxidants as they combine both in a single product and consequently all related health effects.

Considering these arguments, the main objective of this thesis was to produce a blueberry extract, with an elevated anthocyanin concentration, with demonstrated health promoting properties. To accomplish this goal several specific objectives were established: i) selection of a blueberry cultivar to be used in the production of the extracts, as well as the definition of a preferred harvest stage, based on their phenolic and anthocyanin composition. ii) establishment of a simple, relatively easy to scale up, preferably food grade extraction process. iii) characterization of some of the potential biological effects of extracts through the characterization of their effect upon potential probiotic microorganisms as well as their antioxidant, antimicrobial, antiadhesive and antibiofilm activity.

Structure and Thesis Plan

From a general standpoint, (Figure 1) the present thesis can be divided into three major stages: 1) the selection of the blueberries to be used. 2) the definition of an extraction protocol. 3) the characterization of some of the biological properties of the extract.

Stage 1: This step is fully characterized in *chapter 2.1* where four different blueberry cultivars, selected according to their economic relevance, where compared in regards to their antioxidant capacity, phenolic and anthocyanin content throughout. This analysis was made throughout the ripening process in order to ascertain both a preferable cultivar and a preferable harvest time.

Stage 2: This particular stage is characterized in *chapter 3*. Taking into account the benefits and limitations of the anthocyanin extraction methods reviewed in the state of the art, an extraction protocol was defined. Then, after selecting an approach, an optimization process was undertaken (*chapter 3.1.*) analysing the impact of mass/solvent ratios, extraction solvent and ultrasonic treatment. Additionally, the utility of a purification step was also considered.

Stage 3: This last stage was focused on the characterization of some biological properties of the extract (*chapter 4* to *chapter 6*). Chapter 4 describes the antioxidant potential of the extract that was evaluated for its antioxidant potential using a peroxide/DNA system based on the agarose gel electrophoresis method, improved to allow for quantitative measurement of DNA degradation using computational analysis. *Chapter 5* contemplates the extracts potential as antimicrobial agents against common nosocomial agents firstly (*chapter 5.1*) by analysing the crude extract and then by trying to ascertain where the extract's activity comes from (*chapter 5.2* and *5.3*). *Chapter 6* focused on the extract's effect upon food pathogens, potential probiotic microorganisms and pathogen/probiotic systems to determine if the extracts (when in concentrations capable of inhibiting food pathogens) interfered with probiotic growth and metabolic activity (*chapter 6.1.*) or if they impacted on either's adhesion to a surface conditioned to resemble the intestinal epithelium (*chapter 6.2.*).

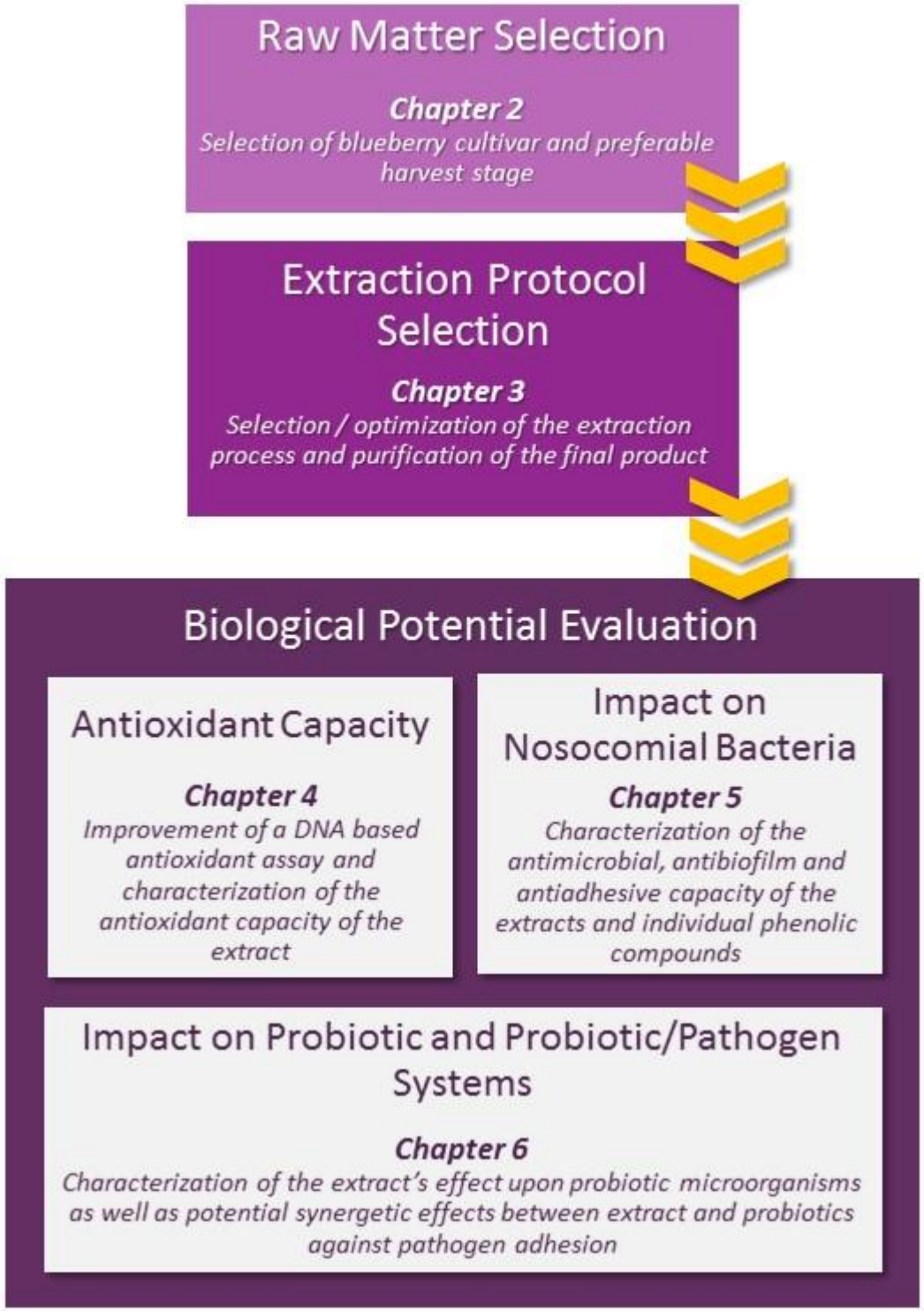


Figure 1. Schematic layout of the thesis structure.

Chapter 2

Raw matter selection

This chapter aimed to select the raw materials to be used as basis for all further assays

"Begin at the beginning – the King said, very gravely – and go on till you come to the end: then stop"

Lewis Carroll *in* ***Alice in Wonderland***

Chapter Preamble

It is widely known that the phenolic composition of a fruit varies significantly from fruit to fruit and accordingly to the environmental context and/or agricultural practices. Several intrinsic and extrinsic factors have been reported to affect both the phenolic profile and overall phenolic content of blueberries. Extrinsic variables, such as solar exposure, water and nutrient availability, have long since been reported as interfering with the phenolic/anthocyanin profile and content. However, as extrinsic factors are harder (and typically more expensive) to control than intrinsic factors, the present chapter focused on evaluating the effect of two of the latter: ripeness and cultivar. It is important to note that, in an attempt to reduce the possible variations caused by extrinsic factors (e.g. solar exposure, nutrient and water availability) a single blueberry producer that had all four cultivars growing in the same plot was used.

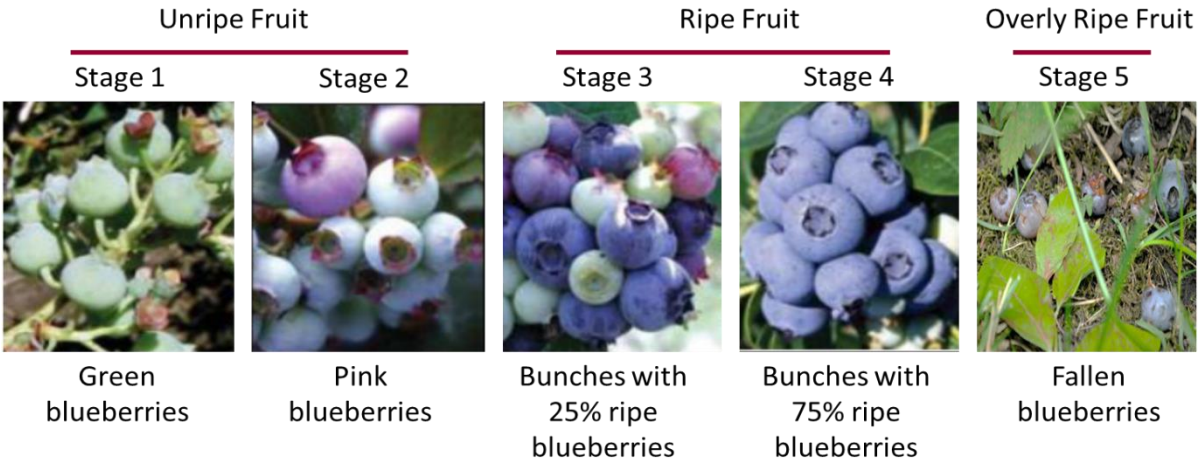


Figure 1. Different blueberry ripeness stages as defined by Mirtilusa S.A.

As the doctoral program counted with the support of Mirtilusa S.A. (one of the largest blueberry producers' association in Portugal), the cultivars selected to be included were those that had the largest harvest areas and highest commercial relevance (higher fruit yields and continuous harvest times).

Ripeness' impact upon the phenolic composition can be observed by the naked eye, as anthocyanin accumulation in the skins explains the colour variation from green unripe fruits to dark blue/purple ripe blueberries. This alone accentuates the need for a better understanding of this variable as anthocyanins are one of the most relevant classes of phenolic present in blueberry extracts. As Mirtilusa S. A. supplied their producers with

information on how to classify the ripeness of blueberries (Figure 1), we chose to follow it as it would simplify the harvesting process for the producer.

Considering the above made arguments, the present chapter aimed to compare the composition of blueberries from four *V. corymbosum* cultivars (Duke, Bluecrop, Goldtraube and Ozarkblue) throughout the ripening process, from unripe to overripe fruits. The results showed a significant variation in total anthocyanins (both overall amount and profile) in regards to the cultivar with Goldtraube blueberries appearing as the richest in both anthocyanins and phenolics. During the ripening of the fruit an increase of anthocyanins was observed up until the overly ripe fruit, and in this stage the amount of anthocyanins either increased (Bluecrop), decreased (Duke and Ozarkblue) or remained stable (Goldtraube). Considering this, Goldtraube blueberries at a latter ripening stage were used throughout to produce the extracts used throughout the present thesis.

Variation of anthocyanins and other major phenolic compounds throughout the ripening of four Portuguese blueberry (*Vaccinium corymbosum* L) cultivars

Sara Silva[1], Eduardo M. Costa[1], Marta C. Coelho[1], Rui M. Morais[1], Manuela E. Pintado[1]

[1] CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

This chapter has been published:

Natural Product Research (2016); doi:10.1080/1478619.2016.1209668

Abstract

Blueberries are widely recognized as one of the richest sources of bioactive compounds, among which are anthocyanins. Though the berries ripeness has been reported as affecting the phytochemical composition of fruits. Therefore, the present work aimed to evaluate the variation of anthocyanins, and other major phenolics, throughout five ripening stages in four blueberry cultivars. The results showed that the antioxidant capacity and anthocyanin content increased during ripening, reaching the highest values when the blueberries are collected from bunches comprised of 75% ripe blueberries. Antagonistically, the amount of phenolic acid decreases while the quercetin-3-glucoside levels remain stable. Furthermore, Goldtraube blueberries appear to possess, systematically, higher amounts of phenolic compounds than the other cultivars studied. Thus, when seeking the highest yield of anthocyanins, the preferred harvest should occur in bunches that contain ca. 75% of ripe blueberries and, considering the cultivars assayed, the Goldtraube cultivar appears the richest in phenolic compounds.

Keywords: *Vaccinium corymbosum*; Anthocyanins; Ripening; Blueberry

1. Introduction

As consumers concern with health grew, so did the industry's attempts to explore this market through the development of functional foods. Primary food producers have focussed on further developing their growth, harvest and storage protocols in an attempt to maximize their products functional potential (Gibson and Williams, 2000). Blueberries are a prime example of a foodstuff that gained a strong health promoting connotation, with general media calling it a "superfruit" and several reports made on their rich phenolic composition. Anthocyanins, a major group of phenolic compounds found in blueberry, have been associated with a vast array of health promoting properties such as protection against oxidative damage, reduction of hepatic damage, anti-inflammatory activity and even as weight management aids (Cristani et al., 2016, Jiang et al., 2016, Sarkar et al., 2014, Cardile et al., 2015, Li et al., 2013). As blueberry phenolic composition varies significantly according to the ripeness of the fruit, and the lack of a compound specific approach analysis of this process, the present work aimed to compare the phenolic composition of four blueberry cultivars throughout the ripening of the fruit, with a particular emphasis on their anthocyanin composition.

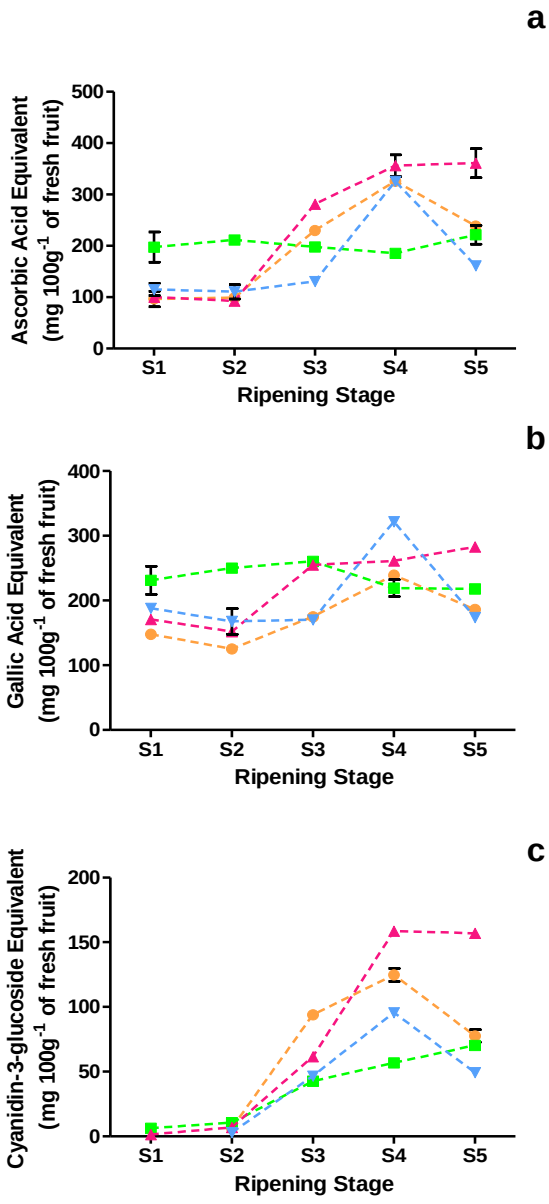


Figure 1. Variation of the total antioxidant capacity (a), total phenolics (b) and anthocyanins (c) throughout the ripening process of Duke (●), Bluecrop (■), Goldtraube (▲) and Ozarkblue (▼) blueberries.

2. Results and discussion

As can be seen in Figure 1 the variation of the total antioxidant capacity (TAC), phenolic content (TPC) and anthocyanin content (ACY) varied differently throughout the ripening stages, though the variation profile differed from cultivar to cultivar. For monomeric anthocyanins, the ACY is significantly ($p < 0.05$) higher in the ripe and over-ripe stages (S3 to S5) than in the unripe fruits (S1 and S2), a fact that stands in line with previous reports (Kalt et al., 2003, Castrejón et al., 2008). The TAC variation was quite similar to that of the anthocyanins for all cultivars with the exception of Bluecrop (Figure 1). In fact, the correlation between these variables (Table 3S) showed that the TAC values observed for Duke, Goldtraube and Ozarkblue blueberries were related to their anthocyanin content as they exhibited a strong, significant ($p < 0.01$), positive relationship (0.980, 0.932 and 0.900, respectively). As the TAC content doesn't vary for Bluecrop and the ABTS method is highly susceptible to interference, it is possible that another compound/factor is responsible for the absence of correlation between these variables. As for the TPC, no overall behavioural trend could be observed, a fact that stands in line to what has been described by Castrejón et al. (2008), who reported that 4 different blueberry cultivars exhibited different phenolic variation trends. Considering the vast array of compounds that are capable of interfering with the Folin-Ciocalteu assay, it is hard to explain TPC variations without looking at the individual compounds (Castrejón et al., 2008, Kalt and McDonald, 1996, Wang and Lin, 2000, Vermerris and Nicholson, 2007). In Figure 2, where the variation of the different anthocyanins along the ripening is expressed, it can be seen that the amount of anthocyanins increased with the ripening. This stands in accordance to the results obtained for the ACY (Figure 1). The most prevalent anthocyanins found were malvidins, which is in accordance with what has been reported by Taruscio et al. (2004). This is particularly interesting when considering the biological potential of the extracts as malvidins have been described as possessing anti-inflammatory activity and, therefore, the extracts may be used to develop nutraceuticals that aid with chronic inflammation (Huang et al., 2014). Delphinidins were found at higher levels than cyanidins which is in accordance with Taruscio et al. (2004) and Lohachoompol et al. (2008) but not with You et al. (2011), who reported similar levels of both families of anthocyanins. The remaining 4 anthocyanins found were petunidin-3-O-glucoside, petunidin-3-O-galactoside, peonidin-3-O-arabinoside (only in Goldtraube) and peonidin-3-O-galactoside.

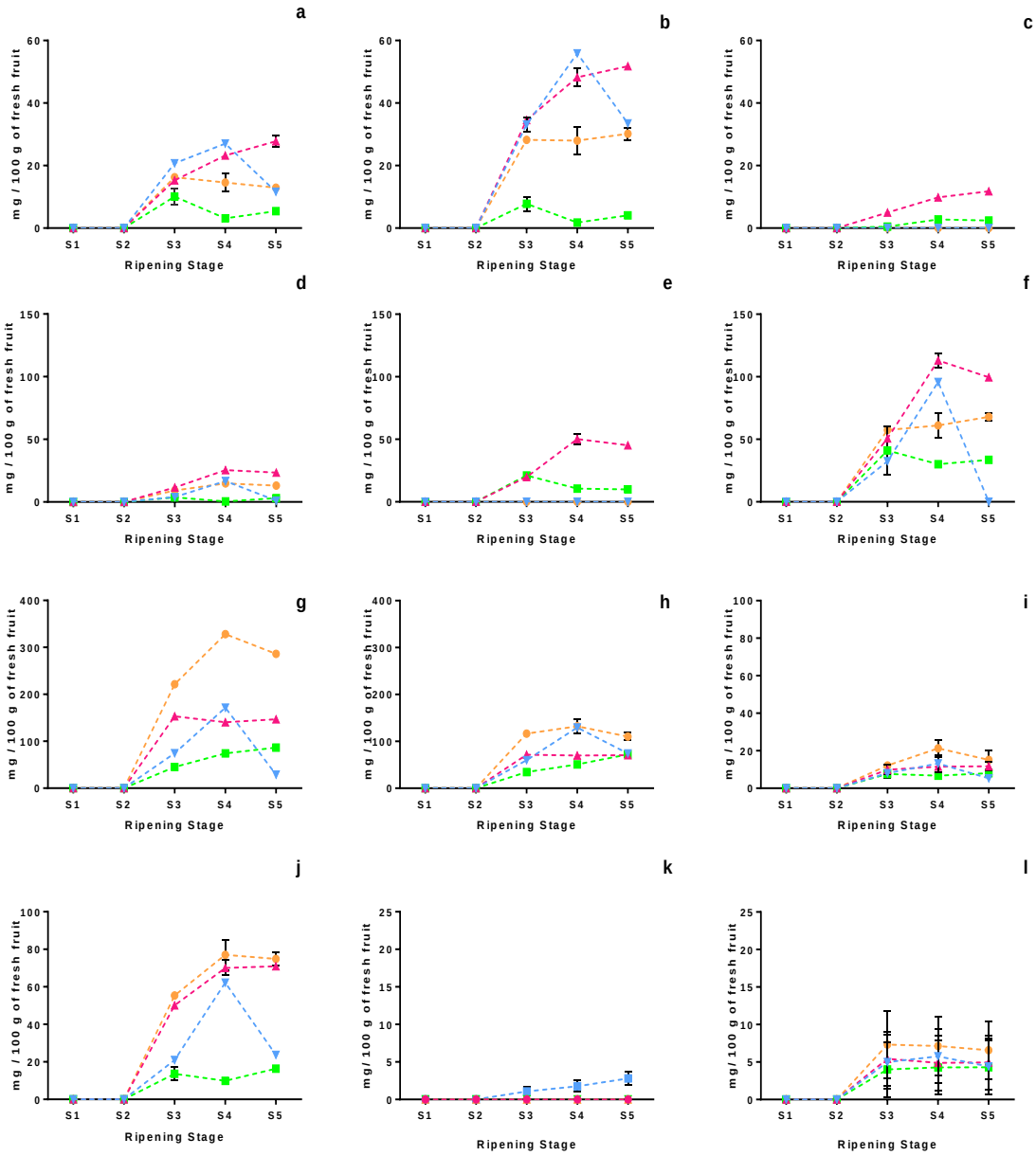


Figure 2. Variation of individual anthocyanins in Duke (●), Bluecrop (■), Goldtraube (▲) and Ozarkblue (▼) blueberries. a, cyanidin-3-arabinoside (C3Ara); b, cyanidin-3-galactoside (C3Gal), c, cyanidin-3-glucoside (C3Glu); d, delphinidin-3-arabinoside (D3Ara); e, delphinidin-3-galactoside (D3Gal), f, delphinidin-3-glucoside (D3Glu); g, malvidin-3-galactoside (M3Gal); h, malvidin-3-glucoside (M3Glu); i, petunidin-3-galactoside (Pt3Gal); j, petunidin-3-glucoside (Pt3Glu); k, peonidin-3-arabinoside (P3Ara); l, peonidin-3-galactoside (P3Gal).

Besides the anthocyanins, other four phenolic compounds were identified, three phenolic acids (chlorogenic, gallic and *p*-coumaric acid) and a flavonol glycoside (quercetin-3-D-glucoside (Q3Glu)) (Figure 4). Cinnamates (such as chlorogenic and hydroxycinnamic acids) have been reported to decrease ($p < 0.05$) as the fruit ripens, a behaviour that stands in accordance to the results observed for *p*-coumaric acid (Figure 4) and for chlorogenic acid

when considering Duke, Ozarkblue and Bluecrop blueberries (Kalt and McDonald, 1996, Castrejón et al., 2008, Kalt et al., 2003). However, as this behaviour is not ubiquitous to all food crops, the stable values observed for Goldtraube are not unexpected. Gallic acid exhibited behaviour similar to the one observed for chlorogenic acid. As a reduction in acidity is one of the main characteristics of the ripening process, the variations observed stand in accordance with what could be expected (Kulkarni and Aradhya, 2005, Ayaz et al., 2001). The Q3Glu values kept relatively stable throughout the assay a fact that is not in accordance to what was reported by Jaakola et al. (Jaakola et al., 2002) that found that quercetin levels varied throughout the ripening process, first increasing and then decreasing. Given the relatively large deviations to the mean found, it is possible that any variations are masked by the dispersion of the data.

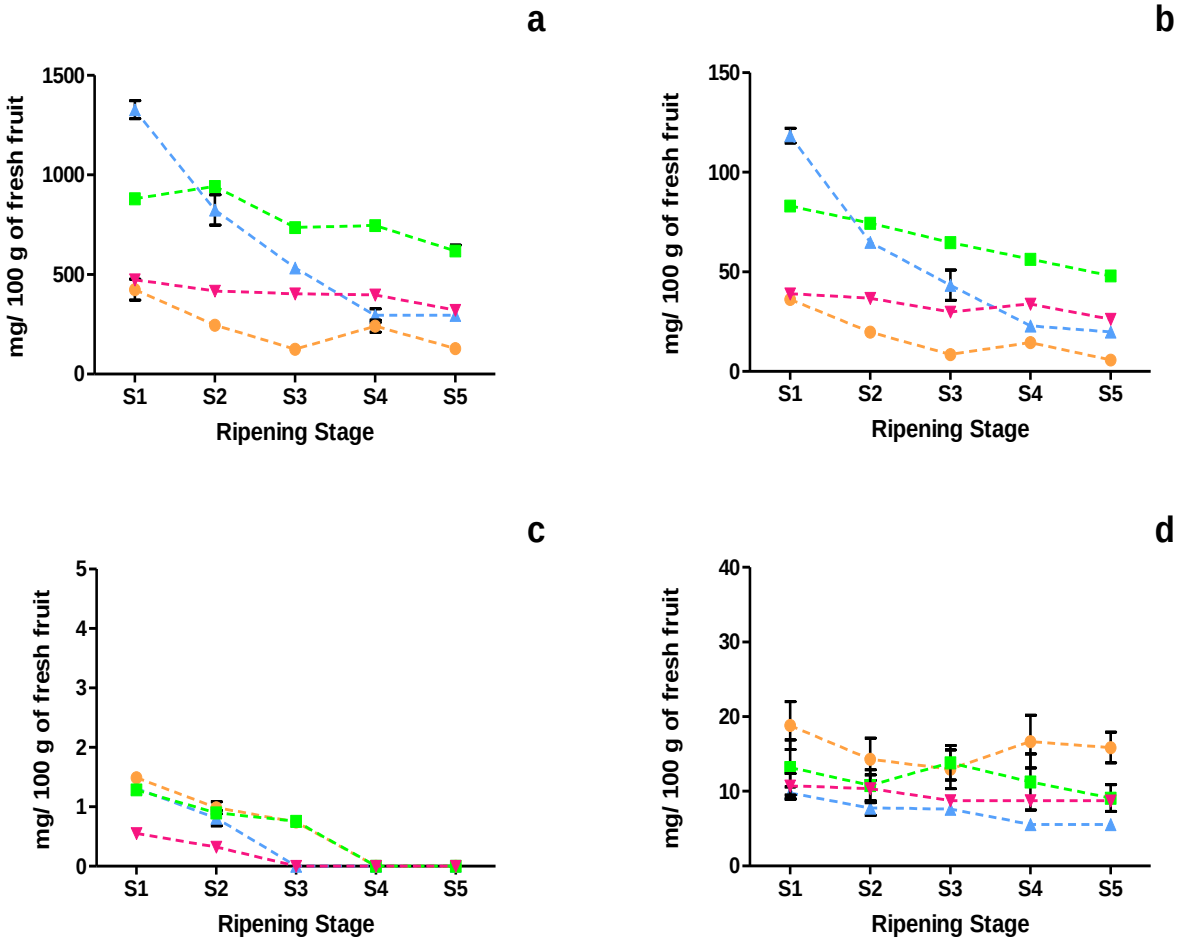


Figure 3. Variation of chlorogenic acid (a, ChA), gallic acid (b, GA), *p*-coumaric acid (c, p-CA) and quercetin-3-glucoside (d, Q3Glu) throughout the ripening process, in blueberries of four different cultivars: Duke (●), Bluecrop (■), Goldtraube (▲) and Ozarkblue (▼).

It is interesting to note that, the sum of each individual phenolic compound (as determined by HPLC) is significantly higher than the TPC or the TAC (Table 5S) which is a consequence of the increased sensitivity of the chromatographic methods by opposition to the spectrophotometric ones (Lee et al., 2008).

3. Conclusions

Despite the various variation profiles found, it can be concluded that the ripening stage S4, i.e. blueberries collected from bunches containing 75% of ripe blueberries, allows for the overall maximization of the anthocyanins in all blueberry cultivars tested.

Acknowledgements: The present work was supported by National Funds from FCT through project Pest-PE/EQB/LA0016/2013 and by funds of the QREN-ADI project 13736 "Myrtillus – Mirtilo com inovação". Additionally, the authors S. Silva and E.M. Costa would like to acknowledge FCT for their Ph.D. grants with the references SFRH/BD/90867/2012 and SFRH/BDE/103957/2014, respectively.

Supplemental Material

4. Experimental section

4.1. Samples

In a production area that possessed all four test cultivars (latitude: 40°47'24.66" N; longitude: 8°23'17,21" W; elevation above the sea level: 549 m), twenty-four blueberry bushes were randomly selected (6 per cultivar with the agronomical characteristics listed in table 1S). Bushes from the different cultivars were arranged in a 2X2 rectangular formation comprised of evenly spaced rows of plants.

Samples were collected from Goldtraube, Duke, Bluecrop and Ozarkblue blueberry bushes at different ripening stages: S1 – green fruits with a reddish crown (unripe fruit); S2 – light pink blueberries with no observable green area (unripe fruit); S3 – blueberries that came from a bunch containing only 25% of ripe blueberries (ripe fruit); S4 – blueberries collected from bunches that had 75% or more of their blueberries ripe (ripe blueberries); S5 – blueberries that fell from the bush (overripe blueberries).

Table 1S. Growth conditions and age of the different blueberry cultivars.

	Duke	Bluecrop	Goldtraube	Ozarkblue
Average soil pH	4.6	5.1	5.1	4.6
Average organic matter content	11%	12%	12%	11%
Soil type	Humic Dystrudept	Humic Dystrudept	Humic Dystrudept	Humic Dystrudept
Average daily light during ripening	ca.9.2 h	ca. 9.2 h	ca. 9.2 h	ca. 9.2 h
Sample harvest period	4^{th} – 8^{th} of June	11^{th} – 16^{th} of June	18^{th} – 23^{rd} of June	25^{th} – 30^{th} of June
Average high temperature (°C)	28 °C	28 °C	28 °C	28 °C
Average low temperature (°C)	12 °C	12 °C	12 °C	12 °C

At each ripening stage, blueberries from the 6 different bushes (20 g collected from each bush) were collected from random areas of each plant and immediately transported to the laboratory for analysis.

4.2. Extracts

The blueberry pulp was homogenized (10% (w/v), 24 000 rpm, 1 min) with acidified methanol (0.01% HCl) using an Ultra-turrax T18 (IKA, Staufen, Germany) and left to extract at room temperature in a light tight container. After 12 h, the mix was centrifuged (6026g, 4 °C, for 15 min) and filtered through a 4-7 µm filter (Prat Dumas, Couze St. Front, France). Each extraction was performed in triplicate.

4.3. Total anthocyanins content (ACY), total antioxidant capacity (TAC) and total phenolic content (TPC)

The total monomeric anthocyanins were assessed using the differential pH method as described by (Jakobek et al. (2007)) and the results, expressed in cyanidin-3-O-glucoside (C3Glu) equivalents, were calculated as described by the same authors (Jakobek et al., 2007).

The ABTS radical cation ($ABTS^{\bullet+}$) assay, to assess the TAC of extracts, and the Folin - Ciocalteu phenol's reagent, to evaluate the TPC, were used as described by Gião et al. (2007).

4.4. Compound identification and quantification

The different phenolic compounds were analysed using reverse – phase high performance liquid chromatography coupled with a diode-array detection system (Waters Series 600, Mildford, Massachusetts) equipped with a reverse phase Symmetry C18 column (250 x 4.6

mm i.d. 5 µm particle size and 125 Å pore size, kept at 30 ºC) and a guard column containing the same stationary phase. Chromatographic separation was carried out using a linear gradient of 2 solvents (A- 5% methanol, 2.5% formic acid, 92.5% ultra-pure water; B- 25% methanol, 25% formic acid, 50% ultra-pure water) varying as illustrated in table 2S. Injection volume was 40 µL. Detection was achieved using a diode array detector measuring at wavelengths ranging from 200 to 600 nm in 2 nm intervals. The different peaks were analysed by comparison of retention times and spectra with that of pure phenolic and anthocyanin glycoside standards all acquired from Extrasynthese (Genay Cedex, France) except for delphinidin-3-arabinoside which was acquired from ChemFaces (Hubei, China). Quantification was performed using calibration curves (Table 4S) of the presumed compounds considering a detection limit of 2.5 mg 100 g^{-1} of fresh blueberries. Three independent analysis were performed for each of the triplicate extracts.

Table 2S. Flow conditions used for HPLC analysis.

Time (min)	% Solvent A	% Solvent B	Flow (mL min^{-1})
0 to 60	100 - 40	0 - 60	0.65
60 to 65	40 – 90	60 – 10	0.50
65 to 70	90 - 100	10 - 0	0.50

4.5. Statistical analysis

The normality of the distribution was assessed using the Shapiro-Wilk normality test. As the results proved to follow a normal distribution, a comparison of the results was performed using One-Way ANOVA coupled with Scheffe's Post-Hoc test (the differences were considered significant when $p < 0.05$). Additionally, the correlation between different variables was assessed using Pearson's correlation (the differences were considered significant when $p < 0.05$).

5. Additional data

Table 3S. Correlation between the total antioxidant capacity, phenolic and anthocyanin content.

		ACY – TAC	ACY – TPC	TAC – TPC
Duke	Pearson's R	0.980	0.898	0.955
	p-value	< 0.01	< 0.01	< 0.01
Bluecrop	Pearson's R	0.051	- 0.464	- 0.058
	p-value	0.857	0.082	0.837
Goldtraube	Pearson's R	0.932	0.896	0,972
	p-value	< 0.01	< 0.01	<0.01
Ozarkblue	Pearson's R	0.900	0.756	0.884
	p-value	< 0.01	0.001	< 0.01

Table 4S. Standards and chromatographic information regarding the calibration curves.

Standard	Retention Time	Maximum absorbance (λ)	Calibration curves		
			Slope	y-Intercept	R^2
Cyanidin-3-arabinoside	42.43	516.2	185958,0	2078,7	0,989
Cyanidin-3-galactoside	37.73	516.2	184409,3	104057,5	0,993
Cyanidin-3-glucoside	40.04	515.6	163200,7	89177,5	0,995
Delphinidin-3-arabinoside	33.41	523.5	124339,2	216344,0	0,996
Delphinidin-3-galactoside	35.17	522.9	166883,9	151461,9	0,979
Delphinidin-3-glucoside	29.67	523.5	190244,0	137988,3	0,979
Malvidin-3-galactoside	38.01	528.1	159352,2	65858,3	0,977
Malvidin-3-glucoside	39.53	527.2	166282,4	13416,4	0,981
Petunidin-3-galactoside	44.28	516.2	141320,5	-609036,5	0,998
Petunidin-3-glucoside	43.90	525.6	198294,5	69479,6	0,991
Peonidin-3-arabinoside	44.77	517.4	129169,8	-9872,4	0.987
Peonidin-3-galactoside	44.93	516.2	141320,49	-609036,5	0,998
Gallic acid	32.85	322.5	5299,2	-9570,7	0,999
Chlorogenic acid	33.5	326.7	218281,8	85772,5	0,999
p-Coumaric acid	45.29	309.8	464264,8	-868221,6	0,994
Quercetin-3-Glucoside	51.40	256.2	100826,0	-559537,5	1,000

Table 5S. Anthocyanin content, in mg per 100 g of fresh fruit, for each stage and cultivar according to the differential pH (TAC) and HPLC analysis.

	Ripening Stage	Anthocyanin content	
		TAC	HPLC
Duke	S1	nd	nd
	S2	6.28 ± 2.87	nd
	S3	93.99 ± 0.81	524.26 ± 16.98
	S4	124.68 ± 5.01	684.37 ± 56.60
	S5	77.65 ± 4.84	617.00 ± 29.37
Bluecrop	S1	6.195 ± 3.47	nd
	S2	10.64 ± 2.63	nd
	S3	42.51 ± 0.01	188.76 ± 41.40
	S4	56.83 ± 2.06	194.57 ± 13.69
	S5	70.27 ± 2.14	245.76 ± 19.66
Goldtraube	S1	1.35 ± 0.59	nd
	S2	6.87 ± 1.46	nd
	S3	61.56 ± 1.67	427.17 ± 11.04
	S4	158.58 ± 3.06	572.76 ± 35.14
	S5	159.97 ± 1.11	564.16 ± 16.06
Ozarkblue	S1	nd	nd
	S2	2.93 ± 1.72	nd
	S3	46.60 ± 2.02	259.6 ± 18.54
	S4	95.63 ± 3.34	523.48 ± 23.13
	S5	49.18 ± 0.53	185.77 ± 8.23

nd, not detected.

6. References

AYAZ, F. A., KADIOGLU, A., BERTOFT, E., ACAR, C. & TURNA, I. 2001. Effect of fruit maturation on sugar and organic acid composition in two blueberries (Vaccinium arctostaphylos and V. myrtillus) native to Turkey. *New Zealand Journal of Crop and Horticultural Science,* 29**,** 137-141.

CARDILE, V., GRAZIANO, A. C. & VENDITTI, A. 2015. Clinical evaluation of Moro (Citrus sinensis (L.) Osbeck) orange juice supplementation for the weight management. *Nat Prod Res,* 29**,** 2256-60.

CASTREJÓN, A. D. R., EICHHOLZ, I., ROHN, S., KROH, L. W. & HUYSKENS-KEIL, S. 2008. Phenolic profile and antioxidant activity of highbush blueberry (Vaccinium

corymbosum L.) during fruit maturation and ripening. *Food Chemistry,* 109**,** 564-572.

CRISTANI, M., SPECIALE, A., MANCARI, F., ARCORACI, T., FERRARI, D., FRATANTONIO, D., SAIJA, A., CIMINO, F. & TROMBETTA, D. 2016. Protective activity of an anthocyanin-rich extract from bilberries and blackcurrants on acute acetaminophen-induced hepatotoxicity in rats. *Nat Prod Res***,** 1-5.

GIÃO, M. S., GONZÁLEZ-SANJOSÉ, M. L., RIVERO-PÉREZ, M. D., PEREIRA, C. I., PINTADO, M. E. & MALCATA, F. X. 2007. Infusions of Portuguese medicinal plants: Dependence of final antioxidant capacity and phenol content on extraction features. *Journal of the Science of Food and Agriculture,* 87**,** 2638-2647.

GIBSON, G. R. & WILLIAMS, C. M. 2000. *Functional foods: Concept to product.,* Cambridge, England, Woodhead Publishing Limited.

HUANG, W. Y., LIU, Y. M., WANG, J., WANG, X. N. & LI, C. Y. 2014. Anti-inflammatory effect of the blueberry anthocyanins malvidin-3-glucoside and malvidin-3-galactoside in endothelial cells. *Molecules,* 19**,** 12827-41.

JAAKOLA, L., MAATTA, K., PIRTTILA, A. M., TORRONEN, R., KARENLAMPI, S. & HOHTOLA, A. 2002. Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant Physiol,* 130**,** 729-39.

JAKOBEK, L., SERUGA, M., MEDVIDOVIC-KOSANOVLC, M. & NOVAK, I. 2007. Anthocyanin content and antioxidant activity of various red fruit juices. *Deutsche Lebensmittel-Randschau,* 103**,** 58-64.

JIANG, Z., CHEN, C., XIE, W., WANG, M., WANG, J. & ZHANG, X. 2016. Anthocyanins attenuate alcohol-induced hepatic injury by inhibiting pro-inflammation signalling. *Nat Prod Res,* 30**,** 469-73.

KALT, W., LAWAND, C., RYAN, D. A., MCDONALD, J. E., DONNER, H. & FORNEY, C. F. 2003. Oxygen radical absorbing capacity, anthocyanin and phenolic content of highbush blueberries (Vaccinium corymbosum L.) during ripening and storage. *Journal of the American Society for Horticultural Science,* 128**,** 917-923.

KALT, W. & MCDONALD, J. E. 1996. Chemical composition of lowbush blueberry cultivars. *Journal of the American Society for Horticultural Science,* 121**,** 142-146.

KULKARNI, A. P. & ARADHYA, S. M. 2005. Chemical changes and antioxidant activity in pomegranate arils during fruit development. *Food Chemistry,* 93**,** 319-324.

LEE, J., RENNAKER, C. & WROLSTAD, R. E. 2008. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry,* 110**,** 782-786.

LI, X., MA, H., HUANG, H., LI, D. & YAO, S. 2013. Natural anthocyanins from phytoresources and their chemical researches. *Nat Prod Res,* 27**,** 456-69.

LOHACHOOMPOL, V., MULHOLLAND, M., SRZEDNICKI, G. & CRASKE, J. 2008. Determination of anthocyanins in various cultivars of highbush and rabbiteye blueberries. *Food Chemistry,* 111**,** 249-254.

SARKAR, B., KUMAR, D., SASMAL, D. & MUKHOPADHYAY, K. 2014. Antioxidant and DNA damage protective properties of anthocyanin-rich extracts from Hibiscus and Ocimum: a comparative study. *Nat Prod Res,* 28**,** 1393-8.

TARUSCIO, T. G., BARNEY, D. L. & EXON, J. 2004. Content and profile of flavanoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of northwest Vaccinium berries. *Journal of Agricultural and Food Chemistry,* 52**,** 3169-76.

VERMERRIS, W. & NICHOLSON, R. L. 2007. *Phenolic Compound Biochemistry,* Springer.

WANG, S. Y. & LIN, H.-S. 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry,* 48**,** 140-146.

YOU, Q., WANG, B., CHEN, F., HUANG, Z., WANG, X. & LUO, P. G. 2011. Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. *Food Chemistry,* 125**,** 201-208.

Chapter 3

Extract production

This chapter sought to establish a simple and efficient extraction method

"Any intelligent fool can make things bigger, more complex, and more violent. It takes a touch of genius – and a lot of courage – to move in the opposite direction"

Ernst F. Schumacher *in* ***The Radical Humanist***

Chapter Preamble

The decision upon an extraction procedure should not be taken lightly for it may condition the real life applicability of any product. Before any selection can be made, it is important to acquire an understanding of the limitations associated with each methodology, particularly when the extraction targets are molecules as finicky as anthocyanins (which are sensitive to temperature and whose form varies according to the environmental pH). Therefore, the first step into accomplishing this chapter's goal (the establishment of a protocol that allowed to obtain an anthocyanin-rich blueberry extract) was to perform a literature review that is, in part, present in the state of the art. Given the lack of straightforward papers comparing the different methods, it is frequently the objective of the extraction the selection of the conditions of the extraction process (though for anthocyanins extraction methods that imply high temperatures are unadvised). Our goal was to produce an extract that, while having some biological potential, could also be used in different types of products, form foodstuffs to cosmetics. This means that our extraction process should be industry-friendly. While complex methodologies using cutting edge technology may be interesting from a scientific standpoint, from an industrial point of view, they represent costly processes and scale-up problems, which are likely to compromise future applications. Therefore, when defining the extraction procedure, a solid liquid extraction (which is relatively cheap and easy to scale up) was used a base to optimize the extraction procedure with an acidified ethanol extract proving to be the most interesting alternative, particularly as ethanol's lack of perceived activity also allows for possible incorporation into foodstuffs. As the dried extract had a relatively low percentage (w w^{-1}) of anthocyanins and total phenolics, the addition of a purification step was deemed necessary. Solid phase extraction columns (that may be re-used) were used and appeared to be an effective means for concentrating the extract.

Production of a food grade blueberry extract rich in anthocyanins: selection of solvents, extraction conditions and a purification method

Sara Silva[1], Eduardo M. Costa[1], Conceição Calhau[2,3], Rui M. Morais[1], Manuela E. Pintado[1]

[1] *CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal*

[2]*Nutrição e Metabolismo, NOVA Medical School, Universidade Nova de Lisboa, Campo Mártires, 130, 1169-056 Lisboa, Portugal*

[3] *CINTESIS, Center for Research in Health Technologies and Information Systems, Porto, Portugal*

Abstract

Blueberries are recognized, by the scientific community and consumers, for their health promoting potential. This fact makes blueberries, and blueberry derived products, prime candidates to aid in the development of healthier foodstuffs that are easily recognized as such by consumers. As blueberries health promoting properties are frequently associated with their phenolic composition, particularly anthocyanins, the present work aimed to establish a simple, food safe, approach to extract these compounds for food industry use. One that, while being food safe also allowed for a relatively easy scale up process. The results obtained demonstrated that ethanol acidified with 0.01% HCl was an effective extractant of both phenolic compounds and anthocyanins and that the extraction of anthocyanins may be further improved with the addition of an ultrasound treatment to the extraction process. Furthermore, if seeking a condensed extract, purification using solid phase extraction columns allowed the production of an extract comprised of ca. 40% (w w^{-1}) anthocyanins.

Keywords: Anthocyanins; Blueberries; Phenolic compounds; Solid liquid extraction; Solid phase extraction

1. Introduction

The increase in life expectancy has led to a rising concern with health and life quality. One of the main consequences of this has been an increase of the consumers' demand for healthier and more nutritive products, preferably without the addition of chemical additives (which have gained a negative connotation). So, as the consumers' search for healthier and 'more natural' products grew, so did the industry's attempts to explore this need, namely through the development of new additives that, while granting foodstuffs some functionality, may also function as natural additives (Gibson and Williams, 2000, Neto, 2007).

Blueberries, dubbed as a superfruit and with health promoting properties valorised by the consumers, present an interesting opportunity. So, adding blueberries to a product the consumers' will easily associate said foodstuff with a healthy diet. Furthermore, as blueberries are rich in phenolic compounds (natural antioxidants), and anthocyanins in particular (water soluble pigments frequently associated with several health promoting activities), the addition of blueberry extracts to a given product may allow for the replacement of some traditional antioxidant and colorant additives (Gibson and Williams, 2000, Wallace and Giusti, 2013). However, the production of blueberry extracts, both safe for food applications and rich in anthocyanins and other phenolics, using relatively simple, low cost and easy to scale up processes has some limitations. Firstly, anthocyanins are notoriously sensitive to heat, with authors recommending temperatures as low as 30 – 35 °C in order to extract anthocyanins without promoting degradation that will lead to alterations of the final extract (Cacace and Mazza, 2003a, Cacace and Mazza, 2003b, Cacace and Mazza, 2002). Secondly, the cost associated with the use of high-tech solutions may make the overall process too expensive to be used in the food industry, especially if further purification processes are required (Silva et al., 2015a). Considering the above made arguments, the present work aimed to define an extraction process that allowed for the production of an anthocyanin/phenolic compound rich extract using a solid liquid extraction (SLE), a relatively simple and low cost approach, combined with solid phase extraction (SPE) a possible solution for purification.

2. Experimental section

2.1. Samples

Blueberries (*Vaccinium corymbosum* cv. Goldtraube) used in the present work were kindly provided by Mirtilusa S.A. (Sever do Vouga, Portugal). The fruits were collected in 2013 and stored at -20 °C until processing.

2.2. Effect of blueberry mass/solvent ratio

Blueberries were pulped using an appliance mill. The pulp was then suspended (1, 5, 10, 15 and 20% (w v^{-1})) in methanol, acidified with 0.01% HCl, homogenized using an Ultra-Turrax T18 (IKA, Staufen, Germany) and left to extract for 1 h at 40 °C in a light-tight container. After, the mixture was centrifuged (6026g, 4 °C, for 15 min) and filtered through a 4 - 7 µm filter (Prat Dumas, Couze St. Front, France) (Silva et al., 2015a). The resulting extracts were assayed for their total phenolic (TPC) and total anthocyanin content (ACY). Each extraction was performed in triplicate.

2.3. Effect of the extraction solvent

Ethanol, acetone, deionized water and methanol (with and without 0.01% HCl) were mixed with blueberry pulp (10% (w v^{-1})) and extracted, in triplicate, as described in section 2.2. The resulting extracts were assayed for their TPC and ACY.

2.4. Effect of an ultrasound treatment

Extracts were prepared using ethanol (with and without 0.01% HCl) as described in section 2.2 with a minor modification viz., before centrifuging the extracts were placed in an ultrasound (US) bath (35 kHz, Bandelin Sonorex, Berlin, Germany) for 15 min. The TPC and ACY were then evaluated. Each extraction was performed in triplicate.

2.5. Purification of the extracts – process audit

The extract produced using acidified ethanol and including US treatment was considered the most effective and, therefore was submitted to purification using Bond Elut Plexa SPE columns (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 powder was ressuspended in deionized water acidified with 0.01% HCl. The resulting solution was

loaded into the SPE columns (previously activated with ethanol and conditioned with acidified water). After loading the extracts, the columns were washed with acidified water and the phenolic compounds were eluted using acidified ethanol (0.01% HCl). In each step the TPC and ACY were determined. The purification was assayed in triplicate. Purification efficacy was evaluated through the comparison of the amount of TPC and ACY per mg of dried extract. Furthermore, eventual losses in TPC and ACY (in µg per g of blueberry) were also monitored at the following steps: i) original extract (original); ii) extract after drying by rotary evaporator and reconstituted in water (dried); iii) extract after SPE purification (purified); iv) extract after drying by rotary evaporator (final).

2.6. Total phenolic content determination

The TPC of the extracts was determined using the Folin-Ciocalteu phenol's reagent method as described by Gião et al. (2007). Briefly, to 50 µL of sample (diluted when needed) 50 µL of Folin-Ciocalteu reagent (Merck, Darmstadt, Germany), 1 mL of a 75 g L^{-1} sodium carbonate solution and 1.4 mL of deionized water were added. After 1 h, the optical density (OD) at 750 nm was measured and the TPC was calculated using a gallic acid calibration curve, with the results being expressed in gallic acid equivalents. All assays were performed in triplicate.

2.7. Total anthocyanin content determination

The determination of the ACY was determined through the calculation of the area under the curve of the extracts chromatogram, at 520 nm, according to the protocol described elsewhere (Lee et al., 2008, Silva et al., 2015b). The ACY was determined using a cyanidin-3-glucoside (Extrasynthese, Geney Cedex, France) standard curve, with the results being expressed in equivalents of cyanidin-3-glucoside.

2.8. Statistical analysis

Statistical analysis of the results was performed using IBM SPSS Statistics v21.0.0.0. (New York, USA). The normality of the distribution was evaluated using the Shapiro-Wilk's test. Since the samples followed a normal distribution, the One - Way ANOVA with Turkey's

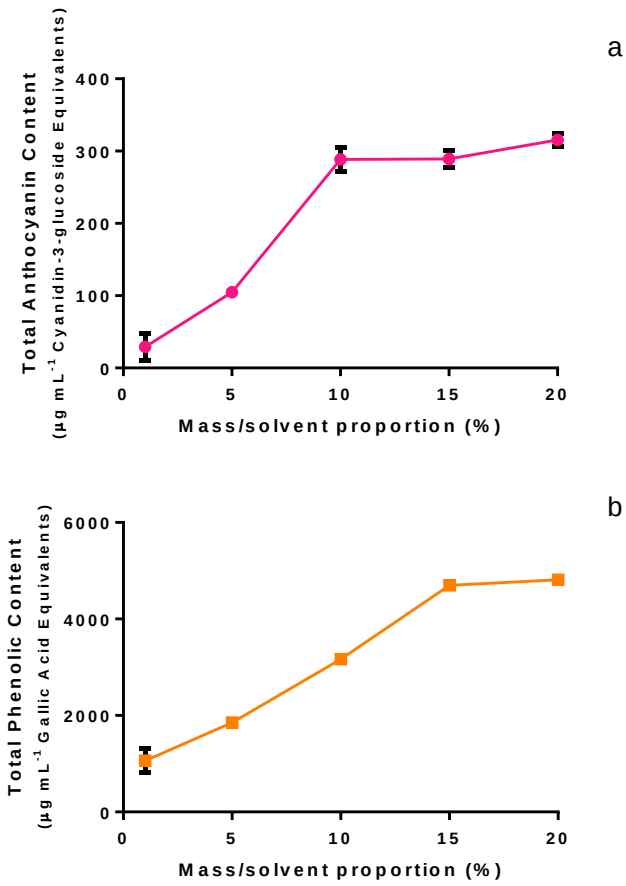


Figure 1. ACY (a) and TPC (b) of methanolic extract produced using varying solvent/mass proportions.

post-hoc test was used. Differences were considered statistically significant for p-values bellow 0.05. The different extraction solvents used were grouped, using a K-means cluster analysis, using standardized TPC and ACY values (z-scores). The clusters were considered significant for p-values bellow 0.05.

3. Results and discussion

Acidified methanol was used to determine the effect of the solvent/mass proportion as it has been classically elected as extraction solvent when seeking to characterize the anthocyanin content of berries (Castañeda-Ovando et al., 2009). As can be seen in Figure 1a, the amount of anthocyanins being extracted appeared to become somewhat stable for mass/solvent proportions above 10% (288.6 ± 16.8 µg mL^{-1}) while for the TPC this stabilization occurred only for solvent/mass ratios of 15% (4695.7 ± 68.9 µg mL^{-1}). This means that the optimum proportion, when seeking to extract both anthocyanins and phenolic compounds would be 15%. However, when concentrations were above 10% the filtration process became significantly longer and constrains feasibility. Fact that, could not only allow for the oxidation of the extracted compounds but also place a significant hamper in any future scale up attempts (Attoe and Von Elbe, 1981). Consequently, the 10% proportion was selected to be used henceforth.

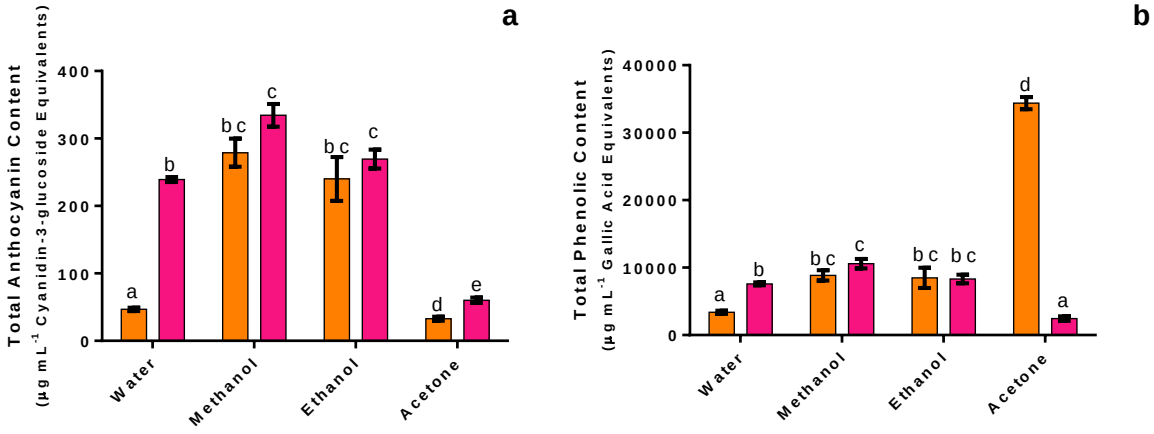


Figure 2. ACY (a) and TPC (b) of extracts produced using ethanol, methanol, acetone or water, with (■) or without (■) 0.01 % (v v^{-1}) HCl. The different letters mark statistically significant differences ($p < 0.05$) between the extraction solvents.

In Figure 2 a comparison between different solvents can be observed. In regards to the non-acidified solvents it can be seen that water and acetone were less effective in extracting anthocyanins (46.7 ± 2.6 and 32.8 ± 2.9 µg mL^{-1} less, respectively). Methanolic and ethanolic extracts exhibited the highest ACY values of the four solvents tested with no statistically significant ($p > 0.05$) differences being found between them. However, when considering the TPC content, ethanol and methanol were not the best extraction solvents. Acetone exhibited the highest TPC values (34388.5 ± 889.3µg mL^{-1}, up to 4 times higher than ethanol or methanol), with water remaining the less effective extraction solvent (3329.9 ± 236.0 µg mL^{-1}). The acidification of water significantly ($p < 0.05$) increased its capacity to extract anthocyanins and phenolic compounds (5.1 and 2.2 times higher for ACY and TPC, respectively). On the other hand, for acetone, while acidification led to an increase of the ACY content (1.8 times higher) it also led to a large (14.1 times) significant ($p < 0.01$) decrease in the TPC value, as acetone is the less polar of the solvents tested the addition of acid, which in turn increases the polarity of the extraction solvent, may compromise the diffusion of the more polar compounds that diffused onto acetone alone (Iloki-Assanga et al., 2015). Additionally, it is interesting to note that no significant differences in TPC and ACY values were observed for either acidified methanol or ethanol. From the analysis of Figure 3 it became clear that the different solvents, whether acidified or not, could be

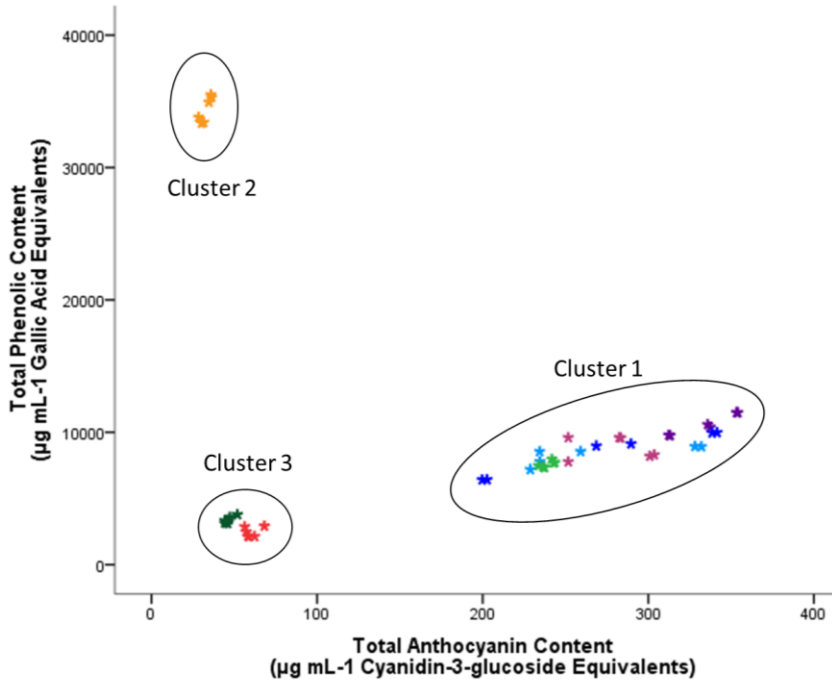


Figure 3. Scatterplot relating the TPC and ACY of the extract obtained using different extraction solvents; Methanol (★), acidified methanol (★), ethanol (★), acidified ethanol (★), water (★), acidified water (★), acetone (★) and acidified acetone (★). The circles mark statistically significant ($p < 0.05$) clusters.

grouped into three different clusters, all statistically significant ($p < 0.01$): cluster 2 that grouped solutions that allowed high amounts of TPC but low anthocyanin contents (cluster 2 comprised only of non-acidified acetone extracts), cluster 3 where relatively low amounts of both phenolics and anthocyanins can be found (cluster 3 comprised of water and acidified acetone extracts) and lastly, cluster 1 that while possessing TPC values not much higher than those of cluster 3, have higher ACY values (cluster 1 comprised of acidified ethanol and methanol, plain ethanol and methanol and acidified water extracts). While this last clustering (cluster 1) indicated that these extracts appeared to be quite similar between them, a closer analysis shows that cluster 1's data is somewhat dispersed in regards to their ACY content. This is reinforced by the fact that, as can be seen in Figure 2, acidified methanol and ethanol exhibited statistically significant ($p < 0.05$) higher values than acidified water. Considering that no significant differences were found between acidified and non-acidified ethanol and methanol, the selection of the best extraction solvent was between these two solvents (acidified or not) and, considering both the toxicity of methanol and the goal of producing food-grade extracts, only ethanol and acidified ethanol were used henceforth (Cabaroglu, 2005).

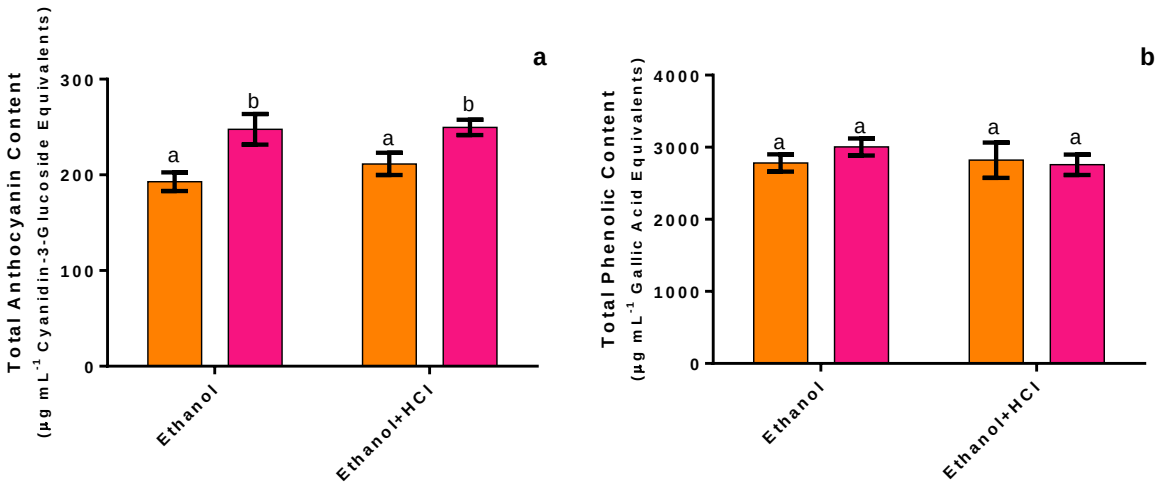


Figure 4. Variation in TPC and ACY content between non-treated (■) and US exposed (■) extracts. Different letters mark statistically significant ($p > 0.05$) differences between the bars.

Previous works have demonstrated that high power US (with frequencies from 20 – 40 kHz) may have different effects when considering the anthocyanin content of an extract, with some reporting increases in the ACY while others stating the opposite (Tiwari et al., 2008, Vieira et al., 2013). Nonetheless, as works with jussara (a berry that is relatively similar to blueberries) demonstrated that it could be effective, the effect of a 32 kHz treatment was assessed. In Figure 4 it can be seen that the US treatment only appeared to have a significant ($p < 0.05$) impact on anthocyanin extraction, with this additional step leading to an increase in ACY of 18.1 to 28.4% (without and with 0.01% HCl, respectively). It is interesting to note that no significant differences ($p < 0.05$) were found between acidified and non-acidified ethanol after US treatment. Consequently, both could be considered the best conditions to produce a food grade extract. Nonetheless, as anthocyanins are reported as considerably more stable under acidic conditions, acidified ethanol was considered the better solution, when coupled to the US treatment to allow for a higher anthocyanin extraction (Castañeda-Ovando et al., 2009, Fleschhut et al., 2006, Seeram et al., 2001).

The purification step led to a reduction of the overall extraction yield for both anthocyanins and phenolic compounds (525.8 and 77.4 µg per g of blueberry, respectively – Figure 5). When considering the anthocyanin variation, it can be seen that the first drying step allows for a significant ($p < 0.05$) reduction of the ACY content (less 22.3 µg g^{-1} of blueberry). As

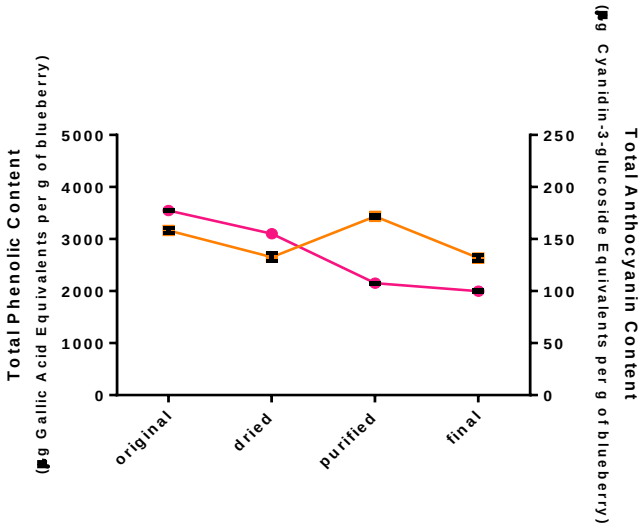


Figure 5. Impact of each purification step upon the extraction yield of TPC (●) and ACY (■).

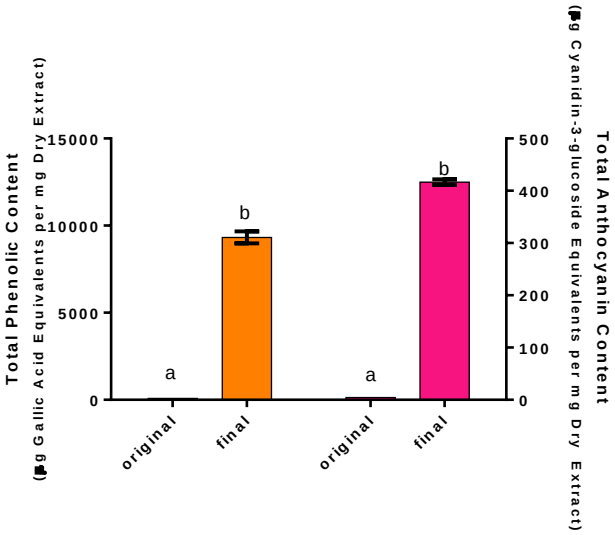


Figure 6. Comparison of the TPC (■) and ACY (■) composition of the dried extract before and after purification. The different letters indicate statistically significant ($p < 0.05$) differences between sets of data.

the extraction was made with acidified ethanol it is possible that, when concentrating the extract, the consequent increase in HCl concentration may promote the hydrolysis of the glycoside bonds (Seeram et al., 2001, Fleschhut et al., 2006, Castañeda-Ovando et al., 2014). The largest reduction in ACY ensued during the purification step with only 69.4% of anthocyanins present after drying of the extract being found after purification. This loss may be due to either the columns' inability to adsorb all anthocyanins present or with the eluent's (acidified ethanol) inability to remove all of the retained anthocyanins (Kraemer-Schafhalter et al., 1998). However, considering the fact that there was a significant increase ($p > 0.05$) in TPC it is possible that some anthocyanin degradation occurred, one where species with higher reducing power (the true measure of the Folin - Ciocalteu method) were formed (Vermerris and Nicholson, 2007). In spite of the reduction in both TPC and ACY yield, when comparing the composition of the extracts prior and after purification, it can be seen that the overall portion of both is significantly higher after purification (Figure 6). In fact, after purification anthocyanins comprised ca. 41.6% (416.5 ± 4.5 μg mg^{-1} extract) of the extracted powder in contrast to the original 0.043% (ca. 0.43 μg mg^{-1} of extract). As for the TPC value it indicates that, after purification, the extract possessed 9323 μg mg^{-1} of extract. However, as this measure is based

upon a non-specific assay that has several different interferents, this value must be considered carefully.

Zheng et al. (2013) described an optimization of a microwave assisted anthocyanin extraction protocol. Although these authors do not provide data regarding the overall anthocyanin concentration of their extract, the conditions that were identified as the best imply the used of 47.1 ºC. At this temperature some anthocyanin degradation may occur meaning that this protocol may allow for artefactual changes of anthocyanin composition (Cacace and Mazza, 2003b). This possible anthocyanin temperature induced degradation of anthocyanins has been reported by Cacace and Mazza (2003b) when assessing the effect of different extraction temperatures upon anthocyanin extraction from blackcurrants viz., temperatures above 30 – 35 ºC led to anthocyanin degradation. Traditionally, authors either focus on the characterization of the extraction process or focus on the biological activity. However, the hereby proposed method, with an extraction process that is simple enough to allow for industrial application and a purification step that implies the use of re-utilizable resins, all while using food grade solvents and obtaining a powder (which simplifies potential application, transport and storage) may be an interesting proposal. Particularly if considering that some biological activity has already been demonstrated (Silva et al., 2016).

4. Conclusions

Out of all solvents tested, acidified ethanol proved to be the best solution when seeking to produce a food grade extract, from blueberries with high anthocyanin and phenolic content. The purification step may be important if seeking to produce a concentrated extract but, as it allows for the loss of some compounds, the use of the non-purified counterpart may also be interesting. Furthermore, the use of a methodology that is relatively simple and non-expensive and that results in an easily manageable powder makes this process particularly interesting for industrial application.

Acknowledgements: The authors would like to acknowledge the project UID/Multi/50016/2013 granted by FCT for the funding provided and Mirtilusa S.A. (Sever do Vouga, Portugal) for providing the blueberries used. Additionally, the authors S. Silva and E.M. Costa would like to acknowledge FCT for their Ph.D. grants, references SFRH/BD/90867/2012 and SFRH/BDE/103957/2014 respectively.

5. References

ATTOE, E. L. & VON ELBE, J. H. 1981. Photochemial Degradation of Betanine and Selected Anthocyanins. *Journal of Food Science,* 46**,** 1934-1937.

CABAROGLU, T. 2005. Methanol contents of Turkish varietal wines and effect of processing. *Food Control,* 16**,** 177-181.

CACACE, J. E. & MAZZA, G. 2002. Extraction of Anthocyanins and Other Phenolics from Black Currants with Sulfured Water. *Journal of Agricultural and Food Chemistry,* 50**,** 5939-5946.

CACACE, J. E. & MAZZA, G. 2003a. Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering,* 59**,** 379-389.

CACACE, J. E. & MAZZA, G. 2003b. Optimization of Extraction of Anthocyanins from Black Currants with Aqueous Ethanol. *Journal of Food Science,* 68**,** 240-248.

CASTAÑEDA-OVANDO, A., GALÁN-VIDAL, C. A., CONTRERAS-LÓPEZ, E. & PÁEZ-HERNÁNDEZ, M. E. 2014. Purification of Anthocyanins with o-Dihydroxy Arrangement by Sorption in Cationic Resins Charged with Fe(III). *Journal of Chemistry,* 2014**,** 9.

CASTAÑEDA-OVANDO, A., PACHECO-HERNÁNDEZ, M. D. L., PÁEZ-HERNÁNDEZ, M. E., RODRÍGUEZ, J. A. & GALÁN-VIDAL, C. A. 2009. Chemical studies of anthocyanins: A review. *Food Chemistry,* 113**,** 859-871.

FLESCHHUT, J., KRATZER, F., RECHKEMMER, G. & KULLING, S. E. 2006. Stability and biotransformation of various dietary anthocyanins in vitro. *European Journal of Nutrition,* 45**,** 7-18.

GIÃO, M. S., GONZÁLEZ-SANJOSÉ, M. L., RIVERO-PÉREZ, M. D., PEREIRA, C. I., PINTADO, M. E. & MALCATA, F. X. 2007. Infusions of Portuguese medicinal plants: Dependence of final antioxidant capacity and phenol content on extraction features. *Journal of the Science of Food and Agriculture,* 87**,** 2638-2647.

GIBSON, G. R. & WILLIAMS, C. M. 2000. *Functional foods: Concept to product.,* Cambridge, England, Woodhead Publishing Limited.

ILOKI-ASSANGA, S. B., LEWIS-LUJÁN, L. M., LARA-ESPINOZA, C. L., GIL-SALIDO, A. A., FERNANDEZ-ANGULO, D., RUBIO-PINO, J. L. & HAINES, D. D. 2015. Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of Bucida buceras L. and Phoradendron californicum. *BMC Research Notes,* 8**,** 396.

KRAEMER-SCHAFHALTER, A., FUCHS, H. & PFANNHAUSER, W. 1998. Solid-phase extraction (SPE)—a comparison of 16 materials for the purification of anthocyanins from aronia melanocarpa var Nero. *Journal of the Science of Food and Agriculture,* 78**,** 435-440.

LEE, J., RENNAKER, C. & WROLSTAD, R. E. 2008. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry,* 110**,** 782-786.

NETO, C. C. 2007. Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition & Food Research,* 51**,** 652-664.

SEERAM, N. P., BOURQUIN, L. D. & NAIR, M. G. 2001. Degradation Products of Cyanidin Glycosides from Tart Cherries and Their Bioactivities. *Journal of Agricultural and Food Chemistry,* 49**,** 4924-4929.

SILVA, S., COSTA, E., CALHAU, C., MORAIS, R. & PINTADO, M. 2015a. Anthocyanin Extraction from Plant Tissues: A Review. *Critical Reviews in Food Science and Nutrition***,** 00-00.

SILVA, S., COSTA, E. M., COSTA, M. R., PEREIRA, M. F., PEREIRA, J. O., SOARES, J. C. & PINTADO, M. M. 2015b. Aqueous extracts of Vaccinium corymbosum as inhibitors of Staphylococcus aureus. *Food Control,* 51**,** 314-320.

SILVA, S., COSTA, E. M., MENDES, M., MORAIS, R. M., CALHAU, C. & PINTADO, M. M. 2016. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic anthocyanin rich blueberry extract purified by solid phase extraction. *J Appl Microbiol*.

TIWARI, B. K., O'DONNELL, C. P., PATRAS, A. & CULLEN, P. J. 2008. Anthocyanin and Ascorbic Acid Degradation in Sonicated Strawberry Juice. *Journal of Agricultural and Food Chemistry,* 56**,** 10071-10077.

VERMERRIS, W. & NICHOLSON, R. L. 2007. *Phenolic Compound Biochemistry*, Springer.

VIEIRA, G. S., CAVALCANTI, R. N., MEIRELES, M. A. A. & HUBINGER, M. D. 2013. Chemical and economic evaluation of natural antioxidant extracts obtained by ultrasound-assisted and agitated bed extraction from jussara pulp (Euterpe edulis). *Journal of Food Engineering,* 119**,** 196-204.

WALLACE, T. C. & GIUSTI, M. 2013. *Anthocyanins in Health and Disease*, Taylor & Francis Group.

ZHENG, X., XU, X., LIU, C., SUN, Y., LIN, Z. & LIU, H. 2013. Extraction characteristics and optimal parameters of anthocyanin from blueberry powder under microwave-assisted extraction conditions. *Separation and Purification Technology,* 104**,** 17-25.

Chapter 4

In vitro *antioxidant capacity*

This chapter aimed to improve an existing, in vitro*, assay for the determination of the antioxidant (or pro-oxidant) capacity using* DNA/H_2O_2 *and* $DNA/H_2O_2/Fe^{2+/3+}$ *systems*

"To be or not to be: That is the question."

William Shakespeare *in* ***Hamlet***

Chapter Preamble

Antioxidants can be perceived as possessing two interesting applications. The first stems from their direct action to prevent oxidation of products (edible, cosmetic or pharmaceutical) the second, as possibly the most controversial, is their use to aid in the maintenance of oxidative homeostasis, a recognized risk factor in the development of diseases. When considering the first application, there are several methodologies that aid in the contextualization of the antioxidant capacity of a compound/extract. Radical scavenging assays like ABTS or DPPH can be useful when looking at an antioxidant from a chemical perspective, however, when passing to a biological context, most of the methods typically imply complex and/or time consuming methodologies. Therefore, the first goal of the present chapter was to modify a pre-existing DNA agarose gel electrophoresis method optimizing it in two different aspects: i) replace the original DNA oxidation system (ascorbic acid and iron) by two that had a higher biological relevance using H_2O_2, (alone and in the presence of Fe^{3+}, the most abundant intracellular iron cation, that reacts with H_2O_2 yielding $HO^{\bullet}$ and $HO_2^{\bullet}$); ii) develop a quantitative approach to measure the degradation of DNA. Gallic and ascorbic acids were used as antioxidant standards to develop the method and evaluate its capacity to assess not only the antioxidant capacity but also any pro-oxidant effect. This last determination (evaluated in the presence and absence of iron cations, as to simulate possible Fenton reactions that may occur) allows for a different approach to the analysis of the antioxidant capacity as it contextualizes it with another property of phenolic compounds have also been associated - their potential pro-oxidant effect.

After developing the method to assess both DNA protection and degradation, the blueberry extract was evaluated to determine its DNA protection capabilities and weather it had any pro-oxidant effect. Furthermore, these results were compared with those obtained using a common *in vitro* assay, the ABTS radical method. This method was chosen to draw the comparison because it measures the antioxidant capacity considering the two different mechanisms for radical quenching, hydrogen and electron transfer (unlike FRAP or ORAC). The results obtained demonstrated that the proposed method allowed for an assessment of both the antioxidant and pro-oxidant effect of solutions, the overall method exhibited a good consistency between repetitions. Moreover, when analysing the antioxidant activity of the blueberry extract it can be seen that it is effective in inhibiting DNA degradation at

concentrations as low as 160 or 200 μg mL^{-1} for the H_2O_2 or the $H_2O_2/FeCl_3$ systems, respectively, while exhibiting no pro-oxidant activity, regardless of the presence of iron.

DNA agarose gel electrophoresis for antioxidant analysis: development of a quantitative approach for phenolic extracts

Sara Silva[1], Eduardo M. Costa[1], Sandra Vicente[1], Mariana Veiga[1], Conceição Calhau[2,3], Rui M. Morais[1], Manuela E. Pintado[1]

[1] *CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal*

[2]*Nutrição e Metabolismo, NOVA Medical School, Universidade Nova de LIsnoa, Campo dos Mártires da Pátria, 130,l 1169-056, Lisboa, Portugal*

[3]*CINTESIS – Centro de Investigação em Tecnologias e Serviços de Saúde, Porto, Portugal*

Abstract

Most of the fast *in vitro* assays proposed to determine the antioxidant capacity of a compound/extract lack either biological context or employ complex protocols. Therefore, the present work proposes the improvement of an agarose gel DNA electrophoresis in order to allow for a quantitative estimation of the antioxidant capacity of pure phenolic compounds as well as a of a phenolic rich extract, while also considering their possible pro-oxidant effects. The result obtained demonstrated that the proposed method allowed for the evaluation of the protection of DNA oxidation (by H_2O_2 and an $H_2O_2/FeCl_3$ system) as well as for the observation of pro-oxidant activities, with the measurements registering interclass correlation coefficients above 0.9. Moreover, this method allowed for the characterization of the antioxidant capacity of a blueberry extract while demonstrating that it had no perceived pro-oxidant effect.

Keywords: Antioxidant assays; Pro-oxidant assays; Phenolic compounds; Blueberry extract, Ascorbic acid, Gallic acid

1. Introduction

As the evidences linking oxidative damage and the risk of chronic disease increases so does the interest in quantifying the efficacy of antioxidant compounds in a biological context (Ghiselli et al., 2000, Frankel and Meyer, 2000, Huang et al., 2005, Zhang and Tsao, 2016, Cabello-Verrugio et al., 2016). Reactive oxygen species (ROS) such as H_2O_2 are endogenous initiators of degenerative processes, as they damage lipids, proteins and DNA, thus favouring the development of a number of degenerative diseases (Jornot et al., 1998, Altıntaş et al., 2016). These species have been linked with an array of DNA lesions, varying from modifications to the deoxyribose base to inducing actual breaks in the DNA strands. In turn, these alterations may compromise metabolic pathways, disturbing the homeostatic balance and increasing the risk of disease (Jornot et al., 1998). Fruits and vegetables contain a plethora of dietary phytonutrients with strong antioxidant capacities, such as phenolics, carotenoids, and vitamins which, in turn, may aid in the maintenance of oxidative balance as these antioxidants have been hypothesized as possible agents that may aid in the maintenance of oxidative balance. Therefore the development of methods that allow for a fast, simple and biological relevant screening of several potentially antioxidant compounds and/or extracts may be of particular use (Ghiselli et al., 2000, Frankel and Meyer, 2000, Huang et al., 2005, MacDonald-Wicks et al., 2006).

Though several antioxidant capacity measuring methods have been reported in literature, one of the most common problems of *in vitro* assays (easier and faster to use when screening for antioxidant potential) is their relative lack of biological context, for even when the methods consider the inhibition of biologically relevant radicals (e.g. hydroxyl radicals; $HO^{\bullet}$), they typically disregard the molecules that the antioxidants could be protecting and the equilibria between antioxidant, pro-oxidant and the body's natural coping mechanisms (Ghiselli et al., 2000, Frankel and Meyer, 2000, Huang et al., 2005, MacDonald-Wicks et al., 2006). In 2005, Rivero et al. (2005) reported an *in vitro* assay that measured the protection of DNA against oxidation using an ascorbic acid/ Cu^{2+} radical generating system. In this methodology, DNA degradation was either measured through electrophoresis or through the quantification of 8-hydroxydeoxyguanosin (8-OHdG) (Rivero et al., 2005). However, both methods possess significant limitations. The electrophoretic evaluation, while simple, as is, allows only for a qualitative evaluation of DNA degradation (i.e. either

$$H_2O_2 + Fe^{3+} \rightleftharpoons Fe^{2+} + HO_2^{\bullet}$$

$$H_2O_2 + Fe^{2+} \rightleftharpoons Fe^{3+} + HO^- + HO^{\bullet}$$

Figure 1. Schematic representation of Fenton reactions (adapted from Jornot et al., 1998 and Perez-Benito, 2004).

there is a band or not). On the other hand, the quantification of 8-OHdG, implies a longer processing of the DNA sample and, therefore, makes it harder to be used in screening assays (Zhang et al., 2013, Rivero et al., 2005). On a different note, the radical generating system proposed (ascorbic acid/ Cu^{2+}), while efficient at promoting DNA degradation, not only lacks in biological context (from a human body perspective) but it has also been hypothesised as being susceptible to bias when analysing antioxidant extracts from plant sources. Particularly, this system's application may be problematic when considering fruits and fruit extracts, as their inherent high levels of ascorbic acid may accentuate the intensity of the initial radical generating system (Jornot et al., 1998, Gião et al., 2008). Therefore, the present paper aimed to improve the existing method by altering the radical system, using both plain H_2O_2 and an H_2O_2/$FeCl_3$ systems (Figure 1) more relevant from a biological standpoint (Cooper et al., 2002), and enable its use as a quantitative method through the measurement of band intensity after agarose gel electrophoresis.

2. Experimental section

2.1. Samples

Phenolic standards ascorbic and gallic acid were acquired from Sigma (Darmstadt, Germany) and dissolved in sterile ultra-pure water (upH_2O). Blueberry extract was produced and purified as described elsewhere and prepared using sterile upH_2O (Silva et al., 2016).

2.2. DNA solution preparation

Sterile upH_2O was mixed with deoxyribonucleic acid (DNA) from calf thymus (Sigma, Darmstadt, Germany) at 0.25 mg mL^{-1} and gently homogenized overnight, using a tilt-and-roll mixer, in a light tight container with no headspace. This solution was stored up to 7 d, at 4 °C in a light tight container.

2.3. Selection of the DNA degradation systems

Two DNA degradation system were considered, both with some physiological context; hydrogen peroxide (H_2O_2) alone and in the presence of cationic iron (to stimulate the production of $HO^{\bullet}$ and $HO_2^{\bullet}$). When considering the impact of H_2O_2 alone, the DNA

solution was mixed with H_2O_2 and phosphate-buffered saline (PBS pH 7.4) to attain final concentrations of 50 µg mL^{-1} for DNA and 7.04, 5.26, 3.52, 2.64, 1.76 or 0.88 M for H_2O_2. When considering the H_2O_2/iron cation systems, an $FeCl_3$ solution (prepared immediately before each assay using sterile upH_2O) was mixed with DNA, H_2O_2 and PBS to attain the following final concentrations: 50 µg mL^{-1} of DNA, 10 mM $FeCl_3$ and 1.76, 0.88, 0.176, 0.088 or 0.0176 M of H_2O_2. All mixtures were incubated for 1 h to allow the reaction to occur and an agarose gel electrophoresis was run as described in section 2.6.

2.4. DNA protection assessment (antioxidant assays)

The DNA was incubated in the presence of the degradation systems selected (7.01 M H_2O_2 and H_2O_2 with $FeCl_3$, 0.05 M and 10 mM respectively) and varying concentrations of compounds, staring with a range of concentrations (400, 300, 200 and 100 µg mL^{-1}) and then further exploring the concentrations that between which the highest variation was observed (Table 1). The DNA solution, without H_2O_2 was used as a negative control (no degradation) for the assays using the H_2O_2 system and a DNA solution with 10 mM $FeCl_3$ was used as a negative control (no degradation) for the assays using the H_2O_2/$FeCl_3$ system. After 1 h incubation, in the dark, at 37 °C and an agarose gel electrophoresis was run as described in section 2.6.

2.5. DNA degradation assessment (pro-oxidant assays)

The DNA solution, with and without 10 mM $FeCl_3$, was incubated (1 h at 37 °C in the dark) in the presence of different concentrations of either ascorbic acid, gallic acid or blueberry extract (Table 1). Afterwards, an electrophoresis was run as described in section 2.6. A negative control (no degradation) was drawn using only the DNA solution or the DNA solution with 10 mM $FeCl_3$, for the H_2O_2 or the H_2O_2/$FeCl_3$ system respectively.

Table 1. Concentrations of antioxidant compounds used in the antioxidant and pro-oxidant assays.

		Concentrations tested (µg mL^{-1})	
		With H_2O_2	Without H_2O_2
Gallic acid	Without $FeCl_3$	0, 100, 200, 225, 250, 275, 300, 400	0, 100, 200, 225, 250, 275, 300, 400
	With $FeCl_3$	0, 20, 40, 60, 80, 100, 200, 300, 400	0, 20, 40, 60, 80, 100, 200, 300, 400
Ascorbic acid	Without $FeCl_3$	0, 20, 40, 60, 80, 100, 200, 300, 400	0, 20, 40, 60, 80, 100, 200, 300, 400
	With $FeCl_3$	0, 20, 40, 60, 80, 100, 200, 300, 400	0, 20, 40, 60, 80, 100, 200, 300, 400
Blueberry extract	Without $FeCl_3$	0, 40, 80, 120, 160, 200, 400, 600, 800	0, 40, 80, 120, 160, 200, 400, 600, 800
	With $FeCl_3$	0, 40, 80, 120, 160, 200, 400, 600, 800	0, 40, 80, 120, 160, 200, 400, 600, 800

2.6. Electrophoresis

Each sample was mixed 1:4 with loading buffer (25 mg bromophenol blue, 10 mL Tris EDTA (TE) buffer 1x pH 8.0 and 20 mL glycerol with pH value adjusted to 8.0) and 10 µL aliquots were transferred into a 0.75% (w v^{-1}) agarose (Nztech, Lisboa, Portugal) gel prepared using Tris-Acetate EDTA buffer (TAE) supplemented with 0.03 µL mL^{-1} GreenSafe Premium (Nztech, Lisboa, Portugal). Electrophoresis was then run for 1.25 h at 150 mV. The gels were analysed using a molecular imager GelDOC XR+ (BioRad, Hercules, California, USA) and the resulting image was processed using Image Lab Software v5.1 (BioRad, Hercules, California, USA). The band area for each positive control was manually defined (to measure the band intensity) and then copied into each sample lane (maintaining the distance to the wells; Figure 2), with the decrease in band intensity being considered as a result of a reduction of the amount of DNA present. The results were given as the percentage of inhibition of the DNA band degradation (for the antioxidant assay) or as percentage of DNA band degradation (for the pro-oxidant assay) both calculated as described in the equations below, in which $Intensity_{sample}$ is the intensity of each sample band, $Intensity_{background}$ refers to the intensity of the background, measured besides the

control bands, and $Intensity_{DNA\ solution}$ refers to the intensity of the intact DNA solution. All incubations were made in triplicate and loaded twice into the gel.

$$Inhibition\ of\ DNA\ degradation\ (\%) = \left(\frac{Intensity_{sample} - Intensity_{background}}{Intensity_{DNA\ solution}}\right) \times 100$$

$$DNA\ degradation\ (\%).100 - \left[\left(\frac{Intensity_{sample} - Intensity_{background}}{Intensity_{DNA\ solution}}\right) \times 100\right]$$

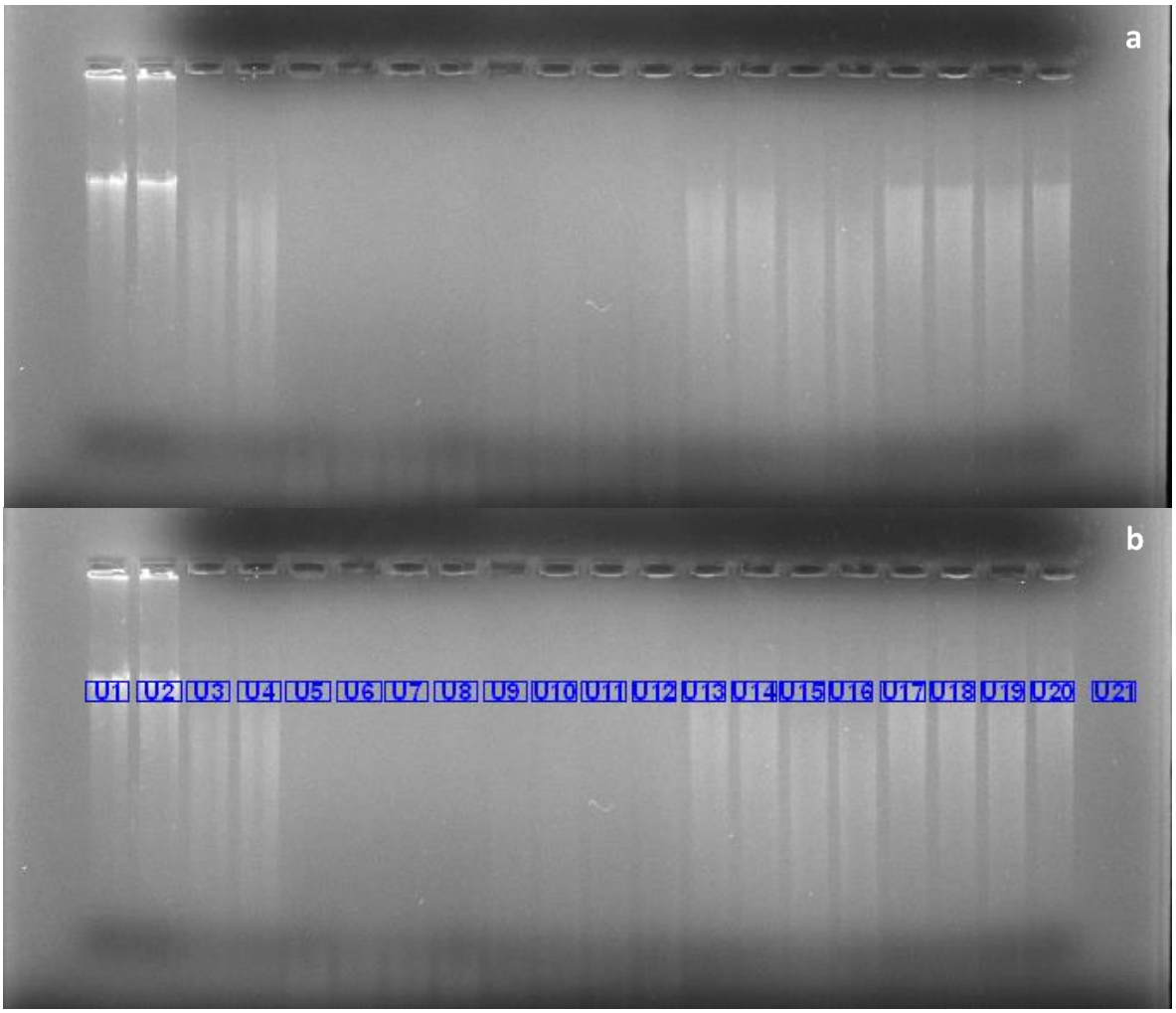


Figure 2. Schematic representation of the processing of the electrophoretic results before (a) and after (b) the integration of each band. U1, U2 – DNA solution; U3, U4 – DNA solution and degradation system; U5 to U20 DNA solution, degradation system; U21 – background noise.

2.7. ABTS radical cation method

In order to compare the presently proposed method's assessment of antioxidant capacity to one that has been widely applied in the characterization of the antioxidant capacity of plant extracts, the 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) method was employed as described by Gião et al. (2007). Briefly, 10 µL aliquots of different extract solutions, diluted when needed, were added an $ABTS^{\bullet+}$ solution with an initial optical density (OD) of 0.700 ± 0.020 measured at 734 nm. After allowing the reaction to occur, the OD was measured with an UV-Vis spectrophotometer (UVmini 1240, Shimadzu, Japan) and the results were presented as relative $ABTS^{\bullet+}$ inhibition, calculated as described below (in which OD_{sample} indicates the OD measured for each assay, DF is the dilution factor and OD_{ABTS} refers to the initial OD of the $ABTS^{\bullet+}$ solution) All assays were performed in triplicate.

$$ABTS^{\bullet+} inhibition\ (\%) = 100 - \left[\left(\frac{OD_{sample} \times DF}{OD_{positive\ control}}\right) \times 100\right]$$

2.8. Statistical analysis

Statistical analysis of the data was performed using IMB SPSS Statistics v21.0.0.0 software (New York, USA). The normality of the data distribution was evaluated using Shapiro Wilk's test with the data proving to follow a normal distribution. To evaluate the reproducibility of the assay the interclass correlation coefficient (ICC, for average measures), comparing the results obtained in two different sets of experiments, each with 6 different replicate values. Additionally, comparisons between different concentrations were evaluated, when needed using a paired samples T-test with the differences being considered significant for p-values below 0.05.

3. Results and discussion

In order alter the ROS generating system used to one based on H_1O_2 and $H_1O_1/FeCl_3$, the first step was to define the concentrations of H_2O_2 to be used. Figure 3 demonstrates the DNA degradation capabilities of both ROS systems considered, H_2O_2 and H_2O_2 in the presence of iron cations. Alone (Figure 3a), H_2O_2 caused DNA degradations that averaged on ca. 35%, with the highest value (46% ± 0.5) having been observed for highest concentration of H_2O_2 tested, i.e. 7.04 M. On the other hand, when considering the iron/ H_2O_2 system (Figure 3a), the DNA degradation percentage obtained ranged from 48 to 92% (0.02 M H_2O_2 with 10 mM $FeCl_3$ and 1.76 M H_2O_2, respectively). Moreover, the fact that the combination of H_2O_2 and $FeCl_3$ was more damaging to the DNA molecule than H_2O_2 alone (with higher degradation rates at lower H_2O_2 concentrations), could be somewhat expected as H_2O_2, in spite of being considered a ROS, has been reported as possessing limited DNA damage inducing capabilities on its own and most of its *in vivo* lethality has been associated with its involvement in Fenton reactions (mostly iron-mediated) (Imlay and Linn, 1988, Mello-Filho and Meneghini, 1991). Regardless, both systems were considered in the

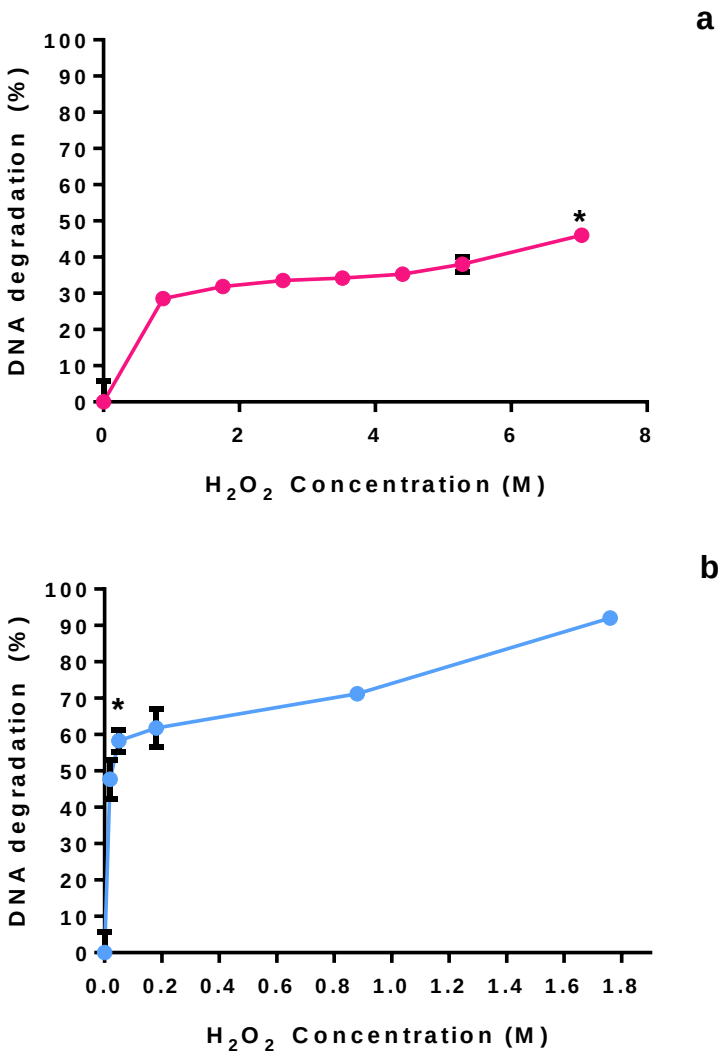


Figure 3. Relative DNA degradation caused by varying concentration of H_2O_2, alone (a) and in the presence of $FeCl_3$ (b). The * mark the H_2O_2 concentrations used in the subsequent assays.

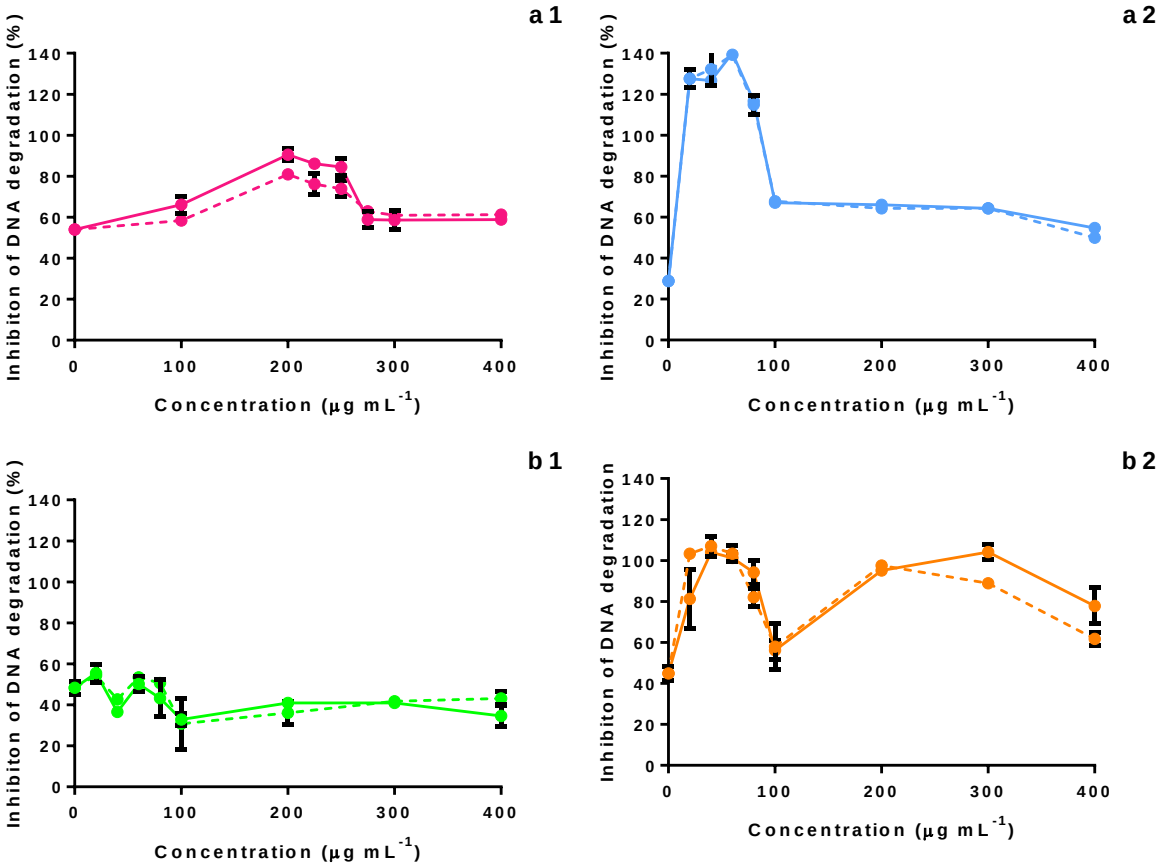


Figure 4. Prevention of DNA oxidation by H_2O_2 (1) or an H_2O_2/ $FeCl_3$ system (2) by gallic (a) and ascorbic (b) acids. The different lines represent independent replicas of each assay.

remaining assays, as both were still capable of inflicting significant DNA damage. On another note, the H_2O_2 concentrations that were selected to be used from this point onward were 7.04 and 0.05 M, alone or in the presence of 10 mM $FeCl_3$, respectively. These concentrations were selected because despite still allowing for a relatively high DNA degradation percentage they still left some room to evaluate if the presence of an antioxidant compound, instead of inhibiting DNA degradation, could accentuate DNA degradation.

When analysing the protective effect of gallic acid against H_2O_2 induced DNA degradation it can be seen (Figure 4a1) that, while relevant protection of the DNA molecule can be observed for concentrations ranging from 200 to 250 µg mL^{-1}, a complete inhibition of DNA (100%) degradation was not observed (Figure 4a1). On the other hand, when DNA degradation was induced by the H_2O_2 iron system, not only were lower concentrations of

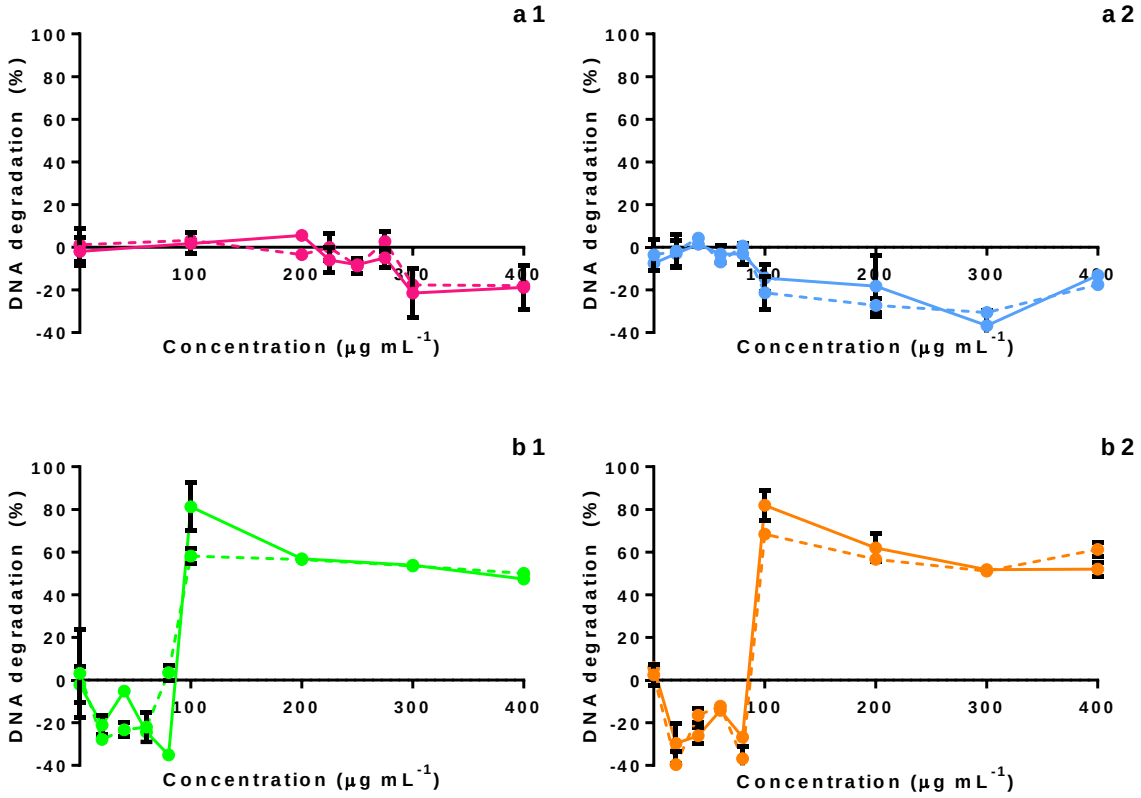


Figure 5. Pro-oxidant effect of gallic (a) and ascorbic (b) acids alone (1) or in the presence of $FeCl_3$ (2). The different lines represent independent replicas of each assay.

gallic acid more effective (20 to 80 µg mL^{-1}) but they were capable of fully protecting the DNA (100%). Although, in some cases, inhibitions of DNA degradation appear above 100%, indicating a higher fluorescence intensity of the band after exposure to the compounds which may hint that some interactions between the compound and DNA occur. Interestingly, when considering the results observed for ascorbic acid it can be seen that it has little to no protective effect when the DNA is exposed to H_2O_2 alone but, at low concentrations (40 to 60 µg mL^{-1}), it is capable of protecting it against damage induced by the $H_2O_2/FeCl_3$ system. Furthermore, while there is an apparent lack in DNA protection at 100 µg mL^{-1} (inhibition of DNA degradation is the same ($p > 0.05$) to that observed in the absence of ascorbic acid), for concentrations above this value the inhibitions rise again. Both ascorbic acid and gallic acid are known antioxidants, however ascorbic acid exhibited little antioxidant activity in the presence of H_2O_2 alone. As ascorbic acid has been used by the industry to remove excess of H_2O_2 that results from some package sterilisation procedures, it could be expected that its capacity to interact with this molecule could aid in the amelioration of H_2O_2 induced DNA oxidation. However, considering that the aerobic oxidation of ascorbic acid to

dehydroascorbic acid (DHAA) results in the production of 2 electrons and 2 hydrogens, it is possible that, while some quenching is occurring, more reactive species are being produced thus promoting DNA oxidation (Harper et al., 1969, Golubitskii et al., 2007). This does not explain the antioxidant effect observed when considering the $H_2O_2/FeCl_3$ systems, particularly as there are additional radical species present ($HO^{\bullet}$ and $HO_2^{\bullet}$). We hypothesise that, the presence of electrons resulting from ascorbic acid oxidation promote the conversion of Fe^{3+} to Fe^{2+}, shifting the equilibria proposed in figure 1 to the production of $HO^{\bullet}$, which in turn may be interacting with hydrogen (that also results from ascorbic acid oxidation to DHAA) resulting in the production of water (Golubitskii et al., 2007, Jornot et al., 1998).

Antioxidant compounds have been reported to act, under certain conditions, as pro-oxidant agents (Procházková et al., 2011). Therefore, in order to ascertain the proposed method's capacity to measure pro-oxidant effect, the DNA was incubated with each of the acids (alone or in the presence of Fe cations to simulate Fenton reactions). As can be seen in Figure 5 (a1 and a2) no DNA degradation was observed for GA, regardless of the presence of Fe^{3+} cations, hinting that this compound had no pro-oxidant effect under the assayed conditions. Nevertheless, considering that some concentrations of gallic acid (300 µg mL^{-1} in the absence of iron, and 100 µg mL^{-1} in the presence of iron) led to higher fluorescence intensities than the control, it is likely that some interactions with the DNA molecule occurred, hinting that while not obvious degradation was observed, some might still be occurring. This stands in line with what has been reported by Yang et al. (2008), who found that gallic acid could interact directly with DNA and cause some damage. Moreover, for ascorbic acid, a similar increase in band intensity (i.e. negative values for DNA band degradation) was also observed below 100 µg mL^{-1}, regardless of the presence of Fe cations on the environment (Figure 5b1 and 5b2). However, for concentrations above, or equal to, 100 µg mL^{-1} there was a significant degradation of the DNA bands which, in turn demonstrates the pro-oxidant effect of ascorbic acid, which is well supported in the literature (Rivero et al., 2005, Podmore et al., 1998). Overall, these results demonstrate that the proposed method appears to be a good alternative for the estimation of both the antioxidant and pro-oxidant activity of an extract. As such, in an attempt to assess the consistency of the analysis, the ICC was calculated for each of the assays and the results can be seen in Table 2. For all assays, the ICC value was above 0.9 therefore demonstrating that the results exhibited a good reliability, fact that is further accentuated by the fact that the lower bound

of the 95% confidence ICC values interval is also above 0.9, meaning that, in 95% of the performed measures exhibit a correlation above 0.9.

Table 2. Test-retest reliability of the proposed antioxidant and pro-oxidant assays.

		ICC	95 % Confidence interval	
			Lower bound	Upper bound
Antioxidant assay	H_2O_2	0.976	0.950	0.990
	$H_2O_2/FeCl_3$	0.980	0.961	0.991
Pro-oxidant assay	Without $FeCl_3$	0.995	0.990	0.998
	With $FeCl_3$	0.990	0.976	0.996

Figure 6 shows the results obtained for the evaluation of the antioxidant (and pro-oxidant) capacity of an anthocyanin rich blueberry extract, whose composition is described elsewhere (Silva et al., 2016). The extract was capable of inhibiting DNA degradation regardless of the degradation system used. For H_2O_2 alone, the lowest concentration of extract used, 40 µg mL^{-1}, was capable of significantly inhibiting the DNA degradation, yielding an inhibition percentage of DNA degradation of 89 ± 1 %. Concentrations above 160 µg mL^{-1}, inclusively, completely inhibited DNA degradation. On the other hand, when considering the H_2O_2 iron degradation system, the extract proved to be less effective, though concentrations of 200 µg $mL^{-1}$and above allowed for a complete inhibition of DNA degradation. As the $HO^{\bullet}$ and $HO_2^{\bullet}$ radicals are considerably more potent oxidants than plain H_2O_2, it stands to reason that in order to reduce their impact upon DNA higher amounts of protective antioxidants are required. Furthermore, when considering that the phenolic compound composition of the extract is 216.8 µg mg^{-1}, the complete inhibition of DNA degradation observed occurs at a phenolic compound concentration of 34.69 and 43.36 µg mg^{-1} (for the H_2O_2 and the H_2O_2 / iron degradation systems, respectively), values that are within the range of activity observed for both gallic and ascorbic acid when in the presence of $H_2O_2/FeCl_3$ system, but lower than

those obtained when considering the prevention of H_2O_2 mediated oxidation (Silva et al., 2016, Regoli and Winston, 1999). These results demonstrate that mixtures of phenolic compounds may be more interesting as protective agents than isolated compounds, particularly as the mechanisms through which they exhort their activity and their affinity to a particular radical may vary from compound to compound, therefore, when in mixtures synergies may be established between the different compounds (De Rosso et al., 2008, Mathew et al., 2015).

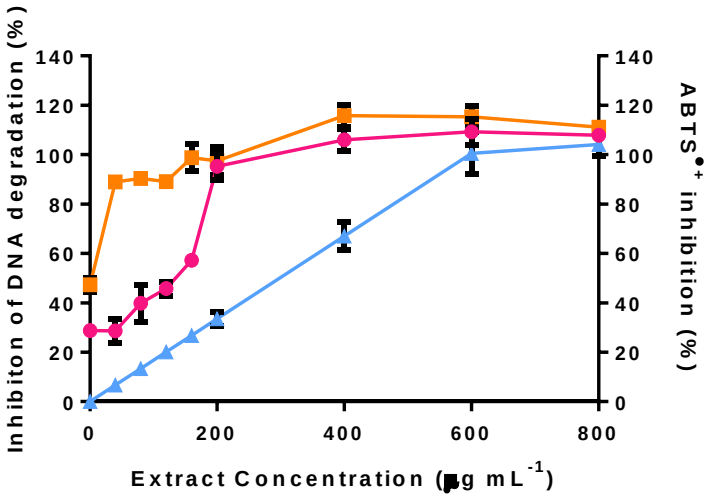


Figure 6. Antioxidant effect of a blueberry extract considering the proposed method (●, protection against H2O2 mediated degradation and ■, protection against H2O2/FeCl3 mediated DNA degradation) in comparison to the one observed using a traditional ABTS●+ method (▲).

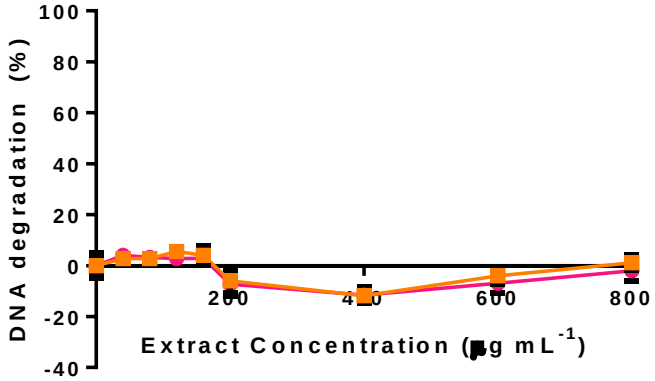


Figure 7. Pro-oxidant effect of a blueberry extract in the absence (●) and presence of FeCl3 (■).

In Figure 7, the evaluation of the potential pro-oxidant activity of the extract is illustrated. Interestingly, the extract, either alone or in the presence of iron, did not induce any DNA degradation in spite of some authors reporting that phenolic compounds may act as pro-oxidants and that anthocyanins may interact with DNA (Decker, 1997, Webb et al., 2008). Moreover, it is interesting to note that, unlike what was observed for both gallic and ascorbic acid, there was little to no increase in fluorescence intensity, hinting that little to no interaction between the compounds and the DNA is occurring. Furthermore, as no significant ($p < 0.01$) differences were found between the extract's effect in the presence, and absence, of iron cations, the likeliness that they will partake in *in vivo* Fenton reactions is relatively low, further accentuating its interest as an antioxidant solution.

On a different approach, the extract's antioxidant capacity was evaluated through the $ABTS^{\bullet+}$ method in order to compare its capacity to quench this radical and its capacity to protect DNA (Figure 6, right axis). As phenolic compounds have been described as capable of quenching radicals either by donating an electron or a hydrogen, the use of an $ABTS^{\bullet+}$ based method allows for a more encompassing comparison than using methods that measure only one of the quenching mechanism, such as the oxygen radical absorbance capacity (ORAC; which measures hydrogen transfer) or the ferric reducing antioxidant power (FRAP; which measures electron transfer) (De Rosso et al., 2008, Mathew et al., 2015, Benzie and Strain, 1996, Ou et al., 2001). As could be expected, the higher the concentration of extract the higher the $ABTS^{\bullet+}$inhibition observed, with this proportion varying linearly up until the concentration of extract is high enough to completely inhibit this radical (600 µg mL^{-1}). It is interesting to note that the extract appears to be much more effective in blocking DNA oxidation that it is at quenching $ABTS^{\bullet+}$ demonstrating that it may be a useful technique to provide some biological context in routine antioxidant capacity screenings.

4. Conclusions

The present work demonstrated that a simple *in vitro* protocol using agarose gel DNA electrophoresis could be an interesting tool for the quantitative evaluation of the antioxidant and pro-oxidant effect of both pure compounds, as well as fruit extracts, while providing some biological context. Moreover, considering the biological relevance of both oxidation systems used and the differences of behaviour observed for each of them, the need for the usage of more than one system when performing an antioxidant or pro-oxidant analysis becomes appropriate. When considering the anthocyanin rich blueberry extract, this method allowed for a demonstration of the extract's capacity to protect DNA against exogenous oxidation while having no pro-oxidant effect and little to no interaction with the DNA itself. Furthermore, it can be seen that the proposed method does not exhibit a behaviour similar to that of the ABTS method (a traditional method used for antioxidant capacity), therefore accentuating its difference from traditional radical quenching assays.

Acknowledgments: The authors acknowledge the financial support given by the national funds from FCT (through project UID/Multi/50016/2013). Additionally, the authors Sara Silva and Eduardo M. Costa wold like to acknowledge FCT for their Ph.D. grants with the references SFRH/BD/90867/2012 and SFRH/BDE/103957/2014, respectively.

5. References

ALTıNTAŞ, A., DAVIDSEN, K., GARDE, C., MORTENSEN, U. H., BRASEN, J. C., SAMS, T. & WORKMAN, C. T. 2016. High-resolution kinetics and modeling of hydrogen peroxide degradation in live cells. *Free Radical Biology and Medicine,* 101**,** 143-153.

BENZIE, I. F. & STRAIN, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry,* 239**,** 70-6.

CABELLO-VERRUGIO, C., RUIZ-ORTEGA, M., MOSQUEIRA, M. & SIMON, F. 2016. Oxidative stress in disease and aging: mechanisms and therapies. *Oxidative medicine and cellular longevity,* 2016.

COOPER, C. E., VOLLAARD, N. B. J., CHOUEIRI, T. & WILSON, M. T. 2002. Exercise, free radicals and oxidative stress. *Biochemical Society Transactions,* 30**,** 280-285.

DE ROSSO, V. V., MORAN VIEYRA, F. E., MERCADANTE, A. Z. & BORSARELLI, C. D. 2008. Singlet oxygen quenching by anthocyanin's flavylium cations. *Free Radicical Research,* 42**,** 885-91.

DECKER, E. A. 1997. Phenolics: prooxidants or antioxidants? *Nutr Rev,* 55**,** 396-8.

FRANKEL, E. N. & MEYER, A. S. 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture,* 80**,** 1925-1941.

GHISELLI, A., SERAFINI, M., NATELLA, F. & SCACCINI, C. 2000. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free radical biology & medicine,* 29**,** 1106-14.

GIÃO, M. S., GONZÁLEZ-SANJOSÉ, M. L., MUÑIZ, P., RIVERO-PÉREZ, M. D., KOSINSKA, M., PINTADO, M. E. & MALCATA, F. X. 2008. Protection of deoxyribose and DNA from degradation by using aqueous extracts of several wild plants. *Journal of the Science of Food and Agriculture,* 88**,** 633-640.

GIÃO, M. S., GONZÁLEZ-SANJOSÉ, M. L., RIVERO-PÉREZ, M. D., PEREIRA, C. I., PINTADO, M. E. & MALCATA, F. X. 2007. Infusions of Portuguese medicinal

plants: Dependence of final antioxidant capacity and phenol content on extraction features. *Journal of the Science of Food and Agriculture,* 87**,** 2638-2647.

GOLUBITSKII, G. B., BUDKO, E. V., BASOVA, E. M., KOSTARNOI, A. V. & IVANOV, V. M. 2007. Stability of ascorbic acid in aqueous and aqueous-organic solutions for quantitative determination. *Journal of Analytical Chemistry,* 62**,** 742-747.

HARPER, K. A., MORTON, A. D. & ROLFE, E. J. 1969. The phenolic compounds of blackcurrant juice and their protective effect on ascorbic acid. *International Journal of Food Science & Technology,* 4**,** 255-267.

HUANG, D., OU, B. & PRIOR, R. L. 2005. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry,* 53**,** 1841-56.

IMLAY, J. & LINN, S. 1988. DNA damage and oxygen radical toxicity. *Science,* 240**,** 1302-1309.

JORNOT, L., PETERSEN, H. & JUNOD, A. F. 1998. Hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions. *Biochemical Journal,* 335**,** 85-94.

MACDONALD-WICKS, L. K., WOOD, L. G. & GARG, M. L. 2006. Methodology for the determination of biological antioxidant capacity in vitro: a review. *Journal of the Science of Food and Agriculture,* 86**,** 2046-2056.

MATHEW, S., ABRAHAM, T. E. & ZAKARIA, Z. A. 2015. Reactivity of phenolic compounds towards free radicals under in vitro conditions. *Journal of Food Science and Technology,* 52**,** 5790-5798.

MELLO-FILHO, A. C. & MENEGHINI, R. 1991. Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 251**,** 109-113.

OU, B., HAMPSCH-WOODILL, M. & PRIOR, R. L. 2001. Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry,* 49**,** 4619-4626.

PEREZ-BENITO, J. F. 2004. Iron(III)−Hydrogen Peroxide Reaction: Kinetic Evidence of a Hydroxyl-Mediated Chain Mechanism. *The Journal of Physical Chemistry A,* 108**,** 4853-4858.

PODMORE, I. D., GRIFFITHS, H. R., HERBERT, K. E., MISTRY, N., MISTRY, P. & LUNEC, J. 1998. Vitamin C exhibits pro-oxidant properties. *Nature,* 392**,** 559-559.

PROCHÁZKOVÁ, D., BOUŠOVÁ, I. & WILHELMOVÁ, N. 2011. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia,* 82**,** 513-523.

REGOLI, F. & WINSTON, G. W. 1999. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicology and Applied Pharmacology,* 156**,** 96-105.

RIVERO, D., PÉREZ-MAGARIÑO, S., GONZÁLEZ-SANJOSÉ, M. L., VALLS-BELLES, V., CODOÑER, P. & MUÑIZ, P. 2005. Inhibition of Induced DNA Oxidative Damage by Beers: Correlation with the Content of Polyphenols and Melanoidins. *Journal of Agricultural and Food Chemistry,* 53**,** 3637-3642.

SILVA, S., COSTA, E. M., MENDES, M., MORAIS, R. M., CALHAU, C. & PINTADO, M. M. 2016. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic anthocyanin rich blueberry extract purified by solid phase extraction. *Journal Applied Microbiology*.

WEBB, M. R., MIN, K. & EBELER, S. E. 2008. Anthocyanin Interactions with DNA: Intercalation, Topoisomerase I Inhibition and Oxidative Reactions. *Journal of food biochemistry,* 32**,** 576-596.

YANG, Z., ZHANG, D., LONG, H. & LIU, Y. 2008. Electrochemical behavior of gallic acid interaction with DNA and detection of damage to DNA. *Journal of Electroanalytical Chemistry,* 624**,** 91-96.

ZHANG, A., WAN, L., WU, C., FANG, Y., HAN, G., LI, H., ZHANG, Z. & WANG, H. 2013. Simultaneous Determination of 14 Phenolic Compounds in Grape Canes by HPLC-DAD-UV Using Wavelength Switching Detection. *Molecules,* 18**,** 14241.

ZHANG, H. & TSAO, R. 2016. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science,* 8**,** 33-42.

Chapter 5

Effect on nosocomial bacteria

This chapter aimed to characterize the antimicrobial effect, upon nosocomial bacteria, of the extract as well as that of pure compounds traditionally found in blueberry

"The trouble of being a hypochondriac these days is that antibiotics have cured all the good diseases"

Caskie Stinnett *in* ***Out of the Red***

Chapter Preamble

Ever since the discovery that bacteria were able to develop resistance to antibiotics, there has been an increasing need to find new sources of antimicrobial compounds. One of the sources that are being looked at as alternative antimicrobial agents are phenolic compounds. One of the families of phenolic compounds that has a perceived antimicrobial activity are anthocyanins, one of the main groups of flavonoids that can be found in blueberries, that are thought to be responsible for the biological properties associated with blueberries.

The main goal of the present chapter was to characterize, and attempt to pinpoint, the activity of a blueberry extract obtained as described in chapter 3. To do so three different sub-chapters were considered:

Chapter 5.1. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction

Nowadays, the traditional antimicrobial approach, i.e. determining the effect of the agents upon planktonic cells, has been found lacking. In nature, bacteria are most commonly found entrapped within survival structures, biofilms, which have been reported to grant bacteria protection against the action of antibiotics and other antimicrobials. Furthermore, when attempting to infect a tissue or colonize a surface, bacteria must adhere to it. Therefore, being able to block either adhesion of biofilm formation may be an interesting property. Considering that there is very little information available regarding the effect of anthocyanin-rich extracts, the present work aimed to gain some insight into this subject. To do so, five different bacteria, which are potential nosocomial pathogens, were selected and, whenever possible, two different strains were used in order to consider possible strain specific effects: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Acinetobacter baumannii*. The bacteria were then exposed to the extracts and their impact upon bacterial growth, biofilm establishment and adherence to surfaces (both plain polysteryne (PS) surfaces and PS surfaces with adsorbed plasmatic proteins) was evaluated. Additionally, as these tests were performed using an extract produced from different blueberries to those of chapter 3, this chapter also contemplates its characterization. The results obtained in this chapter show that the extract (comprised of 8 different anthocyanins, with the most abundant being malvidin-3-glucoside (M3Glu), and neocholorgenic acid (NChA)) while being able to inhibit the growth of *S. aureus* (which overall appeared to be the most susceptible bacteria) and *E. coli*, it did not inhibit the growth

of all other pathogens tested. However, when considering its effect upon biofilm formation all microorganims were susceptible to the extracts though for some the inhibitions fell below 20%. Furthermore, when considering bacterial adhesion to plasmatic protein coated PS surfaces, the inhibition percentages averaged on 88%.

Chapter 5.2. Antibiofilm potential of phenolic acids: The influence of environmental pH and intrinsic physicochemical properties

Phenolic acids have been associated with some antimicrobial and antibiofilm activity, though some authors argue that any inhibitory activity observed may occur due to the reduction of environmental pH and not due to an interaction between the compound and the bacteria. As the extract used in chapter 5.1. possessed a phenolic acid, the present work aimed to determine if blueberry phenolic acids could be responsible for the antimicrobial activity associated with blueberry extracts and evaluate if the environmental pH value was a determining factor. To do so, seven different phenolic acids (at pH 5 and 7, values that did not hamper bacterial growth) were used: chlorogenic (ChA), ferulic (FA), gallic (GA), caffeic (CA), *p*-coumaric (p-CA), syringic (SyrA) and sinapic (SinA) acids. As, at the concentrations tested, no minimum inhibitory concentration was observed only the results for biofilm inhibition were presented. Furthermore, in an attempt to gain some insight into the eventual antibiofilm potential of other phenolic acids effect, some of their intrinsic physicochemical properties were crossed with the inhibitions observed. The results demonstrated that of the three microorganims tested, only *S. aureus* and *Staphylococcus epidermidis* (both methicillin resistant strains) were susceptible to the acids action. Additionally, it was interesting to mark that, despite the phylogenetic proximity of the two bacteria, different conditions favoured the inhibitions depending on the bacterial strain hinting at differences in action mechanism and that, a lower pH value does not necessarily translate into higher biofilm inhibitions (for *S. aureus* lower pH values were more effective while for *S. epidermidis* higher pH values were preferable).

Chapter 5.3. Antiadhesive and antibiofilm effect of malvidin-3-glucoside and malvidin-3-glucoside/neochlorogenic acid mixtures upon *Staphylococcus*

This chapter aimed try to ascertain the origin of the activities observed in chapter 5.1. To do so the major anthocyanin present in the extract (M3Glu) was used as a model for the extract and any potential synergies with NChA (as chapter 5.2 demonstrated that phenolic acids may have some effect) were considered. Furthermore, as *Stahylococcus* appeared to be the most

susceptible to the extract's action, six different staphylococci strains were used: two methicillin sensitive *S. aureus* (MSSA), two methicillin resistant *S. aureus* (S. aureus), a vancomycin resistant *S. aureus* (VRSA) and a methicillin resistant *S. epidermidis* (MRSE). Overall, M3Glu (alone or with NChA) was unable to significantly inhibit the amount of viable cells after 24 h. Nonetheless, significant inhibitions were still observed when considering both biofilm (reductions of biomass, metabolic activity and of the number of viable biofilm entrapped cells) and adhesion inhibition. When comparing the activity to that observed in chapter 5.1. the effect was lower though these compounds may explain some of the inhibitions observed.

Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction

Sara Silva[1], Eduardo M. Costa[1], Marta Mendes[1], Rui M. Morais[1], Conceição Calhau[2,3], Manuela E. Pintado[1]

[1] *CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal*

[2]*Department of Biochemistry, Faculty of Medicine, University of Porto. Portugal*

[3]*CINTESIS – Center for Research in Health Technologies and Information Systems, Porto, Portugal*

This chapter has been published:

Journal Applied Microbiology (2016); doi:10.111/jam.13215

Abstract

Aims: The present work aimed to characterize the impact of an anthocyanin-rich blueberry extract upon the growth, adhesion and biofilm formation of several pathogens including some multiresistant bacteria.

Methods and results: A group comprised of reference strains and clinical multiresistant isolates of *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Acinetobacter baumannii* and *Staphylococcus aureus*, were used to screen for antimicrobial activity. Microbial growth was determined through the measurement of the optical density while adhesion and biofilm formation was determined using the standard crystal violet staining procedure. The results showed that, while blueberry extract was only effective in hindering the growth of *S. aureus* and *E. coli*, it was capable of significantly inhibiting biofilm formation and bacterial adhesion for all microorganisms tested.

Conclusions: The extract demonstrated a considerable potential as a natural, alternative antimicrobial capable of either interfering with microbial growth or hamper the adhesion to surfaces, with *S. aureus* proving to be the most susceptible microorganism.

Significance and impact of the study: The overall study demonstrated the potential of anthocyanin extracts as natural effective alternative antimicrobial agents. Additionally, the

extract's capacity to reduce adhesion without reducing bacterial growth reduces the likeliness of resistance development while reducing the probability of infection.

Keywords: Adhesion inhibition; Anthocyanins; Antibiofilm activity; Antimicrobial activity; blueberry; *Vaccinium corymbosum*

1. Introduction

Blueberries are acknowledged as being rich in phenolic compounds which, in turn, are known for their antimicrobial, antioxidant, anti-carcinogenic, anti-proliferative and metabolism modulating properties. Among all phenolic metabolites present, the most representative, particularly when considering blueberry skins, are anthocyanins (water soluble pigments structurally composed by the α- or β- linkage of an anthocyanidin to a sugar moiety) (Burdulis et al., 2009). In fact, among other anthocyanin containing fruits, blueberries and bilberries exhibit the most complex anthocyanin composition as up to 15 different anthocyanins have been identified in a given cultivar, including monoarabinosides, monoglucosides and monogalactosides of five different anthocyanidins: malvidin, cyanidin, delphinidin, petunidin and peonidin (Kader et al., 1996, Riihinen et al., 2008, Burdulis et al., 2009). Blueberries, in spite of exhibiting lower anthocyanin levels than bilberry, have an interesting innate characteristic; they accumulate mostly malvidins while bilberries accumulate higher amounts of delphinidins and cyanidins. Malvidins possess less hydroxyl groups therefore they are more hydrophobic and more likely transported into the cells and tissues, which may translate into a better bioavailability and consequently bioactivity (Yi et al., 2006, Castañeda-Ovando et al., 2009, Cisowska et al., 2011, McGhie et al., 2003).

Plant extracts' antimicrobial activity against human pathogens has been intensively studied towards various ends, from the development of new food ingredients to potential new applications in medicine (Cisowska et al., 2011). Phenolic compounds are frequently associated with the biological properties exhibited by plant extracts (namely for their antimicrobial activity) and anthocyanins are no exception (Puupponen-Pimiä et al., 2001, Cowan, 1999). Several studies have been reported on the antimicrobial potential of anthocyanin rich extracts though most have been focused on planktonic phase cells. Despite their relevance, these studies are incomplete as bacteria must adhere and even form biofilms in order to colonize and thrive in most matrixes such as foodstuffs, medical devices or even the skin (Donlan, 2001, Shi and Zhu, 2009). Therefore, when contemplating antimicrobial studies, it is important to consider the presence of such complex structures (biofilms) when viewing the cause-effect relations. At this level, studies with anthocyanin rich extracts are relatively rare (Bakkiyaraj and Karutha Pandian, 2010). Considering the arguments above, the present work aimed to evaluate the antimicrobial potential of an ethanolic blueberry extract (purified to maximize anthocyanin

content) against several known pathogens in planktonic state complemented by the evaluation of its capacity to inhibit adhesion and impact upon biofilm formation.

2. Experimental section

2.1. Samples

Blueberries (*Vaccinium corymbosum* L. cultivar Goldtraube), provided by Mirtilusa S.A. (Sever do Vouga, Portugal), were collected in 2014 and stored at -80 °C until processing.

2.2. Extraction

Blueberries were milled and the resulting pulp was mixed (1:9) with acidified ethanol (0.01% HCl) and homogenized (24 000 rpm, 1 min) using an Ultra-Turrax T18 (IKA, Staufem Germany). The mixture was left at 40 °C, for 1 h, in a light tight container. To potentiate the extraction the containers were placed in an ultrasound bath at 35 KHz (RK106 Bandelin Sonorex, Berlin, Germany) for 15 min. The resulting suspension was centrifuged (6026*g*, 4 °C) for 15 min and the supernatant filtered through a 4 - 7 µm filter (Prat Dumas, Couze St. Front, France).

2.3. Purification

Ethanol was removed from the extracts using a rotary evaporator (175 bar, 40 °C; R-210, Buchi, Switzerland) and the resulting powder was suspended in deionized water acidified with 0.01% HCl. The resulting solutions were then purified using Bond Elut Plexa solid phase extraction (SPE) columns from Agilent Technologies (Santa Clara, California, USA). The cartridges were activated with one volume of ethanol and conditioned with two volumes of acidified water. The extracts were loaded into the cartridge, washed with acidified water (two times the volume of extract loaded) and then eluted with one volume of acidified ethanol (0.01% HCl).

The resulting solution was evaporated (using a rotary evaporator under the same conditions) and the resulting powder was used in the remaining assays. Henceforth, whenever extract is mentioned, it refers to the powder obtained in this step.

2.4. Extract characterization

The extract was dissolved in water at 1 g mL^{-1} and its' phenolic composition was determined using reverse phase chromatography as described previously by Silva et al. (2015). Briefly, an

HPLC-DAD system (Waters 600, Milford, Massachusetts, USA) measuring in 2 nm intervals, from 200 to 600 nm, equipped with a C18 Kromasil 100 column (25x0.46 cm; Teknokroma, Barcelona, Spain). Anthocyanin and phenolic composition was determined by comparison with pure analytical standards.

2.5. Microorganisms

Several pathogenic microorganisms were used in this work. Clinical bacterial isolates from urine, all capable of establishing biofilms, were kindly provided by CHTMAD—Hospital Centre of Trás-os-Montes e Alto Douro (through Ph.D. Maria José Alves).This group of isolated strains was comprised of an *Escherichia coli* (*E. coli* CI resistant to ampicillin, nalidixic acid, norflaxin and ciproflaxin), a *Pseudomonas aeruginosa* (*P. aeruginosa* CI intermediately resistant to cefotaxime), an *Acinetobacter baumannii* (*A. baumannii* resistant to cefotaxime, cefepime, ciprofloxacin and trimethoprim/sulfasoxazole), a methicillin resistant *Staphylococcus aureus* (MRSA resistant to oxacillin, ciprofloxacin and levofloxacin), a methicillin sensitive (MSSA) *Staphylococcus aureus* and a *Proteus mirabilis* (*P. mirabilis* resistant to nalidixic acid, ciproflaxin, nitrofurantoin, fosfomycin, trimethoprim/sulfasoxazole and intermediately resistant to gentamicin) (Alves et al., 2012, Alves et al., 2014). Additionally, two reference (R) strains *Pseudomonas aeruginosa* ATCC 10145 (*P. aeruginosa* R) and *Escherichia coli* ATCC 25922 (*E. coli* R) were also considered.

2.6. Time inhibition curves

Extract solutions at 1000, 500, 250, 125 and 50 µg mL^{-1} were prepared using Tryptone Soy Broth (TSB, Biokar Diagnostics, Beauvais, France), sterilized using a 0.22 µm filter (Millipore, Billerica, Massachusetts, USA) and inoculated using an overnight inoculum (ca. 10^8 CFU mL^{-1}). The mixtures were than transposed into a 96 well microtiters (Nunc, Darmstadt, Germany) and the optical density (OD) at 660 nm was assessed for a 24 h period at 37 ºC (1 h intervals) using a microplate reader (Fluostar, Optima, BMG Labtech, Ortenberg Germany), with the increase in OD being considered a consequence of bacterial growth. A positive control was drawn using inoculated TSB without antimicrobial agent and sterile TSB was used as a negative control (Silva et al., 2015, Costa et al., 2012). Each condition was assayed in triplicate.

2.7. Antibiofilm activity

Microtiters for biofilm assessment were prepared by dissolving the extract in TSB supplemented with 1% (w v^{-1}) glucose (Sigma, St. Louis, USA) at 500, 250 and 125 µg mL^{-1} for MRSA and MSSA (which represent the minimum inhibitory concentration (MIC), one half and one fourth of the MIC). For the remaining microorganisms, as no MIC was observed, the three highest concentrations tested were used (1000, 500 and 250 µg mL^{-1}).

Biomass was determined as described by Costa et al. (2014). Briefly, the test solutions were inoculated at 2% (v v^{-1}) using an overnight inoculum (ca. 10^8 CFU mL^{-1}), transferred into 96 wells microtiters (Nunc, Darmstadt, Germany) and incubated for 24 h at 37 °C. Afterwards, the contents of the plate were discarded, each well was carefully washed to remove non-adhered cells and the biofilms were stained using crystal violet. All assays were done in triplicate, a positive control was drawn using inoculated culture media and a negative control was prepared using only sterile media. The results were given in biomass formation inhibition percentage, calculated according to the following formula, in which OD_{assay} is the OD measured in the presence of the extract and $OD_{positive\ control}$ is the OD measured for the positive control.

$$\%\ \text{Biomass inhibition} = 100 - \left(\frac{OD_{assay}}{OD_{positive\ control}} \times 100 \right)$$

2.8. Impact on bacterial adhesion

2.8.1. Adhesion to polystyrene (PS)

Microtiters were prepared using full strength extract (1000 µg mL^{-1}) prepared in sterile saline (0.5% NaCl) solution. This extract was then inoculated at 2% (v v^{-1}) with a 10^8 CFU mL^{-1} inoculum and incubated at 37 °C. After 3 h, the content of each well was discarded and washed with saline solution to remove non-adherent cells. The microplates were then stained with crystal violet as described by Costa et al. (2014). All assays were done in triplicate, a positive control was drawn using inoculated culture media without extract and a negative control was prepared using sterile media. The results were given in adhesion inhibition percentage, calculated according to the following formula, in which OD_{assay} is the OD measured in the presence of the extract and $OD_{positive\ control}$ is the OD measured for the positive control.

$$\%\ \text{Adhesion inhibition} = 100 - \left(\frac{OD_{assay}}{OD_{positive\ control}} \times 100 \right)$$

2.8.2. Adhesion to polystyrene pre-treated with rabbit plasma

Rabbit plasma (rp) was used as to allow the adsorption of plasmatic proteins to the PS surface which in turn allows for a better understanding of the possible activity under physiological conditions. Microtiters were filled with reconstituted, freeze-dried rabbit plasma (Biokar Diagnostics, Beauvais, France) and incubated for 24 h at 37 °C to allow plasma proteins to adsorb to the surface (van Loosdrecht et al., 1990). After this period, the plasma was discarded and each well was carefully washed with saline solution. From this point onward, two different approaches were considered: one where the bacteria were incubated with the extract (PS-rp) and another where the bacteria were exposed to the surface pre-treated with the extract (PS-rp-acy).

In the first approach (PS-rp), the adhesion assay was carried out following the protocol described for adhesion to PS.

In the second approach (PS-rp-acy), after protein adsorption, wells were filled with a 1000 µg mL^{-1} extract solution and incubated (1 h at 37 °C). Afterwards, the extract solution was removed and the wells filled with a saline solution inoculated at 2% (v v^{-1}), with a 10^8 CFU mL^{-1} inoculum. The plates were allowed to incubate at 37 °C for 3 h. The wells were then processed and stained with crystal violet as described by Costa et al. (2014). A positive control was drawn using inoculated saline solution. The results were given in adhesion inhibition percentage, calculated according to the following formula, in which OD_{assay} is the OD measured in the presence of the extract and $OD_{positive\ control}$ is the OD measured for the positive control.

$$\%\ \text{Adhesion inhibition} = 100 - \left(\frac{OD_{assay}}{OD_{positive\ control}} \times 100 \right)$$

2.9. Statistical analysis

Statistical analysis of the results was performed using IBM SPSS Statistics v21.0.0.0. (New York, USA). Normality was assessed using Shapiro-Wilk's test. As all results proved to follow a normal distribution, the differences were evaluated using One - Way ANOVA coupled with Turkey's test. Differences were considered significant when $p < 0.05$. Time inhibition curves

were compared using the Repeated Measures test (to compare the different behaviours along the 24 h period) or One-Way ANOVA (to compare results at the 24 h mark), both coupled with Turkey's post hoc test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Compositional characterization of the extract

Table 1. Phenolic composition of the extract obtained after extraction and purification.

	Concentration (µg mg^{-1})	
	Mean	Standard Deviation
Delphinidin-3-Glucoside	25.7	2.1
Delphinidin-3-Galactoside	16.4	0.7
Cyanidin-3- Glucoside	4.2	0.5
Cyanidin-3-Galactoside	6.5	0.7
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
ni*	10.1	0.7

* ni, non-identified anthocyanin expressed in equivalents of cyanidin-3-glucoside

As can be seen in Table 1, anthocyanins were the major group of phenolics identified in the extract (184.7 µg of anthocyanins per mg of extract) with the only other phenolic compound found being neochlorogenic acid (32.1 µg of neochlorogenic acid per mg of extract). The main fraction of anthocyanins in the extract was comprised of malvidin glycosides (53.7%), particularly malvidin-3-glucoside (M3Glu, 33.6%) which stands in line with what was observed for other blueberry extracts (Cisowska et al., 2011). Overall, phenolic compounds comprise ca. 22% of the extract with the remaining 88% being comprised mostly by sugars (data not shown).

3.2. Time inhibition

Staphylococcus, both MRSA and MSSA, appeared to be the most susceptible microorganisms to the extract action (Figure 1 and Figure 2). From the analysis of Figures 1a and 1b it can be seen that a concentration of 500 µg mL^{-1} completely inhibits staphylococcal growth (it can be considered as the MIC), while all other concentrations tested (below 500 µg mL, i.e. sub-MIC concentrations) were capable of reducing microbial growth. In fact, all sub-MIC concentrations caused a significant increase ($p< 0.05$) of lag phase duration (Figure 1a and 1b), and cause a significant reduction ($p < 0.05$) of bacterial growth at the 24 h mark (Figure 2a and 2b), e.g. for

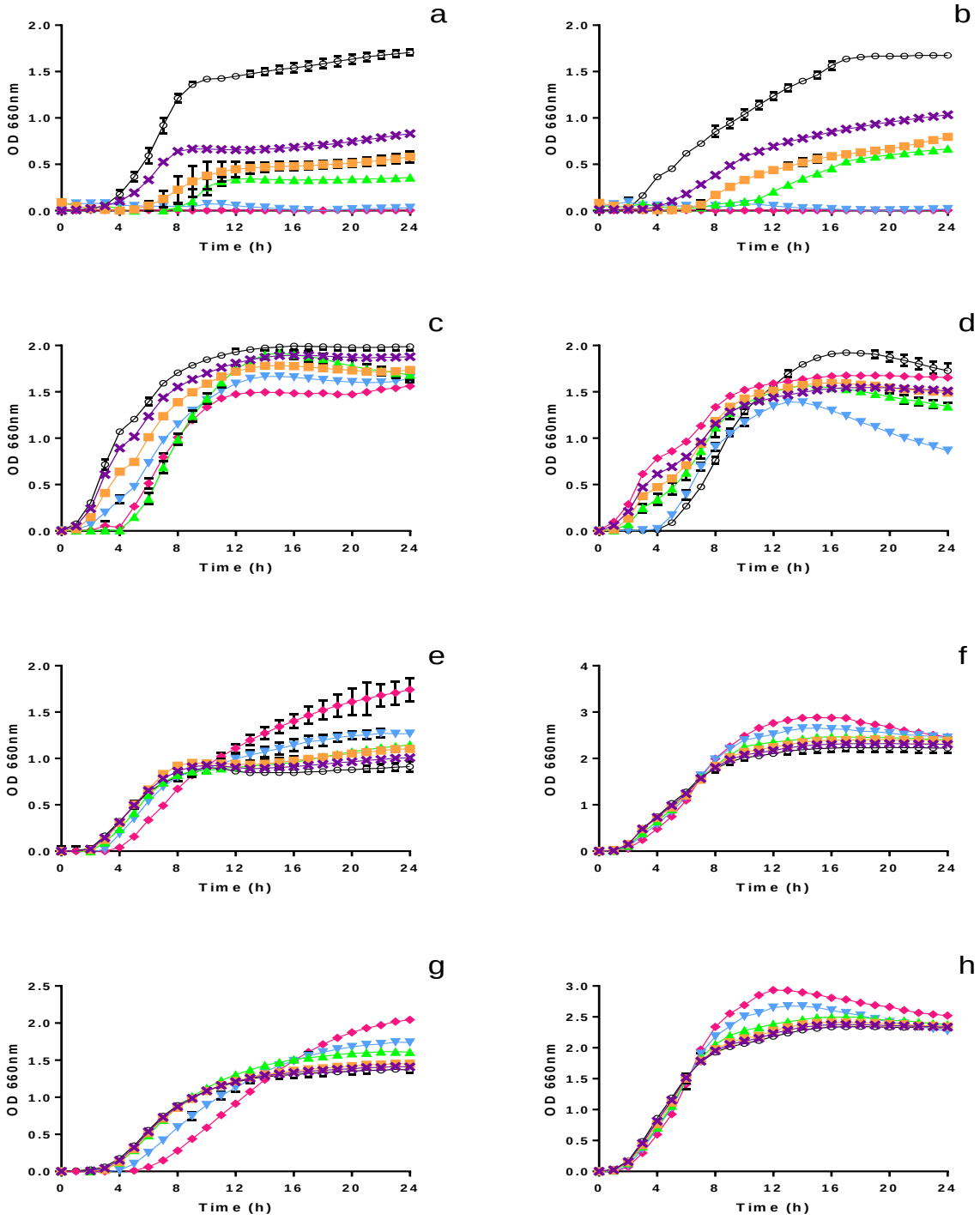


Figure 1. Time inhibition curves drawn at different extract concentrations (○, positive control; ◆, 1000 µg mL^{-1}; ▼, 500 µg mL^{-1}; ▲, 250 µg mL^{-1}; ■, 125 µg mL^{-1}; ✖, 50 µg mL^{-1}) for MSSA (a), MRSA (b), *E. coli* CI (c), *E. coli* R (d), *P. aeruginosa* CI (e), *P. aeruginosa* R (f), *A. baumannii* (g) and *P. mirabilis* (h).

MRSA extract at 50 µg mL^{-1} was capable of delaying the beginning of exponential growth by 3 h with the OD at 24 h being 38% lower.

Considering both *E. coli* strains it is interesting to highlight the different behaviours observed among them (Figure 1c, 1d, 2c and 2d). For *E. coli* CI the extract either had no impact (for 125 and 50 µg mL^{-1}) or caused a significant ($p < 0.05$) delay of the log phase (for concentrations above 125 µg mL^{-1}), while all concentrations induced a reduction in the OD after incubation for 24 h. On the other hand, the reference strain of *E. coli* (*E. coli* R) exhibited lower lag phases when exposed to all concentrations except 500 µg mL^{-1}. It is interesting to note that, this concentration appeared to induce the highest reduction of OD (Figure 2d), while the 1000 µg mL^{-1} exhibited an OD, after 24 h, similar to the one observed for the control ($p > 0.05$), though the maximum OD registered over the 24 h period was lower than the one registered for the control (Figure 1d).

For *P. aeruginosa* (Figure 1e, 1f, 2e and 2f) it can be seen that the extracts either had no impact or caused a significant increase in the OD after 24 h (Figure 2e and 2f). Similarly, the growth curves for concentrations equal or inferior to 250 µg mL^{-1} exhibited no significant differences to the control, while 500 and 1000 µg mL^{-1} exhibited significantly higher OD values than the control (Figure 2e and 2f). In this case, both strains had similar behaviours, i.e. the extract appeared to stimulate bacterial growth, though this was more accentuated for *P. aeruginosa* CI.

When observing the extract's impact upon *A. baumannii*'s growth it can be observed that all concentrations exhibited a statistically similar behaviour to the control for all concentrations except 1000 µg mL^{-1} (Figure 1g). In this concentration a significant increase of the lag phase as well as a considerably higher maximum OD (Figure 1g) was observed. When considering the OD at the 24 h mark (Figure 2g) it can be seen that it is also significantly higher than the one registered for the control when considering the 1000 µg mL^{-1} concentration, though, contrary to what has been observed for the growth curves, all concentrations (except 50 µg mL^{-1}) induced higher OD values after 24 h.

Proteus mirabilis growth curves (Figure 1h) appeared unaffected for concentrations equal or inferior to 250 µg $mL^{-1,}$ while concentrations of 500 and 1000 µg mL^{-1} induced an increase of the maximum OD registered. On the other hand, after 24 h only the 1000 µg mL^{-1} exhibited OD values that were significantly higher ($p < 0.05$) than the control (**Figure**h).

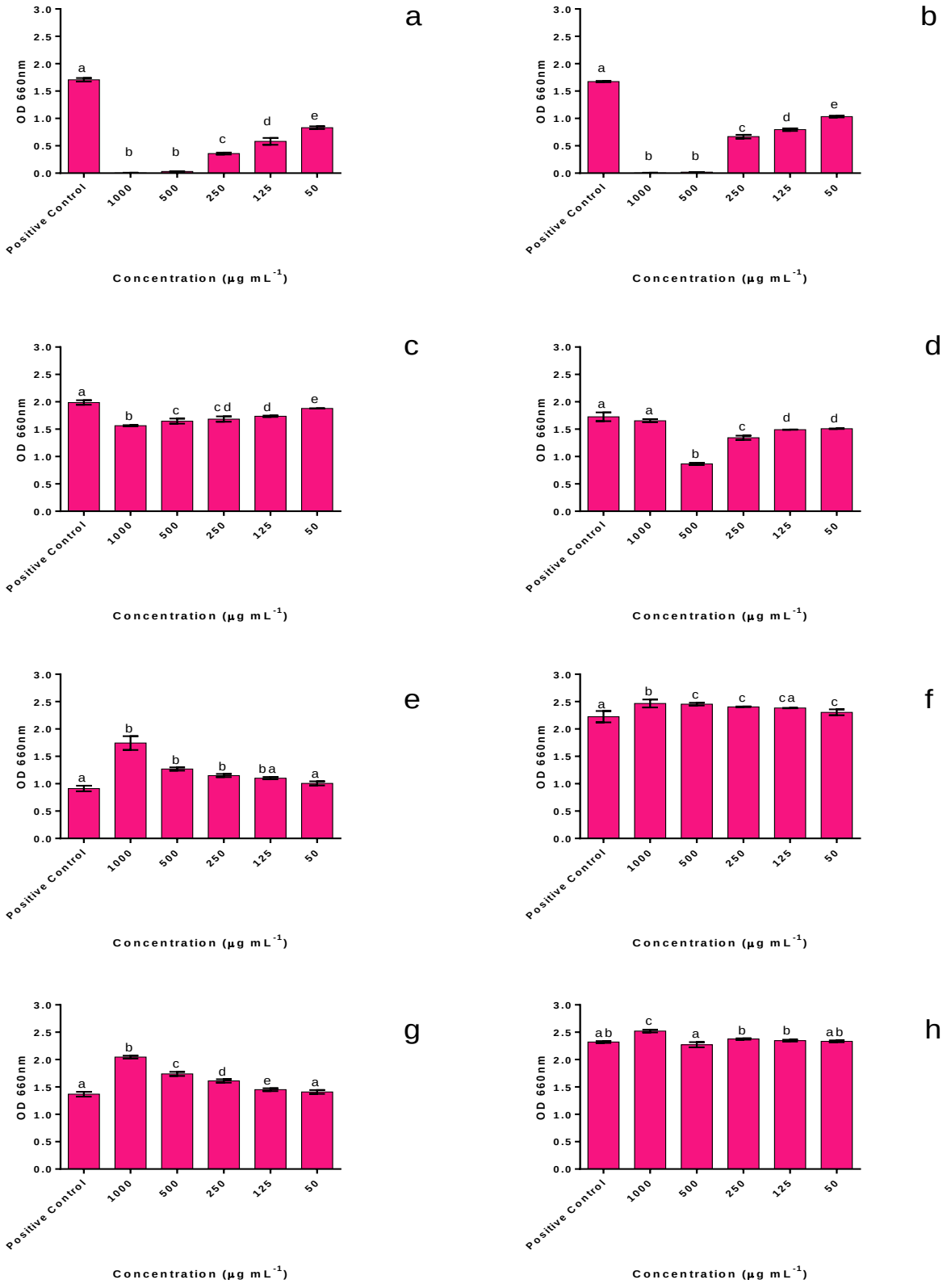


Figure 2. Microbial growth (OD at 660 nm) after 24 h, when exposed to different concentrations of extract (a, MSSA; b, MRSA; c, *E. coli* CI; d, *E. coli* R; e, *P. aeruginosa* CI; f, *P. aeruginosa* R; g, *A. baumannii*; h, *P. mirabilis*). Different letters above each column represent statistically significant differences ($p \leq 0.05$).

Overall, it appeared that *A. baumannii*, *P. mirabilis* and both *P. aeruginosa* strains were impervious to the extract's antimicrobial activity, with little to no inhibition being registered and, in some instances, promoting microbial growth possibly due to the combination of the sugars present in the extract and the lack of growth inhibition.

3.3. Antibiofilm activity

Both *Staphylococcus* strains' biofilm formation (Figure 3a and 3b) was considerably hampered by the presence of the extracts; in fact when exposed to the MIC (i.e. 500 µg mL^{-1}) an inhibition percentage of 49% and 68% for MRSA and MSSA, respectively was observed. In fact, when considering a concentration that was one fourth of the MIC (125 µg mL^{-1}) biofilm inhibition decreased only to 28% and 41 %, for MRSA and MSSA, respectively.

It is interesting to note that the two *E. coli* strains exhibited completely different behaviours when exposed to different concentrations of the extracts: while for *E. coli* CI the increase in extract concentration lead to an increase of biofilm inhibition (from 4% to 25%, Figure 3c), for *E. coli* R only the lowest concentration tested (250 µg mL^{-1}) had an inhibitory (though low, 4%) effect (Figure 3d).

Similarly to what was observed for *E. coli*, both *Pseudomonas strains* exhibited inhibitory behaviours that were somewhat opposites. The *P. aeruginosa* R displayed relatively high inhibition rates, ranging from 38 to 62% for 250 and 1000 µg mL^{-1}, respectively (Figure 3f). On the other hand, the clinical isolate's biofilms were not affected by the action of the highest concentration, with only the lowest concentration tested (250 µg mL^{-1}) exhibiting a slight inhibition of biofilm formation (Figure 3e).

The tendency of lower concentrations of extract induced higher antibiofilm activity was also observed for *P. mirabilis* and *A. baumannii* (Figure 3g and 3h). For *A. baumannii* the maximum inhibition registered was 35% and for *P. mirabilis* was 20% for 250 µg mL^{-1} while at 1000 µg mL^{-1} no effect was observed.

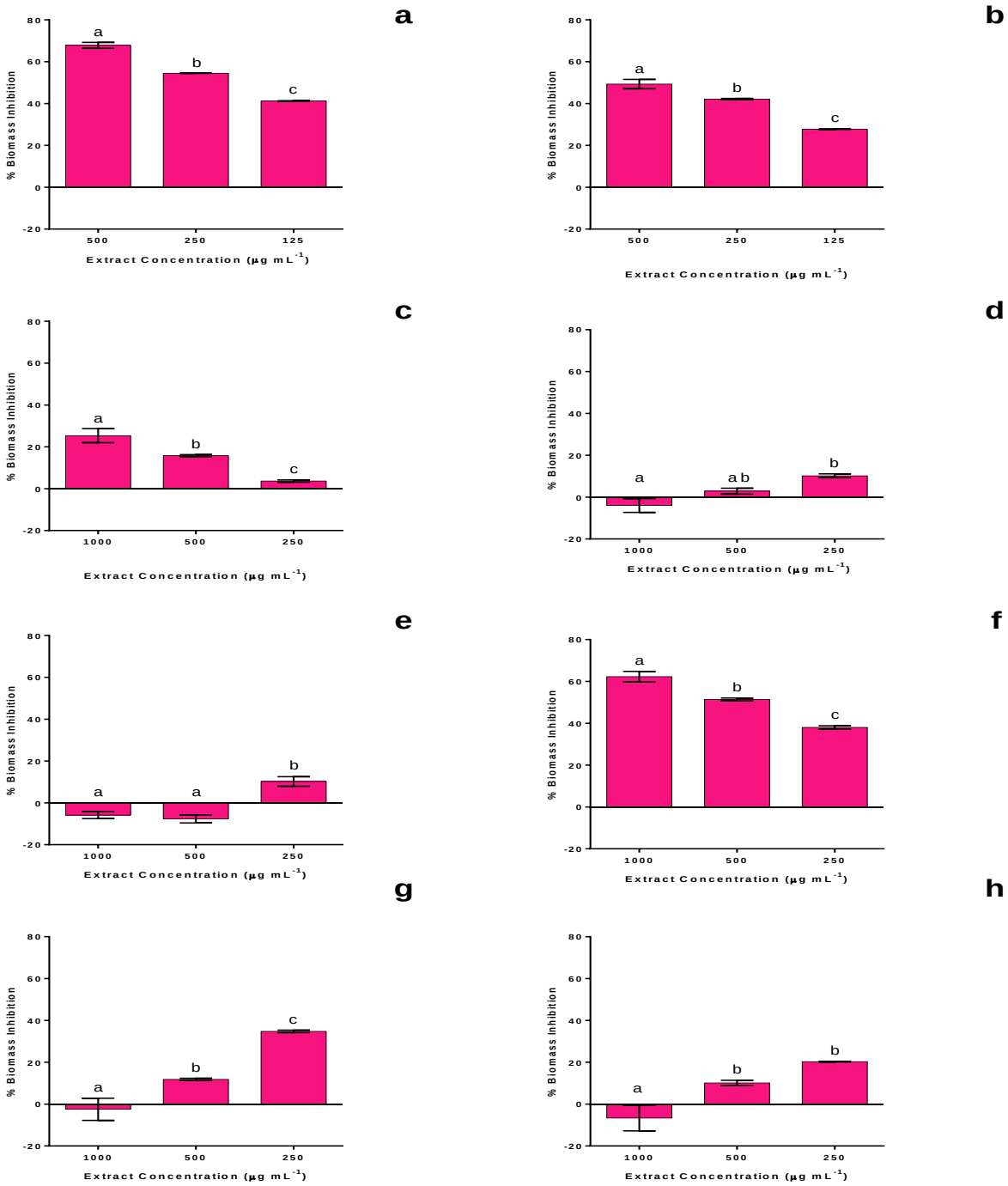


Figure 3. Antibiofilm activity of blueberry extract at 500, 250 and 125 µg mL^{-1} for MRSA and MSSA and 1000, 500 and 250 µg mL^{-1} for the remaining bacteria. a, MSSA; b, MRSA; c, *E. coli* CI; d, *E. coli* R; e, *P. aeruginosa* CI; f, *P. aeruginosa* R; g, *A. baumannii*; h, *P. mirabilis*. Different letters above each column represent statistically significant differences ($p \leq 0.05$).

3.4. Bacterial adhesion

Extracts were capable of inducing a 73% inhibition of MRSA adhesion to PS, with smaller inhibitions being registered for MSSA (18%), *P. aeruginosa* R (30%) and *P. mirabilis* (27%). For all other microorganisms no inhibition was observed (Figure 4).

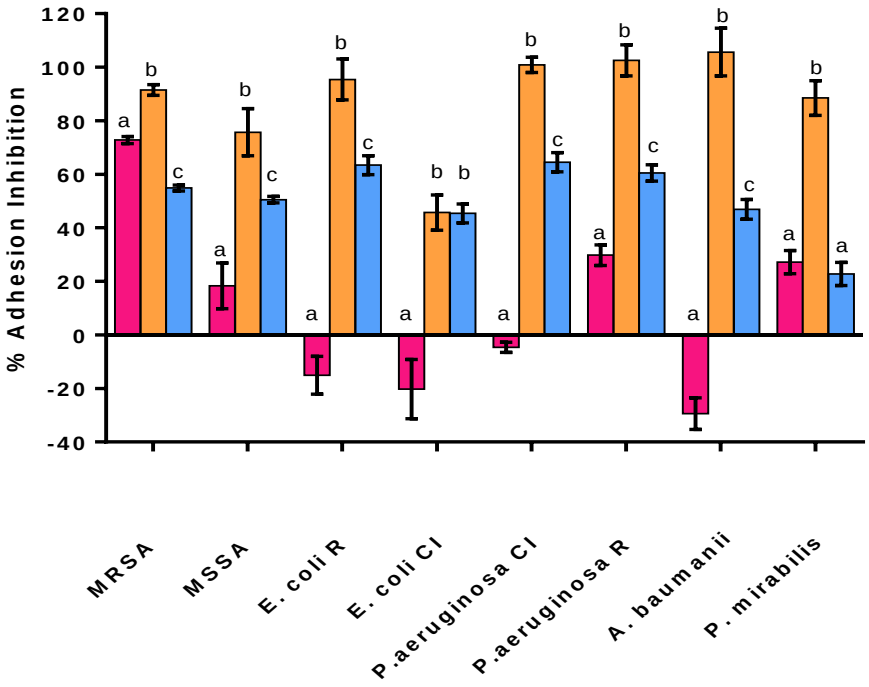


Figure 4. Impact of blueberry extract on bacterial adhesion to PS (■) and PS surfaces exposed to rabbit plasma (■, bacterial adhesion in the presence of the extract (PS-rp); ■, bacterial adhesion after exposing the surface to the extract (PS-rp-acy)). Different letters above each column represent statistically significant differences ($p \leq 0.05$).

The exposure of PS surfaces to rabbit plasma (PS-rp) allowed for significantly higher ($p < 0.05$) inhibitions for all microorganisms (Figure 4). In fact MRSA, *E. coli* R, *A. baumannii* and both *Pseudomonas* strains exhibited inhibition percentages ranging from 91 to 100%. Adhesion of *P. mirabilis* and MSSA was also considerably impaired (88 and 76%, respectively), with the poorest inhibition being observed for *E. coli* CI (46%).

When comparing the results obtained for PS surfaces exposed to rabbit plasma and the extract (PS-rp-acy) with those obtained for PS-rp surfaces it can be seen that all microorganisms, bar *E. coli* CI, (Figure 4) registered significantly lower inhibition percentages, with reduction rates ranging from 25 to 65% (for MSSA and *P. mirabilis*, respectively). On the other hand, when comparing to the results of the adhesion to PS alone, mixed behaviours were observed. For MSSA, *E. coli* R, both *Pseudomonas* strains and *A. baumannii* higher inhibitions were registered in the PS-rp-acy assay, while the opposite was true for MRSA (17% lower) and no significant differences were found for *P. mirabilis* and *E. coli* CI.

4. Discussion

In accordance to what has been previously described by Burdulis et al. (2009), the major anthocyanins present in blueberries, and therefore extracts, from *V. corymbosum* were malvidins. As malvidins are more hydrophobic than other anthocyanins, they can be more easily transported into cells and are, therefore more available to exert any activity (McGhie et al., 2003, Yi et al., 2006).

The extract was capable of inhibiting the growth, adhesion and/or biofilm formation of all microorganisms tested. This is consistent with the results of several authors that demonstrated that anthocyanin rich extracts are capable of inhibiting both Gram negative and Gram positive microorganisms (Burdulis et al., 2009, Silva et al., 2013, Shen et al., 2014). It is interesting to note that while both Staphylococci and *E. coli* strains' growth was impaired by the extract (with concentrations as low as 50 µg mL^{-1}, ca. 11.5 µg mL^{-1} of anthocyanins and 2.0 µg mL^{-1} of neochlorogenic acid, inducing a reduction of the OD after 24 h), for both *Pseudomonas, A. baumannii* and *P. mirabilis* no inhibition of growth was registered; in fact the presence of the extract in some cases led to higher OD values.

When considering sessile cells, and contrary to the results observed for their planktonic counterparts, all microorganisms' biofilm formation was susceptible to the extracts, with the maximum inhibition ranging from 10% (*E. coli* R and *P. aeruginosa* CI) to 68% for MSSA. As biofilms are known as less susceptible to antimicrobials than their planktonic counterparts, it is interesting to note that these inhibitions were all registered for concentrations that were insufficient to completely inhibit microbial growth or, for MSSA and MRSA, the MIC concentration. Similar results have been observed when exposing MSSA and MRSA to dry blueberry extracts where concentrations below the MIC values were capable of reducing biofilms in both total biomass and in the amount of cells present (Silva et al., 2015). Zimmer et al. (2014), also reported a similar behaviour for *P. aeruginosa* when exposed to several blueberry extracts. This potential to inhibit biofilm formation without inhibiting bacterial growth is an interesting aspect as it may hinder the rapid development of bacterial resistance while blocking bacterial adhesion, which is paramount for surface colonization and infection (Zimmer et al., 2014, Costerton et al., 1999, Donlan, 2001). An interesting phenomena registered in the antibiofilm assay was the fact that for four of the microorganisms tested, *E. coli* R, *P. aeruginosa* R, *A. baumannii* and *P. mirabilis*, lower concentrations of extract were

more effective in inhibiting biofilm formation than the higher ones. It is possible that higher concentrations of extract act as a stimulus for biofilm formation, namely through an increase in exopolysaccharide (EPS) production (factor that has been linked with the presence of environmental stress) which could increase the protection of the sessile bacteria as well as the overall amount of biofilm present though no further studies were made to support this possibility (Landini, 2009).

In order to further ascertain the extract's potential to block bacterial binding to surfaces, anti-adhesion studies were carried out considering both a plain PS surface and PS surfaces coated with plasmatic proteins (as to mimic an organic layer). Interestingly, when considering plain PS surfaces, the extracts were only capable of interfering with the adhesion of MRSA, MSSA, *P. aeruginosa* R and *P. mirabilis* but, when contemplating the adhesion to the protein covered surface (PS-rp), not only were all extracts capable of inhibiting adhesion but they did so with inhibition rates that average 88% with almost complete inhibitions being found for both *P. aeruginosa* strains and *A. baumannii*; this may hint that an improved activity when exposed to a physiological environment may occur. To try to ascertain if a direct interaction between the protein-covered surface and the phenolics was responsible for the inhibitions observed, a third surface was considered where the PS was exposed to rabbit plasma and then anthocyanins (PS-rp-acy). All microorganisms, except *E. coli* CI, exhibited adhesion inhibition percentages of 25 to 66% lower than those registered for the adhesion to PS-rp, which suggests that only part of the inhibition observed is caused by the direct interaction of the phenolics with the bacteria, with this effect being stronger for *P. mirabilis* and *A. baumannii* (66 and 59%, respectively). For *E. coli* CI no statistically significant differences were found between PS-rp and PS-rp-acy, which may indicate that most of the inhibition observed is caused by the interaction of the phenolic compounds with the proteins adsorbed to PS.

Overall, the extract exhibited an interesting antimicrobial, antibiofilm and/or antiadhesive potential for extract concentrations ranging from 1000 to 50 mg mL^{-1}, which corresponds to 216.9 to 13.5 µg mL^{-1} of phenolics with the anthocyanin content ranging from 184.7 to 11.5 µg mL^{-1}.

The concentrations of phenolic compounds which exhibited antimicrobial activity are in line with those that have been previously reported. Park et al. (2011) reported that a concentration of berry phenolics of 24 µg mL^{-1} was capable of reducing the growth of *Salmonella enteritidis* and *Listeria monocytogenes*, these results are similar to those observed for MRSA, MSSA and

both *E. coli* strains that were susceptible to an extract concentration of ca. 26 µg mL^{-1} of total phenolics (125 mg mL^{-1} of extract), though both *Pseudomonas* strains, *A. baumannii* and *P. mirabilis* were not susceptible to the extract's action. Previous works with dry blueberry infusions also reported that concentrations of 66-78 µg mL^{-1} of phenolic compounds (50 mg mL^{-1}) of dry blueberry extract were capable of inhibiting the growth of *P. aeruginosa* and *S. aureus*. Further studies upon *S. aureus* revealed that, concentrations as low as 16 µg of total phenolics mL^{-1} were capable of exhorting a considerable inhibitory effect upon biofilm formation of MRSA and MSSA, though in this instance the main components were not anthocyanins but chlorogenic acid (Silva et al., 2015, Silva et al., 2013).

In conclusion, the hereby proposed extract exhibits an interesting antimicrobial activity against *S. aureus*, which appears as the most susceptible microorganisms. Similarly, the extract exhibited a considerable capacity to inhibit biofilm formation and/or adhesion of *P. aeruginosa*, *A. baumannii* and *P. mirabilis*, a group of pathogenic microorganisms that have been associated with biofilm mediated infections and antibiotic resistance (Donlan, 2001, Peleg et al., 2008). Therefore, the presented results bring some insight into the potential of this blueberry extract strengthening the importance of studies to further exploit the anthocyanins potential as new, alternative antimicrobial agents.

Acknowledgements: The authors would like to acknowledge Mirtilusa SA, for kindly providing the fruits used in this work, CHTMAD – Hospital Center of Chaves (Portugal) for providing the clinical isolates and the financial support given by the national funds from FCT (through project Pest-PE/EQB/LA0016/2013) and by funds from QREN-ADI project 13736 "Myrtillus – Mirtilo com inovação". Additionally, the authors S. Silva and E.M. Costa would like to acknowledge FCT for their Ph.D. grants with the references SFRH/BD/90867/2012 and SFRH/BDE/103957/2014, respectively.

5. References

ALVES, M., FERREIRA, I., LOURENÇO, I., COSTA, E., MARTINS, A. & PINTADO, M. 2014. Wild Mushroom Extracts as Inhibitors of Bacterial Biofilm Formation. *Pathogens,* 3**,** 667.

ALVES, M. J., FERREIRA, I. C. F. R., MARTINS, A. & PINTADO, M. 2012. Antimicrobial activity of wild mushroom extracts against clinical isolates resistant to different antibiotics. *Journal of Applied Microbiology,* 113**,** 466-475.

BAKKIYARAJ, D. & KARUTHA PANDIAN, S. T. 2010. In vitro and in vivo antibiofilm activity of a coral associated actinomycete against drug resistant Staphylococcus aureus biofilms. *Biofouling,* 26**,** 711-717.

BURDULIS, D., SARKINAS, A., JASUTIENÉ, I., STACKEVICENÉ, E., NIKOLAJEVAS, L. & JANULIS, V. 2009. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (Vaccinium myrtillus L.) and blueberry (Vaccinium corymbosum L.) fruits. *Acta poloniae pharmaceutica,* 66**,** 399-408.

CASTAÑEDA-OVANDO, A., PACHECO-HERNÁNDEZ, M. D. L., PÁEZ-HERNÁNDEZ, M. E., RODRÍGUEZ, J. A. & GALÁN-VIDAL, C. A. 2009. Chemical studies of anthocyanins: A review. *Food Chemistry,* 113**,** 859-871.

CISOWSKA, A., WOJNICZ, D. & HENDRICH, A. B. 2011. Anthocyanins as antimicrobial agents of natural plant origin. *Nat Prod Commun,* 6**,** 149-56.

COSTA, E. M., SILVA, S., MADUREIRA, A. R., CARDELLE-COBAS, A., TAVARIA, F. K. & PINTADO, M. M. 2014. A comprehensive study into the impact of a chitosan mouthwash upon oral microorganism's biofilm formation in vitro. *Carbohydrate Polymers,* 101**,** 1081-1086.

COSTA, E. M., SILVA, S., PINA, C., TAVARIA, F. K. & PINTADO, M. M. 2012. Evaluation and insights into chitosan antimicrobial activity against anaerobic oral pathogens. *Anaerobe,* 18**,** 305-309.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science,* 284**,** 1318-1322.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews,* 12**,** 564-82.

DONLAN, R. M. 2001. Biofilm formation: a clinically relevant microbiological process. *Clinical Infectious Diseases,* 33**,** 1387-1392.

KADER, F., ROVEL, B., GIRARDIN, M. & METCHE, M. 1996. Fractionation and identification of the phenolic compounds of Highbush blueberries (Vaccinium corymbosum, L.). *Food Chemistry,* 55**,** 35-40.

LANDINI, P. 2009. Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in Escherichia coli. *Research in Microbiology,* 160**,** 259-266.

MCGHIE, T. K., AINGE, G. D., BARNETT, L. E., COONEY, J. M. & JENSEN, D. J. 2003. Anthocyanin Glycosides from Berry Fruit Are Absorbed and Excreted Unmetabolized by Both Humans and Rats. *Journal of Agricultural and Food Chemistry,* 51**,** 4539-4548.

PARK, Y. J., BISWAS, R., PHILLIPS, R. D. & CHEN, J. 2011. Antibacterial Activities of Blueberry and Muscadine Phenolic Extracts. *Journal of Food Science,* 76**,** M101-M105.

PELEG, A. Y., SEIFERT, H. & PATERSON, D. L. 2008. Acinetobacter baumannii: Emergence of a Successful Pathogen. *Clinical Microbiology Reviews,* 21**,** 538-582.

PUUPPONEN-PIMIÄ, R., NOHYNEK, L., MEIER, C., KÄHKÖNEN, M., HEINONEN, M., HOPIA, A. & OKSMAN-CALDENTEY, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* 90**,** 494-507.

RIIHINEN, K., JAAKOLA, L., KÄRENLAMPI, S. & HOHTOLA, A. 2008. Organ-specific distribution of phenolic compounds in bilberry (Vaccinium myrtillus) and 'northblue' blueberry (Vaccinium corymbosum x V. angustifolium). *Food Chemistry,* 110**,** 156-160.

SHEN, X., SUN, X., XIE, Q., LIU, H., ZHAO, Y., PAN, Y., HWANG, C.-A. & WU, V. C. H. 2014. Antimicrobial effect of blueberry (Vaccinium corymbosum L.) extracts against the growth of Listeria monocytogenes and Salmonella Enteritidis. *Food Control,* 35**,** 159-165.

SHI, X. & ZHU, X. 2009. Biofilm formation and food safety in food industries. *Trends in Food Science & Technology,* 20**,** 407-413.

SILVA, S., COSTA, E. M., COSTA, M. R., PEREIRA, M. F., PEREIRA, J. O., SOARES, J. C. & PINTADO, M. M. 2015. Aqueous extracts of Vaccinium corymbosum as inhibitors of Staphylococcus aureus. *Food Control,* 51**,** 314-320.

SILVA, S., COSTA, E. M., PEREIRA, M. F., COSTA, M. R. & PINTADO, M. E. 2013. Evaluation of the antimicrobial activity of aqueous extracts from dry Vaccinium corymbosum extracts upon food microorganism. *Food Control,* 34**,** 645-650.

VAN LOOSDRECHT, M. M., NORDE, W., LYKLEMA, J. & ZEHNDER, A. B. 1990. Hydrophobic and electrostatic parameters in bacterial adhesion. *Aquatic Sciences,* 52**,** 103-114.

YI, W., AKOH, C. C., FISCHER, J. & KREWER, G. 2006. Absorption of Anthocyanins from Blueberry Extracts by Caco-2 Human Intestinal Cell Monolayers. *Journal of Agricultural and Food Chemistry,* 54**,** 5651-5658.

ZIMMER, K. R., BLUM-SILVA, C. H., SOUZA, A. L. K., WULFFSCHUCH, M., REGINATTO, F. H., PEREIRA, C. M. P., MACEDO, A. J. & LENCINA, C. L. 2014. The Antibiofilm Effect of Blueberry Fruit Cultivars Against Staphylococcus epidermidis and Pseudomonas aeruginosa. *Journal of Medicinal Food,* 17**,** 324-331.

Antibiofilm potential of phenolic acids: The influence of environmental pH and intrinsic physicochemical characteristics

Sara Silva[1], Eduardo M. Costa[1], Bruno Horta[1],Conceição Calhau[2,3], Rui M. Morais[1], Manuela E. Pintado[1]

[1] CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

[2] Department of Biochemistry, Faculty of Medicine, University of Porto. Portugal

[3] CINTESIS – Center for Research in Health Technologies and Information Systems, Porto, Portugal

---

This chapter has been published:

Biofouling (2016); doi:10.1080/1478619.2016.1209668

Abstract

Phenolic acids are a particular group of small phenolic compounds which have exhibited some antibiofilm activity, although the link between their activity and their intrinsic pH is not clear. Therefore, the present work examined the antibiofilm activity (inhibition of biomass and metabolic activity) of phenolic acids in relation to the environmental pH, as well as other physicochemical properties. The results indicate that, while *Escherichia coli* was not inhibited by the phenolic acids, both methicillin resistant Staphylococcus aureus and methicillin resistant Staphylococcus epidermidis were susceptible to the action of all phenolic acids with the pH playing a relevant role in the activity: neutral pH favoured MRSE inhibition, while acidic conditions favoured MRSA inhibition. Some links between the molecular polarity and size were associated only with their potential as metabolic inhibitors with the overall interactions hinting at a membrane-based mechanism for MRSA and a cytoplasmatic effect in MRSE.

Keywords: Phenolic acids; Antibiofilm activity; MRSE; MRSA; *Escherichia coli*.

1. Introduction

In nature, while bacteria are found in planktonic state; they are more often present in survival structures known as biofilms. As bacteria in these structures are more impervious to the action of antibiotics and other antimicrobial agents, the search for antibiofilm agents has grown (Stewart and William Costerton, 2001). With the emergence of bacterial resistance to antibiotics, there is an even greater need as antimicrobial agents have to contend not only with the inherent resistance of bacteria but also with resistant due to (Neu, 1992, Stewart and William Costerton, 2001, Costerton et al., 1999).

Phenolic compounds have been associated with an array of health promoting properties, including potential as antimicrobial agents. Several authors have reported on the antimicrobial activity of phenolic acids and phenolic rich extracts (Lacombe et al., 2010, Puupponen-Pimiä et al., 2001, Cowan, 1999, Alves et al., 2014a). Although studies regarding pure compounds and their impact upon biofilm formation are relatively scarce (Cowan, 1999, Jagani et al., 2009, Silva et al., 2015), some phenolic acids, namely chlorogenic acid (ChA), have been reported to possess some antimicrobial and antibiofilm activity, although arguments can be made that their activity may be due to low pH values and not to the compound itself.

The present work study examined the antibiofilm potential of seven phenolic acids against three multiresistant microorganisms at two different pH values (5 and 7). Additionally, the effects of other intrinsic physical properties (concentration, pK_a, molecular weight (MW), molecular surface area (MSA), polar surface area (PSA) and partition coefficient into octanol (logP)) were also evaluated with respect to their potential impact on biofouling prevention.

2. Experimental section

2.1. Microorganisms

Three different pathogens, capable of forming biofilms, were used in this. The test species were a methicillin resistant *Staphylococcus aureus* (MRSA) CCUG 60578, a methicillin resistant *Staphylococcus epidermidis* (MRSE) ATCC 51625 and an *Escherichia coli* clinical isolate (*E. coli* CI) resistant to ampicillin, nalidixic acid, norfloxacin and ciprofloxacin, kindly provided by CHTMAD—Hospital Centre of Trás-os-Montes e Alto Douro (through Ph.D. Maria José Alves). All strains have been used in previous antibiofilm studies (Silva et al., 2015, Alves et al., 2014a, Dewangan et al., 2014).

2.2. Phenolic standard solutions

Standards for ChA, ferulic (FA), gallic (GA), caffeic (CA), *p*-coumaric (p-CA), syringic (SyrA) and sinapic (SinA) acid were purchased from Extrasynthese (Genay, France).

Solutions of each standard were prepared at 500, 250 and 125 mg L^{-1} by dissolving each compound in tryptone soy broth (TSB, Biokar Diagnostics, Beauvais, France) supplemented with 1% glucose (Sigma, St. Louis, USA), and the pH was adjusted to 7.0 ± 0.1 and 5.0 ± 0.1 with HCl and NaOH (0.01 mol L^{-1}) respectively. These pH values were selected because, while they do not affect the microorganisms' growth, they have some biological context as the regular pH of the skin ranges from 4 to 7 and all three microorganisms are possible skin pathogens (Lambers et al., 2006).

2.3. Antibiofilm activity

All phenolic solutions (at pH 5 and at pH 7) were inoculated at 2% (v v^{-1}) with an overnight culture (ca. 10^8 CFU mL^{-1}) grown in TSB with 1% glucose at 37 °C. The mixtures were then transferred into a 24 well polystyrene microtiter (Nunc, Darmstadt, Germany) and incubated at 37 °C for 48 h. After this period, metabolic activity (XTT assay) biomass (crystal violet assay) were assayed to determine biofilm formation.

2.3.1. XTT assay

The metabolic activity of the biofilm was determined using 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide (XTT) colorimetric method as described by Machado et al. (2013). Briefly, biofilms were incubated with a XTT solution for 3 h at 37 °C. The optical density (OD) at 485 nm was then measured using a microplate reader, (FLUOstar, OPTIMA, BMG Labtech). Plain culture medium (TSB) supplemented with glucose was used as a positive control. All assays were done in triplicate and the results were expressed in percentage of activity inhibited (as can be seen in the equation bellow in which the OD_{sample} is the optical density measured for each sample and $OD_{positive\ control}$ is the optical density of the positive control).

$$\%\ \text{Metabolic activity inhibition} = 100 - \left(\frac{OD_{sample}}{OD_{positive\ control}} \times 100 \right)$$

2.3.2. Crystal violet assay

The amount of biofilm formed was determined using the crystal violet method as described by Stepanović et al. (2000). Briefly, after the removal of non-adherent cells, the biofilms were treated with absolute ethanol and then stained with crystal violet. The excess stain was removed, the contents of each well were suspended in 1% (v v^{-1}) acetic acid and the OD at 660 nm was measured. Plain culture medium (TSB) supplemented with glucose was used as a positive control. All assays were done in triplicate and the results given in biomass inhibition percentage calculated according to the equation bellow in which the OD_{sample} is the optical density measured for each sample and $OD_{positive\ control}$ is the optical density of the positive control.

$$\%\ \text{Biomass inhibition} = 100 - \left(\frac{OD_{assay}}{OD_{positive\ control}} \times 100 \right)$$

2.4. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v21.0.0 (New York, USA) software.

2.4.1. Difference tests

One-way ANOVA coupled with Turkey's post hoc test was used to determine the differences between the results observed because the Shapiro-Wilk test ($n < 30$) proved that they followed a normal distribution. Results were considered significantly different for p-values < 0.05.

2.4.2. Correlation analysis

To determine whether the inhibition of biomass was associated with metabolic inhibition, a correlation between both sets of data was performed for each microorganism and pH. As the data followed a normal distribution, evaluated using Kolmogorov-Smirnov test ($n > 30$), Pearson's R was determined with correlations being considered significant at a confidence interval of 95%, though higher confidence intervals were noted.

2.4.3. Multiple linear regression

Table 1. Physicochemical properties of the studied phenolic acids according to Chemicalize.org (Swain 2012).

	pKA	MW	MSA	PSA	logP
ChA	3.33	354.3	450.13	164.75	-0.27
FA	3.77	194.2	264.4	66.76	1.67
GA	3.94	170.1	205.97	97.99	0.72
CA	3.64	180.16	226.17	77.76	1.53
SinA	3.61	224.21	312.22	75.99	1.52
p-CA	4.20	164.2	216.51	57.53	1.83
SyrA	3.93	198.2	279.86	75.99	1.01

Several variables (experimental as well as intrinsic molecular physical properties) were assessed to determine their effect on the observed activity. In addition to pH value and concentration data, variables including acid strength (pKa), size (MW for overall mass and MSA as an estimate of available area for interaction) and lipophilicity (PSA as a measure of overall surface polarity and logP as a measure of the capacity to migrate into a lipophilic environment) were also examined (Table 1). These values were acquired through Chemichalize.org (Swain, 2012). A multiple linear regression was calculated (using only statistically significant data) and the β-weights and p-values were calculated. The homoscedasticity of the residues was determined using the Breuch-Pagan-Koenker homoscedasticity test and, when it was not observed, the values were corrected to accommodate this fact using the procedure described by Hayes and Cai (2007).

3. Results

Table 2. Correlation value between the inhibition of biomass and metabolic activity for each microorganism at different pH values.

	E.coli		MRSE		MRSA	
	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7
Pearson's R	-0.097	-0.363	0.835	0.873	0.583	0.564
p-value	0.417	0.102	<0.001	<0.001	<0.001	<0.001

E. coli CI was less susceptible to the phenolic acids. As can be seen in Figure 1 (and in the supplemental material, Figures S1A to S14A), all the inhibition values, whenever inhibition was registered, were below 30% for metabolic activity and 20% for biomass formation. It is important to note that no other general conclusions can be drawn from these results, as the behaviour varies significantly according to the acid, pH and the concentration. For example, the metabolic inhibition induced by SinA at pH 5 was significantly ($p < 0.05$) greater at 125 mg L^{-1} than at higher concentrations.

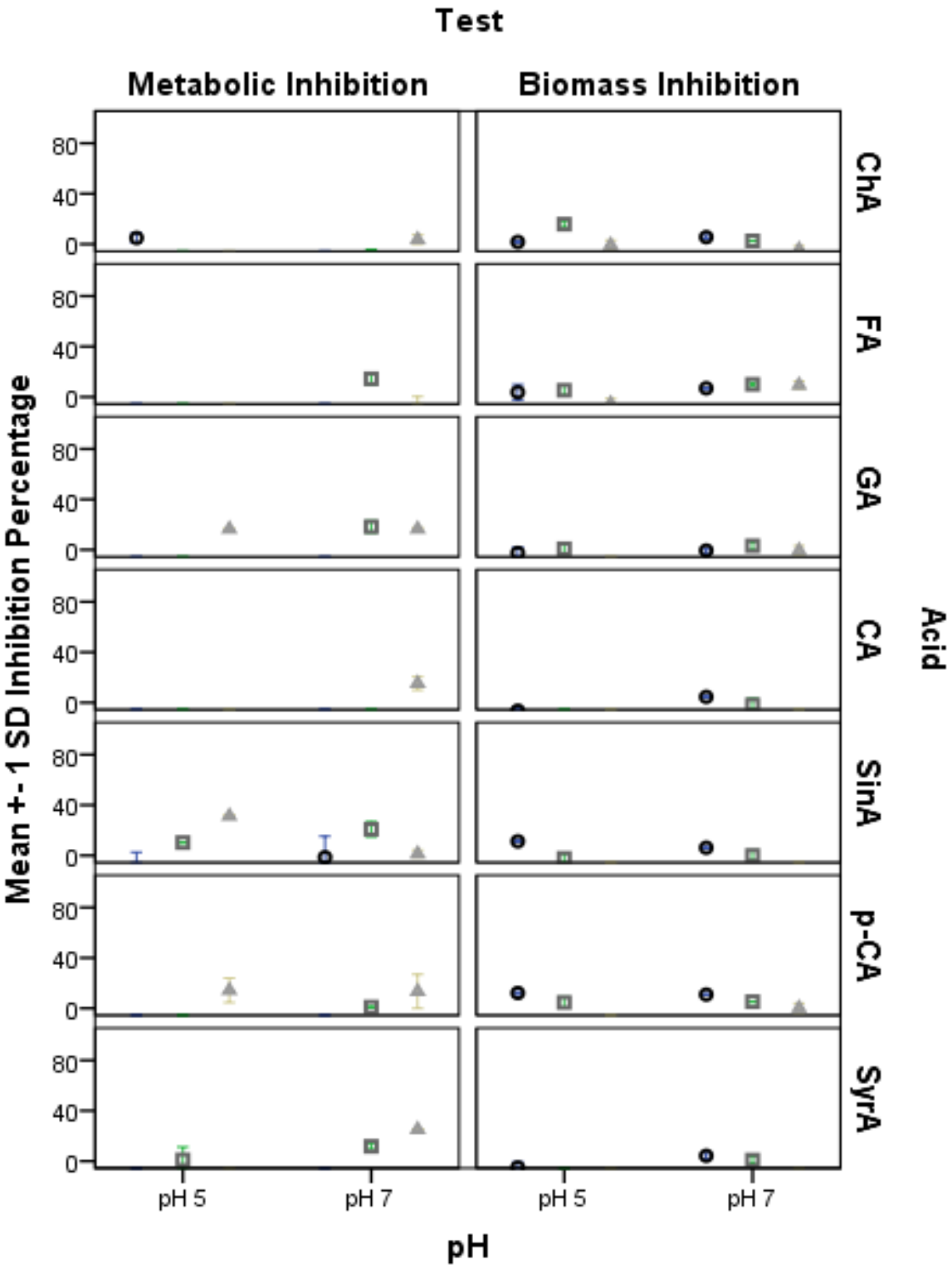


Figure 1. Metabolic and biomass inhibition observed for *E. coli* for each phenolic acid (pH 5 and pH 7) at 500 mg L^{-1} (●), 250 mg L^{-1} (■) and 125 mg L^{-1} (▲).

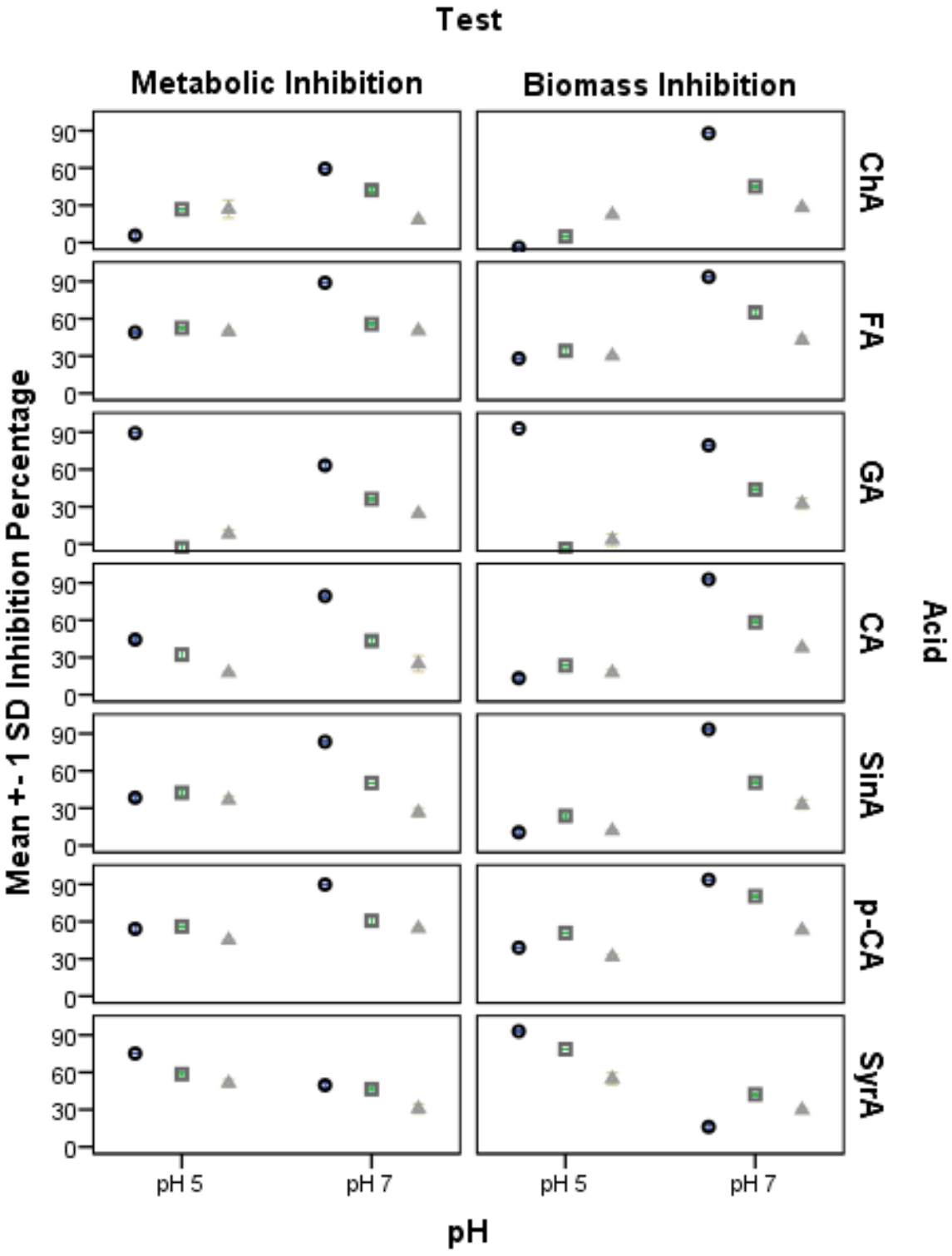


Figure 2. Metabolic and biomass inhibition observed for MRSE for each phenolic acid (pH 5 and pH 7) at 500 mg L^{-1} (●), 250 mg L^{-1} (□) and 125 mg L^{-1} (▲).

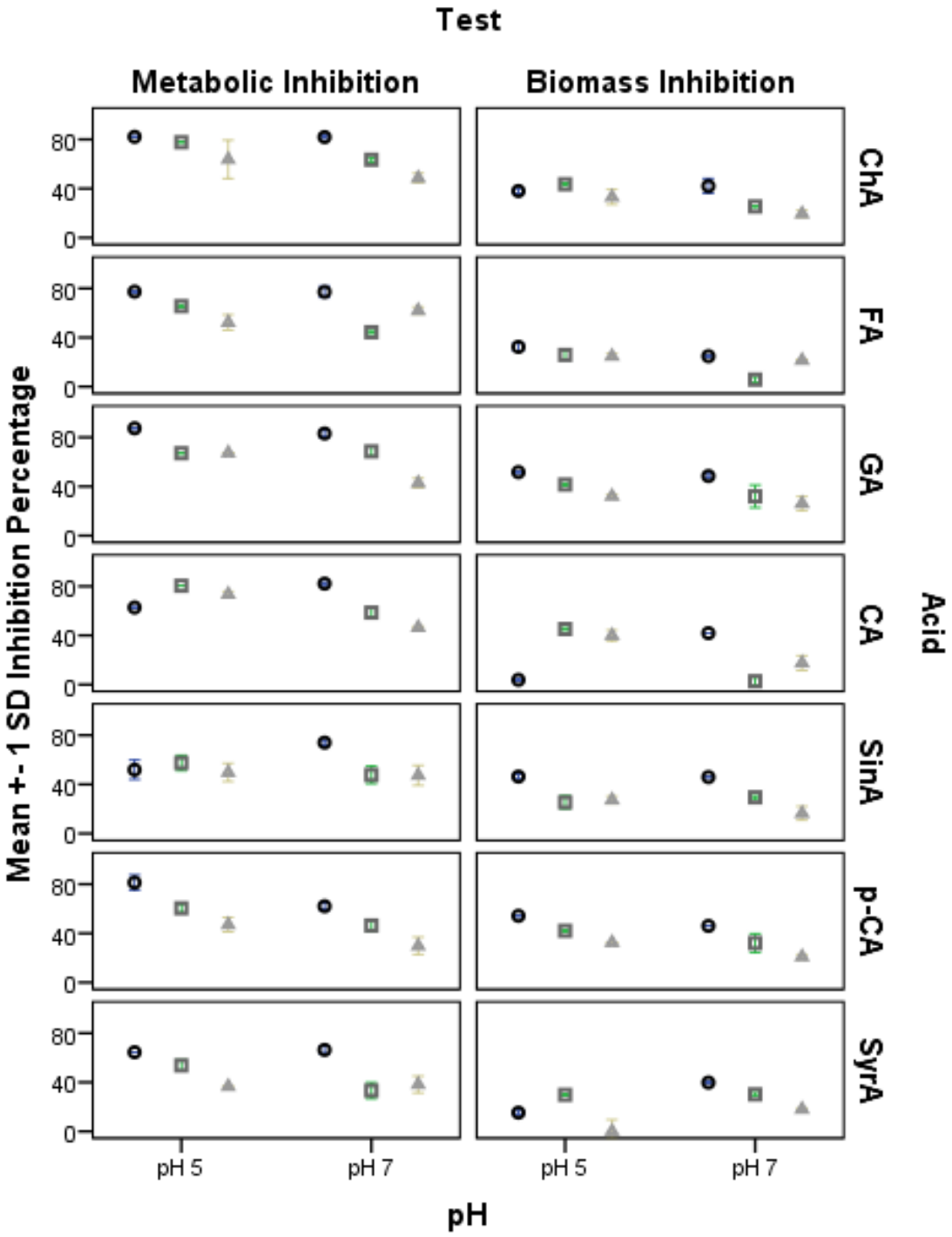


Figure 3. Metabolic and biomass inhibition observed for MRSA for each phenolic acid (pH 5 and pH 7) at 500 mg L^{-1} (●), 250 mg L^{-1} (□) and 125 mg L^{-1} (▲).

However, at pH 7 the greatest inhibition was found at 250 mg L^{-1}. On the other hand, when considering biomass inhibition for the same acid the highest concentration tested (500 mg L^{-1})

resulted in greatest inhibition of biomass formation regardless of the environmental pH. This lack of an overall trend is also apparent in Table 2, where no significant ($p > 0.05$) correlation was found between metabolic and biomass inhibition at pH 5 or at pH 7.

For MRSE (Figure 2 and supplemental material Figures S1B to S14B) at neutral pH, all concentrations were capable of inhibiting both biomass formation and metabolic activity, with reductions ranging from 16-93% and 12-90%, respectively. Furthermore, under these conditions, a dose response curve was observed with higher concentrations resulting in greater inhibition of biomass and metabolism for all acids except SyrA. This is further demonstrated in Table 2 where a (0.873) significant ($p < 0.001$) correlation between biomass and metabolic inhibition is shown. At pH 5, however a (0.835) significant ($p < 0.01$) correlation was still observed between biomass and metabolic inhibition (Table 2). There is a dose response curve at pH 7 but only for the metabolic inhibition induced by CA and both biomass and metabolic inhibition by SyrA (Figure 2 and supplemental material). It is interesting to note that, in some cases, the reduction in pH resulted in a loss of activity, as can be seen for biomass inhibition induced by ChA (all concentrations) and the metabolic and biomass inhibition induced by GA (at 250 and 125 mg L^{-1}).

For MRSA, at neutral pH all acids (except CA and FA for biomass inhibition and SyrA for metabolic inhibition) demonstrated a positive dose response, i.e. higher concentrations resulted in greater inhibition. However, at pH 5 there was no overall trend. As it can be seen in Figure 3 (and supplemental material Figures S1C to S14C), while some of the results demonstrated the existence of the dose response trend observed at pH 7 (e.g. p-CA), others exhibited a dose independent effect (biomass inhibition by CA and FA and metabolic inhibition by SinA). Furthermore, while at pH 7 the most effective concentration was 500 mg L^{-1}, at pH 5 it became the least effective concentration for CA (biomass and metabolic inhibition) and for SyrA (biomass inhibition) (Figure 3 and supplemental material). Biomass and metabolic inhibition are associated through a significant ($p < 0.01$), though moderate (0.583 and 0.564 for pH 5 and 7, respectively) correlation, meaning that increased biomass inhibition is likely to be accompanied by increased metabolic inhibition and vice versa (Table 2). The results from regression analysis indicate that the physicochemical properties anaçysed in this study

Table 3. Impact of the different variables considered upon the variance observed for biomass inhibition.

	Predictors	*β*-weight	p-value	R^2
MRSE[a]	Concentration	0.0749	<0.0001	0.4210
	pH value	12.9406	<0.0001	
	pKa	30.1531	<0.0001	
MRSA[a]	Concentration	0.0376	<0.0001	0.2141
	pH value	-2.3388	.0364	

[a]values adjusted to accommodate heteroscedasticity

Table 4. Impact of the different variables considered upon the variance observed for metabolic inhibition.

	Predictors	*β*-weight	p-value	R^2
E.coli[a]	Concentration	-0.0966	<0.0001	0.3141
MRSE[a]	Concentration	.0770	<0.0001	0.5665
	PSA	-0.4659	0.0001	
	pH value	5.2669	0.0002	
	MW	0.2111	0.0027	
	pKa	24.8444	.0001	
MRSA	Concentration	0.062	<0.0001	0.654
	PSA	0.749	<0.0001	
	MSA	-0.116	<0.0001	
	logP	20.073	<0.0001	
	pH value	-3.700	<0.0001	

[a]values adjusted to accommodate heteroscedasticity

accounted for 21.4 to 39.2% of the variance observed for biomass inhibition (Table 3) and 31.4 to 61.4% of the variance observed for metabolic inhibition (Table 4). The only variable which can be a predictor for the variation of all inhibitions observed is concentration. This variable alone accounts for 31.41% of the variation observed for the metabolic inhibition of *E. coli* CI and curiously, the corresponding *β*-weight is negative (-0.0966 for $p < 0.0001$). This indicates an inverse relationship between concentration and metabolic inhibition (i.e. an increase in concentration leads to a decrease in metabolic inhibition). This is particularly interesting because all other *β*-weights for concentration are positive. The pH value is the next most usefull predictor as it accounts for the variance in all cases except metabolic inhibition of *E.coli* CI. In addition, the *β*-weights which characterize the extracts also have varying results: for MRSA they are positive, whereas for *E.coli* and MRSE they are negative. Similarly, it is interesting to note that, when the pH is associated with a positive *β*-weight the pK_a also becomes a predictor. The variation in metabolic inhibition of MRSE and MRSA (Table 4) is further explained by other predictors (MW, PSA, MSA and logP), resulting in sets of predictor variables that explain 56.65% of the variance observed for MRSE and 65.40% of the variance observed for MRSA.

4. Discussion

Overall, for MRSE and MRSA, when significant reductions in biofilm were observed, the reduction in biofilm biomass was proportional to the reduction in metabolic activity. This indicates that the presence of the phenolic acids induced a reduction in the amount of active bacterial cells within the biofilm. However, for *E. coli* CI no inhibition was observed; therefore the phenolics appear to have little to no effect on the bacterial cells. These results agree with those reported, for *E. coli* by Luís et al. (2014) for FA at 1000 mg L^{-1} (biomass inhibition of 13% and metabolic inhibition of 5%) but not with those reported, by the same authors, for GA at the same concentration (64% and 88% of biomass and metabolic inhibition, respectively). However, as these authors consider higher concentrations it is possible that, the inhibition observed was a result of this fact. Furthermore, it is possible that the different mechanisms of Gram positive and Gram negative bacteria, particularly at membrane level, may explain the different results since these differences have been described as factors when considering the antibiofilm potential of compounds (Gottenbos et al., 2001).

The apparent high susceptibility of both MRSA and MRSE to phenolics observed in this study agrees with what has been previously reported. Luís et al. (2014) reported that GA, CA and ChA were capable of inhibiting biomass formation in *Staphylococcus aureus* biofilms and Zimmer et al. (2014) reported that CA and ChA are both capable of inhibiting biomass formation by *Staphylococcus epidermidis* biofilms.

Although the capability of phenolic acids to inhibit biofilm formation has been reported in literature, to the best of the authors' knowledge, no information can be found regarding the impact of the environmental pH upon this property. At different pH values the acid molecules exhibit diverse protonation levels, which means that they will exhibit different physicochemical properties and, in turn, be prone to different types of interactions. At pH 5 the amount of deprotonated acid molecules is significantly higher than at pH 7. Therefore, it is interesting to note that, while the pH value is a predictor of the variance in both biomass and metabolism inhibition, the effects of their β-weights differ from microorganism to microorganism indicating that the most favourable conditions (acidic or neutral) vary according to the microorganism. Despite this fact, an interesting relationship can be observed; when neutral pH is favourable for biofilm inhibition (positive β-weights), higher pK_a values are also associated with better

inhibition, i.e. when neutral pH is significantly ($p < 0.05$) more effective than an acidic pH, the acids which possess the highest amount of dissociated molecules at pH 7 are more effective.

Metabolic inhibition implies some level of direct interaction between the compounds and the bacterial cells. Therefore it is reasonable to conclude that for MRSA and MRSE (the most susceptible microorganisms to metabolic activity inhibition by phenolic compounds), variables which will affect how the compounds interact with the bacteria (molecular size and polarity) will also be predictors of inhibition. Analysis of these parameters may indicate possible underlying mechanisms. For MRSE, higher PSA values are significantly ($p < 0.01$) associated with acids with lower metabolic inhibitions (negative β-weight, Table 4); since acids with higher PSA values are less likely to passively diffuse through the cellular membrane, it is likely that the metabolic inhibition is caused by intracellular interactions (Ertl et al., 2000). Conversely, it is interesting to note that the metabolic inhibition is also greater at neutral pH which is when the protonated acid molecule, the most likely to pass through the cellular membrane, is predominant (Vattem et al., 2004). Borges et al. (2012) reported that one of the mechanisms by which phenolic compounds may exert antimicrobial activity is due to interactions with intracellular proteins (reactions with sulfhydryl groups or less specific interactions). These interactions may result in the disruption of regular enzyme activity, the production of Quorum sensing markers and of biofilm promoting proteins and therefore explain some of the metabolic inhibition observed for this microorganism.

On the other hand, for MRSA, despite the phylogenetic proximity of the two species, the underlying mechanism appears somewhat different. In fact, while PSA is still a predictor its' β-weight has a positive signal. This means that the greatest metabolic inhibition should be expected for compounds which are less likely to pass through the membrane. Moreover, logP is also a predictor and it exhibits a positive β-weight, i.e. the capacity of the compounds to diffuse into octanol (lipophilic environment), the greater the metabolic inhibition. If both results are considered, it can be perceived that the most effective compounds to inhibit the biofilm metabolic activity of MRSA are those that, while not diffusing through the membrane, diffuse into it. This could possibily alter the physicochemical properties of the membrane and destabilize it, a fact which has been described as a possible action mechanism by which phenolics may exert antimicrobial effects (Borges et al., 2012).

When considering the effect of MSA and MW on the metabolic inhibition, for MRSE, only the MW was found to be a predictor, whereas for MRSA, the MSA was the only significant

predictor. Furthermore, positive β-weights for MW in MRSE and negative β-weights for MSA in MRSA were also predictors. This means that acids with a larger mass (MW) may induce a greater metabolic inhibition of MRSE, while acids with smaller surface areas (MSA) may cause higher inhibitions in MRSA.

In conclusion, phenolic acids are effective agents in the prevention of biofilm formation by MRSA and MRSE, resulting in reductions in biofilm viability > 80% with the most effective acids and, in most cases, the reduction in viability is accompanied by a reduction in the overall biomass of the film. These results, when coupled with the findings of Alves et al. (2014b) (which reported that phenolic compounds may be effective antibiotic adjuvants) demonstrate that phenolic acids may be interesting agents in the treatment/prevention of infections, particularly of the skin. Furthermore, as the intrinsic pH of the skin varies from 4 to 7, an understanding of the effects of this variable upon the inhibitory effects of the compounds may aid in a better selection of the compounds to be used.

Acknowledgements: This *work was supported by Fundação para a ciência e tecnologia [project grant UID/Multi/50016/2013], [S. Silva's PhD grant SFRH/BD/90867/2012], [E.M. Costa's PhD grant SFRH/BDE/103957/2014] and QREN*-AdI [project 13,736 'Myrtillus – Mirtilo com inovação'].

Supplemental Material

5. Additional data

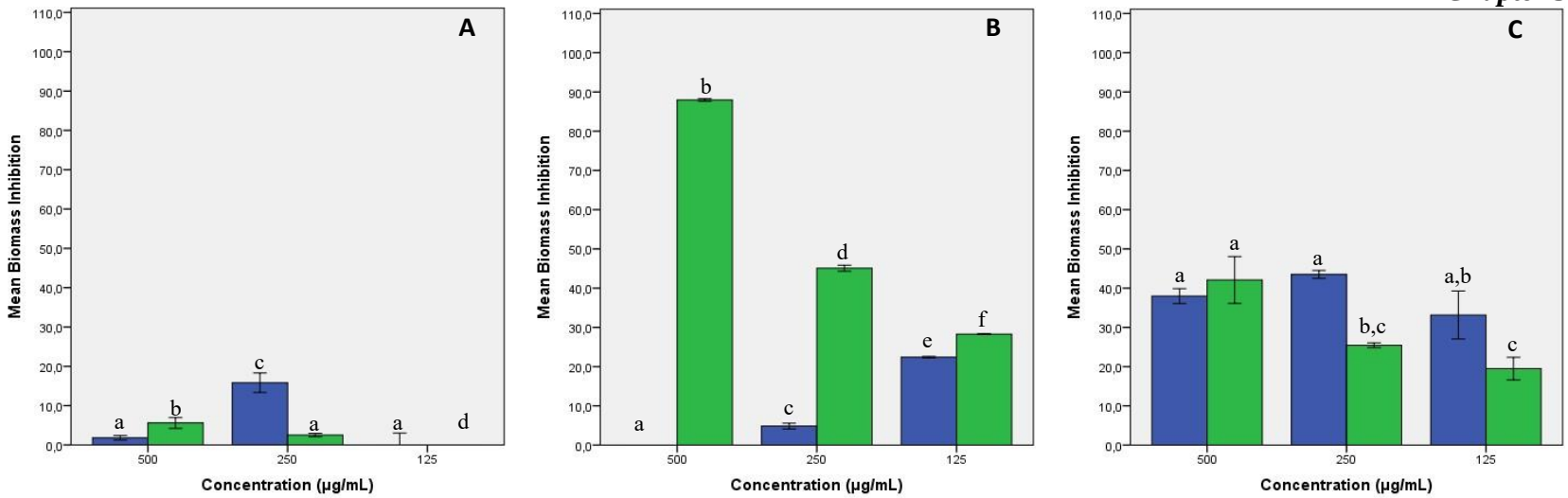


Figure 1S. Inhibition of biomass formation in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by chlorogenic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

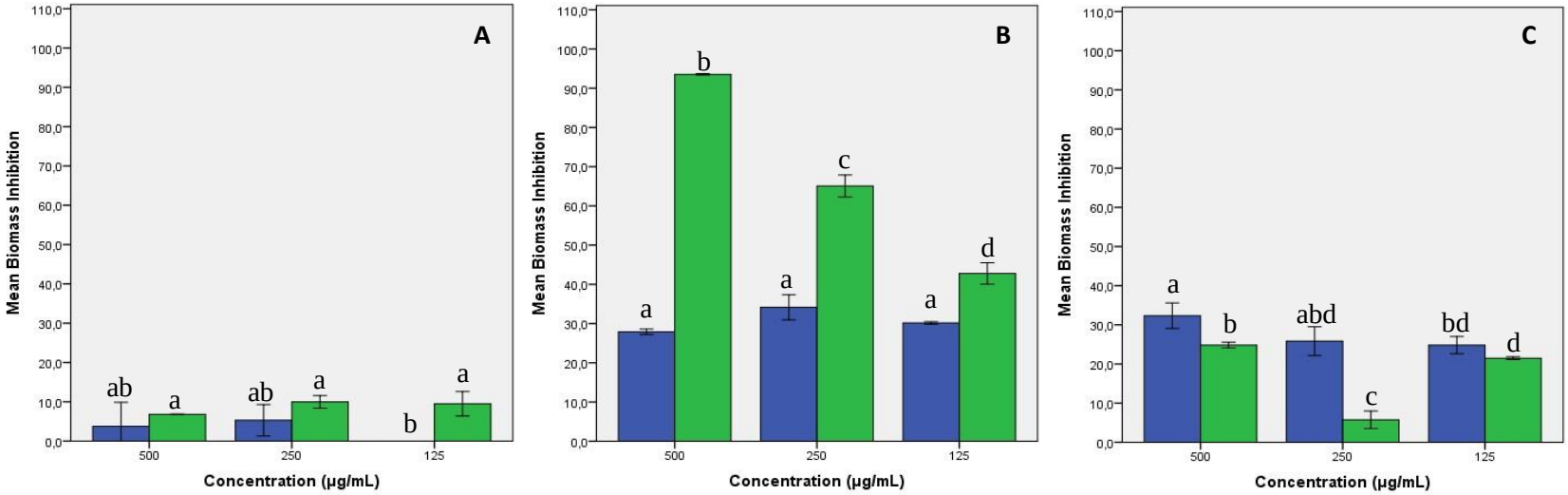


Figure 2S. - Inhibition of biomass formation in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by ferulic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

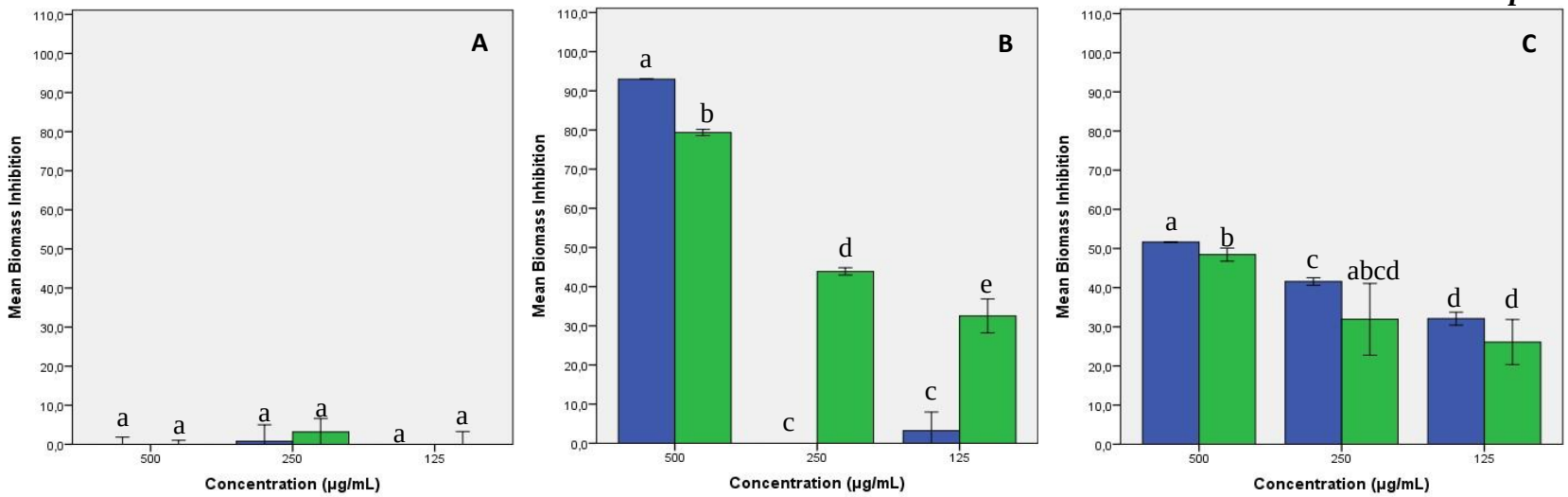


Figure 3S. Inhibition of biomass formation in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by gallic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

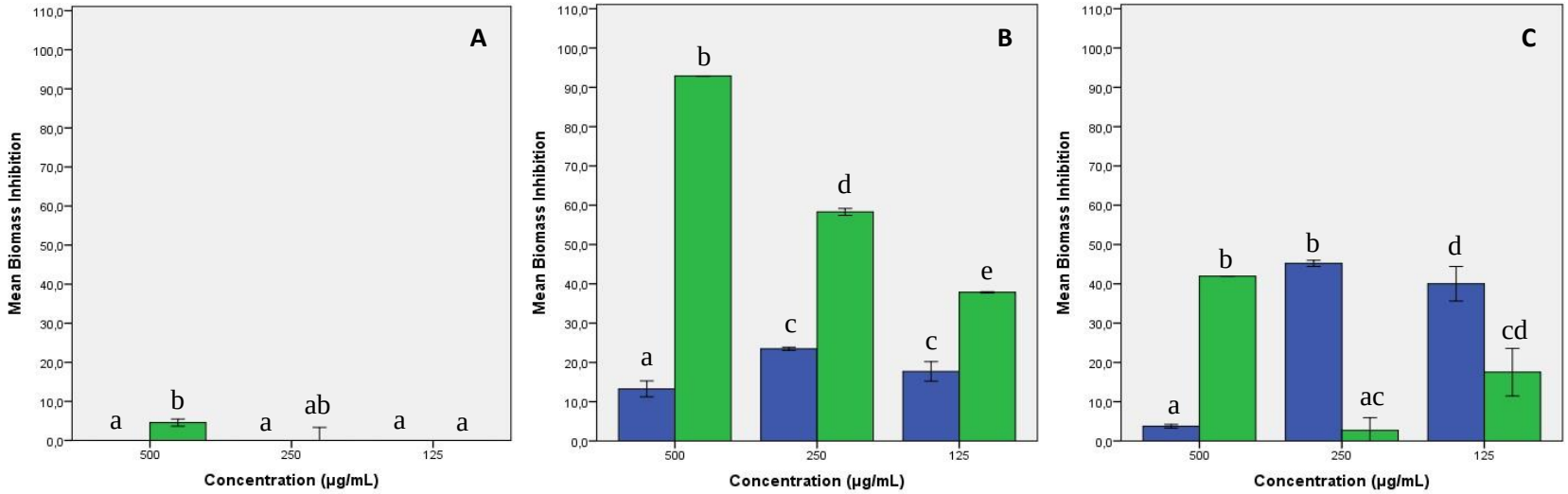


Figure 4S. Inhibition of biomass formation in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by caffeic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

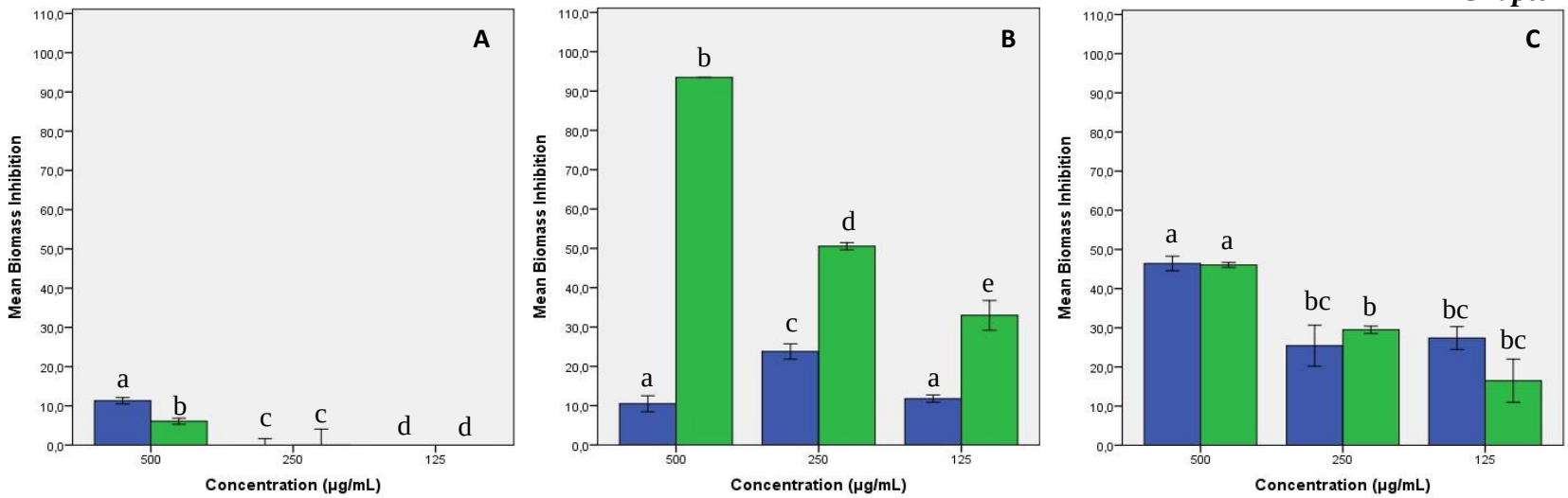


Figure 5S. Inhibition of biomass formation in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by sinapic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

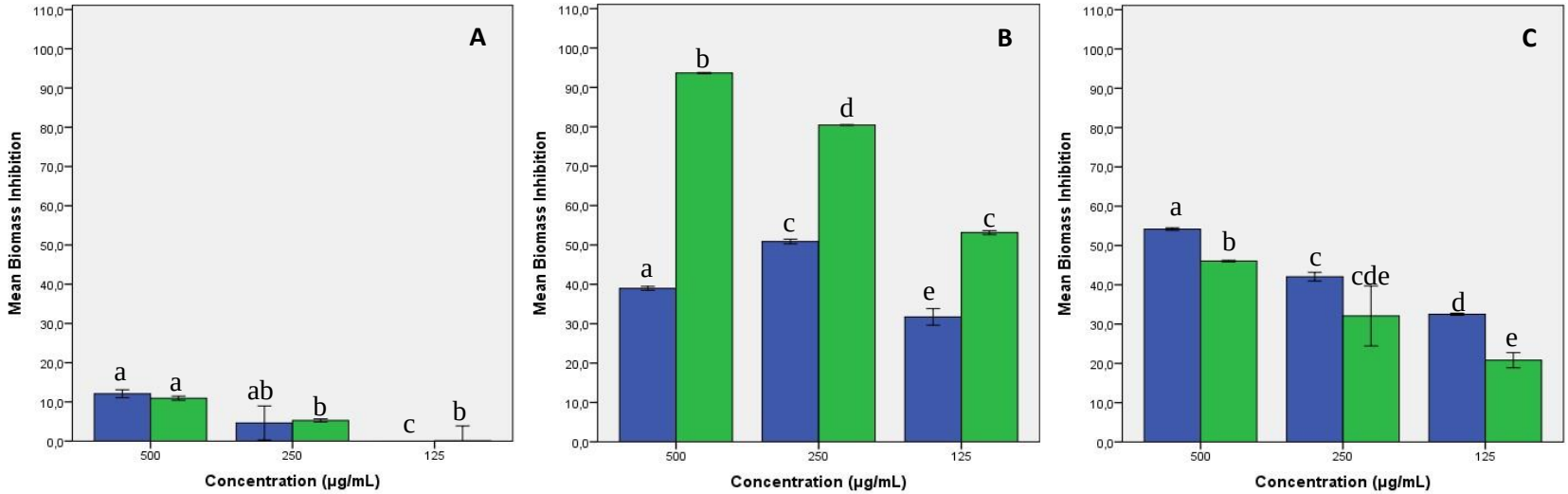


Figure 6S. Inhibition of biomass formation in E. coli (A), MRSE (B) and MSSA (C), biofilms induced by *p*-coumaric acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

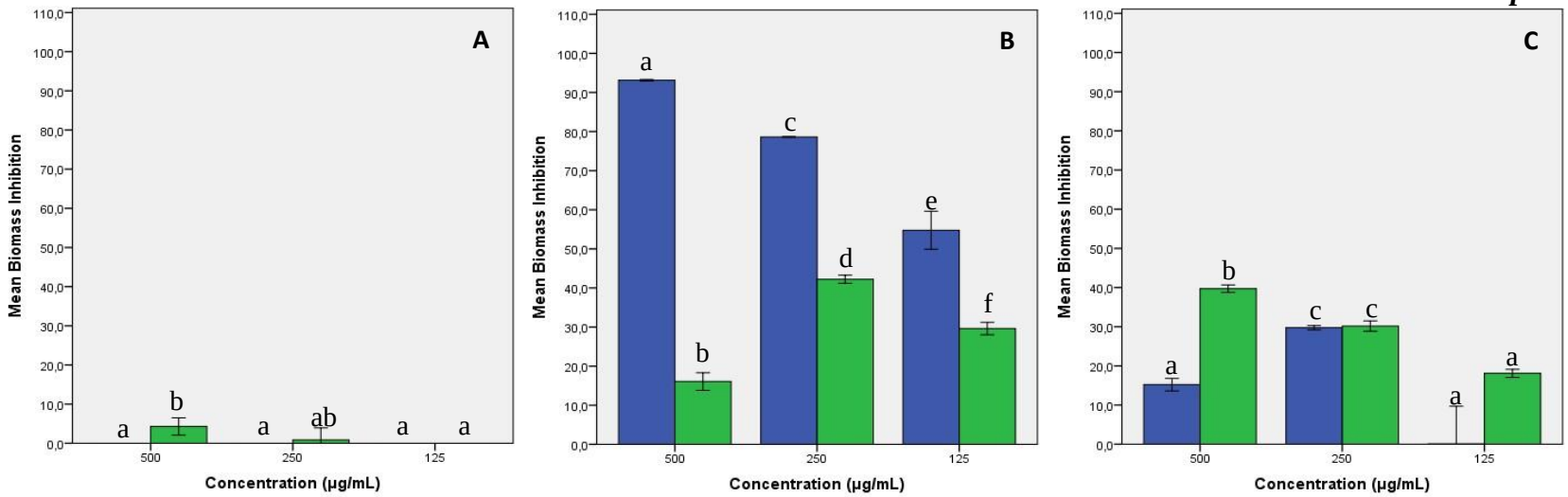


Figure 7S. Inhibition of biomass formation in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by syringic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

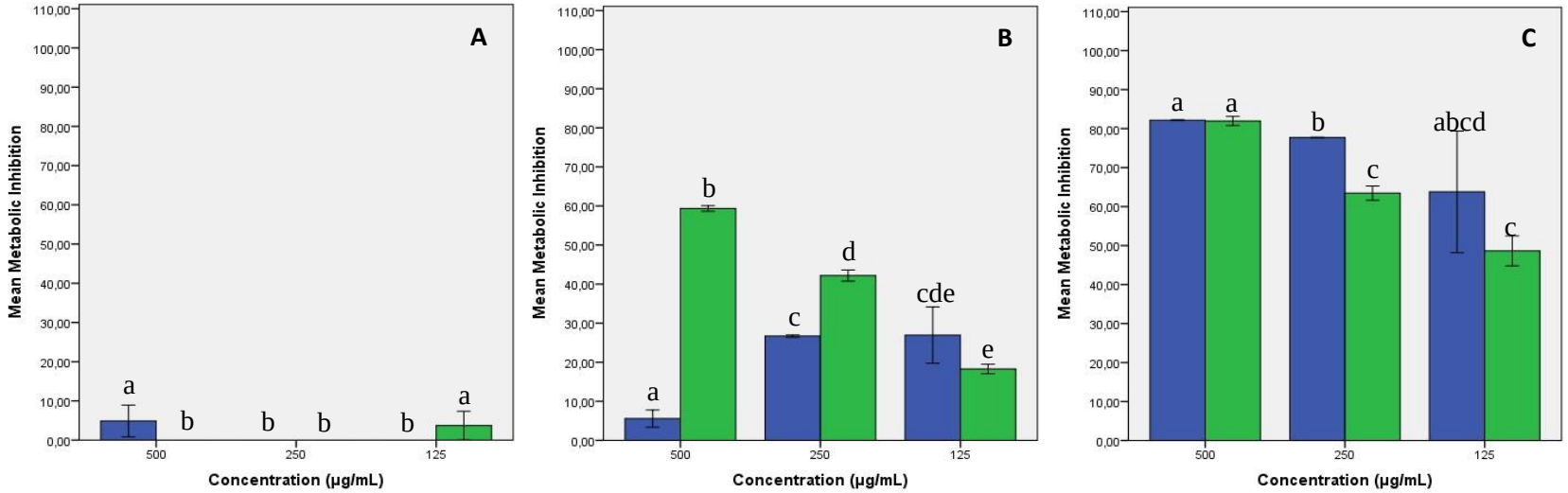


Figure 8S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by chlorogenic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

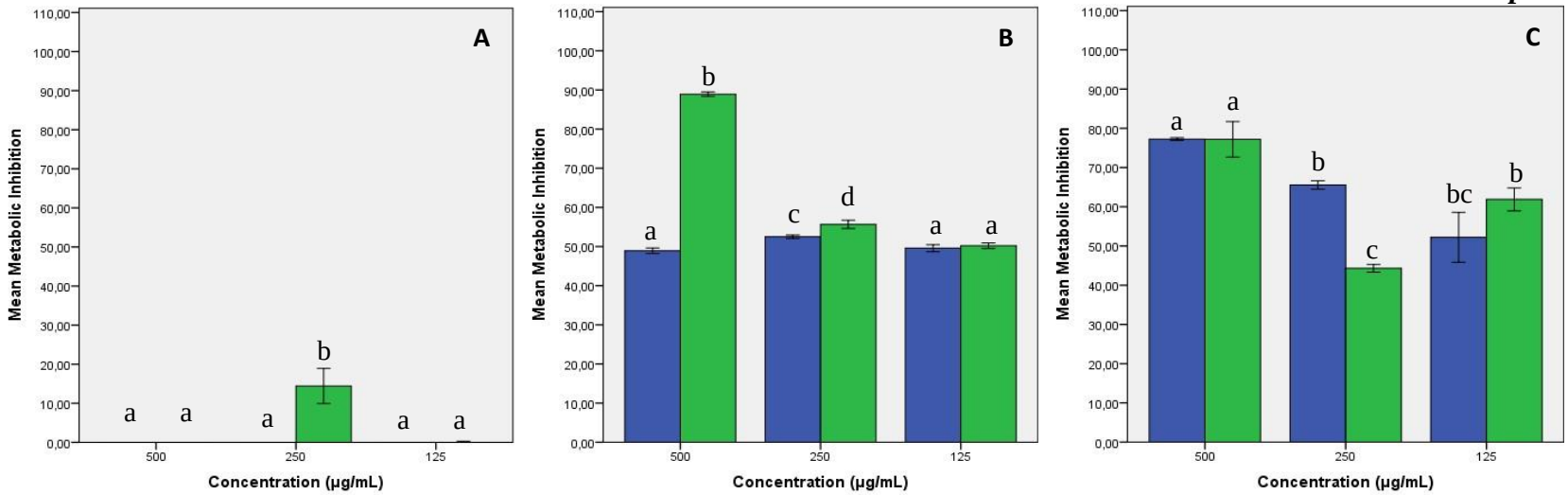


Figure 9S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by ferulic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

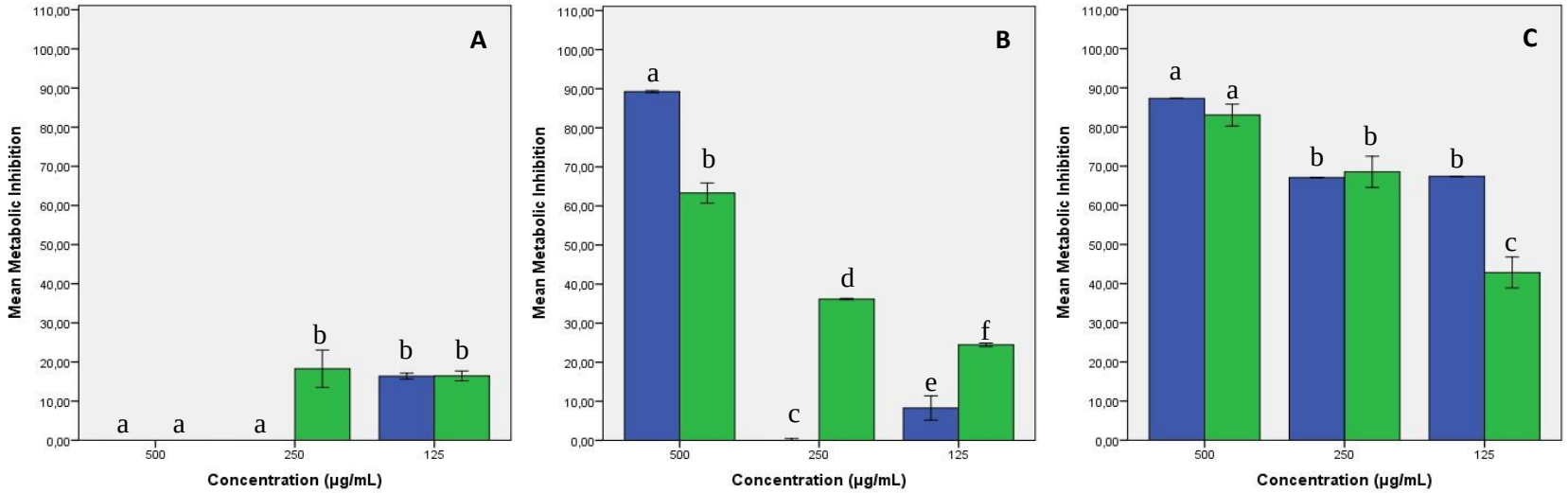


Figure 10S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by gallic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

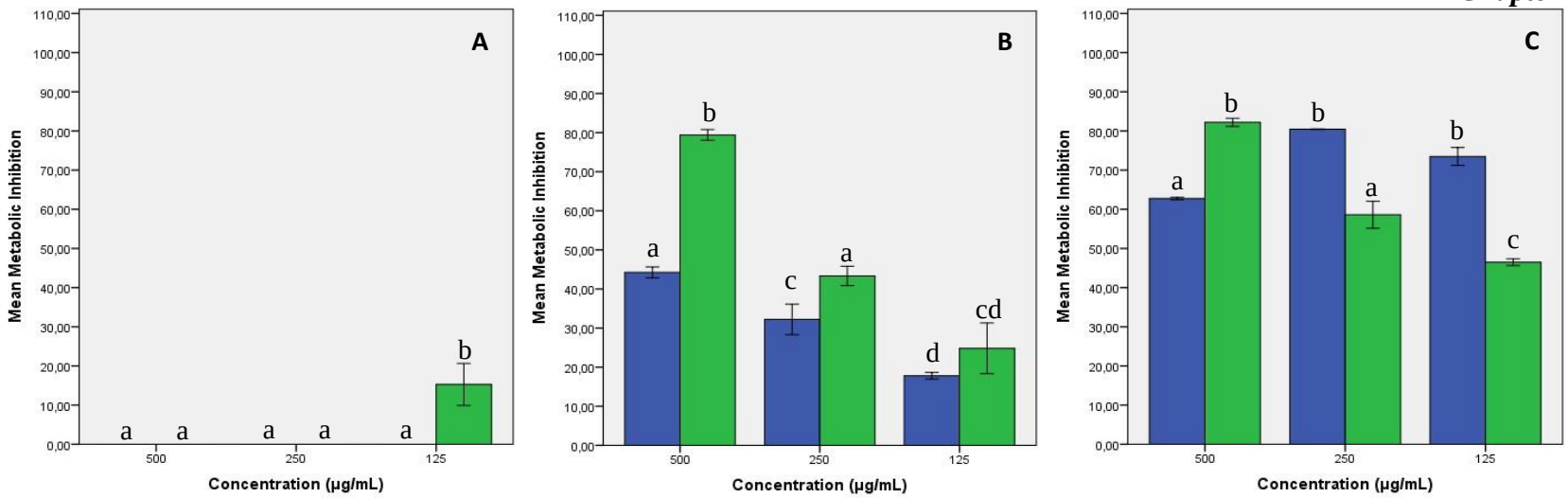


Figure 11S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by caffeic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

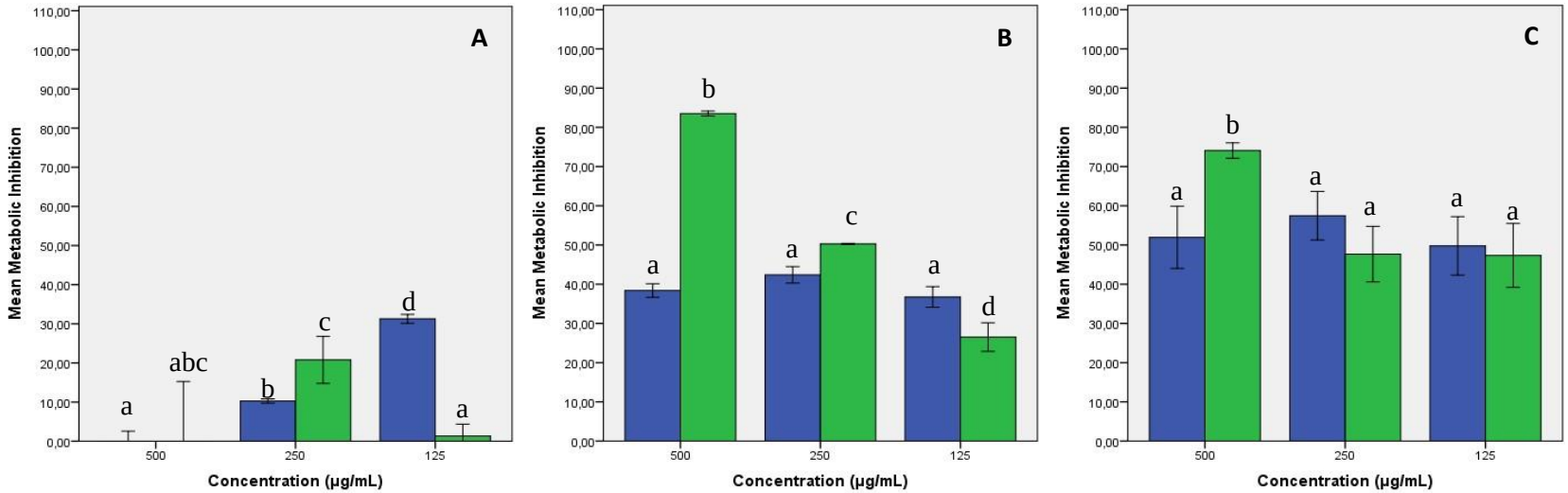


Figure 12S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by sinapic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

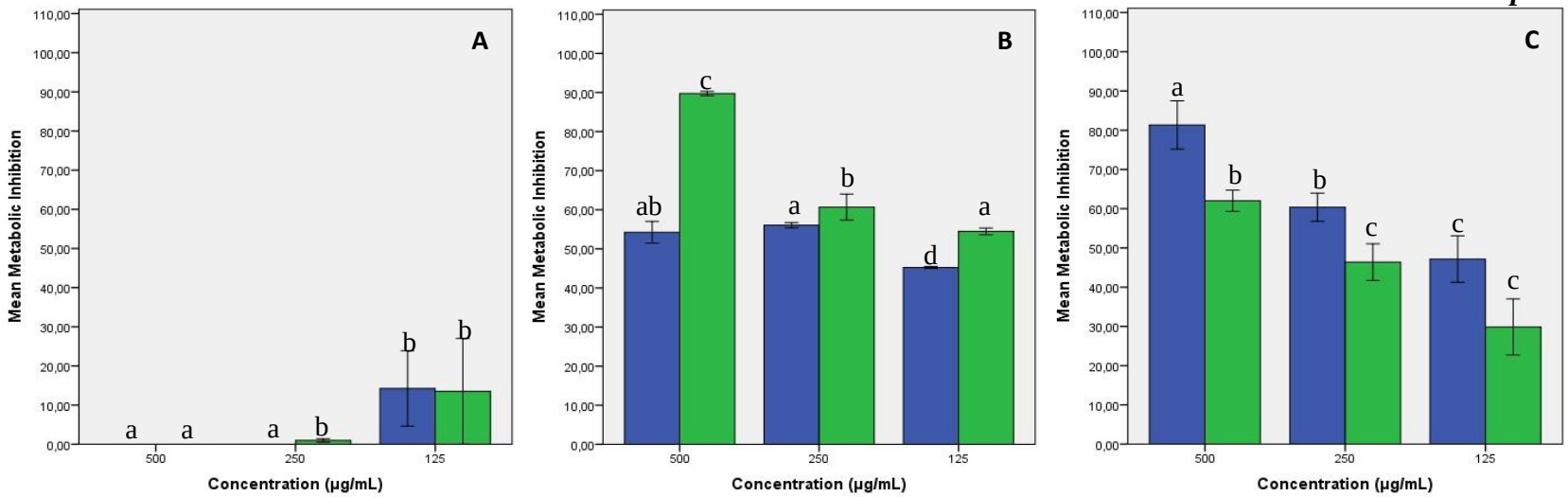


Figure 13S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by *p*-coumaric acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

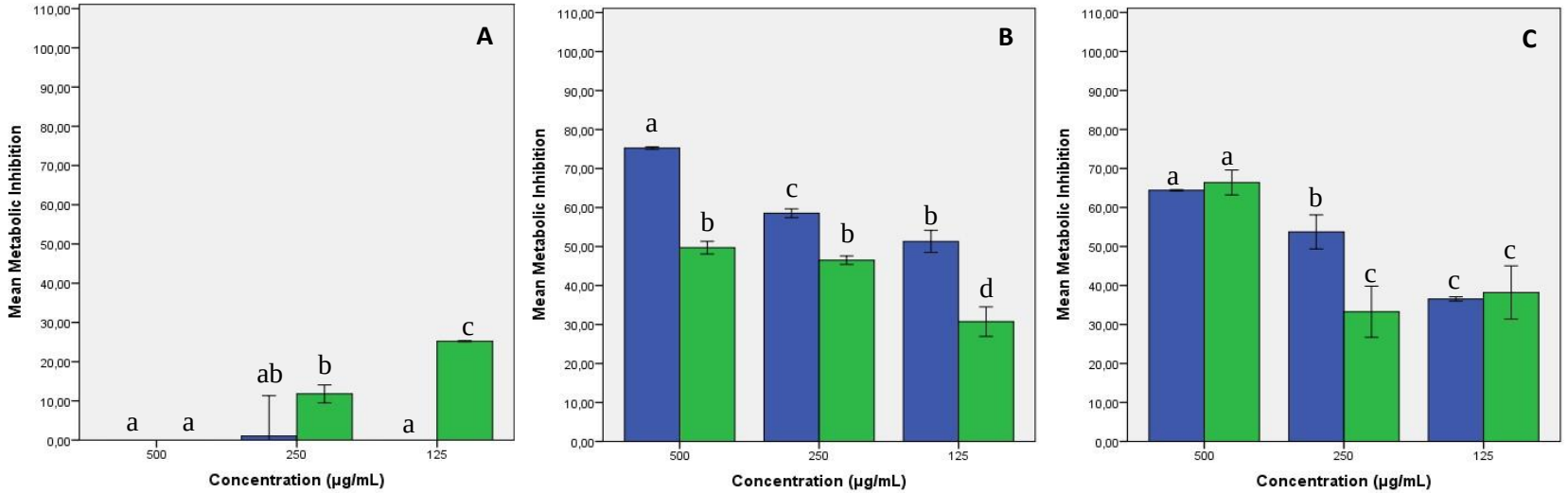


Figure 14S. Inhibition of metabolic activity in *E. coli* (A), MRSE (B) and MSSA (C), biofilms induced by syringic acid at pH 5 (■) and pH 7 (■). Different letters above each bar illustrate the statistically significant differences between bars.

6. References

ALVES, M., FERREIRA, I., LOURENÇO, I., COSTA, E., MARTINS, A. & PINTADO, M. 2014a. Wild Mushroom Extracts as Inhibitors of Bacterial Biofilm Formation. *Pathogens,* 3**,** 667.

ALVES, M., FROUFE, H., COSTA, A., SANTOS, A., OLIVEIRA, L., OSÓRIO, S., ABREU, R., PINTADO, M. & FERREIRA, I. 2014b. Docking Studies in Target Proteins Involved in Antibacterial Action Mechanisms: Extending the Knowledge on Standard Antibiotics to Antimicrobial Mushroom Compounds. *Molecules,* 19**,** 1672.

BORGES, A., SAAVEDRA, M. J. & SIMÕES, M. 2012. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling,* 28**,** 755-767.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science,* 284**,** 1318-1322.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews,* 12**,** 564-82.

DEWANGAN, R. P., JOSHI, S., KUMARI, S., GAUTAM, H., YAR, M. S. & PASHA, S. 2014. N-Terminally Modified Linear and Branched Spermine Backbone Dipeptidomimetics against Planktonic and Sessile Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 58**,** 5435-5447.

ERTL, P., ROHDE, B. & SELZER, P. 2000. Fast Calculation of Molecular Polar Surface Area as a Sum of Fragment-Based Contributions and Its Application to the Prediction of Drug Transport Properties. *Journal of Medicinal Chemistry,* 43**,** 3714-3717.

GOTTENBOS, B., GRIJPMA, D. W., VAN DER MEI, H. C., FEIJEN, J. & BUSSCHER, H. J. 2001. Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy,* 48**,** 7-13.

HAYES, A. & CAI, L. 2007. Using heteroskedasticity-consistent standard error estimators in OLS regression: An introduction and software implementation. *Behavior Research Methods,* 39**,** 709-722.

JAGANI, S., CHELIKANI, R. & KIM, D.-S. 2009. Effects of phenol and natural phenolic compounds on biofilm formation by Pseudomonas aeruginosa. *Biofouling,* 25**,** 321-324.

LACOMBE, A., WU, V. C. H., TYLER, S. & EDWARDS, K. 2010. Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157:H7. *International Journal of Food Microbiology,* 139**,** 102-107.

LAMBERS, H., PIESSENS, S., BLOEM, A., PRONK, H. & FINKEL, P. 2006. Natural skin surface pH is on average below 5, which is beneficial for its resident flora. *Int J Cosmet Sci,* 28**,** 359-70.

LUÍS, Â., SILVA, F., SOUSA, S., DUARTE, A. P. & DOMINGUES, F. 2014. Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids. *Biofouling,* 30**,** 69-79.

MACHADO, I., GRAÇA, J., LOPES, H., LOPES, S. & PEREIRA, M. O. 2013. Antimicrobial Pressure of Ciprofloxacin and Gentamicin on Biofilm Development by an Endoscope-Isolated Pseudomonas aeruginosa. *ISRN Biotechnology,* 2013**,** 10.

NEU, H. C. 1992. The Crisis in Antibiotic Resistance. *Science,* 257**,** 1064-1073.

PUUPPONEN-PIMIÄ, R., NOHYNEK, L., MEIER, C., KÄHKÖNEN, M., HEINONEN, M., HOPIA, A. & OKSMAN-CALDENTEY, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* 90**,** 494-507.

SILVA, S., COSTA, E. M., COSTA, M. R., PEREIRA, M. F., PEREIRA, J. O., SOARES, J. C. & PINTADO, M. M. 2015. Aqueous extracts of Vaccinium corymbosum as inhibitors of Staphylococcus aureus. *Food Control,* 51**,** 314-320.

STEPANOVIĆ, S., VUKOVIĆ, D., DAKIĆ, I., SAVIĆ, B. & ŠVABIĆ-VLAHOVIĆ, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods,* 40**,** 175-179.

STEWART, P. S. & WILLIAM COSTERTON, J. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet,* 358**,** 135-138.

SWAIN, M. 2012. Chemicalize. org. *Journal of Chemical Information and Modeling,* 52**,** 613-615.

VATTEM, D. A., LIN, Y. T., LABBE, R. G. & SHETTY, K. 2004. Phenolic antioxidant mobilization in cranberry pomace by solid-state bioprocessing using food grade fungus Lentinus edodes and effect on antimicrobial activity against select food borne pathogens. *Innovative Food Science & Emerging Technologies,* 5**,** 81-91.

ZIMMER, K. R., BLUM-SILVA, C. H., SOUZA, A. L. K., WULFFSCHUCH, M., REGINATTO, F. H., PEREIRA, C. M. P., MACEDO, A. J. & LENCINA, C. L. 2014. The Antibiofilm Effect of Blueberry Fruit Cultivars Against Staphylococcus epidermidis and Pseudomonas aeruginosa. *Journal of Medicinal Food,* 17**,** 324-331.

Antiadhesive and antibiofilm effect of malvidin-3-glucoside and malvidin-3-glucoside/neochlorogenic acid mixtures upon *Staphylococcus*

Sara Silva[1], Eduardo M. Costa[1], Sandra Vicente[1], Mariana Veiga[1], Rui M. Morais[1], Conceição Calhau[2,3], Manuela E. Pintado[1]

[1] CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

[2]Nutrição e Metabolismo, NOVA Medical School, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130,l 1169-056, Lisboa, Portugal

[3]CINTESIS – Centro de Investigação em Tecnologias e Serviços de Saúde, Porto, Portugal

Abstract

Several reports on the antimicrobial and antibiofouling activity of anthocyanin rich extracts have been made. However, in spite of the association of said activity with their anthocyanins content, to the best of our knowledge, no previous works regarding the antimicrobial, antibiofilm and/or antiadhesive properties of anthocyanins alone. Therefore, the present work aimed to determine the effects of malvidin-3-glucoside, a major component of a previously reported extract and the impact of its association with neochlorogenic acid (the only non-anthocyanin phenolic present in said extract), upon several *Staphylococcus* strains with varying resistance profiles. Results show that, while malvidin-3-glucoside and malvidin-3-glucoside/neochlorogenic acid mixtures were unable to considerably inhibit bacterial growth after 24 h, they still possessed an interesting antibiofilm activity (with reductions of biofilm entrapped cells up to 2.5 log cycles, metabolic inhibition rates up to 81% and up to 51% of biomass inhibition). When considering the bacterias' capacity to adhere to plain polystyrene surfaces the inhibition ranges were considerably lower (21% maximum value). However, when considering polystyrene surfaces coated with plasmatic proteins this value was considerably higher (45 % for adhesion in the presence of extract and 39% for adhesion after the surface was exposed to extract).

Keywords: Anthocyanins; Malvidin-3-glucoside; Antimicrobial activity; Antibiofilm activity; Bacterial adhesion inhibition

1. Introduction

Anthocyanins are natural, water soluble pigments that attracted the interest of the scientific community given their vast array of potential applications, from being used as food additives to serve as base in the development of new photovoltaic energy sources (Silva et al., 2016, Castañeda-Ovando et al., 2009, Silva et al., 2015a). From a biological standpoint anthocyanins have been associated with several health promoting properties such as having antioxidant, anti-inflammatory, anti-proliferative and anti-carcinogenic activity as well as positive effects on blood sugar levels and in the cardiovascular and neurologic systems (Cisowska et al., 2011, Badshah et al., 2015, Wallace and Giusti, 2013). One of the potential biological activities of anthocyanins includes antimicrobial activity. In literature several different reports may be found on the antimicrobial capacity of different types of anthocyanin rich extracts against a considerable wide range of microorganisms. However, the available literature regarding the effect of individual anthocyanins is considerably scarce and focused upon planktonic cells. In nature, bacteria are scarcely found in this state, rather they are most often present in biofilms, survival structures notorious for their high resistance to antimicrobial agents. Therefore, the literature available regarding the antimicrobial potential of pure anthocyanins is not only scarce but, to the best of our knowledge, disregarding one very important factor – the impact upon bacterial biofilms (Puupponen-Pimiä et al., 2001).

Staphylococcus are one of the most commonly found pathogenic agents and possess several inherent characteristics that help them being so, namely their ubiquitous presence in humans and their propensity to develop antimicrobial resistance (Chambers, 2001, Livermore, 2000). *S. aureus*, one highly recognized pathogen, has long since been split into two different groups regarding their antibiotic resistance; methicillin sensitive *S. aureus* (MSSA) or methicillin resistant *S. aureus* (MRSA). Recently, a new resistant group has emerged, the vancomycin resistant *S. aureus* (VRSA) (Livermore, 2000, Carbon, 2000). Furthermore, *S. epidermidis*, usually regarded as a commensal microorganism of human skin, has also emerged as an important pathogen as it has acquired resistance to methicillin (methicillin resistant *S. epidermidis*; MRSE) (Carvalho et al., 2015).

A previous work showed that an anthocyanin rich blueberry extract (whose most abundant anthocyanins were malvidins, particular malvidin-3-glucoside (M3Glu)) with neochlorogenic acid (NChA) was capable of significantly inhibit the growth, adhesion and biofilm formation

of *S. aureus* (Silva et al., 2016). Therefore, in attempt to better understand the real effect of each compound and establish a compound-effect relationship, the present work aimed to determine the antimicrobial potential of M3Glu (alone and in the presence of NChA) against an array of staphylococci strains with clinical relevance (MSSA, MRSA, VRSA and MRSE).

2. Experimental section

2.1. Microorganisms

Several *Staphylococcus* strains were used in this work. Clinical isolates (CI, from urine), capable of establishing biofilms, of a methicillin sensitive (MSSA CI) and a methicillin resistant (MRSA CI) *S. aureus* were kindly provided by CHTMAD – Hospital Centre of Trás-os-Montes e Alto Douro (through Ph.D. Maria José Alves). Additionally, three reference (R) strains of *S. aureus* and one of *S. epidermidis* were also considered: MSSA ATCC 25923 (MSSA R), MRSA CCUG 60578 (MRSA R), vancomycin resistant *S. aureus* ATCC 700699 (VRSA) and methicillin sensitive *S. epidermidis* ATCC 51625 (MRSE).

2.2. Test solutions

Malvidin-3-glucoside was acquired from Extrasynthese (Lyon, France) and NChA was acquired from Sigma (St. Louis, USA). Four different test solutions were prepared, two using only M3Glu (500 and 250 μg mL^{-1}) and two using a mixture of M3Glu and NChA: 500 μg mL^{-1} M3Glu with 100 μg mL^{-1} NChA (cM3Glu/NChA) and 250 μg mL^{-1} M3Glu with 50 μg mL^{-1} NChA (M3Glu/NChA). All solutions were prepared using sterile saline (0.5% (w v^{-1}) NaCl) for the adhesion assays or tryptic soy broth (TSB, Biokar Diagnostics, Beauvais, France) (supplemented with 1% (w v^{-1}) glucose (Sigma, St. Louis, USA) for the antibiofilm assays) and sterilized using a 0.22 μm sterile filter (Millipore, Billerica, USA).

2.3. Time inhibition curves

Time inhibition curves were drawn as described elsewhere (Silva et al., 2016). Briefly, the test solutions were inoculated with an overnight inoculum and transposed into a 96-well microtiter (Nunc, Darmstadt, Germany) and incubated at 37 °C. The optical density (OD) at 660 nm was assessed for a 24 h period, at 1 h intervals, using a microplate reader (Fluostar Optima; BMG Labtech, Ortenberg, Germany). A positive control was drawn using plain TSB without

antimicrobial agent and TSB was added as a negative control. Each condition was assayed in triplicate.

2.4. Total planktonic viable cell determination

After 24 h incubation in the presence of the test solutions, the total viable cells were determined using the drop method as described by Miles et al. (1938). Briefly, decimal dilutions were plated in plate count agar (PCA, Biokar Diagnostics, Beauvais, France) and incubated at 37 °C for 24 h. Plain TSB was used as a positive control and each assay was performed in triplicate.

2.5. Antibiofilm activity

The test solutions were inoculated at 2% (v v^{-1}) with an overnight inoculum and aliquots were distributed into 96-well microtiters (Nunc, Darmstadt, Germany) and incubated for 24 h at 37 °C. Afterwards, the content of each well was carefully discarded, washed to remove non-adherent cells and then used to determine the biofilm entrapped cells, biomass, metabolic activity and protein content. Plain TSB supplemented with 1% (w v^{-1}) glucose was used as a positive control. Each assay was performed in triplicate.

2.6. Biofilm entrapped viable cells

To quantify the biofilm embedded bacteria a method described previously was used (Silva et al., 2015b). Briefly, the content of each well was scraped and suspended in sterile phosphate-buffered saline pH 7.4 (PBS). The total viable counts were determined using serial dilutions and the drop method described by Miles et al. (1938), and the results given in log reduction of viable cells calculated according to the equation below:

$$\log \text{ viable cells reduction} = \log \text{CFU}_{\text{positive conttrol}} - \log \text{CFU}_{\text{assay}}$$

2.7. Biofilm biomass

Total biofilm biomass was determined using the crystal violet method as described by Stepanović et al. (2000). Briefly, after the removal of non-adherent cells, the biofilms were fixed using absolute ethanol (Panreac, Barcelona, Spain), stained with crystal violet and, after a thorough washing, the content of each well was resuspended in acetic acid (0.1% (v v^{-1})) and the OD at 660 nm was read using a microplate reader (Fluostar Optima; BMG Labtech,

Ortenberg, Germany). Results were given in biomass formation inhibition percentage according to the following formula:

$$\%\ \text{Biomass inhibition} = 100 - \left(\frac{OD_{assay}}{OD_{positive\,control}} \times 100 \right)$$

2.8. Biofilm metabolic activity

The metabolic activity was assessed using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium—caboxanilide (XTT) colorimetric method as described by Machado et al. (2013). After a 3 h incubation at 37 °C the OD at 485 nm was measured using a Fluostar Optima microplate reader (BMG Labtech, Ortenbeg Germany). The results were given in metabolic activity inhibition percentage calculated according to the following formula:

$$\%\ \text{Metabolic activity inhibition} = 100 - \left(\frac{OD_{sample}}{OD_{positive\,control}} \times 100 \right)$$

2.9. Impact on bacterial adhesion

2.9.1. Adhesion to polystyrene (PS)

The test solutions prepared using saline were inoculated at 2% (v v^{-1}), aliquoted into sterile polystyrene microtiters (Nunc, Darmstadt, Germany) and allowed to incubate for 3 h at 37 °C. After this period, the content of each well was discarded and washed with sterile saline and processed as described above to determine biofilms' biomass (Silva et al., 2016). Inoculated saline was used as a positive control and all assays were performed in triplicate. The results were given in percentage of adhesion inhibition according to the equation below.

$$\%\ \text{Adhesion inhibition} = 100 - \left(\frac{OD_{sample}}{OD_{positive\,control}} \times 100 \right)$$

2.9.2. Adhesion to polystyrene pre-treated with rabbit plasma

Aliquots of rabbit plasma (rp) were used to fill 96-well PS microtiters (Nunc, Darmstadt, Germany) and incubated for 24 h at 37 °C to allow for protein adsorption to the PS surface. After this period, the plasma was discarded and the wells washed with saline solution(Silva et al., 2016, van Loosdrecht et al., 1990). From this point forward two different approaches were used. In the first bacteria were incubated, for 3 h at 37 °C, with the test solutions (PS-rp). In the

second the bacteria were exposed to the test solutions first (37 °C, 1 h) and only then to the microorganims (37 °C, 3 h) (PS-rp-ts). Both protocols are fully described elsewhere (Silva et al., 2016). Inoculated saline was used as a positive control and all assays were performed in triplicate. The results were given in percentage of adhesion inhibition according to the equation below.

$$\%\ \text{Adhesion}\quad \text{inhibition}\quad = 100 - \left(\frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{positivecontrol}}} \times 100 \right)$$

2.10. Statistical analysis

Statistical analysis of the results was performed using IBM's SPSS Statistics v21.0.0.0 (New York, USA). The normality of the distributions was assessed using Shapiro-Wilk's test. When comparing between the different test solutions, One-Way ANOVA coupled with Turkey's post hoc test was used. The time inhibition curves were compared using the Repeated Measures test (to compare the behaviour throughout the 24 h period). Differences were considered significant for p-values inferior to 0.05.

3. Results and discussion

3.1. Impact upon Staphylococcus *growth*

As can be seen in Figure 1, all tested solutions (comprised of M3Glu alone or M3Glu mixed with NChA) were capable of reducing the growth of all staphylococci tested. For MRSE (Figure 1e), higher concentrations of anthocyanins allowed for higher inhibitions of bacterial growth, with the presence of NChA having no significant ($p > 0.05$) impact upon the results. When considering MSSA R (Figure 1a) no significant ($p > 0.05$) differences were found between the inhibitions observed for the most concentrated M3Glu and NChA mixture (500 µg mL^{-1} M3Glu with 125 µg mL^{-1}NChA, cM3Glu/NChA), M3Glu at 500 µg mL^{-1} and at 200 µg mL^{-1}, thus indicating that the presence of NChA didn't grant any additional inhibition when the anthocyanin was present at 500 µg mL^{-1}. When considering the less concentrated M3Glu and NChA mixture (200 µg mL^{-1} M3Glu with 50 µg mL^{-1} NChA, M3Glu/NChA) it can be seen that

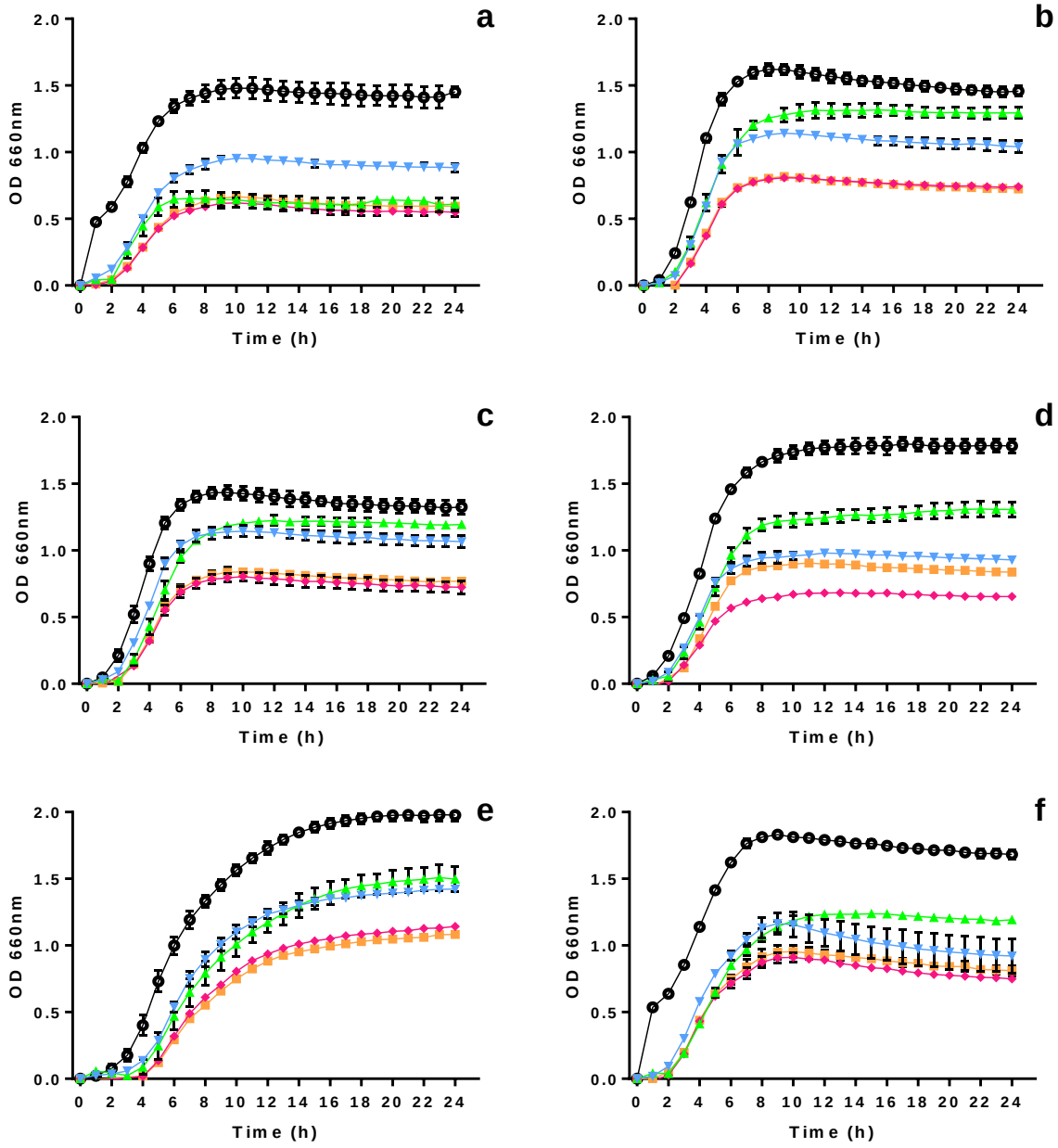


Figure 1. Time inhibition curves drawn for MSSA R (a), MSSA CI (b), MRSA R (c), MRSA CI (d), MRSE (e) and VRSA (f) when exposed to M3Glu at 500 µg mL^{-1} (■), M3Glu at 250 µg mL^{-1}(▲), cM3Glu/NChA (◆;500 µg mL^{-1} M3Glu with 100 µg mL^{-1} NChA), M3Glu/NChA (▼;250 µg mL^{-1} M3Glu with 50 µg mL^{-1} NChA) and for the positive control (O).

the inhibition induced by the anthocyanin alone (at 250 µg mL^{-1}) was hindered in the presence of NChA, as this combination allowed for higher OD values and an earlier beginning of the log phase. Interestingly, for MSSA CI, MRSA R and VRSA (Figure 1b, 1c and 1f), while for the highest concentration of M3Glu (M3Glu at 500 µg mL^{-1} and cM3G/NChA) no significant ($p > 0.05$) differences were observed, the presence of NChA led to an increase in the inhibitory activity when the anthocyanin was present at 250 µg mL^{-1}. On the other hand, for MRSA CI

(Figure 1d) the presence of NChA in the test solution increased the inhibition observed in comparison to the counterpart solution with M3Glu alone. In an earlier work, it was reported that an anthocyanin rich blueberry extract (whose main anthocyanin was M3Glu) that contained NChA was capable of fully inhibiting the growth of MSSA R and MRSA R. This activity was observed for 1000 (184.7 μg mL^{-1} total anthocyanins and 32.2 μg mL^{-1} NChA) and 500 μg mL^{-1} (92.35 μg mL^{-1} total anthocyanins and 16.1 μg mL^{-1} NChA) extract (Silva et al., 2016). However, in the present work the test solutions, even when at similar or higher concentration of pure compounds, were unable to exert an inhibition as strong has that of the extract. As it is comprised of several different compounds and not only the two compounds tested it is likely that some of the loss of activity may be explained by their absence.

In spite of the overall inhibitions in OD observed (after 24 h) in Figure 1, when contemplating the total viable cells (Figure 2) it can be seen that not all *Staphylococcus* were effectively inhibited by the test solutions. For instance, MSSA CI, MRSA R, MRSE and VRSA growth was not significantly ($p > 0.05$) inhibited (Figure 1b, 1c, 1e and 1f). In fact, in some cases the total viable counts were higher than those registered for the control with the increase ranging from 0.44 log (MSSA CI exposed to 500 μg mL^{-1} of M3Glu) to 0.91 log (MRSE exposed to M3Glu/NChA). Nevertheless, some inhibitions were still observed for MSSA R and MRSA CI viz. M3Glu (500 μg mL^{-1}) and cM3Glu/NChA (500 μg mL^{-1} M3Glu with 100 μg mL^{-1} NChA) were capable of inducing reductions up to 1.4 log of MSSA R viable cells and both mixtures with NChA caused an average of 0.6 log reduction of the total viable cells of MRSA CI. It is interesting to note the lack of parity between the inhibitions observed after 24 h in OD and the ones for total viable cells. As the spectrophotometric method measures the overall capacity to absorb light and anthocyanins are pigments it is possible that some interferences may occur. Furthermore, as the anthocyanins' chemical nature allows them to shift between forms (with different colours and absorbance spectra) depending on the environmental pH values which, in turn, vary as a result of the bacterial metabolism it is possible that this measurement is, somewhat biased (Castañeda-Ovando et al., 2009).

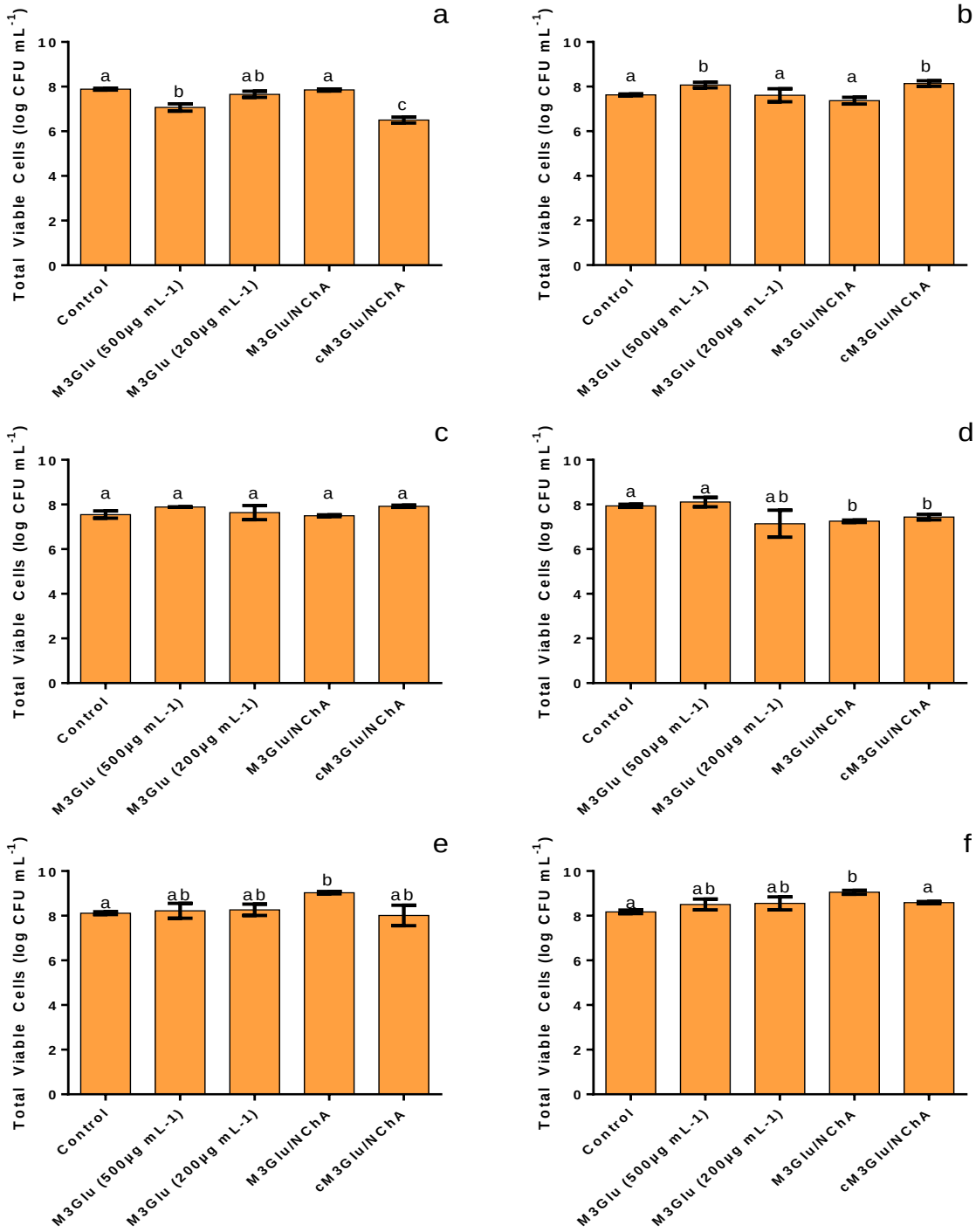


Figure 2. Total viable cells after 24 h exposure of MSSA R (a), MSSA CI (b), MRSA R (c), MRSA CI (d), MRSE (e) and VRSA (f) to the different test solutions. The different letters indicate the statistically significant ($p < 0.05$) differences between the bars.

3.2. Impact upon Staphylococcus *biofilms*

As can be seen in Figure 3a, overall, all test solutions were capable of inducing a reduction of the viable, biofilm entrapped, cells though some exceptions were observed. Namely, for MSSA R exposed to 250 µg mL^{-1} a small increase in viable cells was observed (0.29 log), while for MRSE (500 µg mL^{-1} M3Glu and cM3Glu/NChA), VRSA (500 µg mL^{-1} M3Glu, cM3Glu/NChA and M3Glu/NChA) and MSSA CI (250 µg mL^{-1} M3Glu) no significant ($p > 0.05$) variations were observed. For MSSA and MRSA (both R and CI strains), as could be expected, a direct relation between concentration an inhibition was observed with the most concentrated M3Glu solution (500 µg mL^{-1}) being more effective (by an average of 1.0 log cycles) in reducing the amount of biofilm entrapped cells than its diluted counterpart (250 µg mL^{-1}). However, when considering the M3G solutions with NChA, no statistically significant ($p > 0.05$) differences were found between both solutions regardless of their concentration (cM3Glu/NChA and M3Glu/NChA). The only exception was observed for MSSA R where the most diluted solution was more effective than

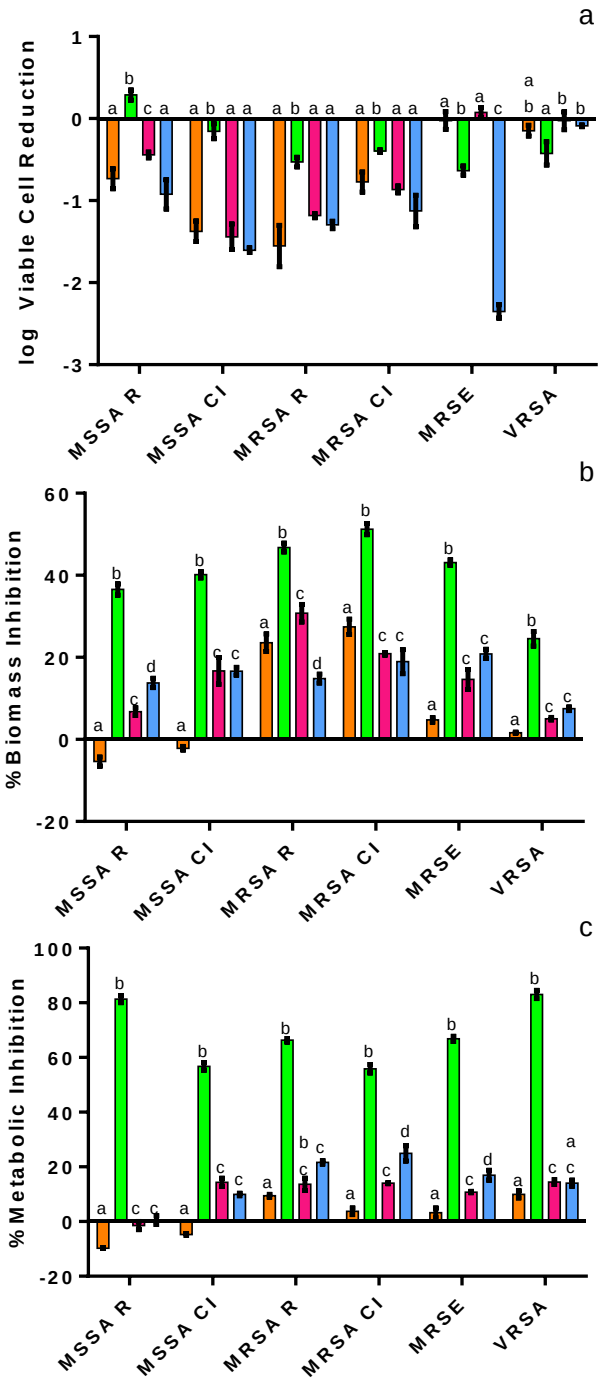


Figure 3. Effect of the test solutions upon biofilms' viable cells (a), biomass (b) and metabolic activity when exposed to 500 µg mL^{-1} M3Glu (■), 250 µg mL^{-1} M3Glu (■), cM3Glu/NChA (■; 500 µg mL^{-1} M3Glu with 100 µg mL^{-1} NChA) and M3Glu/NCh3 (■; 250 µg mL^{-1} M3Glu with 50 µg mL^{-1} NChA). The letters above each bar indicate the statistically significant ($p < 0.05$) differences found for each microorganism.

the concentrated one, an effect also observed for MRSE in the presence of M3Glu and NChA mixed solutions.

Interestingly, when observing the results pertaining to the biofilms biomass (Figure 3 b) and metabolic activity (Figure 3c) M3Glu at 250 µg mL^{-1} (the overall less effective in reducing the biofilm entrapped cells) produced the highest inhibitions observed. For instance, for MSSA R the 250 µg mL^{-1} solution while promoting a 0.29 log increase of viable cells also allowed for a 40% inhibition of total biomass and 81% inhibition of metabolic activity. This scenario, that is similar to the one observed for all other staphylococci (except VRSA, where the 250 µg mL^{-1} solution is the most effective in reducing the biofilm entrapped cells) indicated that while the overall amount of cells was not be the lowest, they were less active and less surrounded by the polymeric matrix that helps in the protection of biofilm imbedded bacterial cells (Chaignon et al., 2007). Considering that one of the mechanisms through which biofilms are thought to grant resistance is by obstructing the antimicrobials access to the bacterial cells, this reduction in biomass may be particularly interesting to explore in combination with other antibiotics/antimicrobials (Chaignon et al., 2007, Fux et al., 2005).

Higher concentration of anthocyanin (M3Glu at 500 µg mL^{-1}) exerted little to no metabolic inhibition upon any of the strains tested (less than 10%) and the same was observed for biomass inhibition (less than 5%) for all bacteria, with the exception of MRSA R and MRSA CI (23.6% and 27.4% inhibition, respectively). These results are particularly interesting if considering that the overall amount of biofilm entrapped cells were lower than in the control (e.g. MSSA R), meaning that as there were fewer viable cells present in the biofilm they had to be both more metabolically active and produce more extracellular matrix to compensate for the difference. It is possible that the bacteria, perceiving the presence of a less favourable environmental conditions, increased their metabolic activity in order to compensate for this stress namely through the increase in production exopolysaccharide (EPS) (Landini, 2009). On another note, the addition of NChA led to significantly ($p < 0.05$) lower inhibition values for the less concentrated mixed solution (M3Glu/NChA). Conversely, when considering cM3Glu/NChA, the addition of NChA caused a significant ($p < 0.05$) increase in the inhibitions observed for biomass (all staphylococci except MRSA CI) and metabolic (all bacteria bar VRSA). This demonstrated the importance of the overall chemical context when considering the activity of natural plant extracts and potential synergies established between compounds in order for them to exhort a given activity.

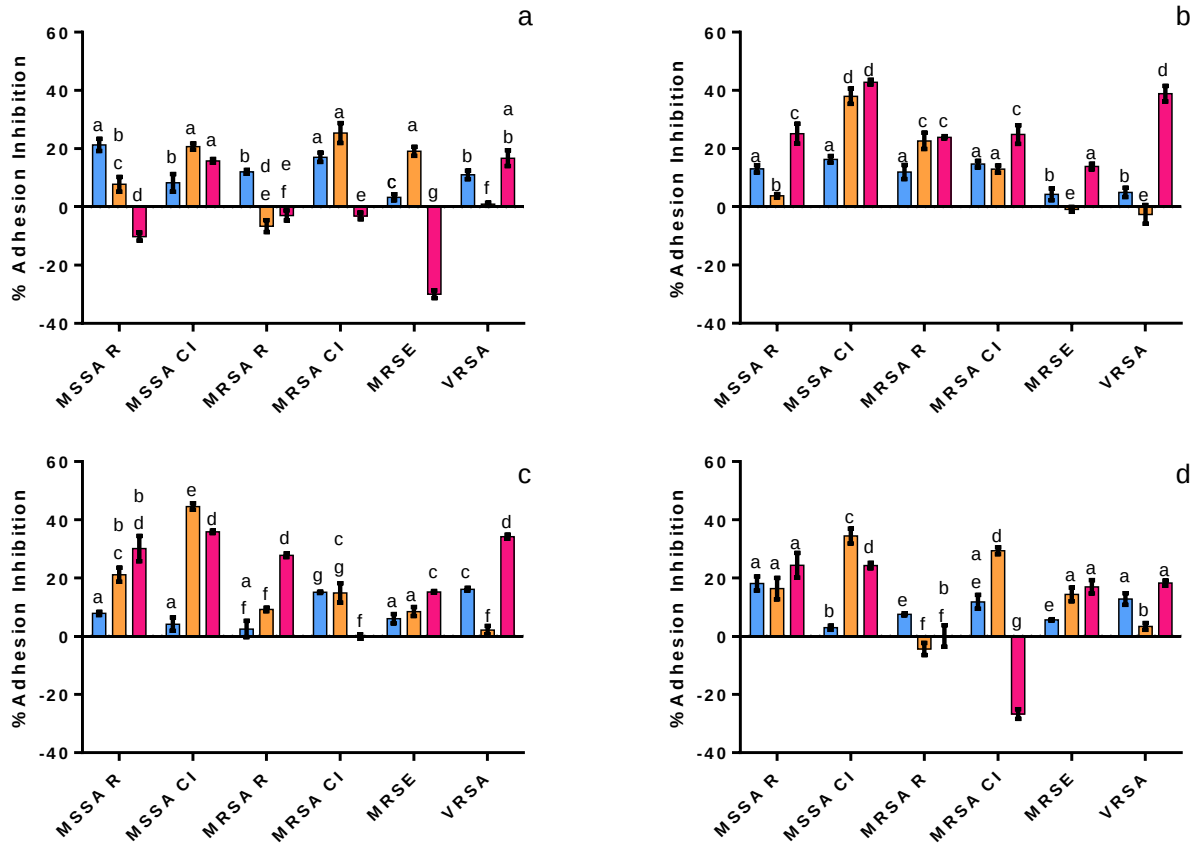


Figure 4. Effect of M3Glu (500 µg mL^{-1}(a) and 250 µg mL^{-1} (b)), cM3Glu/NChA (c; 500 µg mL^{-1} M3Glu with 100 µg mL^{-1} NChA) and M3Glu/NChA (d; 250 µg mL^{-1} M3Glu with 50 µg mL^{-1} NChA) upon bacterial adhesion to PS (■), PS-rp (■) and PS-rp-ts (■). The different letters indicate the statistically significant ($p < 0.05$) differences between the bars.

Overall, the test solutions were capable of reducing the biofilms formed (Figure 3) by the different staphylococci strains and they did so at concentrations that had little to no impact upon the number of viable cells (Figure 2). Furthermore, it appears to reduce bacterial adhesion to surfaces without promoting the development of bacterial resistance (Costerton et al., 1999, Donlan, 2001, Zimmer et al., 2014).

3.3. Impact upon Staphylococcus *adhesion*

To ascertain the anthocyanin and anthocyanin/NChA mixtures potential to block staphylococcal binding to surfaces, anti-adhesion studies were carried out considering both a plain polystyrene (PS) surface and a PS surface coated with plasmatic proteins (PS-rp as to mimic exposure to a cytoplasmatic environment) (van Loosdrecht et al., 1990). All of the tested solutions were capable of inhibiting bacterial adhesion to PS surfaces (fugure 4(with a range of

inhibitions from 2.9 (M3Glu/NChA against MSSA CI) to 21.3% (500 µg mL^{-1} M3Glu against MSSA R), with the only exception being MRSA R exposed to cM3Glu/NChA. When considering the PS-rp assay, more cases where no inhibition was observed were registered; VRSA for all solutions except M3Glu/NChA, MRSA R for M3Glu (500 µg mL^{-1}) or M3Glu/NChA and M3Glu (250 µg mL^{-1}) for MRSE. Moreover, it is interesting to note that while more microorganims appear to be less susceptible to the compounds, the maximum inhibition values are also significantly ($p < 0.05$) higher (ca. 40%), though that did not always translate into higher inhibition values for PS-rp (when comparing to PS). This is unlike what has been observed in a previous work with a blueberry extract (rich in anthocyanins, particularly M3Glu and possessing NChA) where the pre-treatment of PS surfaces with rabbit plasma did not always led to higher inhibition percentages(Silva et al., 2016). These differences are a possible consequence of the extracts' complexity as the array of anthocyanins may allow for synergistic activities that M3Glu alone, even when in the presence of NChA, is not able to reproduce.

On another note, to try and ascertain how the interaction of the compounds with the adsorbed proteins could affect the adhesion process, a third surface, sequentially exposed to rabbit plasma, test solutions and microorganims, was considered (PS-rp-ts). Overall four different behaviours were observed: i) no significant differences ($p > 0.05$) were found between the adhesion to PS-rp and PS-rp-ts surfaces indicating that the inhibitory effect observed is a consequence of the interactions between M3Glu, NChA and the adsorbed proteins (Soares et al., 2007). ii) adhesion inhibition was higher for PS-rp than for PS-rp-ts, however the presence of inhibition in the latter indicates that at least part of the inhibition observed was mediated by interactions between the compounds and the adsorbed proteins. iii) inhibition of adhesion was lower for PS-rp than for PS-rp-ts. This occurs only for VRSA, which may indicate that the compounds are less effective in blocking bacterial adhesion when in the presence of VRSA, either because the bacteria are faster to adhere than the compounds are to interact or because VRSA interacted with the compound reducing its effectiveness. iv) PS-rp inhibition values are higher than those observed for PS-rp-ts and the latter are negative, thus signalling that the interaction of the compounds with the adsorbed proteins may accentuate bacterial adhesion.

Overall, when considering the activity of the isolated compounds it is significant lower than that observed for a similar extract. Considering the lack of evidence on the antimicrobial activity of pure anthocyanins and the lack, to the best of our knowledge, of mechanistic papers

we can only hypothesise why this occurs. The addition of NChA proved to cause a significant variation in M3Glu activity, thus demonstrating that one of the possible explanations for the differences is the absence of synergies with other compounds. Another reason that may explain these differences, particularly when considering the effect of the addition of an acid, is the environmental pH values. In solution, anthocyanins may be found in several different forms depending on the environmental pH; at low pH the main form is the flavylium cation which is positively charged while at the pH in which these inhibitions were observed (ca. 6.5 – data not shown) the major form is the neutral quinoidal base (Lopes et al., 2011). Bacterial cells are electronegative entities which makes them more prone to interact with positively charged compounds, thus the environmental pH values may explain the comparatively lower activity observed as well as prove to be an important subject to be studied in the future (Costa et al., 2012).

4. Conclusions

While not effective in inhibiting the amount of planktonic staphylococci after 24 h, M3Glu (alone and in the presence of NChA) was effective in inhibiting biofilm formation and *Staphylococcus* adherence. This capacity to reduce adhesion and biofilms without reducing bacterial growth is an important characteristic as it reduces the likeliness of resistance development making M3Glu an interesting alternative antimicrobial compound. Furthermore, their capacity to reduce bacterial adhesion makes them interesting additives to be used when bacterial infection/colonization is an important factor to control, though further studies seeking to understand the environmental pH role may be of importance as it may allow for better inhibitions.

Acknowledgements: The authors acknowledge CHTMAD – Hospital Centre of Chaves (Portugal) for providing the MRSA and MSSA clinical isolates and the financial support given by the national funds from FCT (through project UID/Multi/50016/2013). Additionally, the authors Sara Silva and Eduardo M. Costa wold like to acknowledge FCT for their Ph.D. grants with the references SFRH/BD/90867/2012 and SFRH/BDE/103957/2014, respectively.

5. References

BADSHAH, H., KIM, T. H. & KIM, M. O. 2015. Protective effects of Anthocyanins against Amyloid beta-induced neurotoxicity in vivo and in vitro. *Neurochemistry International,* 80**,** 51-59.

CARBON, C. 2000. MRSA and MRSE: is there an answer? *Clinical Microbiology and Infection,* 6, Supplement 2**,** 17-22.

CARVALHO, F. B., GUTIERRES, J. M., BOHNERT, C., ZAGO, A. M., ABDALLA, F. H., VIEIRA, J. M., PALMA, H. E., OLIVEIRA, S. M., SPANEVELLO, R. M., DUARTE, M. M., LOPES, S. T. A., AIELLO, G., AMARAL, M. G., PIPPI, N. L. & ANDRADE, C. M. 2015. Anthocyanins suppress the secretion of proinflammatory mediators and oxidative stress, and restore ion pump activities in demyelination. *The Journal of Nutritional Biochemistry,* 26**,** 378-390.

CASTAÑEDA-OVANDO, A., PACHECO-HERNÁNDEZ, M. D. L., PÁEZ-HERNÁNDEZ, M. E., RODRÍGUEZ, J. A. & GALÁN-VIDAL, C. A. 2009. Chemical studies of anthocyanins: A review. *Food Chemistry,* 113**,** 859-871.

CHAIGNON, P., SADOVSKAYA, I., RAGUNAH, C., RAMASUBBU, N., KAPLAN, J. B. & JABBOURI, S. 2007. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Applied Microbiology and Biotechnology,* 75**,** 125-132.

CHAMBERS, H. F. 2001. The changing epidemiology of Staphylococcus aureus? *Emerging Infectious Diseases,* 7**,** 178-182.

CISOWSKA, A., WOJNICZ, D. & HENDRICH, A. B. 2011. Anthocyanins as antimicrobial agents of natural plant origin. *Nat Prod Commun,* 6**,** 149-56.

COSTA, E. M., SILVA, S., PINA, C., TAVARIA, F. K. & PINTADO, M. M. 2012. Evaluation and insights into chitosan antimicrobial activity against anaerobic oral pathogens. *Anaerobe,* 18**,** 305-309.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science,* 284**,** 1318-1322.

DONLAN, R. M. 2001. Biofilm formation: a clinically relevant microbiological process. *Clinical Infectious Diseases,* 33**,** 1387-1392.

FUX, C. A., COSTERTON, J. W., STEWART, P. S. & STOODLEY, P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol,* 13**,** 34-40.

LANDINI, P. 2009. Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in Escherichia coli. *Research in Microbiology,* 160**,** 259-266.

LIVERMORE, D. M. 2000. Antibiotic resistance in staphylococci. *International Journal of Antimicrobial Agents,* 16, Supplement 1**,** 3-10.

LOPES, T. J., YAGINUMA, S. R., QUADRI, M. G. N. & QUADRI, M. B. 2011. Evaluation of red cabbage anthocyanins after partial purification on clay. *Brazilian Archives of Biology and Technology,* 54**,** 1349-1356.

MACHADO, I., GRAÇA, J., LOPES, H., LOPES, S. & PEREIRA, M. O. 2013. Antimicrobial Pressure of Ciprofloxacin and Gentamicin on Biofilm Development by an Endoscope-Isolated Pseudomonas aeruginosa. *ISRN Biotechnology,* 2013**,** 10.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J Hyg (Lond),* 38**,** 732-49.

PUUPPONEN-PIMIÄ, R., NOHYNEK, L., MEIER, C., KÄHKÖNEN, M., HEINONEN, M., HOPIA, A. & OKSMAN-CALDENTEY, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* 90**,** 494-507.

SILVA, S., COSTA, E., CALHAU, C., MORAIS, R. & PINTADO, M. 2015a. Anthocyanin Extraction from Plant Tissues: A Review. *Critical Reviews in Food Science and Nutrition***,** 00-00.

SILVA, S., COSTA, E. M., COSTA, M. R., PEREIRA, M. F., PEREIRA, J. O., SOARES, J. C. & PINTADO, M. M. 2015b. Aqueous extracts of Vaccinium corymbosum as inhibitors of Staphylococcus aureus. *Food Control,* 51**,** 314-320.

SILVA, S., COSTA, E. M., MENDES, M., MORAIS, R. M., CALHAU, C. & PINTADO, M. M. 2016. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic anthocyanin rich blueberry extract purified by solid phase extraction. *J Appl Microbiol*.

SOARES, S., MATEUS, N. & DE FREITAS, V. 2007. Interaction of Different Polyphenols with Bovine Serum Albumin (BSA) and Human Salivary α-Amylase (HSA) by Fluorescence Quenching. *Journal of Agricultural and Food Chemistry,* 55**,** 6726-6735.

STEPANOVIĆ, S., VUKOVIĆ, D., DAKIĆ, I., SAVIĆ, B. & ŠVABIĆ-VLAHOVIĆ, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods,* 40**,** 175-179.

VAN LOOSDRECHT, M. M., NORDE, W., LYKLEMA, J. & ZEHNDER, A. B. 1990. Hydrophobic and electrostatic parameters in bacterial adhesion. *Aquatic Sciences,* 52**,** 103-114.

WALLACE, T. C. & GIUSTI, M. 2013. *Anthocyanins in Health and Disease*, Taylor & Francis Group.

ZIMMER, K. R., BLUM-SILVA, C. H., SOUZA, A. L. K., WULFFSCHUCH, M., REGINATTO, F. H., PEREIRA, C. M. P., MACEDO, A. J. & LENCINA, C. L. 2014. The Antibiofilm Effect of Blueberry Fruit Cultivars Against Staphylococcus epidermidis and Pseudomonas aeruginosa. *Journal of Medicinal Food,* 17**,** 324-331.

Chapter 6

Effect on probiotics & probiotic/pathogen systems

This chapter aimed to characterize the extract's impact upon probiotic bacteria, when present at concentrations effective against food pathogens.

"To declare war on ninety-nine percent of bacteria when less than one percent of them threaten our health makes no sense. Many of the bacteria we're killing are our protectors."

Sandor Katz *quoted in* ***Cooked: A Natural History of Transformation***

Chapter Preamble

When looking at the antimicrobial potential of a natural extract it is common to focus only on potential pathogens ignoring the fact that, as not all bacteria are harmful not all inhibitions are positive. Probiotic and lactic acid bacteria are a prime examples of groups of bacteria whose inhibition may be detrimental, not only because it may limit the use of the extracts to non-fermented foods, but also because it may compromise potential health benefits they have (e.g. interfering with transient adherence and presence in gut microbiota and the production of short chain fatty acids (SCFA)). Hence, the main goal of the present chapter was to evaluate the effect of the extract upon five different probiotic bacteria as well as its impact upon some of their potential beneficial characteristics: organic acid production and inhibition of intestinal pathogens. To accomplish this, the chapter was divided in two.

Chapter 6.1: Selective activity of a purified blueberry extract upon pathogenic and probiotic bacteria

Some authors have reported that anthocyanins and anthocyanin rich extracts while capable of inhibiting the growth of potential pathogens they had little to no inhibitory effect upon lactic acid bacteria and potential probiotics. Therefore, this chapter intended to evaluate the impact of blueberry extract upon five different probiotic microorganims' (*Lactobacillus plantarum* 299V, *Lactobacillus acidophilus* Ki, *Lactobacillus rhamnosus* R11, *Bifidobacterium animalis* Bo and *Bifidobacterium animalis* Bb12) growth and metabolic activity at a concentration that was effective in inhibiting the growth of potential four potential food pathogens (*Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enteritidis*). To do so, different extract concentrations were assessed for their capacity to inhibit pathogen growth over a 24 h period using and absorbance measuring method. The concentration identified as the most interesting (1000 µg mL^{-1}) was used to determine the extract's impact upon the total viable pathogen counts. As this concentration proved to be effective in inhibiting pathogen growth, it was used to determine the extract's impact upon the growth and metabolic activity of probiotics. Overall, the extract had little to no inhibitory effect upon the overall growth of the probiotic microorganisms though it did appear to stimulate the overall production of organic acids.

Chapter 6.2. Impact of a purified blueberry extract on probiotic mucin adhesion and its effect on probiotic/intestinal pathogen systems

The production of organic acids by probiotics has long since been describes as one of the mechanism through which probiotics may aid in the protection against gastrointestinal infections. Another mechanism is associated with the probiotics' capacity to hinder probiotic colonization of the gut either by aiding in the removal of, occupying or competing with pathogens. As the extract has been found to be an effective inhibitor of pathogen adhesion (chapter 5.1), it is possible that its presence aid in the reduction of pathogen adhesion to the intestinal epithelium. To test this hypothesis an *in vitro* system that used mucin and bovine serum albumin (BSA) was used to determine the extracts impact upon the probiotics' capacity to either displace adhered pathogen cells, compete with pathogens for adhesion spots or impede their adhesion to the treated surface while also evaluating the extract's effect upon probiotic colonization of the surfaces. The results demonstrated that, while the extracts may cause some reduction of the viable adhered probiotic cells, they generally cause a reduction in the amount of adhered pathogens with some combinations of extract with *B.* Bo being able to almost completely inhibiting the adhesion of pathogen cells.

Selective activity of a purified blueberry extract upon pathogenic and probiotic bacteria

Sara Silva[1], Eduardo M. Costa[1], Hélder Oliveira[2], Mariana Veiga[1], Bruno Horta[1], Sandra Vicente[1], Victor de Freitas[2], Rui M. Morais[1], Conceição Calhau[3,4], Manuela E. Pintado[1]

[1] *CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal*

[2]*REQUINTE/LAQV, Department of Chemistry and Biotemistry, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal*

[3]*Department of Biochemistry, Faculty of Medicine, University of Porto. Portugal*

[4]*CINTESIS – Center for Research in Health Technologies and Information Systems, Porto, Portugal*

Abstract

Blueberry extracts have been widely recognized as possessing antimicrobial activity against several potential pathogens. However, the contextualization of the interaction of these extracts with beneficial bacteria (i.e. probiotics), particularly when considering food applications of these products, may be of importance, not only because their presence is important in the regular gut microbiota but also because they are important constituents of regular and functional foodstuffs. Therefore, the present work firstly sought to demonstrate the inhibitory effect of a blueberry extract upon four potential food pathogens and, after identifying the active concentrations, evaluated their impact upon the growth and metabolic activity (organic acid production and sugar consumption) of five potential probiotic microorganisms. Results showed that the extract, at a concentration that inhibited *L. monocytogenes*, *B. cereus*, *E. coli* and *S. enteritidis* (1000 μg mL^{-1}), had no inhibitory effect on the growth of the potential probiotic stains used. However, it had a significant impact on metabolic activity of all probiotic strains resulting in higher amounts of organic acid production (acetic, citric and lactic acids) and an earlier production of propionic acid.

Keywords: Probiotic; Pathogen; Antimicrobial activity; Blueberry extract; Organic acids; Short chain fatty acids

1. Introduction

Blueberries are recognized as being rich in phenolic compounds, particularly anthocyanins. This trait has made them the focus of several studies, which aim to better understand the health promoting properties of blueberry-based extracts. One of the most common attributes associated with phenolic compounds is their potential as antimicrobial agents, with several authors having demonstrated it (Silva et al., 2016b, Shen et al., 2014, Deng et al., 2014, Lacombe et al., 2012, Park et al., 2011, Burdulis et al., 2009). However, in a previous work it has been reported that a blueberry extract, while capable of inhibiting the growth of several potential pathogens, had no inhibitory effect upon *Lactobacillus rhamnosus*, *Lactococcus lactis* and *Lactobacillus bulgaricus* growth (Silva et al., 2013). This has raised an interesting question - if a blueberry extract is capable of inhibiting a pathogen, while simultaneously not inhibiting potentially probiotic microorganisms, can it be used as potential antimicrobial additive for fermented foods or as a coadjuvant for the treatment of intestinal infections? Furthermore, although a few works may be found on anthocyanin rich extracts' lack of inhibitory effect against potential probiotics, to the best of our knowledge none has considered the impact that the extracts' presence may have upon their metabolic activity when present at the concentrations needed to have an antimicrobial effect upon pathogenic microorganisms (Lee et al., 2008, Lacombe et al., 2012, Silva et al., 2013, Puupponen-Pimiä et al., 2001). Therefore, the present work aimed to assess the impact of a purified, phenolic rich, blueberry extract upon probiotic growth and metabolic activity (in particular production of organic acids) while simultaneous demonstrating the extract capacity to inhibit the growth of four potential food pathogens: *B. cereus*, *S. enteritidis*, *L. monocytogenes* and *E. coli*.

2. Experimental section

2.1. Extract production and purification

Goldtraube blueberries, harvested in 2015, were kindly provided by Mirtilusa S.A. (Sever do Vouga, Portugal) and were kept at -20 °C until processing. The extracts were produced using ethanol, purified using Bond Elut Plexa solid phase extraction (SPE) columns from Agilent technologies (Santa Clara, California, USA) and dried to obtain a powder according to the protocol described elsewhere (Silva et al., 2016b). Henceforth, whenever extract is mentioned it refers to the powder obtained in this step.

2.2. Extract characterization

The extract was dissolved in methanol at 1 mg mL^{-1} and the total anthocyanin content was determined through the measurement of the area under the curve at 520 nm using the HPLC-DAD method described elsewhere (Silva et al., 2016a, Lee et al., 2008). Compound identification was carried out by HPLC-MS as described by Fernandes et al. (2012). Briefly, a C18 reverse phase HPLC column (25 cm) was used and separation carried out using 2 distinct solvents (A: 10% formic acid in water; B: 10% formic acid and 30% acetonitrile in water). Each chromatographic analysis occurred using a 0.5 mL min^{-1} flow under the following gradient: 0 to 70 min, 80-20% of A; 70 to 80 min, 100% B; from 80 to 90 min 80% of solvent A.

2.3. Microorganisms

Four potential food pathogens and five different probiotic strains were considered in the present work. The probiotics considered were *Lactobacillus acidophilus* Ki, *L. plantarum* 299V, *L. rhamnosus* R11, *Bifidobacterium animalis* Bb12 (B. Bb12) and *B. animalis* Bo (B. BO) and the pathogens were *Escherichia coli* NCTC 9001, *Salmonella enteritidis* ATCC 13076, *Listeria monocytogenes* ESB 3562 (a food isolate from Escola Superior de Biotecnologia's culture collection, Porto, Portugal) and *Bacillus cereus* NCTC 2599.

2.4. Effect on pathogenic bacteria

2.4.1. Time inhibition curves

Extracts at 1000, 500, 250 and 125 µg mL^{-1} were prepared using Tryptone Soy Broth (TSB, Biokar Diagnostics, Beauvais, France), sterilized using a 0.22 µm filter (Millipore, Massachusetts, USA) and inoculated using an overnight inoculum (ca. 10^8 CFU mL^{-1}). The mixtures were incubated in a 96 well microplate (Nunc, Darmstadt, Germany) for 24 h at 37 °C with the optical density (OD) at 660 nm being assessed at 1 h intervals (Fluorostar Optima Microplate Reader, BMG Labtech, Ortenberg, Germany) and the increase in OD being considered as a consequence of bacterial growth. A positive control was drawn using inoculated culture media and sterile TSB was used as a negative control. Each condition was assayed in triplicate (Silva et al., 2016b).

2.4.2. Impact on pathogenic viable counts

A 1000 µg mL^{-1} extract solution in TSB was inoculated with an overnight inoculum of each of the pathogenic microorganisms and incubated at 37 °C for 24 h. At the 0, 6, 12 and 24 h mark the total viable cells were determined using decimal dilutions and plated in Plate Count Agar (PCA, Biokar Diagnostics, Beauvais, France) (Miles et al., 1938, Silva et al., 2015). The PCA plates were then incubated at 37 °C for 24 h. Each condition was assessed in triplicate.

2.5. Effect on probiotic bacteria

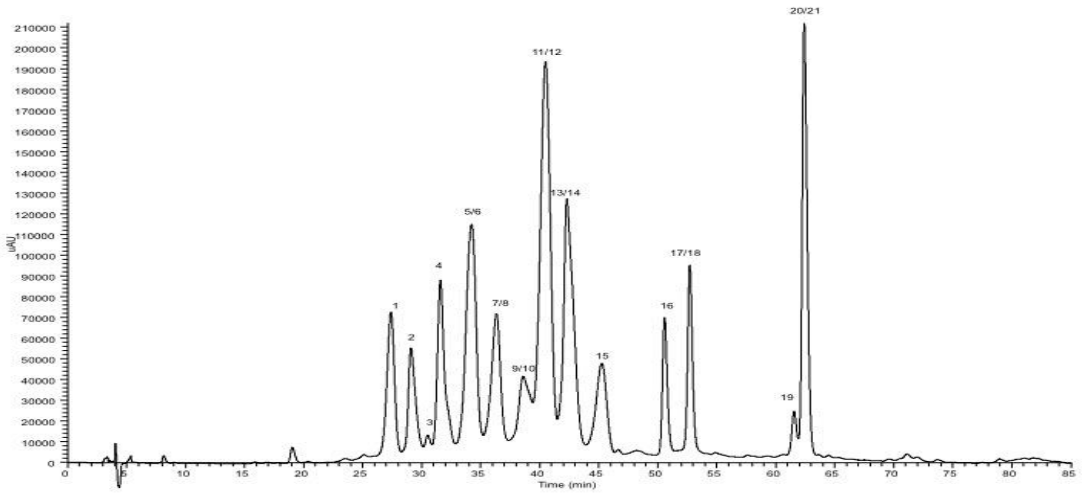
The effect of a 1000 µg mL^{-1} extract solution prepared using de Mann Rogosa and Sharpe Broth (MRS broth, Biokar Diagnostics, Beauvais, France) for lactobacilli or MRS broth supplemented with 0.5 g L^{-1} L-cysteine-HCl (Sigma, St. Louis, USA) (MRS+CYS broth) for bifidobacteria. This mixture was inoculated using an overnight inoculum and incubated at 37 °C for 24 h (bifidobacteria were incubated in anaerobiosis). At 0, 6, 12 and 24 h the total viable cells, environmental pH values and organic acid production/sugar consumption were assessed. The total viable probiotic counts were determined using decimal dilutions and plated in either MRS (48 h at 37 °C) or MRS+CYS (48 h at 37 °C under anaerobic conditions) agar. The culture media pH values were measured using a crison micropH 2002 (Crison Instruments S. A., Barcelona, Spain) pH reader. Sugar consumption/organic acid production was evaluated using an HPLC-RI-UV system, following the analytic conditions described by Sousa et al. (2015). Positive controls were drawn through inoculation of the respective culture media without extract and non-inoculated culture media (with and without extract) was used as a negative control. Each condition was assessed in triplicate.

2.6. Statistical analysis

Statistics analysis of the results was performed using IBM SPSS Statistics v21.0.0.0 (New York, New York, USA). Normality was assessed using Shapiro-Wilks's test. As the results proved to follow a normal distribution, the differences in were evaluated using One-Way Anova coupled with Turkey's test. The exception being when comparing between the different times in a given condition, in this case a One Way Repeated Measures ANOVA

coupled with Turkey's test was used. Differences were considered significant for p-values below 0.05.

Table 1. Compositional characterization of the blueberry extract by HPLC-MS.



Peak number	Anthocyanin	m/z (M^+)	Fragments (m/z)
1	Delphinidin-3-galactoside	465	303; 162
2	Delphinidin-3-glucoside	465	303; 162
3	Cyanidin-3-galactoside	449	287; 162
4	Delphinidin-3-arabinoside	435	303; 132
5	Cyanidin-3-glucoside	449	287; 162
6	Petunidin-3-galactoside	479	317; 162
7	Cyanidin-3-arabinoside	419	287; 132
8	Petunidin-3-arabinoside	479	317; 162
9	Peonidin-3-galactoside	463	301; 162
10	Petunidin-3-arabinoside	449	331; 162
11	Malvidin-3-galactoside	493	331; 162
12	Peonidin-3-glucoside	463	301; 162
13	Malvidin-3-glucoside	493	331; 162
14	Peonidin-3-arabinoside	433	301; 132
15	Malvidin-3-arabinoside	463	331; 162
16	Cyanidin	287	174; 213; 231; 259
17	Delphinidin	303	157; 229; 257
18	Petunidin	317	302
19	Peonidin	301	286
20/21*	Malvidin	331	270; 287; 299; 316

*peak 21 is s non-specific fragment of peak 20

3. Results

The extract was comprised of 637 mg g^{-1} of anthocyanin and, as can be seen in Table 1, all fifteen anthocyanins typically reported as being present in blueberries were identified in the extract, as well as their aglycone counterpart (Routray and Orsat, 2011).

Overall all microorganisms' growth was affected by the presence of the extract at 1000 µg mL^{-1} (Figure 1), though total OD inhibition throughout the 24 h period was never achieved. From the analysis of Figure 1a, it can be seen that *L. monocytogenes* growth was significantly ($p < 0.05$) reduced in the presence of the extract at 1000 and 500 µg mL^{-1} (57.4% and 19.8% lower than the control, respectively). Furthermore, all concentrations of extract were capable of inducing both a reduction of the maximum OD as well as a reduction of the overall growth rate of *L. monocytogenes*. For *E. coli* (Figure 1b), extract at 1000 µg mL^{-1} had an OD value

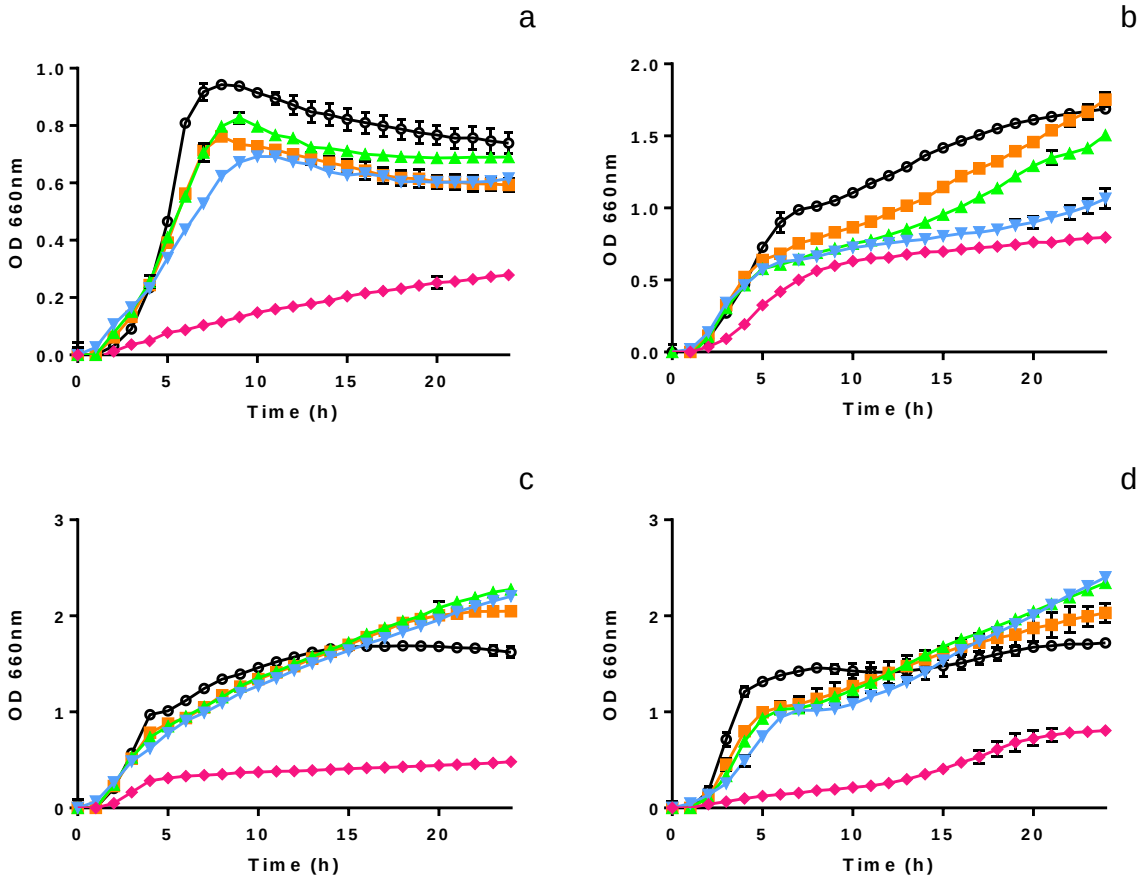


Figure 1. Time inhibition curves for *L. monocytogenes* (a), *E. coli* (b), *S. enteritidis* (c) and *B. cereus* (d) when exposed to different concentrations of extract; 1000 µg mL^{-1} (◆), 500 µg mL^{-1}(▼), 250 µg mL^{-1}(▲), 125 µg mL^{-1}(■) and 0 µg mL^{-1}(○).

that was 52.9% lower than that of the control, at the 24 h mark, while when considering 500 µg mL^{-1} this reduction was of only 36.9%. The remaining two concentrations, while still being capable of significantly ($p < 0.05$) hindering the growth of the bacteria, at the 24 h mark had little to no inhibitory effect. Considering *S. enteritidis* (Figure 1c), it is interesting to note that the extract only inhibited bacterial growth at 1000 µg mL^{-1} (70.3% reduction in OD, compared to that of the control, after 24 h). All other concentrations lead to OD values (after 24 h) that were 26.3 to 40.4% higher than those of the control. A similar behaviour was observed for *B. cereus* (Figure 1d). For this microorganism the extract was only capable of inhibiting growth at the highest concentration tested (53.1% lower OD than in the control, after 24 h) while the remaining concentrations led to final OD values that were higher than those registered for the control (from 18.1 to 39.6%).

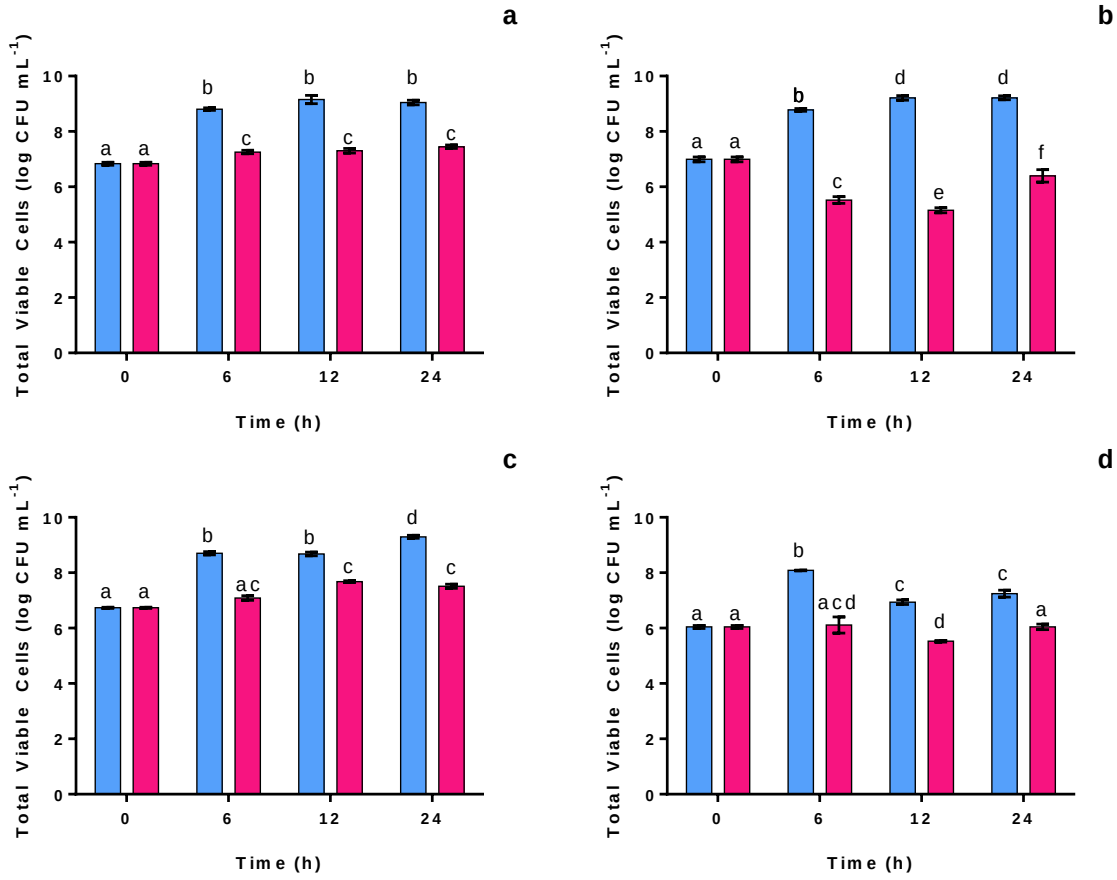


Figure 2. Total viable cells for *L. monocytogenes* (a), *E. coli* (b), *S. enteritidis* (c) and *B. cereus* (d) in the presence ((■)1000 µg mL^{-1}) and absence (■) of extract. The different letters represent statistically significant ($p < 0.05$) differences between each bar.

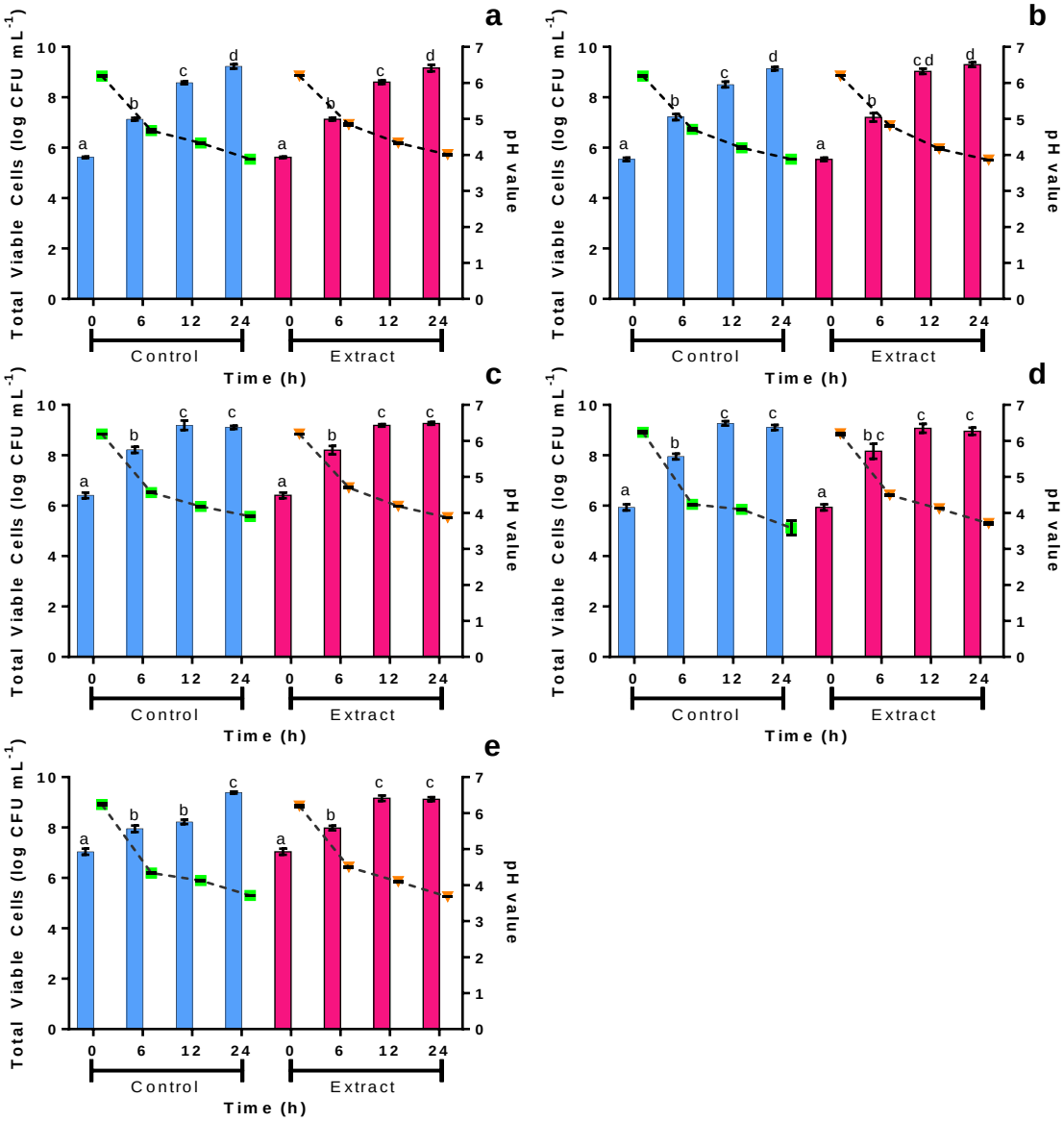


Figure 3. Total viable cells (bars) and pH values (lines) for *L. rhamnosus* (a), *L. plantarum* (b), *L. acidophilus* (c), *B.* Bo (d) and *B.* Bb12 (e) when exposed (■), or not (■), to 1000 µg mL^{-1} of extract. Different letters mark statistically significant ($p < 0.05$) differences between the bars.

In Figure 2, the impact of the extract at 1000 µg mL^{-1} (concentration that appeared to be the most effective in inhibiting bacterial growth (Figure 1)), upon the total viable cells was assessed. As can be seen for both *L. monocytogenes* and *S. enteritidis* (Figure 2a and 2c), the extract did not allow bacteria to grow as much as they did in the control, as viable cells counts were, in average, ca. 18 and 16% lower, respectively. However, when comparing with the initial bacterial counts, some significant ($p < 0.05$) growth was observed, though it fell below one logarithmic (log) cycle. The same was not observed for *E. coli* (Figure 2b). In this case, there was a significant ($p < 0.05$) reduction in the initial viable cell, which

reached 1.82 log of CFU after 12 h. Between 12 and 24 h the total viable cells increased by 1.25 log of CFU, though the overall amount still positioned below the one observed in the beginning (0.59 log of CFU lower). For *B. cereus* (Figure 2d) the extract appears to have a bacteriostatic effect, as between 0 and 24 h no significant growth was observed. However, it is interesting to note that, at 12 h, the total viable cell counts had dropped 0.52 log of CFU ($p < 0.05$), meaning that the bacteria counts were actually being reduced in this time frame.

The extract's impact upon potential probiotic microorganisms was evaluated and, as can be seen in Figure 3, the extract had no significant ($p > 0.05$) impact upon the growth of *L. rhamnosus, L. plantarum, L. acidophilus* and *B.* Bo (Figure 3a to 3d; bars). The only exception was found for *B.* Bb12. For this microorganism (Figure 3; bars), after 12 h, the presence of extract at 1000 μg mL^{-1} led to a viable count value that was 0.94 log of CFU higher than for the positive control. However, it is important to note that this difference was not observed after 24 h. Furthermore, Figure 3 also displays the acidification of media throughout the assay (Figure 3; lines) and no significant variations were registered between the extract and the control.

The evaluation of the extract's impact upon the probiotics' metabolic activity demonstrated that, overall, there was an increase in the amount of acid present (Figure 4), particularly after 24 h. Four different species of acids were identified, lactic, citric and two different short chain fatty acids (SCFA) viz. acetic and propionic acids. The addition of the extract to the culture media resulted in a ca. 34% reduction in acetic acid concentration at the starting point (Figure 4a1 and 4a2). In spite of this, after 24 h the amount of acetic acid found when bacteria were incubated in the presence of extract was 1.5 to 3.16 times higher (for *B.* Bb12 and *B.* Bo, respectively) than that of the control. It is interesting to note that these higher values appeared to be (for all probiotics except *L. rhamnosus*) due to a lack of acetic acid consumption from 12 h onwards because, at that time point, the amount of acetic acid present in the extract is similar or lower than that of the control. As for the effect on citric acid production (Figure 4b1 and 4b2) it is interesting to note that, in the case of *L. acidophilus*, the presence of extract appeared to delay the increase in citric acid concentration but, at 12 and 24 h no statistically significant differences ($p > 0.05$) were found. Similarly, for *B.* Bb12, no significant ($p > 0.05$) differences were found in citric acid concentration after 12 or 24 h. For all other probiotics the presence of the extract led to a significant ($p < 0.05$) increase of citric acid levels, ranging from 1.1 to 1.9 times higher than those of the control (for *L. rhamnosus* and *B.* Bo, respectively). Regarding the production of lactic acid (Figure 4c1 and

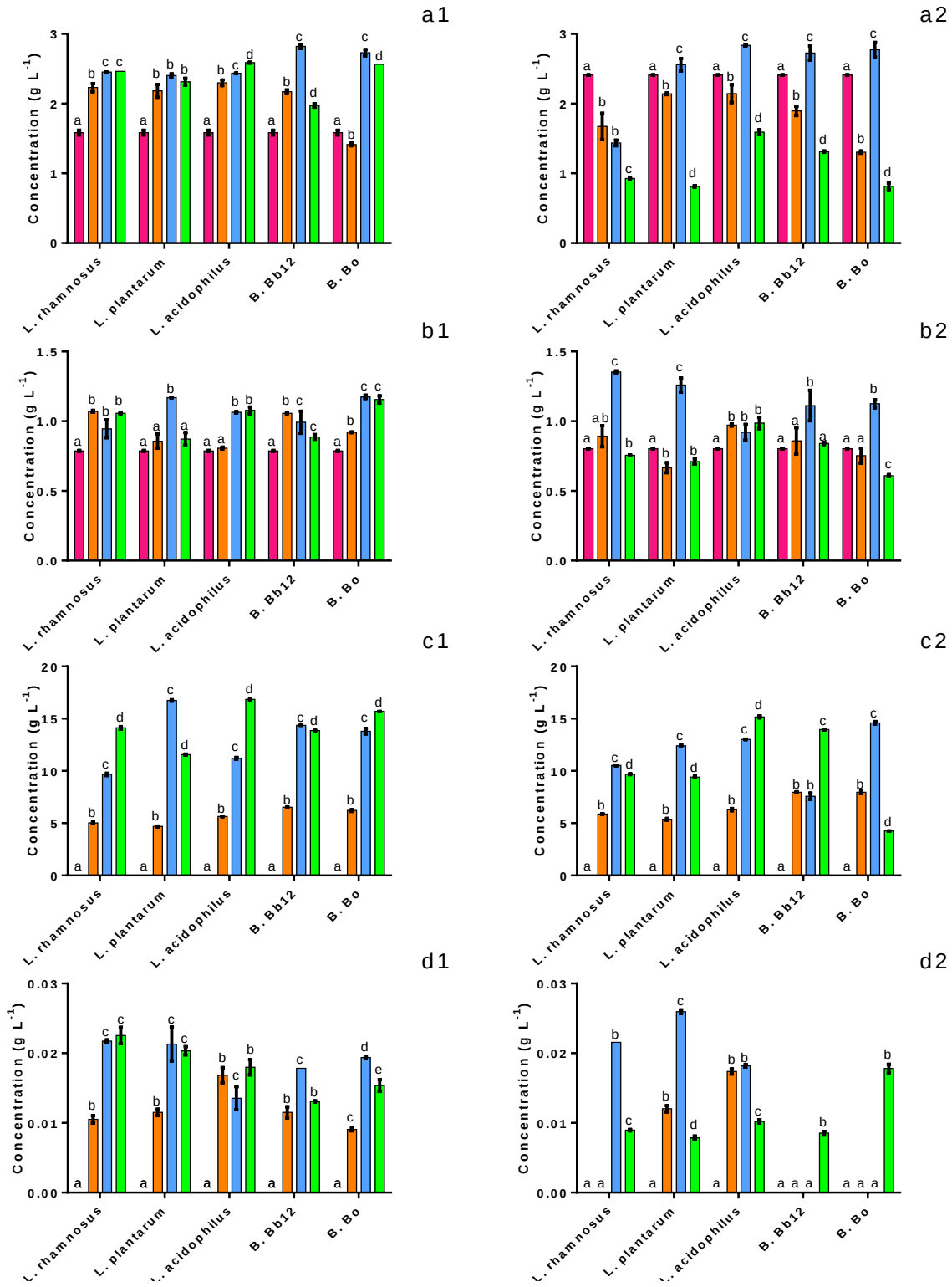


Figure 4. Concentration of acetic (a), citric (b), lactic (c) and propionic (d) acids in the presence (1) and absence (2) of extract at 0 (,)■6 ,)■(12■( ) and 24 (■) h. The different letters mark statistically significant ($p < 0.05$) differences between the times for each individual microorganism assayed.

4c2) the extracts presence led to an increase in the amount of acid produced after a 24 h period (ranging from 1.1 to 3.7 times higher for *L. acidophilus* and *B.* Bo, respectively). The only exception was found for *B.* Bb12, where no statistically significant ($p > 0.05$) differences were found, between extract and positive control, after 24 h. Propionic acid production was also significantly affected by the presence of the extract (Figure 4d1 and 4d2). When considering the 24 h mark alone, it can be seen that, for all probiotics, there was an increase in propionic acid, ranging from 1.6 to 2.5 times higher for *B.* Bb12 and *L. plantarum*. The only exception was observed for *B.* Bo. In this case, the amount of propionic acid found after 24 h was 1.2 times lower in the presence of extract. However, the presence of the extract in the media appeared to anticipate the timeframe where this acid was produced, e.g. in the positive control, for both *Bifidobacterium*, propionic acid was only observed at 24 h while, when exposed to the extract, propionic acid was detected after 6 h of incubation.

Figure 5 illustrates the effect of the extract upon probiotic sugar consumption. In regards to glucose consumption (Figure 5a1 and 5a2), after 24 h the presence of the extract either had no significant ($p > 0.05$) impact on the leftover glucose (for *L. acidophilus* and *B.* Bb12) or it led to higher values than the control (4.4 and 1.5 times higher for *L. plantarum* and *L. rhamnosus*, respectively). The exception being *B.* Bo for whom the leftover glucose levels were 2.9 times lower than those observed for the control. As for maltose (Figure 5b1 and 5b2), and with the exception of *L. rhamnosus*, at the 12 h mark no maltose was detected regardless of the presence of the extract. In the case of *L. plantarum* and *L. acidophilus* no significant ($p > 0.05$) differences between extract and control were found at 6 h, hinting at a larger consumption of maltose in the positive control as it had an initial amount of maltose ca. 34% higher than of the media with extract. However, it is interesting to note that, for *B.* Bb12 the opposite appears to be true, i.e. the reduction of maltose concentration at the 6 h mark is significantly ($p < 0.05$) lower in the presence of the extract (70% less in the presence

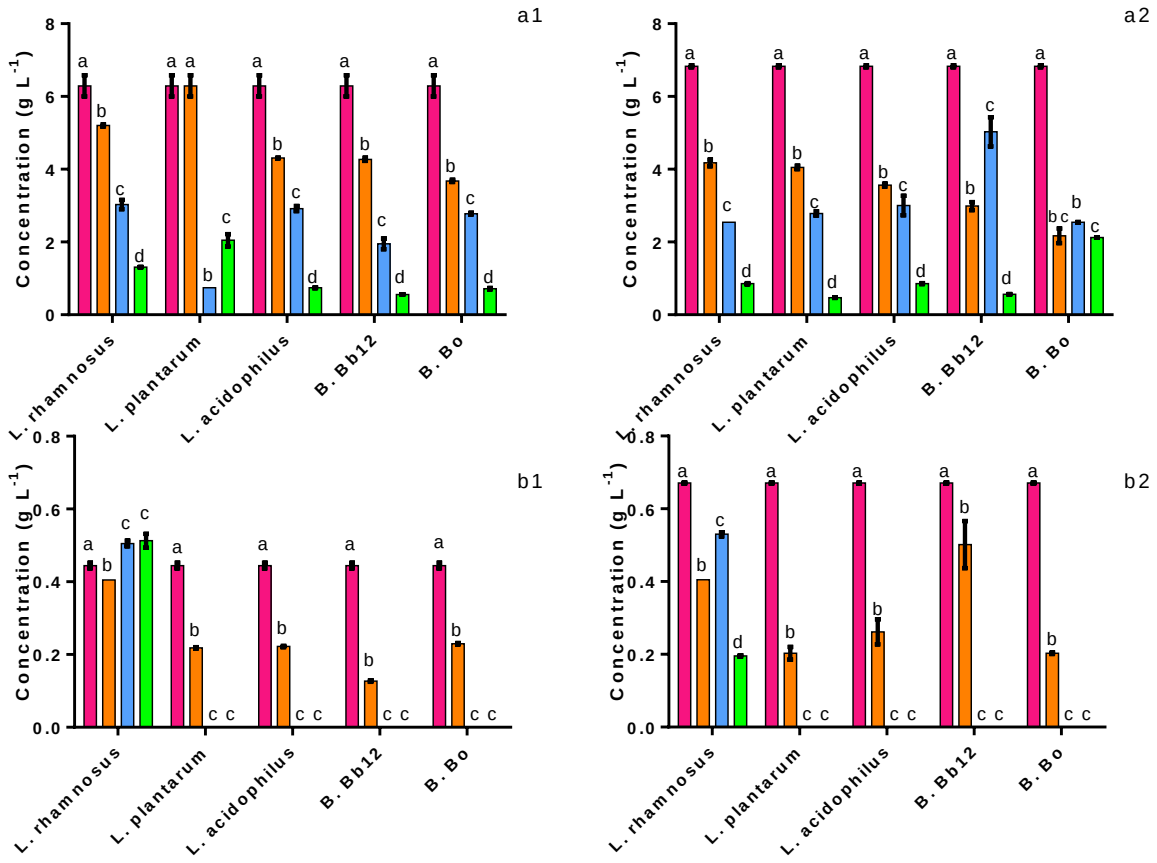


Figure 5. Concentration of glucose (a) and maltose (b) in the presence (1) and absence (2) of extract at 0 (,) ■6 ,)■(12■( ) and 24 (■) h. The different letters mark statistically significant ($p < 0.05$) differences between the times for each individual microorganism assayed.

of the extract vs. 25% less in the control). *L. rhamnosus* exhibited a response to the extract presence, in regards to maltose degradation, which was dissimilar to all other microorganisms. More specifically, while in the positive control its fermentative process led to a significant decrease in maltose after 24 h, the amount of maltose found in the presence of the extract after 24 h was 1.2 times higher than that found at the beginning.

4. Discussion

The extract, concentrated at 1000 µg mL^{-1}, was effective at inhibiting the growth of all food pathogens tested, which stands in accordance to what has been previously reported for an extract, obtained using the same methodology, in regards to other potential pathogens (Silva et al., 2016b). Additionally, these results are also in line with those reported by Shen et al. (2014), who found that *L. monocytogenes* and *S. enteritidis* were susceptible to the action of a blueberry extract. It interesting to note the disparities between the OD measurements and the quantification of viable cells, as for *L. monocytogenes* and *S. enteritidis* the apparent OD

growth did translate into an increase in viable cells, for *B. cereus* and *E. coli* while the OD hinted at a reduced bacterial growth, the total viable cells either demonstrated no growth (*B. cereus*) or a slight reduction in comparison to the initial bacterial load (*E. coli*). Some differences between both methods have been described early on and may be explained by several reasons, one of which is the accumulation of metabolic products that interfere with the OD measurement or the fact that unviable cells may still be measured by OD but not in the viable cell determination (Dalgaard et al., 1994).

Anthocyanin rich extracts have been reported as being effective against both Gram negative and Gram positive bacteria by several authors (Lacombe et al., 2012, Cisowska et al., 2011, Lacombe et al., 2010, Burdulis et al., 2009, Shen et al., 2014). However, while inhibiting potential pathogens is always interesting, there are bacteria whose inhibition might present a disadvantage, e.g. probiotics. In the present work, none of the probiotic strains' growth was negatively affected by the extracts presence hinting at a selective inhibitory activity. This is similar to what was observed by Lacombe et al. (2012) who reported that *Vaccinium angustifolium* blueberry extracts (at 34.75 or 17.4 mg L^{-1} equivalents of cyanidin-3-glucoside) were capable of inhibiting the growth of *E. coli* 157:H7, *L. monocytogenes* and *S. typhimurium*, while having little to no impact on the growth of *L. rhamnosus*. However, it is interesting to note that, while the blueberry extract proposed in the present work exhibited an antimicrobial activity at significantly higher anthocyanin concentrations (637 mg L^{-1}), the higher concentration of anthocyanins had no impact upon probiotic growth. Conversely, it is important to note that the authors did not use an HLPC based assay (as the one used in this work) but use the differential pH method to quantify anthocyanins, which has been demonstrated to significantly underestimate the anthocyanin values of extracts, so the value considered could be significantly higher than the proposed 34.75 or 17.4 mg L^{-1}(Lee et al., 2008, Lacombe et al., 2012). Puupponen-Pimiä et al. (2001) evaluated the effect that a pure cyanidin-3-glucoside had upon several potential probiotics, among which stand a *L. plantarum* and two *L. rhamnosus* stains, and found that the anthocyanin alone had no inhibitory effect (at concentrations up to 28 µg $well^{-1}$), therefore standing in line with the lack of inhibitions observed in the present work.

Despite the evidences that anthocyanins and anthocyanin rich extracts have no inhibitory effect upon probiotics, to the best of our knowledge, no work has focused on the possible metabolic consequence of their presence at levels capable of inhibiting pathogenic growth. Overall, the extracts appeared to cause an increase in the amount of acids produced by the

probiotics, at the 24 h mark, which stands in line with the results of Mousavi et al. (2013), who reported that the fermentation of an anthocyanin rich pomegranate juice resulted in higher levels of both acetic and propionic acids. Organic acids, particularly SCFA, are probiotic metabolites that have been widely associated with an array of health promoting properties (Stanton et al., 2005, Lankaputhra and Shah, 1998). Namely, *L. plantarum* 299v has been described as producing propionic and acetic acids, SCFA that have been associated with the inhibition of pathogenic microorganims (Makras and De Vuyst, 2006). This might mean that, the antimicrobial activity of the extract could be accentuated by the presence of the probiotic bacteria, as both acid levels were significantly higher when probiotics were exposed to the extract. However, the increase in acetic acid absorption has been linked with an increase in serum cholesterol levels while propionic acid has been reported as an inhibitor of cholesterol biosynthesis (Mack et al., 1999, Saarela et al., 2000, Wolever et al., 1991). As the proposed extract leads to both acetic acid accumulation and an earlier production of propionic acid, to speculate on its potential impact on cholesterol levels is precocious. From a different perspective the conservation of these acids after 24 h may be interesting for fermented products as it may allow for the extension of product shelf life as the acids may act not only as antimicrobials, but also as antioxidants and texture/colour stabilizers (Brul and Coote, 1999, Pizzocaro et al., 1993, Eswaranandam et al., 2004, Sallam, 2007, Di Cagno et al., 2011).

From a sugar consumption standpoint, it can be seen that the presence of extract caused a reduction in the consumption of glucose. Considering that higher amounts of acids were produced from a smaller amount of sugars, it stands to reason that some other compounds are acting as substrate for the potential probiotics, and as anthocyanins have been demonstrated to act as a possible carbon source, hypothetically they may also be acting as substrate in this case (Cheng et al., 2016).

It is interesting to highlight that the addition of the extract to the culture media affected both the amount of acetic acid and maltose present in the beginning of the incubation. While interactions between extract and culture media is out of the scope of the present work, the existing literature provides some insights into why this may be observed. Anthocyanin's acetylation is a relatively well described process and it may explain the reduction of acetic acid as it would be sequestered by the anthocyanin molecules (Fong et al., 1971, Anderson et al., 1970). Maltose reduction, however, may not be as easy to explain though other authors have also found that the addition of this sugar to anthocyanin rich extracts causes a small

reduction in the total anthocyanins (Mousavi et al., 2013, Kopjar et al., 2012). However, Jackman et al. (1987) reported that some sugars, and their degradation products (resulting from Maillard reactions and other oxidation reactions) may cause a reduction in the detected anthocyanin values.

5. Conclusion

The hereby proposed extract, while effectively inhibiting the growth of four potential pathogenic microorganisms did not hamper probiotics growth. Moreover, when observing the extract's potential effect on probiotic metabolism it was observed that, overall, higher amounts of organic acids were accumulated in the media when in comparison to the control. While the accumulation of organic acids may be interesting from a food production standpoint, the accumulation of acetic acid may not be as interesting from a health promotion standpoint (as acetate functions as a precursor for cholesterol synthesis). However, this accumulation of acetic acid is also accompanied by an increase in the production of propionic acid which has some interesting health promoting potential thus demonstrating the need of further studies in order to better elucidate the blueberry extract potential effects.

Acknowledgements: This work was supported by Fundação para a Ciência e Tecnologia (Portugal) through the project UID/Multi/50016/2013 and the individual Ph.D. grants of Sara Silva (SFRH/BD/90867/2012), Eduardo M. Costa (SFRH/BDE/103957/2014) and Hélder Oliveira (SFRH/BD/106062/2015).

6. References

ANDERSON, D., GUEFFROY, D., WEBB, A. & KEPNER, R. 1970. Identification of acetic acid as an acylating agent of anthocyanin pigments in grapes. *Phytochemistry,* 9**,** 1579-1583.

BRUL, S. & COOTE, P. 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology,* 50**,** 1-17.

BURDULIS, D., SARKINAS, A., JASUTIENÉ, I., STACKEVICENÉ, E., NIKOLAJEVAS, L. & JANULIS, V. 2009. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (Vaccinium myrtillus

L.) and blueberry (Vaccinium corymbosum L.) fruits. *Acta poloniae pharmaceutica,* 66**,** 399-408.

CHENG, J.-R., LIU, X.-M., CHEN, Z.-Y., ZHANG, Y.-S. & ZHANG, Y.-H. 2016. Mulberry anthocyanin biotransformation by intestinal probiotics. *Food Chemistry,* 213**,** 721-727.

CISOWSKA, A., WOJNICZ, D. & HENDRICH, A. B. 2011. Anthocyanins as antimicrobial agents of natural plant origin. *Nat Prod Commun,* 6**,** 149-56.

DALGAARD, P., ROSS, T., KAMPERMAN, L., NEUMEYER, K. & MCMEEKIN, T. A. 1994. Estimation of bacterial growth rates from turbidimetric and viable count data. *International Journal of Food Microbiology,* 23**,** 391-404.

DENG, Y., YANG, G., YUE, J., QIAN, B., LIU, Z., WANG, D., ZHONG, Y. & ZHAO, Y. 2014. Influences of ripening stages and extracting solvents on the polyphenolic compounds, antimicrobial and antioxidant activities of blueberry leaf extracts. *Food Control,* 38**,** 184-191.

DI CAGNO, R., MINERVINI, G., RIZZELLO, C. G., DE ANGELIS, M. & GOBBETTI, M. 2011. Effect of lactic acid fermentation on antioxidant, texture, color and sensory properties of red and green smoothies. *Food Microbiology,* 28**,** 1062-1071.

ESWARANANDAM, S., HETTIARACHCHY, N. S. & JOHNSON, M. G. 2004. Antimicrobial Activity of Citric, Lactic, Malic, or Tartaric Acids and Nisin-incorporated Soy Protein Film Against Listeria monocytogenes, Escherichia coli O157:H7, and Salmonella gaminara. *Journal of Food Science,* 69**,** FMS79-FMS84.

FERNANDES, I., DE FREITAS, V., REIS, C. & MATEUS, N. 2012. A new approach on the gastric absorption of anthocyanins. *Food Funct,* 3**,** 508-16.

FONG, R. A., KEPNER, R. E. & WEBB, A. D. 1971. Acetic-Acid-Acylated Anthocyanin Pigments in the Grape Skins of a Number of Varieties of Vitis Vinifera. *American Journal of Enology and Viticulture,* 22**,** 150-155.

JACKMAN, R. L., YADA, R. Y., TUNG, M. A. & SPEERS, R. A. 1987. ANTHOCYANINS AS FOOD COLORANTS —A REVIEW. *Journal of Food Biochemistry,* 11**,** 201-247.

KOPJAR, M., JAKŠIĆ, K. & PILIŽOTA, V. 2012. Influenceof Sugars And Chlorogenic Acid Addition On Anthocyanin Content, Antioxidant Activity and Color of Blackberry Juice During Storage. *Journal of Food Processing and Preservation,* 36**,** 545-552.

LACOMBE, A., WU, V. C. H., TYLER, S. & EDWARDS, K. 2010. Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157:H7. *International Journal of Food Microbiology,* 139**,** 102-107.

LACOMBE, A., WU, V. C. H., WHITE, J., TADEPALLI, S. & ANDRE, E. E. 2012. The antimicrobial properties of the lowbush blueberry (Vaccinium angustifolium) fractional components against foodborne pathogens and the conservation of probiotic Lactobacillus rhamnosus. *Food Microbiology*, 30**,** 124-131.

LANKAPUTHRA, W. E. V. & SHAH, N. P. 1998. Antimutagenic properties of probiotic bacteria and of organic acids. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 397**,** 169-182.

LEE, J., RENNAKER, C. & WROLSTAD, R. E. 2008. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry,* 110**,** 782-786.

MACK, D. R., MICHAIL, S., WEI, S., MCDOUGALL, L. & HOLLINGSWORTH, M. A. 1999. Probiotics inhibit enteropathogenic *E. coli a*dherence in vitro by inducing intestinal mucin gene expression. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 276**,** G941-G950.

MAKRAS, L. & DE VUYST, L. 2006. The in vitro inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. *International Dairy Journal,* 16**,** 1049-1057.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J Hyg (Lond),* 38**,** 732-49.

MOUSAVI, Z. E., MOUSAVI, S. M., RAZAVI, S. H., HADINEJAD, M., EMAM-DJOMEH, Z. & MIRZAPOUR, M. 2013. Effect of Fermentation of Pomegranate Juice by Lactobacillus plantarum and Lactobacillus acidophilus on the Antioxidant

Activity and Metabolism of Sugars, Organic Acids and Phenolic Compounds. *Food Biotechnology,* 27**,** 1-13.

PARK, Y. J., BISWAS, R., PHILLIPS, R. D. & CHEN, J. 2011. Antibacterial Activities of Blueberry and Muscadine Phenolic Extracts. *Journal of Food Science,* 76**,** M101-M105.

PIZZOCARO, F., TORREGGIANI, D. & GILARDI, G. 1993. Inhibition of Apple Polyphenoloxidase (PPO) by Ascorbic Acid, Citric Acid and Sodium Chloride. *Journal of Food Processing and Preservation,* 17**,** 21-30.

PUUPPONEN-PIMIÄ, R., NOHYNEK, L., MEIER, C., KÄHKÖNEN, M., HEINONEN, M., HOPIA, A. & OKSMAN-CALDENTEY, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* 90**,** 494-507.

ROUTRAY, W. & ORSAT, V. 2011. Blueberries and Their Anthocyanins: Factors Affecting Biosynthesis and Properties. *Comprehensive Reviews in Food Science and Food Safety,* 10**,** 303-320.

SAARELA, M., MOGENSEN, G., FONDEN, R., MATTO, J. & MATTILA-SANDHOLM, T. 2000. Probiotic bacteria: safety, functional and technological properties. *J Biotechnol,* 84**,** 197-215.

SALLAM, K. I. 2007. Chemical, sensory and shelf life evaluation of sliced salmon treated with salts of organic acids. *Food Chemistry,* 101**,** 592-600.

SHEN, X., SUN, X., XIE, Q., LIU, H., ZHAO, Y., PAN, Y., HWANG, C.-A. & WU, V. C. H. 2014. Antimicrobial effect of blueberry (Vaccinium corymbosum L.) extracts against the growth of Listeria monocytogenes and Salmonella Enteritidis. *Food Control,* 35**,** 159-165.

SILVA, S., COSTA, E. M., COELHO, M. C., MORAIS, R. M. & PINTADO, M. E. 2016a. Variation of anthocyanins and other major phenolic compounds throughout the ripening of four Portuguese blueberry (Vaccinium corymbosum L) cultivars. *Natural Product Research***,** 1-6.

SILVA, S., COSTA, E. M., COSTA, M. R., PEREIRA, M. F., PEREIRA, J. O., SOARES, J. C. & PINTADO, M. M. 2015. Aqueous extracts of Vaccinium corymbosum as inhibitors of Staphylococcus aureus. *Food Control,* 51**,** 314-320.

SILVA, S., COSTA, E. M., MENDES, M., MORAIS, R. M., CALHAU, C. & PINTADO, M. M. 2016b. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic anthocyanin rich blueberry extract purified by solid phase extraction. *J Appl Microbiol*.

SILVA, S., COSTA, E. M., PEREIRA, M. F., COSTA, M. R. & PINTADO, M. E. 2013. Evaluation of the antimicrobial activity of aqueous extracts from dry Vaccinium corymbosum extracts upon food microorganism. *Food Control,* 34**,** 645-650.

SOUSA, S., PINTO, J., PEREIRA, C., XAVIER MALCATA, F., BERTOLDO PACHECO, M. T., GOMES, A. M. & PINTADO, M. 2015. In vitro evaluation of yacon (Smallanthus sonchifolius) tuber flour prebiotic potential. *Food and Bioproducts Processing,* 95**,** 96-105.

STANTON, C., ROSS, R. P., FITZGERALD, G. F. & SINDEREN, D. V. 2005. Fermented functional foods based on probiotics and their biogenic metabolites. *Current Opinion in Biotechnology,* 16**,** 198-203.

WOLEVER, T. M., SPADAFORA, P. & ESHUIS, H. 1991. Interaction between colonic acetate and propionate in humans. *The American Journal of Clinical Nutrition,* 53**,** 681-7.

Impact of a purified blueberry extract on, *in vitro*, probiotic mucin-adhesion and its effect on probiotic/intestinal pathogen systems

Sara Silva[1], Eduardo M. Costa[1], Mariana Veiga[1], Bruno Horta[1], Sandra Vicente[1], Rui M. Morais[1], Conceição Calhau[2,3], Manuela E. Pintado[1]

[1] CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

[2]Nutrição e Metabolismo, NOVA Medical School, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130,l 1169-056, Lisboa, Portugal

[3]CINTESIS – Center for Research in Health Technologies and Information Systems, Porto, Portugal

Abstract

Several arguments have been made to support the need to develop natural antimicrobial alternatives to be used by the food industry. With blueberry extracts, the most compelling arguments are not only their healthy connotation but also the possibility to obtain a multipurpose solution that can either be an antioxidant, a coloring agent and an antimicrobial. From an antimicrobial perspective, as blueberry/anthocyanin rich extracts have been associated with both a capacity to inhibit harmful bacteria while causing little to no inhibition upon potential probiotic microorganisms, the study of potential benefits that come from synergies between the extract and probiotics may be of particular interest. Therefore, the present work aimed to evaluate the effect of anthocyanin rich extract upon the adhesion of five different probiotics as well as their effect upon the probiotics' capacity to compete with or block pathogen adhesion to a mucin/BSA treated surface. The results showed that, in spite of some loss of probiotic adhesion, the combined presence of extract and probiotic is more effective in reducing the overall amount of adhered viable pathogen cells than the probiotic alone, regardless of the probiotic/pathogen system considered. Furthermore, in some instances, the combination of extract with *Bifidobacterium animalis* Bo allowed for an almost complete inhibition of pathogen adhesion.

Keywords: Probiotic; Pathogen; Adhesion; Blueberry; Anthocyanins

1. Introduction

As the consumers' perception of the importance of food in health grew, so did the demand for healthier and health-promoting foodstuffs. This, coupled with the negative connotation associated with some traditional food additives, has given relevance to the use of plant extracts as replacements of traditional additives (namely antioxidants), while still conferring some functionality to the foodstuff. As blueberries have been advertised as being a superfruit, they are perceived by the consumers as possessing health promoting capabilities which makes their addition to a foodstuff (either directly or as an extract) a way to increase their perceived value (Gibson and Williams, 2000). Blueberry phenolic compounds, and anthocyanins in particular, may be of particular interest, as they not only act as antioxidant additives but also as colouring agents. Furthermore, as blueberry extracts (and other anthocyanin rich extracts) have been described as possessing antimicrobial activity while causing little to no inhibition of the growth of potential beneficial probiotics (though the information regarding probiotic inhibition is relatively scarce), their possible incorporation into a food matrix as an antimicrobial may pose an interesting alternative, not only for food control but also as a potential co-adjuvant to the prevention or control of gastrointestinal infections (Silva et al., 2013, Lee et al., 2008, Lacombe et al., 2012, Puupponen-Pimiä et al., 2001, Shen et al., 2014).

Probiotic bacteria, have long been thought to aid in the amelioration of intestinal imbalances (Isolauri et al., 2002). Though several possible mechanisms through which a probiotic may exhort a positive effect upon a host have been identified, their capacity to prevent, anticipate or remove adhered pathogens from the intestinal surface stands as one of its most interesting effects. Considering that blueberry extracts have been described as capable of inhibiting pathogen adhesion, it is possible that its presence could have a symbiotic effect with probiotics leading to reduced pathogen adhesion to the intestinal tract. This might mean that their addition to a fermented food product may not only aid in its preservation, but also potentiate one of their possible health benefits (Lacombe et al., 2012, Shen et al., 2014, Silva et al., 2013, Puupponen-Pimiä et al., 2001, Silva et al., 2016, Isolauri et al., 2002). However, to the best of our knowledge, no report has been made on the potential effect of exposing, simultaneously, potential intestinal pathogens to probiotics and anthocyanin or anthocyanin rich extracts.

Therefore, the present work aims were threefold: ascertain if the presence of an anthocyanin rich blueberry extract (that inhibited food pathogens without inhibiting probiotic growth) had any impact upon probiotic adhesion; assess a possible probiotic/extract synergy when competing with potential pathogens in adhering to a mucin (glycoproteins that are abundant in the mucosa of the gastrointestinal tract) treated surface and evaluate the extract's capacity to remove adhered pathogens and replace them with potential probiotics.

2. Experimental section

2.1. Extract production and purification

Goldtraube blueberries (harvested in 2015), kindly provided by Mirtilusa SA (Sever do Vouga, Portugal), were stored at – 20 °C until processing and extracted as described elsewhere (Silva et al., 2016). Briefly, ethanolic extracts were produced and purified using solid phase extraction columns (Bond Elut Plexa, Agilent Technologies, Santa Clara, California, USA). The resulting powder was then dissolved in deionized water (2000 µg mL^{-1}) and sterilized using a 0.22 µm sterile filter (Millipore, Massachussetts, USA). The chemical composition of the extract has been previously described elsewhere (chapter 6.1.). Henceforth, whenever extract is mentioned it refers to the solution obtained in this step.

2.2. Microorganisms

Four Five potential probiotics as well as three known intestinal pathogens were used in the present work: *Lactobacillus plantarum* 299v, *Lactobacillus acidophilus* Ki, *Lactobacillus rhamnosus* R11, *Bifidobacterium animalis Bo* (B. Bo)*, Bifidobacterium animalis* Bb12 (*B.* Bb12), *Escherichia coli* NCTC 9001, *Salmonella enteritidis* ATCC 13076 and *Listeria monocytogenes* ESB 3562 (a food isolate from Escola Superior de Biotecnologia's culture collection, Porto, Portugal).

2.3. Adhesion studies

2.3.1. Microtiter preparation

The extract's effect on bacterial adhesion was carried out by adapting the protocol described by Valeriano et al. (2014). Briefly, 100 µL of a 1 mg ml^{-1} sterile mucin solution (mucin from porcine stomach; Sigma, Darmstadt, Germany) was aliquoted into 96 well microtiters (Nunc, Darmstadt, Germany) and allowed to incubate overnight at 4 °C. Afterwards, each well was carefully washed using sterile phosphate-buffered saline solution (PBS, pH 7.4), filled with

100 µL of a 20 mg mL^{-1} sterile bovine serum albumin (BSA, Nzytech, Lisbon, Portugal) solution and the plates were then incubated at 4 °C. After 1 h, the excess BSA was removed and each well was carefully washed with PBS. From this point onward the microplates were used to carry out all the remaining assays. Henceforth, when a method describes the use of a microplate it refers to the microplates prepared in this step.

2.3.2. Bacterial suspension preparation

Overnight inoculums, incubated at 37 °C (bifidobacteria under an anaerobiotic atmosphere comprised of 10% CO_2, 10% H_2, and 80% N_2 contained within an Whitley D6250 anaerobic workstation (don Whitley Scientific, West Yorkshire, United Kingdom)) were prepared using tryptic soy broth (TSB, Biokar Diagnostics, Beauvais, France) for *E. coli, L. monocytogenes* and *S. enteritidis*, de Mann, Rogosa and Sharpe broth (MRS broth, Biokar Diagnostics, Beauvais, France) for *Lactobacillus* and MRS supplemented with 0.5 g L^{-1} L-cysteine-HCl (Sigma, St. Louis, USA) was used for *Bifidobacterium*. The inocula (10 mL) were centrifuged and washed twice and ressuspended (5 mL) using sterile PBS (Valeriano et al., 2014).

2.3.3. Impact on single species adhesion

Each bacterial suspension was mixed (1:1) with either extract or sterile deionized water (positive control), 100 µL aliquots were transferred into the previously prepared microtiters and then incubated at 37 °C in an anaerobic environment. After 1 h, the contents were carefully discarded and each well was washed twice (with sterile PBS) to remove non-adherent cells. The adhered cells were ressuspended using in 200 µL of triton-x100 (0.5 % (v v^{-1}); Sigma, Darmstadt, Germany) and the total viable counts were determined using the drop method described by Miles et al. (1938) and growing conditions described in Table 1 (Valeriano et al., 2014). All assays were performed in sextuplicate. The results were given in percentage of relative adhesion calculated according to the equation bellow in which $CFU_{initial}$ refers to the viable counts present in each of the wells and log $CFU_{adhered}$ refers to the amount of cells adhered to the surface.

$$\%\ Relative\ adhesion = \frac{\log CFU_{initial}}{\log CFU_{adhered}} \times 100$$

Table 1. Culture conditions for each microorganism.

Microorganism	Culture Media	Incubation Conditions
L. monocytogenes	Palcam Selective Agar (Biokar Diagnostics, Beauvais, France)	24 h, at 37 °C under aerobiosis
E. coli	MacConkey Agar (Biokar Diagnostics, Beauvais, France)	24 h, at 37 °C under aerobiosis
S. enteritidis	MacConkey Agar (Biokar Diagnostics, Beauvais, France)	24 h, at 37 °C under aerobiosis
L. rhamnosus, *L. acidophilus* *L. plantarum*	MRS agar (Biokar Diagnostics, Beauvais, France)	48 h, at 37 °C under aerobiosis
B. Bo *B.* Bb12	MRS agar with cysteine (0.5 g L^{-1}; Sigma, Darmstadt, Germany)	48 h, at 37 °C under anaerobiosis

2.3.4. Impact on dual species (probiotic / pathogen) adhesion

Probiotic suspension was mixed (1:1:2) with pathogen suspension, extract or sterile deionized water (positive control) and the resulting solution was aliquoted (100 µL) into microplates. After 1 h incubation (at 37 °C, under anaerobiosis) the contents were discarded, the wells were washed with PBS. The wells' contents were ressupended in a sterile triton x100 (0.5 % (v v^{-1})) and plated (under the conditions described in Table 1), using the drop method described by Miles et al. (1938). All assays were performed in sextuplicate (Valeriano et al., 2014). The results for the effect upon probiotic cells were given in percentage of relative adhesion described as calculated above (section 2.3.3.). The results regarding pathogen adhesion were presented as an inhibition percentage, calculated according to the equation below in which $CFU_{control\ pathogen}$ refers to the viable pathogen cells adhered in the single species assay and CFU_{sample} refers to the pathogen viable cells for each condition.

$$\%\ inhibition = \frac{\left(\log CFU_{control\ pathogen} - \log CFU_{sample}\right)}{\log CFU_{control\ pathogen}} \times 100$$

2.3.5. Impact on pathogen displacement by probiotics

The pathogen suspensions were mixed (1:1) with sterile deionized water and aliquoted (100 µL) into microplates. After 1 h incubation (under anaerobiosis), the wells' content was discarded and they were washed twice with sterile PBS. Afterwards, probiotic suspensions (mixed 1:1 with either extract or sterile deionized water (positive control) were aliquoted

(100 µL) into the wells and the microplates were, once again, incubated for 1 h at 37 °C (under anaerobiosis). The adhered cells were ressuspended using 200 µL of triton-x100 (0.5 % (v v^{-1}) and the total viable counts were determined using the drop method described by Miles et al. (1938) with plating being performed in the appropriate medium (for probiotic and pathogen alike) and growing conditions described in Table 1. All assays were performed in sextuplicate. The results regarding pathogen exclusion were presented as the percentage of displaced cells, calculated according to the equation below in which $CFU_{control\ pathogen}$ refers to the viable pathogen cells adhered in the single species assay and CFU_{sample} refers to the pathogen viable cells for each condition.

$$\%\ displaced\ pathogen\ cells = \frac{\left(\log CFU_{control\ pathogen} - \log CFU_{sample}\right)}{\log CFU_{control\ pathogen}} \times 100$$

2.3.6. Impact on pathogen exclusion by probiotics

The probiotic suspensions were mixed (1:1) with sterile deionized water and aliquoted (100 µL) into the microplates. After 1 h incubation (under anaerobiosis), the wells' content was discarded and they were washed twice with sterile PBS. Afterwards, pathogen suspensions (mixed 1:1 with either extract or sterile deionized water (positive control) were aliquoted (100 µL) into wells and the microplates were, once again, incubated for 1 h at 37 °C (under anaerobiosis). The adhered cells were ressuspended using in 200 µL of triton-x100 (0.5 % (v v^{-1}) and the total viable counts were determined using the drop method described by Miles et al. (1938) with plating being performed in the appropriate medias (for probiotic and pathogen alike) and growing conditions described in Table 1. All assays were performed in sextuplicate. The results for the effect upon probiotic cells were given in percentage of relative adhesion described as calculated above (section 2.3.3.). The results regarding pathogen exclusion were presented as the percentage of displaced cells, calculated according to the equation below in which $CFU_{control\ pathogen}$ refers to the viable pathogen cells adhered in the single species assay and CFU_{sample} refers to the pathogen viable cells for each condition.

$$\%\ excluded\ pathogen\ cells = \frac{\left(\log CFU_{control\ pathogen} - \log CFU_{sample}\right)}{\log CFU_{control\ pathogen}} \times 100$$

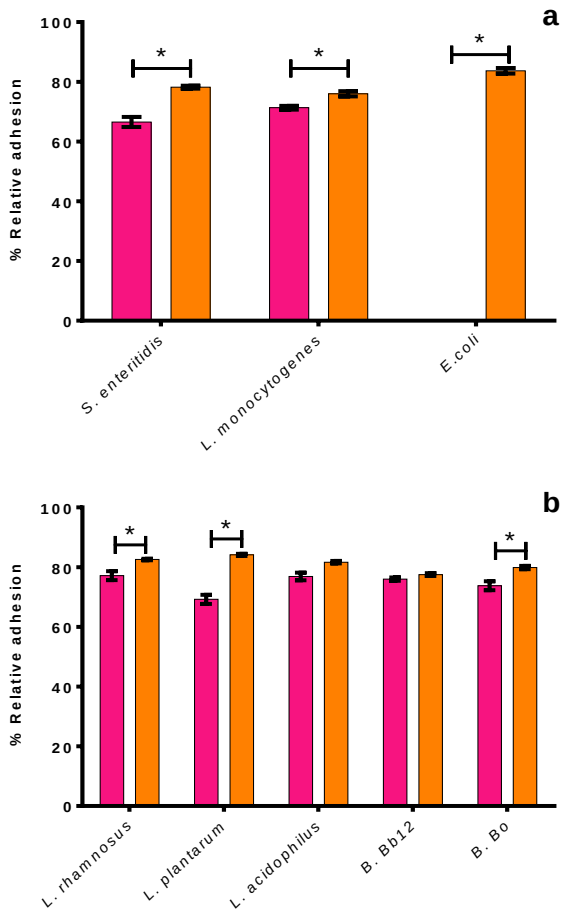


Figure 1. Percentage of relative adhesion of probiotic (a) and pathogenic (b) bacteria in the presence (■) and absence (■) of blueberry extract. The asterisks (*) mark statistically significant differences between sets of data ($p < 0.05$).

2.4. Statistical analysis

The statistical analysis of the experimental data was carried out using IBM SPSS Statistics Software V21.0.0.0 (IBM, New York, USA). Kolmogorov Smirnoff's test was used ($n < 30$) to confirm the normality of the distributions. One way ANOVA, coupled with Turkeys's post hoc test, was used to evaluate the differences between sample sets. Furthermore, scatter plots were drawn, using the same software, in order to better ascertain the effects of extract in the mixed pathogen/probiotic populations according to the species of probiotic used.

3. Results and discussion

All probiotics were capable of adhering to the mucin treated surfaces exhibiting relative adhesion levels that ranged from 77.5 to 84% for *B.* Bb12 and *L. plantarum* (Figure 1b). The presence of extract caused no significant ($p < 0.05$) inhibition of *L. acidophilus* and *B.* Bb12 though it significantly reduced the adhesion of all other probiotics. *Lactobacillus plantarum* was the most susceptible to the extracts activity, with its' presence leading to relative adhesion percentages that were, on average, 15% lower. For *L. rhamnosus* and *B.* Bo the reduction in adhesion observed, while statistically significant ($p < 0.05$), was only 5 to 6% lower resulting in relative adhesion values of 74% and 77%, respectively. Valeriano et al. (2014) reported that a potentially probiotic *Lactobacillus mucosae* (*L. mucosae*) had relative adhesion values of ca. 75%, which is lower than the results observed for *L. rhamnosus*, *L. acidophilus* and *B.* Bb12 in the presence of extract. This indicates

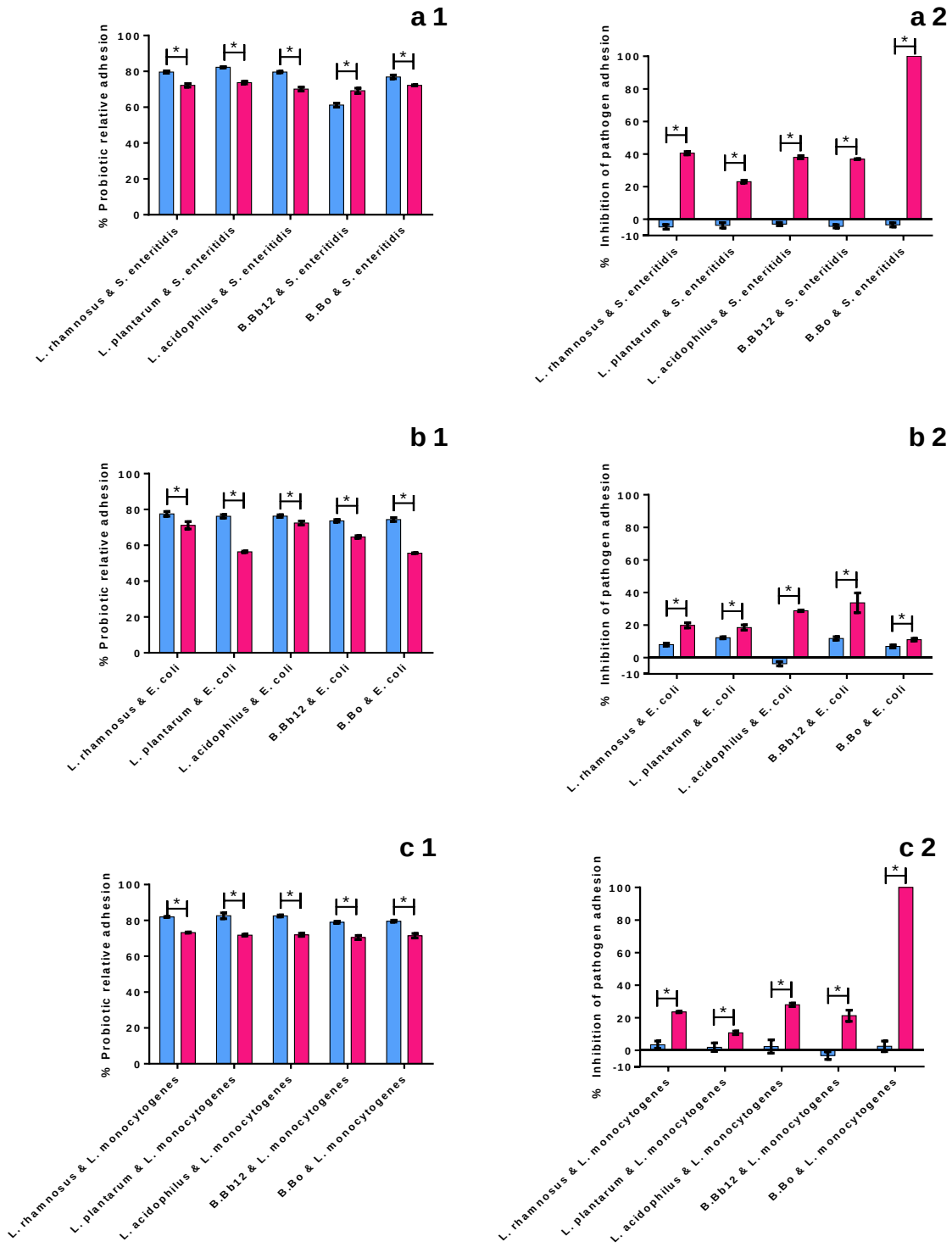


Figure 2. Impact of blueberry extract (presence (■) and absence (■) of extract) upon bacterial adhesion considering pathogen/probiotic systems: Relative probiotic adhesion (1) and inhibition of pathogen adhesion (2). (a) S. enteritidis /probiotic system, (b) E. coli/probiotic system and (c) L. monocytogenes/probiotic system. The asterisks (*) mark statistically significant differences between sets of data ($p < 0.05$).

that, while the extract may cause slight adhesion inhibition it may not do so at levels that may compromise the probiotics' action. When considering pathogen adhesion (Figure 1a), it can be seen that the mucin treated surfaces allowed for all pathogens adhesion with values averaging on 79.3% and the highest value being observed for *E. coli* (84%). For all pathogens, the addition of extract lead to lower percentages of relative adhesion ($p < 0.05$) with *L. monocytogenes* being the less susceptible (5% less adhesion) and *E. coli* the most susceptible (no viable cells were detected – 0% relative adhesion). An earlier work reported that a blueberry extract (at the same concentration and obtained using the same methodology) was capable of inhibiting *E. coli* adhesion to plasma treated surfaces, though the level at which the inhibition was observed varied according to the strain used (either ca. 90% or ca. 50%). Although, to the best of our knowledge, no studies have been performed on the antiadhesive effect of blueberry extracts against *S. enteritidis* and *L. monocytogenes*, some authors have described that they possess some inhibitory effect upon these bacteria, namely Lacombe et al. (2012) reported that extracts were capable of inhibiting the growth of both strains at 1.1 and 2.23 g L^{-1} of total phenolics (in gallic acid equivalents) for *L. monocytogenes* and *E. coli*, respectively, while not causing significant reductions of *L. rhamnosus*, and Shen et al. (2014) reported that blueberry extracts were capable of inhibiting the growth of both bacteria at concentrations ranging from 112.5 to 900 mg mL^{-1}.

Blueberry extracts, as well as other anthocyanin rich extracts, have been described as possessing antimicrobial activity against pathogens while being unable to effectively inhibit the growth of potential probiotics and lactic acid bacteria. Therefore, it may be interesting to see if the inhibitions in adhesion observed for the individual species remain the same i.e. if the extract poses a competitive advantage to probiotics or if probiotics compromise the action of the extract upon the pathogens (Puupponen-Pimiä et al., 2005, Shen et al., 2014). As can be seen in Figure 2, the inhibition of pathogen adhesion in the presence of extract is significantly ($p < 0.05$) higher than of the control (Figure 2a2, 2b2 and 2c2). In fact, for both *L. monocytogenes* and *S. enteritidis*, while the probiotics alone had little to no effect upon pathogen adhesion, the presence of extract allowed for inhibition percentages that ranged from 11% (adhesion of *L. monocytogenes* in the presence of *L. plantarum*) to 100% (adhesion of both *L. monocytogenes* and *S. enteritidis* in the presence of *B.* Bo). On the other hand, for *E. coli*, the presence of probiotics led to a reduction in the activity previously observed when the extract was used alone, i.e. while alone the extract appeared to completely inhibit the adhesion of *E.* coli, the simultaneous exposure to probiotics led to inhibition

percentages below 50%. This reduction in activity may be due to an eventual metabolization of the extract by the probiotics, as some lactic acid bacteria have been described as being capable of metabolizing anthocyanins, the major group of phenolic compounds that constitute the used extract (Vivas et al., 1997, Tabasco et al., 2011). Simultaneously, *E. coli* appears to be the only pathogen whose adhesion is affected by the presence of all probiotics (except *L. acidophilus*) regardless of the presence of extract, exhibiting adhesion inhibition percentages that range from 7 to 12% in its absence and from 11% to 34% in its presence. When considering the effects of the extract upon probiotic adhesion (Figure 2a1, 2b1 and 2c1), it can be seen that the extract's presence led to lower relative adhesion percentages (ranging from 4.7 to 19.8% for *B.* Bo with *S. enteritidis* and for *L. plantarum* with *E. coli*, respectively) for all probiotic/pathogen combinations, except for *B.* Bb12 adhesion in the presence of *S. enteritidis* in which the extract's presence led to an 8% increase ($p < 0.05$) in probiotic relative adhesion. As the capacity to adhere to the intestinal epithelium is an important functional characteristic of probiotics, the reduction in relative adhesion could hamper their action. However, probiotic relative adhesion values did not fall below 50%, averaging on 71.5, 64.2 and 71.8% when in the presence of *S. enteritidis*, *E. coli* and *L. monocytogenes*, respectively. Overall, while in the presence of the extract adhesion inhibition occurred for both probiotic and pathogen, an apparent symbiotic effect could be observed between *B.* Bo and the extract's action when considering the inhibition of *S. enteritidis* and *L. monocytogenes*. In these cases, neither the extract (relative pathogen adhesions above 65%) nor *B.* Bo alone were capable of fully inhibiting *L. monocytogenes* or *S. enteritidis* adhesion, while the combination *B.* Bo/extract led to ca. 100% inhibition percentages.

Figure 3 illustrates the probiotics' capacity to remove adhered pathogens on their own and in the presence of extract (Figure 3a2, 3b2 and 3c2) as well as their capacity to adhere to the pathogen colonized surfaces (Figure 3a1, 3b1 and 3c1). For all probiotic / pathogen combinations tested, the probiotics were capable, on their own, of displacing some of the adhered pathogens with the percentage of displaced cells ranging from 9.9 to 33.3% for *S. enteritidis*, 10.2 to 25.0% for *E. coli* and 4.1 to 17.9% for *L. monocytogenes*. These results are somewhat similar to those reported by Valeriano et al. (2014) for a potential probiotic *L. mucosae*'s displacement of *E. coli* (ca. 22%) and *Salmonella enterica* (*S. enterica;* ca. 11%) adhered to a surface that underwent the same treatment as the one employed in the present work. Moreover, these results are also in line with those reported by Collado et al. (2007)

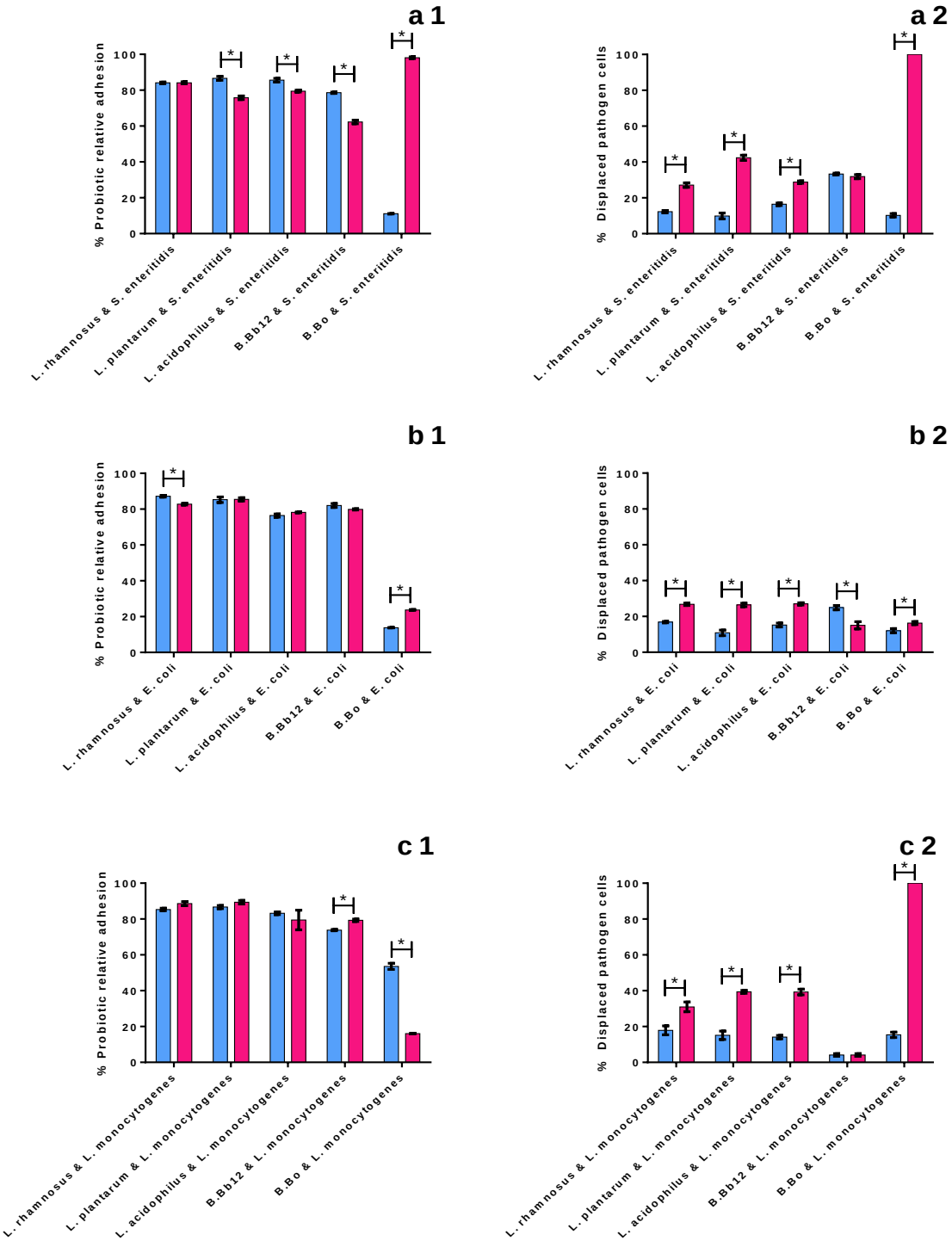


Figure 3. Impact of blueberry extract (presence (■) and absence (■) of extract) upon bacterial adhesion considering pathogen/probiotic systems: Relative probiotic adhesion (1) and displacement of pathogen cells (2). (a) S. enteritidis /probiotic system, (b) E. coli/probiotic system and (c) L. monocytogenes/probiotic system. The asterisks (*) mark statistically significant differences between sets of data ($p < 0.05$).

for *E. coli* (an average of ca. 25%) but not with those reported for *S. enterica* (an average of ca. 74%). However, as these authors use piglet mucosa and mucus in opposition to a surface treated with mucin and BSA, comparisons between both sets of results may not be straightforward. Overall, extract addition led to higher ($p < 0.05$) percentages of pathogen displaced cells in all pathogen/probiotic combinations (except *B.* Bb12) exhibiting displacement percentages ranging from 14.9 to 89.75%, 13.1 to 84.6% and 4.3 to 11.6% for *S. enteritidis*, *L. monocytogenes* and *E. coli*, respectively (Figure 3a2, 3b2 and 3c2). For *B.* Bb12, the addition of extract either had no significant ($p > 0.05$) impact upon *S. enteritidis* and *L. monocytogenes* displacement or led to a significant ($p < 0.05$) reduction in the displacement percentage of *E. coli* (ca. 10% lower displacement percentage in the presence of extract when comparing to the effect of *B.* Bb12 alone). Additionally, it is interesting to note that, similarly to what was observed in the dual species adhesion assay, the combined presence of *B.* Bo and extract led to *L. monocytogenes* and *S. enteritidis* displacement percentages of ca. 100%. However, when considering the relative probiotic adhesion for these two combinations (Figure 3a1 and 3c1), it can be seen that while for the combination *S. enteritidis* / *B.* Bo the presence of extract led to a 98% relative adhesion of probiotic (87.1% increase in comparison to the control; $p < 0.05$), for *L. monocytogenes* / *B.* Bo the presence of extract led to a significant decrease (37.5%, $p < 0.05$) in probiotic relative adhesion. This behaviour, a decrease in probiotic relative adhesion coupled with an increase in pathogen displacement when in the presence of extract, was also observed for several other pathogen/probiotic combinations *L. plantarum* / *S. enteritidis*, *L. acidophilus* / *S. enteritidis* and *L. rhamnosus* / *E. coli*. Moreover, this means that the extract, while not always promoting the replacement of pathogens by probiotic cells, still aids in pathogen removal from the mucin treated surfaces while allowing for probiotic relative adhesions averaging on 73.5%.

In regards to the pathogen exclusion assay it can be seen that, for most cases the presence of the probiotic alone is not enough to cause a significant reduction of pathogen adhesion (Figure 4a2, 4b2 and 4c2). *Salmonella enteritidis* appears to be the most susceptible microorganism to the action of probiotics alone, but the percentages of pathogen exclusion range from 1.1 to 19.9% for *L. acidophilus* and *B.* Bb12. *Escherichia coli* adhesion was inhibited by the presence of *L. rhamnosus*, *L. plantarum* and *B.* Bb12 (6.1, 7.1 and 16.6%, respectively), but *B.* Bo and *L. acidophilus* had no

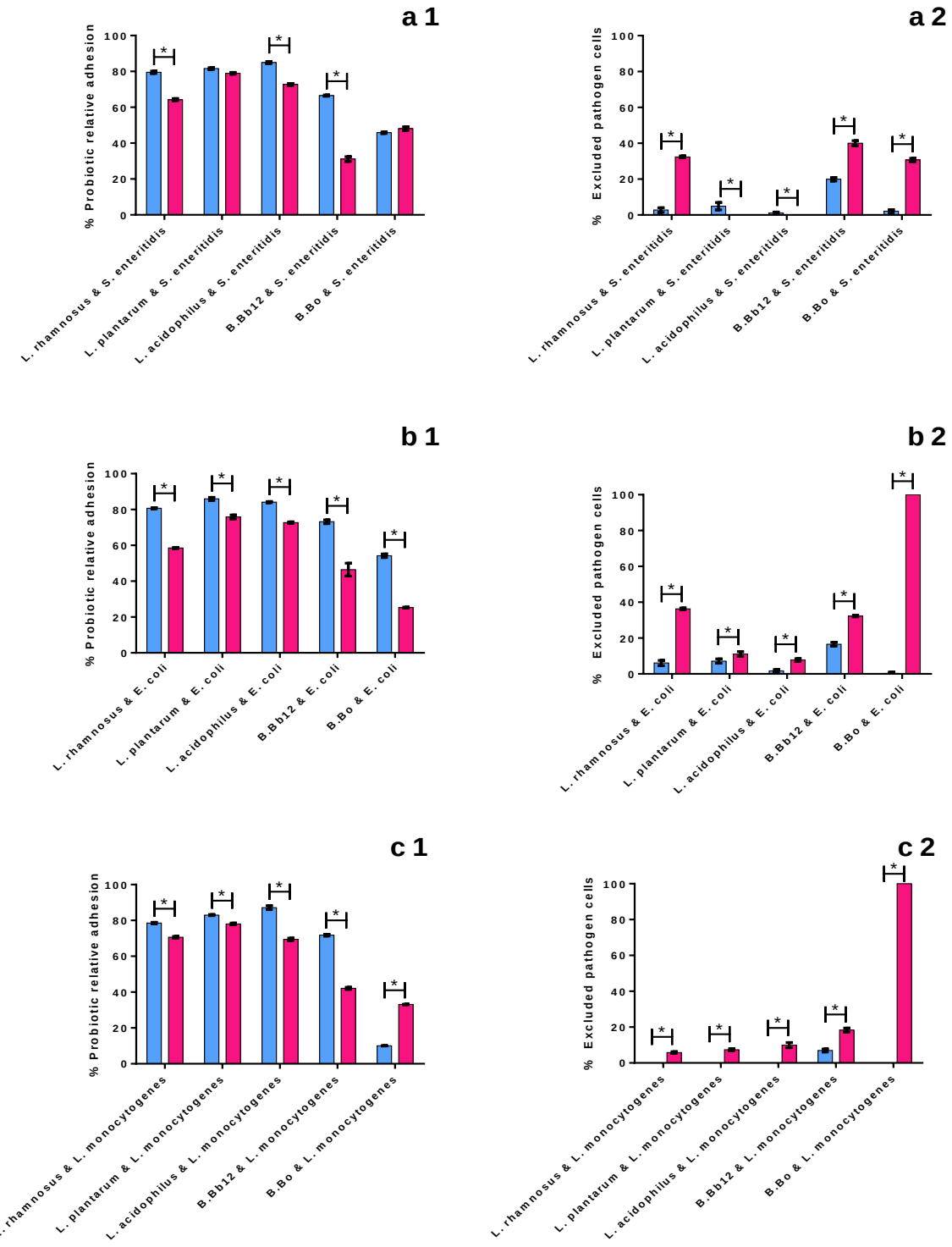


Figure 4. Impact of blueberry extract (presence (■) and absence (■) of extract) upon bacterial adhesion considering pathogen/probiotic systems: Relative probiotic adhesion (1) and exclusion of pathogen cells (2). (a) S. enteritidis /probiotic system, (b) E. coli/probiotic system and (c) L. monocytogenes/probiotic system. The asterisks (*) mark statistically significant differences between sets of data ($p < 0.05$).

effect. In turn, *L. monocytogenes* appeared to be less susceptible to the action of probiotics alone with the presence of all (bar *B.* Bb12 which, alone, was capable of inducing a small inhibition of *L. monocytogenes*) appearing to promote pathogen adhesion (exclusion percentages ranging from -11.5 to -6.3%). As coaggregation between pathogens and probiotics has been described, it is possible that some of the pathogen cells coaggregated with the adhered probiotic cells thus increasing the amount of adhered pathogen cells (Collado et al., 2008, Collado et al., 2007). Nevertheless, the values observed for pathogen exclusion by probiotics stand in line with those reported by Valeriano et al. (2014) for *E. coli* (exclusion percentages ranging from 5 to 20%) and for *Salmonela enterica* (-3.4 to 2%) for pathogen exclusion by probiotics (*Lactobacillus mucosae, Lactobacillus johnsonii* and *L. rhamnosus*) using a similar mucin/BSA treated surface. The addition of extract typically allowed for higher levels of pathogen exclusion, with exclusion percentages ranging from 5.8 to 100% for *L. monocytogenes* and 7.1 to 100% for *E. coli*. The only exception to this behaviour (i.e. the presence of extract leading to higher percentages of pathogen exclusion) was observed for *S. enteritidis*. In this case, while the extract led to higher pathogen exclusion percentages in the presence of *L. rhamnosus*, *B.* Bb12 and *B.* Bo (ca. 34% on average) it also allowed for a loss of exclusion capacity by *L. acidophilus* and *L. plantarum* (ca. -4%, on average). When considering probiotic relative adhesion (Figure 4a1, 4b1 and 4c), it can be seen that, in the absence of extract, *Lactobacillus* were more capable of remaining adhered to the surface in the presence of pathogens than *Bifidobacterium* and that relative adhesion values averaged on 81.5% for lactobacilli and 53.6% for bifidobacteria. The addition of extract had mixed effects upon the relative adhesion of probiotics: it had no significant impact ($p > 0.05$) on the relative adhesion of *L. plantarum* and *B.* Bo, it led to a significant increase ($p < 0.05$) in the adhesion of *B.* Bo in the presence of *L. monocytogenes* and, in all other cases, it led to a reduction of probiotic relative adhesion (with reductions ranging from 7.9 to 35.5%) with *B.* Bb12 being the most susceptible to the extract, as it exhibited probiotic relative adhesion percentages that were 35.5, 26.4 or 29.7% lower than those of the control for *S. enteritidis*, *E. coli* and *L. monocytogenes*, respectively. However, despite the reductions in relative adhesion caused by the extract, it is important to highlights that the values, in average, were never below 50%. Furthermore, it is interesting to note that while *B.* Bo registered some of the lowest probiotic relative adhesions to surface after pathogen exposure, when in the presence of extract it also exhibited a ca. 100% pathogen exclusion percentage for *E. coli* and *L. monocytogenes*.

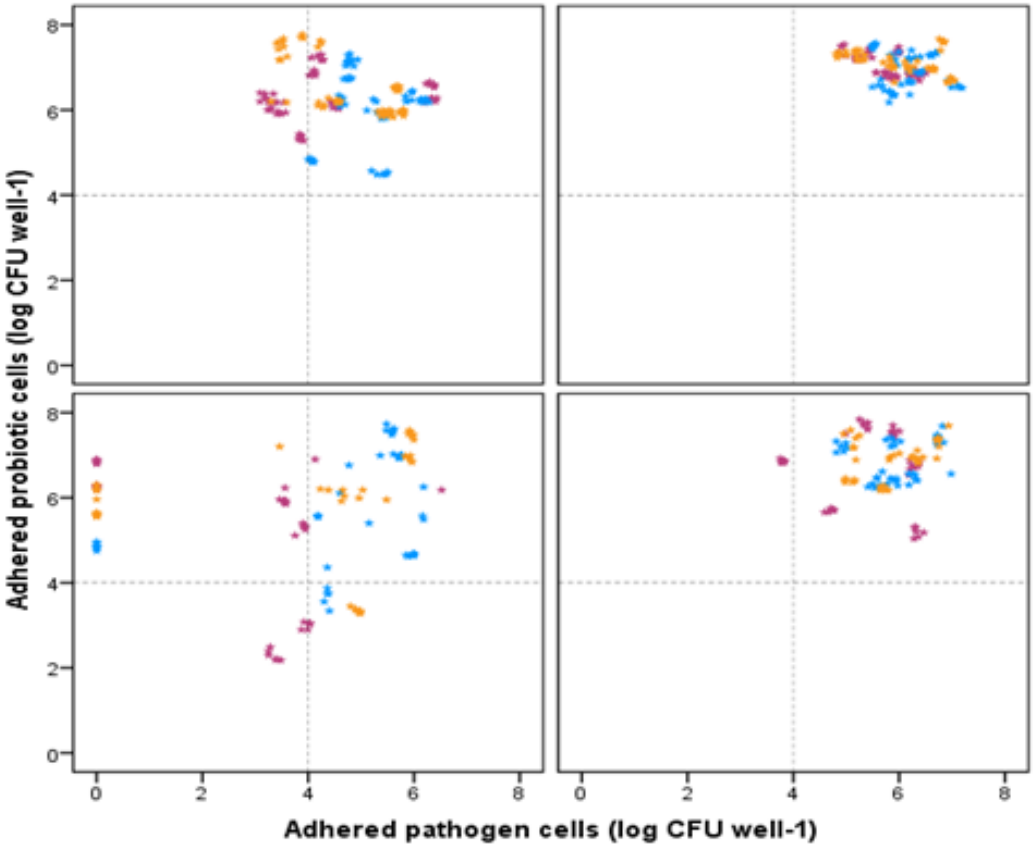


Figure 5. Effect of blueberry extract (presence (1) and absence (2)) upon the adhered probiotic and pathogen viable cells when considering *Lactobacillus*' (a) and *Bifidobacterium*'s (b) adhesion in the presence of *E. coli* (★), *S. enteritidis* (★) and *L. monocytogenes* (★).

In Figure 5 the impact of the extract upon the overall amount of adhered viable cells of both pathogen and probiotics can be seen. When observing the results for *Lactobacillus*, before extract addition the overall data was tightly clustered both when considering pathogen (5.96 ± 0.58 log CFU well^{-1}, on average) and *Lactobacillus* (7.03 ± 0.32 log CFU well^{-1}, on average) viable cells (Figure 5a1). In turn, the presence of extract led to less condensed data, though the overall intervals were similar or smaller in range, with the amount of pathogen and *Lactobacillus* adhered cells averaging on 4.71 ± 0.95 and 6.31 ± 0.74, respectively. Generally, the presence of extract led to lower amounts of both probiotic and pathogen cells adhesion to the mucin/BSA treated surfaces with reductions that averaged on 1.2 and 0.72 log CFU well^{-1}, respectively. Moreover, it is interesting to note that when considering the combination of lactobacilli with each individual pathogen, the presence of extract and *E. coli* leads to lower *Lactobacillus* adhesions than when the other pathogens are present (intervals

of *Lactobacillus* adhesion of [4.48, 7.32] log CFU well^{-1} in the presence of *E.* coli versus [5.28, 6.69] and [5.83, 6.64] CFU well^{-1} for *S. enteritidis* and *L. monocytogenes*, respectively).

As for *Bifidobacterium* in the absence of extract (Figure 5b2) it can be seen that, baring 3 small groups of data observed for the incubation of *Bifidobacterium* in the presence of *S. enteritidis*, the adhesion values appeared to be clustered together, with average values for pathogen and *Bifidobacterium* adhesion of 5.73 ± 0.76 and 6.81 ± .65 log CFU well^{-1}, respectively. When comparing the results observed for *Bifidobacterium* with those of *Lactobacillus* it can be seen that, in the absence of extract, the data was more disperse than what was observed for *Lactobacillus*, with higher inhibitions of pathogen and probiotic adhesions being observed particularly in the *S. enteritidis*/*Bifidobacterium* systems (the most dispersed data). The presence of extract in the environment (Figure 5b1) led to a set of disperse data that exhibited pathogen and *Bifidobacterium* adhesion values that averaged on 3.59 ± 2.27 or 5.55 ± 1.49 log CFU well^{-1}. *Salmonella enteritidis* was the most susceptible to the combined effects of *Bifidobacterium* and extract allowing for a reduction of adhered pathogen cells of, on average, ca. 3 log CFU well^{-1}. Furthermore, this combination of extract with *Bifidobacterium*, in some cases, led to an apparent complete absence of pathogen viable cells (regardless of the probiotic/pathogen system considered) while still allowing for some *Bifidobacterium* to adhere. Nevertheless, while these observations make the combination of extract with bifidobacteria appear more effective than the extract/lactobacilli combination, the range of probiotic adhesion is considerably wide (from 2.18 to 6.90, 3.34 to 7.3 or 3.28 to 7.58 log CFU well^{-1}, in the presence of *S. enteritidis*, *E. coli* and *L. monocytogenes*, respectively) possibly due to the different behaviours observed for *B.* Bb12 and *B.* Bo which, in turn, demonstrated the need for further studies with wider arrays of pathogens and bifidobacteria.

4. Conclusions

In spite of the eventual loss of probiotic adhesion to the mucin/BSA treated surfaces, the combined presence of extract and probiotic, overall, causes a reduction in pathogen adhesion regardless of the pathogen/probiotic system and the type of assay: simultaneous pathogen/probiotic adhesion, pathogen displacement or exclusion by probiotics. Furthermore, B. Bo appears to be one of the most interesting probiotics tested as it was the only one which, when combined with extract, allowed for ca. 100% pathogen inhibition

percentages even when *B.* Bo alone had no inhibitory effect. On another note, the extract was never fully capable on inhibiting the adhesion of probiotic microorganisms, regardless of the presence of pathogens, meaning that while compromised, some probiotic adhesion always occurred. Overall, these results point at a possible synergy between blueberry extracts and probiotic microorganisms that may have interesting repercussions when considering the prevention of pathogen colonization of mucin rich surfaces like the intestinal tract.

Acknowledgements: This work was supported by Fundação para a Ciência e Tecnologia (Portugal) through the project UID/Multi/50016/2013 and the individual Ph.D. grants of Sara Silva (SFRH/BD/90867/2012) and Eduardo M. Costa (SFRH/BDE/103957/2014).

5. References

COLLADO, M. C., GRZESKOWIAK, L. & SALMINEN, S. 2007. Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa. *Curr Microbiol*, 55**,** 260-5.

COLLADO, M. C., MERILUOTO, J. & SALMINEN, S. 2008. Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology*, 226**,** 1065-1073.

GIBSON, G. R. & WILLIAMS, C. M. 2000. *Functional foods: Concept to product.*, Cambridge, England, Woodhead Publishing Limited.

ISOLAURI, E., KIRJAVAINEN, P. V. & SALMINEN, S. 2002. Probiotics: a role in the treatment of intestinal infection and inflammation? *Gut*, 50**,** iii54-iii59.

LACOMBE, A., WU, V. C. H., WHITE, J., TADEPALLI, S. & ANDRE, E. E. 2012. The antimicrobial properties of the lowbush blueberry (Vaccinium angustifolium) fractional components against foodborne pathogens and the conservation of probiotic Lactobacillus rhamnosus. *Food Microbiology*, 30**,** 124-131.

LEE, J., RENNAKER, C. & WROLSTAD, R. E. 2008. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry*, 110**,** 782-786.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J Hyg (Lond),* 38**,** 732-49.

PUUPPONEN-PIMIÄ, R., NOHYNEK, L., HARTMANN-SCHMIDLIN, S., KÄHKÖNEN, M., HEINONEN, M., MÄÄTTÄ-RIIHINEN, K. & OKSMAN-CALDENTEY, K. M. 2005. Berry phenolics selectively inhibit the growth of intestinal pathogens. *Journal of Applied Microbiology,* 98**,** 991-1000.

PUUPPONEN-PIMIÄ, R., NOHYNEK, L., MEIER, C., KÄHKÖNEN, M., HEINONEN, M., HOPIA, A. & OKSMAN-CALDENTEY, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* 90**,** 494-507.

SHEN, X., SUN, X., XIE, Q., LIU, H., ZHAO, Y., PAN, Y., HWANG, C.-A. & WU, V. C. H. 2014. Antimicrobial effect of blueberry (Vaccinium corymbosum L.) extracts against the growth of Listeria monocytogenes and Salmonella Enteritidis. *Food Control,* 35**,** 159-165.

SILVA, S., COSTA, E. M., MENDES, M., MORAIS, R. M., CALHAU, C. & PINTADO, M. M. 2016. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic anthocyanin rich blueberry extract purified by solid phase extraction. *J Appl Microbiol*.

SILVA, S., COSTA, E. M., PEREIRA, M. F., COSTA, M. R. & PINTADO, M. E. 2013. Evaluation of the antimicrobial activity of aqueous extracts from dry Vaccinium corymbosum extracts upon food microorganism. *Food Control,* 34**,** 645-650.

TABASCO, R., SÁNCHEZ-PATÁN, F., MONAGAS, M., BARTOLOMÉ, B., VICTORIA MORENO-ARRIBAS, M., PELÁEZ, C. & REQUENA, T. 2011. Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: Resistance and metabolism. *Food Microbiology,* 28**,** 1345-1352.

VALERIANO, V. D., PARUNGAO-BALOLONG, M. M. & KANG, D. K. 2014. In vitro evaluation of the mucin-adhesion ability and probiotic potential of Lactobacillus mucosae LM1. *Journal of Applied Microbiology,* 117**,** 485-497.

VIVAS, N., LONVAUD-FUNEL, A. & GLORIES, Y. 1997. Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. *Food Microbiology,* 14**,** 291-299.

Chapter 7

Final remarks

"The Road goes ever on and on. Down from the door where it began. Now far ahead the Road has gone, and I must follow, if I can"

J. R. R. Tolkien *in* ***The Hobbit***

General conclusions

This experimental work allowed us to conclude that, in general, it is possible to attain an extract that, while produced using a simple and relatively inexpensive approach, may possess a high anthocyanin content and exhibited some relevant antioxidant and antimicrobial properties. On a closer look, chapter 2 allowed us to confirm that blueberry cultivar and fruit ripeness play a significant impact upon the anthocyanin (and other phenolics) composition of the fruits and, therefore we were able to select the best possible matrix from where to begin the extraction process. Chapter 3 demonstrated that while acidification and solvents play an important role in the composition of the extract, a solvent as common as ethanol may be an interesting alternative, especially when coupled with the solid phase extraction procedures (which use re-usable columns), to attain an anthocyanin rich extract whose final form, a powder, makes it easy to store, transport and incorporate unto products. In chapter 4 an existing method used for determining the protective effect of antioxidants against oxidative damage of DNA was improved in order to allow for a quantitative assessment and the use new oxidant systems that closer resembling biological systems. This method demonstrated that the proposed extract had an interesting antioxidant activity at concentrations ranging from 160 to 200 µg mL^{-1} while having no pro-oxidant effect. Furthermore, this method allowed for the establishment of a dose response curve, that did not mimic the one obtained using the traditional ABTS radical cation method, while using biologically relevant degradation systems, therefore making it an interesting and relatively fast means to screen for a potentially, biologically relevant, antioxidant effect. Chapter 5 focused on the potential antimicrobial activity of the extract against known nosocomial agents and it demonstrated that the extract, while not necessarily inhibiting microbial growth, affected bacterial capacity to adhere and colonize surfaces, which makes it interesting as a possible antimicrobial coadjuvant. Furthermore, while we demonstrated that the individual constituents may possess some activity, it is considerably less than that observed for the extract, hinting that synergies between the different compounds may play an important role in the inhibitions observed. Chapter 6, which focused on the interactions between the extract and probiotic organisms, allowed for two very interesting conclusions: i) the extract while effective against food pathogens appeared to possess no inhibitory effect against probiotics. ii) the extract appeared to establish an interesting synergetic effect with *Bifidobacterium animalis* Bo, which led to almost complete inhibitions of pathogen adhesion to an *in vitro* system that simulated the intestinal tract.

Overall the present work employed an exploratory approach towards the evaluation of some biological properties, that we perceived as interesting, when considering the production of an extract with a possible commercial value viz. its antioxidant and antimicrobial capacity. While the antimicrobial potential of the extract, against an array of potential of nosocomial pathogens (some with multiple antibiotic resistance) made the extract interesting to be used as a coadjuvant, particularly at skin level, where the digestive process will not interfere. As the extract proved to be effective in inhibiting some potentially pathogenic microorganisms (while not inhibiting potentially beneficial ones), exhibited an interesting antioxidant capacity and possessed an intrinsic colouring, it combined three characteristics that may allow for the replacement of colouring and preserving agents which makes one of the main possible applications for the extract proposed in the current thesis, its incorporation into food products.

Future work

As the present work employed an exploratory approach toward the evaluation of some of the relevant biological properties of blueberries, there is a wide space for further characterization of these extracts. When considering only this thesis as a base, two research lines appear as of particular interest, the effect upon multiresistant bacteria and the synergies between extract and probiotic microorganisms.

Given the extract's inhibitory effect upon several multiresistant microorganims it would be interesting to determine whether it had any implication in the resistance profile, possibly acting as a coadjuvant for traditional antibiotics. Furthermore, it could be interesting to further attempt to characterize this activity namely by evaluating if the extract had any impact upon the expression of virulence factors; if (and how) they interacted with the bacterial membrane and whether that interaction was linked with the inhibitions in adhesion observed; if the incorporation of the extract into a matrix (such as a hydrogel or a film) would compromise its activity when contemplating skin applications and how digestion would affect the extract's activity when considering eventual systemic applications (through oral ingestion).

As the extract demonstrated an interesting synergy with *Bifidobacterium animalis* Bo when contemplating the inhibition of adhesion of pathogens, it would be interesting to attempt to further characterize this effect. Firstly, a metabolome analysis could be performed in order to determine if the presence of extract causes this bacterium to produce some inhibitory compound(s). Additionally, it could be interesting to determine if this antimicrobial activity remained when considering progressively more complex matrixes, from a multispecies probiotic mixture to a gut microbiota analysis. On a different note it could be interesting to see if how the higher levels of acid production by probiotics (induced by the extracts in all probiotics tested) could affect intestinal cells (e.g. inflammatory responses). Moreover, it would be interesting to exploit the proposed research lines contextualizing the impact that the digestion has on the extract's observed effects.

When considering the existing information regarding the overall potential of blueberries, it could also be interesting to observe if the DNA protection observed actually translated into the protection of cells against oxidative damage and if this activity remained after the extract is digested and how limitations imposed by the extracts bioavailability could interfere with any potential effect.